Working with rhizobia

J.G. Howieson and M.J. Dilworth (Eds.)
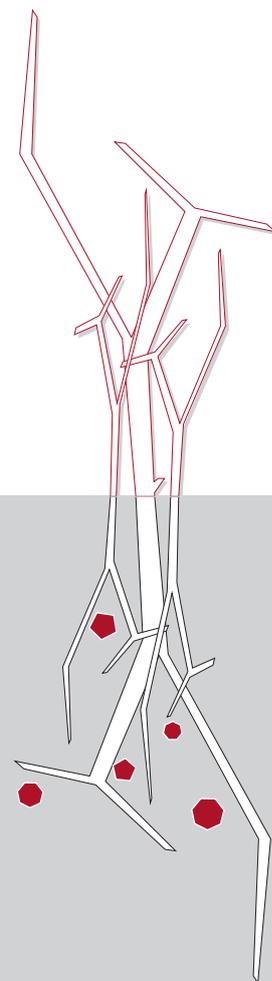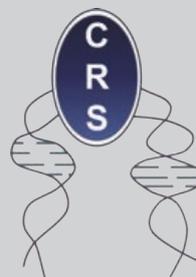
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Centre for *Rhizobium* Studies
Murdoch University

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The legume symbiosis with rhizobia (root nodule bacteria) has been acknowledged as fundamental to sustainable agriculture because this intimate relationship between soil bacteria and flowering plants can alleviate the need to provide manufactured nitrogen for farming systems. Prior to the invention of the Haber–Bosch process, which led to the manufacture of fertiliser nitrogen early last century, rhizobial nitrogen fixation was the dominant source of nitrogen in agriculture. We now understand that much of the change to our climate has resulted from the burning of fossil fuels, which is essential for generating the high temperature and pressure for the Haber–Bosch process. Any anthropomorphic activity that can limit consumption of fossil fuels must therefore be embraced. This has brought a renewed focus to the science of biological nitrogen fixation because the reductant and metabolic energy needed for the key enzyme nitrogenase to function in rhizobia are instead derived from solar radiation.

The Australian Centre for International Agricultural Research (ACIAR) has a fundamental commitment to the development of sustainable agricultural practices. Further, many of the ACIAR aid projects are directed at landscapes that are infertile. Nitrogen infertility is a global production constraint for farmers who cannot afford manufactured fertiliser, and thus adoption of legumes inoculated with appropriate rhizobia in their farming systems can lead to greatly increased food production.

This manual provides scientists and technicians with modern guidelines for ensuring that the legume symbiosis with rhizobia is optimised for nitrogen fixation in their environments. It builds upon similar manuals produced over the last hundred years and updates our knowledge of this fundamental biological process and our ability to use it in agriculture.

Nick Austin
Chief Executive Officer, ACIAR

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Preface

About this manual

Study of the legume/rhizobium symbiosis necessitates an understanding of methods to isolate and characterise the bacteria. Since the publication of '*A manual for the practical study of root-nodule bacteria*' by Jim Vincent (1970) a number of sequels have been published, such as the NifTAL, CIAT and CIMMYT manuals, which are now out of date and out of print. Discoveries of a much wider range of root-nodulating bacteria than previously known means that even simple isolation methods need revisiting to ensure unusual types of bacteria are not discarded. Drawing on the rich experience from earlier publications, this manual brings together state-of-the-art methods for the study of root-nodule bacteria, both in the free-living state and in symbiosis with legumes. In each chapter, we introduce the topic and provide guidance on how study of the symbiosis might best be tackled. We then provide a detailed description of protocols that need to be followed and highlight potential problems and pitfalls. Topics covered include acquiring, recognising, growing and storing rhizobia, experimenting with strains in the laboratory, glasshouse and field, and applying contemporary molecular and genetic methodologies to assist in the study of rhizobia. We include a chapter that describes the current taxonomy and physiological understanding of rhizobia, and another on the production of inoculants and quality control in the supply chain.

About the authors and compilation of the manual

The lead authors for each chapter were selected on the basis of their current expertise in working with rhizobia. They were invited to an initial meeting to conceive the contents of the manual at Rottnest Island, Western Australia in March 2011. Each authorship group provided early drafts which were then circulated to selected co-authors for revision (listed next page) before being finalised by the editors.

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The legume-rhizobia symbiosis and assessing the need to inoculate

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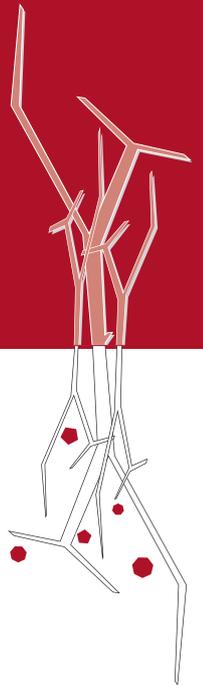
1.1 Legumes in agriculture, society and the environment

Legumes are a major component of all agrarian systems throughout the world. They are particularly attractive to low input systems of agriculture because they take inert nitrogen from the air and (through rhizobia) transform it into proteins in a process that leaves no carbon footprint. But beware, legumes differ in their adaptation to infertile soils, and the right legume must be chosen for each environment. Legumes are many and varied. The grain legumes provide protein-rich food, and soybean and groundnut are also important oilseed crops (Table 1.1). Pasture or fodder legumes are important for livestock feed in various forms: in grazed systems, as feed concentrates made from their grains, or in cut-and-carry systems where animals are kept in stalls. Woody or tree legumes produce a number of useful products apart from poles and construction materials; they are important sources of feed and browse for livestock and several of them produce edible fruits. The other major uses of legumes are for soil fertility improvement, through cover crop protection of the soil from erosion, and as green manures contributing nitrogen to improve soil fertility.

Table 1.1 The major uses of legumes, together with some examples

Use	Products	Examples
Food	Grain	Chickpea (*Cicer arietinum*), common bean (*Phaseolus vulgaris*), cowpea (*Vigna unguiculata*), faba bean (*Vicia faba*), groundnut (*Arachis hypogaea*), pea (*Pisum sativum*), pigeonpea (*Cajanus cajan*), soybean (*Glycine max*), white lupin (*Lupinus albus*), lablab (*Dolichos lablab*).
	Oil	Groundnut (*Arachis hypogaea*), soybean (*Glycine max*)
Fodder	Grazed pastures	White clover (*Trifolium repens*), sub clover (*T. subterraneum*), serradella, (*Ornithopus* spp.), biserrula (*Biserrula pelecinus*), medic (*Medicago* spp.), Townsville stylo (*Stylosanthes humilis*), desmodium (*Desmodium intortum*), Wynn cassia (*Chamaecrista rotundifolia*), crown vetch (*Onobrychis vicifolia*).
	Fodder crops	Alfalfa (*Medicago sativa*), birdsfoot trefoil (*Lotus corniculatus*), red clover (*Trifolium pratense*), lebeckia (*Lebeckia ambigua*), pink serradella (*Ornithopus sativus*)
	Feed concentrates	Soybean (*Glycine max*) cake, narrow-leaf lupin (*Lupinus angustifolius*) seed, Velvet bean (*Mucuna pruriens* var. *utilis*) seed
	Fruits	Edible pods (*Inga edulis*)
Society	Fuel, dyes and poles	Multi-purpose trees (*e.g. Calliandra calothyrsus*, *Leucaena* spp.), *Acacia mangium*, *Acacia karroo*, *Indigofera* spp.
	Shade and gum	*Acacia senegal*, *Erythrina* spp.
Environment and soil fertility	Cover crops	Mixtures of *Calopogonium caeruleum*, *Centrosema pubescens* and *Pueraria phaseoloides* used in plantations of rubber (*Hevea brasiliensis*) and oil palm (*Elaeis guineensis*), crimson clover (*Trifolium incarnatum*) in vineyards
	Green manures	Velvet bean (*Mucuna pruriens* var. *utilis*), sunnhemp (*Crotalaria juncea*), Lablab (*Dolichos purpureus*), common vetch (*Vicia sativa*, *Vicia benghalensis*)

1.2 Rhizobia and nodules

The bacteria associated with legumes (and which produce the enzymatic mechanisms that reduce atmospheric di-nitrogen (N_2) to ammonia) are collectively termed rhizobia or root-nodulating bacteria. These bacteria are the subject of this manual. At the time of writing, rhizobia are found in seven bacterial families, divided into 15 genera (Table 1.2). A recent major advance, since publication of manuals that preceded this one, is the discovery of nitrogen fixation in the β-Proteobacteria. The regularity of these organisms in nodules of subtropical Mimosa (although not from indigenous species in Mexico) and in many herbaceous legumes of the South African fynbos is notable. These organisms grow very quickly and have almost certainly previously been ignored, possibly considered as contaminants by many rhizobiologists. The β-Proteobacteria are treated comprehensively in this text, particularly in Chapters 3, 6 and 7. All rhizobia are common Gram-negative soil-inhabiting bacteria containing genes required for nodulation (*e.g. nod*, *rhi*) and N_2 fixation (*e.g. nif*, *fix*) as described in Chapter 7.

Table 1.2   The currently described rhizobia and the number of species in each genus (see also Chapter 7)

Family	Genus	Number of described species
α-Proteobacteria		
Bradyrhizobiaceae	*Bradyrhizobium*	15
Brucellaceae	*Ochrobactrum*	2
Hyphomicrobiaceae	*Azorhizobium*	3
	Devosia	1
Methylobacteriaceae	*Methylobacterium*	1
	Microvirga	3
Phyllobacteriaceae	*Phyllobacterium*	1
	Aminobacter	1
	Mesorhizobium	29
Rhizobiaceae	*Rhizobium*	43
	Neorhizobium	3
	Sinorhizobium/Ensifer	13
	Shinella	1
β-Proteobacteria		
Burkholderiaceae	*Burkholderia*	6
	Cupriavidus	2

1.2.1 The nodule

The structure in which the reduction of N_2 gas to ammonia takes place is called the nodule. Nodules are found mostly on legume roots but occasionally on stems. Nodules vary in shape and size (Sprent 2009) but all have structures and modifications from normal root cells that protect the oxygen-sensitive rhizobial enzymes from inactivation. They accomplish this while both delivering an energy supply to the bacteria and removing N-rich products. The main external features of nodule morphology are illustrated in Chapter 2 (Figure 2.2).

1.3 The legumes and their nodule characteristics

Legumes are the third largest family of dicotyledonous plants and have traditionally been divided into three subfamilies associated with distinct flower types Caesalpinioideae, Mimosoideae and Papilionoideae (Figure 1.1). Few of the Caesalpinioideae are able to nodulate and most of the Mimosoideae and an even larger proportion of Papilionoideae can nodulate; the latter includes most of the familiar crop and forage plants, such as peas, beans and clovers. Although legume taxonomy is currently undergoing major revision, at present it is convenient to retain the traditional subdivisions until the various legume working groups have an agreed alternative. A general reference that covers the important points noted below is Sprent *et al.* (2013). At the time of writing, the total number of genera in the

family was 732, with 19,321 species, but by the time this work is published both these numbers will have increased. In addition to the qualities noted below, many legumes are important for what may be summarised as ethnobotanical purposes, such as medicines, poisons and fibres (van Wyk *et al.* 1997).

1.3.1 Subfamily Caesalpinioideae

Nine genera within this subfamily are currently known to nodulate. One, *Chamaecrista*, is the eighth largest genus of legumes, with 330 species. It is one of the few that has extended into temperate regions and is the only one of agricultural significance, as exemplified by 'Wynn Cassia' a forage legume of warm temperate and subtropical regions. *Chamaecrista* may represent a separate evolutionary event for nodulation. All nodules are branched, often woody, and with the exception of some species of *Chamaecrista*, house their bacteroids (the N_2 fixing form of rhizobia) in fixation threads. A number of nodulating caesalpinioid trees are of great economic importance for their pigmented wood, for example *Erythrophleum fordii* in South-West China and Vietnam.

1.3.2 Subfamily Mimosoideae

In generic terms, this is the smallest of the subfamilies but it houses the second (*Acacia*), fifth (*Mimosa*) and ninth (*Inga*) largest legume genera, all of which form root nodules. A few basal members of the subfamily cannot nodulate. All species studied have indeterminate nodules, often branched, with root hair infection and with the central region containing both infected and uninfected cells. Most mimosoids, which are predominantly shrubs and trees, are important in their ecosystems, which range from tropical rainforests to arid areas. Three tribes have been generally recognised, Acacieae, Ingeae and Mimoseae, but recent work on acacias has changed the composition of these. The former genus *Acacia* has now been divided into six genera (Sprent 2009) with the type genus *Acacia* being largely Australian and placed in the tribe Ingeae. Most of the African and South American species are now in *Senegalia* and *Vachellia* genera. These genera include species important for products such as animal fodder, gum arabic, honey and many important timbers. Not all of the new genera have been fully accepted and new specific combinations are still being made. African workers commonly retain the generic name *Acacia.* As a result of these changes, the tribe Acacieae will probably cease to exist.

1.3.3 Subfamily Papilionoideae

This is the largest of the three subfamilies and the most widely studied for its N_2 fixation processes. It contains about 25 tribes, although this number is likely to change. Fortunately these legumes can generally be grouped in ways that are consistent with nodule characters. The principal tribes in the genistoid group are Genisteae and Crotalarieae. Their nodules are characterised by having an epidermal infection process and a central region that is uniformly infected. Nodules are

indeterminate, often branched and occasionally encircling the subtending root (lupinoid) as in *Lupinus* and *Listia.* Some lupin species are important grain legumes. Species of *Genista*, *Ulex* and others are major components of Mediterranean ecosystems as well as being invasive species in countries such as New Zealand. The dalbergioid group is characterised by having nodules associated with lateral (occasionally adventitious) roots and rhizobial infection is via cracks where these roots emerge. Nodules have determinate growth and the central region is uniformly infected. Included in this group are important grain legumes such as *Arachis hypogaea* (groundnut or peanut), forage plants such as *Stylosanthes*, highly-prized coloured timbers such as species of *Dalbergia*, whose wood may be more valuable that ivory or rhinoceros horn. Many *Dalbergia* species are on the CITES endangered list. The Phaseoleae tribe and close relatives, Desmodieae and Psoraleae (these three tribes will likely be merged) are characterised by nodules having determinate growth with surface lenticels, root hair infection and central tissue containing both infected and uninfected cells. Because of the great importance of soybeans and *Phaseolus* beans, this group of legumes has been the subject of extensive research. Unlike most nodules which export the amides glutamine and asparagine as products of fixation, this group exports ureides which can be used as an assay for N_2 fixation (see Chapter 10). The Indigofereae tribe houses the third largest legume genus *Indigofera* whose nodules are like an extended soybean nodule but have been little studied. Some species are important for production of dyes such as indigo, some are toxic to animals and many are used for fodder, green manure and cover crops. With the exception of *Lotus* that has determinate nodules but does not export ureides, and *Sesbania* which is in its own tribe and has many unique features, all other legumes studied, both temperate and tropical, have indeterminate nodules, root hair infection and central nodule tissue containing both infected and uninfected cells. Included in this group are important grains such as peas, lentils and chickpeas, and forages such as clovers and medics. The largest of all legume genera, *Astragalus*, is in this group and now gives its name to the tribe Astragaleae, formerly Galegeae. This name change has occurred because the type genus *Galega* is now known not to belong here.

Figure 1.1 Flowers formed by Subfamilies of the Leguminosae; a) Caesalpinioideae, b) Mimosoideae, c) Papilionoideae

1.3.4 Actinorhizal plants

A completely different group of plants, known as actinorhizal plants, is nodulated not by rhizobia but by the filamentous bacterial genus *Frankia.* These plants occur in a number of different dicotyledonous families. Examples are the genera *Alnus*, *Casuarina* and *Ceonothus*. Nodules are root-like in structure with a central rather than a peripheral vascular system, as in legumes, and like legumes they vary in other features such as mode of infection. A detailed comparison of legume and actinorhizal nodules can be found in Pawlowski and Sprent (2008). So far, it has not been possible to culture *Frankia* from all actinorhizal species.

1.4 Assessing the need to inoculate

The establishment of a N_2-fixing legume/rhizobia symbiosis needs the presence of the legume in association with a compatible rhizobial strain. However, there is very substantial specificity between legumes and rhizobia. When an exotic legume is introduced into an area in which it has not been previously grown, it is likely that its growth and yield can be improved by inoculating with rhizobia. Other cases where inoculation may be necessary are where the soil contains a large population of rhizobia that are able to nodulate but are ineffective (*i.e.* incapable of N_2 fixation) with the legume of interest. So how can we identify whether or not inoculation is needed?

The only sure way to know if inoculation is necessary is to conduct 'need-to-inoculate' trials. These are simple trials with a minimum of three treatments:

1. An uninoculated control without N fertiliser (–I, –N)

2. A treatment inoculated with the best-quality inoculant, without N fertiliser (+I, –N)

3. An uninoculated control treatment with N fertiliser added (–I, +N)

The uninoculated treatment without N fertiliser added will reveal the status of the background rhizobia, if present. The uninoculated treatment with N is needed to show whether legume growth is N-limited or indeed if other nutrients (such as P or K) are limiting growth (see Figure 1.3). The inoculated treatment shows whether the N-limitation can be overcome by establishment of an effective symbiosis through inoculation with rhizobia. Ideally all three treatments should be compared at background soil fertility (*i.e.* that currently used by the farmer) and with optimal fertility (all limiting nutrients added). The treatments with optimal fertility are needed to ensure that the full N_2-fixation potential of the legume/rhizobia symbiosis can be expressed, and is not limited due to deficiencies of other nutrients. Possible observations of the different treatment plots are given in Table 1.3 together with an explanation of why such effects may occur.

Table 1.3   Possible field observations made in different plots of need-to-inoculate trials and explanations of the underlying causes, with suggestions for where further research may be required*.

	Plant growth	On the roots	Explanation	Further research required
Uninoculated control	Poor growth, plants yellow	No nodules	No native rhizobia capable of infecting the test legume	
	Poor growth, plants yellow	Many small ineffective nodules	Native rhizobia ineffective in N_2 fixation with the test legume	
	Good growth, plants dark green	No nodules	Soils rich in mineral N, no native rhizobia capable of nodulating the test legume	
	Good growth, plants dark green	Many small ineffective nodules	Soils rich in mineral N, native rhizobia may be effective or ineffective	
	Good growth, plants dark green	Many large nodules, red inside	Large soil population of effective compatible rhizobia	
Inoculated plants	Poor growth, plants yellow	No nodules	Inoculum does not contain a compatible strain or inoculum dead, or strain not adapted to edaphic conditions	Select better rhizobial strain, ensure careful production, QA, transport and handling of inoculants, improved carrier
	Poor growth or growth not as vigorous as N-fed plants, plants light green or yellow	Few nodules or many small ineffective nodules	Poor quality inoculant not delivering enough cells, or rhizobial strain not highly effective, or not competitive, or strain not adapted to edaphic conditions	Select better rhizobial strain, ensure careful production, QA, transport and handling of inoculants, improved carrier
	Poor growth, plants pale	Many nodules, red inside	Other nutritional factors limiting growth	Conduct investigations to identify other limiting nutrients
	Good growth, plants dark green	Many large nodules, red inside	Highly effective inoculant	
N fed plants	Poor growth, plants yellow	No nodules or few nodules	Other factor limiting plant growth	Conduct investigations to identify other limiting nutrients or biotic factors
	Good growth, plants dark green	No nodules or few nodules	Availability of mineral N inhibits nodule formation	

*Modified from Date (1982); further detail may be found in Sessitsch *et al.* (2002)
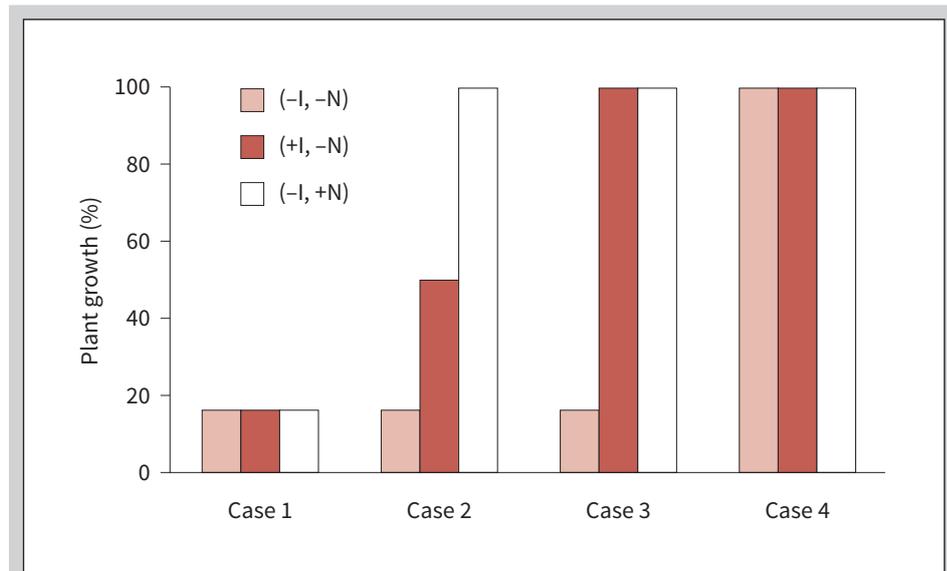
Figure 1.2 Possible results obtained from 'need-to-inoculate' trials

A combination of treatments is needed to understand whether N is the principal factor limiting growth and yield of the legume and whether inoculation is necessary (Figure 1.2). In all cases, the roots must be carefully examined for nodulation.

In Case 1, there is no response to inoculation or N fertiliser, suggesting that some other factor is limiting plant growth. This could be limitations of nutrients such as P, K or other nutrients, and a set of treatments with all nutrients added would allow understanding of whether this is the case (Figure 1.3). If water is limiting growth, this is likely to be obvious but biotic stresses may be more difficult to diagnose.

In Case 2, legume growth is clearly N-limited but inoculation leads to only partial success in N_2 fixation. This suggests that a better inoculant strain is required or that inoculation was only partially successful due, for example, to problems with the production and application of the inoculant or competition from resident background rhizobia that fix nitrogen poorly. This is illustrated in Figure 1.4.

Case 3 displays successful inoculation with rhizobia, where the inoculant has delivered a high-quality strain and it has achieved nodulation in the absence or presence of a background ineffective strain.

Case 4 illustrates a situation where either the soil can supply sufficient mineral N for optimal growth or where the indigenous rhizobial population is both large and effective so that optimal N_2 fixation occurs in the unfertilised control.

Figure 1.3   Inoculated and nodulated lablab growing on an apatitic soil without added K in the foreground, and with 18 kg/ha K_2SO_4 in the background, illustrating how lack of macro-nutrients can limit plant growth in the presence of an effective symbiosis (Case 1)

Figure 1.4   The results of a need to inoculate trial on a farm in Ethiopia, where the inoculant MAR1495 was suboptimal (Case 2) compared with 'Legumefix', a commercial inoculant using strain 532c (Case 3), either because of containing an inferior strain, a poorer carrier or lower numbers of rhizobia. Missing here is a +N control.

1.5 Selecting inoculant quality strains

Where this simple trial indicates that inoculation is necessary, yet a suitable inoculant is unavailable or the carrier is inadequate, rhizobial strains must be acquired, isolated and purified (Chapters 2–4), evaluated (Chapters 5–8) then manufactured in a suitable carrier (Chapter 9). Frequent scenarios that necessitate research to provide well-adapted strains or suitable carriers include the following:

▶ **Acid soils**, which are common in subtropical regions of the world, decrease the survival of many inoculants, either in the year of application or in subsequent seasons. Where a regenerating pasture legume is reliant on this inoculant to persist in the soil, research must be undertaken to select adapted strains. Techniques applicable to this are discussed in Chapter 8.

▶ **Hot and dry environments** where farmers cannot refrigerate peat cultures of rhizobia. Research into carriers that are stable at ambient temperature, or strains that do not require refrigeration, is required. Chapter 9 discusses some approaches to this issue.

▶ **Ineffective background populations** which preclude the desired inoculant from forming the majority of the nodules. This is a common occurrence where a legume has been cultivated for many years, and the original inoculant strains deteriorate in effectiveness, or where new cultivars are developed that have a more specific rhizobial requirement than the original inoculant.

This manual covers methodologies which will allow those working with rhizobia to achieve strain selection and manufacture to deliver high-quality inocula, to overcome the above constraints to nitrogen fixation from legumes, and with which to assess the success of the inoculation procedure.

1.6 References

Date R.A. 1982. Collection, isolation, characterization and conservation of Rhizobium. Pp 95–109 in 'Nitrogen fixation in legumes', ed. by J.M. Vincent. Academic Press: New York.

Pawlowski K. and Sprent J.I. 2008. Comparison between actinorhizal and legume symbiosis. Pp 261–288 in 'Nitrogen fixation: origins, applications, and research progress', ed. by K. Pawlowski and W. Newton. Springer: The Netherlands.

Sessitsch A., Howieson J.G., Perret X., Antoun H. and Martínez-Romero E. 2002 Advances in *Rhizobium* research. Critical Reviews in Plant Science 21, 323–78.

Sprent J.I. 2009. Legume nodulation: a global perspective. Wiley-Blackwell, Oxford: UK.

Sprent J.I., Ardley J.K. and James E.K. 2013. From north to south: A latitudinal look at legume nodulation processes. South African Journal of Botany 89, 31–41.

van Wyk B.E., van Outsdhoorn B. and Gericke N. 1997. Medicinal plants of South Africa. CABI.

Collecting nodules for isolation of rhizobia

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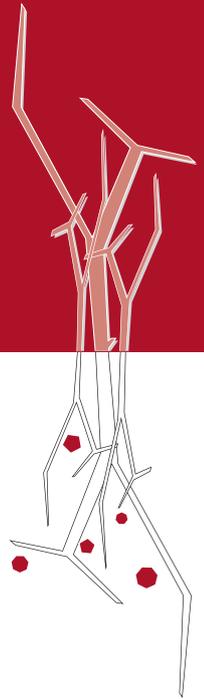
2.1 Introduction

For the legumes widely used in commerce, rhizobial strains that are well matched to these for nitrogen fixation (*i.e.* highly effective at N_2 fixation) are usually available from manufacturers or from gene banks. Some of these sources are listed in Table 2.1. However, new acquisitions of rhizobia may be required for many reasons, for example: to overcome poor N_2 fixation from an existing symbiotic relationship as described in Case 2, Chapter 1; to select well-adapted strains for a difficult environment (*e.g.* acid soil); to assist in legume domestication programs; to match with sequenced legumes for genetic studies of N_2 fixation (*e.g.* Terpolilli *et al.* 2008); or to undertake biodiversity studies.

If researchers feel that currently available strains may not satisfy their research program, then a broader range of rhizobium germplasm must be sought. Strains of rhizobia for many legumes have long been collected from their natural environments for this purpose. These environments represent *in situ* repositories of rhizobium genetic resources (Date 1982). After the collection, isolation and evaluation of new strains they should be deposited in curated gene banks. The bulk of this chapter covers methods to acquire nodule bacteria from *in situ* sources.

Table 2.1   Some curated repositories of nodule bacteria

Rhizobia Gene banks	Working collections of rhizobia
Ghent University, Belgium (LM)	WSM, CB, CC, WU, Centre for *Rhizobium* Studies (CRS), Murdoch University, Australia
USDA ARS, USA	IITA, Ibadan, Nigeria
Murdoch University, Australia (WSM)	SARDI, University of Adelaide, Australia
EMBRAPA, Brazil (SEMIA)	ICARDA, Aleppo, Syria
NIAS, Japan	SU, Sydney University, Australia
ICRISAT, India	NAK, Nairobi University, Kenya

2.2 Collecting new strains of rhizobia

Rhizobia are best sourced from nodules collected directly from the target legume growing in its natural environment (*in situ*). However, if this is not possible, strains may be recovered indirectly from soil collected near the target legume (preferably in its rhizosphere) using a trap host grown under controlled conditions in the glasshouse (see Chapter 5).

2.2.1 The *in situ* repository

If nodules are to be collected *in situ* (with a view to solving an agricultural challenge) the target environment for the eventual application of the legume should influence the locality from which the rhizobial germplasm is sourced. This is because rhizobial success in nodulation can be greatly influenced by soil properties such as clay content, pH, cation exchange saturation and climate (Graham 1998). Hence, the collection site should reflect the target site as far as possible in its edaphic properties. Prior information on the edaphic properties of the potential collection area may be derived from a geological atlas that identifies parent rock materials (Figure 2.1, left panel) or published soil surveys and rainfall charts. These may be overlayed with floral keys and knowledge of legume distribution to help guide and focus the collection expedition.

Often the richest *in situ* repositories are in rocky regions where cropping cannot be practiced (Figure 2.1, right panel) or in national parks where land disturbance has been minimised. In both scenarios, permits for exploration must be obtained in advance.

Figure 2.1 A soil map (left) illustrates granitic regions within the Cyclades group of Greek islands that give rise to acidic and infertile soils (in red colour, *e.g.* Ikaria top right). Surveying the granitic region on Ikaria (right) for legumes growing on soils in a region which receives less than 400 mm annual rainfall. This approach has produced high-quality inoculants for similar environments in southern Australia (*e.g.* Howieson *et al*. 2000; Loi *et al*. 2012).

2.3 Respecting the international biodiversity convention

The 1992 United Nations Convention on Biological Diversity (CBD- http://www.cbd.int/) sets out a series of articles that assign principles of conservation of genetic resources and rights of ownership. CBD assigns sovereignty over natural resources to States, and also suggests scientific experiments be undertaken within the country of origin of the genetic resources, where possible. Within the MOSAICS framework (http://bccm.belspo.be/projects/mosaics/) a voluntary and guiding code of conduct exists and covers access to, and circulation of, genetic resources, a pathway that tracks utilisation and potential commercial benefits arising from their exploitation. Permission must be gained from the local authorities for collection activities. Some legal and policy issues surrounding ownership of rhizobia have been discussed by Howieson and Fox (2012).

2.4 Collecting nodules

2.4.1 Equipment

Where the collection site is remote from the laboratory, the following equipment should be assembled to accompany the expedition:

► small spade or lever (*e.g.* screwdriver) to prise legume roots from soil

► scissors to remove nodules

▶ plastic screw-topped vials with desiccant (*e.g.* silica gel) for long term (> two days) nodule storage

▶ plastic bags to store whole plants with soil attached to roots

▶ permanent marker pens to label vials or bags

▶ pH kit or pH meter

▶ bulk aqueous diluent for pH measurement (*e.g.* 0.01 M $CaCl_2$)

▶ file containing passport data recording sheets and pens

▶ small envelopes for collecting seeds if they can be found

▶ camera for recording legume and flower parts for identification, nodule morphology and site characteristics (a scrap book for pressing plant parts is also useful)

▶ 50 mL vials for collecting soil (if nodules cannot be found)

▶ GPS for recording site location.

2.4.2 Timing of collection

Nodules are easiest to collect when the soil is moist, as dry conditions can cause the legume to shed its nodules or the nodules to desiccate and rupture. Soils are usually moist during winter and spring in temperate and Mediterranean climates or in the wet season(s) in subtropical and tropical environments.

When collecting nodules, the target legume must be identified, at least to the genus level. A sample of leaf, flower or pod may be pressed for future identification of the species and lodged in a local herbarium. This is often logistically difficult but, at the very least, the general taxonomic indicators (flower, pod, leaf) of the legume should be photographed and recorded along with other passport data (see Figure 2.6). The researcher should be able to recognise general taxonomic characters of the Leguminosae, particularly the different flower types of the three subfamilies and their variations (Figure 1.1, Chapter 1). A secondary advantage of collecting nodules in spring is the presence of flowers (to aid identification). Healthy, vigorous and green plants are most likely to have fully effective symbioses (see Chapter 5) and resultant nodule isolates may be at the upper end of the effectiveness scale for N_2 fixation.

2.4.3 Excavation of plant roots

The plant roots must be carefully removed from the soil, as (except in very sandy soils) nodules will be dislodged easily if the plant is pulled from the soil. A lever (*e.g.* screwdriver) or a small trowel can be very useful to loosen the soil, and a small volume of water can be carried to wash adhering material away from the exposed roots. For perennial and shrub legumes, where nodules may be very deep (>1 m) excavation of only a portion of the root system may be possible. However, the researcher must be satisfied that the exposed piece of root originates from the target legume. Because many perennial legumes have mature roots which are often devoid of nodules, fine surface roots or lateral roots must sometimes be sought. It is often easier to look for a nearby young seedling of the same species because perennial species often form tap root nodules in the first year of growth. However, for trees, it may be necessary to go much deeper, as there are reports of nodules found below 4 m in species such as *Prosopis* (mesquite) (Felker and Clark 1982).

2.4.4 Healthy nodules

Functional nodules which are firm, and when cut in half are red or pink inside, are the best to collect because isolation of rhizobia from these is usually more successful. Shrunken, flaccid or desiccated nodules indicate senescence and should be avoided. Nodules are best excised leaving a piece of root 2–5 mm in length to facilitate manipulation during the isolation steps. If healthy nodules cannot be found, techniques for isolation from ruptured nodules are described in Chapter 2, Section 2.7.

2.4.5 Nodule description

A general description of the nodule morphology can later be useful when working with novel symbioses. Sprent *et al.* (2013) have defined nodule types as summarised in Figure 2.2.
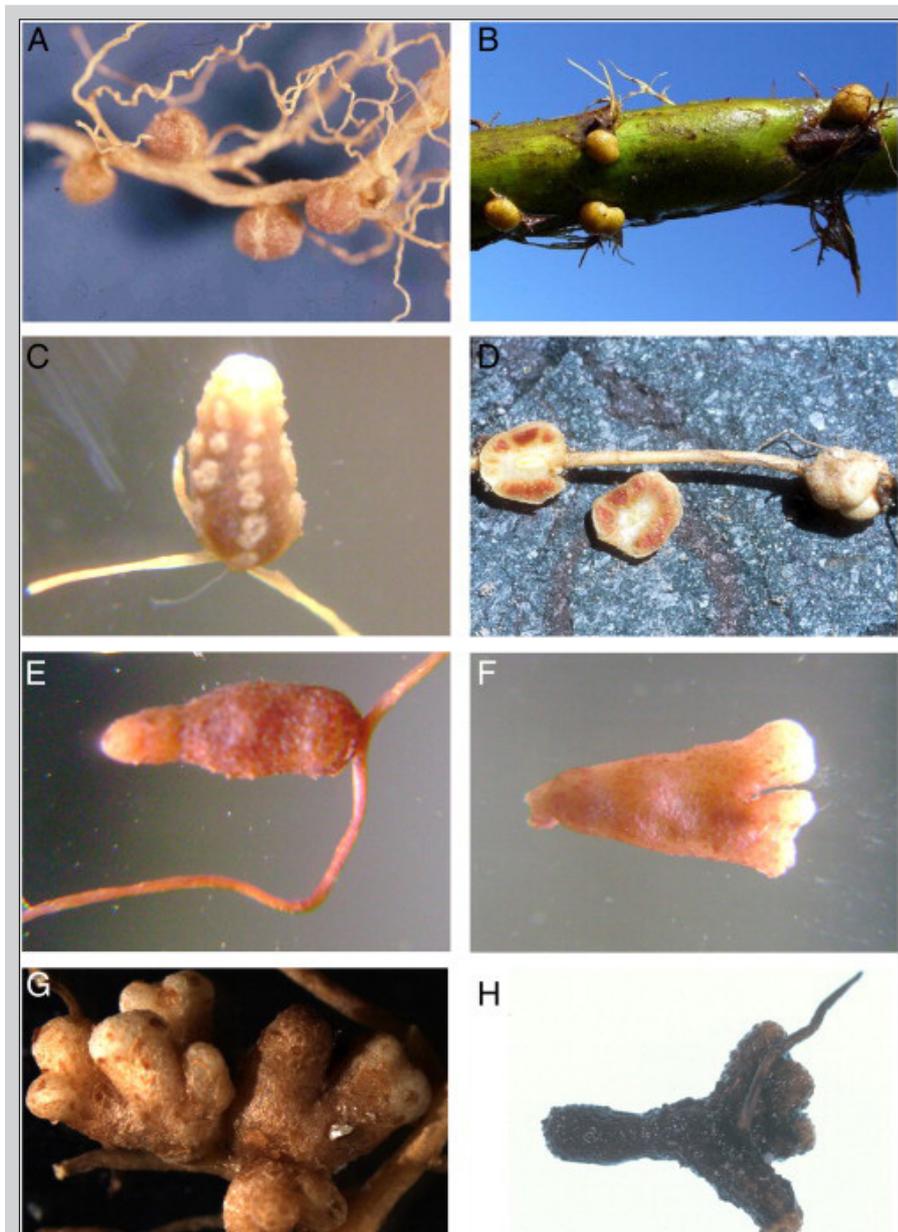
Figure 2.2   Legume nodule morphology. A: determinate, desmodioid. These nodules are more or less spherical and have lenticels, usually as stripes, but occasionally as stars (see C). B: determinate, aeschynomenoid. These nodules are always associated with lateral or adventitious roots and do not have lenticels. C: Indigoferoid, rather like an indeterminate desmodioid nodule, its detailed structure has not yet been examined and has so far only been reported from Indigofereae. D: lupinoid. E: indeterminate unbranched nodules, common in mimosoids and other groups. F: indeterminate with one or few branches, common in many papilionoids. G: indeterminate with many branches, found in all subfamilies. In some genera, such as *Ormosia* and *Crotalaria*, there may be more branching than shown here. Note that many nodules of types F and G are unbranched when young. H: woody. Most nodules that have fixation threads, both caesalpinioid and papilionoid, have a woody scleroid outer layer when mature. Reproduced from the Interactive Legume Database of Nodulation (ILDON) with permission.

2.5 Many nodules from one plant or a few nodules from many plants?

Isolation from nodules (see Chapter 3) is not always successful, so it is prudent to collect several nodules from any root system. Tropical pulse legumes can have more than 500 nodules, so an early decision must be made about the purpose of the collection. Whether a researcher should source rhizobia from a single plant, a diversity of plants in a defined region, or from a diversity of regions depends on the purpose of the collection. A biodiversity study may wish to cover as many regions as possible, but to describe the complete nodulating population in a discrete niche, all the nodules from a single plant may need to be collected. In practice, the researcher has to balance obtaining a representative selection of strains for the target legumes with the time and resources available. It is important to recognise that legumes do not necessarily form nodules with the most effective strains *in situ* (Howieson 1999) although this is disputed theoretically (Denison 2000) and so a reasonably large pool of nodules should be collected if a strain that fixes nitrogen optimally is required.

2.6 Storage of nodules

Nodules can be stored and preserved in screw-capped plastic tubes containing desiccant material, such as anhydrous calcium chloride or silica gel, with a cotton plug separating nodules from the desiccant in the bottom (Figure 2.3).

Figure 2.3 Plastic screw-capped bottles of 5 mL (left) and 20 mL (right) with cotton wool overlying desiccated silica gel for storage of nodules

Remember to label the tube (not only its cap) with a permanent marker to record the location of the collection site and link it to the passport information (Figure 2.6). An alternative is to place a piece of paper with pencilled identification inside the tube. Vials of 5 mL volume are adequate for storing approximately

three to six pasture legume nodules and 20 mL vials can be used for larger (*e.g.* soybean) nodules. Some silica gel desiccants are blue when dry and pink when moist; others are orange when dry but dark pink when moist. It is best to test the silica gel by drying a sample at 60°C overnight. The desiccant is designed to quickly remove water from the nodules and keep them dry, preventing the growth of other microorganisms. Nodules should be kept in this way for a maximum of three months, however successful isolations have been made from nodules stored for longer periods. A disadvantage of anhydrous calcium chloride desiccant is that vials of white powder can be mistaken for illicit drugs when transiting borders.

If the legumes are collected in close proximity to the laboratory and can be handled within a few days, roots may be bagged with soil attached and stored in a cool box for transport. The plants can then be shaken free of soil, washed and the nodules removed, again leaving a small portion of root attached. Fresh nodules excised from roots may be stored for a few days in the refrigerator in a sealed plastic bag but must not be frozen because internal water crystals formed by freezing can kill the bacteroids.

2.6.1 Collection of soil for trapping nodule bacteria

Sometimes nodules cannot be found on the target legume, or they may be desiccated and ruptured, and there is little choice but to collect soil then attempt to 'trap' the rhizobia when back in the laboratory. A small amount of soil (*e.g.* 10 g) from the rhizosphere of the legume will suffice. If the soil is to represent a larger geographic area, as in a biodiversity study, then an unbiased sampling procedure is needed. Subsamples, randomly and spatially distributed, should be taken to make a composite sample that is representative of areas of up to several hectares. As when collecting nodules, the sampling location should be recorded using a reference map or GPS. Information about vegetation, previous cropping history and historical application of chemicals are always very useful.

If the researcher elects to trap rhizobia from the soil, a further decision must be made: with which legume to trap the rhizobia? The target legume for the study is clearly the most appropriate (assuming seed is available) but a broad host range legume such as siratro (*Macroptilium atropurpureum*) might be appropriate for some studies.

2.6.1.1 Do trapped rhizobia resemble those that might form the nodule *in situ*?

There is some evidence that trapped strains represent a different population of nodule bacteria to those isolated directly from nodules. One example is shown in Figure 2.4 from a study with common bean plants (Alberton *et al.* 2006). The genetic diversity of strains as assessed by BOX-PCR (see Chapter 11, Section 11.3) varied according to whether they were sourced directly from nodules on the legume or from soil around the roots of the legume.
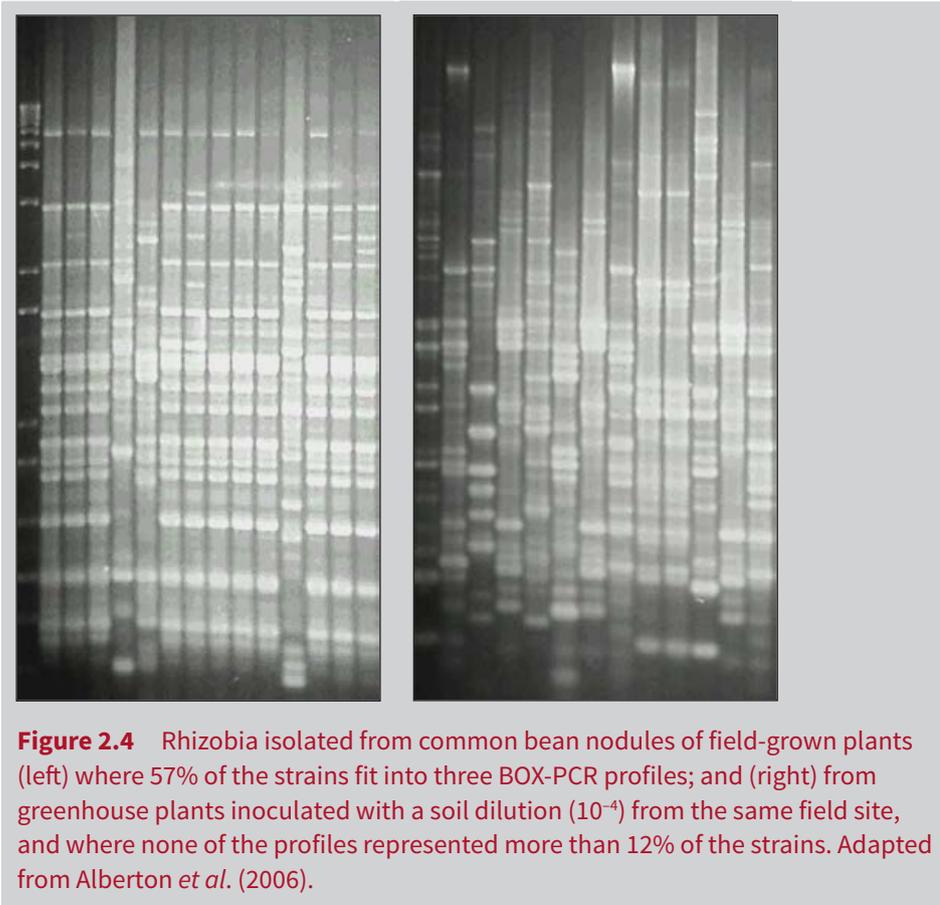
Figure 2.4 Rhizobia isolated from common bean nodules of field-grown plants (left) where 57% of the strains fit into three BOX-PCR profiles; and (right) from greenhouse plants inoculated with a soil dilution (10^{-4}) from the same field site, and where none of the profiles represented more than 12% of the strains. Adapted from Alberton *et al.* (2006).

There is also evidence that trapped strains possess less of the desirable characteristics (success in nodulation, effectiveness for N_2 fixation, ability to colonise soil) than strains sourced from the nodules of vigorous legumes growing in soils that resemble the target edaphic characteristics (Howieson 1999). Thus, the source and method of isolation should be described in each study. For example, more competitive strains may be more readily obtained from field-grown plants while a higher diversity of strains may be obtained when inoculating soil dilutions onto plants grown under greenhouse conditions.

For soils (or ruptured nodules) collected from remote locations, the samples should be stored in a suitable container for return to the laboratory. If an estimate of rhizobial number is required (see Chapter 6) the samples should be kept cool. At the laboratory, samples should be stored in the dark in a refrigerator (4–7°C). When bacterial counts are to be performed, soil samples must be stored for no longer than 30 days.

2.7 Trapping rhizobia from soil or ruptured nodules

Once back in the laboratory:

1. place the soil (or ruptured nodule) carrying the target rhizobia above a sterile medium (sand or vermiculite) in a sterile pot or vial filled to 60% of its capacity

2. cover the rhizobia-rich material with a shallow layer of sterile soil(Figure 2.5)

3. wet the soil with sterile DI water

4. sow the trap seedling into the sterile layer (see Chapter 5)—the radicle then emerges and grows down through the rhizobium-rich layer of soil to nodulate

5. cover with alkathene beads and insert watering tube for nutrient (see Chapter 5)

6. after four to five weeks, remove plants from the soil and isolate rhizobia from the fresh nodules (see Chapter 3).
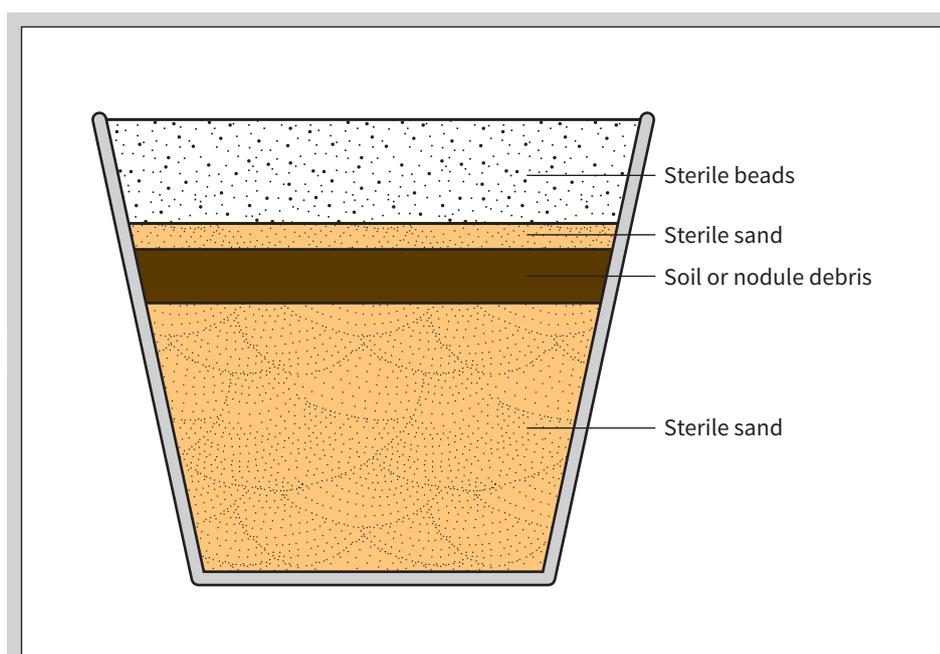
Figure 2.5   The layering technique used in the CRS laboratories for trapping rhizobia from small quantities of soil or from ruptured nodules

2.8 Specific host *in situ* trapping

A variation on trapping rhizobia from soils in the glasshouse is to sow the (surface sterilised) target legume directly into the field soil, *in situ*. This might overcome the tendency for different populations to be accessed from soil dilutions when

applied to legume roots *in vitro*. This technique was successfully applied to a project seeking acid soil-tolerant rhizobia from acidic Sardinian soils, specifically for *Pisum sativum.* This species does not naturally occur widely in rangeland settings in Sardinia, hence surface sterilised seed was sown into a wide number of rangeland sites before the onset of seasonal rains, and surviving plants excavated 10 weeks later. One further advantage of this approach is that authentication can be undertaken on the same source of seed as that which was planted.

2.9 Collection of passport data

A minimum data set, such as that referred to in Figure 2.6, is recommended. The type of soil, the altitude and rainfall, reference to a photo of the host or environment and latitude and longitude are all valuable parameters when describing the source of nodule bacteria. An estimation of the land use, grazing pressure, slope, water run-off and associated plant species is often also very valuable.

Figure 2.6   An example of the passport data that should accompany nodule collection

A laboratory soil analysis that quantifies the environment from which the strains were collected is often also very useful in later studies that seek to understand adaptation of rhizobia to new environments. For this, a 100 g sample of soil will be required.

An important parameter in rhizobial ecology is pH, and this can be determined at the collection site with a portable universal indicator dye kit or by carrying small quantities of diluent (DI water or 0.01 M CaCl$_2$) and a portable pH probe attached to an electronic meter (Figure 2.7).

Figure 2.7   Soil pH is a valuable parameter to record. Note the yellow colour produced by the Universal Indicator pH kit which approximates to a soil pH of 4.69 as registered on the portable electronic pH meter after dilution of soil in 0.01 M CaCl$_2$. This represents the lower limit for nodulation of annual species of *Medicago*, shown here collected in a natural environment on the Cyclades island of Tinos in Greece.

2.10 Collection of seed (for authentication)

Isolation of rhizobia from nodules and subsequent authentication are described in Chapter 3. However, authentication of isolates is greatly expedited if seed can be collected from the same plant as the nodule. The conundrum is that if nodules are collected in spring, when soils are moist, seeds are rarely mature. Conversely, if nodules are collected when seeds are mature, they are often desiccated and ruptured.

Several resolutions are possible. Nodules can be collected in late spring, when early maturing seeds may be found on the plant, or on the same species growing

in nearby pockets of dry soil. Alternatively, and perhaps most conveniently, annual legumes often have multiple 'hard' seeds in pods (ungerminated seed) from which a single soft seed has germinated. The residue pod containing hard seed can often be found still attached to the legume root, and thus seeds representing siblings of the plant containing the nodule can be prised from the pod. This is common for subterranean clover and most annual species of *Medicago*. Many other legumes form hard seeds and these can be recovered from the legume pod residues on the surface soil, particularly where these pods do not fully dehisce (*e.g. Lotus ornithopodioides*, *Ornithopus compressus*, *Lebeckia ambigua*). Ensure that collected seed is stored in a manner that maximises its viability. If seed cannot be collected at the time of nodule collection, a GPS record will guide a subsequent site visit. After nodules have been collected, the task is to isolate, grow and authenticate strains recovered from inside them. These activities are discussed in the following Chapter.

2.11 References

Alberton O., Kaschuk G. and Hungria M. 2006. Sampling effects on the assessment of genetic diversity of rhizobia associated with soybean and common bean. Soil Biology and Biochemistry 38, 1298–1307.

Date R.A. 1982. Collection, isolation, characterization and conservation of Rhizobium. Pp 95–109 in 'Nitrogen fixation in legumes', ed. by J.M. Vincent. Academic Press: New York.

Denison R.F. 2000. Legume sanctions and the evolution of symbiotic cooperation by rhizobia. American Naturalist 156, 567–576.

Felker P. and Clark P.R. 1982. Position of mesquite (*Prosopis* spp.) nodulation and nitrogen fixation (acetylene reduction) in 3 m-long phraetophytically simulated soil columns. Plant and Soil 64, 297–305.

Graham P.H. 1998. Symbiotic nitrogen fixation. Pp 325–347 in 'Principles and applications of soil microbiology', ed. by D. Sylvia, *et al.* Prentice Hall.

Howieson J.G. 1999. The host-rhizobia relationship. Pp 96–106 in 'Genetic resources of Mediterranean pasture and forage legumes', ed. by S.J. Bennett and P.S. Cocks. Kluwer Academic Publishers: The Netherlands.

Howieson J.G., O'Hara G.W. and Carr S.J. 2000. Changing roles for legumes in Mediterranean agriculture: developments from an Australian perspective. Field Crops Research 65, 107–122.

Howieson J.G. and Fox S.L. 2012 Plant growth promotion with micro-organisms. Pp 138–149 in 'Beneficial microorganisms in agriculture, food and the environment: safety assessment and regulation', ed. by I. Sundh, A. Wilcks and M.S. Goettel. CABI.

Loi A., Nutt J., Howieson J.G., Yates R. and Norman H.C. 2012. Preliminary assessment of bladder clover (*Trifolium spumosumn* L.) as an annual legume for ley farming systems in southern Australia. Crop and Pasture Science 63, 582–591.

Sprent J.I., Ardley J.K. and James E.K. 2013. From North to South: a latitudinal look at legume nodulation processes. South African Journal of Botany 89, 31–41.

Terpolilli J.T., O'Hara G.W., Tiwari R.T., Dilworth M.J. and Howieson J.G. 2008. The model legume *Medicago truncatula* A17 is poorly matched for N2 fixation with the sequenced microsymbiont *Sinorhizobium meliloti* 1021. New Phytologist 179, 62–66.

CHAPTER 3
Isolation and growth of rhizobia

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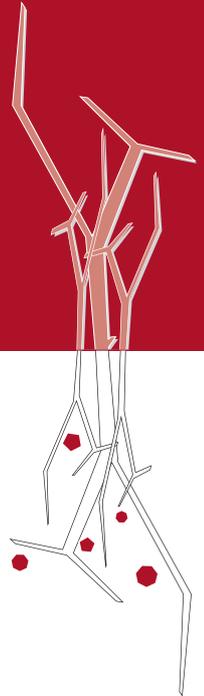
3.1 Introduction

This chapter describes basic techniques for the isolation and growth of rhizobia, some of which have been used for more than a century. While these techniques retain their importance, the success of current and future rhizobiology studies and enterprises will depend on the training, skills and techniques described in this chapter. A note of caution: nodules (particularly those collected from the field) are not always occupied by a single rhizobial isolate nor even by a single micro-organism. Nodules of pea and lupin, for example, have been described as containing both the nitrogen-fixing symbiont and associative organisms such as *Micromonospora* (Trujillo *et al*. 2010). Hence, we must be prepared for a range of organisms to appear on growth plates during isolation procedures. Recognition of rhizobia when growing on a solid medium is an essential skill in rhizobiology.

3.2 Preparing solid growth media

A wide range of growth media are available for rhizobia and these are listed in Section 3.7 of this chapter. For isolation from nodules, it is sufficient to choose one of the routine (undefined) media such as YMA or ½ LA as these are inexpensive, simple to prepare and able to support a broad range of nodule bacteria. It is recommended that isolations be made upon solid media as contaminants are more readily discernible than in liquid media. A further decision should be made as to incorporation of Congo Red dye, which is readily adsorbed by Gram positive bacteria, but also some nodule bacteria (see Section 3.8.1).

3.3 Preparing the nodules

1. Nodules may have been either stored desiccated or still attached to the legume roots in the cold room as described in Chapter 2. When attached to roots, these should be washed free of soil then nodules excised from the root (leaving a small root section attached) and left in water until ready for isolation (within 12 hours).

2. Desiccated nodules should be re-hydrated by immersion in DI water for three to four hours in a labeled Petri dish. Best results have been obtained with desiccated nodules stored for less than six months.

3.4 Isolating bacteria from nodules

The nodules must be surface sterilised to remove as many contaminants as possible because it is difficult to discern rhizobia growing on a plate if there are many other bacterial contaminants present. A convenient technique to transfer the nodule through the sterilising then rinsing solutions is to use forceps locked onto the small piece of adjoining root. If many nodules are to be worked with, then they can be held together in a tea strainer (Figure 3.1) for bulk immersion in the solutions.

1. Prepare sterilising solutions of 70% (v/v) ethanol and 4% (v/v) sodium hypochlorite, and six changes of sterile water in vessels that can accommodate the forceps (*e.g.* 20 mL) or tea strainer (100 mL). If working with many nodules (>20) then prepare a second set of rinsing solutions which can be changed to decrease the chances of transferring contaminants. Tween 80 (10 µL/L) can be added to the sodium hypochlorite solution as a wetting agent. *NB:* commercial hypochlorite can vary in sodium hypochlorite concentration between 2 and 6% (w/v).

2. Surface sterilise nodules to remove microorganisms on the surface by immersing them for one minute in 70% (v/v) ethanol, followed by up to three minutes in sodium hypochlorite. Nodules vary in their resilience to surface sterilisation and it may be necessary to vary this treatment. For example, to sterilise the surface of *Arachis* nodules, we immerse in ethanol for only 10 seconds and replace sodium hypochlorite with 3% (v/v) hydrogen peroxide.

3. Carefully rinse the nodules six times in sterile water by transferring the tea strainer or locked forceps from vessel to vessel, ensuring the solution is drained from the strainer each time.

4. From the last rinse, aseptically crush individual nodules with blunt-nosed forceps held directly over the growth medium, allowing the contents to drop onto the plate. Alternatively, the nodule can be macerated into a drop of sterile

water in the lid of a Petri dish with the flattened end of a flamed glass rod or sterile wooden stick.

5. Using a new plate for each nodule, streak the drop, or take a loopful of the macerate and aseptically streak onto plates containing an appropriate growth medium. Use a dilution streaking pattern to isolate single colonies (Figure 3.2).

6. Incubate the inoculated plates at 28°C and check every 24 hours to observe growth of the rhizobia and contaminant bacteria. Young colonies of most species of rhizobia are translucent when young (Figure 3.3).

7. A high percentage of isolations will result in mixed cultures (Figure 3.3) and care will be needed to identify and purify rhizobia.

8. Select single colonies that resemble rhizobia for subculture and then purify by touching the edge of the loop to the colony then streak on a fresh plate.

9. At this point, a decision must be made as to how many cultures from a nodule should be progressed. If more than one, select colony types that have different morphologies and re-streak.

10. After the single colony subcultures have grown, store them for the short term at −80°C in a glycerol medium (see Chapter 4, Section 4.5). A RAPD PCR or a partial 16S rRNA sequence can later assist their differentiation (Chapters 11 and 12). To expedite this, take one loopful of the pure culture and place it into 0.1 mL sterile 0.89% (w/v) saline, then refrigerate for later creation of the DNA template.

Figure 3.1   A tea strainer can be used to transfer hydrated nodules between sterilizing agents

Figure 3.2 A dilution streaking pattern for isolation of single colonies. This pattern would suit a left-handed person. The inoculating loop should be flamed when starting each new set of strokes to ensure adequate dilution of the culture and development of well-separated colonies.
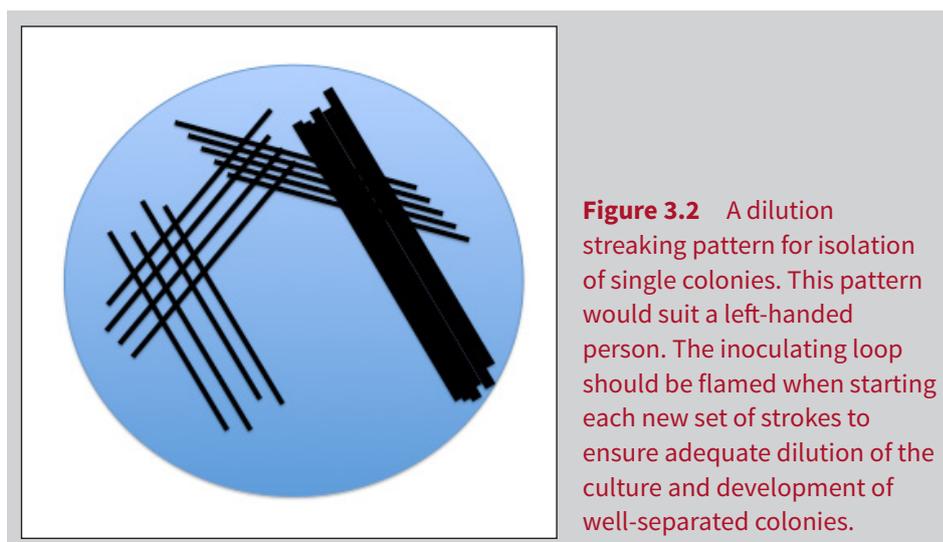
3.4.1 Isolation from damaged nodules

It is difficult to obtain cultures from a damaged nodule because the sterilants can enter the nodule tissue and kill the rhizobia. If the nodule contains a potentially valuable strain, we suggest macerating the nodule in a drop of sterile water and then inoculating onto a seedling growing under aseptic conditions, as described in Chapter 2, Section 2.8. Of course, this approach cannot preclude rhizobia on the outside of the damaged nodule from forming any new nodules on the trap plants.

3.5 Recognising nodule bacteria growing on solid media

Rhizobia are generally slightly raised (although *Burkholderia* and *Bradyrhizobium elkanii* are nearly flat) and with entire margins, but the growth rates of the major genera differ substantially. More details of the physiology of rhizobia are given in Chapter 7, and their appearance in Table 7.3, but here we briefly cover growth rates and colonial morphology to aid in selection of the correct colonies from the isolation exercise.

The recognised factors affecting the growth rate of a rhizobial species are culture medium, pH and temperature. On ½ LA (see Section 3.7.2) at 28°C we expect to see growth from nodule squashes begin within:

► one to two days for very fast growers, such as *Burkholderia* and *Cupriavidus*

► two days for *Microvirga*

► two to four days for classic fast growers, such as *Rhizobium* and *Sinorhizobium*

► three to five days for *Mesorhizobium*

▶ six to 14 days for *Bradyrhizobium*

Helpful notes

▶ Growth rates from a nodule are generally slower than from subcultures, presumably as the bacterial metabolism for growth on artificial media has to be expressed.

▶ Nearly all colonies of nodule bacteria are translucent when first visible and viewed with a dissecting microscopic with light from below (Figure 3.3). If plates are viewed regularly, dominant patches of translucent bacterial growth can be identified and marked with a felt-tipped pen.
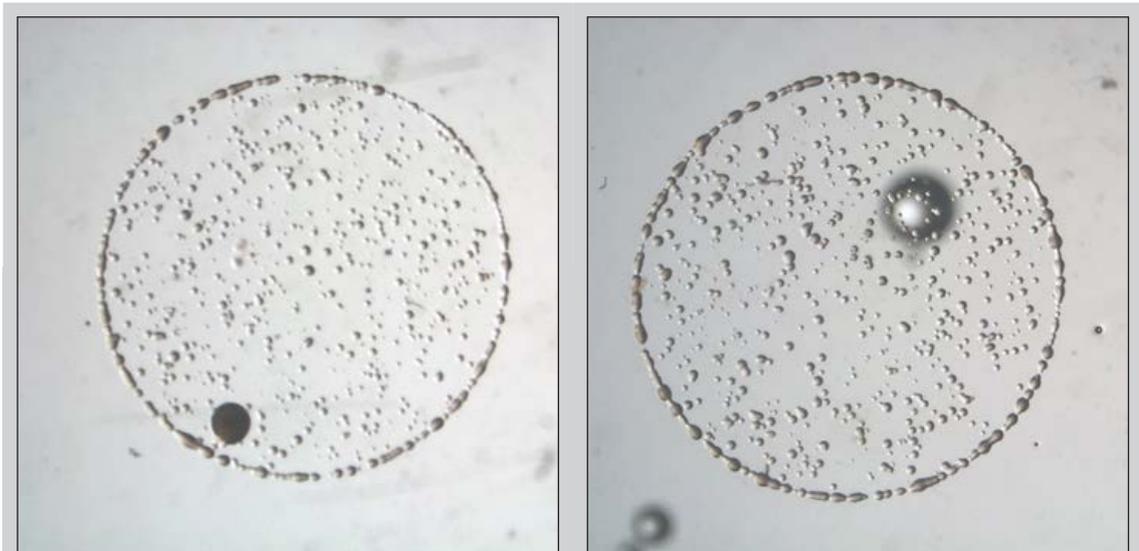
Figure 3.3 Young colonies appear translucent for most nodule bacteria; bradyrhizobium 7d (left), rhizobium 2d (right).

More often than not, a wide diversity of colony types will grow on the isolation plates, and it is very helpful in diagnosis of the colonies to record growth every 24 hours and to circle colonies that appear in each 24-hour period. Figure 3.4 illustrates a range of common nodule bacteria growing on a medium containing Congo Red. Colonies that are not rhizobia tend to absorb the dye strongly.
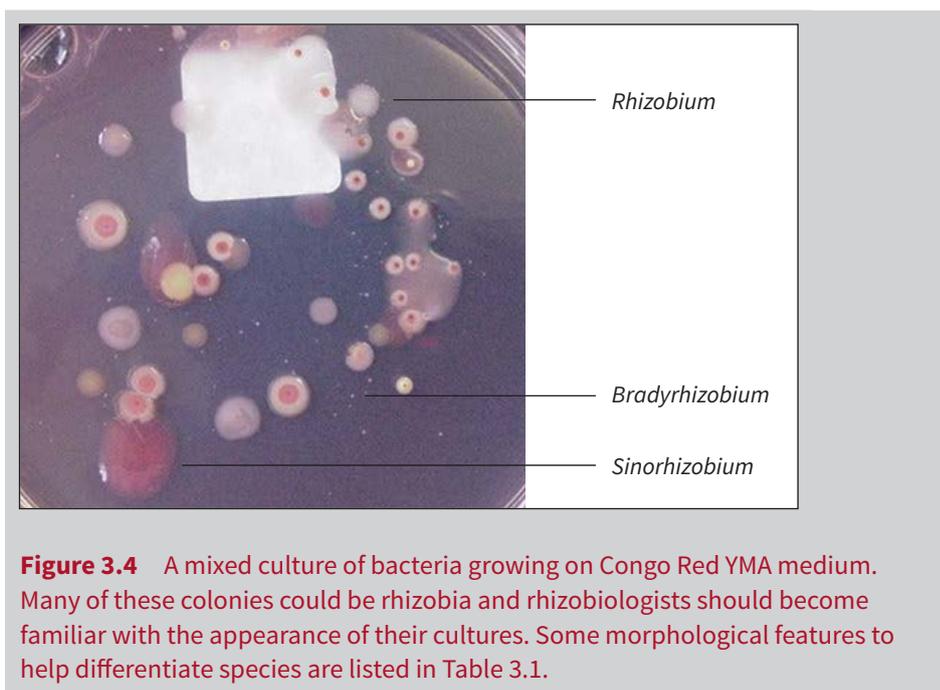
Figure 3.4 A mixed culture of bacteria growing on Congo Red YMA medium. Many of these colonies could be rhizobia and rhizobiologists should become familiar with the appearance of their cultures. Some morphological features to help differentiate species are listed in Table 3.1.

3.5.1 Viewing colonies through a dissecting microscope

It is helpful to visualise bacteria growing on solid medium with the aid of a dissecting microscope. With light from below, and with low magnification, an experienced microbiologist can become familiar with the subtle differences between the major genera in shape and hue of the colonies. *Burkholderia* and *Cupriavidus* often have a light yellow-brown hue in early growth, but this becomes more opaque with age (Figure 3.5). *Burkholderia sprentiae* has less of a brown tinge than *Burkholderia dilworthii* and grows faster. The beta-rhizobia can have colonies whose margins are slightly irregular.
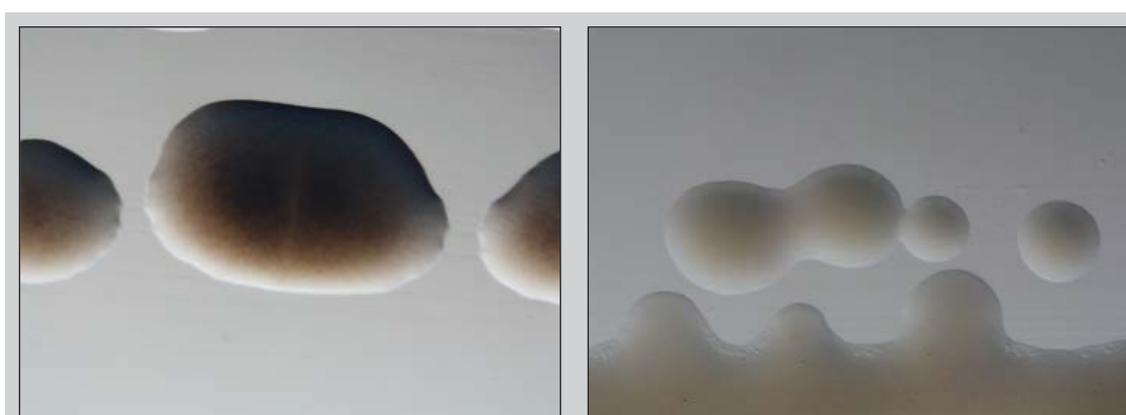
Figure 3.5 Colonies of *Burkholderia* after 24 hour growth on ½ LA. *Burkholderia dilworthii* (left) and *B. sprentiae* (right), as described in De Meyer *et al.* (2013). Note the brownish tinge of *B. dilworthii* when viewed with light from below, and very slightly irregular margins.

Sinorhizobium medicae nearly always produces colonies with a "doughnut" morphology when isolated directly from nodules, but this may disappear after prolonged subculture. Figure 3.6 illustrates the different colonial morphology of *S. medicae* compared with *S. meliloti* when grown on ½ LA medium.

Other photographs of colonies of different species of rhizobia can be accessed at the Centre for *Rhizobium* Studies (CRS) website: www.crs.murdoch.edu.au/
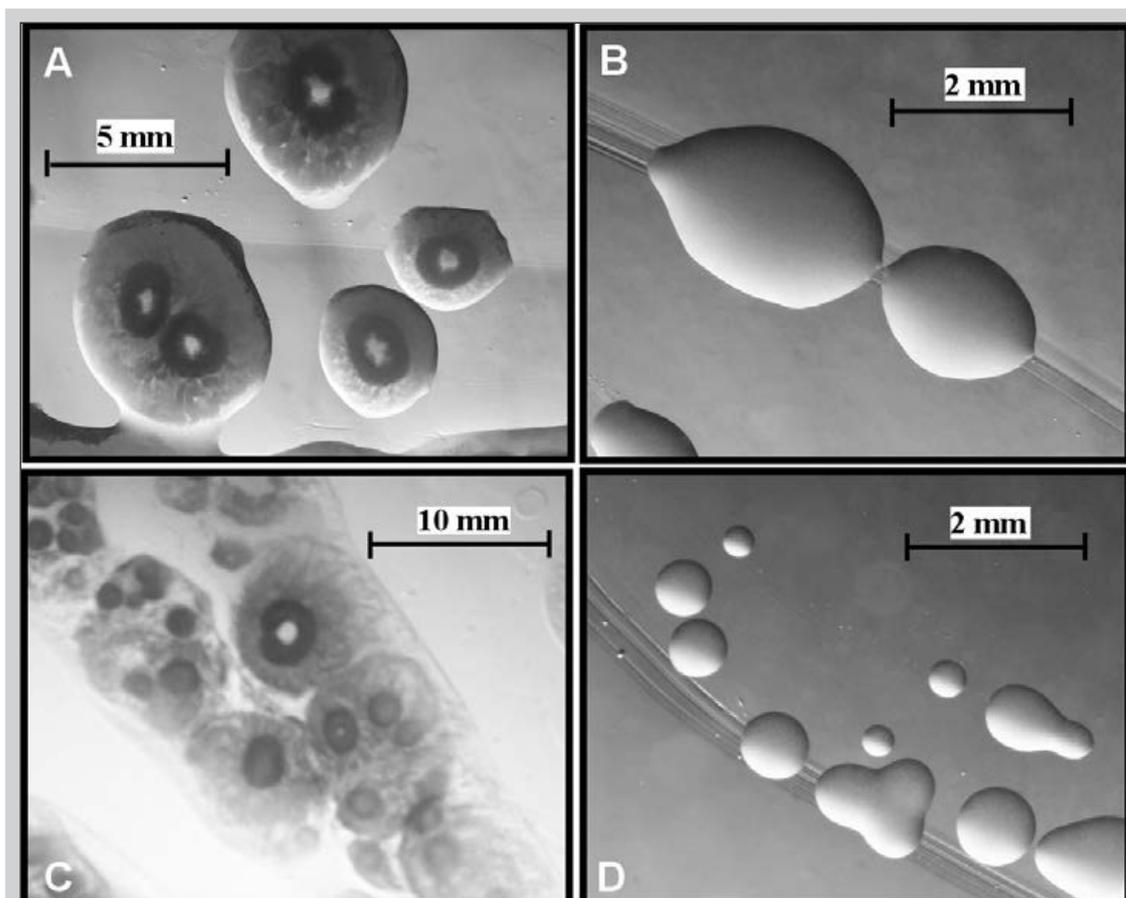
Figure 3.6 *S. medicae* (A, C) and *S. meliloti* (B, D) colonial morphology and gum production when grown on ½ LA medium and lit from below with an incandescent globe. The morphology of *S. medicae* is referred to as doughnut (Chatel DL pers. comm.)

3.5.2 Pigmented rhizobia

Root nodule bacteria are rarely pigmented but some specific exceptions have emerged. *Methylobacterium* isolates from *Listia bainesii* (formerly *Lotononis bainesii*) produce red-pigmented colonies (Norris 1958; Godfrey 1972; Kleinig and Broughton 1982) (Figure 3.7). *Microvirga*, a recently identified genus of nodulating bacteria, may produce pink or light orange colonies after 48 hour incubation on ½ LA (Ardley *et al.* 2011) (Figure 3.7).

Figure 3.7   Red colonies of *Methylobacterium* spp. from nodules of *Listia bainesii* (left) and pink-orange colonies of *Microvirga* spp. from nodules of *Listia angolensis* (right)

Pigmented colonies (as for very rapidly growing colonies) should not automatically be discarded.

3.6 Some useful diagnostic features of species of nodule bacteria

The following table lists diagnostic features of various nodule bacteria, (based on long experience with them) which can assist in the identification of isolates from nodules. All colonies are non-pigmented, convex (or elevated) and with entire margins unless stated otherwise.

Table 3.1 Some features of the common nodule bacteria that aid in working with them. This table will be regularly updated at the CRS website: www.crs.murdoch.edu.au/

Species	Features
Bradyrhizobium spp.	Colonies slow growing and translucent at first appearance when less than 1 mm in diameter (approx. six to ten days) but then strongly opaque and tending to dark grey with further maturity. Rarely gummy.
Bradyrhizobium japonicum	As above, but may reach 1–3 mm after five to eight days, immediately opaque and elevated.
Bradyrhizobium elkanii	As above, colonies irregular and non-elevated.
Burkholderia spp.	Colonies visible in 24–36 hours, flat, margin regular except *B. dilworthii*, colonies may acquire a yellow-brown tinge after 48 hours when viewed with light from below (see Figures. 3.5, 3.11a and 3.11b).
Burkholderia sprentiae	As above, but colonies more grey and opaque than brown-yellow, and more raised (see Figures 3.5 and 3.10). Growth slightly faster that *B. dilworthii.*
Cupriavidus spp.	Colonies visible in 24 hours, flat, margin not entire, grey tinge when viewed with light from below.
Mesorhizobium spp.	Colonies as for *Rhizobium* but visible 24 h later, weakly opaque after 72 h, increasing with age.
Methylobacterium	Colonies visible in four to seven days. Strongly opaque and not gummy.
Microvirga lotononidis (from *Listia angolensis*)	Colonies visible in 24 h; light pink develops 48–72 h later (Figure 3.7), weakly opaque and mildly gummy. Optimal growth at 41°C.
Microvirga zambiensis (from *Listia angolensis*)	Colonies visible in 24 h. Weakly opaque and mildly gummy. Optimal growth at 35°C.
Microvirga texensis (from *Listia angolensis*)	Colonies visible in 24 h. Light orange coloration develops 72 h later, opaque and not gummy.
Methylobacterium spp. (from *Listia* spp. other than *L. angolensis*)	Colonies visible in four to seven days. Colonies develop strong pink pigmentation within 24 h of becoming visible (Figure 3.7).
Rhizobium leguminosarum bv. *trifolii*	Colonies visible in 48 hours. Weakly opaque at 72 hours. Gummy strands adhere to loop.
Rhizobium leguminosarum bv. *viceae*	Colonies visible in 48 hours. Weakly opaque at 72 hours. Gummy strands generally do not adhere to loop but variation evident according to host genus.
Sinorhizobium medicae	Colonies visible in 48 hours. Doughnut morphology when first isolated from nodules (Figure 3.6A and 3.6C), weakly opaque after 72 hours and gummy.
Sinorhizobium meliloti	Colonies visible in 48 hours. Do not form doughnut colonies, weakly opaque after 72 hours (Figures. 3.6B and 3.6D) often (but not always) less gum than *S. medicae*.

3.7 Routine culture media for rhizobia

Of a variety of media described in the literature for growing rhizobia, only the most commonly used will be listed here. Specific strains or species may require modifications to each medium.

The culture media can be classified as undefined or defined. The composition of undefined media is not completely known, for example when a generic yeast extract is added to provide vitamins. Defined media are completely chemically specified, and are used for growing rhizobia with specific nutrient requirements, or to determine metabolic behaviour and growth products. Many molecular studies utilise generic bacterial media such as TY (3.7.2.3) for growing rhizobia, as these have been developed over the last several decades, but they tend to distort the unique colonial morphology of individual species.

3.7.1 pH Control

Bacterial growth on either liquid or solid medium can result in large and rapid changes in pH as a result of metabolism (see Chapter 7, Section 7.2.6). In general, growth on sugars results in acidification, while growth on organic acids or amino acids results in alkalinisation (Figure 3.8). Where a constant pH is important, for example when acid-tolerant bacteria are being evaluated, addition of an appropriate non-toxic buffer is essential to maintain pH. The buffer should be selected so that the desired pH for the medium is within 0.7 pH unit of the pK_a listed for the buffer (see Table 3.2). Growth in a medium with the buffer at the desired concentration (usually 20–40 mM) needs to be as good as that in its absence, otherwise the buffer is having an effect on bacterial growth outside of effects upon pH control. This usually means testing growth at a range of buffer concentrations at normal pH. For many rhizobia, growth rate decreases as pH decreases below 6.5, as shown in Figure 3.9.
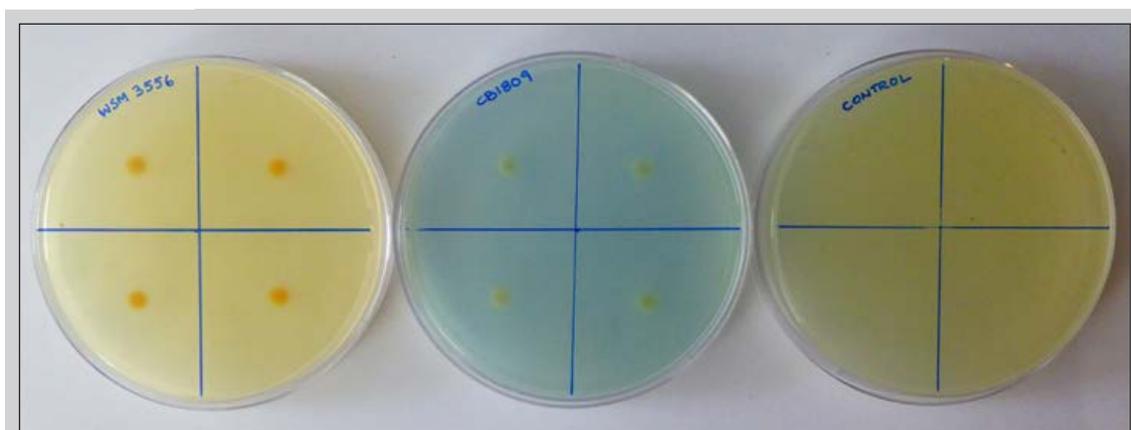
Figure 3.8 The pH change on unbuffered ½ LA medium (with bromthymol blue indicator) caused by rhizobial metabolism. The control medium is light green (far right). Colonies spotted onto the plates in four replicates have acidified the medium, turning it from light green to yellow (far left WSM3556) or have caused an alkaline reaction (central plate CB1809) turning the indicator blue. The pH change has permeated the whole plate, but it is strongest under the colonies themselves. Species are *Burkholderia dilworthii* WSM3556 and *Bradyrhizobium japonicum* CB1809.
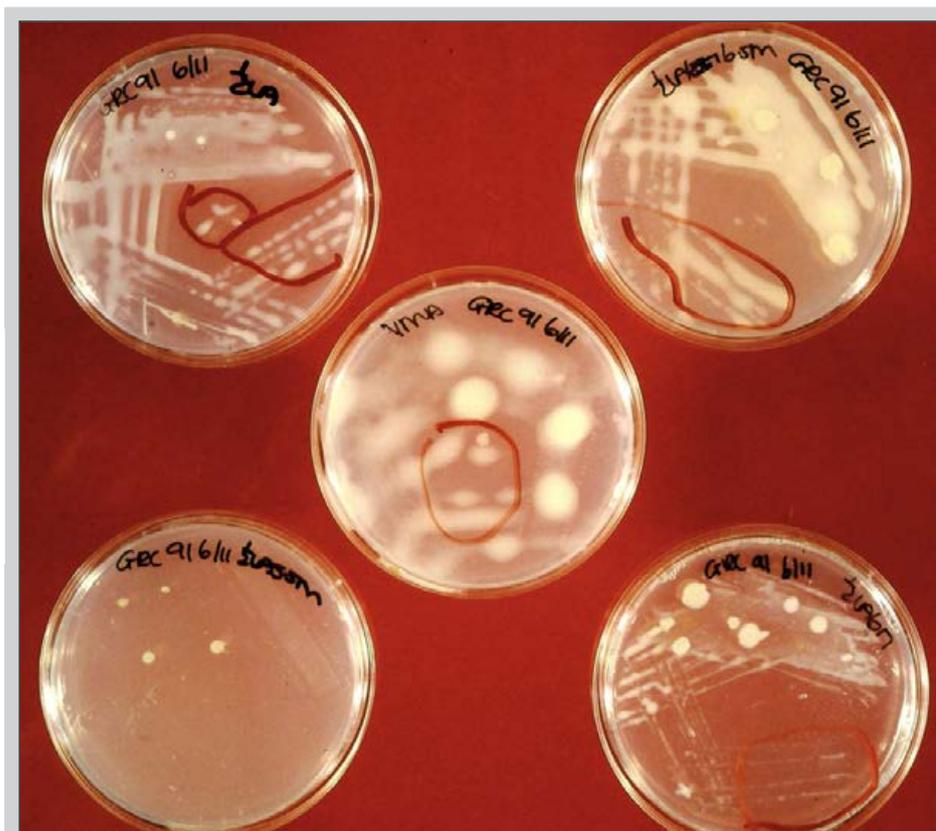
Figure 3.9 *S. medicae* grows well at pH 7 (top left) and at pH 6.5 in MES buffered media (top right) but decreases at pH 6 (bottom right) and barely grows at pH 5.5 (bottom left). Growth on YMA at pH 7 (middle plate) is gummier than on ½ LA.

Table 3.2 Buffers used in rhizobial growth media and their pK_a

Chemical name	Abbreviation	pK_a at 25°C
3-(Cyclohexylamino)-2-hydroxy-1-propansulfonic acid	CAPSO	9.6
N-(2-hydroxyethyl)piperazine-N'-(2-ethansulfonic acid)	HEPES	7.5
2-N-Morpholino-ethansulfonic acid	MES	6.1
Homopiperazine-1,4-*bis*(2-ethansulfonic acid)	HOMOPIPES	4.55

3.7.2 Undefined media

3.7.2.1 Yeast mannitol agar

The universal culture medium used to grow rhizobia in laboratories worldwide is YMA, as described by Fred and Waksman (1928) and Vincent (1970) with slight modifications through the decades.

Component	Quantity/L
K_2HPO_4	0.5 g
$MgSO_4.7H_2O$	0.2 g
NaCl	0.1 g
Mannitol[1]	5 g
Yeast extract[2]	0.4 g
Distilled water to complete 1 L	
Agar	12–15 g

For liquid medium (YMB), omit the agar. The pH should be adjusted to 6.5 to 6.8 before autoclaving and before addition of agar. Autoclave at 121°C for 15 minutes.

[1] The original recipe calls for 10 g of mannitol, but growth of the great majority of rhizobia is adequate with only 4 g or 5 g. Other cheaper or preferred C-compounds may be used.

[2] Can be substituted by the addition of 100 mL of yeast water, prepared from 100 g of bakers´ compressed yeast mixed with 1 L of cold water, allowed to stand at room temperature for one to two hours, autoclaved for 40 to 60 minutes at 121°C, allowed to settle (or centrifuged) and the clear supernatant adjusted to pH 6–8 (Vincent 1970).

3.7.2.2 ½ LA (Howieson and Ewing 1986)

This is a useful medium for isolation from nodules that allows differential colonial morphology of the different species to be expressed. The concentration of P is low by normal standards and serves to reduce culture gumminess in genera such as *Sinorhizobium* (Figure 3.9). However, some rhizobial species (*e.g.* those that nodulate *Hedysarum spinosissimum*) require a fivefold increase in P concentration to grow on this medium.

Component	Quantity/L
D-glucose	5 g
Mannitol	5 g
$MgSO_4.7H_2O$	0.8 g
NaCl	0.1 g
Yeast extract	1.25 g
$CaCl_2.2H_2O$	0.2 g
Agar	12–15 g

Liquid solutions*	Final quantity/L
K_2HPO_4	0.16 mg
KH_2PO_4	0.13 mg
$FeSO_4.7H_2O$	0.1mg

*make stocks at 50× concentration

Trace elements solution**	
$Na_2B_4O_7.10H_2O$	2.34 mg
$MnSO_4.4H_2O$	2.03 mg
$ZnSO_4.7H_2O$	0.22 mg
$CuSO_4.5H_2O$	0.08 mg
$Na_2MoO_4.2H_2O$	0.126 mg

**make stock at 1000× concentration

Weigh out components (except agar) and add into 800 mL of distilled water while stirring.

To this, add the three liquid stock solutions made at 50-fold higher concentration (20 mL of K_2HPO_4, 20 mL of KH_2PO_4, 10 mL of $FeSO_4$) and 1 mL of trace element stock (made at 1000× concentration). Adjust the pH to 6.8 using 0.1 M NaOH. Add agar if required and make volume up to 1 L before autoclaving at 121°C for 15 minutes.

3.7.2.3 Tryptone yeast extract—TY
(for molecular studies, Beringer 1974)

Component	Quantity
Tryptone	5.0 g
Yeast extract	3.0 g
$CaCl_2.6H_2O$ (or $2H_2O$)	1.3 g (0.87 g)
Distilled water to complete 1 L	
pH adjusted to 6.8–7.0	

3.7.2.4 Peptone (Hirsch *et al*. 1980)

Component	Quantity
Peptone	4.0 g
$MgSO_4.7H_2O$	0.5 g
Distilled water to complete 1 L	
pH adjusted to 6.8–7.0	

3.7.2.5 Luria-Bertani (Hirsch *et al*. 1980)

Component	Quantity
Tryptone	10.0 g
Yeast extract	5.0 g
NaCl	3.0 g
Distilled water to complete 1 L	
pH adjusted to 7.5	

3.7.2.6 Sucrose glucose broth (for fast-growing rhizobia, Date and Halliday 1979)

Component	Quantity
K_2HPO_4	0.75 g
$MgSO_4.7H_2O$	0.4 g
$CaCO_3$	0.4 g
Sucrose	2.5 g
Glucose	2.5 g
Yeast extract	3.0 g
Distilled water to complete 1 L	

3.7.2.7 Glycerol broth (for slow-growing rhizobia, Date and Halliday 1979)

Component	Quantity
K_2HPO_4	0.5 g
$(NH_4)_2HPO_4$	0.3 g
KNO_3	0.8 g
$MgSO_4.7H_2O$	0.2 g
Yeast extract	4.0 g
$MnSO_4$	0.1 g
$FeCl_3$	0.1 g
Glycerol	10 g
Distilled water to complete 1 L	

3.7.3 Defined media

3.7.3.1 BSM (modified from Bergersen 1961)

Component	Quantity
Mannitol	10 g
$Na_2HPO_4.12H_2O$	0.45 g
$MgSO_4.7H_2O$	0.1 g
Fe solution[2]	0.6 mL
Glutamic acid	1.1 g
Thiamine solution[1]	1 mL
Biotin solution[1]	1 mL
Distilled water to complete 1 L	
pH adjusted to 7	

[1] Vitamin stock solutions (1 mg/mL) should be prepared in water separately, filter sterilised through a 0.2 μm Millipore membrane and mixed when the medium is at a temperature of 45–55°C).

[2] 100 mL of distilled water, 0.67 g $FeCl_3.6H_2O$, 0.42 mL conc. HCl.

3.7.3.2 Defined medium (Brown and Dilworth 1975)

Component	Quantity/L
Glucose	2.5 g
KH_2PO_4[1]	0.36 g
K_2HPO_4[1]	1.4 g
N-source[2]	0.7 g
$MgSO_4 \cdot 7H_2O$	0.25 g
$CaCl_2.2H_2O$	0.02 g
NaCl	0.2 g
Liquid stocks[3]	Final quantity/L
$FeCl_3$	6.6 mg
EDTA	0.15 mg
$ZnSO_4.7H_2O$	0.16 mg
$Na_2MoO_4.2H_2O$	0.2 mg
H_3BO_3	0.25 mg
$MnSO_4.4H_2O$	0.2 mg
$CuSO_4.5H_2O$	0.02 mg
$CoCl_2.6H_2O$	1.0 µg
Vitamins	Final quantity/L
Thiamine-HCl	1 mg
Ca pantothenate	2 mg
Biotin	1 µg
Distilled water to complete 1 L	
pH adjusted to 7.0	

[1] Phosphates are sterilised separately to avoid precipitation.
[2] N source can be either KNO_3 (0.7 g), NH_4Cl (0.7 g) or L-glutamate (1.0 g).
[3] Make stock solutions at 1000× concentration and then add 1 mL per L of medium.

3.7.3.3 JMM—a minimal defined medium (O'Hara *et al*. 1989)

Component	Quantity/L
D-galactose	1.8 g
L-arabinose	1.5 g
L-glutamate	0.51 g
$MgSO_4.7H_2O$	0.25 g
$CaCl_2.2H_2O$	0.15 g
Na_2SO_4	0.1 g
Liquid stocks[1]	Final quantity/L
$FeSO_4.7H_2O$	5.5 mg
$ZnSO_4.7H_2O$	1.1 mg
$Na_2MoO_4.2H_2O$	1.0 mg
$MnSO_4.4H_2O$	1.1 mg
$CuSO_4.5H_2O$	0.5 mg
KH_2PO_4[2]	0.22 g
K_2HPO_4[2]	0.26 g
Biotin[2]	20 µg
Thiamine-HCl[2]	1 mg
Ca pantothenate[2]	1 mg
Distilled water to complete 1 L	

[1] These liquid stocks are made up at 1000× concentration.
[2] The phosphates and vitamin liquid stocks are prepared as separate solutions, filter sterilised and added to the cooled medium after autoclaving to give the required final concentration.

Buffers: Depending on the desired pH an appropriate buffer (HEPES, MES, HOMOPIPES, CAPSO) is added at 20–40 mM (see Section 3.7.1)

Adjust pH to required level. Autoclaving does not usually alter the pH of buffered JMM medium but it can significantly alter the pH of unbuffered JMM medium.

Add 12–15 g/L of agar for solid medium.

All media described in this chapter should be autoclaved at 121°C at 1 atm pressure for 20 minutes. Ideally, wait at least two days before using any medium to be sure that the sterilisation process was successful.

3.8 Antibiotics and indicators

Antibiotics and indicators can be very useful for avoiding contaminants and for helping in the authentication of strains. The antibiotics can be added through a sterile filter from stock solutions after autoclaving the medium, while the dyes can generally be added before autoclaving.

3.8.1 Preparation

Actidione (cycloheximide) can be used to suppress fungal growth. For the stock solution, add 25 mg of cycloheximide in 300 µL of ethanol. Add 200 µL of the stock solution per 300 mL of culture medium (final concentration, 55 mg/L).

Vancomycin is useful for inhibiting growth of Gram-positive bacteria. For the stock solution, dissolve 9 mg of vancomycin chloride in 3 mL of distilled water. Add 100 µL of stock solution per 300 mL of culture medium (final concentration, 1 mg/L).

Congo Red is an indicator that may be very useful for differentiating rhizobia from contaminants, especially Gram-positive bacteria, as nodule bacteria do not tend to adsorb the dye. Thus, it is a useful addition to media for isolation of rhizobia from nodules, where contaminants are common. Routine addition of Congo Red to media is not recommended. Stock solution should be prepared by adding 0.25 g/100 mL. Add 10 mL of stock solution per L of culture medium (final concentration: 25 mg/L) immediately before autoclaving.

Some nodule bacteria, such as *Sinorhizobium*, do adsorb Congo Red, and others will over time, so growth must be carefully monitored. Figure 3.10 shows a culture of *Burkholderia sprentiae* which adsorbs Congo Red (right plate) with white colonies on the left in the medium without Congo Red.
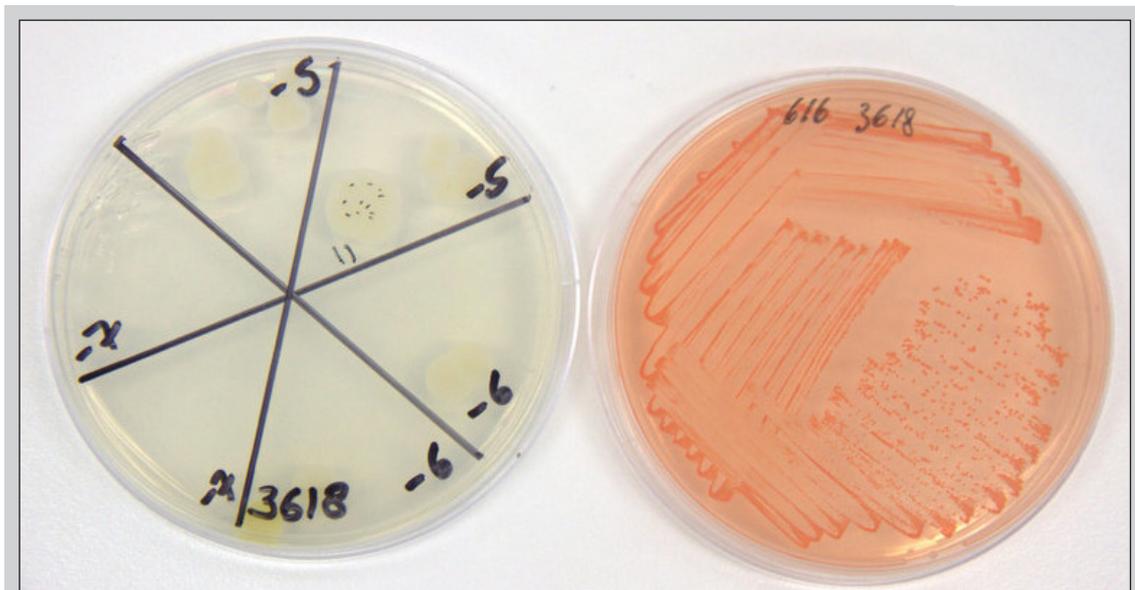
Figure 3.10   A culture of rhizobia (*Burkholderia sprentiae* strain WSM3618) on ½ LA medium with Congo Red (right) where colonies have absorbed the dye. Opaque colonies are seen on the ½ LA medium without Congo Red (left). See also Chapter 7.

Because rhizobial strains generally do not absorb Congo Red, the use of this dye has been a practical way of identifying contaminants on agar. However, the species of nodulating *Burkholderia* can be separated by their slightly differential adsorption of Congo Red and the reaction of the medium to their growth (Figure 3.11a and 3.11b)
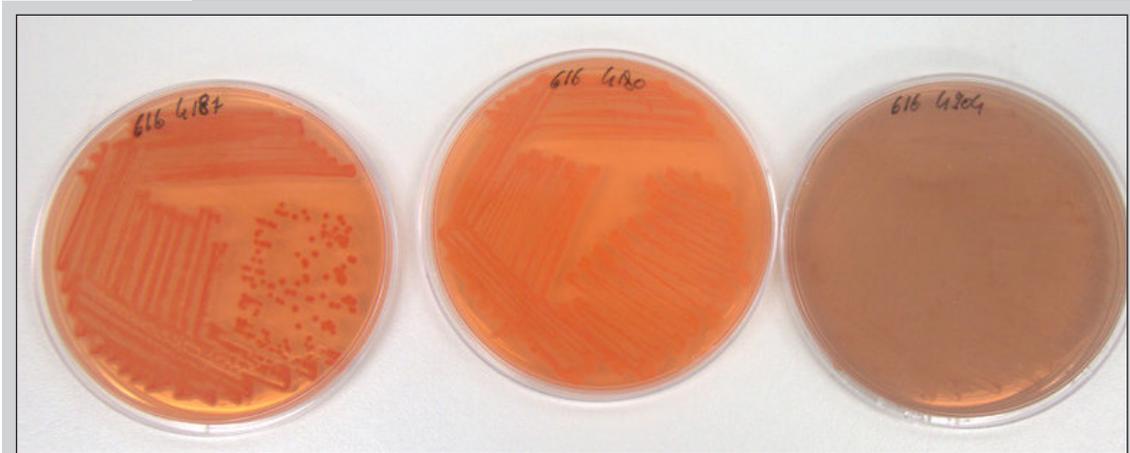
Figure 3.11a Variable reactions of *Burkholderia* spp. on ½ LA with Congo Red. *B. sprentiae* (left) adsorbs the dye strongly while *B. tuberum* (centre) less so. *B. dilworthii* (right) causes purpling of the medium and adsorbs the dye after approximately eight days. A close up of *B. tuberum* WSM4180 is reproduced below.

Figure 3.11b *B. tuberum* strain WSM4180 grown on ½ LA with Congo Red and showing the older growth which does not strongly adsorb the dye

Bromothymol blue is a broadly used indicator of acid or alkaline reaction in culture medium.

Prepare a stock solution of 0.5% (w/v) bromothymol blue in 0.2 M KOH. Add 5 mL/L of the stock solution to obtain a green coloration just before autoclaving. Figure 3.8 shows species of rhizobia giving acid and alkaline reactions when grown on ½ LA containing bromothymol blue.

Morphological characterisation should be undertaken with freshly grown cultures, *i.e.* after one to two days for the *Burkholderia*, three to five days for fast growers, six to nine days for slow growers and more than 10 days for extra-slow growers.

3.9 Avoiding contamination

Basic microbiological principles should be followed to exclude contaminants when pouring agar plates or inoculating them. The most important precautions are:

▶ rinse hands with ethanol or wear sterile gloves

▶ work in a laminar flow cabinet or adjacent to a flame

▶ flame inoculating loops and needles to red heat but allow to cool before use

▶ flame the necks of flasks, bottles and vials

▶ keep the room clean and free of dust

▶ avoid strong air currents, such as air conditioning fans.

The sterilisation performance of the autoclave should be checked periodically by commercially-available spore test strips (containing spores from *Bacillus*). Alternatively, plates or small volumes of broth cultures of sporulating bacteria (*Bacillus subtilis* is a useful species) can be placed at several points in the autoclave, to verify if adequate sterilisation is occurring.

Flow hoods facilitate microbiological work under sterile conditions but if the filters are not changed according to the manufacturer's recommendation they can represent a source of contaminants. The hoods should be cleaned with 70% (v/v) ethanol and if UV lights are fitted, they should be turned on for about 15–20 minutes, always screening the opening with paper, appropriate glass or a dark plastic to protect the operator against UV damage to the eyes. Hoods should be allowed to run for about 10 minutes before use and for about two to three minutes after each stage of manipulation so that aerosols can be flushed from the system.

Verification of air filtration in cabinets should be checked periodically by leaving exposed plates with culture media in several positions throughout the hood while in operation. Colony growth on media indicates the presence of contaminants in the air flow.

If access to a commercial hood is not possible, a cabinet can be built (Figure 3.12) or microbiological work can be carried out on a bench adjacent to a Bunsen burner where convection causes air to rise away from the work area.
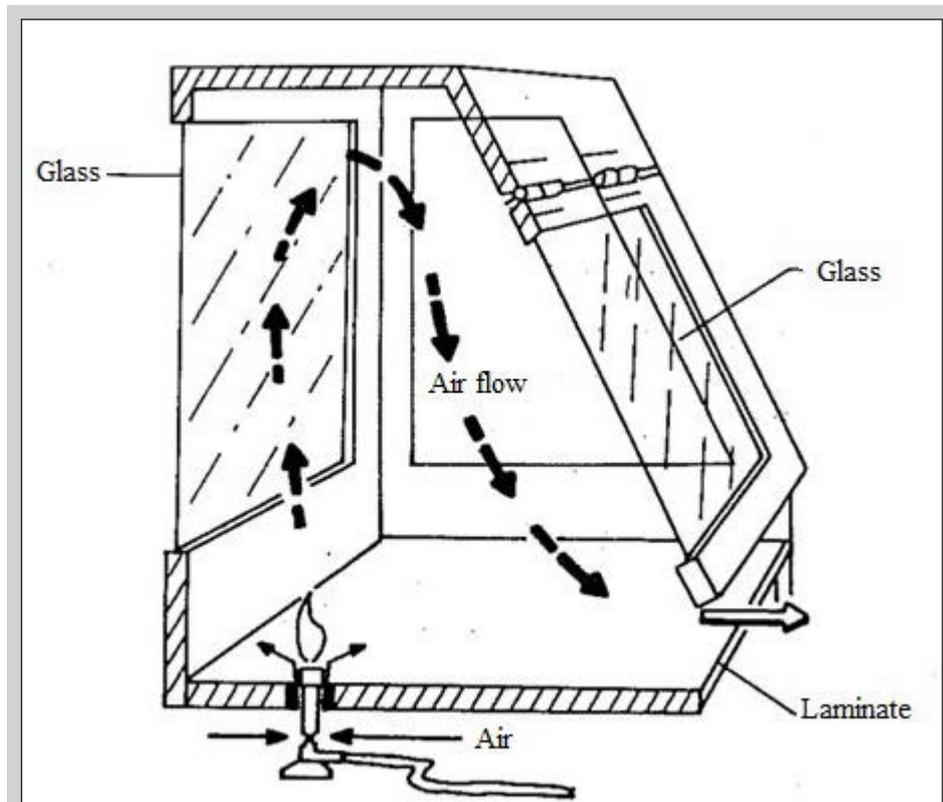
Figure 3.12 A cross sectional view of a laminar flow cabinet that is simple to construct and that protects microbiological work from contamination. Approximate dimensions are 1 m wide × 1.25 m high × 1.5 m long (from Somasegaran and Hoben 1994).

3.10 Authentication of isolates and Koch's postulates

Authentication is the term given to obtaining proof that the selected isolate is indeed a nodulating bacterium. There have been many examples where substantial research has been undertaken on bacteria whose origin is uncertain, and the authentication process avoids some of this confusion. However, non-nodulating variants of rhizobia can always emerge or some rhizobia can lose the symbiotic plasmid and stop nodulating. There is also increasing evidence of dual nodule occupancy, sometimes with bacteria that require assistance to enter the nodule. A recent example of this is the non-nodulating strains of *Burkholderia* associated with *Lebeckia ambigua* (Howieson *et al.* 2013).

Techniques for preparing plants for authentication are discussed in Chapter 5.

There is always a compromise between storing isolates permanently (to avoid prolonged subculture on media) while awaiting evidence that the cultures are nodule bacteria, and the time and resources required for acquiring this evidence. A useful approach when dealing with large numbers of strains is as follows.

▶ Keep a set of non-inoculated plants of the host legume always available, growing in vials or pots (Chapter 5) with supplied N to keep them healthy. Unusual colonies can be immediately applied to the legume and nodulation can be observed relatively rapidly.

▶ Be prepared to preserve unauthenticated material in glycerol vials for the medium term.

Methods for short-term preservation are detailed in Chapter 4, and for plant infection tests in Chapter 5.

3.10.1 Koch's postulates

The fundamental principles for isolation of rhizobia are the same as for the isolation of any infective bacterium (pathogenic or beneficial) as outlined by Koch (1884). Koch's postulates need to be fulfilled to provide the evidence that rhizobia have been isolated. The postulates recommend the following steps:

1. the infecting organism (rhizobia) should always be present in the host when the 'disease' (nodulation) occurs

2. the probable cause of the infection (rhizobia) must be isolated from the host (nodule) and grown in pure culture

3. the organism obtained in pure culture, when inoculated back on the host, must infect (nodulate) the host

4. the organism believed to be the cause of the infection (nodule) must be isolated again, grown in pure culture and compared with the initial isolate.

Since Koch wrote these guidelines, we have obtained a slightly wider view in the legume symbiosis.

▶ Pseudo nodules can arise as legume roots perceive 'nod factor' from rhizobia and in response form nodule-like growths. These nodules do not contain rhizobia and may thus be in conflict with step 1.

▶ Nodules may be crushed and the occupants subjected to a PCR-based identification technique, such as RAPD or ERIC that can provide convincing evidence of the identity of the strain in the nodule without the need to culture the organism. This is somewhat in conflict with step 3.

▶ Similarly, utilizing knowledge from whole genome sequences, a mass spectrometry profile of proteins through MALDI-TOF can identify some rhizobial species to strain level from a nodule crush. The same approach can give corroboration to the nodulating organism, as required in step 4.

A variety of techniques can add value to the authentication process, and most of these are discussed in this manual. Depending on the laboratory equipment and expertise available, these can range from a careful morpho-physiological characterisation through assay of serological properties to a variety of molecular

techniques, such as DNA analysis by rep-PCR (*e.g.* Menna *et al.* 2009) or sequence analysis. The full characterisation of strains is described in other chapters of this manual; morpho-physiology (Chapter 7) and genetic characterisation (Chapters 11 and 12).

Methods to infect legumes with the isolated nodule bacteria and to confirm nodulation are covered in Chapter 5.

3.11 References

Ardley J.K., Parker M.A., De Meyer S., Trengove R.D., O'Hara G.W., Reeve W.G., Yates R.J., Dilworth M.J., Willems A. and Howieson J.G. 2011. *Microvirga lupine* sp. nov., *Microvirga lotonoides* sp. nov. and *Microvirga zambesiensis* sp. nov. are Alphaprotobacterial root nodule bacteria that specifically nodulate and fix nitrogen with geographically and taxonomically separate legume hosts. International Journal of Systematic and Evolutionary Microbiology 62, 2579–2588.

Bergersen F.J. 1961. The growth of *Rhizobium* in synthetic medium. Australian Journal of Biological Sciences 14, 349–360.

Beringer J.E. 1974. R factor transfer in *Rhizobium leguminosarum*. Journal of General Microbiology 84, 188–198.

Brown C.M. and Dilworth M.J. 1975. Ammonia assimilation by *Rhizobium* cultures and bacteroids. Journal of General Microbiology 86, 39–48.

Date R.A. and Halliday J. 1979. Selecting Rhizobium for acid infertile soils of the tropics. Nature 277, 62–64.

Fred E.B. and Waksman S.A. 1928. Laboratory manual of general microbiology. McGraw-Hill Book Company: New York.

Godfrey C.A. 1972. The carotenoid pigment and deoxyribonucleic acid base ratio of a *Rhizobium* which nodulates *Lotononis bainesii* Baker. Journal of General Microbiology 72, 399–402.

Hirsch P.R., Van Montagu M., Johnston A.W.B., Brewin N.J. and Schell J. 1980. Physical identification of bacteriocinogenic, nodulation and other plasmids in strains of *Rhizobium leguminosarum*. Journal of General Microbiology 120, 403–412.

Howieson J.G. and Ewing M.A. 1986. Acid tolerance in the *Rhizobium meliloti – Medicago* symbiosis. Australian Journal of Agricultural Research 37, 55–64.

Howieson J.G., de Meyer S., Vivas-Marfisi A., Ratnayake S., Ardley J.K. and Yates R.J. 2013. Novel *Burkholderia* bacteria isolated from *Lebeckia ambigua*—a perennial suffruticose legume of the fynbos. Soil Biology and Biochemistry 60, 55–64.

Kleinig H. and Broughton W.J. 1982. Carotenoid pigments in a red strain of *Rhizobium* from *Lotononis bainesii* Baker. Archives of Microbiology 133, 164.

Koch R. 1884. Die Aetiologie der Tuberculose. Mittheilungen aus dem Kaiserlichen Gesundheitsamt 2, 1–88.

Menna P., Pereira A.A., Bangel E.V. and Hungria M. 2009. rep-PCR of tropical rhizobia for strain fingerprinting, biodiversity appraisal and as a taxonomic and phylogenetic tool. Symbiosis 48, 120–130.

De Meyer S.E., Cnockaert M., Ardley J.K., Maker G., Yates R.J., Howieson J.G. and Vandamme P. 2013. *Burkholderia sprentiae* sp. nov., isolated from *Lebeckia ambigua* root nodules. International Journal of Systematic and Evolutionary Microbiology 63, 3950–3957.

Norris D.O. 1958. A red strain of *Rhizobium* from *Lotononis bainesii* Baker. Australian Journal of Agricultural Research 9, 629–632.

O'Hara G.W., Goss T.J., Dilworth M.J. and Glenn A.R. 1989. Maintenance of intracellular pH and acid-tolerance in *Rhizobium meliloti*. Applied and Environmental Microbiology 55, 1870–1876.

Somasegaran P. and Hoben H. 1994. Handbook for rhizobia. Springer-Verlag: New York.

Trujillo M.E., Alonso-Vega P., Rodríguez R., Carro L., Cerda E., Alonso P. and Martínez-Molina E. 2010. The genus *Micromonospora* is widespread in legume root nodules: the example of *Lupinus angustifolius*. The ISME Journal 4, 1265–1281.

Vincent J.M. 1970. A manual for the practical study of root-nodule bacteria. (International Biological Programme Handbook 15). Blackwell: Oxford, U.K.

CHAPTER 4
Preservation of rhizobia

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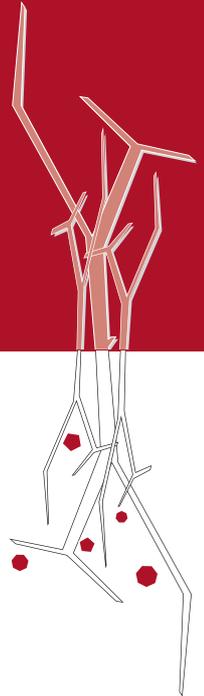
4.1 The need to preserve cultures

Experimentation with strains of rhizobia can last for many decades; hence there must be a reliable and efficient means of storing the bacteria. While many repositories of rhizobia have been developed since the symbiosis was scientifically understood, few remain available for exploitation. This is because strains were commonly stored on agar and remained the responsibility of an enthusiast, who may not necessarily have been replaced by his institution upon retirement. Agar slope-borne cultures have a relatively finite life. For this reason we recommend long-term preservation of valuable cultures lyophilized in glass tubes which will ensure survival over long periods of inattention.

4.2 Options for preservation

There is a trade-off, however, between the requirement for short-term storage after strains have been isolated from a nodule and passed through the purification process, and long-term storage to preserve the integrity of the strain. One way to manage this logistically is to have a short-term system based upon agar or glycerol storage (with strains labeled in a temporary code) and then long-term preservation after authentication (Chapter 5). Storage methods to keep rhizobia alive need to minimise the opportunity for variation or mutation, because strains may lose desirable properties during storage, or after repeated subculture. Careful maintenance of stock cultures, and periodic testing of their symbiotic efficiency, are recommended.

Several methods for short- and long-term storage are listed in Table 4.1, with notes on their characteristics. It is recommended that the survival of strains be examined earlier rather than later.

Table 4.1   Method of maintenance, main characteristics and cell viability related to each method (Hungria *et al*. 2005).

Method	Main characteristics	Viability
Agar slopes	Medium usually undefined, kept at 5–7°C for periodic transfer; simple and low-cost	1 year
Agar slopes	As above, covered with sterilised mineral or paraffin oil, kept at 5–7°C; simple and low-cost	2 years
Porcelain beads	Dry suspension of cells on sterilised porcelain beads, kept in a tube with dehydrated silica at 5–7°C	2 years
Soil, peat or clays	Preferably material with high water activity, ground, corrected for chemical properties and sterilised at 5–7°C.	2–4 years
Lyophilization	Viability depends on the physiological state of the culture, cell concentration, medium and lyophilization rate; can be kept at room temperature for decades, but little information is available	Several decades
Freezing	Storage in temperatures ranging from –70°C to –190°C in deep freeze or liquid nitrogen. Viability depends on the culture medium, freezing speed, freezing temperature and type of cryoprotectant used; good viability has been shown in a number of collections after 15–20 years.	Months to several years

4.2.1 Storage on agar slopes

1. Select preferred growth medium (Chapter 3).

2. Add 15–20 g agar per litre and stir vigorously with heating to dissolve the agar and ensure it is thoroughly mixed.

3. Dispense mixture into a screw-capped container to fill 33% of the volume (*i.e.* 10 mL into a 30 mL McCartney bottle or 1 mL into a 3 mL plastic vial).

4. Place vials into a rack. After autoclaving, rest the rack at an angle of 45–60 degrees until set.

5. Take a loopful of culture and streak across the surface of the agar slope; allow to grow until visible.

6. Store at 5–7°C.

7. Cover with sterile paraffin or mineral oil to decrease the rate of desiccation.

4.2.2 Storage on porcelain beads

1. Pack (autoclavable) vial to 30% of volume with desiccated silica gel covered by cotton wool, top with cleansed porcelain beads, screw cap on loosely then autoclave.

2. Grow culture in selected liquid medium (Chapter 3) to visible turbidity or wash from solid medium with diluent.

3. Add drops of culture to porcelain beads under sterile conditions, screw top tight and store at 5–7°C

4. When needed, one bead is removed and dropped into a broth medium. This can be allowed to grow or can be shaken then streaked on solid medium to check for purity.

4.3 Longer-term preservation

4.3.1 Lyophilization (vacuum drying)

The main objective of the lyophilization process is to preserve living organisms without modifications in their physiological, biochemical or genetic properties. The principle of the process is to remove moisture from the culture under a strong vacuum, with the evaporation of moisture producing a drop in temperature. The culture becomes desiccated but enough cells survive, protected by the low temperature and preserving medium. Cultures are then sealed in glass ampoules which maintain the very low vacuum. Several cryoprotectors have been employed including dimethylsulfoxide, glycerol, albumin, skim milk and peptone (Hubálek 2003; Day and Stacey 2007). We recommend a 50:50 mixture of 10% (w/v) peptone with 10% (w/v) Na-glutamate.

Glass ampoules are typically of the dimensions 4 mm × 50 mm, made of high-quality glass and open at one end (Figure 4.1).

4.3.1.1 Preparation of ampoules

1. Place a small swab of cotton wool at the bottom of the tube by pushing it down the tube with a wooden stick or the handle of an inoculating loop.

2. Prepare labels, written with a pen or printed on copy paper using a laser printer or photocopier, and indicating strain information, batch number and date. Place the label into the ampoules above the cotton wool.

3. Form a plug with non-absorbent cotton wool by rolling it tightly between the fingers, and place in the top of the tube. Do not push it too far down because it must be removed before inoculation. Alternatively, a cigarette filter may be used in place of the cotton wool plug.

4. Place prepared ampoules in a glass beaker or similar vessel and cover with aluminium foil prior to autoclaving.

5. Sterilise the ampoules at 121°C, 1 atm., for 30 minutes.
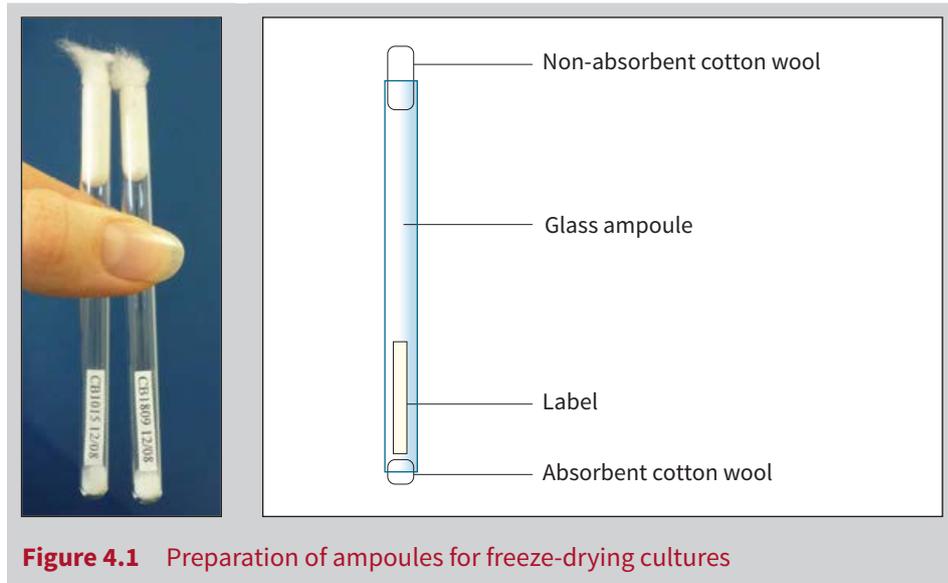
Figure 4.1 Preparation of ampoules for freeze-drying cultures

4.3.1.2 Preparation of the cultures

1. Grow the strain on the preferred medium (Chapter 3). We use a solid medium at this point to allow us to check for contamination.

2. All steps are to be performed under sterile conditions in a laminar flow cabinet. Dispense 2 mL of the lyophilisation mixture (*e.g.* a 50:50 mixture of 10% peptone and 10% Na-glutamate) into screw-topped vials and autoclave.

3. Take a loopful of the rhizobial strain and place it into the cooled lyophilisation mixture, replace lid and shake well or vortex. Alternatively, take 1 mL of a broth suspension of rhizobia and mix well into the lyophilisation medium.

4. Check the label before transferring the suspension to the ampoule.

5. Using a sterile Pasteur pipette, transfer 0.1 mL of suspension to the ampoule. We make 10 ampoules per strain, requiring 1–2 mL. It is important not to add too much fluid to each ampoule as this can lead to problems with removal of the liquid during lyophilisation. If less than 0.1 mL is added, the primary drying process (below) can be omitted.

6. Push the cotton wool plug halfway down the ampoule, below the nominated constriction point, to close the vial and protect the culture during the lyophilization process.

4.3.1.3 Primary drying process

1. Turn on the lyophilizer (Figure 4.2), introduce the ampoules and follow the manufacturer's instructions. The first drying stage (90% to 95% of dehydration) usually takes about 90 to 120 minutes in most lyophilizers.

2. Pressure at the end of primary drying should be approximately 13.3 Pascals.

Figure 4.2 Components of a standard lyophilizer
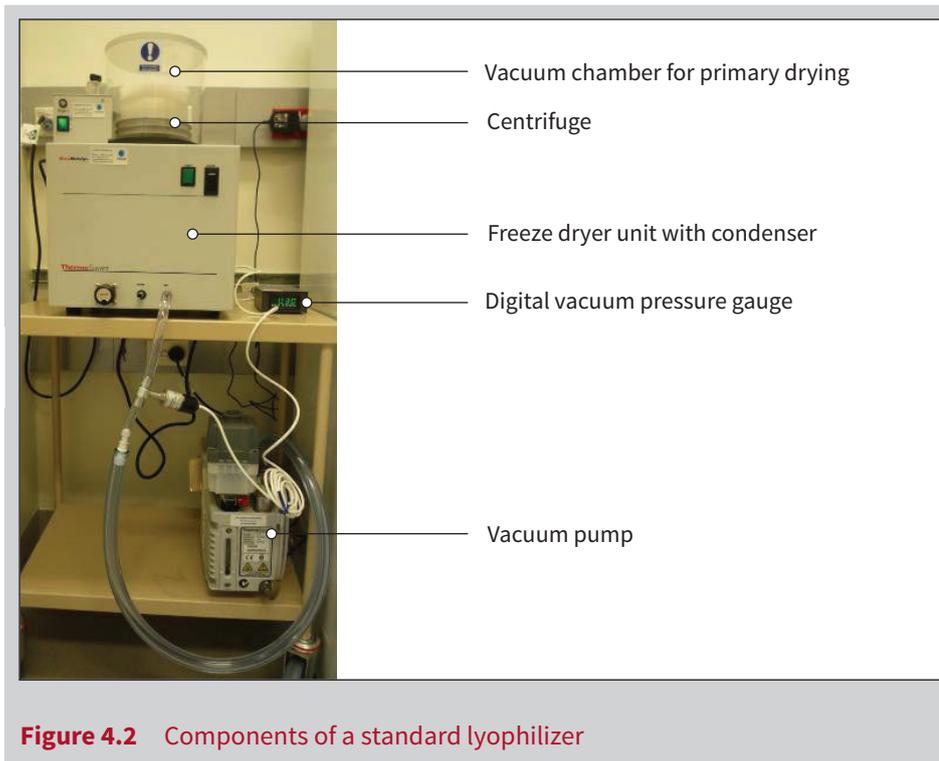
After the first drying stage, constrict the ampoules with the aid of a burner to draw out a heavy-walled capillary section on the ampoules.

4.3.1.4 Ampoule constriction

1. Before ampoule constriction, cotton wool plugs should be trimmed and pushed down the tubes to a position above the label using a sterile stick.

2. Turn on the gas and light the torch. Open the valve to incorporate air into the flame and adjust both gas and air mixture to produce a small, fine-pointed flame which allows the heat to be concentrated on a section of the ampoule.

3. Ampoules are then constricted in the middle, above the inserted plug, by gently turning ampoules while holding the centre point over a flame, allowing the glass to melt.

4. Ampoules should be held horizontally by the ends, resting on the index and second fingers. Slowly roll between the thumb and first finger on both hands, using thumbs on top. Care should be taken at this point as the tendency is for one hand to roll more quickly than the other. As the glass melts, the two halves of the ampoule become only weakly connected. Rolling should allow the glass to flow into the middle of the ampoule at the point where the flame is directed. As the glass flows into the middle, remove the ampoule from the flame while continuing to roll and gently stretch the middle section to 1–1.5 cm. It is important not to stretch the narrow neck too far as the glass walls can become thin and brittle. Care should be taken not to seal ampoules during this process.

5. Mechanical ampoule constrictors (Figure 4.3) provide an easier solution if available. Ampoule constrictors have angled rollers that apply a gentle outwards pressure while the ampoules are turned over a fixed flame.

Figure 4.3 Ampoule constrictor with butane-or propane-fueled flame and angled rollers to assist with stretching of tube for constriction

4.3.1.5 Secondary drying

1. Constricted ampoules are applied to a manifold by pushing over rubber adapters or into a rubber sleeve (Figure 4.4).

2. When the vacuum is re-started, the ampoules will be held tightly, although it is important that the rubber sleeves have not perished.

3. It is also important that ampoules make good contact with rubber adapters and that there are no cracks or chips in the glass allowing leaks to occur.

4. Secondary drying should continue for approximately one hour until a vacuum of approximately 6.7 Pascals is reached. Drying over a desiccant, such as phosphorus pentoxide, is particularly effective for the removal of any remaining moisture but care should be taken not to inhale or touch phosphorus pentoxide.

5. Once the vacuum has been achieved, ampoules may be sealed by holding a flame to the constricted part of the ampoule. When melting this narrow neck, care must be taken to avoid burning a hole in the ampoule, which can occur if the glass walls have been stretched too thin during the constriction process. If this happens, there will be a loss of vacuum. The damaged ampoule needs to be removed and replaced. The vacuum pump is then re-started until a vacuum of 6.7 Pascals is once again reached.

6. The ampoule is held at the end and twisted, after the narrow neck has melted, until it can be separated from the top half of the ampoule (Figure 4.5).

7. Remember to burn off any remaining needles of glass at the point of constriction to avoid injury.
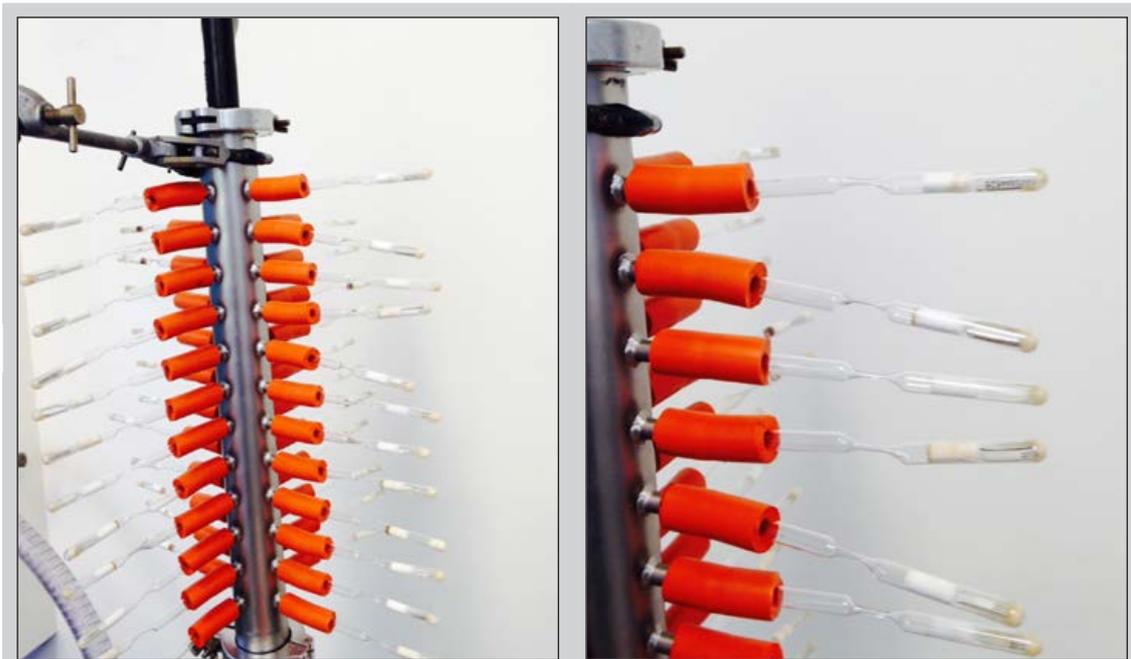
Figure 4.4   Constricted ampoules attached to a manifold that can evacuate four rows of 12 ampoules (left) and showing the insertion *inside* rubber sleeves (right panel). In Figure 4.5, the ampoule is placed *over* a rubber sleeve. Either system works but the rubber can perish over time and must be periodically replaced.
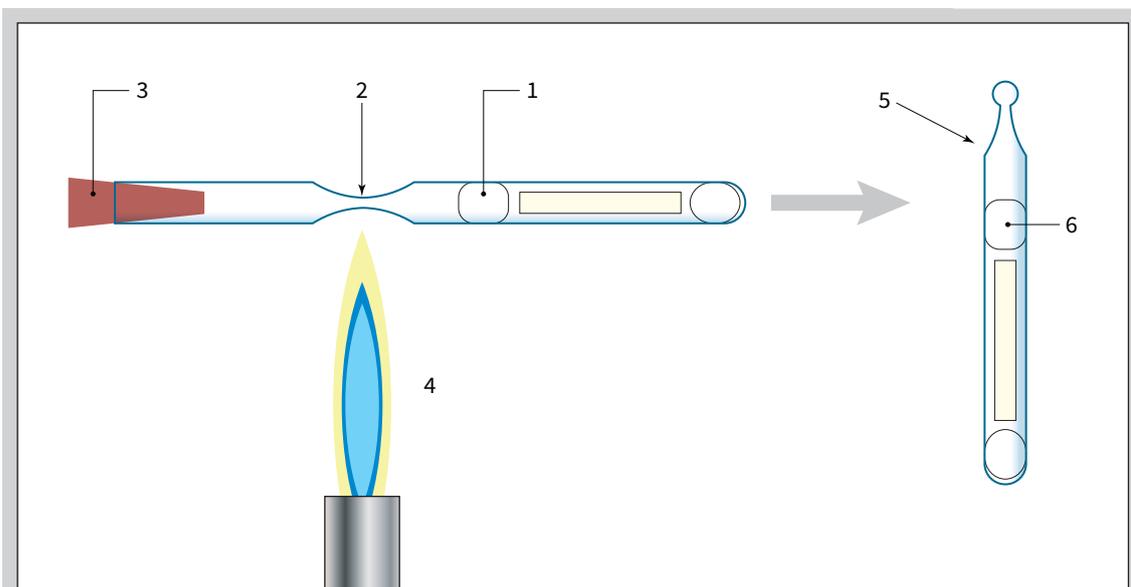
Figure 4.5   Description of parts and flow of operations for freeze drying after primary drying: 1. cotton wool, 2. narrow neck, 3. rubber nipple, 4. flame, 5. constriction melted above the cotton wool, 1 and 6.

4.3.1.6 Checking vacuum and survival of dried cells

1. For long-term survival of strains, ampoules need to retain a vacuum.

2. A high-frequency spark tester or generator can be used to check if a vacuum has been maintained within the sealed ampoule and that there are no leaks.

3. If a vacuum is present, the inside of the ampoule will show a faint glow like a neon light (Figure 4.6).
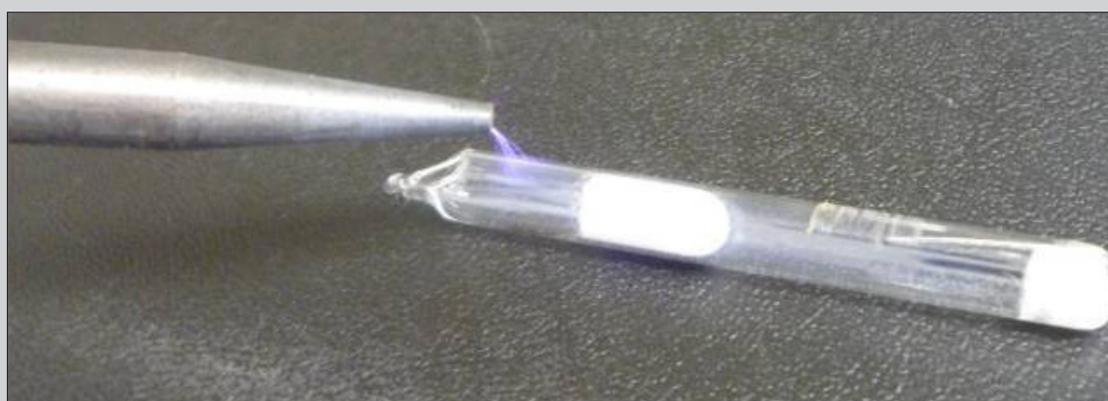
Figure 4.6 Checking for a vacuum with a high-frequency spark tester

4.4 Quality control

Cell viability should be checked in at least one ampoule of each culture per batch soon after the lyophilization process.

4.4.1 Recovery from lyophilized ampoules

1. In a laminar flow cabinet, score the glass above the cotton plug with a steel file, heat the score mark, then gently bend the glass allowing it to crack across the score.

2. Alternatively, and more safely, wet the score mark and touch a softened hot glass rod across the wet score to crack the ampoule.

3. Remove the fragmented part and the cotton with sterile forceps. Add 0.1–0.3 mL growth medium or lyophilizing mixture with a Pasteur pipette, or a sterile tip on a micro-pipette.

4. Homogenise by sucking up and expelling through pipette.

5. Transfer a drop or two to a Petri plate containing growth medium.

6. Spread and allow bacteria to grow (Chapter 3).

4.5 Cryopreservation

Cryopreservation refers to the biological maintenance of living organisms at low temperature (–80°C or below) in a way that allows them to survive after thawing. Electric freezers allow storage at –80°C to –150°C. Because there are some reports that the viability of cultures maintained at –80°C decreases over time, the culture should ideally be checked every five years. For the preservation of bacteria, the OECD (2007) recommends they be stored at temperatures below –140°C.

4.5.1 Preparation

Select an appropriate preservation medium, such as the glycerol-peptone medium of Gerhardt *et al.* (1981) modified in Table 4.2 to be suitable for rhizobia by removal of a meat extract component.

Table 4.2   A glycerol-peptone medium for use with rhizobia

Component	Amount/100 mL
Peptone	1.0 g
Yeast extract	0.5 g
NaCl	0.5 g
Glycerol	12.5 g

pH adjusted to 7.2

4.5.2 Procedure

The following is a basic description of the CRS method for cryopreservation for rhizobia.

1. Prepare a standard culture medium without indicator or dyes, mix 85:15 with 80% (v/v) glycerol (final concentration = 12% glycerol) and autoclave.

2. In a laminar flow cabinet, dispense the culture medium + glycerol mix into sterile cryotubes (1–2 mL).

3. After confirming purity of the culture to be preserved, take the plates to the laminar flow hood, add a loopful of culture to the cryotube and vortex to resuspend the culture.

4. Alternatively, if the culture is a broth, add 150 μL of sterile 80% (v/v) glycerol to the cryotube, make up to 1 mL with broth culture and shake to mix.

5. Decrease the temperature at a rate of 1.0°C/minute until it reaches –50°C and then leave at the final temperature.

Some laboratories have better cell recoveries by plunging cryotubes into liquid nitrogen, allowing a rapid drop of temperature. Glycerol cell suspensions may also be added to vials containing small sterile glass beads, removing excess liquid so

that beads are coated with cell suspensions. Coated beads can then be removed individually from frozen cultures without the need to thaw the whole suspension before re-culturing.

4.5.3 Recovery of cultures from freezing

Preserved cultures should be checked at least every five years. The CRS uses the following approach.

1. If frozen cell suspensions are to be returned to cold storage, they should be kept on ice while being handled and not allowed to thaw.

2. A warm sterile loop is used to transfer a small amount of frozen culture to an agar plate.

3. Once thawed, the culture is then streaked onto the plate.

To check the purity of the frozen culture, it is advisable to streak to obtain single colonies (Chapter 3).

When rhizobia are being recovered, either from ampoules or from cultures stored at low temperatures, growth may be much slower than that of cultures streaked out from routine slopes or plates, and longer incubation times may be needed.

4.6 Developing a parent/working-lot system

With commonly used cultures, genetic drift can be minimised by implementing a parent/working-lot system (Figure 4.7). This allows early cultures to be available for production of new working stock. The first set of working cultures can be made from the initial culture. A set of parent cultures is made from the same initial culture, and these can be used subsequently to make further parent and working cultures. If the culture collection is to be stored frozen, it is recommended that parent lots are stored in separate refrigerators to minimize losses from failure of either the electricity supply or the compressor.
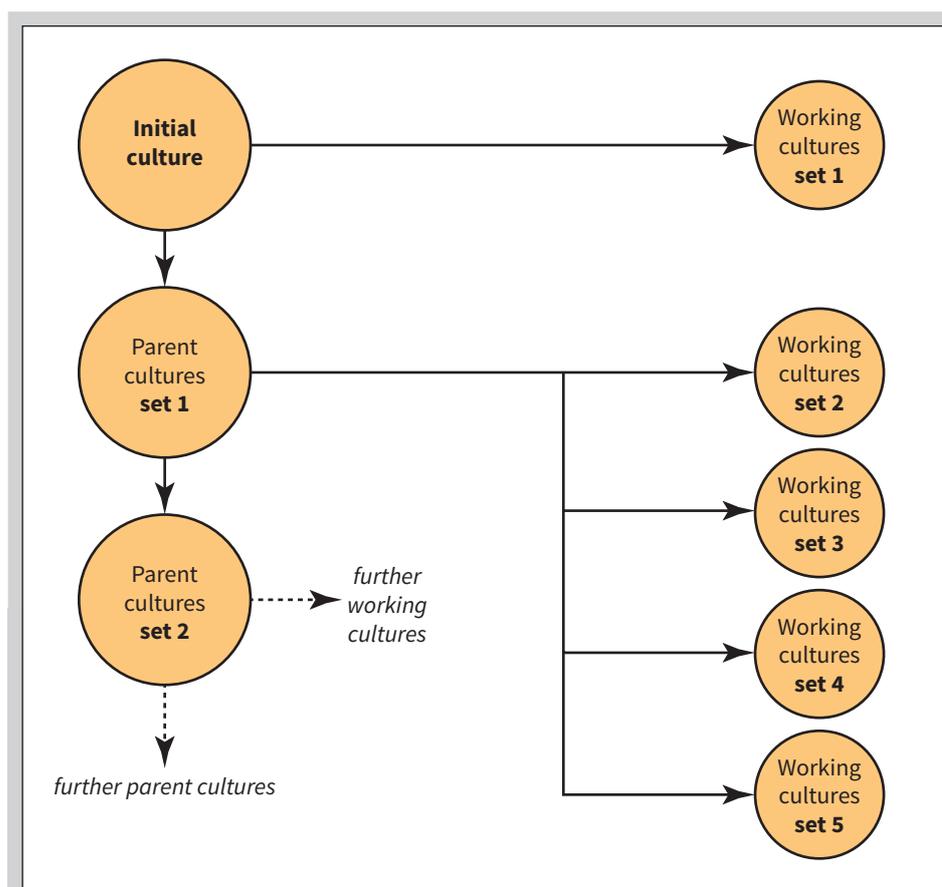
Figure 4.7   A parent/working-lot system for culture preservation

The OECD best practices guidelines for biological resource centers (BRCs) recommends that bacteria should be kept in at least two different ways, and copies should be kept in two different places, preferably in two different buildings (OECD 2007).

4.7 References

Day J.G. and Stacey G.N (eds) 2007. Cryopreservation and freeze-drying protocols, 2nd edition. Methods in molecular biology series 368. Humana Press: Totowa, New Jersey.

Gerhardt P., Murray R.G.E., Costilow R.N., Nester E.W., Wood W.A., Krieg N.R. and Phillips G.B. (eds) 1981. Manual of methods for general bacteriology. American Society of Microbiology: Washington.

Hubálek Z. 2003. Protectants used in the cryopreservation of microorganisms. Cryobiology 46, 205–229.

Hungria M., Loureiro M.F., Mendes I.C., Campo R.J. and Graham P.H. 2005. Inoculant preparation, production and application. Nitrogen fixation in agriculture, forestry, ecology and the environment. Nitrogen Fixation: Origins, Applications and Research Progress 4, 223–263.

OECD 2007. OECD best practice guidelines for biological resource renters. (Organization for Economic Cooperation and Development (OECD): Paris.

Authentication of rhizobia and assessment of the legume symbiosis in controlled plant growth systems

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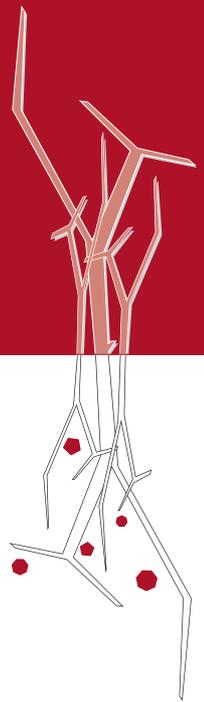
5.1 Introduction

After strains of rhizobia have been isolated from nodules (Chapter 3), and (ideally) before long-term preservation (Chapter 4), the strains should be examined to ensure they retain the essential features of nodule bacteria. The first step in this process is termed 'authentication', which examines the ability of the strain to infect a legume to form a nodule. Following this, strains may be evaluated for their ability to fix nitrogen. This latter characteristic is sometimes termed 'effectiveness'; it is an assessment of the genetic compatibility between the host plant and the rhizobium strain for nitrogen fixation. If a strain can nodulate a legume and fix N_2 effectively in the glasshouse environment, the researcher may wish to proceed further, to assessment in the field. However, if the strain is to be released to the field, then 'duty of care' requires that we have an understanding of its host-range (Section 5.2). This is because releasing strains into the general environment that might be detrimental to existing legumes either agricultural or natural would be negligent. The techniques described in this chapter allow a researcher to compare strain symbiotic performance across a spectrum of plant genotypes to fulfil this duty of care.

5.2 Host-range, cross nodulation and effectiveness

The group of legumes with which a strain of rhizobia is able to nodulate is often described as its host-range. Cross-nodulation within this host-range was a concept developed to record nodulation relationships and thus became a tool with which to group strains (Fred *et al*. 1932; Vincent 1970). This has been very useful, for example, when developing commercial inoculant groups.

In describing host-range it is important to distinguish between specificity for *nodulation* and effectiveness for *N_2 fixation*. Whenever a strain achieves nodulation with a legume, the association may have one of several possible outcomes for N_2 fixation; varying anywhere from no N_2 fixation to maximum N_2 fixation (Terpolilli *et al*. 2008). Too often researchers ignore the fact that many symbioses are not optimal for N_2 fixation. This becomes very important when developing commercial inoculant recommendations, or investigating regulation of genes for N_2 fixation.

5.3 Plant growth systems with which to assess nodulation and effectiveness

A wide range of growth systems are available with which to evaluate strain nodulation and effectiveness under controlled conditions (Table 5.1). Selecting the most appropriate system is a critical decision. It will depend on seed size, the duration of the experiment and the type of information required. For the larger-seeded pulses, the legume may need to grow for up to seven or more weeks to exhaust cotyledon nitrogen before the expression of the symbiosis becomes evident. For very large seeds, one cotyledon can be excised after seedling emergence to expedite the reliance of the legume upon N_2 fixation.

A summary of the advantages and disadvantages of a range of apparatus for screening strains for nodulation or effectiveness is provided in Table 5.1.

Table 5.1 Apparatus for screening rhizobia for nodulation and effectiveness with a guide to their advantages and disadvantages

Assembly and growth medium	Advantages	Disadvantages
Glass test tubes with a vermiculite, sand or agar support medium.	Can be sterilised as a whole unit. Strong control of hygiene. Space efficient.	Not all legumes fix N_2 in glass or polycarbonate tubes.
Plastic growth pouches provided with a liquid medium.	Space efficient. Can easily visualise root systems.	Can be expensive. Less control of airborne contaminants.
Leonard jars containing sand or vermiculite, with added nutrient solution.	Can screen large seeded legumes (*e.g.* soybean).	Time and labour intensive.
Enclosed, polycarbonate 'O'Hara' vials.	Easy to set-up. Can be sterilised as a unit. Recyclable and space efficient.	Not all legumes fix N_2 in enclosed vials.
Sand with nutrient solution in plastic pots, with surface-applied beads.	Can screen large or small legumes, with several species per vessel. Sand better reflects field conditions, allows deep drainage and is cheap to procure.	Requires adequate space and a clean glasshouse.
Intact soil cores.	Can mimic soil physical and chemical conditions.	Time and labour intensive in core collection.
Hydroponic solution	Can closely monitor nutrient flows. Space efficient and roots are easily examined.	Difficult to control contamination. Limited rhizosphere development

5.3.1 Growing seedlings in glass tubes for authentication of rhizobia

For small seeds, such as clovers (*Trifolium* spp.) and alfalfa (*Medicago sativa*), it is possible to conduct authentication tests in glass or polycarbonate test tubes of 200 mm × 25 mm dimensions, with one plant per tube.

5.3.1.1 Procedure for setting up the glass tubes containing agar slants

1. Select the appropriate nutrient solution (see Section 5.7) and add agar powder at 15–20 g/L. Adjust the pH to 6–7, otherwise the agar does not set solidly.

2. Microwave the mixture to melt the agar, stir to homogenise and then dispense into each tube, to approximately one-third of its volume before it cools and sets. If a microwave is not available, evenly mix the agar powder into the mineral solution by vigorous stirring then dispense evenly to each tube.

3. Allow sufficient tubes for a 10% failure rate, and for both positive (+N) and negative (-I) controls.

4. Close tubes with individual plastic caps, or by inserting a cotton wool bung (in this case, cover with a sheet of aluminium foil or moisture-proof paper to prevent the cotton wool from becoming wet) then place in a rack and sterilise at 121°C for 20 minutes.

5. Remove from the autoclave before the agar solidifies and place the rack at an angle of approximately 60 degrees to let the agar set.

6. In the laminar flow cabinet, and using sterile tweezers, gently grasp the pre-germinated seedling around the seed (see Figure 5.6) and plant one into each tube, with the radicle penetrating into the agar. Ensure the radicles do not dry out and are oriented downwards. *NB:* sometimes it helps to push the tip of the tweezers into the agar to make a crack prior to inserting the radicle.

7. Inoculate the seedlings (see Section 5.9) as soon as possible with 1 mL of suspension as this also prevents drying. Replace the cap or cotton wool.

8. Place the tubes in a shallow box or cover the base with aluminium foil to protect the roots from light.

9. Place the tubes in the glasshouse or near a window to allow sufficient light for photosynthesis.

10. Evaluation of nodulation can be performed 25 to 35 days after inoculation.

11. Authentication is positive if isolates nodulate the roots of inoculated plants while uninoculated plants remain nodule free.

12. A measure of effectiveness* can be obtained by cutting the tops, drying at 60°C then weighing (*NB:* see Section 5.12.1 for qualifications).

*NB: some legumes (*e.g. Biserrula pelecinus*) do not readily fix N_2 when enclosed in glass tubes and therefore caution should be exercised if assessing N_2 fixation with this approach. The reasons for this are unclear.

5.3.2 Growing seedlings in O'Hara vials for authentication of rhizobia

O'Hara vials (Figure 5.1) are essentially mini-pots. They are screw-topped polycarbonate (100 mL) vials that may be autoclaved after the addition of growth medium (usually sand) and nutrients (Section 5.7) to approximately 50% of capacity. Germinated seedlings are lowered into the medium with forceps in the laminar flow cabinet and the lid rested on the top to allow exchange of gases during the growth of the seedling. This system is suitable for small-seeded legumes (1–20 mg) such as siratro (*Macroptilium atropurpureum*) but larger vials (500 mL) have been used for larger seeded legumes (20–50 mg). If necessary, sterile water or nutrient solution can be added to the vials during plant growth.

The advantages of the O'Hara vials include ease of preparation, cleaning and storage, and they can be recycled. They also represent a closed system that minimises contamination. As for glass tubes, not all legumes fix N_2 to the maximum of their ability when enclosed in polycarbonate.

Figure 5.1   Sterilised O'Hara growth vials containing a sand mixture, with adhesive labels

5.3.3 Growth pouches for authentication of rhizobia

Growth pouches are made of plastic (polyethylene) bags in which it is possible to grow legumes of small and medium seed size, such as mungbean (*Vigna radiata*), *Trifolium*, *Medicago* and vetch (*Vicia sativa*). Although the pouches are not completely sealed after inoculation, with care, cross-contamination is rare and they may be manufactured in the laboratory. The pouches have a thickness of approximately 0.12 mm, must be autoclavable and are self-supporting. The average dimensions of the base and height are of 130 mm × 140 mm, respectively. Germination paper supports the seedlings in the top of the pouch and also provides a wick that draws up moisture. The roots grow into the pouch to further access nutrients. Light entering the root zone is reduced by an inner brown paper lining and by filing the pouches close together (Figure 5.2).

Figure 5.2   Plant growth pouches are adequate for observing nodulation and authenticating strains, but not all legumes will fix N_2 optimally in these conditions so alternative growth assemblies may be needed if N_2 fixation is to be quantified

5.3.3.1 Procedure for setting up the growth pouches

1. Place pouches into a grid or holder (Figure 5.2).

2. Add 80–100 mL of sterile nutrient solution (Section 5.7) to the pouches.

3. The germination paper is then folded and perforated, and added to each pouch to support the seedlings.

4. Place one pre-germinated seedling per pouch, with the roots immersed in nutrient solution and properly oriented.

5. Inoculate at the base of the seedling after two days.

6. Positive (+N) and negative (–I) control pouches should always be included.

7. If necessary, sterile water or nutrient solution can be added to the pouches during plant growth.

8. The evaluation can be performed between 25 and 35 days after inoculation, with nodules visible through the bag (Figure 5.2).

5.4 Screening for N$_2$ fixation

In our experience, and that of others, diffusion barriers exist in glass tubes where the plant is supported in either agar or vermiculite, and this can affect N$_2$ fixation. In extreme cases, N$_2$ fixation is completely suppressed. Important assumptions governing the screening of rhizobial strains for effectiveness under controlled conditions are as follows.

1. No plant-available mineral nitrogen enters the system, except that contained in the seed. This ensures that N$_2$ fixation can be monitored as a direct function of plant growth. This precludes the necessity for costly analysis of N in the tops to assess symbiotic performance.

2. The system must be free of rhizobia that are capable of nodulating the legume, other than that applied as inoculum, implying that hygiene must be maintained throughout the duration of the experiment.

3. Control treatments of uninoculated (–I) and uninoculated but with mineral N (+N) must be applied.

4. The +N control treatment must have N available at a non-limiting rate, such that growth can be compared with the N$_2$-fixing treatments and relative effectiveness of strains can be assessed by comparison. Obtaining the right rate for the +N control may necessitate a preliminary experiment.

5. Other nutrients required for plant growth must be non-limiting. This may also require a preliminary experiment as legumes differ in their nutrient requirements.

6. The growth substrate must be non-limiting for plant development (*i.e.* does not limit diffusion of gases, light or nutrients, become water-logged, too dry, salty, anoxic, toxic or compacted).

7. The container must not limit plant growth in any way (*e.g.* by constricting the roots).

8. The external environment must not limit plant growth through inappropriate temperature or day-length, or amount of light.

5.4.1 Leonard jars

Leonard jars are suitable for larger-seeded legumes, such as the tropical pulses that cannot be grown for long periods in pouches or glass tubes. Leonard jars are suitable for quantification of N$_2$ fixation but not recommended for competition experiments as a rhizosphere does not establish in the normal manner. Leonard jars consist of two separate parts: the base providing a reservoir for nutrient solution, and the top supporting the plants. Leonard jars can be built from common commercial bottles (Figure 5.3).
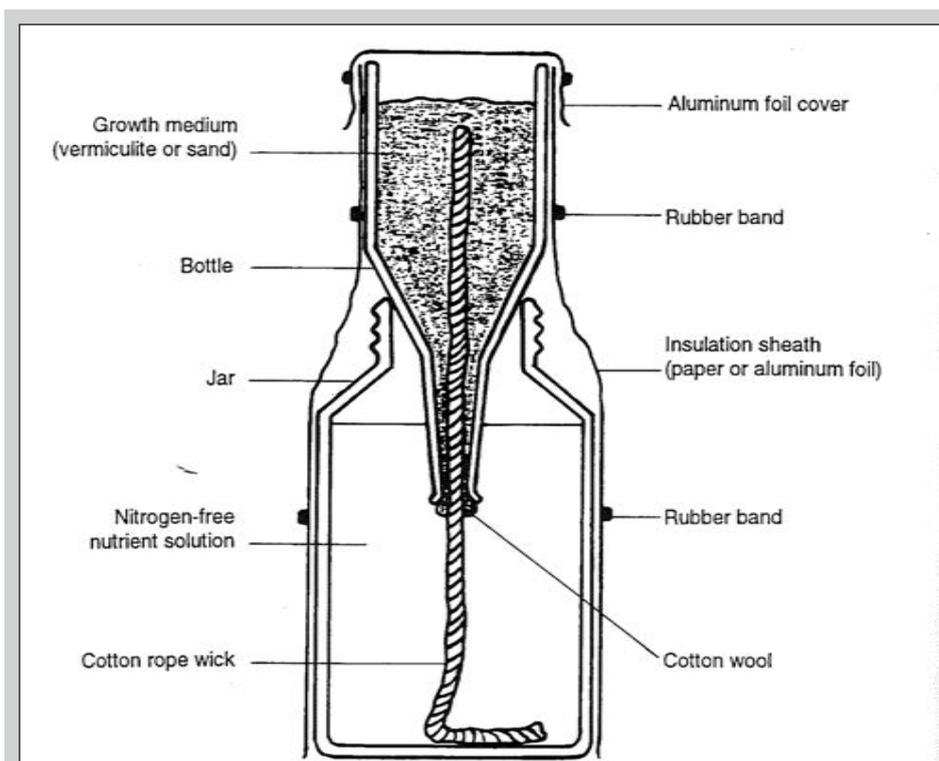
Figure 5.3   A schematic diagram of a Leonard jar (from the NifTAL manual, Somasegaran and Hoben 1994).

5.4.1.1 Procedures for setting up Leonard jars

1. The top half of the assembly consists of an inverted glass bottle (round beer or spirit bottle) of about 700 mL capacity that has had its bottom removed. The CIAT manual (Sylvester–Bradley and Kipe-Nolt 1988) suggests the bottom may be removed by heating at the appropriate place with a wire resistor then plunging into cool water if necessary.

2. The inverted glass bottle sits on the lower half, which is in effect a glass jar that holds the reservoir for N-free nutrient solution. The assembly is such that the inverted bottle sits snugly on the rim of the jar and the neck of the bottle comes to within 2–4 cm of the bottom of the jar.

3. A wick is placed in the neck of the inverted bottle so that the nutrient solution in the jar is brought up to the top of the growth vessel by capillarity. The wick is secured in the bottle neck by cotton wool.

4. Add coarse, well-washed river sand or other substrate (see Section 5.5.1) to the bottle units to within 2 cm of the top. In doing so, keep the wick near the centre of the vessel and have it reaching almost to the surface.

5. Moisten the jar from the top with N-free nutrient solution (see Section 5.7) until it begins to drain into the reservoir. Fill the reservoir with the nutrient solution to within 2 cm of the junction of the two parts.

6. Cover the open end of the inverted bottle with aluminium foil (or a glass Petri dish cover) and the whole unit with moisture-proof paper secured with rubber bands or heat-resistant tape.

7. Autoclave complete units at 121°C for two hours. Do not vent the autoclave after sterilisation but let it come down to zero pressure before opening the door. Remove the units to a clean place and keep the covering intact until planting.

8. Sterilise sufficient gravel (3–4 mm diameter size) or alkathene beads to cover the substrate in the vessels to a depth of 2 cm by dry heating in an oven at 200°C for one hour.

9. Place the assembly in a laminar flow cabinet or on a clean bench and remove the foil cover or Petri lid. Using sterile forceps make holes in the substrate into which pre-germinated seeds are placed. If the treatment is to be inoculated, do so before covering with sand using forceps and tamp the sand in place over the seed to provide a firm seed bed.

10. Cover the vessel opening with half a Petri dish or clear plastic film to protect against contamination, and set the units in a greenhouse or growth chamber. Remove the covers when the seedlings reach a height of about 2 cm and cover the soil surface with sterile gravel or beads. Inoculation (see Section 5.9) can be carried out just prior to this step, directly onto the surface of the sand at the base of the plant, if it has not previously been done. The units are now left open.

11. Uninoculated and mineral N controls are provided as previously described. The assembly is maintained for five to eight weeks during which N-free nutrient solution in the reservoir may be depleted, depending on plant size and growth conditions. If necessary, new nutrient solution (1:4 dilution) may be transferred aseptically into the reservoir.

12. At the end of the growth period, the plant shoot is harvested and measured for dry weight while the nodules are counted and the dry weights of roots and nodules is measured.

5.4.2 Draining pots with the surface covered by sterile beads

If the researcher has access to coarse sand, vermiculite or other substrate and a steaming assembly to pasteurise it, then draining pots can be adopted for the growth of legumes of any seed size. Up to six small-seeded legumes may be grown in a small plastic pot (5 cm radius, 1.5 kg sand) which represents an experimental unit. Larger pots (10 cm radius, up to 5 kg sand) are required for large-seeded pulse legumes, such as pea, soybean, lupin and beans, which must be grown for five or six weeks before seed reserves of nitrogen are exhausted. For these, a support assembly to keep the plants upright is usually required (Figure 5.4).

A feature of this assembly is that more than one species of legume can be grown in each pot (*e.g.* Figure 5.7) which enables the researcher to assess the host range of a single rhizobial strain across genotypes, species or genera, in a split-plot design (Howieson *et al.* 1995). Hygiene is maximised by a layer of sterile alkathene beads applied to the surface of the pot, with sterile nutrients added through a capped watering tube (see Section 5.5.4).

Figure 5.4 Free-draining pots containing coarse sand in a system for assessing effectiveness. Small 1 kg pot (left) growing Siratro and *Acacia saligna*, and large 5 kg pot (right) growing both black-eyed bean and climbing bean.

5.4.3 Hydroponic systems

Some researchers have eliminated the substrate as a support structure for growing legumes, and instead suspend the legumes immediately above hydroponic solutions that provide all the nutrients required for legume growth (except N). Mechanisms to suspend the legumes include foam (which floats on the surface of the nutrient solution, with holes for the roots to extend through) or lids that fit over the vessels and that contain slits into which foam sleeves holding the roots are inserted (Figure 5.5). Contamination by rhizobia can be controlled by altering the pH, according to the principles described by Munns (1968) and refined by Ewing and Robson (1990). Briefly, as infection of legumes by rhizobia is pH sensitive, the solution may be kept at a pH that precludes infection but does not affect root growth. The pH can be raised to allow infection at the time of inoculation, and lowered again two days post-inoculation to preclude further infection. Vessels must be aerated, and this is usually achieved with an aquarium pump.

5.4.3.1 Procedure

1. Determine appropriate nutrient solution for growing the legume (Section 5.7). The CRS uses one-quarter strength CRS solution (Section 5.7.3).

2. Make sufficient concentrated solution for all vessels, ensuring adequate $CaSO_4$ is available (CRS maintain a separate 20 L concentrated (10×) solution of $CaSO_4$ continuously aerated).

3. Adjust pH to below that where infection will occur for the target symbiosis but that does not damage plant roots. CRS add 1 mM MES buffer to provide some management of pH control that results from plant metabolism (see Chapter 3, Table 3.2).

4. Thoroughly cleanse all equipment in 4% (w/v) hypochlorite solution and rinse. Note that chlorine residues dissipate in full sunshine so residuals are not damaging if left exposed to the sun for several days.

5. Fill vessels to the required volume with deionised water (DIW) allowing space for the addition of concentrated nutrients.

6. Insert aeration device (usually an aquaponic aerator with plastic pipes to each vessel running from a manifold; Figure 5.5) and ensure each pot has visible bubbles.

7. Add nutrients and check pH. Adjust with 0.1 N NaOH or HCl solution if buffer present or 0.01 N solution if not.

8. Germinate seedlings after appropriate measures for ensuring seed viability and hygiene (Section 5. 8). It is convenient for hydroponic experiments to germinate seeds in bubbling solution by suspending them on the surface of the solution on fibreglass wire, down through which the radicles penetrate. Ensure pH is below that required for infection during this process.

9. Several legume cultivars or species can be assessed in a single vessel by having a different set of plants held in each sleeve (Figure 5.5).

10. When roots are 2–3 cm long gently place seedlings in foam sleeves, insert sleeves into slits in the lid of the apparatus and suspend over the hydroponic solution (Figure 5.5), ensuring roots are fully immersed.

11. Raise the pH to that required for infection of roots by rhizobia for the particular symbiosis under study (see Chapter 7 for discussion of pH sensitivity in rhizobia).

12. Inoculate with appropriate rhizobial treatments (Section 5.9). CRS add 10 mL of a 10^9 suspension per 10 L vessel, providing 10^6 cells per mL.

13. After 48 hours reduce the pH to that below which infection can occur, and monitor daily.

14. Change the nutrient solution every week, taking precautions to ensure pH is optimised. At this point, it is convenient to have a second set of vessels prepared, and to transfer the lids carrying the seedlings to this set.

15. Maintain the experiment until sufficient growth is achieved to examine the symbiotic effectiveness of the legume–rhizobial association.

NB: pH and calcium are somewhat interchangeable in their effects on nodulation (Ewing and Robson 1990) so attention must be paid to levels of both.

Figure 5.5 An hydroponic assembly for assessing effectiveness in *Medicago* spp. This arrangement has 12 sleeves and two aeration manifolds per vessel (above). Each foam sleeve holds five plants (below). Note the presence of abundant young nodules at many infection points on the roots.
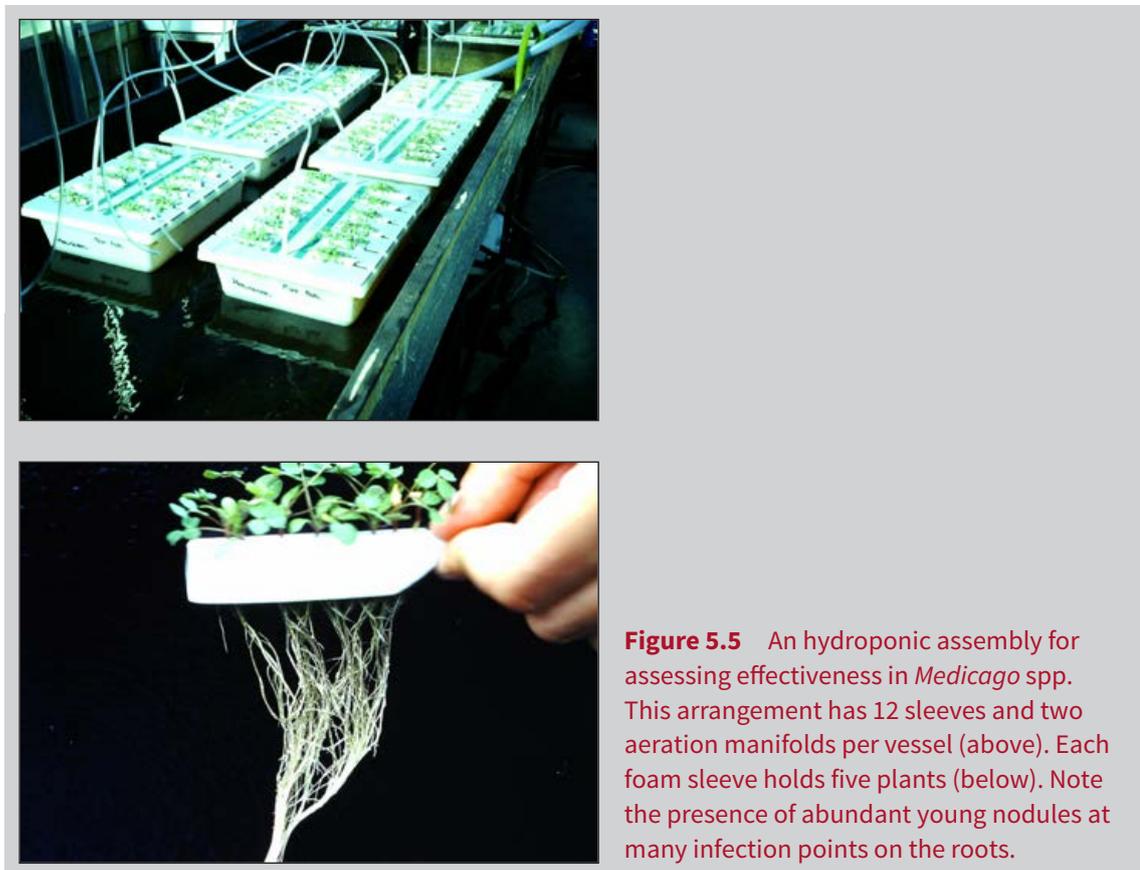
5.5 Substrates for effectiveness experiments

5.5.1 Coarse sand

The key requirement for sand to be used as a medium to support legume growth is that it is coarse and does not compact or become waterlogged once in the vessel. This is because compaction can affect diffusion of gases and nutrient uptake. Coarse river sand containing less than 3% clay is often a good choice, if available. Sand should be free of N or any element that may be toxic to plants. Manganese and aluminium toxicity has been observed in sands of some origins, and in these cases it should be washed with hydrochloric acid (HCl) and then rinsed with running water until it is clear. To complete the process, the sand is then dried in an oven or in the open air. Good quality coarse sand seldom requires washing and it is worth the effort to locate a reliable and clean source. The sand, washed and dried, can be stored in plastic bags. Ensure pH is near neutrality.

5.5.2 Vermiculite

Vermiculite is a 2:1 clay mineral with the capacity for expansion and contraction, conferring high plasticity and stickiness. Depending on the source, vermiculite may be very alkaline. Therefore it is good advice to wash then soak the vermiculite for about eight hours in water, then dry at 90–100°C for 24 hours at room temperature for about a week. The vermiculite must be screened with mesh between 1.0 mm and 3.0 mm dimension. The pH should be checked and if necessary corrected to approximately 6.5.

5.5.3 Mixtures

Legumes can be grown in a mixture of sands with differing clay content, or sand and vermiculite (1:1, v:v), or sand and charcoal (3:1, v:v). It is recommended that the researcher makes a preliminary assessment of the substrate that most suits their biological material and needs to ensure that plant growth is optimal.

5.5.4 Procedure for setting up draining pots with coarse sand

1. Prepare substrate, such as a mix of coarse river sand and yellow sand, with pH adjusted to neutrality. Select a mix of sand that allows free drainage and does not compact.

2. Steam sand for four hours to remove live cells of rhizobia and transfer to sterile bulk bags.

3. Soak pots of required dimension in 4% hypochlorite solution overnight then rinse and dry in a clean room.

4. Autoclave paper towels and pad the bottom of the pot to completely cover the drainage holes.

5. Fill pots to the rim with steamed sand mix (1.5 or 5 kg) and place in a clean glasshouse or growth room. The sand settles to leave a small lip of the pot exposed.

6. Wash twice with boiled water to leach N, allow to drain and cool (this step may be omitted if soil has very low N).

7. Cover with a plastic sheet to avoid aerosol landing on soil surface.

8. Immediately before planting, wet the soil with a liberal application of sterile water until it begins to drain.

9. When seeds are germinated (Section 5.8.2) transfer pots for each experimental treatment to the sterilised laminar flow bench.

10. Make holes of the required number, dimension and depth in the soil surface around the perimeter of the pot with a sterilised rod.

11. Gently grasp the pre-germinated seed (not the radicle) with sterile forceps and place into the holes with the radicle facing down (Figure 5.6). Ensure there is excellent root-soil contact to avoid drying. At this stage, it may be necessary to spray sterile DI water over the seedling to slightly collapse the hole.

12. If the experiment is large, sow only sufficient pots for one rhizobial treatment, spray the remaining seedlings with sterile water and close the Petri dish to avoid further drying.

13. Inoculate planted seedlings with 0.5 mL of rhizobial suspension (Section 5.9) then cover the seed with sand using a sterilised rod and bed down with a small quantity of sterile water.

It is critical that the radicle is not damaged or allowed to dry during this process

14. Cover the pot with clear plastic wrap and place in the glasshouse.

15. Thoroughly sterilise the laminar flow bench and rod before moving to the next inoculant treatment. Repeat steps 8–14 for each rhizobial strain.

16. When the legume has emerged and grown 1–2 cm high, remove the plastic wrap, and insert a sterilised polyvinyl-chloride watering tube (20 mm × 150 mm) into the centre of the pot, approximately 30 mm deep and loosely cap (Figure 5.7).

17. Cover the surface of the pot with sterilised alkathene beads in a layer 1 cm deep, taking care not to bury the cotyledons of small-seeded legumes (Figure 5.7). For large-seeded legumes, the beads can be applied immediately after step 13, eliminating step 14.

18. Provide water and nutrients as required, including N for the control treatment (see Section 5.7) through the watering tube.

19. Two weeks after emergence, thin seedlings to the required number by carefully removing excess plants with scissors or forceps, sterilising utensils from one pot to the next. Particular attention should be paid to removing variants.

20. Water sufficiently for pots to begin to drain, or to a measured weight (if pots become hard to squeeze, then the soil is likely to be too dry). As a guide, 30 mL of water is added to a 1.5 kg pot every second day once the seedlings have emerged and begun to transpire. Daily inspection is recommended as the plants become larger, as it is likely that more water will be required, *e.g.* after six weeks of growth *Phaseolus vulgaris* may require 50–80 mL every second day.

21. Randomise the pots after inoculation.

Figure 5.6 Placing the pre-germinated seed gently into holes in the sand growth medium

Figure 5.7 The sterile coarse sand mixture in these pots, topped with alkathene beads, allowed the expression of differential N$_2$ fixation in eight species of *Trifolium* by strain WU95 (left) and the same clover species by strain WSM1325 (right). The photos show all four replicates (front to back) with four species of *Trifolium* in each pot, in a split-pot design.

NB: when grown in sand culture, small-seeded pasture legumes and *Phaseolus vulgaris* need a pulse of N (5 mL per pot of 1% (w/v) KNO$_3$) at the first (and perhaps second) watering because the growth medium often lacks sufficient N to sustain the legume after seed reserves are exhausted, and before N$_2$ fixation begins.

5.6 Assessment of N$_2$-fixing potential in non-sterile soil cores

After strains have been screened for symbiotic effectiveness under sterile conditions, their performance can be assessed in non-sterile soils in the glasshouse before final evaluation in the field. At this stage, biological control is lost but the experimenter is still able to control other environmental influences, such as soil moisture and temperature. Glasshouse evaluation of the strains in non-sterile soils takes account of the compounding effects of other factors, such as microbial flora and soil herbicide status, and soil chemical status, such as pH, and can provide useful information about the symbiotic potential of the test strains under these conditions. However, collection of the cores is labour-intensive.

5.6.1 Collecting intact soil cores

1. Clean and sterilise the corer (Figure 5.8) prior to travelling to the field.

2. Select the site and drive the core into the soil to just below the surface by applying weight to the step and slowly rotating the core.

3. Remove the corer by lifting the whole apparatus out of the soil. With the plunger, push the core out into a ziplock plastic bag.

4. Place the bags into a cooler box for transfer to the glasshouse.

5. In the glasshouse, place the ziplock bag into an appropriate-sized plastic pot (Figure 5.9)

6. Sow directly into the undisturbed cores utilising seed and inoculant appropriate for the experimental purpose (see Sections 5.8 and 5.9).

7. Acquire data appropriate for the experiment (*e.g.* top dry weights, nodulation) (Section 5.10). For example, the effect of herbicide residues on reducing nodulation in clover is also reflected in growth of the plant tops (Figure 5.9) so tops should be dried then weighed.

For intact cores, attention to the field capacity of the soil is vital to ensure the pots are neither over- nor under-watered. Depending on the fertility of the soil, apply nutrients as for glasshouse experiments with soil (Section 5.7.3).

Figure 5.8   A metal soil corer with cross-bar for stepping on, and showing extracted core

Figure 5.9 Soil cores in ziplock bags placed into pots with three replicates. Experiment showing effects of residual herbicides on clover growth, left to right: residues of trisulfuron, pyroxasulfone, control, chlorsulfuron and clopyralid.

5.7 Nutrient media for legume cultivation in glasshouse experiments

Several different nutrient solutions have been used to provide mineral nutrition to legumes grown in effectiveness experiments. Although they differ in the amounts and types of nutrient sources, they all have a similar composition and can be applied to most legumes. However, over the years the different media have tended to be applied in different systems. For example, Norris and Date (1976) nutrient formulation works well in aquaponic systems (*e.g.* Leonard jars) with tropical legumes, while Jensen's medium is suited to small-seeded legumes grown in agar slopes, and the CRS medium has been successful with Mediterranean and temperate legumes grown in sand or in hydroponics. It is prudent to conduct a preliminary experiment to assess the adequacy of the nutrients supplied, and if necessary amend the constituents to suit the circumstances of the research. For instance, *Phaseolus vulgaris* has a higher nutrient requirement than many other legumes but 300 mL per week of the N-free Broughton and Dilworth medium (Section 5.7.4) has been successful for growing this legume in a vermiculite system (Mwenda 2016). The composition of some of the common nutrient solutions is given below. Unless otherwise stated, all solutions are prepared and diluted in boiled DIW

5.7.1 Norris and Date (1976), *e.g.* for tropical legumes grown in Leonard jars

Solution	Reagent stock	Quantity (g/L)
A	KCl	29.8
B	K_2HPO_4	69.6
C	$MgSO_4.7H_2O$	98.6
D	Micronutrients	
	$CuSO_4.5H_2O$	0.078
	$ZnSO_4.7H_2O$	0.22
	$MnSO_4.4H_2O$	2.03
	$(NH_4)_6Mo_7O_{24}.4H_2O$	0.01
	H_3BO_3	1.43
E	Ferric citrate	1.795

Take 2.5 mL each of stock solutions: A, B and C; 0.5 mL of D and 1.0 mL of E to prepare a 1 L solution. Add 0.344 g of $CaSO_4.2H_2O$ per litre of medium. It does not dissolve so mix well when dispensing.

5.7.2 Jensen's medium (1942), *e.g.* for growing small seedlings in agar in test tubes

Reagents	Quantity (g/L)
$CaHPO_4$	1.0
K_2HPO_4	0.2
$MgSO_4.7H_2O$	0.2
NaCl	0.2
$FeCl_3$	0.1
Trace element solution*	
H_3BO_3	0.5
$Na_2MoO_4.2H_2O$	0.05
$CuSO_4.5H_2O$	0.02
$MnSO_4.4H_2O$	0.5
$ZnSO_4.7H_2O$	0.05

Add stock trace element solution*(5 mL) and distilled water to make up to 1000 mL. Add 1 g agar for solid medium.

5.7.3 CRS Plant Growth Nutrient Solution, *e.g.* for plants grown in sand

The advantage of this solution is that it provides adequate calcium with low chloride content and chelated iron. Make five stock solutions, the fifth being for trace elements, and a sixth for calcium.

Reagent	Quantity (g/L)
1 $MgSO_4.7H_2O$	12.3
2 KH_2PO_4	6.8
3 K_2SO_4	17.5
4 Fe-EDTA	2.5
5 trace element solution (store at 4°C)	
H_3BO_3	0.464
$Na_2MoO_4.2H_2O$	0.018
$ZnSO_4.7H_2O$	0.539
$MnSO_4.4H_2O$	0.042
$CoSO_4.7H_2O$	0.141
$CuSO_4.5H_2O$	0.125
6. $CaSO_4$ agitated solution	2.04
+N control	5 mL weekly of 10 g/L KNO_3

5.7.3.1 Procedure

1. Combine equal parts of the stock solutions (1–4) to make up a nutrient concentrate of 200 mL in a 2 L glass screw-capped bottle.

2. Add 0.5 mL of trace element solution (5) to this, and make the volume up to 1600 mL with de-ionised water, then autoclave.

3. After cooling, add 400 mL of autoclaved and well-agitated $CaSO_4$ solution (6) to make to a final volume of 2 L (this limits precipitation of the Ca and provides adequate Ca without the chloride salt).

4. Add weekly to the sand culture systems for pulse and forage legumes in 20 mL aliquots (for 1.5 kg pots) or 40 mL for larger pots.

NB: some vigorous legumes respond to bi-weekly applications of CRS nutrients in their fifth week of growth and onwards.

5.7.4 Broughton and Dilworth (1970), *e.g.* for *Phaseolus vulgaris* grown in vermiculite

Reagent groups	Quantity (g/L)
1. $CaCl_2.2H_2O$	294.1
2. KH_2PO_4	136.1
3. $FeC_6H_5O_7.3H_2O$	6.7
$MgSO_4.7H_2O$	123.3
K_2SO_4	87
$MnSO_4.H_2O$	0.338
4. H_3BO_3	0.247
$ZnSO_4.7H_2O$	0.288
$CuSO_4.5H_2O$	0.10
$CoSO_4.7H_2O$	0.056
$Na_2MoO_4.2H_2O$	0.048
+ N control (KNO_3)	5.0 g per L in full strength solution

Prepare stock solutions of reagent groups 1–4 using warm water to dissolve the ferric citrate. To make 10 L of full-strength solution, add 5 mL of each stock to 5 L DI water and mix. Dilute to 10 L by adding another 5 L of DI water. Adjust pH to 6.6–6.8 with 1 N NaOH.

5.8 General procedures for handling seed in preparation for authentication or for an effectiveness experiment

Seed preparation to ensure viability and uniformity is very important for effectiveness experiments because seed size and seedling vigour greatly affect plant development. Ensure fresh seed is acquired and test the level of germination. Because of seed dormancy mechanisms in some legumes, different species may require different treatments before their seeds germinate, often refined by trial and error. Examples are provided below.

5.8.1 Enhancement of germination for impermeable seeds

5.8.1.1 Scarification

For species with an impermeable (hard) seed coat:

► Place seeds on a sheet of sandpaper in a plastic tray.

► Strap a second sheet of sandpaper around a wooden block and rub firmly over the seeds until they are visibly scratched (test different grades of sand paper to find the most efficient for the different species of legume). The thickness of the seed coat can vary 10-fold between legume species, so the degree of rubbing to

achieve scarification varies for different species. This must be determined for the species under examination.

► Assess the seeds for imbibition by placing on water agar or wet filter paper for two hours, and if a high proportion imbibe then they are ready for germination. Proceed to step 1 in General Procedures (Section 5.8.2) for germination.

5.8.1.2 Boiling

As an alternative to scarification:

► Place seeds in metal or polycarbonate beaker.

► Pour boiling water over hard seeds and allow to cool.

► Check for imbibition and if a satisfactory proportion imbibe proceed to surface sterilisation and germination (Section 5.8.2).

5.8.1.3 Acid treatment

As a second alternative to disrupting hard seed coats:

► Place seeds in a glass or polycarbonate vessel.

► Cover seeds with concentrated sulphuric acid and stir (cautiously) for three to five minutes.

► Carefully drain off the bulk of the acid and wash the seeds at least six times with sterile water.

► Leave some of the seeds in the final change of sterile water for two hours to assess imbibition. If a satisfactory proportion imbibe, these seeds are now surface sterilised. Proceed with germination (Section 5.8.2).

5.8.1.4 Smoke treatment

For some legumes, mainly from Australia and South Africa, germination levels are increased when seeds are exposed to chemicals contained in smoke from burnt plant residues (Staden *et al*. 2000). 'Smoke water' containing these chemicals is now available commercially. Soak the seeds in smoke water overnight prior to germination.

5.8.1.5 Vernalisation

Some legumes of Mediterranean and temperate climate origin require exposure to a period of cold temperature before germination. Place non-scarified seeds into a cool room set at between 1–5°C for two weeks prior to the experiment.

5.8.2 General procedures for surface sterilisation and germination of seeds

1. From a fresh batch of germinable seed, select the appropriate number or weight required and remove any damaged or irregular variants.

2. Sort seeds to an even size by rejecting excessively small or large seeds and pre-test the level of germination. Enhance germination by the techniques described above if necessary.

3. Remove live cells of rhizobia from the seeds by immersion in 70% ethanol for 1 min then wash twice in sterile water. Some labs transfer from ethanol to a 3–4% sodium hypochlorite solution for 1 min followed by six changes in sterile water. *NB:* some legumes, such as *Arachis*, are sensitive to this procedure and the surface sterilisation regime must be diminished.

4. Germinate small seeds (1–20 mg) on sterilised water agar in Petri dishes wrapped in aluminium foil to exclude light. After overnight imbibition, invert the plate to allow downward development of the radicles (Figure 5.10). This assists in the planting step.

5. Some legume seeds carry pathogens below their seed coats and these cannot be reached by sterilant. For these species, place surface-sterilised seed on a sterile moist filter in Petri dishes or sterile paper towel, and after germination sow the seed before the radicals reach 5 mm. This minimises fungal and bacterial development.

6. Germinate larger-seeded species, such as soybean or lupin, on sterilised, moistened paper towel in sterilised plastic containers. Ensure there is adequate free water in the container for seeds to imbibe.

7. When the radicle is fully emerged, plant the seedlings into the target growth medium or system. Take great care not to damage the radicle while sowing and ensure that the radicle is projecting downwards and the seed head is just below the surface then covered by the growing medium.

> *Seedlings should not be allowed to dry at any stage during germination or in transfer to the experimental vessel.*

8. If a large number of plants are required, and the time to sow them is extensive, then the seedlings can be regularly sprayed with a fine mist of sterile water during the sowing period. Similarly, in warm weather the surface of the substrate may need additional moisture.

Figure 5.10   An inverted Petri plate with germinated seedlings. At this stage of germination it is easy to transfer a seedling to the chosen growth medium.

5.9 General methods for inoculation

1. Assemble the full range of root-nodule bacteria to be tested and ensure they are equally fresh and well grown. If broth cultures, keep agitated or on ice.

2. If inocula are agar-grown cultures, in the laminar flow cabinet add 0.5–1 mL sterile 1% (w/v) sucrose solution or DI water to agar plate or slope and scrape the colonies off with a flamed spatula to produce a suspension. Phosphate buffer or saline are not used as they can affect plant growth.

3. Transfer the suspension to a sealable bottle, close the lid, label and shake vigorously or vortex to ensure uniformity. Keep on ice. The volume of the bottle can be adjusted to between 10 and 100 mL (according to how many seeds are to be inoculated) with either DI water or 1% (w/v) sucrose solution. This will dilute the inocula, but each mL should contain between 10^6 and 10^8 cells, which is sufficient for nodulation. If an accurate count of cell number is required, see Chapter 6.

4. With a sterile syringe or pipette tip place 0.5–1 mL of the shaken suspension directly over the seedlings (Figure 5.11). Inoculate every replicate of the particular rhizobium treatment.

5. Change the syringe or pipette tip between each rhizobium treatment and take care not to suck inoculant into the body of the pipette.

6. After completion of inoculation, pipette 0.5 mL of sterile 1% (w/v) sucrose or DI water to the seedlings of the –I and +N controls. If these controls are nodulated at harvest then the microbiological procedures have been defective and the results are invalid.

Inoculant can also be supplied as liquid broth culture (Chapter 3, Section 3.1). The advantage of liquid culture is that strains can be produced in a more uniform

concentration, adjusted by optical density, for example to OD 0.1. The disadvantage is that contaminants are more difficult to detect as they are not as visible as when they are growing on nutrient agar plates.

Figure 5.11 Inoculation of the planted seed using a disposable tip attached to a pipette gun

5.10 Growing conditions and facilities

Typical facilities for housing the growth vessels include:

1. open glasshouses where contamination is managed at the individual container level, such as described in Section 5.5.4

2. temperature-controlled glasshouses and phytotrons, where opportunities for contamination are lessened, but control of contamination must remain at the container level

3. closed growth chambers that permit little air exchange and maximum control of hygiene.

In facilities where the temperature can be regulated, optimal temperature settings for temperate legume species range from 15–25°C and for tropical legume species from 25–30°C. Nodulation and N_2 fixation can be markedly depressed in some symbioses by temperatures higher than 28°C, hence a glasshouse must have an appropriate cooling system, such as evaporative air blowing through it, or a refrigerated air recycling system. Painting of windows with water-soluble whitener and use of shading (*e.g.* shade cloth) reduces the problem of high temperature during the summer and allows enough brightness for plant growth. On the other hand, in temperate regions in the winter it may be necessary to heat the glasshouse and supply extra lighting to grow subtropical species.

A closed growth chamber often provides a superior facility for controlled conditions. It is an aseptic small room with automatic temperature control that can supply consistent brightness from 100 to 1,000 lumens, with a timer to control the photoperiod (corresponding to the legume requirements) and automatic maintenance of humidity to about 70%.

5.10.1 Hygiene and preparation

Cleanliness of the facility is crucial to the success of the experiment and a high level of hygiene must be practised at all times. A clean glasshouse floor and bench are essential; the facility must be free of excessive dust, old potting mixture, soil and other debris. Fixed structures should be regularly washed with bleach (4% NaClO) or ethanol (70%, v/v). The bench top is ideally constructed of a wire mesh to allow free-draining systems to drain excess solution onto the floor and avoid contamination from pot to pot through the base.

All equipment, such as test tubes, vials, jars, pots, tubes, lids, alkathene beads, filter paper, syringes and containers must be cleansed and sterilised or disposed of between experiments. Common practices include soaking equipment in ethanol or bleach followed by rinsing in sterile water or autoclaving where appropriate.

5.11 Data acquisition for quantification of effectiveness

5.11.1 Top dry weight

In most scenarios, if the −I control plants have characteristic symptoms of N-deficiency (viz. small plants with pale green or yellow leaves) and are not nodulated, and the inoculated plants are two or three times as large, have formed nodules and produced healthy green leaves, it can be assumed that the symbiosis is effective. However, this must be quantified.

The shoot dry weight of plants harvested after significant plant biomass accumulation is an accepted criterion for N_2-fixing effectiveness in systems that are free of mineral nitrogen. After four to 10 weeks (depending upon growth conditions) tops are cut at the pot surface, or at the hypocotyl, placed in a paper bag, labelled then dried (60–70°C) for three to four days then weighed.

If −I controls are not nodulated, the capacity for N_2 fixation can be assessed by comparing yields of inoculated plants with the +N controls as well as with the commercial inoculant strains. Strains can be ranked by comparing yield as a percentage of that achieved by the + N treatment or by the best strain or by the commercial strain, as required.

This approach requires some qualifications, however. Firstly, when working with seeds from wild plants, or even outcrossing plants such as lucerne, there can be substantial plant to plant variation in top growth even though the seeds have been sorted for uniformity. Further, many woody legumes put more resources into their roots than into their tops. The researcher must be aware of these anomalies and should determine an appropriate parameter to suit their circumstances.

Researchers can consider the symbiosis effective (E) where plant weight exceeds 75% of the +N treatment. Where plant weight is less than 20% of the +N treatment, the symbiosis is deemed ineffective (I). Between these parameters, the symbiosis is considered partially effective (P). However, one must be aware that the +N controls must be at an acceptable level of growth to make such comparisons. In some species it is difficult to estimate adequate rates of mineral N for good growth and a preliminary experiment is sometimes required.

5.11.2 Root nodulation

It is often a very valuable exercise to look at the nodules and assess their size, position on the root system, colour and shape. Carefully remove the roots from the pots, wash them free of the growth medium and score them for nodulation based on the number, size and position of nodules on the root system (Figure 5.12). Plants collected from field experiments can also be scored with this chart but it works best where there are less than 50 nodules per root system. Once the researcher has become familiar with the root systems in the experiment, the scoring process can be much more time efficient than counting the number of nodules, and 20 to 30 plants can be scored quickly to provide an average score per treatment. This approach works well for agricultural legumes but many woody root systems form very few nodules, and hence it may be better for the researcher to simply count the number present on woody plants and shrubs. Nodule dry weight may also be assessed but, as for nodule scoring, it is not a reliable indicator of strain effectiveness.

For detailed descriptions of nodule morphology (see Chapter 2, Figure 2.2) it is valuable to have a camera and lighting system set-up to capture images of the nodules.

The researcher may also wish to assess nodule occupancy as a final part of adhering to Koch's postulates. This is commonly achieved with molecular tools such as RAPD or RFLP PCR techniques (Chapter 11).

0	**0.5**	**1**	**2**	**3**	**4**
Absent	Ineffective	Rare (Effective)	Scarce	Moderate	Adequate
No nodules	White ineffective nodules	≤ 5 small pink nodules	< 10 small pink nodules	10–20 small pink nodules	> 20 small and/or large pink nodules
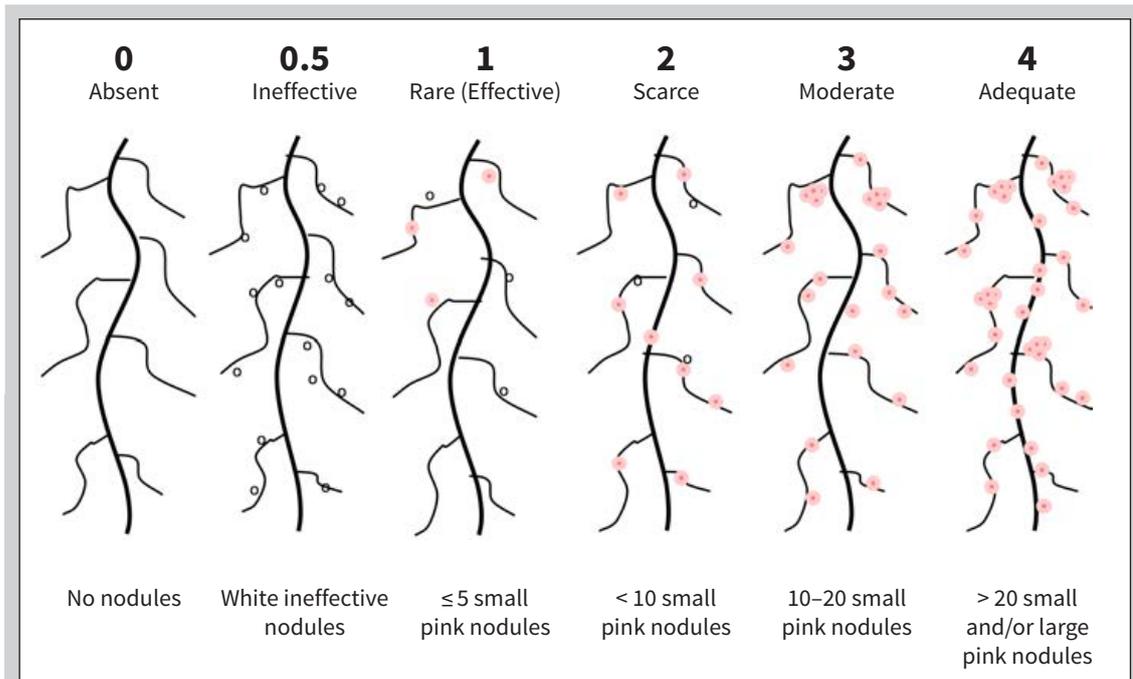
Figure 5.12   A nodule scoring chart than can be applied to both pulse and pasture legumes where the root systems have less than 50 nodules per plant

5.11.3 Acetylene reduction by nitrogenase

Reduction of acetylene to ethylene can provide an assay for the activity of the rhizobial enzyme nitrogenase at a point in time. It is particularly useful to assess whether a strain is genetically compatible for N_2 fixation with a given legume host. In the nodule environment it is the host that protects nitrogenase from denaturation in the presence of oxygen through production of leghemoglobin (Section 5.11.4 and Figure 5.14). Acetylene reduction is not particularly useful for quantification of N_2 fixation, although if handling procedures are optimised it can provide relative quantification data for pot experiments.

The protocol below is used at the CRS and is a simplified routine because it uses modern columns that allow a more clear separation of acetylene and ethylene peaks than when the technique was first developed in the 1960s (Dilworth 1966).

<div style="text-align:center">SAFETY INFORMATION</div>

Since explosive gases and high-pressure cylinders are used in this protocol, you must comply with all local safety rules before commencing. You must also ensure that your experimental set-up and the location of gas cylinders has been inspected and approved by the relevant health and safety authority in your institution.

1. Grow plants in a system where they can be harvested with minimal root disturbance. Ideally, a system using fine vermiculite is best as this can be easily removed from roots at time of harvest without washing the root extensively.

2. Once harvested, transfer intact plants into an appropriately-sized bottle that can comfortably contain the plant without damage. For approximately four-week-old pea plants, 250 mL Duran bottles are of an adequate size (Figure 5.13).

3. Place a moist piece of paper towel at the bottom of the bottle to ensure that plants do not dry out during the assay.

4. Seal the Duran bottle with rings (GL-45, 34 mm aperture size, fit all standard Duran tops, catalogue number 292271007) and seals (Silicone rubber seals VMQ for piercing GL-45 cat # 292461002).

5. Within one hour add a total of 2% acetylene gas to each bottle using a timed staggered-start method.

   NB: acetylene should be taken from the gas storage cylinder in a way that adequately vents the gas into a fume cupboard. One way is to have a piece of silicone tubing attached to the cylinder regulator which then vents directly into the fume cupboard. The unattached end of the silicone tubing is placed into a 1 L plastic beaker which is approximately half-filled with water.

6. Procedure for adding 2% acetylene to a 250 mL Duran bottle.

   ▷ The total volume of a standard 250 mL Duran bottle (*i.e.* when filled to the brim) is 320 mL, which is the value from which the 2% calculation is taken. To ascertain the total volume, fill the bottle to the brim with water and measure the volume of the water by transferring it to a measuring cylinder.

   ▷ If you use a bottle with a different volume, all subsequent calculations will need to be scaled accordingly.

   ▷ It is important to check that the integrator on your gas chromatograph (GC) can measure the acetylene and ethylene peaks.

   The procedure is as follows:

   a. open the acetylene cylinder lid fully allowing the acetylene to bubble through the water in the 1 L beaker

   b. using a 22 G syringe, remove 8 mL air from the bottle

   c. take 6.4 mL of acetylene from silicone tubing and add it to the bottle (Figure 5.13)

   d. the difference in the volume of air removed and acetylene added creates a small amount of negative pressure on the bottle.

7. Incubate the bottles for a known amount of time at a known, constant temperature which is usually 20–25°C. When running the assay for the first time, it is important to perform a time course experiment to ensure that you are accumulating ethylene in the sample bottles and that the rate of accumulation is linear. To begin with, you could sample at 30 minute, 60 minute, 90 minute

and 120 minute intervals. Sampling beyond 120 minutes is not recommended as longer exposure of nitrogenase to acetylene ultimately inactivates the enzyme.

8. At each time point, take 1 mL samples from bottles using a 12.7 mm 29 G (0.33 mm) syringe, which is the correct size to adequately transfer samples into a GC. Once sampled, the syringe can be placed into a labelled rubber bung to prevent any loss of gas.

9. Once all bottles have been sampled they can then be run on a GC.

Figure 5.13   Whole plants stored in Duran bottles (left) and acetylene being added to start the assay (right)

5.11.3.1 Protocol for gas chromatography

The precise step by step protocol for separating and quantifying ethylene and acetylene in gas samples will vary widely between GCs and operating systems. However, a set of parameters that work well to separate these gases on a Varian CP-3800 gas chromatograph with a Flame Ionisation Detector (FID) are given below.

▶ A Restek RT-U-BOND 15 m × 0.32 mm internal diameter × 10 μm column (catalogue number 19751).

▶ Oven temperature at 40°C.

▶ Carrier gas is H_2 set at a constant flow rate of 1 mL/minute.

▶ Compressed air supply for FID.

▶ FID set at 190°C with a supply of 30 mL/minute H_2 and 300 mL/minute air.

▶ 1 mL samples are injected with a 5:1 split ratio (*i.e.* 20% of the sample is loaded onto the column).

▶ Program runs for 48 sec.

These parameters allow for clear peak resolution of ethylene and acetylene peaks, with ethylene and acetylene retention times of 24 sec and 32 sec respectively.

NB: other columns could also be used to separate ethylene and acetylene peaks, such as the HaySep N range of columns available from Agilent, Restek or Perkin Elmer. Check with a technician to ensure your column and operating parameters are adequate to clearly separate these two gases.

5.11.3.2 Data handling

To calculate the rate of acetylene reduction as amount of ethylene produced/plant/ unit time, the following information is required:

1. the amount of acetylene present in the bottle at the start of the assay (A_0)

2. the ratio of the ethylene peak area to the total peak area (E_R)

3. time of incubation with acetylene (t).

With this information, rate of acetylene reduction (AR) can then be calculated as follows:

$$AR = \frac{A_0 E_R}{t}$$

Below, we give an example of how to derive the rate of acetylene reduction using the information given in the above protocol, where 6.4 mL (0.0064 L) of acetylene was added to a 250 mL Duran (of total volume 320 mL) at a standard pressure of 1 atm and a temperature of 293.15 K (*i.e.* 20°C). This approach can be easily modified by substituting in alternative values for temperature and volume of acetylene, as appropriate.

1. Determining the amount of acetylene at the start of the assay (A_0)

   To calculate the initial amount of acetylene added at the start of the assay you can use the ideal gas law which states:

   $PV = nRT$

   Where P = Pressure, V = volume (L), n = moles, R = gas constant defined as 0.0821 atm.L mol^{-1} K^{-1} and T= Temperature (in Kelvin). In this instance, P = 1 atm and V=0.016 L, so rearranging for n and substituting in values:

   $$n = \frac{PV}{RT}$$

   $$n = \frac{1 \times 0.0064}{0.0821 \times 293.15}$$

   $n = 2.66 \times 10^{-4}$ moles

   $n = 266$ μmoles

   Shows that A_0 = 266 μmoles of acetylene

2. Determining the ratio of the ethylene peak area to the total peak area (E_R)

    Derive the ratio of the ethylene peak area to the total peak area (E_R) using the data obtained from the GC chromatogram in the following way.

    a. Firstly, integrate the area under the ethylene and acetylene peaks. This can be expressed in a number of ways, either area under the curve or percentage area, and should be done using a GC analysis software package specific for your GC (such as the Varian MS Data Review software package).

    b. Sum the area under the ethylene and acetylene curves to yield a value for the total area

    c. Calculate the ratio of the ethylene peak area to the total area to yield E_R.

    So, if the percentage area for:

    ethylene = 0.4508 units

    acetylene = 16.0036 units

    total area = 16.4544 units

    Then the ratio of the ethylene peak to total area (E_R) would be:

    $$E_R = \frac{0.4508}{16.4544}$$

    $E_R = 0.0274$

3. Time of incubation

    Determining the time of incubation was discussed above. We have found that for peas, incubation at 20°C for one hour generates measurable quantities of ethylene and that accumulation is linear.

    Using these values derived for A_0, E_R and t, the rate of acetylene reduction in this case would be:

    $$AR = \frac{A_0 E_R}{t}$$

    $$AR = \frac{226 \times 0.0274}{1}$$

    AR = 7.29 µmoles/hour/plant

5.11.4 Estimation of leghemoglobin in legume root nodules

Many a field researcher has sliced open a nodule to seek evidence that it contains leghemoglobin (Figure 5.14) and indeed the leghemoglobin content of nodules is correlated with the ability of the nodule to fix N_2 (Riley and Dilworth 1985). Sometimes researchers like to quantify the amount of leghaemoglobin in nodules and this is best expressed per unit of nodule or bacteroid tissue.

Figure 5.14   A cross section through multiple 'lupinoid' nodules on the root of *Lupinus angustifolius* showing strong leghemoglobin content

Leghemoglobin can be most conveniently measured by the pyridine hemochromogen assay (Appleby and Bergersen 1980; Dilworth 1980). The methods described below are used in the CRS for extraction of leghemoglobin from nodules (Dilworth 1980; Riley and Dilworth 1985; Melino *et al.* 2012), and estimation of leghemoglobin concentration (Dilworth 1980; Bergersen *et al.* 1973; Bisseling *et al.* 1978).

5.11.4.1 Extraction of leghemoglobin from nodules

1. Mix fresh root nodules (usually 100–200 g) with about four volumes of cold (4°C) 100 mM sodium phosphate buffer (6.8 pH).

2. Macerate in a mortar.

3. For extraction from soybean and lupin nodules, the addition of polyvinylpyrrolidone in a ratio of 0.3 g per g nodules is recommended to remove polyphenols (Dilworth 1980).

4. For extraction from faba bean (*Vicia faba*) nodules, the addition of 2.5 mM EDTA is recommended to inhibit polyphenol oxidase activity (Dilworth 1980).

5. Filter the macerate through two layers of muslin (miracloth or cheesecloth). This step is useful to remove a lot of plant debris when extracting from large nodules (*e.g.* lupin, soybean, pea, faba bean) but may not be necessary when extracting from small nodules (*e.g.* clovers, medics, serradella).

6. Centrifuge the macerate at 100 g for 15 minutes to pellet plant debris.

7. Discard the nodule debris and collect the supernatant then centrifuge at 20,000 g for 20 minutes at 5°C to remove bacteroids and other suspended material.

8. Measure the heme concentration of the resulting supernatant by the hemochrome method as described below.

5.11.4.2 Measurement of leghemoglobin concentration

1. To a volume of the supernatant add an equal volume of 4.2 M pyridine in 0.2 M NaOH and mix. *NB: Pyridine is very toxic*

2. The resulting mixture is divided between two 1 mL cuvettes.

3. The hemochrome in one cuvette sample is reduced by adding a few crystals of sodium dithionite ($Na_2S_2O_4$). *Very little dithionite is needed.*

4. The hemochrome in the other cuvette sample is oxidised by adding a few crystals of potassium hexacyanoferrate (III) aka. potassium ferricyanide $K_3[Fe(CN)_6]$. *Very little is needed.* Alternatively, add 0.01 mL of 3 M $K_3[Fe(CN)_6]$.

5. The differential absorbance at 556 nm (ΔA_{556nm}) and 539 nm (ΔA_{539nm}) is determined and the heme concentration (mM) is calculated using a millimolar extinction coefficient of 23.4.

Leghemoglobin concentration (mM) of the supernatant is calculated using the following formula:

$$\text{Lb concentration (mM)} = \frac{(\Delta A_{556mm} - \Delta A_{539mm}) \times 2}{23.4}$$

5.11.4.3 Purified preparations of leghemoglobin

Specific methods are described in Dilworth (1980) for isolation of purified leghemoglobin from soybean, broad bean, kidney bean and yellow lupin. These preparations can be assayed without needing to use an oxidised sample. Simply, equal volumes of 4.2 M pyridine in 0.2 M NaOH and purified leghemoglobin solution are mixed and the resulting hemochrome reduced by adding a few crystals of sodium dithionite ($Na_2S_2O_4$).

Absorbance at 556 nm is measured against a reagent blank, the heme concentration (mM) is calculated using a millimolar extinction coefficient of 34.6:

$$\text{Lb concentration (mM)} = \frac{A_{556mm} \times 2}{34.6}$$

5.12 Outcomes from authentication and effectiveness experiments

There can be a range of outcomes in any authentication experiment. There may be specificity exhibited for nodulation or for effectiveness and this may be expressed *within* a legume species (*i.e.* between cultivars) *between* legume species or *between* legume genera.

5.12.1 Specificity between cultivars *within* a legume species

A trait often sought in strains for commercial application in agriculture is an ability to fix N_2 optimally with a range of cultivars of the target host legume species. For example, USDA110 was considered broadly effective across a wide range of soybean breeding lines and cultivars and was sown widely on a number of continents (Hungria *et al.* 2006). However, strains like USDA110 are the exception rather than the rule. Clover strain TA1 was used commercially in Australia for many years but did not nodulate clover cv. Woogenellup effectively (Gibson 1968). We must be aware that legume cultivars within species may differ in their reaction to rhizobial strains. This is clearly illustrated below; in the early phase of the domestication of *Biserrula pelecinus* (Howieson *et al.* 1995) it was discovered that some strains of mesorhizobia could fix N_2 with the white flowered form, but not with the blue flowered form of this legume (Figure 5.15).

Figure 5.15　Specificity for N_2 fixation within a legume species. A contaminant seed of the white flowered form of *Biserrula pelecinus* (on the right of the pen in this row of plants) has nodulated and grown well when inoculated with a single strain of *Mesorhizobium ciceri* bv *biserrulae* whereas the many seedlings of the blue flowered form (all other plants) are small and N-deficient, indicating lack of effective nodulation.

5.12.2 Broad specificity across legume species *within* a genus

Some legume genera are widely exploited commercially, and the clovers and medics are good examples. Valuable rhizobial strains for these genera are those that fix optimally across the wide range of the species that have been exploited in agriculture. Figure 5.7 illustrates a clover nodulating strain (WSM1325) that is highly effective for N$_2$ fixation across a wide range of species of *Trifolium*. It is compared with a former inoculant quality strain (WU95) that was adequate in an era prior to the wider domestication of the *Trifolium* spp. in Australia (Howieson *et al.* 2000).

5.12.3 Broad specificity *across* legume genera

Researchers and manufacturers may also require strains that are effective across a range of legume genera. A relevant example here is that *Lens*, *Vicia*, *Lathyrus* and *Pisum* all nodulate with *Rhizobium leguminosarum* bv *viceae* and manufacturers of inoculants prefer to produce one strain to suit all these genera wherever possible. Such strains are rare but do exist. Similarly, certain strains of bradyrhizobia may effectively nodulate *Cajanus*, *Lablab* and *Macrotyloma*. Selecting inoculant strains with a broad effective host-range, which thus reduces the cost of manufacture, is always an important consideration in any selection program.

5.13 References

Appleby C.A. and Bergersen F.J. 1980 Preparation and experimental use of leghemoglobin. Pp 315–335 in 'Methods for evaluating biological nitrogen fixation', ed. by F.J. Bergersen. Wiley-Chichester.

Bergersen F.J., Turner G.L. and Appleby C.A. 1973. Studies of the physiological role of leghaemoglobin in soybean root nodules. Biochimica et Biophysica Acta 292, 271–282.

Bisseling T., Van Den Bos R.C. and Van Kammen A. 1978. The effect of ammonium nitrate on the synthesis of nitrogenase and the concentration of leghemoglobin in pea root nodules induced by *Rhizobium leguminosarum*. Biochimica et Biophysica Acta 539, 1–11.

Broughton W.J. and Dilworth M.J. 1970. Methods in legume-rhizobium technology: plant nutrient solutions. Pp 245–249 in 'Handbook for rhizobia', ed. by P. Somasegaran and H.J Hoben. Springer-Verlag: New York.

Dilworth M.J. 1966. Acetylene reduction by nitrogen-fixing preparations from *Clostridium pasteurianum*. Biochimica et Biophysica Acta 127, 285–94.

Dilworth M.J. 1980. Leghemoglobins. Methods in Enzymology 69, 812–823.

Ewing M.A. and Robson A.D. 1990. The effect of solution pH and external calcium concentration on the early growth and nodulation of several annual *Medicago* species. Australian Journal of Agricultural Research 41, 933–939.

Fred E.B., Baldwin I.L. and McCoy E. 1932. Root nodule bacteria and leguminous plants. University of Wisconsin Press: Madison, WI.

Gibson A.H. 1968. Nodulation failure in *Trifolium subterraneum* L. cv. Woogenellup (Sum. Marrar). Australian Journal of Agricultural Research 19, 907–918.

Howieson J.G., Loi A. and Carr S.J. 1995. *Biserrula pelecinus* L.—a legume pasture species with potential for acid, duplex soils which is nodulated by unique root-nodule bacteria. Australian Journal of Agricultural Research 46, 997–1009.

Howieson J.G., O'Hara G.W. and Carr S.J. 2000. Changing roles for legumes in Mediterranean agriculture: developments from an Australian perspective. Field Crops Research 65, 107–122.

Hungria M., Franchini J.C., Campo R.J., Crispino C.C., Morales J.Z., Sibaldelli R.N.R., Mendes I.C. and Arihara J. 2006. Nitrogen nutrition of soybean in Brazil: contributions of biological N_2 fixation and N fertilizer to grain yield. Canadian Journal of Plant Science 86, 927–939.

Jensen H.L. 1942 Nitrogen fixation in leguminous plants. I. General characteristics of root nodule bacteria isolated from species of *Medicago* and *Trifolium* in Australia. Proceedings of the Linnean Society NSW 67, 98–108.

Munns D.N. 1968. Nodulation of *Medicago sativa* in nutrient solution. I Acid sensitive steps. Plant and Soil 28, 129–146.

Norris D.O. and Date R.A. 1976. Legume Bacteriolgy. Pp 134–74 in 'Tropical Pasture Research—Principles and Methods', ed. by N.H. Shaw and W.W. Bryan. Commonwealth Bureau Pastures & Field Crops Bulletin No.51. Tropical Pasture Research—Principles and Methods.

Melino V.J., Drew E.A., Ballard R.A., Reeve W.G., Thomson G., White R.G. and O'Hara G.W. 2012 Identifying abnormalities in symbiotic development between *Trifolium* spp. and *Rhizobium leguminosarum* bv. *trifolii* leading to suboptimal and ineffective nodule phenotypes. Annals of Botany 110, 1559–1572.

Mwenda G. 2016. Diversity, effectiveness and competitiveness of *Phaseolus vulgaris* nodulating bacteria from Kenya. PhD thesis, Murdoch University.

Riley I.T. and Dilworth M.J. 1985. Cobalt requirement for nodule development and function in *Lupinus angustifolius* L. New Phytologist 100, 347–359.

Somasegaran P. and Hoben H. 1994. Handbook for rhizobia. Springer-Verlag: New York.

Staden J.V., Brown N.A.C, Jäger A.K. and Johnson A. 2000. Smoke as a germination cue. Plant Species Biology 15, 167–178.

Sylvester–Bradley R. and Kipe-Nolt J. 1988. Legume–Rhizobium Symbiosis. International Center for Tropical Agriculture (CIAT): Colombia.

Terpolilli J., O'Hara G.W., Tiwari R.P., Dilworth M.J. and Howieson J.G. 2008. The model legume *Medicago truncatula* A17 is poorly matched for N_2 fixation with the sequenced microsymbiont *Sinorhizobium meliloti* 1021. New Phytologist 179, 62–66.

Vincent J.M. 1970. A manual for the practical study of root-nodule bacteria. IBP handbook 15. Blackwell Scientific Publications: Oxford.

CHAPTER 6
Counting rhizobia

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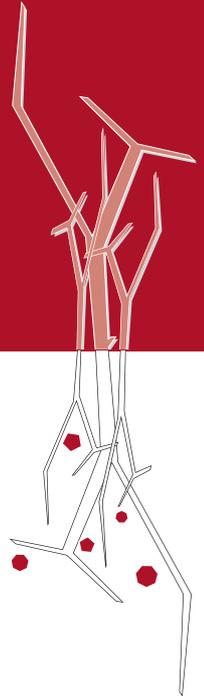
6.1 Introduction

The enumeration of rhizobia is valuable for the assessment of rhizobial populations in soil and how they vary, to follow the growth of cultures in the laboratory or to assess the number and viability of rhizobia in commercial inoculants for quality control. The number of rhizobia in soil is dynamic and varies within and between seasons, so any enumeration must be set in context. Enumeration of rhizobia is labour and resource intensive and should only be undertaken when the information is vital.

There are few new techniques with which to quantify rhizobia. Despite the molecular and genomic eras, there has emerged no robust DNA-based technology to replace serial dilutions and direct or indirect quantification. The qPCR and MISEQ methodologies cannot currently differentiate rhizobial strains reliably nor distinguish sufficiently between DNA from live cells and DNA from dead cells to make them effective substitutes for the standard viable counting techniques discussed below, although this may change. This chapter, therefore, reiterates appropriate and well-utilised approaches to enumeration of rhizobia in soil, inoculants and in vitro.

6.2 Serial dilution

The principle in counting bacteria by dilution is to serially dilute them to reduce the bacterial density to the level where individual cells can be differentiated. This may be, for example, as live cells under the microscope, as colonies that grow on plates from single cells, or estimated in the plant-infection technique (with the principle that a single cell can multiply to initiate an infection). Serial dilution can be applied to liquids, solids or soils. A 10-fold serial dilution is most often used

(Figure 6.1) but if the number of rhizobia is expected to be low then a five-fold dilution can be adopted.

For dilution of soils or solids (such as a clay inoculant) a large mass (*e.g.* 10 g) is usually selected for initial dilution as quite often this will represent a subsample from a larger source. Diluents must be sterile and can be high-purity water, rhizobial growth medium (Chapter 3) or a more osmotically-friendly solution such as 0.85% (w/v) NaCl or dilute phosphate buffer (*e.g.* Chapter 13, Section 13.4) as required.

6.2.1 Procedure for serial dilution

1. Autoclave chosen diluent in bulk (*e.g.* 2 L in a 5 L bottle) and allow to cool.

2. Aseptically dispense 90 mL diluent into a pre-sterilised 250 mL flask or bottle and five to eight lots of 9 mL in a 30 mL McCartney (pre-sterilised) for each sample that is to be enumerated*.

3. Place a 10 g subsample or 10 mL of rhizobial suspension into the 90 mL to form a 10^{-1} dilution and shake for five to 30 minutes on a shaker. Glass beads can be added to the vial or flask to help the release of rhizobia from the solid matrix.

4. With a sterile pipette or micro-pipette with a sterile tip, transfer 1.0 mL of the 10^{-1} dilution to a bottle containing 9.0 mL of diluent, forming the 10^{-2} dilution. Shake well or use a vortex mixer for 20 sec to mix the suspension. It is sometimes convenient to cut off the tip of the pipette for this initial dilution if solids are present that might block the opening.

5. With a new pipette or pipette tip, transfer 1.0 mL of the 10^{-2} dilution to another bottle containing 9.0 mL of diluent, to form a 10^{-3} dilution, and so on.

6. For soils, it is rare to encounter populations of rhizobia >10^7 cells/g. For inoculants, this concentration may be 10^9 cells/g. If counting a broth culture, a slightly turbid suspension contains approximately 10^7 cells/mL.

7. At the completion of the dilution, and starting with the most dilute sample, apply the dilutions to the medium that provides enumeration. If the dilutions contain only rhizobia, they can be directly plate counted (see Section 6.3 and Figure 6.2). For counting non-sterile systems, the diluents may be inoculated onto a seedling growing on an agar slant, O'Hara vial or growth pouch (see Chapter 5 and Figure 6.1). This method of enumeration is called the MPN (Most Probable Number) count.

*Some laboratories use smaller volumes of sample and diluent, especially for laboratory cultures. A 10-fold decrease will be obtained by using Eppendorf tubes containing 0.9 mL of diluents to which 0.1 mL samples are added. However, the procedure should be verified before adoption, as micro-pipettes must be checked regularly for accuracy. The step of mixing the samples is even more critical under

those circumstances and it is also recommended that the sterile diluent should be distributed into sterile Eppendorf tubes under the hood, as any evaporation will significantly affect the dilution.
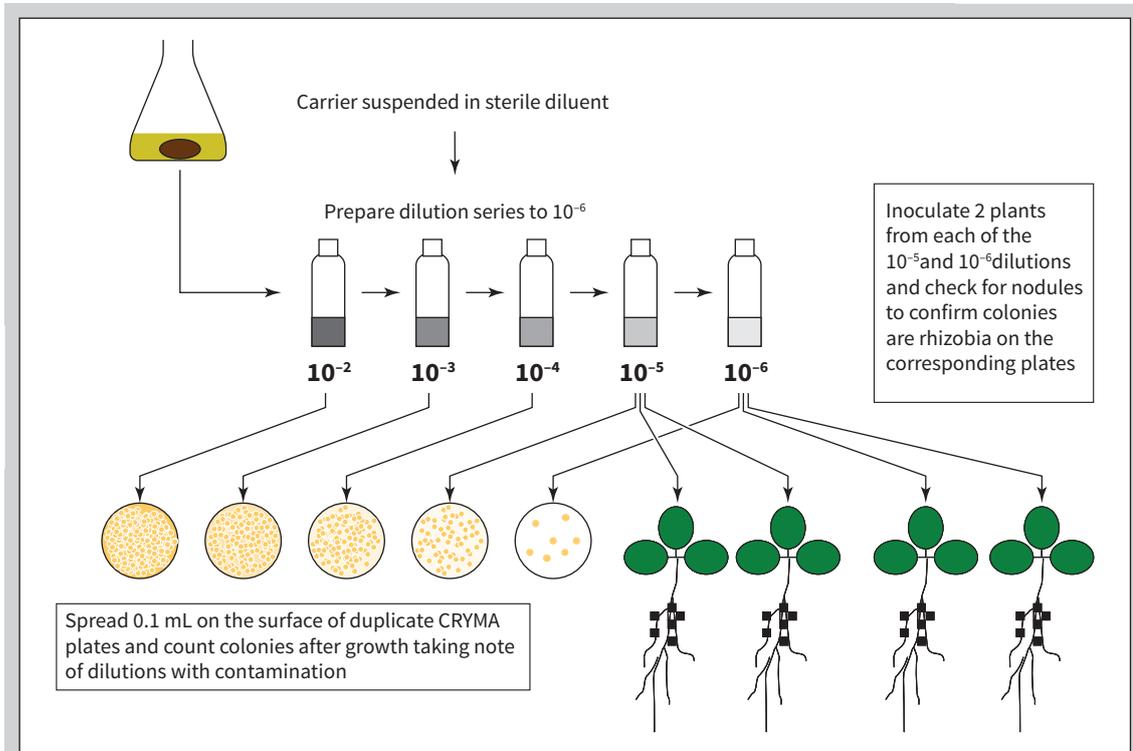
Figure 6.1   Scheme of a serial dilution series as might be applied to an inoculant carrier or to inoculated seed (see Chapters 8 and 9). A sample of the carrier (*e.g.* peat, granule or seed) is shaken in a bulk diluent which represents the first level of dilution. This is then serially diluted with a sample at each level of dilution directly plated or inoculated onto the legume to achieve enumeration via plant infection.

6.3 Plate counts of rhizobia in sterile diluent

The counting of microorganisms on plates, following dilution, is also called direct counting. Two main methods can be used: the drop-plate method and the spread-plate method. For rhizobia, the usual culture medium for counting viable cells is the traditional YMA (Chapter 3) with 15–20 mL of medium per plate. Other media can be used, *e.g.* containing specific antibiotics to suppress contaminants, Congo Red to observe contaminants, or ½ LA to reduce gum production (Chapter 3). The latter may be important when colonies overgrow and thus obscure each other in lower dilution plates.

6.3.1 Spread-plate counting

1. Inoculate 0.1 mL of the serial dilutions desired (Section 6.2.1) (*e.g.* 10^{-5}, 10^{-6} and 10^{-7}) on the surface of the culture medium in Petri dishes (Figure 6.1).

2. Spread the 0.1 mL aliquot over the culture medium with a sterilised L-shaped glass spreader (or equivalent, *e.g.* a Drigalski loop). There should be at least three separate replicate plates for each dilution.

3. After inoculation and absorption of the inoculum into the agar, the plates are placed in an incubator at approximately 28°C, inverted and allowed to grow for a period of two to eight days (according to the growth rate of the species; see Chapter 3).

4. Count the number of colonies on plates where colonies are well separated. If colony numbers are low, variation between plates and errors may be large. If colony numbers are too high, overcrowding may result in an underestimation of numbers. Many texts recommend counting between 30 and 300 CFU (Colony Forming Units) per plate to give statistical robustness.

Calculation: Multiply the average number of CFU on the three Petri dishes by the inverse of the dilution that gave the reading in the range 30 to 300 CFU by 10 (to correct for the 0.1 mL used).

Example: Assuming that the average of three plates was 75 CFU and that the dilution that gave this reading was 10^{-6}, calculate:

Average = 75

Correction factor = 10

Dilution of the suspension = 10^{-6}

CFU = $75 \times 10 \times 10^6 \Rightarrow 7.5 \times 10^8$ CFU/mL

6.3.2 Drop-plate counting

A drop of approximately 25 µL of the desired serial dilutions (*e.g.* 10^{-5}, 10^{-6} and 10^{-7}) is deposited on a sector of a Petri dish containing solid culture medium.

NB: if the plate is too wet, the drop will spread uncontrollably and if the plate is too dry, the drop may bounce. To avoid these scenarios, cool the agar growth medium to approximately 50°C before pouring plates. This reduces condensation on the lid which can then fall onto the agar surface. Pre-incubate plates for one hour at 28°C before use.

1. As a suggestion, plates can be divided into six sectors, using two sectors of each plate as replicates per dilution. In this way, one plate is sufficient to count three dilutions with two replicates, each with two drops per replicate (Figure 6.2).

2. Calculate the number of plates required for the exercise.

3. Label the base of the plates before inoculation.

4. Using a sterile micro-pipette tip, or a Pasteur pipette, and working from the most dilute suspension, place a drop from a height of approximately 1–2 cm

onto the appropriate sector of the plate. Be careful to avoid splashing from drops that can cause cross contamination between dilutions.

5. Repeat for replicate plates then move to the next lower dilution step and repeat the procedure.

6. There is no need to change the pipette, or tip, but the weight of the drop should be calibrated on a sensitive scale before the tip is discarded.

7. After absorption of the inoculum, the plates are inverted and placed in an incubator at approximately 28°C and allowed to grow for one to eight days, depending on the strain (see Chapter 3 for growth rates). Inspect daily for emergence of colonies.

8. Count the colonies in the sector that produces 5–50 CFU per drop (sectors 2 or 3 in Figure 6.2) either directly or through a dissecting microscope. It is convenient to mark each colony with a fine-tipped marker pen while counting to avoid confusion.
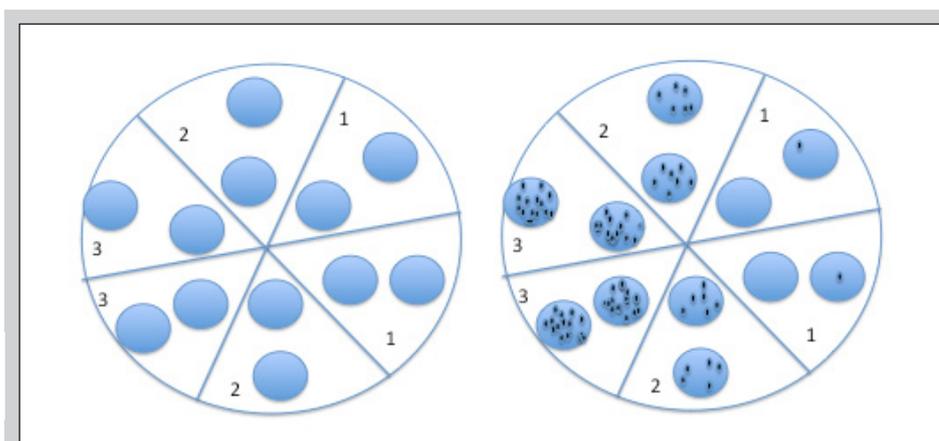
Figure 6.2a   An outcome of counting using the drop-plate method. The fresh drops applied to the plate (left) illustrate how to position the drops for maximum efficiency. After incubation (right) showing the expected dilution in number of colonies from sector 3 through to sector 1.
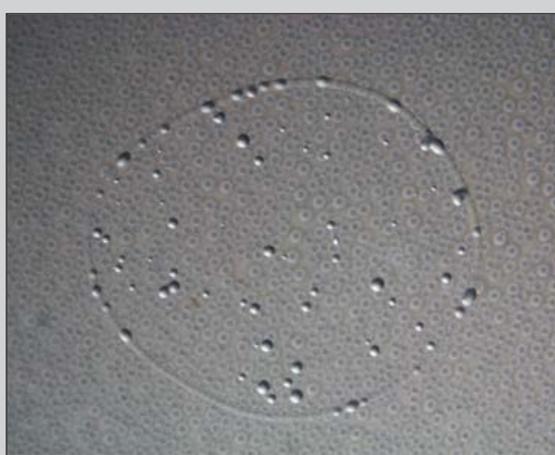
Figure 6.2b   The emergence of colonies in a drop at approximately the correct stage for counting. Colonies often concentrate on the circumference of the drop with this technique.

Calculation: Multiply the average number of CFU per drop × the inverse of the dilution of the two sectors that provided these readings × the correction factor for volume of the drop (40, for the aliquot of 25 μL), to give live cells per mL of original suspension.

Example: Assume that the average of the two drops in sector 2 above was 6.25 CFU/drop for the 10^{-6} dilution:

Average = 6.25

Correction factor = 40 (for a drop weight of 0.025g = 25 μL)

Dilution factor = 10^{-6}

CFU = $6.25 \times 10^6 \times 40 \Rightarrow 2.5 \times 10^8$ CFU/mL

6.4 Evaluating rhizobial survival on seed

Survival of rhizobia on seed may be assessed by applying inoculant to seed at the recommended rate (see Chapter 8) then counting viable cells over time. For this purpose, seeds should be surface-sterilised prior to inoculation (Chapter 5, Section 5.8.2) to reduce the effect of competition or antagonism by microorganisms already present on the seed surface. The recommended rate of inoculant applied to seed will vary according to the number of rhizobia per unit weight of inoculant and the desired number per seed. As an example, the recommended rate of application of Australian peat inoculant for subterranean clover containing $>10^9$ CFU/g is 250 g moist peat inoculant to 50 kg seed.

Survival on seed of rhizobia from peat cultures may be tested in the laboratory using the following protocol (also see Figure 6.1).

1. Prepare a slurry by mixing 25 g peat inoculant with 100 mL adhesive solution (rate will vary depending on manufacturer's recommendations and rhizobial number per g of inoculant). The peat culture should be homogenised before sampling by rolling the packet between the fingers. The quantity of peat used should be sufficient to adequately represent the total rhizobial population within the packet.

2. Apply 1 mL of slurry to 50 g seed (or equivalent proportions) in a small beaker, container or plastic bag and mix or shake thoroughly to distribute inoculant over the seed surface. Note that if the adhesive solution is viscous and difficult to pipette, it is better to weigh the quantity of slurry onto the seeds.

3. If coating inoculated seed with limestone or another dry powder, the powder should be applied while seeds are wet and stirring or shaking should be continued for another 5–10 secs until the seeds are evenly coated.

4. Sample seeds as soon as possible after coating, noting the time, and count the viable number of rhizobia on the seed surface by dilution.

5. Remaining seeds should be spread on a clean surface to air dry and samples taken at intervals to count viable rhizobia during drying and storage (*e.g.* one hour to 72 hours).

In general, the number of cells per seed counted immediately after coating provides an actual inoculum number, and counts after one hour may indicate natural death of rhizobia, incompatibilities with water-soluble toxins from the natural seed coat, seed applied chemicals (*e.g.* fungicide) or polymer adhesive. Some strains of *Burkholderia* can lose viability completely within four hours after coating onto their legume host or onto glass beads (Howieson *et al.* 2013) so timing of measurements for survival on seed may vary for each strain–seed combination.

6.5 Indirect counts by plant infection to estimate Most Probable Number (MPN)

The Most Probable Number (MPN) method is an indirect way of counting rhizobia in any non-sterile medium such as soil, inoculants or on seed. It involves inoculation of a compatible legume grown under aseptic conditions with the increasing dilutions. Nodulation indicates that infective rhizobia were present in the inoculum; no nodulation indicates they were absent. The MPN relies on the assumption that one viable rhizobial cell can give rise to a nodule but it is implicit that this cell must multiply after the inoculation event to produce sufficient nod-factor to begin the nodulation process. For the method to give valid results, all replicates of the last dilution should be negative.

After the addition of the inoculum, the test requires 10 to 30 days until the nodules form. With basic microbiological skills, three replicates give adequate results, although many laboratories use four replicate plants at each dilution step.

The MPN assays can be conducted in test tubes, plastic bags, pots of sand or Leonard jars (Chapter 5) or adaptations of these vessels that allow cell multiplication in the rhizosphere, with one seedling growing per unit and at least three replicates.

The level of dilution required for enumeration of viable rhizobia will depend on the number of microorganisms per g of sample. For example, if counting cells in a high-quality peat containing 10^9 CFU/g peat, the 10^{-7} and 10^{-8} dilutions should be sufficient to reduce colony numbers to 10 and one respectively when 1 mL is used to inoculate plants. If a pre-inoculated seed product contains 10^3 CFU/seed then the 10^{-1} and 10^{-2} dilutions should reduce colony numbers to 10 and one respectively (where initial suspension of 100 seeds in 100 mL of diluent is considered the 10^0 dilution).

6.5.1 Counting multiple species of rhizobia simultaneously

More often than not soils contain several different species/genera of rhizobia and these may be of interest to the researcher. An adaptation in the CRS laboratories at Murdoch University is that the MPN is often used to count two or three species of rhizobia in the soil simultaneously (*e.g. S. medicae*, *M. ciceri* bv *biserrulae*, *R. leguminosarum* bv *trifolii*). The three host seedlings are planted in the one vessel. One mL of inoculant is placed onto the roots of each seedling which are sown as spaced plants in a 5 cm pot containing steamed sand (Chapter 5).

1. Sow seedlings into chosen vessel (Chapter 5) one week before commencing the MPN allowing 10% extra for mortality and for controls.

2. Prepare diluents and undertake dilution series (Section 6.2).

3. Inoculate 1 mL from each dilution onto the roots of each seedling(s), repeating for each plant replicate.

4. Place inoculated plants into glasshouse and take steps to avoid contamination (Chapter 5).

5. After three to six weeks, inspect the plants in each unit for the presence or absence of nodules.

6. Record nodulation on each plant in every dilution. The presence of at least one nodule indicates a positive result.

7. Utilising MPN tables or the MPNES computer program* (Woomer 1994) calculate the most probable number of cells in the original sample.

Figure 6.3   A multiple MPN with each unit containing a seedling of biserrula and sub-clover (left). The labels a-e, represent soil dilutions of 10^{-2}–10^{-6} with four replicates of each. The units are watered through the pipette tip and further protected from contamination by Perspex shields (right).

Example

An example of a calculation for a three replicate experiment of the type shown in Figure 6.3 is given below. For purposes of calculation, three dilutions inoculated onto sub-clover (a: 10^{-2}; b: 10^{-3}; and c: 10^{-4}) are nodulated; the next dilutions (d: 10^{-5} and e: 10^{-6}) have resulted in no nodules.

Search Table 6.1 columns D1 (a), D2 (b) and D3 (c) with the number of units (reps) testing positive for each dilution to determine factor (f). This factor from Table 6.1 is then multiplied by the lowest dilution before all units were negative for nodulation.

Number of rhizobia = f × d

Where:

f = factor from Table 6.1

d = lowest dilution before all units were negative

Assuming that a soil gave three positive units at D1 (a), three at D2 (b), one at D3 (c) and none at d or e (10^{-5} or 10^{-6})

Factor (f; Table 6.1) for 3–3–1 = 46.208

Lowest dilution = 10^{-4}

Number of cells = $46.208 \times 10^4 = 4.62 \times 10^5$ cells per g of soil

However, the biserrula in the example above was nodulated at d (the 10^{-5} dilution) giving three positive reps in b, two in c and one in d.

The calculation for biserrula rhizobia in the soil is:

Factor (f, Table 6.1) for 3–2–1 = 14.938

Lowest dilution = 10^{-5}

Number of cells = $14.938 \times 10^5 = 1.5 \times 10^6$ cells /g soil

NB: there are further tables for estimating the number of viable cells in Vincent (1970), Brockwell *et al.* (1975) and Woomer *et al.* (1990). Table 6.1 is from Andrade and Hamakawa (1994) estimated according to Cochran (1950). Andrade and Hamakawa (1994) also provide tables for three, four and five replicates.

Table 6.1 Estimation of number of cells of rhizobia evaluated by the Most Probable Number infection method in plants. Adapted from Andrade and Hamakawa (1994).

| Dilution | | | Factor | Prob.% | Interval of confidence | |
D1	D2	D3	MPN		minimum	maximum
0	0	1	0.300	0.332	0.073	1.675
0	0	2	0.601	0.001	0.186	2.176
0	0	3	0.904	0.000	0.329	2.645
0	1	0	0.305	3.365	0.074	1.698
0	1	1	0.611	0.045	0.189	2.208
0	1	2	0.917	0.000	0.334	2.684
0	1	3	1.224	0.000	0.498	3.140
0	2	0	0.620	0.156	0.192	2.239
0	2	1	0.930	0.004	0.338	2.722
0	2	2	1.242	0.000	0.505	3.185
0	2	3	1.555	0.000	0.686	3.636
0	3	0	0.944	0.004	0.343	2.762
0	3	1	1.261	0.000	0.512	3.233
0	3	2	1.579	0.000	0.695	3.693
0	3	3	1.898	0.000	0.890	4.141
1	0	0	0.357	39.203	0.087	2.058
1	0	1	0.723	0.624	0.225	2.711
1	0	2	1.098	0.006	0.399	3.336
1	0	3	1.482	0.000	0.601	3.948
1	1	0	0.736	6.445	0.228	2.762
1	1	1	1.118	0.179	0.407	3.401
1	1	2	1.510	0.002	0.612	4.026
1	1	3	1.911	0.000	0.838	4.644
1	2	0	1.138	0.627	0.414	3.468
1	2	1	1.538	0.025	0.623	4.108
1	2	2	1.950	0.000	0.854	4.740
1	2	3	2.370	0.000	1.104	5.356
1	3	0	1.568	0.030	0.635	4.194
1	3	1	1.988	0.002	0.871	4.842
1	3	2	2.418	0.000	1.126	5.486
1	3	3	2.860	0.000	1.397	6.126
2	0	0	0.917	31.927	0.288	3.773
2	0	1	1.432	1.120	0.522	4.791
2	0	2	1.990	0.019	0.802	5.832
2	0	3	2.600	0.000	1.123	6.887
2	1	0	1.469	11.963	0.535	4.951
2	1	1	2.046	0.632	0.823	6.037
2	1	2	2.680	0.015	1.156	7.143
2	1	3	3.359	0.000	1.529	8.261
2	2	0	2.106	2.318	0.847	6.261

Table 6.1 (cont'd)   Estimation of number of cells of rhizobia evaluated by the Most Probable Number infection method in plants. Adapted from Andrade and Hamakawa (1994).

Dilution			Factor	Prob.%	Interval of confidence	
D1	D2	D3	MPN		minimum	maximum
2	2	1	2.763	0.170	1.191	7.420
2	2	2	3.478	0.005	1.579	8.598
2	2	3	4.240	0.000	2.009	9.782
2	3	0	2.855	0.216	1.229	7.726
2	3	1	3.602	0.021	1.632	8.966
2	3	2	4.408	0.001	2.082	10.217
2	3	3	5.254	0.000	2.574	11.478
3	0	0	2.312	34.098	0.871	12.822
3	0	1	3.850	3.099	1.511	17.662
3	0	2	6.348	0.158	2.434	22.749
3	0	3	9.538	0.004	3.706	27.927
3	1	0	4.272	37.433	1.664	21.327
3	1	1	7.488	6.579	2.777	28.088
3	1	2	11.520	0.649	4.380	35.159
3	1	3	15.878	0.032	6.485	42.502
3	2	0	9.324	32.817	3.331	38.555
3	2	1	14.938	12.507	5.569	50.581
3	2	2	21.470	2.470	8.652	64.067
3	2	3	29.170	0.235	12.543	79.220
3	3	0	23.970	36.594	9.128	139.550
3	3	1	46.208	42.767	17.836	240.763
3	3	2	109.849	44.442	38.227	478.767

6.6 Estimate of cell number by optical density

It is possible to determine the cell density of a suspension of a pure culture of rhizobia spectrophotometrically but this method does not allow an assessment of cell viability nor does it distinguish cell types. The advantage of measurement of cell growth by optical density is that it is very rapid. An example is the standardisation of suspensions of bacteria for competitiveness studies. However, a calibration curve has to be constructed for each strain by measuring optical density and determining CFU per mL by plate counts. Calibration curves may vary between strains, particularly if they produce exopolysaccharides.

1. Select 1 cm path length cuvettes (plastic or glass).

2. Set spectrophotometer to 600 nm and allow five minutes to warm up.

3. Insert cuvette containing 3 mL growth medium in the correct orientation and adjust to zero.

4. Replace growth medium with suspension to be assayed, preferably a log-phase culture.

5. Record OD, repeat with a second replication.

6. If OD is above 0.2*, dilute the sample to read in the range 0.05 to 0.2.

7. Construct calibration curve by diluting original culture with growth medium to produce at least five points between 0.01 and 0.4, and determine plate count at each dilution.

* Because the measured optical density of a suspension is due to light scattering by the suspended bacteria rather than light absorption, optical density figures above 0.2 at 600 nm are not linear with cell number and cultures should be diluted so that optical densities are less than 0.2. It is important to note that cell death starting from the stationary phase will reduce the relationship between viable cell number obtained in plate counts and the optical density which will include live and dead cells.

6.7 Direct counts under the microscope

It is possible to enumerate rhizobia directly in pure cultures and high-standard inoculants. There are some modern specialised techniques and equipment (*e.g.* Coulter counters) for counting cells directly from a culture medium but this section only describes standard laboratory methods.

Counts under the microscope produce total rhizobial numbers but do not differentiate between viable and non-viable cells. Results from microscope counts can be very useful to make quick decisions without having to wait for bacterial growth.

The use of calibrated chambers is well described in several textbooks (Vincent 1970; Somasegaran and Hoben 1994). The Petroff-Hausser chamber has a depth of 0.002 cm, with intermediate (4×10^{-4} cm^2) and small (2.5×10^{-5} cm^2) squares (Figure 6.4). With a known volume derived from the depth and the area of the squares, the number of rhizobia per mL can be calculated. The shallow depth allows a direct count of cells with normal bright field illumination. However, phase contrast microscopy gives better definition because unstained cells in bright field microscopy have essentially the same refractive index as the counting medium. A special coverslip is required for the use of 20× to 40× objectives; the 40× magnification phase contrast objective usually gives the better results. The counting limit in these chambers is eight to ten cells per intermediate square, corresponding to 10^6 cells/mL.
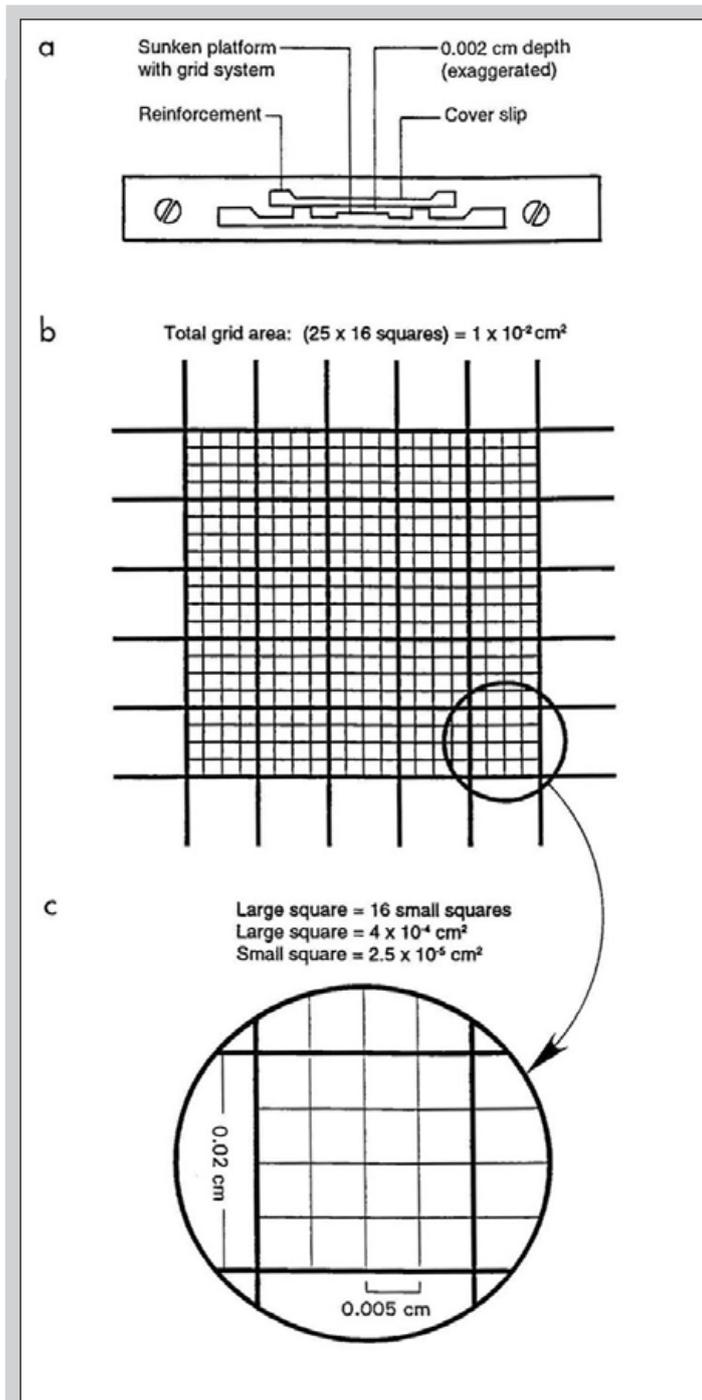
Figure 6.4 A representation of the components of a Petroff-Hausser counting chamber
(a) Cross section showing location of the sunken platform etched with the grid system.
(b) Top view of complete grid.
(c) Magnified view of an intermediate square containing 16 small squares.
(Image from Somasegaran and Hoben 1985)

6.7.1 Petroff-Hausser chamber

There are several precautions to be observed in the use of a Petroff-Hausser chamber.

1. The chamber and cover glass should be washed in detergent, rinsed several times in distilled water and dried in a dust-free environment. This will ensure that the liquid spreads evenly and prevents the formation of air bubbles during counting.

2. Bacterial cultures should be diluted several times (Section 6.2.1) to determine the best dilution to be used during the tests at each stage of growth. After dilution, the chamber should be washed again.

3. The diluted culture is added to the slide (after the coverslip is in place) with the aid of a micro-pipette or Pasteur pipette. The pipette should only touch the chamber and the liquid will flow by capillarity, thus avoiding leakage. If excess culture does escape, it should not be dried-off with filter paper. The coverslip should be removed and, along with the chamber, washed again, dried and the whole process restarted.

4. The image should be observed at a 40× magnification under phase contrast and the number of cells counted.

5. For cells that overlap a border, a criterion should be established, *e.g.* count a cell as 'in' if it overlaps the top or right border, and 'out' if it overlaps the bottom or left border.

Logarithmic growth phase cultures, where few cells are dead, give good results for counting viable cells; the advantage being the speed of obtaining results. It is only appropriate for liquid media without particles and requires trained people to make the meticulous observations needed for quick and reproducible counts.

Calculation: Table 6.2 can be used for planning counts of rhizobia in Petroff-Hausser chambers.

Table 6.2 Number of cells that can be counted without and with dilution (Vincent 1970)

Expected number of bacteria/mL ($\times 10^6$)	Dilution
<10	No need
10–100	No need
100–1,000	10-fold dilution
1,000–10,000	100-fold dilution

Calculation: Bacteria per mL of the initial suspension: Multiply d (dilution of the counted suspension) × n (number of cells per square) × f (factor for the size of square).

Example: Assume an average of 33 bacteria are counted in an intermediate square from 10^{-2} dilution.

Dilution (d) = 10^{-2}

Number of cells (n) = 33

Factor (f) = 1.25×10^6

Number of cells = $33 \times 10^2 \times 1.25 \times 10^6 \Rightarrow 4.125 \times 10^9$ cells/mL

Other useful information for counting in Petroff-Housser chambers is given in Tables 6.3 and 6.4.

Table 6.3 Area, corresponding volume and factor for sizes of squares in the Petroff-Hausser chamber

Area	Corresponding volume (mL)	Factor (f) (1/vol.)
Total enclosed squares ($25 \times 16 = 1 \times 10^{-2}$ cm²)	2×10^{-5}	5×10^4
Intermediate square (=16 smallest squares = 4×10^{-4} cm²)	8×10^{-7}	1.25×10^6
Smallest square (2.5×10^{-5} cm²)	5×10^{-8}	2×10^7

Table 6.4 Number of fields that have to be counted according to the number of bacteria per field of the Petroff-Hausser chamber (Vincent 1970)

Average number per square	Number of fields to be counted for the coefficient of variability	
	10%	5%
10	10	
20	5	20
40	3	10
80	2	5
100	1	4

6.7.2 Neubauer haemocytometer

For more dilute samples, a haemocytometer (0.01 cm depth) with Neubauer rulings can be used. In this methodology, bacteria can be stained with methylene blue (1 mL of 1% (w/v) methylene blue, freshly prepared) and counted after approximately 20 minutes. The main divisions of the Neubauer haemocytometer separate the grid into nine large squares, each of which has a surface area of 1.0 mm² and the depth of the chamber is 0.1 mm. Thus, the entire counting grid lies under a volume of 0.9 mm³. In this chamber, cells in squares have to be selected so that the total count is around 100 cells; the number needed for statistical significance if several cells are counted. Table 6.5 gives the parameters to estimate cell number in the haemocytometer.

Table 6.5   Area, corresponding volume and factor for size of square in the Neubauer haemocytometer

Area	Volume (mL)[1]	Factor (f) (1/vol.)
Total squares—9 large squares × 1 mm^2 (area) × 0.1 mm (depth) = 0.9 mm^3	9×10^{-4}	1.1×10^3
Large square—1 mm^2 (area) × 0.1 mm (depth) = 0.1 mm^3	1×10^{-4}	1×10^4
Small squares—1/25 mm^2 – 0.04 mm^2 (area) × 0.1 mm (depth) = 0.004 mm^3	4×10^{-6}	2.5×10^5

1,000 mm^3 = 1 cm^3 = 1 mL

The same formula used for the Petroff-Hausser can be also used for the haemocytometer.

Calculation: To determine the number of bacteria per mL for the initial suspension, multiply d (dilution of the counted suspension) × n (number of cells per square) × f (factor for the size of square).

Example: Assuming that 1 mL of a rhizobia culture of the 10^{-2} dilution gave an average of 38 bacteria in the small squares (*e.g.* five small squares resulting in 190 bacteria, therefore higher than 100 and statistically valid).

Dilution (d) = 10^{-2}

Number of cells (n) = 38

Factor (f) = 2.5×10^5

Number of cells = $38 \times 10^2 \times 2.5 \times 10^5 \Rightarrow 9.5 \times 10^8$ cells/mL

6.8 References

Andrade D.S. and Hamakawa P.J. 1994. Estimativa do número de células viáveis de rizóbio no solo e em inoculantes por infecção em plantas. Pp 63–94 in 'Manual de métodos empregados em estudos de microbiologia agrícola', ed. By M. Hungria and R.S. Araujo. EMBRAPA-SPI: Brasília, Brazil.

Brockwell J., Diatloff A., Grassia A. and Robinson A.C. 1975 Use of wild soybean (*Glycine ussuriensis* Regel and Maack) as a test plant in dilution-nodulation frequency tests for counting *Rhizobium japonicum*. Soil Biology and Biochemistry 7, 305–311.

Cochran W.X. 1950. Estimation of bacterial density by means of the "most probable number". Biometrics 6, 105–116.

Howieson J.G., De Meyer S., Vivas-Marfisi A., Ratnayake S., Ardley J.K. and Yates R.J. 2013. Novel *Burkholderia* bacteria isolated from *Lebeckia ambigua*—a perennial suffruticose legume of the fynbos. Soil Biology and Biochemistry 60, 55–64.

Somasegaran P. and Hoben H.J. 1994. Handbook for rhizobia—methods in legume rhizobium technology. Springer-Verlag: New York, USA.

Vincent J.M. 1970 Manual for the Practical Study of Root Nodule Bacteria. (International Biological Programme Handbook 15). Blackwell: Oxford, UK.

Woomer P.L. 1994. Most probable number counts. Pp 59–79 in 'Methods of soil analysis. Part 2. Microbiological and biochemical properties', ed. by R.W. Weaver, S. Angle, P. Bottomley, D. Bezdicek, S. Smith, A. Tabatabai and A. Wollum. (SSSA Book Series 5). Soil Science Society of America: Madison.

Woomer P. Bennett J. and Yost R. 1990. Overcoming the inflexibility of most-probable-number procedures. Agronomy Journal 82, 349–353.

Taxonomy and physiology of rhizobia

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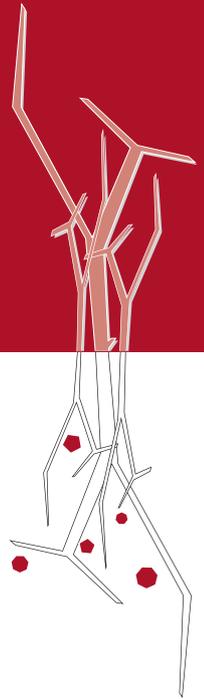
7.1 Taxonomy of rhizobia

Rhizobia are common Gram-negative soil-inhabiting bacteria distinguished by the feature that they contain genes required for nodulation (*e.g. nod*, *rhi*) and nitrogen fixation (*e.g. nif*, *fix*). These genes enable them to form a symbiotic association with legumes. Currently there are 15 genera of root nodule bacteria (Table 7.1) containing more than 120 described species.

7.1.1 Species concept for bacteria

The current species concept used for bacteria is necessarily rather subjective, with a species being considered as a distinct group of strains that share many stable distinguishing features and differ significantly from other groups of strains (Brenner *et al*. 2005a). The reader is referred to 'Bergey's manual of systematic bacteriology', in particular Volume 2, Part A Introductory Essays (Brenner *et al*. 2005b) for detailed information on current understanding and principles of prokaryotic taxonomy.

For prokaryotic organisms, the International Committee on Systematics of Prokaryotes (ICSP) defined in 2002 the 'Gold Standard' for species assignment based on a polyphasic approach using the following four characteristics.

1. Phenotypic and/or morphological similarity.

2. Genome similarity.

3. G+C content similarity.

4. 16S rRNA sequence similarity.

A prokaryotic species is thus recognised as a collection of strains that have: phenotypic and/or morphological similarity; genome similarity, as shown by DNA-DNA hybridisation >70%; G+C content similarity, as indicated by melting temperatures of DNA within 5°C; and 16S rRNA sequence similarity as indicated by <3% divergence. Established practice is that new species of prokaryotic organisms are formally published in the International Journal of Systematic and Evolutionary Microbiology (http://ijs.sgmjournals.org/), the official journal of record for bacterial names of the ICSP.

A further taxonomic sub-division of biovar has been used for some species of rhizobia, where the biovar is defined by the host range for nodulation as determined by *nod* and related genes being carried on the accessory genome (Jordan 1984). More recently the term 'symbiotic variant' or 'symbiovar' has been proposed to replace biovar, as a parallel term to pathovar in pathogenic bacteria (Rogel *et al.* 2011). At present, there are numerous recognised symbiovars in four genera of nodule bacteria: *Sinorhizobium* (*e.g. S. meliloti* sv. *ciceri*, *S. meliloti* sv. *meliloti*); *Rhizobium* (*e.g. R. leguminosarum* sv. *viciae*, *R. leguminosarum* sv. *trifolii*); *Mesorhizobium* (*e.g. M. ciceri* sv. *ciceri*, *M. ciceri* sv. *biserrulae*); and *Bradyrhizobium* (*e.g. B. japonicum* sv. *genistearum*, *B. japonicum* sv. *glycinearum*).

7.1.2 Polyphasic approach for taxonomy of rhizobia

The polyphasic taxonomic approach (Graham *et al.* 1991; Vandamme *et al.* 1996) used for determining species of rhizobia is overseen by the ICSP Subcommittee on the taxonomy of *Rhizobium* and *Agrobacterium* (Lindstrom and Young 2011). The subcommittee's website (http://edzna.ccg.unam.mx/rhizobial-taxonomy) provides useful information on:

1. up-to-date taxonomic information about all validly published and forthcoming rhizobial/agrobacterial taxa

2. hands-on tutorials on how to perform rigorous phylogenetic analyses using sequence data, from SSU rRNA sequence alignment and model fitting up to ML tree searches

3. information on current issues in rhizobial taxonomy, systematics and genomics, including scientific meetings and workshops

4. advice and recommendations from the subcommittee for microbiologists on good taxonomic/systematic practice.

The designation of species of rhizobia is based on the combination of classical microbiological characteristics (morphology, physiology, biochemistry) with the genome characteristics of DNA base composition (G + C content), DNA-DNA hybridization, sequences of the 16S ribosomal RNA genes and Fatty Acid Methyl Ester (FAME) profiles.

The usefulness of the 16S rRNA gene as a molecular marker for assessing phylogeny and taxonomy of prokaryotes has been broadly demonstrated, and the gene has also been applied to rhizobial taxonomy. Information about amplification of these genes is given in Chapter 11. However, there are problems with solely relying on 16S rRNA sequences, including: the issues of plesiomorphy; multiple copies of the 16S rRNA gene with small intragenomic differences (up to 5%) (Kampfer and Glaeser 2012); the conserved structure of the 16S rRNA gene sequence limiting resolution power below genus level (*e.g.* Willems *et al.* 2001; Gevers *et al.* 2005); and reports that genetic recombination and horizontal gene transfer occur among 16S rRNA genes (*e.g.* Gevers *et al.* 2005). Other regions of DNA have been proposed as alternative phylogenetic markers (Stackebrandt *et al.* 2002), such as the 16S-23S rRNA ITS region and genes (termed 'housekeeping genes') located in the core genome. These have a faster evolution rate than 16S rRNA but are conserved enough to retain genetic information. These genes need to be both broadly distributed among taxa and also be present in single copies within a given genome; the current consensus is that at least five genes are necessary for reliable taxonomic classification (Stackebrandt *et al.* 2002). Among the most used genes are *dnaK*, *dnaJ*, *glnA*, *gyrB*, *recA*, *gltA*, *glnII*, *rpoA*, *rpoB*, and *atpD*. There is also increasing discussion about the adoption, together with the 16S rRNA, of MLSA (Multi Locus Sequence Analysis) of housekeeping genes (Martens *et al.* 2007; Kampfer and Glaeser 2012) to replace the high cost and intensive work required for the DNA-DNA hybridization still required for species definition. Increased use of MLSA with rhizobia suggests that it may soon be an accepted tool to define new species; identities of 94–96% have been proposed to replace the 70% of DNA-DNA homology.

With wider availability of whole genome sequences, their use for species definition has been proposed. A first proposal was based on the ANI (Average Nucleotide Identity) of the whole genome by Konstantinidis and Tiedje (2005a,b) who suggested that the level of 94% identity of the shared genes between two strains would be equivalent to the 70% level of DNA-DNA hybridization. Further developments will follow as more rhizobia genomes are sequenced and available for analysis.

Further information on the current taxonomy of rhizobia can be found on Bevan Weir's New Zealand rhizobia website (http://www.rhizobia.co.nz/taxonomy/rhizobia).

7.1.3 Current rhizobia are in the Proteobacteria (Gram-negative bacteria)

All the current known rhizobia are in the Proteobacteria (the Gram-negative bacteria), a large phylum of more than 500 genera containing more than 2000 species. Prior to 2001, rhizobia were not known to occur outside the α-Proteobacteria, but

Moulin and collaborators (Moulin *et al.* 2001) reported the isolation from *Mimosa* spp. of rhizobia of the genus *Burkholderia* in the β-Proteobacteria, a result confirmed more broadly in *Mimosa* spp. by others (Gyaneshwar *et al.* 2011; Bournaud *et al.* 2013). The host range of beta-rhizobia has been extended further with their isolation from root nodules of South African shrub (Elliott *et al.* 2007) and forage legumes (Garau *et al.* 2009; Howieson *et al.* 2013; De Meyer *et al.* 2013a,b,c). It is important to be aware that the genera and species containing rhizobial strains also contain non-rhizobial strains and species (non-symbiotic bacteria lacking symbiotic genes) that can be isolated from soils and plants. To avoid confusion and misunderstandings, the status of a strain as rhizobial must be assessed by nodulation tests (Chapter 3).

The Alpha-Proteobacteria contains about 20 families of predominantly aerobic bacteria; they are characteristically oligotrophic (able to grow at very low substrate concentrations) and characterised by having some unusual metabolic modes such as methylotrophy, chemolithotrophy and N_2 fixation. There are currently 13 genera of Alpha-Proteobacteria containing legume-nodulating species: *Aminobacter*, *Azorhizobium*, *Bradyrhizobium*, *Devosia*, *Ensifer (Sinorhizobium)*, *Mesorhizobium*, *Methylobacterium*, *Microvirga*, *Neorhizobium*, *Ochrobactrum*, *Phyllobacterium*, *Rhizobium* and *Shinella*.

The Beta-Proteobacteria contains about 12 families of bacteria that are somewhat similar to the Alpha-Proteobacteria but tend to use substrates that diffuse from organic decomposition in anoxic zones in soils/sediments. Bacteria in this group are often characterised by having greater catabolic versatility than the Alpha-Proteobacteria. There are currently two nodulating genera of Beta-Proteobacteria: *Burkholderia* and *Cupriavidus*.

Table 7.1   Families and genera of root nodule bacteria and the approximate number of described species (Also shown are genera containing species used as commercial legume inoculants.)

Family	Genus	Number of described species
α-**Proteobacteria**		
Bradyrhizobiaceae	*Bradyrhizobium#*	15
Brucellaceae	*Ochrobactrum*	2
Hyphomicrobiaceae	*Azorhizobium*	3
	Devosia	1
Methylobacteriaceae	*Methylobacterium#*	1
	Microvirga	3
Phyllobacteriaceae	*Phyllobacterium*	1
	Aminobacter	1
	Mesorhizobium#	29
Rhizobiaceae	*Rhizobium#*	43
	Neorhizobium#	3
	Sinorhizobium/Ensifer#	13
	Shinella	1

Table 7.1 (cont'd)   Families and genera of root nodule bacteria and the approximate number of described species (Also shown are genera containing species used as commercial legume inoculants.)

Family	Genus	Number of described species
β-**Proteobacteria**		
Burkholderiaceae	*Burkholderia#*	6
	Cupriavidus	2

\# Indicates genera that contain species with strains used as commercial inoculants in agriculture.

Currently the number of formally described species of rhizobia is increasing at a rate of more than 10 species per year. However it is important to note that strains in only five genera of rhizobia are currently used as inoculants in agriculture, as indicated in Table 7.1.

7.2 Physiology of root nodule bacteria

Root nodule bacteria are predominantly aerobic chemoorganotrophic organisms with an oxidative metabolism. They are relatively easy to culture and grow very well in the presence of oxygen when utilizing simple carbohydrates and amino acids. Optimal growth for most strains occurs at a temperature range of 25–30°C and at a pH of 6–7 (Table 7.2).

7.2.1 Metabolic diversity

Being predominantly chemoheterotrophic organisms, most rhizobia are not particularly fastidious in their nutritional requirements. However, there are exceptions and some species do not grow on particular classes of carbon compounds; many species of *Bradyrhizobium* fail to grow on disaccharides. It is also important to recognise that some strains have quite particular metabolic activities, such as facultative autotrophy (*e.g. B. japonicum*, Boursier *et al.* 1988), denitrification (O'Hara and Daniel 1985), phototrophy (Evans *et al.* 1990; Young *et al.* 1991) and in a few strains an ability to fix N_2 *ex planta* (*e.g. Azorhizobium*, Dreyfus *et al.* 1988; *Bradyrhizobium*, McComb *et al.* 1975).

7.2.2 Growth rates in laboratory culture vary with genus and species

The growth rates of rhizobia in laboratory culture vary widely and can be used to differentiate them from contaminants, as outlined in Chapter 3. Table 7.2 below provides a guide to common growth characteristics of each genus.

Table 7.2 Growth rate, generation time, temperature and pH ranges for the genera of nodule bacteria

Genus	Growth rate	Mean generation time (h)	Optimal temp. range (°C)	Minimum growth temp. (°C)	Maximum growth temp. (°C)	Common pH range
α-**Proteobacteria**						
Aminobacter	fast	3–5	25–30	nr[1]	37–39	5–11
Azorhizobium	fast	3–5	25–30	12	43	5–8
Bradyrhizobium	slow	8–18	25–30	10–15	33–35	4–8
Devosia	fast	2–4	25–30	nr[1]	nr[1]	5.5–7
Mesorhizobium	moderate	4–15	25–30	4–10	37–42	4–10
Methylobacterium	moderate	5–7	30–37	nr[1]	37–39	5–7
Microvirga	fast	2–4	30–35	10	43	5.5–9.5
Neorhizobium	fast	2–4	25–30	10	37–40	5–10
Ochrobactrum	fast	3–4	25–30	20	39	5–10
Phyllobacterium	fast	2–4	25–30	4	37	6–8
Rhizobium	fast	2–4	25–30	10	40	4–10
Sinorhizobium	fast	2–4	25–30	10	44	5–10.5
Shinella	fast	2–4	25–30	nr[1]	nr[1]	5–11
β-**Proteobacteria**						
Burkholderia	very fast	1	25–30	10	40	4.5–9
Cupriavidus	very fast	1	25–30	nr[1]	39	nr[1]

nr[1] = not recorded

7.2.3 General description of rhizobia

Most colonies of nodule bacteria are white or creamy and opaque, are rarely translucent (except when young) rarely pigmented and do not adsorb Congo Red. However there are rare exceptions and some species of *Burkholderia* and *Sinorhizobium* can adsorb the dye, while other species are pigmented (Chapter 3, Section 3.5) so this general description is equivocal.

Colony characteristics change with media, time and conditions of incubation. The colony texture may be buttery or elastic, and can be determined by touching the colony surface with a loop. Colony appearance may be gelatinous, dry or wet. In general, colonies are flat or rounded, with a few strains producing colonies with a conical or fried egg-shaped elevation. Some strains of *S. medicae* will produce colonies with a 'doughnut' appearance (Howieson *et al.* 1988) as shown in Chapter 3, Figure 3.6. Table 7.3 provides general descriptions of the colony characteristics of different genera and species of nodule bacteria.

Table 7.3 Colony morphology and characteristic features for nodule bacteria grown on routine media (usually YMA unless otherwise noted) at 28°C

Genus	Colony morphology and characteristic features
Aminobacter	Colonies appearing on YMA within two to three days of incubation at 28°C are circular, opaque, convex, have a creamy colour and are usually a diameter of 2–3 mm.
Azorhizobium	Colonies on agar are circular and have a creamy colour.
Bradyrhizobium	Colonies are circular, convex and often translucent at first appearance, and when less than 1 mm in diameter (approx. five to eight days), but then strongly opaque and tending to dark grey with further maturity. Colonies of some strains are 3 mm in diameter or greater after four to seven days. Rarely gummy.
Devosia	Colonies are mucoid and pearl white on YMA medium; typical of fast growing rhizobia.
Mesorhizobium	Colonies as for *Rhizobium* but visible 24–48 hours later, weakly opaque after 72 hours, increasing with age.
Methylobacterium	Colonies visible in four to seven days. Colonies are glistening, smooth, raised, with entire margins, 0.5–1 mm in diameter after four to seven days incubation. After 7 days, colonies of some strains from species of *Listia* are pale pink to bright red-orange.
Microvirga	Colonies visible in 24 hours. Many colonies are light pink or pale orange to brown, convex, smooth, round and colouration develops 72 hours after visible colonies emerge, weakly opaque and mildly gummy. Some strains may show brown spot after seven to-10 days.
Neorhizobium	Colonies are circular, convex, white or cream coloured, semi-translucent or opaque, with a diameter of 1–4 mm within two to four days on YMA at 28°C.
Ochrobactrum	Cells form white mucoid colonies on YMA medium.
Phyllobacterium	Colonies are small, punctiform or circular, pearl white with a regular edge and colony diameter <1 mm after one to two days growth on YMA at 28°C.
Rhizobium	Colonies usually visible in 48 hours. Colonies are usually white or beige, circular, convex, semi-translucent or opaque, raised and mucilaginous, 2–4 mm in diameter within three to five days on YMA.
Sinorhizobium/Ensifer	Colonies visible in 48 hours, weakly opaque after 72 hours and gummy. Doughnut morphology (Figure 3.6) in *S. medicae* when first isolated from nodules.
Shinella	Colonies are circular, cream coloured, semi-translucent and 2–4 mm in diameter after three days incubation at 28°C on YMA medium
Burkholderia	Colonies visible in 24 hours. Colonies are white, smooth, round, convex, mostly with entire margins, 0.5–2.0 mm in diameter after 24 hours incubation. Colonies may acquire a yellow-brown tinge after 48 hours when viewed from below.
Cupriavidus	Colonies visible in 24 hours. Colonies are white, smooth, round, convex with entire margins, 0.5–4.0 mm in diameter after 24 hours incubation. Colonies may acquire a grey tinge after 48 hours when viewed from below (Figure 3.5).

7.2.4 Colony dimorphism

Some strains of rhizobia show two colony types (*e.g. Bradyrhizobium*, Sylvester-Bradley *et al.* 1988; *Sinorhizobium*, Bloem *et al.* 2002). In these situations it is important to take special care to eliminate the possibility of contamination. Sub-culturing of each colony type several times may be required to verify purity; plant testing to confirm authenticity is recommended (Chapter 4).

7.2.5 Purity tests for rhizobial cultures

Rhizobial cultures can be easily contaminated and it is essential in all stages of strain management that purity checks, as described below, are regularly used.

7.2.5.1 Colony characteristics

The easiest purity check is the recognition of typical colony morphology of a particular strain. A very important practical skill for a rhizobiologist to develop is an ability to recognise what are rhizobia and what are contaminants. With regular practice, and by repeated examination, individual strain characteristics can be readily recognised by the experienced researcher, and the presence of contaminants easily distinguished. It is essential that cultures are incubated for the appropriate period for the particular strain (*e.g.* incubation for more than seven days for *Bradyrhizobium* colonies to develop, especially those isolated from nodules). Table 7.4 lists a series of observations that can assist in differentiating common species of rhizobia.

Table 7.4 Colony morphology and characteristic features useful for distinguishing between species of some common for nodule bacteria

Genus and species	Colony morphology and characteristic features
Rhizobium leguminosarum bv trifolii	Colonies usually visible in 48 hours. Colonies are usually white or beige, circular, convex, semi-translucent or opaque, raised and mucilaginous, 2–4 mm in diameter within three to five days on YMA. When grown on ½ LA gummy strands from colony adhere to a loop.
Rhizobium leguminosarum bv viceae	As for Rlt described above, and weakly opaque at 72 hours. When grown on ½ LA gummy strands generally do not adhere to a loop.
Burkholderia dilworthii	Colonies visible in 24 hours. Colonies are white, smooth, round, convex with entire to slightly irregular margins, 0.5–2.0 mm in diameter after 24 hours. incubation. Colonies acquire a yellow-brown tinge with a brown centre after 48 hours when viewed from below. When grown on ½ LA containing Congo Red the agar shows a purple colour after eight to 10 days incubation (Figure 3.11).
Burkholderia sprentiae *Burkholderia phymatum*	As for Bd described above. Colonies are creamier and do not develop brown centre after 48 hours. When grown on ½ LA medium containing Congo Red colonies show dark red colour after eight to 10 days incubation.
Methylobacterium spp. (from Listia bainesii)	Colonies visible in four to seven days. Colonies are glistening, smooth, raised, with entire margins, 0.5–1 mm in diameter after four to seven days incubation. Colonies develop pink pigmentation within 24 hours. of becoming visible. After seven days colonies are 1–3 mm in diameter and pale pink to bright red-orange.
Methylobacterium spp. (from other legumes)	As above, except colonies are strongly opaque, not gummy and do not develop pink or orange colour.
Sinorhizobium medicae	Colonies visible in 48 hours. Doughnut morphology when first isolated from nodules, weakly opaque after 72 hours and gummy.
Sinorhizobium meliloti	Colonies visible in 48 hours. Do not form doughnut colonies, weakly opaque after 72 hours, often (but not always) drier than *S. medicae*.
Bradyrhizobium spp.	Colonies slow growing and translucent at first appearance when less than 1 mm in diameter (approx. seven to 10 days) but then strongly opaque and tending to dark grey with further maturity. Rarely gummy.
Bradyrhizobium japonicum	As above, but may reach 1–3 mm after five to eight days, immediately opaque, and elevated.
Bradyrhizobium elkanii	As for B spp., colonies irregular and non-elevated.

7.2.5.2 Gram stain and motility

Several characteristics are important and discriminating. Firstly, cells are usually motile in fresh broth cultures which can be examined under the microscope. While bacterial flagella are not visible with the light microscope unless stained, motility caused by movement of flagella is seen by direct microscopy of wet preparations. A fresh broth culture is required for best observations of motility. Freshly prepared slides should be viewed promptly since motility can be easily lost with prolonged exposure on a slide.

1. Using a sterile Pasteur pipette, take a sample of the broth culture.

2. Transfer a drop of the culture to the centre of a slide, cover with a coverslip.

3. Examine under the microscope with the 40× objective (you may need to reduce the illumination of your specimen in order to see the unstained bacteria).

4. Note that cells showing simple vibration—Brownian motion—are NOT motile; they need to show long 'swims' across the field.

Secondly, all rhizobia are Gram-negative, rod-shaped bacteria and do not produce spores. The Gram stain devised by the Danish scholar, Christian Gram, in 1884 is the most widely used differential stain in bacteriology and divides common bacteria into two groups: Gram-negative (which appear pink after Gram staining) and Gram-positive (which appear purple).

Protocol for the Gram stain

NB: use forceps to hold the slide for all manipulations involving dyes.

1. Take a clean slide out of the alcohol in the storage tub, flame off any alcohol and allow the slide to cool. Label the slide with pencil in the frosted area so that you know which sample it is.

2. Using a sterile loop, take a small amount of a bacterial colony from the agar plate and place it on the slide. Emulsify it with a small drop of sterile saline and spread the liquid over an area of about 1 cm square.

3. Air dry the smear or dry it gently high above a Bunsen burner flame.

4. Heat fix the dried slide by passing it three times through the hot part of the Bunsen flame with the smear side up. Use forceps to hold the slide. Allow the slide to cool.

5. At the staining rack, cover the smear with a few drops of crystal violet for 60 seconds.

6. Pour off the excess crystal violet and rinse with a gentle stream of running tap water.

7. Cover the smear with iodine and leave for 45 seconds.

8. Pour off the excess iodine, wash down the front and back of the slide with the acetone/alcohol solution until purple stain stops eluting and then immediately wash under a gentle stream of running tap water for 5–10 seconds.

9. Counterstain with safranin for 30 seconds.

10. Wash off any excess stain with water and gently blot the slide dry between sheets of blotting paper. Always remember which side of the slide is up.

11. View your slide under the microscope (40× magnification) to describe the Gram stain and morphology of the bacterial cells.

Gram-positive bacteria retain the crystal violet stain and appear purple. Gram-negative bacteria, such as rhizobia, do not, and are thus colourless until counter stained with a basic dye such as safranin, which stains colourless bacteria pink but does not change the purple colour of the Gram-positive bacteria. It is essential to use fresh broth or agar cultures, as older cultures of Gram-positive bacteria may fail to retain the crystal violet and falsely appear to be Gram-negative.

Crystal violet stain

▶ Crystal violet 10 g

▶ Ammonium oxalate 4 g

▶ Ethanol 100 mL

▶ Water 400 mL

Iodine solution

▶ Iodine 1 g

▶ Potassium iodide 2 g

▶ Ethanol 25 mL

▶ Water 100 mL

Counterstain

▶ 2.5% (w/v) Safranin in ethanol 10 mL

▶ Make up to 100 mL with water

There are also a range of molecular methods which can aid in the identification of rhizobia. These are covered in Chapters 11 to 13.

7.2.6 Physiology of rhizobia

Rhizobia commonly use the Entner-Doudoroff and Pentose Phosphate pathways for sugar metabolism, and normally have a fully functional TCA cycle. They can

often use a variety of nitrogen sources (see below), and ammonia assimilation is via the GS/GOGAT system.

In general, rhizobia can catabolise a wide array of carbon compounds and obtain iron from a range of sources; some strains use a variety of siderophores for iron uptake (Poole *et al.* 2008). However, some species of rhizobia can be auxotrophic for specific vitamins such as biotin, thiamin, and pantothenate (*e.g. M. loti*, Sullivan *et al.* 2001; Graham 1963; *S. meliloti*, Watson *et al.* 2001) and these need to be included in defined media for growth of these strains.

7.2.6.1 Physiology of rhizobia in relation to carbon sources

Summarising the carbon metabolism of such a diverse range of organisms as the root nodule bacteria is obviously difficult and individual exceptions to many of the 'generalisations' listed below can be readily found. Nevertheless, these generalisations are useful as background to devising media for specific organisms and purposes.

The three major physiological groupings are essentially the fast- and slow-growing rhizobia, and the very fast-growing β-rhizobia. Growth of the root nodule bacteria is generally heterotrophic; in a few cases autotrophic growth on H_2/CO_2 has been observed in the slow-growing group. Genes for C_1 metabolsim, like ribulose *bis*-phosphate carboxylase, are nevertheless conserved, and autotrophic growth in the soil under particular conditions may be possible.

While there may be occasional differences with particular strains, the following generalisations about carbon nutrition can be made for the α-rhizobia.

1. In very broad terms, the range of substrates on which the fast-growing group will grow is wider than that for the slow-growing group.

2. Both groups metabolise a wide range of C_5- and C_6- sugars and sugar alcohols, often resulting in acidification of solid and liquid media due to organic acid production. The major sugar degradation route appears to be the Entner-Doudoroff pathway.

3. Only the fast-growing groups metabolise C_{12} sugars like sucrose; the slow-growing groups apparently lack uptake systems and the appropriate disaccharidases. Metabolism of C_{12} sugars by fast-growers also results in acidification of media (see Chapter 4 for pH control).

4. The metabolic pathways for the metabolism of the C_5- sugar L-arabinose differ between the fast- and slow-growing groups.

5. C_4-Dicarboxylic acids (malate, fumarate, succinate) are usually good substrates for both fast- and slow-growing groups. Malic enzyme is an essential enzyme for degradation of these dicarboxylic acid substrates, both in laboratory cultures and in N_2-fixing nodule bacteroids. Growth on organic acids

as sole carbon sources results in alkalinisation of unbuffered solid or liquid media.

6. Growth on amino-acids as sole carbon sources is often strain-specific, so growth of particular strains has to be checked individually. Growth is often inhibited by concentrations normally used for sugars or organic acids like 10–20 mM but growth may occur at lower concentrations. Their metabolism results in marked alkalinisation of unbuffered media and significant ammonia (NH_3) accumulation. Apart from its alkalinising effect, this ammonia can be tolerated to quite high concentrations (50 mM or more). Growth on some amino-acids as sole source of carbon can cause marked changes in cell morphology. For example, growth of *Rhizobium leguminosarum* on histidine produces almost coccoid cells.

7. The ability of root nodule bacteria to utilise aromatic compounds (those containing the benzene ring) varies widely, though the range of compounds used is usually wider with the fast-growing than with the slow-growing groups. The catechol and/or the protocatechuate branches of the 3-oxoadipate pathway may occur in a particular strain. In most cases, the enzymes of the 3-oxoadipate pathway are inducible in the fast-growing groups and constitutive in the slow-growing, though in the latter one key enzyme is usually inducible.

8. Terminal oxidation of substrates typically involves the tricarboxylic acid cycle. Oxidation of C_2- substrates like acetate via the glyoxylate cycle is well documented but in many organisms, the activities of the key enzymes, isocitrate lyase and malate synthetase, are low and growth on acetate is poor.

9. Terminal electron transport is generally to O_2 via a variety of cytochrome-based systems. Some strains can use nitrate as a terminal electron acceptor and achieve limited anaerobic growth.

10. Metabolic regulation of the utilisation of mixed sources of carbon is more like that in *Pseudomonas* than in the enterobacteria. Thus, root nodule bacteria will co-utilise mixed carbon sources (though obviously the rates may be widely different) rather than fully utilise a preferred carbon source before starting on a less-favoured one. The classical catabolite repression typical of substrate utilisation in *Escherichia coli* is not a major aspect of regulation in root nodule bacteria.

7.2.6.2 Physiology in relation to nitrogen source

A very limited range of root nodule bacteria show nitrogenase activity in the laboratory and only under highly selective conditions; only a few strains have been shown to fix enough N_2 (usually only under very stringent conditions) to meet their growth requirements. A fixed nitrogen source is therefore necessary for growth in laboratory culture.

The ability to use particular compounds as nitrogen sources varies widely. Most complex media contain a sufficiently wide range of compounds to meet growth requirements. Trying to grow an unstudied strain in defined medium may therefore require assessment of whether it will grow using urea, ammonium salts, nitrates or amino-acids as sources of N. Many strains utilise all these forms of N, but some do not. Growth may also be faster with a particular form and this can only be determined experimentally.

Because a dense culture ($A_{600\ nm}$ = 1 to 2) may use all the N from a 5–10 mM N-source, pH changes associated with N utilisation may also be significant, *i.e.* acidification with ammonium salts or alkalinisation with nitrate or amino-acids. Accumulation of nitrite may be a (not commonly recognised) consequence of growth on nitrates.

7.2.6.3 Physiology in relation to pH change

As mentioned above, catabolism of carbon substrates and utilisation of N-compounds during growth results in pH changes. Growth on compounds like sugars (*e.g.* glucose or sucrose) or sugar alcohols (*e.g.* mannitol) results in acidification (for fast growers) with pH in unbuffered media commonly falling into the pH 4–5 range as a result of organic acid production. Growth on organic acids (*e.g.* fumarate or succinate) can in unbuffered media lead to pH rises to pH 8.5 or higher as a result of what is essentially a release of hydroxide ions coupled to carboxylate uptake, usually terminating growth. Growth on amino-acids (*e.g.* histidine or glutamate) as sole source of carbon results in alkalinisation, partly from hydroxide exchange as with the organic acids and partly from release of NH_3 into the medium. Since the quantitative demand for C is much greater than that for N, very high concentrations of NH_3 may be released; for an N-rich amino-acid like histidine, growth on this amino-acid alone can result in up to 100 mM ammonia release.

If growth at a particular pH is to be assessed, adequate pH maintenance through buffering is essential (Howieson and Ewing 1986). Buffers which generally do not effect growth rate can be selected from the so-called 'good' biological buffers, though each must be tested at the concentration range required (usually 20–30 mM).

Each buffer has a particular pH range of usefulness, depending on the pK_a value(s) of the buffer chemical. It is important to recognise that the ability to buffer depends on the ratio of the two forms of the buffer; where the pH is the same as the pK_a, the ratio is one and buffering is maximal. At a pH one unit removed from the pK_a (either up or down) *ca.* 90% of the buffer is in a form incapable of contributing to further buffering. Accordingly, this represents an extreme departure from the pK_a for using that buffer effectively. Ideally, the pH at which a buffer is to be used should be less than one unit from its pK_a.

Four buffers which are extremely useful for pH control in media were given in Chapter 3 and these are listed again in Table 7.5.

Table 7.5 Buffers recommended for studies of rhizobia where pH control is important.

Chemical name	Abbreviation	pK$_a$ at 25°C
3-(Cyclohexylamino)-2-hydroxy-1-propansulfonic acid	CAPSO	9.6
N-(2-hydroxyethyl)piperazine-N'-(2-ethansulfonic acid	HEPES	7.5
2-[N-morpholino] ethansulfonic acid	MES	6.1
Homopiperazine-1,4-*bis*(2-ethansulfonic acid)	HOMOPIPES	4.55

7.2.6.4 Physiology in relation to mineral nutrient deficiencies

There are a wide range of specialised techniques and procedures that have been developed for studying the physiology and growth of rhizobia, and it is beyond the purpose of this manual to provide full details. The reader is referred to the rich scientific literature and previous manuals and publications, such as Bergersen (1980) for guidance and information. Nutritional studies require the use of purified defined media (Abreu *et al.* 2012; O'Hara *et al.* 1987) and specialised culture techniques (Cassman *et al.* 1981; Smart *et al.* 1984) as discussed in O'Hara *et al.* (1988). In particular, the use of specific anionic or cationic resins to purify media components of the nutrient being studied, and the addition of metal chelators, such as EDTA and nitrilotriacetate (NTA) to remove residual nutrient contaminants from media and solutions, have proven essential for studies determining roles of micronutrients in nodule bacteria.

7.3 Studying membrane transport systems

Nodule bacteria must acquire all substrates and nutrients from external sources and consequently membrane transport is essential in both free-living and symbiotic life styles. Studies of membrane transport systems have been essential for revealing both the common metabolic features and differences rhizobia have developed to deal with the challenges associated with nutrient acquisition in complex soil and plant environments (Udvardi and Poole 2013). This protocol describes how to undertake a membrane transport assay using the rapid filtration technique. It applies to the use of ^{14}C and ^{3}H labelled compounds, although others can be used. You will need to comply with local regulations about the use of radioactivity. The assay is based on bacteria accumulating a radioactive compound inside cells which are then collected on a membrane filter. Radioactivity not inside cells is washed off with a salts solution. The filter is then removed and added to a vial with scintillation fluid.

Preparation

Previous day:

1. Set up 50 mL of growth medium with appropriate carbon and nitrogen sources in a 250 mL conical flask. (If strain contains a plasmid then add required

antibiotic). Inoculate with 600 μL of freshly washed slope culture that is 2–3 days old. This may vary with different rhizobial strains as this protocol was developed for *R. leguminosarum* that has a mean generation time on minimal medium of approximately 4 hours;

Day of assay:

2. Fill wash bottle with transport wash.

3. Take 40–50 mL of overnight culture and centrifuge cells at 4000 rpm for 20 min in a bench centrifuge.

4. Wash cells by re-suspending in the same volume of transport wash. Spin at 4000 rpm for 20 min.

5. Re-suspend cells in 5 mL of transport wash. Take OD_{600} of 1/10 dilution. Re-suspend in transport wash to $OD_{600} \approx 1$. Take OD_{600} and note for final calculation.

6. Leave culture to starve for 1 hour in a large shaking water bath at 28°C.

7. Prepare 25 μM working concentration solutions of the 'cold' (unlabelled) compounds in the assay. Make up a 0.1 M stock (to store at 4°C for future assays). From this, make up 5 mL of 0.5 mM solution (25 μL of 0.1 M in 5 mL HEPES buffer pH 7.0).

8. Dilute 'hot' labelled compound to a specific activity of 4.625 kBq (0.125 μCi) in 50 μL. *i.e.* for six assays you will need 300 μL 'hot' solute.

Procedure

1. Put tips on three Gilson type pipettes (100–200 μL sample volumes). To the first pipette, add 25 μL 'cold substrate' (0.5 mM). To the second, add 50 μL 'hot' substrate (4.625 kBq). The third is a sampling pipette.

2. Assemble a vacuum manifold with a filter disc that will trap bacteria (see below). Filters such as nitrocellulose are used or you can save costs by cutting your own using Whatman GFF glass fibre filters. Use a standard cork borer to cut sheets of Whatman GFF into the appropriate-sized discs.

3. Add 225 μL of 28°C TRANSPORT WASH to a sampling tube (Universal or Falcon as appropriate). Add 200 μL cells.

4. Start a stop-watch.

5. At 55 sec on the clock, add 25 μL 'cold' substrate (0.5 mM) and mix.

6. At 60 sec, add 50 μL 'hot' substrate (4.625 kBq) and mix.

7. At 1 min 10 sec, take 100 μL of the mix and squirt into a vacuum manifold. Wash twice with transport wash, remove filter to scintillation tube and cover with scintillation fluid (this will be the time zero value).

8. Take further samples at 2 min 10 sec, 3 min 10 sec and 4 min 10 sec (to give 1 min, 2 min and 3 min samples). Take 50 µL standard from the sampling tube without filtration. When this standard is counted, you will be able to check that the correct level of radioactivity was added to the sample.

9. Count all samples in a liquid scintillation counter. The radioactivity in the bacteria enables the calculation of a rate of uptake as follows:

10. nmole transported = [CPM in sample/(standard × 2)] × 2.5 nmole

11. This can be converted to a rate by dividing by the sample time. Use the OD_{600} reading to convert the rate to per unit OD_{600}. By preparing a standard curve of bacterial OD_{600} versus protein or dry weight it is also possible to express rates per unit bacterial protein or dry weight.

Notes

There are a number of different vacuum manifolds that can be used. Companies such as Millipore produce a vacuum manifold that can take several 25 mm nitrocellulose filters. You can make your own filtration device out of polycarbonate. This trap contains a Sintered Disc from VWR (10 mm diameter, P40 (232/0005/14)). It is wise to periodically (weekly or monthly depending on use) invert the sintered glass disc as it becomes clogged with glass fibres. Scintillation vials are typically polyethylene tubes/ polypropylene cap and are available from a number of suppliers (*e.g.* 215/0092/02 from VWR). Scintillation fluid should be bought commercially and is available from a number of suppliers.

The above rate calculation is based on 2.5 nmole of cold solute being in each 100 µL sample. The hot sample will add some extra solute and for greatest precision this amount should be calculated from the specific activity on the stock bottle. All radiochemical suppliers will specify KBq mmole^{-1} (or equivalent) and from this it is possible to calculate how many nmoles of solute are added in the 4.625 kBq of hot solute. This amount should be added to the 2.5 nmole above *e.g.* if 0.1 nmole is present in 4.625 kBq of hot solute then 2.6 rather than 2.5 should be used in the above formula. You may wish to alter the starting solute concentration or the assay time and these will require appropriate alterations to the calculations.

Transport wash

Ingredients:

1.4 g	K_2HPO_4
0.36 g	KH_2PO_4
0.25 g	$MgSO_4.7H_2O$
0.2 g	NaCl
1 mL	Solution A (below)
2 mL	Solution B
1 mL	Solution C
1000 mL	FINAL VOLUME

Method:

Dissolve ingredients in slightly less than final volume; adjust pH to 7.0, make up to 1 L.

DO NOT autoclave but keep in dark bottle at 4°C. It should store for several weeks.

Rhizobium Solution A

Ingredients:

15 g	EDTA-Na$_2$
0.16 g	ZnSO$_4$.7H$_2$O
0.2 g	Na$_2$MoO$_4$.2H$_2$O
0.25 g	H$_3$BO$_3$
0.2 g	MnSO$_4$.4H$_2$O
0.02 g	CuSO$_4$.5H$_2$O
1 mg	CoCl$_2$.6H$_2$O

[Dissolve 100 mg in 100 mL GDW (Glass Distilled Water) and add 1 mL.]

Method:

Dissolve each ingredient in turn before adding the next.

Make up to 1 L with GDW.

Store at 4°C.

Rhizobium Solution B

Ingredients:

1.28 g	CaCl$_2$.2H$_2$O
0.33 g	FeSO$_4$.7H$_2$O

Method:

Dissolve each ingredient in 50 mL water then combine.

Store at 4°C for no more than one week.

Rhizobium Solution C

Ingredients:

1 g	Thiamine hydrochloride
2 g	D-Pantothenic acid Ca salt
1 mg	Biotin

[Dissolve 100 mg in 1000 mL GDW and add 10 mL. Store rest of biotin at −20°C]

Method:

Dissolve each ingredient in turn before adding the next.

Make up to 1 L with GDW.

Filter, sterilise and store at 4°C

Add aseptically to media at 1 mL per litre.

7.4 Final comments

The description of the nodule bacteria is a rapidly expanding field as scientists continue to examine roles for legumes in sustainable production systems, and understand legumes in their natural environments. Fundamental to working efficiently with legumes is being aware of the varied forms of their symbionts, and recognising them in the laboratory. The unique capacity of rhizobia to harness inert dinitrogen will mean their physiology and biochemistry will always be the subject of intensive study. The material in this chapter will expedite these pursuits.

7.5 References

Abreu I., Cerda M.E., Perez de Nanclares M., Baena I., Lloret J., Bonilla I., Bolanos L. and Reguera M. 2012. Boron deficiency affects rhizobia cell surface polysaccharides important for suppression of plant defense mechanisms during legume recognition and for development of nitrogen-fixing symbiosis. Plant and Soil 361, 385–395.

Bergersen F.J. 1980. Methods for Evaluating Biological Nitrogen Fixation. John Wiley & Sons: Chichester.

Bloem J.F., Botha W.J., Law I.J. and Steyn P.L. 2002. Colony variation in *Sinorhizobium meliloti* inoculant strain U45. Microbiological Research 157, 283–292.

Bournaud C., de Faria S.M., dos Santos J.M.F., Tisseyre P., Silva M., Chaintreuil C., Gross E., James E.K., Prin Y. and Moulin L. 2013. Burkholderia species are the most common and preferred nodulating symbionts of the Piptadenia Group (Tribe Mimoseae). PLoS ONE 8(5): e63478. doi:10.1371/journal.pone.0063478

Boursier P., Hanus F.J., Papen H., Becker M.M., Russell S.A. and Evans H. 1988. Selenium increases hydrogenase expression in autotrophically cultured *Bradyrhizobium japonicum* and is a constituent of the purified enzyme. Journal of Bacteriology 170(12), 5594–5600.

Brenner D.J., Staley J.T. and Krieg N.R. 2005a. Classification of prokaryotic organisms and the concept of bacterial speciation. Pp 27–32 in 'Bergey's Manual of Systematic Bacteriology. Volume 2 The Proteobacteria, Part A Introductory essays', ed. by G. Garrity. Springer: New York, USA.

Brenner D.J., Krieg N.R. and Staley J.T. 2005b. Bergey's Manual of Systematic Bacteriology. Volume 2 The Proteobacteria, Part A Introductory essays, ed. by G. Garrity. Springer: New York, USA.

Cassman K.G., Munns D.N. and Beck D.P. 1981. Growth of *Rhizobium* strains at low concentrations of phosphate. Journal Soil Science Society America 45, 520–523.

De Meyer S.E., Cnockaert M., Ardley J.K., Trengove R.D., Garau G., Howieson J.G. and Vandamme P. 2013a. Burkholderia rhynchosiae sp. nov., isolated from *Rhynchosia ferulifolia* root nodules. International Journal of Systematic and Evolutionary Microbiology 63, 3944–3949.

De Meyer S.E., Cnockaert M., Ardley J.K., Maker G., Yates R., Howieson J.G. and Vandamme P. 2013b. *Burkholderia sprentiae* sp. nov., isolated from *Lebeckia ambigua* root nodules. International Journal of Systematic and Evolutionary Microbiology 63, 3950–3957.

De Meyer S.E., Cnockaert M., Ardley J.K., Van Wyk B-E., Vandamme P. and Howieson J.G. 2013c. *Burkholderia dilworthii* sp. nov., isolated from *Lebeckia ambigua* root nodules. International Journal of Systematic and Evolutionary Microbiology 64, 1090–1095.

Dreyfus B., Garcia J.L. and Gillis M. 1988. Characterization of *Azorhizobium caulinodans* gen. nov. sp. nov., a stem-nodulating nitrogen-fixing bacterium isolated from *Sesbania rostrata*. International Journal of Systematic and Bacteriology 38, 89–98.

Elliott G.N., Chen W.M., Chou J.H., Wang H.C., Sheu S.Y., Perin L., Reis V.N., Moulin L., Simon M.F., Bontemps C., Sutherland J.M., Bessi R., de Faria S.M., Trinick M.J., Prescott A.R., Sprent

J.I. and James E.K. 2007. *Burkholderia phymatum* is a highly effective nitrogen-fixing symbiont of Mimosa spp. and fixes nitrogen ex planta. New Phytologist 173(1), 168–180.

Evans W.R., Fleischman D.E., Calvert H.E., Pyati P.V., Alter G.M. and Subba Rao N.S. 1990. Bacteriochlorophyll and photosynthetic reaction centers in Rhizobium sp. strain BTAi1. Applied and Environmental Microbiology 56, 3445–3449.

Garau G., Yates R.J., Deiana P. and Howieson J.G. 2009. Novel strains of nodulating Burkholderia have a role in nitrogen fixation with papilionoid herbaceous legumes adapted to acid, infertile soils. Soil Biology & Biochemistry 41, 125–134.

Graham P.H. 1963. Vitamin requirements of root nodule bacteria. Journal of General Microbiology 30, 245–248.

Graham P.H., Sadowsky M.J., Kersters H.H., Barnet Y.M., Bradley R.S., Cooper J.E., De Ley D.J., Jarvis B.D.W., Roslycky E.B., Strijdom B.W. and Young J.P.W. 1991. Proposed minimal standards for the description of new genera and species of root-and stem-nodulating bacteria. International Journal of Systematic Bacteriology 41, 582–587.

Gevers D., Cohan F.M., Lawrence J.G., Spratt B.G., Coenye T., Feil E.J., Stackebrandt E., de Peer Y.V., Vandamme P., Thompson F.L. and Swings J. 2005. Re-evaluating prokaryotic species. *Nature Reviews Microbiology* **3,** 733–739.

Gyaneshwar P., Hirsch A.M., Moulin L., Chen W.M., Elliott G.N., Bontemps C., Estrada-de Los Santos P., Gross E., Dos Reis F.B., Sprent J.I., Young J.P. and James E.K. 2011. Legume-nodulating betaproteobacteria: diversity, host range, and future prospects. Molecular Plant Microbe Interactions 24(11), 1276–1288.

Howieson J.G. and Ewing M.A. 1986. Acid tolerance in the *Rhizobium meliloti – Medicago* symbiosis. Australian Journal of Agricultural Research 37, 55–64

Howieson J.G. Ewing M.A. D'Antuono M. 1988. Selection for acid tolerance in *Rhizobium meliloti*. Plant and Soil 105, 179–188.

Howieson J.G., De Meyer S.E., Vivas-Marfisi A., Ratnayake S., Ardley J.K. and Yates R.J. 2013. Novel Burkholderia bacteria isolated from *Lebeckia ambigua*—a perennial suffrutescent legume of the fynbos. Soil Biology & Biochemistry 60, 55–64.

Jordan D.C. 1984. Genus I. Rhizobium. Pp. 235–242 in 'Bergey's Manual of Systematic Bacteriology, 1st Ed., Vol. 1', ed. by Kreig and Holt. The Williams & Wilkins Co.: Baltimore.

Kampfer P. and Glaeser S.P. 2012. Prokaryotic taxonomy in the sequencing era—the polyphasic approach revisited. Environmental Microbiology 14(2), 291–317.

Konstantinidis K. and Tiedje J.M. 2005a. Genomic insights that advance the species definition for prokaryotes. Proceedings National Academy Science USA 102, 2567–72.

Konstantinidis K.T. and Tiedje J.M. 2005b. Towards a genome-based taxonomy for prokaryotes. Journal of Bacteriology 187, 6258–6264.

Lindström K. and Young J.P. 2011. International Committee on Systematics of Prokaryotes Subcommittee on the taxonomy of Agrobacterium and Rhizobium: minutes of the meeting, 7 September 2010, Geneva, Switzerland International Journal of Systematic and Evolutionary Microbiology 61(12), 3089–93.

McComb J.A., Elliot J. and Dilworth M.J. 1975. Acetylene reduction by *Rhizobium* in pure culture. Nature (London) 256, 409–410.

Martens M., Delaere M., Coopman R., de Vos P. Gillis M. and Willems A. 2007. Multilocus sequence analysis of *Ensifer* and related taxa. International Journal of Systematic and Evolutionary Microbiology 57, 489–503**.**

Moulin L., Munive A., Dreyfus B. and Boivin-Masson C. 2001. Nodulation of legumes by members of the β-subclass of Proteobacteria. Nature 411, 948–950.

O'Hara G.W. and Daniel R.M. 1985. Rhizobial denitrification: a review. Soil Biology & Biochemistry 17, 1–9.

O'Hara G.W., Franklin M. and Dilworth M.J. 1987. Effect of sulfur supply on sulfate uptake, and alkaline sulfatase activity in free-living and symbiotic bradyrhizobia. Archives of Microbiology 149, 163–167.

O'Hara G.W., Boonkerd N. and Dilworth M.J. 1988 Mineral constraints to nitrogen fixation. Plant and Soil 108, 93–110.

Poole P.S., Hynes M.F., Johnston A.W.B., Tiwari R.P., Reeve W.G. and Downie J.A. 2008. Physiology of root nodule bacteria. Pp 241–292 in 'Nitrogen fixing Legume Symbioses', ed. by W.E. Newton and M.J. Dilworth. Springer Sciences.

Rogel M.A., Ormeno-Orrillo E. and Martinez Romero E. 2011. Symbiovars in rhizobia reflect bacterial adaptation to legumes. Systematic and Applied Microbiology 34, 96–104.

Smart J.B., Robson A.D. and Dilworth M.J. 1984. A continuous culture study of the phosphorus nutrition of *Rhizobium trifolii* WU96, *Rhizobium* NGR234 and *Bradyrhizobium* CB756. Archives of Microbiology 140, 276–280.

Stackebrandt E., Frederiksen W., Garrity G.M., Grimont P.A.D., Kampfer P., Maiden X., Nesme M.C.J., Rossello-Mora R., Swings J., Truper H.G., Vauterin L., Ward A.C. and Whitman W.B. 2002. Report of the ad hoc committee for the re-evaluation of the species definition in bacteriology. International Journal of Systematic and Evolutionary Microbiology 52, 1043–1047.

Sullivan J.T., Brown S.D., Rogers Yocum R. and Ronson C.W. 2001. The *bio* operon on the acquired symbiosis island of *Mesorhizobium* sp. Strain R7A includes a novel gene involved in pimeloyl-CoA synthesis. Microbiology 147, 1315–1322.

Sylvester-Bradley R., Thornton P. and Jones P. 1988. Colony dimorphism in *Bradyrhizobium* strains. Applied and Environmental Microbiology 54, 1033–1038.

Udvardi M. and Poole P.S. 2013. Transport and metabolism in legume-rhizobia symbioses. Annual Review of Plant Biology 64, 781–805.

Vandamme P., Pot B., Gillis M., De Vos P., Kersters K. and Swings J. 1996. Polyphasic taxonomy; a consensus approach to bacterial systematics. Microbiology and Molecular Biology Reviews 60, 407–438.

Watson R.J., Heyes R., Martin T. and Savard M. 2001. *Sinorhizobium meliloti* cells require biotin and either cobalt or methionine for growth. Applied and Environmental Microbiology 67, 3767–3770.

Willems A., Coopman R. and Gillis M. 2001. Comparison of sequence analysis of 16S–23S rDNA spacer regions, AFLP analysis and DNA–DNA hybridizations in *Bradyrhizobium*. International Journal of Systematic and Evolutionary Microbiology 51, 623–632.

Young J.P.W., Downer H.L. and Eardly B.D. 1991. Phylogeny of the phototrophic rhizobium strain BTAil by polymerase chain reaction-based sequencing of a16S rRNA gene segment. Journal of Bacteriology 173, 2271–2277.

CHAPTER 8
Field experiments with rhizobia

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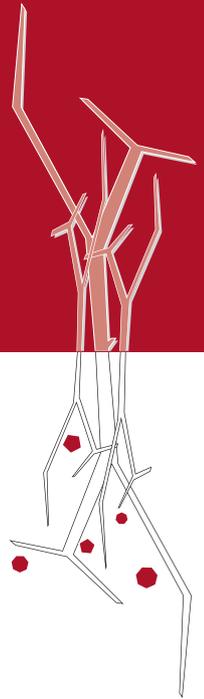
8.1 Introduction

In this chapter, we discuss methodologies to select rhizobial strains for their adaptation to field conditions as this is an important trait when developing inoculant-quality strains. Techniques to assess nitrogen fixation *per se* are described in Chapters 5 and 10, and traits related to manufacturing in Chapter 9.

For many legume symbioses, the greatest challenge in developing inoculants is to select strains that confer consistent nodulation upon the legume in the target soil environment. Edaphic stresses after inoculation, such as dry heat and acidity, can be detrimental to rhizobial survival, so selecting strains tolerant of these stresses is very useful. Some legumes regenerate annually from hard (impermeable) seeds in the soil and therefore, like their rhizobia, are only sown once in several decades. Hence, assessment of the ability of strains to persist in the field environment through several seasons is required. This trait cannot be reliably predicted without field experimentation (Howieson *et al.* 2000). Many legume evaluation programs over the years have been compromised because inoculants suitable to the field conditions have not been available.

8.2 Defining the aim of the field evaluation

It is important to clearly define the aim of the activity as this will influence the site selection and the design of the field experiment. Examples of common aims in field experiments are to select rhizobial strains that:

► are competitive with resident (but less-effective) nodulating bacteria (see need to inoculate experiment in Chapter 1)

▶ have limited persistence (these strains would rely heavily on a successful inoculation procedure but ultimately provide the opportunity to be displaced if a superior strain were to be developed)

▶ colonise difficult soils and persist through several seasons, such that legumes regenerating from hard seed 'banks' after cropping can promptly nodulate

▶ tolerate difficult soil conditions, such as dry and hot soil, long enough to nodulate the host

▶ have optimal nodulation performance across different delivery systems (*e.g.* achieve high cell number, long shelf life and compatibility with other bacteria in multiple strain inoculants).

8.3 Experimental design

For the data from field experiments to be statistically robust, several fundamental aspects of experimental design must be followed.

▶ Treatments must be randomly assigned to plots.

▶ Treatments must be replicated.

▶ Trial layout must take account of (and try to minimise) natural variation in the site.

▶ Controls must be included in the treatments.

A simple design that is often applicable to field experiments is the randomised block. In this form of design, treatments are allocated into replicates, which are sown in blocks, and the blocks are arranged such that any evident variation in the field is exposed to a discrete replicate. An Excel spreadsheet can be designed to record the treatment randomisation and allocate these to plots, as shown in Figure 8.1.

plot #	1	2	3	4	5	6	7	8	9	10	
trt #	J	E	B	H	C	I	F	A	D	G	rep 1
plot #	11	12	13	14	15	16	17	18	19	20	
trt #	F	A	J	C	B	G	D	H	I	E	rep 2
plot #	21	22	23	24	25	26	27	28	29	30	
trt #	A	E	I	B	D	H	G	C	J	F	rep 3
plot #	31	32	33	34	35	36	37	38	39	40	
trt #	H	D	I	A	G	J	F	B	E	C	rep 4

Figure 8.1   A randomised complete block layout for 10 treatments (A-J) with four replicates, to guide the layout of a field experiment as shown in Figure 8.2.

8.4 Site selection

One of the most critical aspects of field work, often neglected, is to select appropriate sites for the experiment. The key elements of site selection are to:

► maintain relevance to the ultimate aim of the experiment (*e.g.* an understanding of farming systems and soil types likely to be encountered by the symbiosis)

► ensure that previous management of the site (*e.g.* herbicide history) will not compromise rhizobia or legume performance

► reduce variability in plant or microbial performance across the plots

► choose sites with suitable texture to avoid damage to the roots and loss of nodules when collecting data (*i.e.* avoid fine textured, hard-setting clay soils if possible)

► minimise rhizobial contamination through water movement and soil erosion by choosing sites that are on a slope gradient of less than 1:100 and where good water drainage is retained

► avoid sections of the field that may have been subjected to heavy machinery or animal traffic as resulting compaction may result in uneven plant performance

► avoid specific locations in the field where header-trails, burnt residues, hay, fertiliser, gypsum or lime have been accumulated, as residues of these can affect the consistency of plant performance

► avoid sites where top soil depth is variable.

8.4.1 Land and seed preparation

Particular attention should be given to preparing the site in such a way that there is a minimal requirement for physical intervention (*e.g.* human, animal or machinery movement) once the experiment has been sown. This is essential to reduce cross-plot contamination. The site must be prepared to be agronomically suitable for growth of the legume.

► Weeds should be controlled in the season prior to the experiment, and again before planting, by herbicide or cultivated by machinery, or by manual raking.

► The site should be fertilised with all necessary macro- and micro- nutrients, except nitrogen.

► The site should be fenced to ensure that there is no traffic movement on the site, particularly from farm or wild animals.

Sites that have produced cereal crops (*e.g.* wheat in temperate/Mediterranean climates, sorghum or maize in subtropical areas) in the preceding season are useful, as they often have reduced weed burden, may homogenise the soil and deplete soil nitrogen. However, the historical herbicide regime must be clear. Breakdown

of the sulfonyl-urea herbicides occurs more rapidly in acid soils, wet soils and at higher temperatures (Koopman *et al.* 1995). There is mounting evidence that minute residues of these herbicides can reduce legume nodulation (Figure 5.9) and this can be problematic after drought (Farquarson 2010).

If biological yield of the legume is to be an important parameter, then the depth of the soil profiles and its variability can be gauged from soil sampling across a grid, or by using ground-penetrating radar (GPR) to map changes in soil texture with depth. Data from GPR can be a useful covariate for statistical analysis of yield (Burgos *et al.* 2009).

High-quality seed needs to be sourced for experiments, and scarification of hard seeds undertaken to ensure they germinate (Chapter 5). A germination test should precede the trial; many field experiments have failed due to poor-quality seed.

Figure 8.2   Land preparation and layout; in this example, the site is relatively flat, uniform and fenced, with individual plots marked with pegs.

8.4.2 Matching site characteristics to the aim of the experiment

There are a few key considerations when selecting the site for a field experiment.

► **For low numbers of background rhizobia.** Avoid sites where the inoculated legume has previously been sown. An MPN estimate (Chapter 6) prior to sowing the experiment is often indicative, but for some symbioses even low background counts (<20 cells of infecting rhizobia per gram of soil) can be very

competitive with seed applied inoculants, and thus remove any response to inoculation (Thies *et al.* 1991).

▶ **For high numbers of background rhizobia.** To research selective nodulation, the impact of different inoculant carriers or the capacity to introduce superior strains on seed, seek sites where the nodulating rhizobia have previously been established. It is possible to introduce rhizobial strains on unrelated legumes the previous season (*e.g. Ornithopus*-nodulating rhizobia may be introduced as inoculants on *Lupinus*; *Pisum* rhizobia may be introduced in association with *Vicia*, *Lathyrus* or *Lens;* many strains of bradyrhizobia can be introduced as inocula on *Macroptilium purpureum*) to avoid plant disease pressures from continuous cultivation of the legume under investigation.

▶ **To assess abiotic stress.** The range of stressful soils to be encountered (*e.g.* waterlogged, dry or desiccating, acid, alkaline, low clay content) should form the basis of the selection criteria. If seeking acid tolerance, then be aware that acidity combined with low clay content and low organic matter content is more stressful than low pH in isolation (Howieson and Ballard 2004). Acidity can also be associated with toxicity due to soluble Al^{3+} and Mn^{2+} ions (Helyar and Porter 1989). If resources permit, a control set of plots should be established where the stress is ameliorated (*e.g.* by liming an acidic soil).

▶ **To assess biotic stress.** If acting directly upon the nodule bacteria, the agents of stress, such as predators or antibiotic-producing agents, need to be encouraged, perhaps by reduced tillage. The level of organic matter and the microbial biodiversity of the site will be important parameters. Biotic stress associated directly with nodulation or nodule function may need prior history of the legume to encourage appropriate levels of the stress parameter. For example, nodule predation by sitona weevil larvae is very specific. *Medicago* but not *Trifolium* nodules are predated by *Sitona discoideus* in Australia. A healthy stand of medic would need to be developed, and the *Sitona* encouraged to colonise the site prior to introducing an experiment to control the effects of this biotic stress.

▶ **To assess strain persistence through rotations.** Annually regenerating pasture legumes are iconic in Australian agriculture. If selecting rhizobia that need to persist in the absence of the host legume, ensure the field site is suitable for both the target legume and the rotational species, and that a long-term lease of the site is possible. For example, rhizobia that effectively nodulate *Biserrula pelecinus* often need to persist through three to five successive crops (*e.g.* wheat, canola, barley, wheat, canola) before the legume (regenerating from hard seed buried in the soil) requires them for nodulation (Loi *et al.* 2005).

8.5 Selecting the appropriate carrier and adhesive for application of rhizobia to the seed

The aim of many field experiments with rhizobia is to introduce a spectrum of strains, or species, into the soil to select one or two that are well adapted to the conditions. A decision must be made on which form of inoculant carrier is to be used as it may affect the outcome. The carrier supports live cells of rhizobia and protects them from stress, such as desiccation that can lead to death (Vincent *et al*. 1962, see Figure 8.3). There are many inoculant carriers available (Table 8.1) and the characteristics of these are discussed in more detail in Chapter 9. However, it is important that the carrier selected must deliver a known quantity of live cells to the target legume under the prevailing conditions.

Figure 8.3   Unthrifty, yellow and poorly-nodulated seedlings of *Lebeckia ambigua* (left) contrast with stronger, nodulated plants at six weeks of age (right). The poor growth and yellowing was due to N deficiency as a result of the death of the peat-based inoculant after application to the seed, and prior to nodulation in this sandy soil (Howieson *et al*. 2013).

Table 8.1   Carriers available to deliver rhizobial strains in field experiments (see also Chapter 9)

Carrier	Characteristics
Finely-ground organic substrates, such as peat, coir dust, filter or mud residues applied to the legume seed coat as a slurry with an adhesive (Table 8.2)	Moist (30–45% water) can deliver high cell count (10^9 cells/g) but must be applied to wet soils as death rate is 90% per day, and must not contact fungicides or other seed-applied chemicals.
Peat-based granules—sown alongside seed at planting	As above, but can be used where the inoculant must be separated from the seed to avoid chemical toxicity.
Clay granules—sown alongside seed at planting, or mixed with the fertiliser	Dry (5–10% water) can deliver lower cell count (10^5–10^7 cells/g) but stable with slow death rate, and can be used to sow legumes into dry soil before rains.
Liquid inoculants—sprayed onto seed or in furrow	Broth cultures stabilised in their growth phase (at approx. 10^9 cells/mL) by low temperature or nutrient exhaustion, but generally less robust than peats or clays.
Freeze dried inoculants	Very high count (10^{12} cells/mL) that are diluted before sowing and used in the same manner as liquid inoculants.
Pre-coated seeds—usually a peat-based matrix applied to seed with polymers to aid longevity	Can deliver stable high count live cells of *Sinorhizobium* (10^3/seed) but have the same death rate as peat inoculants for other rhizobial species (Deaker *et al*. 2004)

Any of these carriers can establish the experiment in small plots sown by hand (as swards or lines) but all may contaminate machinery when used to sow larger plots (>20 m^2). Dry clay carriers have a large dust component that can cause contamination in machine parts, such as the seed bin or tubes that carry the inoculants to the sowing tynes. Peat carriers can loosen from the surface of seed as they dry, but coating with lime or dolomite can minimise this. When coating seeds, adhesive concentrations can be increased to ensure high pellet integrity and minimise formation of dust during sowing. To minimise contamination, machine parts may be swabbed with ethanol (70%) and fertilisers can be passed through the machinery between rhizobial treatments to dislodge dust.

8.6 Adhesives for application of inoculants to legume seeds

Several additives are available to increase adherence of rhizobial carriers to seed, to protect them from desiccation and toxic chemicals, and to allow a longer window for sowing (Table 8.2).

Table 8.2   Adhesives for applying rhizobial carriers to seed for field experiments

Adhesive	Form	Characteristics
Sugars	Sucrose, maltose often used as 10% (w/v) solutions	Not as effective as some other adhesives, but can aid survival of rhizobia.
Natural polymers	Gum Arabic, xanthan gum at 2–5% (w/v) solutions	Usually very effective, but can be variable. Require pre-preparation to solubilise.
Semi-synthetic polymers	methyl cellulose used at 1–2% (w/v)	Prepared the day before use by dissolving in hot water. Avoid preparations with fungicides.
Synthetic polymers	polyvinylpyrrolidone, polyvinyl acetate, vinylacetate/vinylpyrrolidone co-polymer used at 10–20% (w/v)	Have been shown to assist cell survival. Often used at 10–20% but may become expensive if used on a broad scale.

The properties of these adhesives or additives can be species-specific, hence some pre-experimentation is recommended to assess their efficacy against the rhizobial species under evaluation.

8.7 General procedures for application of inoculants

1. Grow rhizobial strains to be investigated on appropriate medium (Chapter 3) and introduce strains individually into the chosen carrier. Mix well and incubate if required.

2. Enumerate inoculant product after period of maturation (Chapter 6).

3. Ensure similar numbers of each strain are compared by varying the amount of carrier applied to seed or soil. For seed-applied inoculants, refine the inoculation procedure by testing trial batches.

4. For peat and similar carriers, mix inoculant with a pre-sterilised adhesive (at a rate recommended for the product) in a sterilised vessel, such as a 20 mL plastic McCartney vial for small seeded legumes or a 500 mL beaker for larger seeds. Use a new vial for each rhizobial strain and sterilise utensils (spatula, spoon) in ethanol and flame (allow to cool) between each strain.

5. Surface-sterilise seed (Chapter 5).

6. Mix seed thoroughly with the inoculant /sticker slurry in the vial to deliver approximately commercial rates of inoculant per gram of seed (see below and Chapter 9).

7. It is important to ensure the seeds are not so moist after inoculation that they clump together, as this makes sowing very difficult. Surface-inoculated seeds, particularly small pasture seeds, may be dried and separated into single units by application of any benign substance, such as lime, clay or talc to the vial with gentle stirring or shaking.

8. Weigh inoculated seed into aliquots for each replicate and arrange packets into sowing order. Small seed envelopes are useful.

9. If using a granular inoculant, weigh granules into aliquots for each replicate, again taking care to ensure similar numbers of cells are compared for each strain and avoid contamination. Add appropriate weight of surface-sterilised seed to the packet if granules and seed are to be sown in the one operation.

10. Store aliquots of inoculated seed immediately in a secure cool, dark and dry area to ensure survival of inoculant.

11. Sow as soon as possible (unless measuring cell deaths over time) with chosen apparatus and approach (see below).

12. In the laboratory, keep a sample of inoculated seed and/or carrier and store at 1–5°C for later assessment of rhizobial numbers at time of inoculation (Chapter 6).

13. In the field, collect a sample of inoculated seed and/or carrier at the time of sowing, store in cold box and enumerate when back at the laboratory to estimate rhizobial numbers at the time of sowing.

8.8 Laying out plot dimensions for field experiments

Determine the experimental design and calculate the number of plots per replicate, the number of replicates, their shape and the dimensions of each individual plot.

Develop a printed trial plan showing the plots and the preferred layout (Figure 8.1).

At the field site, calculate the full dimensions of the trial, survey the site and select a space sufficient for the trial.

1. Using a tape measure or notched string, lay out a baseline for one dimension of the trial site.

2. Place pegs at intervals along the tape, appropriate to the plot size, leaving a buffer between each plot.

3. Select one corner of the baseline and form a right angle (using an optical square, or calculate using a 3–4–5 triangle).

4. Lay out the plot sizes along this second dimension to delineate blocks or replicates. Peg for all replicates.

5. Ensure space for a buffer is provided between each replicate to minimise the risk of contamination and to allow the passage of sowing and spraying machinery, and operators.

6. Repeat the right angle from the other end of the baseline and peg all replicates.

7. Lay the tape or notched string along a line parallel with the baseline delineating the second replicate and place pegs to form each individual plot.

8. Repeat by moving the tape or string in parallel blocks for the number of replicates required.

9. Number each plot in each replicate on the plot pegs with a permanent marker.

8.8.1 Sowing swards

In the simplest scenario, inoculated seed can be sprinkled by hand evenly across a small sward (*e.g.* dimensions of 2 m × 2 m). A significant advantage of hand-sown plots is that hygiene can be optimised, whereas for machine-sown plots, strains can transfer from plot to plot through dust or loose carrier, adhering to machinery parts.

1. Calculate the weight of seed required, surface sterilise, inoculate and weigh out the seed required for each plot into a small envelope as outlined in Section 8.7.

2. Lay out the field experiment as described in Section 8.8 (Figure 8.4).

3. Select the first treatment and mark its plot in each replicate*.

4. Distribute seeds evenly over the plot surface in each replicate.

5. Gently cover by light raking (treatment by treatment) if the species needs to be buried to assist germination.

6. Rinse hands and any implements in 70% ethanol and repeat for all subsequent treatments.

7. For large seeds to be sown in lines, trenches can be established to the required depth in the sward with a simple tyned implement, if not provided earlier during the land preparation.

*   The un-inoculated treatment is often sown first, with additional un-inoculated controls sown last.

Figure 8.4 Hand-sowing seed into lines in small plots delineated by strings, and covering with a rake. Note that both scientists are standing in the buffers between plots.

8.8.2 The 'cross-row' technique

This technique has been very successful in selecting inoculant-quality strains. It requires two seasons to complete. The cross-row approach identifies strains that are saprophytically competent in the target soil (Chatel and Parker 1973) and that can colonise the soil away from the point of inoculation with some reliability (Howieson and Ewing 1986; Yates *et al.* 2005).

Year 1

1. Plots are sown as 1.5 m lines, with seed sown at a rate of 0.5 g/m for small seeds and 20 g/m for large seeds. Calculate the weight of seed required, surface sterilise and inoculate as described in Section 8.6.

2. Weigh out the seed required for each plot (line) into a small envelope and place the envelopes in treatment order in a tray. Store in cold room.

3. Experimental design should be as randomised blocks, as described in Section 8.7. Individual lines are separated by 1 m buffers and banks are separated by 1.5 m buffers.

4. After pegging the plots, make a shallow indentation (2 cm deep) in the soil, usually with a length of angle iron or a vegetable planter (Figure 8.5). It is helpful if a length of chain is welded to each end of the iron to facilitate lifting between plots.

5. Lay out the envelopes containing the appropriate treatment for each plot and cross check with the randomisation chart (Figure 8.6).

6. To sow the seed, open the top flap or alternatively tear the bottom off the appropriate envelope then place a crease in the main surface of the envelope to guide the seed out.

7. Apply a gentle tapping motion to the envelope with the forefinger and encourage the seed to flow from the envelope evenly along the row. If the sowing is not uniform, seed may be spread along the row using the tip of the envelope, which is then discarded.

8. Cover the seeds by collapsing the side of the indentation with the envelope or an inverted rake, making sure not to make contact with the seed.

9. Allow the plants to grow through the season. Above-ground production parameters may be scored or measured, if required, but the root system and soil should not be disturbed. If a first year assessment of nodulation is required, samples can be taken from plants at the end of the row.

10. Annual legumes are allowed to senesce naturally between the wet seasons while crop legumes are carefully cut at 1 cm height and herbage removed, dried and weighed. Perennial legumes may be sprayed with herbicide at the completion of the first year growing season.

Figure 8.5 Angle iron with chains attached to aid lifting (above left) and vegetable planter (right) have been used to make indentations into soil for a cross-row trial.

Figure 8.6 Packet envelopes containing seed laid out ready for sowing into the furrow.

Year 2

1. Allow weeds to germinate and control these with herbicide without disturbing the soil.

2. Package the seed for each cross-row (pasture 0.4 g/m, pulses 8 g/m) into individual envelopes. Allow for 10% extra packets.

3. Before sowing, with a short (40 cm) piece of angle iron, create the indentations for the cross rows (Figure 8.7). Equipment to prepare the indentation in the soil, to a depth of 1–2 cm, must be cleansed in 70% ethanol between treatments.

4. Sow un-inoculated seed (surface sterilised) across the original row in a perpendicular pattern at two or three points, with the cross row extending 20 cm each side of the original row (Figure 8.8).

5. Ensure a relatively constant number of seeds are placed into each plot. If a cross row is accidentally sown unevenly, seed cannot be moved with the tip of the envelope as described in year 1, as this may move rhizobia laterally. It is better to apply extra seed to cross rows under these circumstances, so it is necessary to pack extra packets of fresh seed for this eventuality.

Figure 8.7   Making the cross row furrow. On this coarse textured soil, the sand has been pre-wet to aid this step, but the implement must be cleaned in ethanol between plots.

Figure 8.8   Sowing the un-inoculated seed, taking care not to disturb the soil laterally.

8.8.3 Data collection from a cross-row experiment

1. In the first year of the cross-row, it is particularly successful if you see plots of yellow plants from un-inoculated treatments (Figure 8.9) but the major parameter to assess the colonisation of the soil by rhizobia is the proportion of nodulated seedlings in each of the cross rows in year 2.

2. After allowing sufficient time for seedlings to nodulate (six to10 weeks) divide the cross-rows into sections (0–10 cm and 11–20 cm) from the original row as shown in Figure 8.10.

3. Excavate plants from each section using a plastic 'T' to guide the sections as shown in Figure 8.10. The whole 10 cm section can often be lifted in one spade-full to a depth required to access the main part of the root section. For small seeded legumes, this is often approximately 15 cm deep after six weeks of growth.

4. Shake the plants to remove excess soil then bag for transportation. Sections (*e.g.* the 0–10 cm group) within a plot may be bulked and then plants selected randomly from them for assessment. At least 15 plants from each section should be evaluated (see Section 8.10.1).

5. Strains that have persisted over the dry period then achieved nodulation in the cross-rows in the second year (particularly in the 11–20 cm sections) are considered suitable for further development.

Figure 8.9 The first year of a cross-row experiment with *Pisum sativum* and *R. leguminosarum* bv *viceae* after five weeks of growth. The un-inoculated treatment (left row) lacks nitrogen and is pale in comparison to the surrounding inoculated rows, and to the inter-row weeds.
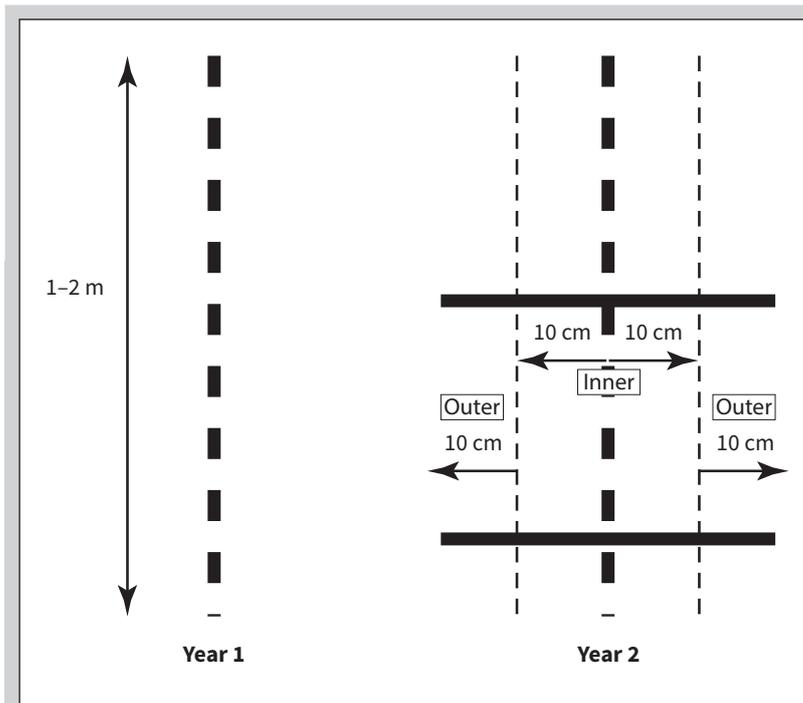
Figure 8.10 Schematic diagram of the cross-row technique describing the layout in years 1 and 2.

Where strains have colonised the soil away from the original placement, and survived over the dry period, good nodulation should be reflected in strong legume growth across the cross-row, as illustrated in Figure 8.11.
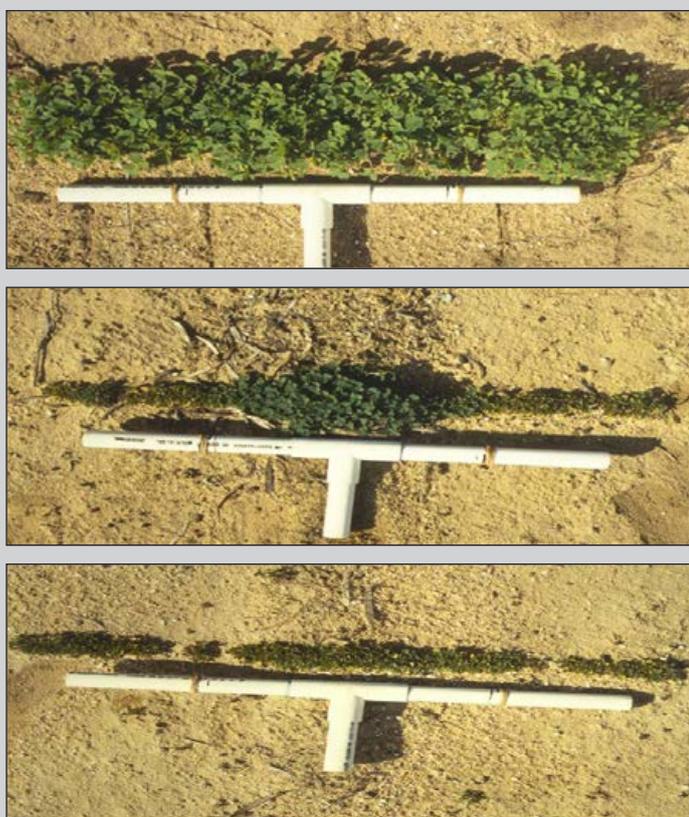
Figure 8.11 Possible outcomes in the second season of a cross-row experiment with *Medicago polymorpha* and *Sinorhizobium medicae*. In the top plate, the rhizobial strain has colonised at least 20 cm either side of the original row (along the handle of the poly pipe). In the middle plate, the strain has survived in its original position but not moved laterally. In the bottom plate, there has been no survival and hence plant growth is greatly compromised by N deficiency. Note the high seeding rate required to generate sufficient plants for nodulation assessment in this example.

8.9 Plots established with precision seeding machinery

Where the aim of the experiment is to investigate response to inoculation in an 'on-farm' scenario, it may be unrealistic to sow plots by hand. A reliable estimate of seed production requires a larger plot size than can be uniformly sown by hand, and destructive sampling for nodule parameters and early growth can reduce the amount of material available. Further, many grain legumes (*e.g.* soybean, common bean, lupin) must be sown at 5 cm depth or deeper which is sometimes difficult to achieve uniformly by hand. Under these circumstances, machine-drawn precision seeders may be the best option for establishment of the plots. Precision seeding machines (Figure 8.12) have been designed to sow small plots (5–25 m length) uniformly, with defined seeding rates per hectare and defined distances between seeds and between rows to ensure plant to plant competition is uniform. Machines typically have a sowing width of 1.2–1.8 m, with row spacing around 15–25 cm apart. If replicates are to be sown in blocks, ensure there is sufficient buffer area between blocks for turning the machinery.

Figure 8.12 A precision seeding machine for sowing larger plots, such as for grain legume experiments.

8.9.1 Inoculation of large-seeded legumes for plots to be sown by machine

For sowing rates of 100 kg per ha, standard plots sown with a precision seeder (*e.g.* 10 m long) require up to 100 g of seed.

1. Surface sterilise the bulk seed (Chapter 5).

2. Split seed into treatments for each plot and pack into cardboard boxes (*e.g.* unused milk containers) or waterproofed paper bags.

3. For inoculation with a liquid (*e.g.* peat slurry or broth) it is possible to inject the required volume (1–5 mL) into the container using a disposable syringe.

4. For granular inoculants, a measured amount (usually at 10 kg/ha) can be delivered to each container, calculated for the plot size.

5. After inoculation, shake the container to ensure even distribution of the inoculant, allow to dry enough that the individual seeds flow, and then place in sowing order.

6. As for hand-sown experiments, each inoculant treatment must be prepared in a separate container and all equipment should be sterilised between treatments.

7. Once inoculation has been completed, sowing should occur within 24 hrs.

8. Mark out and peg plots as described in Section 8.7.

8.9.2 Hygiene at sowing

As for hand-sown plots, all replicates for a given treatment should be sown before moving to the next treatment. However, for the un-inoculated control, four replicates may be sown first to enable assessment of nodulation and plant growth achieved by the resident soil population of rhizobia, and then additional un-inoculated treatments may be sown last. The latter plots can aid in understanding whether there has been contamination in the sowing procedure, by assessing nodule occupancy (Chapter 11). Thus, if logistics permit, eight replicates of the un-inoculated control can be sown. The machinery should be thoroughly cleansed between treatments to avoid transfer of inoculant strains between plots. Parts should be wiped with ethanol (70% v/v) and allowed to air dry. Ethanol can also be washed down sowing tubes and collected in a bucket for reuse. Take care when swabbing any Perspex sections of the seeder with ethanol as this can cause parts to crack. Fertiliser granules can also be dropped through the seeding tubes after each treatment and before cleaning with ethanol to dislodge material that may have been caught in the machine.

8.10 Data collection and assessment of nodulation

Field experiments are large undertakings so it is important to gather the appropriate information from them. This does not infer the need to gather maximum information but rather to understand the purpose of the experiment and sample accordingly. For example, biomass accumulation is not particularly relevant in the second year of the cross-row experiments described previously, as these are designed to assess the adaptation of rhizobial strains to the soil.

8.10.1 Nodule counts and scores

Nodulation is evident within three weeks for many symbioses, but for field experiments it is advisable to wait four to eight weeks before the first assessment of nodulation, depending upon the temperature. Representative samples for nodulation

assessment (*e.g.* 40 plants) can be removed from random positions within the plots, avoiding the edges. In the cross-row experiments, all plants in the 0–10 cm and 11–20 cm region are excavated. Twenty to 30 plants are randomly scored from both areas and the average score recorded. In machine-sown plots, a second nodulation assessment may be taken after 12 weeks to give an indication of the dynamics of nodulation. Nodule abundance may be either counted or scored (see Figure 8.13). Scores can be analysed statistically if they conform to rules of continuity in data. For nodule scores, continuous data is acquired if a number of plants are sampled in each replicate, and the mean score then developed. Ultimately, the decision on what data to accumulate must be made based on the quality of the data required and the resources available.

Nodule score is closely correlated with nodule weight, which can be assessed if nodules are picked from the root system, cleaned, dried then weighed. Where soil cannot be fully removed from the nodules, it may not be possible to generate accurate weight data.

0	0.5	1	2	3	4	5	6	7	8
Absent	Ineffective	Rare (Effective)	Scarce	Moderate	Adequate	Ample	Abundant	Very Abundant	Extremely Abundant
No nodules	White ineffective nodules	0–5 small pink nodules	6–10 small pink nodules	11–20 small pink and/or 1–2 large pink nodules	21–40 small pink and/or 3–4 large pink nodules	< 40 small pink and/or 5–9 large pink nodules	≤ 10 large pink nodules	Crown nodulation <5 mm diameter	Crown nodulation ≥5 mm diameter

Figure 8.13   A nodule-scoring chart that can be applied to both pulse and pasture legumes that have grown more than 12 weeks in the field

8.10.2 Nodule occupancy

Nodule occupancy is an important parameter when assessing the impact of inoculation in the face of competition by strains resident in the soil. The identification of strain(s) within nodules can be determined by isolation from a representative number of these nodules (Chapter 3) and identification by an appropriate technique (*e.g.* agglutination, ELIZA, PCR RAPD). Molecular techniques to identify strains are discussed in Chapter 11. MALDI-TOF analysis of ribosomal proteins is rapid and does not require culturing of bacteria. This has been applied to nodules to determine occupancy, and can be discriminative at strain level for some species of rhizobia (Zeigler *et al.* 2015).

8.10.3 Rhizosphere counts by MPN

The colonisation of the rhizosphere by inoculant strains can be an important parameter with which to judge strain success, yet counts by MPN (Chapter 6) are a time-consuming exercise and results vary both temporally and spatially. The value of the knowledge gained by an MPN estimation needs to be balanced against the effort and resources required, and the likely nature of its variability. For fresh root samples, rhizosphere counts of nodule bacteria may be undertaken by immersion of the roots in a volume of liquid and using this as the primary source of material to begin the dilution series (Chapter 6). The rapid development in next generation sequencing technologies, combined with the growing availability of whole genome sequences for rhizobia, holds promise for the development of a quantitative sequencing-based enumeration method. This would greatly expedite studies of rhizobial ecology.

8.10.4 Plant yield

Plant yield can be very valuable to demonstrate the benefits of inoculation to increased growth, or the benefits of an improved strain relative to controls (Chapter 10, Section 10.4.1). Biomass can be estimated by removing plants within a defined quadrat (*e.g.* 0.25 m^2) placed randomly several times within each plot, usually across the tillage lines. Plants can be cut at ground level using a sharp knife or secateurs, dried in loosely-packed bags at 65–80°C then weighed. Roots from within the quadrat can also be excavated, washed free of soil then dried and weighed, although root biomass is usually highly correlated with plant biomass (Russell and Fillery 1996; McNeill *et al*. 1997). An exception may be in un-inoculated plots, or nitrogen-fed plots, where root systems may be more substantial if they need to explore soil for uptake of mineral nitrogen.

8.10.5 Seed yield

Seed yield can demonstrate the value of an effective symbiosis. However, under conditions of terminal drought, a plant that grows larger because of an improved symbiosis can sometimes yield less seed because of premature senescence. For larger plots, a machine harvester can accurately determine yield, and this can be sub-sampled for analysis. Alternatively, yield on a sub-set of the plot can be determined by quadrats cut at random within the plot. Several quadrats of at least 0.5 m^2 are recommended per 10 m of plot length.

8.10.6 Nitrogen fixation

Plant nitrogen at peak biomass is often a useful parameter to fully understand and describe the benefits of inoculation. N analysis, including measurements on ^{15}N natural abundance, is covered fully in Chapter 10. Plant parts must be harvested, dried at 60–70°C then stored before analysis. Note that in many legume crops, seed protein is not a good indication of N$_2$ fixation per se, as legumes are very

efficient at translocating N to the seeds. In addition, nodule number is not always a good indicator of N_2 fixation. Where molybdenum is deficient, or the strain is poorly effective, nodule number can be greatly increased.

The above parameters (Sections 8.10.1 to 8.10.6) may all be measured to aid in interpretation of the impact of inoculation. Sample containers, such as plastic bags for fresh roots and tops, screw capped vials for MPN counts, and paper bags for dry plant parts and seed, must be clearly labelled with the experiment number, date, treatment and plot number.

8.11 Long-term experiments

Inoculants have several fates: they may remain in a stable form and become part of the soil microflora, such as for soybean in Brazil (Boddey and Hungria 1997); they may be displaced and eventually disappear altogether (Graham 1992); or they may mutate and be altered in their symbiotic properties (Kucey and Hynes 1989; Louvrier *et al.* 1996; Ochman *et al.* 2000). Inoculants may also donate their symbiotic genes to resident (but non-nodulating) bacteria, which can then emerge as competitive, nodulating organisms. Nandasena *et al.* (2009) described the emergence of new species of mesorhizobia within five years of sowing *Biserrula pelecinus* in Western Australia, after their acceptance of a symbiotic plasmid from the inoculant strain. Long-term experiments that wish to follow the fate of introduced strains need to be well marked, preferably with a GPS coordinate. The corners of the main plots can be pegged or metal can be buried beneath the corners for later detection with a metal detector or with GPR.

8.11.1 Following the fate of fixed N and 'free N farming'

In many situations, the profit from incorporating legumes into farming systems is realised in the rotational crop, and much of this benefit is in the fixed N. Where the legume growth has been successful, there is often sufficient fixed N to grow one or more subsequent non-legume crops without additional inorganic N. This we have termed 'free N farming'. The economic benefit can be estimated by carrying the experiments described in Section 8.9 into a second season, with the plots sown to the target non-legume. The benefit of fixed N to a subsequent crop can be estimated as follows.

1. Ensure legume × rhizobium treatments sown in the first year are in plots of sufficient size to split for N rates in a second season. Plots 4m × 20m are usually sufficient.

2. Include an un-inoculated treatment, as well as several rhizobia treatments of reduced effectiveness relative to the best strain, as this will provide a set of comparisons of N fixed.

3. Carefully mark the corner of each plot in the first year (legume × rhizobia) of the experiment.

4. In the next season, manage weeds with a knockdown herbicide, then over-sow the plots with the non-legume crop (cereal or canola) and within each plot, split for levels of inorganic N. Usually zero, 25 and 50 units of N will suffice.

5. Thus, the original 4 m wide plot is split into three sub-plots, each of 1 m × 20 m with 30 cm gaps between sub-plots.

6. The rotational crop can be sampled for biomass yield and N concentration through the season, as well as seed yield, N concentration and protein when the crop has matured.

7. By comparing the response to applied N, both within plots and between strains, the value of the fixed N from the most effective strain of rhizobia can be estimated.

8.12 References

Burgos F.A., O'Hara G.W., Kobryn H. and Howieson J.G. 2009. The application of remote sensing to estimate nitrogen levels across field pea genotypes. In 'Proceedings 15th Australian Nitrogen Fixation Conference, Margaret River', ed. by M.J. Dilworth and L. Brau.

Boddey L.H. and Hungria M. 1997. Phenotypic grouping of Brazilian *Bradyrhizobium* strains which nodulate soybean. Biology Fertility Soils 25, 407–415.

Chatel D.L. and Parker C.A. 1973. Survival of field-grown rhizobia over dry summer period in Western Australia. Soil Biology & Biochemistry 5, 415–423.

Deaker R., Roughley R. and Kennedy I.R. 2004. Legume seed inoculation technology- a review. Soil Biology and Biochemistry 36, 1275–1288.

Farquarson R. 2010. The impact of acetohydroxyacid synthase inhibiting herbicides on symbiotic nitrogen fixation of grain and pasture legumes. PhD thesis. University of Adelaide, Australia.

Graham P.H. 1992. Stress tolerance in *Rhizobium* and *Bradyrhizobium*, and nodulation under adverse soil conditions. Canadian Journal of Microbiology 38, 475–84.

Helyar K.R. and Porter W.M. 1989. Soil acidification, its measurement and the processes involved. Pp. 61–101 in 'Soil Acidity and Plant Growth', ed. by A.D. Robson. Academic Press: Sydney.

Howieson J.G. and Ewing M.A. 1986. Acid tolerance in the *Rhizobium meliloti-Medicago* symbiosis. Australian Journal of Agricultural Research 36, 55–64.

Howieson J.G., O'Hara G.W. and Carr S.J. 2000. Changing roles for legumes in Mediterranean agriculture: developments from an Australian perspective. Field Crops Research 65, 107–122.

Howieson J.G. and Ballard R. 2004. Optimising the legume symbiosis in stressful and competitive environments within southern Australia—some contemporary thoughts. Soil Biology & Biochemistry 36, 1261–1273.

Howieson J.G., De Meyer S.E., Vivas-Marfisi A., Ratnayake S., Ardley J.K. and Yates R.J. 2013. Novel Burkholderia bacteria isolated from *Lebeckia ambigua*—a perennial suffrutescent legume of the fynbos. Soil Biology and Biochemistry 60, 55–64.

Koopman D.J., Tow P.G., Reeves T.G. and Gibson A.H. 1995. Soil acidification, chlorsulfuron application and *Rhizobium meliloti* as factors in lucerne yield decline. Soil Biology & Biochemistry 27, 673–677.

Kucey R.M.N. and Hynes M.F. 1989. Populations of *Rhizobium leguminosarum* biovars *phaseoli* and *viciae* in fields after bean or pea in rotation with non-legumes. Canadian Journal of Microbiology 35, 661–667.

Loi A., Howieson J.G., Nutt B.J. and Carr S.J. 2005. A second generation of annual pasture legumes and their potential for inclusion in Mediterranean-type farming systems. Australian Journal of Experimental Agriculture 45, 289–299.

Louvrier P., Laguerre G. and Amarger N. 1996. Distribution of symbiotic genotypes in *Rhizobium leguminosarum* biovar viciae populations isolated directly from soils. Applied Environmental Microbiology 62, 4202–4205.

McNeill A.M., Zhu C. and Fillery I.R.F. 1997. Use of *in situ* ^{15}N-labelling to estimate the total below-ground nitrogen of pasture legumes in intact soil-plant systems. Australian Journal of Agricultural Research 48, 295–304.

Nandasena K.G., O'Hara G.W., Tiwari R.P., Willems A. and Howieson J.G. 2009. *Mesorhizobium australicum* sp. nov. and *Mesorhizobium opportunistum* sp. nov., isolated from *Biserrula pelecinus* L. in Australia. International Journal Systematic Evolutionary Microbiology 59, 2140–2147.

Ochman H., Lawrence J.G. and Groisman E.A. 2000. Lateral gene transfer and the nature of bacterial innovation. Nature 405, 299–304.

Russell C.A. and Fillery I.R.P. 1996. In situ labelling of lupin below-ground biomass N. Australian Journal of Agricultural Research 47, 1035–1046.

Thies J.E., Cook S.E. and Corner R.J. 1991. Influence of the size of indigenous rhizobial populations on establishment and symbiotic performance of introduced rhizobia on field-grown legumes. Applied Environmental Microbiology 57, 19–28.

Vincent J.M., Thompson J. and Donovan K. 1962. Death of root-nodule bacteria on drying. Australian Journal of Agricultural Research 13, 258–270.

Yates R.J., Howieson J.G., Real D., Reeve W.G., Vivas-Marfisi A. and O'Hara G.W. 2005. Evidence of selection for effective nodulation in the *Trifolium* spp. symbiosis with *Rhizobium leguminosarum* biovar trifolii. Australian Journal of Experimental Agriculture 45, 189–198.

Ziegler D., Pothier J.F., Ardley J., Fossou R.K., Pflüger V., Meyer S.E., De Vogel D., Tonolla M., Howieson J., Reeve W. and Perret X. 2015. Ribosomal protein biomarkers provide root nodule bacterial identification by MALDI-TOF MS. Applied Microbiology and Biotechnology (DOI 10.1007/s00253-015-6515-3).

CHAPTER 9
Inoculant production and quality control

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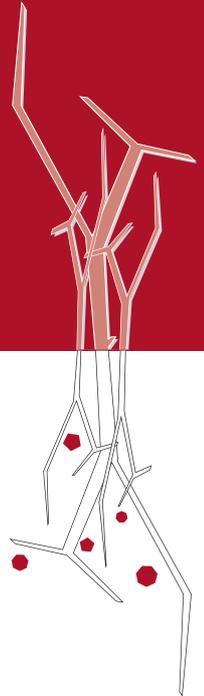
9.1 Introduction

The manufacture of high-quality inoculants increases the potential for maximum nitrogen fixation in inoculated legumes (see Chapters 1, 5, 8 and 10). If legume inoculation is successful, the inoculant strain will colonise the rhizosphere and compete with resident soil rhizobia for nodulation sites on the host legume root. Researchers too must prepare high quality inoculants for experimental field trials, similar to those described in Chapter 8.

Large-scale production of high-quality legume inoculants is complex. It requires expert skills in aseptic handling of rhizobia, an understanding of the conditions in which rhizobia grow and survive, research and development to find suitable carrier materials for inoculant formulation, and a program of quality control and quality assurance to maintain the manufacturing process and product integrity. There are several other publications describing methods involved in legume inoculant quality control; comprehensive texts have been published by Vincent (1970) and Somasegaran and Hoben (1994).

The inoculant 'supply chain' can be summarised as:

► rhizobial strains are selected that are highly effective in fixing nitrogen with the target legume host (Chapters 2, 5 and 8)

► batch cultures of these strains are grown in liquid medium (Chapter 3)

► the cells are maintained either as liquids, concentrated cell masses or incorporated into solid carriers to form inoculant products

► the inoculants are distributed to farmers

▶ the farmer inoculates the legume, by coating directly onto the seed, or apply-
ing as liquids or granules to the soil in close proximity to the seed.

At each point along this chain, quality control protocols should be implemented
to ensure strain purity, growth, survival and functional stability (Figure 9.1).
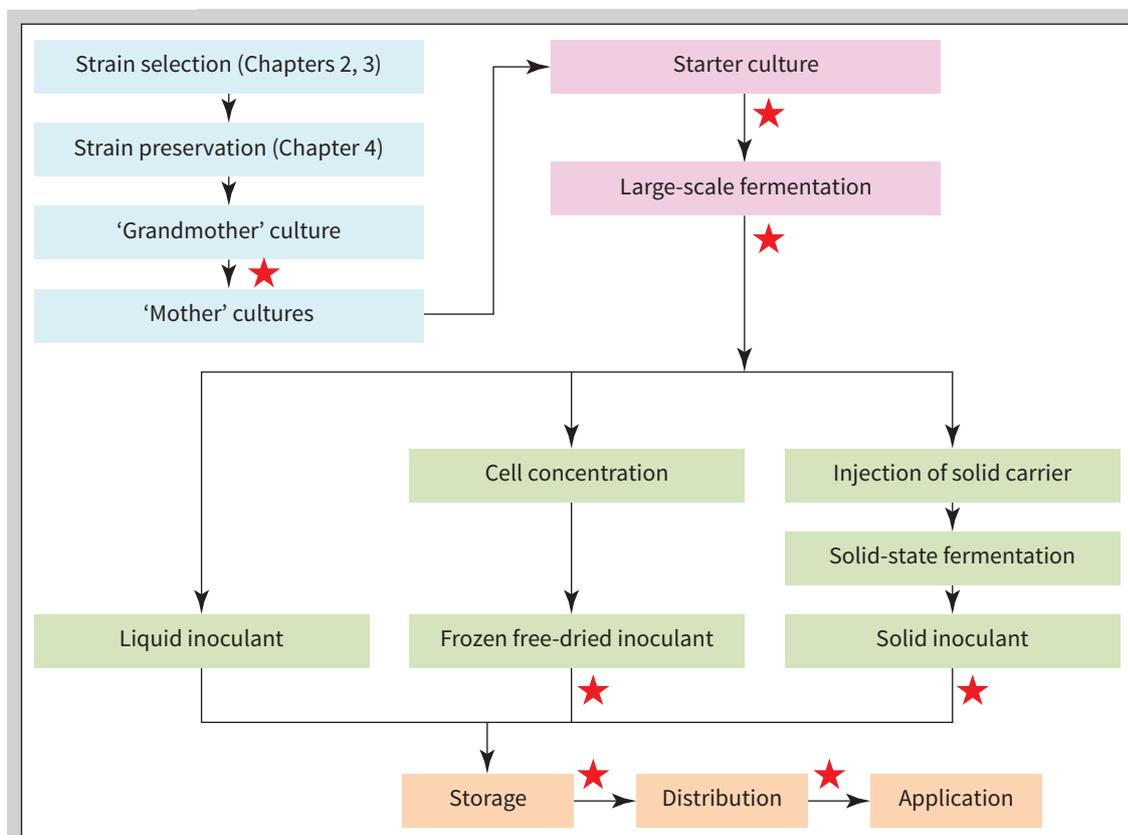
Figure 9.1 Stages in production of legume inoculants, their distribution and application. Colours indicate different stages in the process: 1. strain selection and preservation (blue); 2. Large-scale production of batch cultures (pink); 3. formulation of inoculants (green); 4. storage, distribution and application (orange). Red stars indicate points for quality control to determine strain purity, growth, survival and functional stability.

9.2 Inoculant production

The first step in inoculant production is the growth of liquid starter cultures from a 'mother' culture.

9.2.1 Growth in batch culture

The most widely adopted method for growing cultures of 20 mL to 1,000 mL is the regular batch or discontinuous culture. The key requirements are maintenance of culture sterility during inoculation and provision of oxygen for respiration of

the rhizobial cells. Volumes less than 1 L are aerated on a shaking platform; larger volumes require filtered air or oxygen.

1. Select culture medium (Chapter 3) and dispense into appropriate-sized vessels to no more than 20% of the vessel volume. For laboratory purposes this might be a 250 mL Erlenmeyer flask containing 50 mL of liquid.

2. Close with screw cap (not fully tightened) or cotton bung covered with aluminium foil to keep the bung dry during sterilisation in the autoclave.

3. Autoclave at 121°C (15 psi) for 20 minutes. Larger volumes (>1 L) take longer to reach the required temperature so the sterilisation time must be increased.

4. Place media in a laminar flow cabinet to cool and close screw caps when vessels reach room temperature.

5. Inoculate small volumes (<1 L) in the laminar flow cabinet. Remove the cap or cotton bung and pass the neck of the vessel through a Bunsen flame. Tilt to one side to bring the liquid near the neck, then using a sterile inoculating loop, transfer a loopful of culture from an agar slope or plate into the liquid, or insert a porcelain bead coated with rhizobia. Take care not to touch the top of the open vessel. Replace cap or cotton bung and aluminium cover.

6. Inoculate larger volumes by pipette with a broth culture at log phase to a final volume of 10% of the growth medium.

7. Place small vessels on a platform shaker set at 150 rpm, or provide filtered oxygen / air to large vessels (*e.g.* 5 L/L/hour) and monitor pO_2.

8. Monitor the growth characteristics of the strain by regular inspection. There are about 10^7 cells per mL when the growth medium is slightly opaque (OD = 0.1).

After reaching the target cell density (usually 10^9 cells/mL), strains can be introduced directly into the carrier of choice to make small-scale inoculants for researchers, or as a starter culture in a scale-up process, with the batch culture providing a proportion (*e.g.* 10%) of the volume to the next vessel.

There are alternatives to preparing a starter culture in liquid broth. One such method uses two plugged Buchner flasks with side arms joined by silicon tubing (described in Thompson 1983). One contains agar medium which can be sloped for culturing cells, the other has liquid medium, and the whole system is sterilised. After growing a culture on the agar slope, the liquid is decanted through the silicon tubing to the sloped agar culture. The cells are then washed from the slope and transferred directly to a fermentation unit without opening any bungs. The advantage of this method is that growth of any contaminant microorganism on the slope is easily observed.

9.2.2 Scaling up broth cultures for manufacture

Vessels to produce large volumes of liquid cultures (>5 L) are generically termed fermenters. They vary from simple to highly complex in design, but it is essential that they are readily sterilised, allow access for inoculation and sampling, and provide aeration for the culture. Because large fermentors are bulky and heavy, they usually have capacity for sterilisation *in situ*.

Continuing with the example above, the 50 mL broth culture can provide the inoculant for 450 mL held in a 2.5 L flask that can be constructed from readily available laboratory materials (Figure 9.2a). Fermentors larger than this generally have the same main components (Figure 9.2b). The broth is aerated via an inlet tube fitted with a sterile air filter. This filtered air pumped through the medium acts to both aerate and mix the broth culture. Where the fermentor volume exceeds the capacity of the air to provide adequate mixing, mechanical paddles can be added to the design to stir the medium. Fermentors also require a gas outlet and a port for sampling broth during the growth period. All components and fittings shown in Figure 9.2b must be sterilised to ensure the broth does not become contaminated. This unit can be sterilised as a whole in an industrial autoclave, or it can be set on a gas ring burner and the contents steamed for at least 30 minutes, with all ports vented and exposed to the steam.

After inoculation, the cultures should be monitored for cell number and pH during growth, taking great care not to introduce contaminants in the process. Unusual changes in pH in complex media (*i.e.* < 6 or > 8) can be a convenient way of detecting possible contamination, however, as is microscopic visualisation of a sample from the fermentor in the early phases of growth (see Chapter 7, Section 7.2.5.2). Common contaminants may be detected in broth samples by plating a sample on peptone agar (Chapter 3, Section 3.7.2.4) and observing growth and pH change after 24–48 hours at 30°C (Chapter 7).

Figure 9.2a A simple fermentor –adapted from Somasegaran and Hoben (1994). A: aquarium pump; B: non-absorbent cotton wool; C: plastic syringe barrel; D: clamp; E: silicon and glass tubing air inlet; F: silicon and glass tubing air outlet; G: glass Erlenmyer flask; H: broth culture; and I: broth sampling tube.
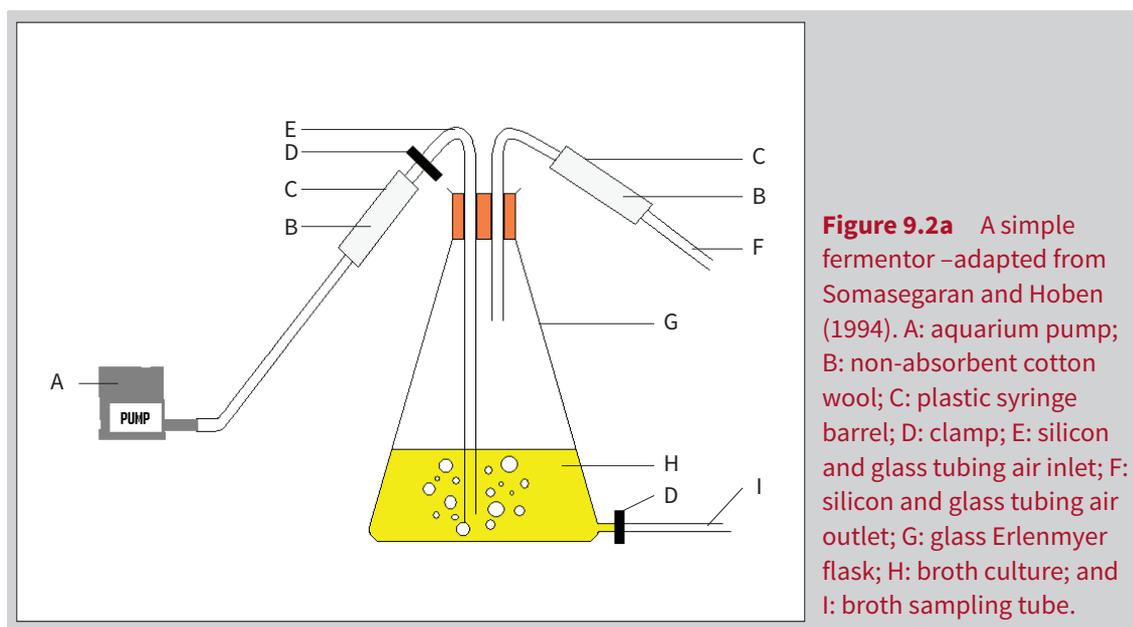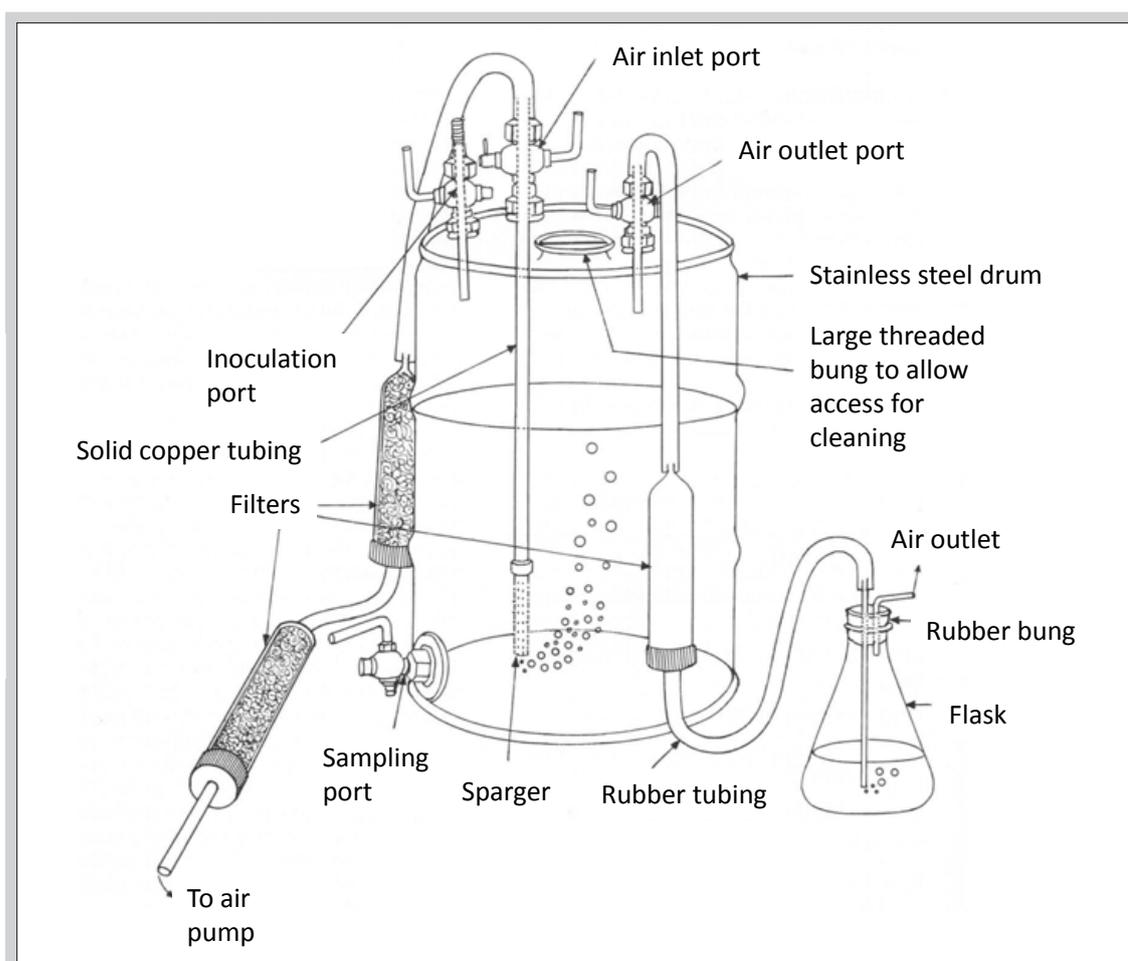
Figure 9.2b A simple stainless steel fermentor (reproduced from Thompson 1983).

9.3 Carrier materials for inoculants

After reaching the required cell density, the inoculant is then 'formulated' with several possible different carriers and extenders. These are solid or liquid media that support live bacteria after broth culture substrates are exhausted. The broth culture from the fermentor is introduced into the carrier, and for most carriers (after an initial 'curing' period) there is little subsequent active growth of bacteria. The carrier is then stored, distributed to growers and finally applied to either the legume seed or the soil.

Finely milled peat is a well-researched and widely adopted carrier in many countries but inoculants may also be available as liquid, granular, frozen pastes and freeze-dried preparations (Figure 9.3). Suitable peat sources are not always available and many alternatives have been investigated, including organic carriers such as compost, bagasse, farmyard manures, sugarcane filter mud and coconut coir dust; and inorganic carriers such as perlite, polymers and clays (reviewed by Roughley and Pulsford 1982; Burton 1984; Bashan 1998). Composted materials

from the cork industry and perlite were superior to peat in maintaining survival of different rhizospheric bacteria (Albareda *et al.* 2008).

Desirable characteristics of solid carrier materials are listed below (from Thompson 1980).

1. A capacity to be finely ground to allow thorough mixing with other components and to allow ease of application.

2. Adjustability of pH to 6.5–7.0.

3. Good moisture-holding capacity.

4. Able to be sterilised to favour survival of rhizobia.

5. Free of toxic materials and low salinity.

Figure 9.3 Alternatives to peat inoculant products. (a) Peat granules (left), bentonite clay granules (middle), attapulgite clay (right); (b) Liquid; (c) Freeze-dried powders.

9.3.1 General preparation of a solid carrier, *e.g.* peat

1. Drain any excess moisture from the peat for ease of handling.

2. Grind peat in a hammer mill until particle size is less than 75 μm.

3. Homogenise the peat.

4. Adjust pH to near neutral with weak acid or $CaCO_3$.

5. Apportion peat into desired volumes in containers.

6. Adjust moisture content to desired level (often 10–20%).

7. Reduce resident contaminating microflora by sterilisation, *e.g.* autoclaving, heating, radiation or other treatment.

9.3.2 Adjusting the pH of carriers

If $CaCO_3$ is required to raise the pH of the carrier, it should be well mixed and reacted carefully, allowing time for equilibration. The amount of $CaCO_3$ or acid required to change the pH will depend upon organic matter and clay content as well as the buffering capacity of the carrier.

9.3.3 Reducing contaminants in the carrier

Ideally, the carrier material should contain very low numbers of contaminant microorganisms before introducing rhizobial strains, as resident microflora can restrict the growth of rhizobia and reduce their final number in the inoculant. The amount of restriction depends upon the rhizobial strain (Roughley and Vincent 1967). Autoclaving of organic carriers is an effective means of sterilisation but can result in the release of toxic polyphenols, and this should be assessed. A small amount of moisture is required for the production of steam within the carrier for optimum sterilisation. If a carrier is too dry, sterilisation will not be complete. The efficacy of sterilisation can be measured by injecting the 'sterile' carrier with sterile growth medium and measuring growth of contaminants over time (*e.g.* for one month).

It is better to autoclave organic materials several times at temperatures lower than 121°C (*e.g.* 100°C), allowing them to cool between runs, as toxic products from the breakdown of organic matter may accumulate. Repeated cycles of heating will ensure spore-forming microorganisms are sufficiently reduced. Otherwise, gamma-radiation (at approximately 75 KGy) is a well-proven means of sterilisation but is not without limitations; its effectiveness depends on the initial contaminant load in the carrier which is related to moisture content and packaging conditions before exposure to radiation (Bullard *et al.* 2005).

Other means of reducing contaminants in the carrier can be tested. Exposure to UV light, hydrogen peroxide, ethylene oxide, acidification and solarisation have all been explored for this purpose.

If sterilisation is not possible, broth culture can be injected at a higher cell concentration resulting in little dependence on further growth. However, carriers must have a high moisture holding capacity if large volumes of broth are added. After injecting sterile carriers with broth cultures of rhizobia, the number of viable cells per gram of carrier should be measured at several time intervals to indicate relative growth, survival and 'shelf life'. Rhizobial strains can be differentiated from other microorganisms using techniques described in Chapters 3, 7 and 11.

9.3.4 Moisture content

The moisture content of the carrier is important as it affects rhizobial growth and survival. Particle size, organic matter and clay content of peat contribute to its water holding capacity. For example, Vietnamese peat inoculants with low organic matter content have been amended with materials such as coconut coir dust and fish pond filter mud to increase moisture holding capacity (unpublished data from AusAID CARD project 01306VIE). Increasing the water holding capacity of carriers allows larger volumes of concentrated rhizobial broth culture to be introduced before incubation while maintaining optimum moisture content for cell growth.

9.3.5 Determination of optimum moisture content in solid-based carriers

Optimum moisture content for good growth and survival of rhizobia can be determined by measuring viable rhizobial numbers in carriers with different moisture contents over time. Examples of the amount of liquid required to adjust peat to 40, 50 and 60% moisture content are listed in Table 9.1. The volumes are based on an initial moisture content of 20%. Peat carriers that have different moisture holding characteristics will require different volumes of liquid to adjust to the same moisture content.

Table 9.1 Examples of treatments for measuring optimum moisture content for microbial inoculants. Calculations based on 70 g dry peat after adjusting to 20% moisture content for sterilisation.

Moisture content (%)	Total liquid added (broth + water, mL)	Volume of broth (mL)	Volume of sterile water (mL)
40	37.3	37.3	0
50	56.0	37.3	18.7
60	84.0	37.3	46.7

9.3.5.1 Calculating the amount of liquid to add to peat or carrier mixture

The equation below is used to calculate the moisture content of 70 g dry peat, where x is the amount of liquid added and y is the final percent moisture. The same equation can be used for any quantity of peat, but as peat will invariably already contain some moisture, the mass of dry peat must first be determined.

$$\frac{x}{70 + x} = \frac{y}{100}$$

Therefore, to adjust 70 g of dry peat to 20% for sterilisation 17.5 mL of water should be added.

Sample calculation of liquid added to peat to obtain specific moisture content

How much liquid (*e.g.* broth) should be added to 70 g peat with 20% moisture to achieve a moisture content of 40%?

Mass of dry peat

$$\frac{20}{100} \times 70g = 14g$$

$$70g - 14g = 56g$$

Therefore, the mass of dry peat is 56 g

Volume of liquid to add to dry peat

$$\frac{x}{54 + x} = \frac{40}{100}$$

$$x = 0.40\,(56 + x)$$

$$0.60x = 22.4$$

$$x = 37.3\text{g}$$

Therefore, 37.3 g moisture should be added to 70 g dry peat to achieve 40% after adjusting to 20% moisture.

9.4 Peat inoculants

Pre-sterilised (gamma-radiated) finely ground peat sealed in plastic packets is widely utilised as a carrier for rhizobia. Peat inoculants consist of finely ground peat injected with a single strain of rhizobia at a high concentration. The final preparation has relatively high moisture content when compared with other solid formulations. They are usually prepared as a slurry to coat the seed and are most effective when sown into moist soils. They can also be diluted in potable water and applied as a liquid directly into the soil.

The preparation procedures described below may also be applied to any finely ground organic carrier.

1. Prepare a broth culture of rhizobia in a suitable vessel or flask.

2. Estimate the amount of broth culture to be added to the packet to obtain the correct moisture content for optimal rhizobial growth and survival.

3. At the site of injection, surface sterilise the packet containing pre-sterilised peat (or other carrier) with a swab containing 70% ethanol.

4. Using a sterile syringe and needle, carefully draw up the required volume of broth from the flask. Flame the neck of the flask, tilt it to one side and insert the needle into the broth culture. Draw the broth into the syringe. Take care not to touch the open neck of the flask.

5. Inject the required amount of broth culture into the packet, ensuring that none of the culture escapes from the injection site. Eject the last drop of liquid to ensure the needle is not blocked by organic matter if it is to be reused. Manufacturers can automate this step using an injection system.

6. Swab the injection site with ethanol and allow it to evaporate before covering the hole with a sticky label displaying the rhizobial strain name and the date of injection.

7. Multiple peat packets can be injected using the one syringe and needle when researchers are producing multiple inoculants, but needles and syringes must be discarded before working with a second or subsequent strain.

8. To check for contaminants in the broth culture prior to injection into the carrier, streak a drop of the broth culture onto general agar medium (*e.g.*

glucose-peptone medium) in a Petri dish and incubate at 26–28°C and check for growth after 24–48 hours. Check the growth every 24 hours to verify purity of the broth culture.

9. Carefully massage the peat packets until the broth culture is evenly distributed within the packet, making sure that the broth reaches each of the four corners.

10. Incubate the inoculant for one to two weeks at 26–28°C.

Some inoculant manufacturers dilute broth cultures (*e.g.* 1:10 or 1:100 dilutions) to make peat-based inoculants. However, it is necessary to check growth and survival of rhizobia with each carrier over time to determine suitable dilutions.

9.4.1 Counting viable rhizobia in peat inoculants

If the carrier is sterile, before injection of broth, the Miles and Misra drop plate count (Chapter 6) can be used as an alternative to the spread plate count method for enumeration. If numbers differ widely between strains then the quantity of inoculants to be utilised in the experiments (Chapter 8) will have to be varied.

If the peat carrier is non-sterile, it is recommended to use a plant infection – frequency of nodulation test that provides a most probable number estimate of viable rhizobia per gram of inoculant (Chapter 6).

9.5 Granular inoculants

Granular inoculants are convenient to use as they can be applied directly to soil and there is no need to prepare adhesive slurries to inoculate the seed. Granules can be mixed with seed and then distributed from the seed box, or through a separate fertiliser box available on some planters. Granules generally have lower moisture contents than peat inoculants and therefore have the advantage of not requiring refrigeration and can be loaded into seeders in bulk when required. Some dry granules have proven effective for introducing inoculants to dry soils by protecting rhizobial cells until rains fall (Loi *et al.* 2012). However, the requirement for high application rates (because of generally lower numbers of rhizobia per gram) can be a disadvantage.

Granules can be manufactured by:

▶ prilling an existing carrier such as clay (usually dry) or organic matter (usually moist); this may be done by either extrusion or sorting of naturally formed granules

▶ impregnating broth cultures onto pre-formed granules (such as peat, alginate, hydrophilic minerals or polymers) which absorb the inoculant into the granular matrix.

Granular products are graded into a range of particle sizes from 0.5 mm to 5 mm in diameter. The smaller particles are better for distribution of rhizobia over a cropping area. If a large proportion of particles are less than 125 μm, flowability of the granules through the seeder or other distribution equipment may be reduced and cause blockages.

9.5.1 Granulated clays

A recent invention is the dry clay granule which holds rhizobial cells protected from desiccation within a clay and organic matrix, analogous to how rhizobia exist naturally in many soils. Survival in the clay carrier has been related to formation of a protective layer around cells by clay minerals as a result of surface charge interactions (Marshall 1968, 1969).

Procedures for making dry-clay granules

1. Prepare a quality peat inoculant as described in Section 9.4.

2. Source appropriate clay, and if practicable, reduce numbers of contaminant microbes.

3. Dilute peat inoculant 1:5 in sterile water to produce a slurry.

4. Add slurry 1:10 to clay and mix thoroughly.

5. Pour the clay slurry into a shallow (1–5 cm) containment vessel and allow to air dry.

6. Crush or mill the dry product to the required particle size.

7. Bag, label and store at room temperature.

Clays may be alkaline or acidic, and rhizobial species react differently to pH. Bradyrhizobia in general survive in greater numbers in acidic clays, while the faster growing species of rhizobia prefer alkaline clays. However, the *Burkholderia* inoculants for *Lebeckia ambigua* did not survive the drying process in manufacturing dry clay inoculants based upon bentonite (Howieson *et al.* 2013); hence the researcher must assess the suitability of the carrier for the target rhizobial species.

Numbers of rhizobia per gram are generally lower in dry clay granules than in moist granules or peat, decreasing from approximately 10^9 per g at mixing to 10^7 per gram when dry. However, as the granules are dry, further death of the inoculants upon exposure to a drying environment is potentially minimised.

9.5.2 Freeze-dried powders

Freeze-dried inoculants are produced from cell pastes that have been concentrated from large quantities of broth, usually by centrifugation. The cells are mixed with cryoprotectant agents (*e.g.* skim milk powder) before drying in commercial freeze-dryers. Depending on the volume and capacity of the freeze-dryer, drying

may take several days. Freeze-dried cells are reconstituted with a protective liquid polymer to form a slurry to inoculate seed and can also be applied as a liquid directly into the soil.

Not all strains of rhizobia respond well to freeze-drying and rhizobial numbers may be reduced. However, freeze-dried inoculants generally have very high numbers of rhizobia per gram and their shelf life under vacuum over a range of temperatures can be many years.

9.6 Storage of inoculants

The different carriers and rhizobial species have varying requirements for shelf life and storage depending upon:

► the growth characteristics of the rhizobial strain

► whether the strain originated from a tropical or temperate climate

  ▷ rhizobial peat inoculants should be stored in a cool place (ideally 4–8°C) and should never be frozen

  ▷ there are exceptions for inoculants prepared for tropical legume species *e.g. Desmodium*, *Centrosema*, *Lablab* and *Stylosanthes* which should be stored between 20 and 25°C

► the type of carrier (solid, liquid or freeze-dried)

► the form of packaging.

Table 9.2 below provides a matrix showing recommended storage conditions for different inoculant formulations and strain origins.

Table 9.2 General storage conditions for different rhizobial inoculants

Inoculant formulation	Origin of strain	
	Tropical	**Temperate**
Peat-based	Ambient (20–25°C)	Refrigerated (2–4°C)
Granular	Ambient (20–25°C)	Ambient (10–20°C)
Freeze-dried	Refrigerated(4–10°C)	Refrigerated (4–10°C)
Liquid	Refrigerated (4–6°C)	Refrigerated (4–10°C)

9.7 Inoculant quality control

9.7.1 What is quality?

Quality refers to the delivery of high numbers of viable and effective rhizobial cells to nodulate the target legume at sowing. The term 'quality' is often associated

with the number of live cells delivered and the ratio of these to contaminants. The efficacy of inoculants may not be the same at all sites because of the manner in which the inoculant is used, and the great diversity of environmental, biotic and edaphic conditions they may confront. For some symbioses, very few inoculant cells are required to achieve optimal nodulation (less than 10 per seed), whereas for others this number may be 1,000 times greater. However, the number of live cells delivered is a convenient descriptor of quality, and one which the industry has come to promote.

Without adequate practical means of determining the symbiotic interactions at each site, standards are based largely on the maximum possible inoculum potential (number of live rhizobia in inoculant × application rate) with each formulation. The numerical standards for Australian legume inoculants are listed in Table 9.3. These standards have been recommended for many years and are sourced from the Australian National Code of Practice, 'Quality Trademark for Microbial Inoculant Products used in Australian Crops and Pastures' compiled by the Australian Inoculants Research Group (AIRG) and the Legume Inoculant Industry, and implemented in 2010.

Table 9.3 Current numerical standards for Australian inoculants

Product	Fresh count	Expiry count	Expiry (months from test date)
Peat (cfu/g)	$\geq 1 \times 10^9$	$\geq 1 \times 10^8$	12–18
Liquid* (cfu/mL)	$\geq 5 \times 10^9$	$\geq 1 \times 10^9$	6
Granules (MPN/g)	$\geq 1 \times 10^7$	$\geq 1 \times 10^6$	6
Freeze dried** (cfu/vial)	$\geq 1 \times 10^{12}$	$\geq 5 \times 10^{11}$	6

*   Standard for liquids based on a 3 L bottle used to treat one tonne of seed.
**Standard for freeze-dried product based on vial used to treat 500 kg seed for large-seeded legumes. Contaminants in peat, liquid and freeze-dried products should be absent at the 10^{-6} dilution when counted according to AIRG protocols.

Despite technical advances in inoculant production systems over the years, and the expertise of commercial inoculant manufacturers in producing large volumes of legume inoculants, there have been challenges with inoculant production and utilisation.

Challenges associated with the production and use of peat inoculants between 1953 and 2003 in Australia were listed by Bullard *et al.* (2005) and are presented in Table 9.4 to illustrate some of the issues that may occur. Some challenges remain unresolved, such as poor survival for some genera of rhizobia on pre-inoculated seed.

Table 9.4 Problems associated with the manufacture of peat inoculants in Australia 1953–2003

Problem	Description	Year
Quality of peat cultures	Low numbers and poor survival of some strains of rhizobia in peat due to contaminants	1953–1966
Quality of peat carrier	Overheated peat caused poor viable counts of clover strains of rhizobia	1963
Quality of peat carrier	Ethylene oxide residue after sterilisation treatment caused low numbers of rhizobia in peat cultures	1964
Quality of peat carrier	High salt content due to changes in climate and water table levels caused low numbers of rhizobia in peat cultures	1974
Peat culture quality	Death in peat culture of some rhizobia for tropical legumes due to cold storage	1978
Quality of peat carrier	Ineffective sterilisation using gamma irradiation caused problems with contaminants	1978–2003
Plastaid® seed coating calcium carbonate	Low counts of rhizobia on seed due to high pH of Plastaid® (calcium carbonate) material used for coating seeds	1990–2002
Pre-inoculated seed quality [A]	Low viable numbers of clover rhizobia on seed	1959–ongoing

[A] Pre-inoculated seed refers to seed that is commercially inoculated and coated prior to sale, and then stored for up to six months. (Table adapted from Bullard *et al*. 2005).

While there is no global standard, there is general agreement that inoculants should deliver a recommended number of rhizobia per seed and this varies according to seed size (Table 9.5).

Table 9.5 The number of cells recommended for legumes of different seed size*

Seed size	Cells per seed
<2 mg *e.g.* biserrula, white clover	500
2–5 mg *e.g.* sub clover, lotus,	1,000
5–10 mg *e.g.* hedysarum, siratro	10,000
>10 mg *e.g.* lupin, pea, soy, bean	100,000

*(from Lupwayi *et al*. 2000; Herridge *et al*. 2002)

Field evaluation of the different inoculant products is recommended (Chapter 8). As a guide, in Australia the recommended rate of application of inoculants has been designed to deliver $> 1 \times 10^{10}$ live cells per ha within the shelf life of the inoculant.

In addition to rhizobial numbers, inoculants should be correctly labelled with information about the recommended plant host, the rhizobial strain, methods for application, batch number, conditions for storage and expiry date.

9.7.2 Numbers of contaminating organisms

Ideally, peat, liquid and freeze-dried inoculants should not contain a large number of any organism (contaminants) other than the selected rhizobial strains. However, if this is not possible, contaminants should be at least 100 times fewer in number than the rhizobial strain. Numbers of contaminants and moisture content

of peat–based inoculants are good indicators of potential shelf life and should be checked routinely. If inoculants are retested just prior to expiry and the rhizobial numbers have been maintained without contaminants, shelf lives can be extended.

9.7.3 Quality control protocols

The objectives of quality control protocols are to determine the number of viable rhizobia in an inoculant formulation at a point in time and to measure parameters that indicate the likelihood that quality will be maintained throughout shelf life. The key activities and parameters are:

- ► monitoring of batch variability

- ► recovery, enumeration and positive identification of rhizobial cells from the carrier

- ► effectiveness in fixing nitrogen of inoculant strains

- ► enumeration of contaminants

- ► measurement of moisture content or relevant conditions that support growth and survival in the carrier

- ► assessment of products from different points in the supply chain.

9.7.3.1 Batch variability

Both liquid and solid inoculants are usually produced from single strain fermentation in batches. Each batch represents a different set of conditions that may affect the quality of the resultant products. The number of packets that should be tested per batch depends on variability of the product. For example, in Australia, seven packets are submitted from each batch of legume inoculant produced. A subset of five packets is tested, and if all five pass the standards, the batch is reported as passed. If one of these five packets fails, the other two packets are tested. If these two additional packets pass the standards, then the batch is passed. However, the batch is reported to have failed if two or more of the original five packets fail the set standards, or one of the two extra packets tested fails.

There should be an adequate recording system so that testing of each batch can be tracked and reported back to manufacturers. Consideration should be given to the logistics of product recall should product release be based on presumptive tests only.

It is useful if the QC testing laboratory is notified ahead of time of the number of samples being sent to enable the necessary materials (media *etc.*) to be prepared. After receipt of products in the laboratory, they should be stored in appropriate conditions (*e.g.* at 4°C) and quality control tests should be carried out promptly.

9.7.3.2 Strain identity

To ensure the correct strain is present in the inoculant, the identity of the strain must be established. Refer to Chapters 3, 6, 7 and 11 for methods of identification of rhizobia.

9.8 Determination of shelf life

9.8.1 Testing inoculants in a distribution network

The number of live rhizobia in inoculants should be determined from products in the distribution network (*e.g.* from retail outlets and storage warehouses). Survival of inoculant strains is variable and is dependent on environmental factors, such as temperature fluctuations. Rhizobial numbers in Australian peat inoculants counted at the point of manufacture (fresh) and throughout the distribution chain (retail) demonstrates the variable response of some rhizobial species to conditions during distribution of inoculants (Figure 9.4). Results from studies of long-term cold storage (4°C) of peat inoculants revealed less variation in survival, and while interesting, may not be a true representation of shelf life in the distribution chain. As well as temperature, shelf life of inoculants is affected by changes in moisture content of the carrier. Loss of moisture can be reduced by selecting less permeable packaging materials, but packaging should also allow adequate exchange of gases.

In Australia, standards were originally set by consciously rejecting inoculants with rhizobial counts in the bottom 20% of the distribution. The boxplots in Figure 9.4 allow visualisation of the distribution of viable rhizobial numbers in inoculants above the 25th percentile. With the exception of white clover, 75% of the inoculants have counts above the minimum standard of $\log_{10}$ 8.0 at expiry. In this dataset there are several inoculants that fall outside the normal distribution, as indicated by the red and green crosses. Therefore, when setting numerical standards, an acceptable minimum number should be recommended, bearing in mind that numerical standards are often a compromise between what is agronomically desirable and commercially realistic.
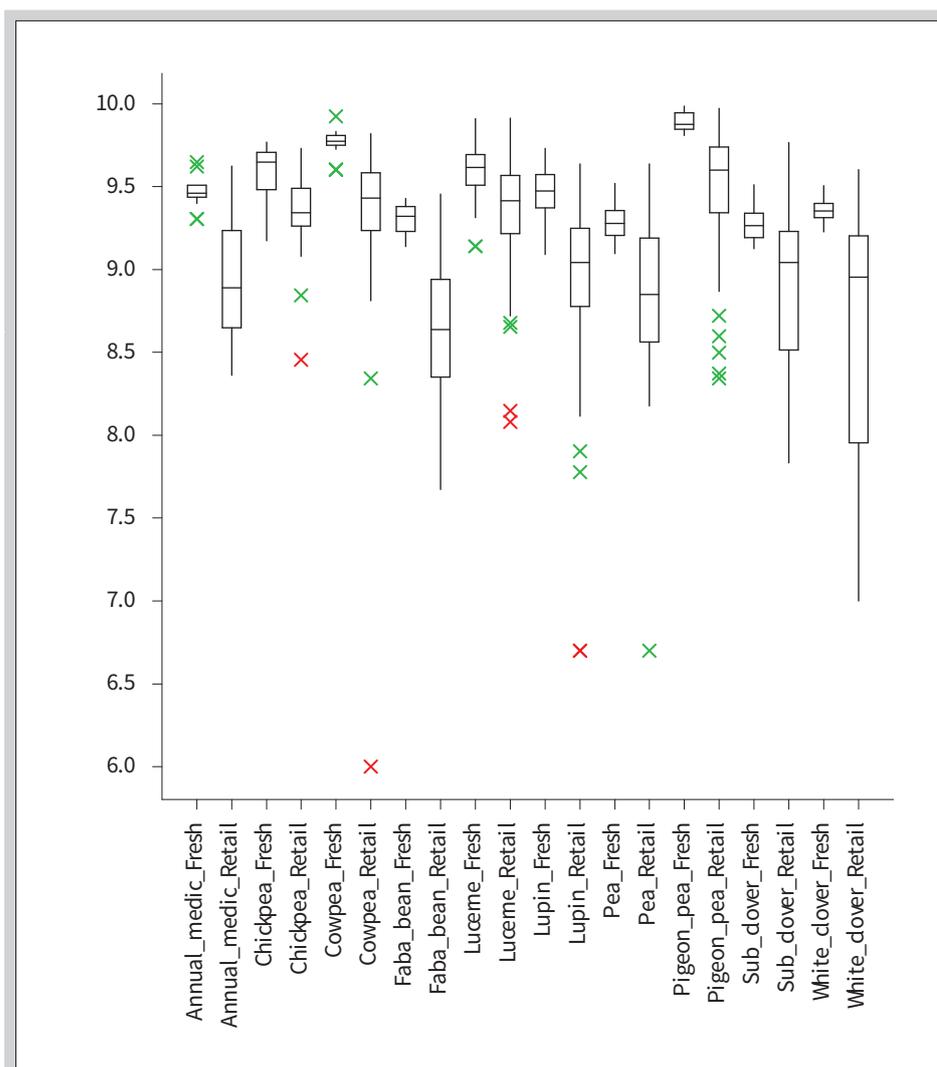
Figure 9.4 Boxplot of $\log_{10}$ transformed number of rhizobia per gram of peat inoculant counted at point of sale (retail) and at point of manufacture (fresh). Data are from the Australian Inoculant Research Group inoculant surveys. The box represents 50% of data or inter-quartile range (IQR). The line in the box represents the median and whiskers extend no more than 1.5 × IQR. Data points outside this range may be considered outliers in an analysis. The boxplot gives a useful summary of the distribution of data, whether it is symmetrical or skewed.

9.9 Criteria for the selection of strains for use in commercial manufacture of legume inoculants

The underlying principles for the selection of strains for commercial inoculant production relate to functional characteristics, such as effective N_2 fixation and the ability to grow and survive during manufacture, distribution and application.

The selection criteria for commercial quality strains are:

1. effectiveness and appropriate host range (Chapter 5)

2. genetic stability in culture, storage and manufacture

3. potential for scale up of production

4. ability to survive in carrier and on seed

5. Field performance and (where required) persistence (Chapter 8)

Criteria 2–4 are briefly covered here.

9.9.1 Genetic stability

Rhizobia can alter their characteristics when cultured or stored for long periods (Bullard *et al.* 2005; Ochman *et al.* 2000). Some outcomes from the Australian quality control evaluations are presented in Table 9.6 as examples. Isolates of some strains may exhibit genetic instability, hence strains recommended for commercial inoculants should be selected to be stable in culture with no loss of symbiotic capacity.

Table 9.6 Some genetic stability problems associated with commercial rhizobial strains in Australia 1953–2003. (Table adapted from Bullard *et al*. 2005).

Rhizobium strain(s)	Strain description	Description of problem	Year(s) reported
NA30	Clover strain from 1956–1960 (to 1962 in WA)	Loss of effectiveness on white and red clovers	1959–1960
TA1	Current commercial strain for white clover	Produced variants ineffective on white and red clovers	1963
NA34	Clover strain from 1953–1955	Loss of effectiveness and production of non-invasive isolates	1956
UNZ29	Clover strain from 1963–1966	Poor competitive ability and loss of effectiveness	1966
CB756	Strain for mung bean (1963–1977), peanut (1963–1989) and several others	Reduction of, or variation in, effectiveness	1975
WSM826	Lucerne, *Medicago littoralis* and *Medicago tornata* strain (1992–2000)	Variation in colony types	1992
W118	*Medicago rugosa* strain (1970–1980)	Partial loss of invasiveness	1980
WU425	Lupin (1970–present) and serradella strain (1970–1996)	Variation in time to form nodules	1971

Of the problems listed in Table 9.6, loss of effectiveness is the most serious. Strains should be tested every season for their ability to form nodules and effectively fix nitrogen after being cultured and stored under different conditions (Chapters 3 and 4). To test for this, undertake a glasshouse experiment (Chapter 5) and follow the procedures below.

1. Prepare legume seedlings for nodulation in the preferred authentication medium (Chapter 5). If the legume can fix nitrogen optimally in glass tubes or pouches, these are most convenient assemblies, as the time to nodulation can be observed.

2. Streak out strain from mother culture to give isolated colonies.

3. Select five to 20 isolated colonies and add each to a diluent (Chapter 6) to provide sufficient inocula for the number of test plants (usually four repetitions per colony).

4. Use diluents as inocula, changing pipette or sterile tip for each source, and monitor nodulation, and N_2 fixation.

As it is likely that loss of effectiveness is related to loss of symbiotic DNA, a well-equipped laboratory may also choose to assay the single colonies selected above for known symbiotic genes such as *nif*, *nod* and *fix*, or the presence of the symbiosis island using directed primers and PCR or sequencing techniques (Chapters 11–13).

9.9.2 Potential for scale-up production

The growth rate of rhizobial strains in broth culture varies and this can affect their suitability for manufacture. The criteria relating to growth characteristics can be examined by counting cells at appropriate times in the manufacturing cycle. Methods for counting rhizobia are described in Chapter 6.

If strain growth or survival characteristics have been compromised during storage, an alternative inoculant strain should be used.

9.9.3 Strain variation in survival on seed, in the carrier, or in soil

Strains vary considerably in their survival in soil (Chapter 8) and there is a large volume of literature on this subject, particularly in relation to stress tolerance (*e.g.* O'Hara *et al.* 2002). The cross-row technique (Chapter 8) provides a bio-assay for strain success in soil in which nodulation is the criterion for selection rather than cell number. The MPN determined by the plant infection test (Chapter 6) is an alternative method to enumerate strains in soil, and this can be applied to assess spatial and temporal variation in cell number.

There is considerably less assessment of strain variation in survival in carriers or when applied to seed, but this is still a very important criterion when selecting

strains for commercial use (Roughley and Vincent 1967). Poor survival on seed can have serious consequences for legume success (Materon and Weaver 1984) and strains do vary in their ability to tolerate desiccating conditions on seed and survive (*e.g.* Deaker *et al.* 2007; Howieson *et al.* 2013). The dilution and enumeration techniques outlined in Chapter 6 are appropriate to determine viable numbers in carriers or on seed, particularly where there is likely to be a low level of contamination.

9.10 References

Albareda M., Rodríguez-Navarro D.N., Camacho M. and Temprano F.J. 2008. Alternatives to peat as a carrier for rhizobia inoculants: solid and liquid formulations. Soil Biology and Biochemistry 40, 2771–2779.

Bashan Y. 1998. Inoculants of plant-growth promoting bacteria for use in agriculture. Biotechnology Advances 16, 729–770.

Bullard G.K., Roughley R.J. and Pulsford D.J. 2005. The legume inoculant industry and inoculant quality control in Australia : 1953–2003. Australian Journal of Experimental Agriculture 45, 127–140.

Burton J.C. 1984. Legume inoculant production manual. University of Hawaii, Dept. of Agronomy and Soil Science, College of Tropical Agriculture and Human Resources: Hawaii.

Deaker R., Roughley R.J. and Kennedy I.R. 2007. Desiccation tolerance of rhizobia when protected by synthetic polymers. Soil Biology and Biochemistry 39, 573–580.

Herridge D., Gemell G. and Hartley E. 2002. Legume Inoculants and Quality Control, Pp 105–115 in 'Inoculants and Nitrogen Fixation of Legumes in Vietnam' ed by D. Herridge. ACIAR Proceedings 109e, Canberra.

Howieson J.G., De Meyer S., Vivas-Marfisi A., Ratnayake S., Ardley J.K. and Yates R.J. 2013. Novel *Burkholderia* bacteria isolated from *Lebeckia ambigua*—a perennial suffrutescent legume of the fynbos. Soil Biology and Biochemistry 60, 55–64.

Loi A., Nutt B., Yates R. and D'Antuono M. 2012. Summer sowing: a new alternative sowing technique to introduce annual legumes into mixed farming systems. Proceedings of the 16th Australian Agronomy Conference, Armidale: Australia.

Lupwayi N.Z., Olsen P.E., Sande E.S., Keyser H.H., Collins M.M., Singleton P.W. and Rice W.A. 2000. Inoculant quality and its evaluation. Field Crops Research 65, 259–270.

Marshall K. 1968. Interaction between colloidal montmorillonite and cells of rhizobium species with different ionogenic surfaces. Biochimica et Biophysica Acta 156, 179–186.

Marshall K. 1969. Studies by microelectrophoretic and microscopic techniques of the sorption of illite and montmorillonite to rhizobia. Journal of General Microbiology 56, 301–306.

Materon L. and Weaver R. 1984. Survival of *Rhizobium* on toxic and non-toxic arrowleaf clover seeds. Soil Biology and Biochemistry 16, 533–535.

Ochman H., Lawrence J.G. and Groisman E.A. 2000. Lateral gene transfer and the nature of bacterial innovation. Nature 405, 299–304.

O'Hara G.W., Howieson J.G. and Graham P.H. 2002. Nitrogen fixation and agricultural practise. Pp 391–410 in 'Nitrogen fixation in the millenium', ed by G.J. Leigh. Elsevier.

Roughley R.J. and Vincent J.M. 1967. Growth and survival of *Rhizobium* spp. in peat culture. Journal of Applied Bacteriology 30, 362–376.

Roughley R. and Pulsford D. 1982. Production and control of legume inoculants. Pp 193–209 in 'Nitrogen fixation in legumes', ed. by J. Vincent. Academic Press Australia: Sydney.

Somasegaran P. and Hoben H. 1994. Handbook for rhizobia. Springer-Verlag: New York.

Thompson J. 1980. Production and quality control of legume inoculants. Pp. 489–533 in 'Methods for evaluating biological nitrogen fixation', ed. by F.J. Bergersen. John Wiley & Son Ltd.

Thompson J. 1983. Production and quality control of carrier-based legume inoculants. Information bulletin no. 17. International Crops Research Institute for the Semi Arid Tropics (ICRISAT).

Vincent J. 1970. A Manual for the practical study of root-nodule bacteria. Blackwell Scientific: Oxford.

Measurement of nitrogen fixation

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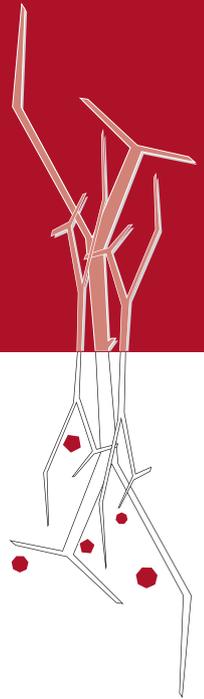
10.1 Introduction

Previous chapters have described how to assess the symbiotic effectiveness of rhizobia with legume genotypes in a range of experimental systems, from growth pouches and small tubes containing single plants in a growth room, to large replicated field trials. What is important is to measure the extent of effective nodulation and the yield of biomass and/or grain by the legume.

Rhizobial treatments can be evaluated without quantifying the amount of nitrogen being fixed. Measuring the amount of N fixed by single plants will not add much greater insight to that provided by nodulation and yield data. However, quantifying N_2 fixation is achieved by simply multiplying the plant biomass by tissue %N for each treatment, then subtracting the N in the uninoculated control (N difference method, see later).

In the field, there are many reasons for estimating N_2 fixation. These estimates can provide information that is critical to understanding the cycling of N in the soil-plant system and what effect the treatments are having on it. Given sufficient numbers and locations, the individual values can be aggregated to provide information about N inputs from legumes and N cycling in systems at regional, country and global scales. Field-based estimates of N_2 fixation can also assist in determining if different rhizobial treatments have improved N_2 fixation in the face of environmental challenges.

The potentially beneficial economic and environmental impacts of effective exploitation and management of legume N_2 fixation can only be assessed by acquiring quantitative data on N_2 fixation. As a cautionary note however, measuring N_2 fixation is often a resource-intensive activity and the researchers must satisfy themselves that it is necessary.

10.2 Methods currently available

There are five current methodologies for quantifying N_2 fixation by nodulated legumes:

1. N balance

2. N difference

3. ^{15}N isotope dilution

4. ureide concentration

5. acetylene reduction.

The principles behind these methods and their effective use are detailed in Unkovich *et al.* (2008), a book published by the Australian Centre for International Agricultural Research (ACIAR) and available free from the Centre's website (http://aciar.gov.au/publication/MN136). Another recent, comprehensive treatise on measurement of N_2 fixation is Peoples *et al.* (2009). Other useful publications include Chalk (1985), Shearer and Kohl (1986), Witty and Minchin (1988), Danso *et al.* (1993), Vessey (1994), Unkovich and Pate (2000), Giller (2001) and Peoples *et al.* (2002).

The N balance method estimates N_2 fixation on an area basis, *i.e.* kg N/ha, while the N difference method can be used for either single plants or an area. The ^{15}N and ureide methods provide estimates of the percentage of total N of the plant or crop that is derived from N_2 fixation (%Ndfa). The N fixed per unit area or unit of production is then calculated as the product of %Ndfa and the total amount of N accumulated by the legume. Finally, the acetylene reduction technique assays the activity of nitrogenase, the enzyme catalysing N_2 fixation.

10.3 Which method to use?

There is no single 'correct' way to measure N_2 fixation and since all current methodologies have limitations, measuring the exact amount of N fixed continues to be a challenge. Ideally, several different methods should be used simultaneously, particularly if they do not rely on the same assumptions. Because this is not always practical, the choice of method should be made carefully and will often depend on the resources and expertise available.

The particular analytical equipment need not always be in the researcher's laboratory. Mass spectrometers for ^{15}N isotope analysis, for example, are costly and have very high skill and maintenance requirements. It may be wiser to establish collaborative activities with specialised groups that have suitable equipment and expertise. The International Atomic Energy Agency (IAEA) in Vienna, Austria offers services and training facilities and has special programs in agriculture (http://www.iaea.org/OurWork/ST/index.html).

Table 10.1 Characteristics of methods to quantify biological N_2 fixation. The '√' signifies that the particular method has the characteristic. In the last row, the more '$' the higher the cost (adapted from Unkovich *et al*. 2008).

Characteristics	Non-isotopic methods				Isotopic methods	
	N balance	**N difference**	**Ureide**	**C$_2$H$_2$ reduction**	**^{15}N natural abundance**	**^{15}N enrichment**
Time integrated	✓	✓			✓	✓
Reference plant needed		✓			✓	✓[c]
Non-destructive			✓[a]		✓[a]	
%Ndfa measured		✓[d]	✓		✓	✓
Quantify kg N/ha fixed	✓	✓	✓		✓	✓
Laboratory			✓	✓		✓
Glasshouse	✓	✓	✓	✓	✓	✓
Field	✓	✓	✓		✓	✓
Possibility to assess fate of fixed N in system						✓
Short-term			✓	✓	✓	✓
Long-term	✓	✓	✓		✓	✓
Precision	low	low-medium	good	low	low–good[b]	medium-good
Costs	$$	$	$	$	$	$$

[a] If only %Ndfa is required
[b] Depending on natural enrichment of soil
[c] Not when cultivated in N-free media
[d] Can be calculated indirectly

The characteristics and potential suitability of the various methods for quantifying N_2 fixation in different plant-growth systems, *i.e.* glasshouse or field, are summarised in Table 10.1. It is not an absolute guide but an aid to help the researcher decide which method to use.

10.4 Sampling for biomass and biomass N

10.4.1 Sampling in space

Accurately estimating plant (crop) biomass and N is critical to measuring N_2 fixation. Insufficient and biased sampling of plants will result in errors. Plant samples should be collected from the field using a predetermined pattern for all plots or sampling areas. Generally, samples are taken from replicates as a fixed row length (*e.g.* 1 m) or quadrat area (*e.g.* 1.0×0.5 m). Quadrat sizes should reflect the size of the plots or field and the extent of variation within them. Although it may sometimes be necessary to use individual plants in field studies, reduced accuracy may result, and if small numbers are involved, (*i.e.* <10/replicate) bias (Hunt *et al*. 1987; Swan *et al*. 2003).

Table 10.2   Effects of sampling area of field-grown common bean and faba bean on estimates and variability of shoot dry matter (DM) and shoot %N. Coefficients of variation (CVs) are given in brackets (adapted from Peoples *et al*. 2009).

	Quadrat size		
	0.2 m²	0.5 m²	1.0 m²
Shoot DM (t/ha)			
Common bean	2.17 (41%)	1.86 (22%)	1.81 (20%)
Faba bean	10.0 (52%)	8.70 (38%)	7.78 (29%)
Shoot %N			
Common bean	3.59 (8%)	3.61 (11%)	3.63 (8%)
Faba bean	2.32 (10%)	2.31 (8%)	2.33 (10%)

In Table 10.2, typical sampling strategies are compared. For both legumes, the largest sampling area (1 m²) gave the lowest estimates of biomass, while the smallest areas (0.2 m²) gave the highest estimates. The coefficients of variation (CVs) for DM (plant dry matter) also increased substantially as the sampling area became smaller. Ideally, at least 0.5 m² should be harvested from each replicate plot to provide a reasonable estimate of DM. The number of replicates required depends on sample size and field variability. With a 1 m² sample, four to six replicates are sufficient, while up to 10 replicates may be necessary for smaller sampling areas. The shoot %N values did not vary with sampling area and their CVs were uniformly small (generally 10% or less).

10.4.2 Sampling in time

The researcher must also decide when to sample for maximum crop N accumulation. With grain legumes, sampling should be at late-pod filling, close to physiological maturity. This usually works well with determinate, bush varieties but unfortunately not for indeterminate varieties, with climbing beans being an extreme example. Sampling too early can result in the grain N being greater than N in the biomass samples thought to have been taken at maximum N accumulation (Kipe-Nolt and Giller 1993). However, if sampling is left too late, *i.e.* between physiological maturity and grain harvest, substantial leaf fall can lead to an underestimate of total N. Judging exactly when to sample for peak N content is difficult and the ideal time for sampling may vary between varieties (Figure 10.1). For very long-duration, indeterminate varieties, one may have to sample at or very near to final grain harvest and estimate the amount and N content of fallen leaves. In many experiments, different sampling times may be needed to accommodate different durations of different legumes/varieties.
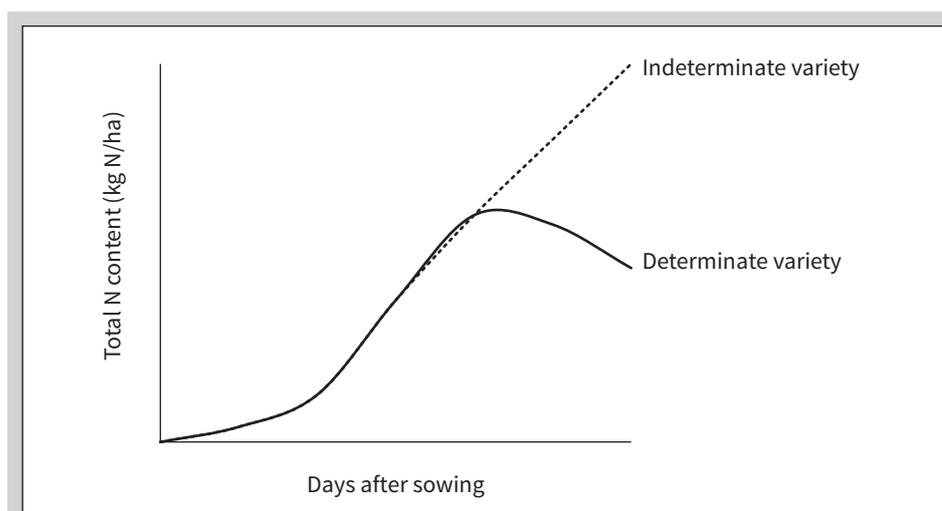
Figure 10.1   N accumulation in a determinate legume variety and an indeterminate legume variety. The optimum time for sampling for N accumulation in the determinate variety is 80 days after sowing. In the indeterminate variety it is at final harvest.

10.5 Preparing and analysing plant samples for %N and ^{15}N

10.5.1 Drying and weighing plant material

Sample bags should be clearly labelled with the date (including year), plot or treatment number or name, the plant species, and the part of the plant harvested. Plant samples are best kept in paper bags and allowed to 'breathe' prior to drying. Plastic bags are not recommended. Avoid tight packing of samples as this can result in 'stewing' of material and subsequent changes in plant N forms. Where plants have been labelled with isotopes it is important to keep labelled and unlabelled material in separate boxes to avoid contamination. The same applies for oven drying: labelled and unlabelled material should be kept separate, especially when dry and brittle material is easily detached. Many laboratories maintain separate systems (rooms, ovens, balances, storages) for ^{15}N-labelled and unlabelled samples.

Samples should be oven-dried as soon as possible after collection. If this cannot be arranged within a few hours, plant samples should be kept in a coolroom or cool place overnight. The objective of drying is to remove water from the plant material without loss of organic compounds, and without the growth of bacteria and fungi. Where there is insufficient oven space for a large amount of sample material, all samples should receive the same treatment. Preliminary air drying in a glasshouse may be possible in dry environments. If plant samples have attached soil or dust, it should be washed off while the plants are fresh as it cannot be separated from dried material.

Plant material must be dried to constant weight at 65–80°C, usually for at least 48 hours, in an air-circulating oven. Very bulky samples can be spread on metal trays and returned to the sample bags when dry. Sample bags must not be packed tightly into an oven as this can cause a fire. Drying plant material at 100°C is not recommended as rapid proteolysis (protein breakdown), thermal decomposition and loss of N may occur. High temperatures also increase the risk of an oven fire.

The dry weight of the total sample should be recorded immediately after drying, before any grinding and subsampling. Samples can conveniently be weighed in their paper bags with the bag weight (the average weight of similarly oven-dried bags) subtracted from the sample weight. Once dried and weighed, the samples can be stored indefinitely provided they are kept in dry, airtight conditions. However, ground-up samples require less storage space and they can be kept in containers to prevent damage from insects and rodents. Samples should be stored in paper bags and cardboard boxes for only short periods (days to weeks) to avoid possible sample damage or loss.

10.5.2 Grinding and subsampling plant material

As the amounts of sample used for %N and ^{15}N analysis are very small with respect to the sample harvested in the field, great care is needed in preparing subsamples for analysis. In particular, the relative proportion of the different plant parts (*e.g.* stems, leaves and immature pods) in the subsample must be representative. After chopping, or at different phases of subsampling, 'quartering' can be useful. The plant sample is spread thinly on a sheet of plastic/paper or other clean surface, one quarter taken and the rest discarded. This procedure is repeated until the subsample has the required size, the aim being to prevent the inevitable settling (layering) of samples according to particle size in upright containers.

Although the amount milled depends on constraints of time and the capacity of the mill, the larger the sample that can be hammer milled and then subsampled for analysis the better. To produce a fine powder for ^{15}N analysis, a small amount of sample needs to be ball milled. Additional details on preparation of samples for analysis of %N and ^{15}N can be found in Unkovich *et al.* (2008).

10.5.3 Analysis of plant material for %N and ^{15}N

Plant material can be analysed for %N using one of three standard methods— Kjeldahl digestion, dry combustion or near infra-red spectroscopy (NIR). Analysis of ^{15}N requires an isotope-ratio mass spectrometer or emission spectrometer for higher-level enrichments, *i.e.* greater than 0.05 atom % ^{15}N excess.

The Kjeldahl method involves digesting the plant sample with a catalyst in hot sulfuric acid, converting the organic N to ammonium (NH_4^+) then determining the concentration of NH_4^+. The method uses limited resources and allows analysis of total N and ^{15}N from a single sample distillation. Full details of the methodology are given in Peoples *et al.* (1989). Unkovich *et al.* (1993) provide details

of modifications required for using the sample distillates for high-precision ^{15}N analysis.

Dry combustion avoids the 'wet chemistry' of the Kjeldahl method and is the choice of most laboratories. Its use, however, requires access, directly or via collaboration, to a combustion analyser. This instrument provides rapid analysis of large numbers of samples but is expensive and requires a skilled operator. In recent years, the dry combustion instruments have been coupled to isotope-ratio mass spectrometers so that both total N and ^{15}N can be determined on a single sample. A number of commercial services for such analyses are listed in Appendix 9 of Unkovich *et al.* (2008).

One such service is at the University of California Davis Stable Isotope Facility http://stableisotopefacility.ucdavis.edu/. Samples for %N and ^{15}N analysis have to be weighed into tin cups using a balance that can measure accurately to ±0.00001 g. Samples need to be dried in an oven at 60–70°C and kept in a desiccator prior to weighing. The amount of sample depends on the %N in the sample; about 2.5 mg for legume seeds (4% N) and pods, about 5 mg for legume shoots (2% N) and 10 mg for cereal straw (1% N). As the tin cups are very full with the 10 mg samples, care must be taken to ensure they are closed well so that they do not leak in transit.

The third method for %N analysis, NIR, is rapid, inexpensive, non-destructive and is repeatable in routine use (Williams *et al.* 1983) but its reliability must be constantly validated by cross-referencing data against Kjeldahl or dry combustion analyses. This ability to make multiple determinations (*e.g.* plant moisture, carbohydrates) further enhances the value of the NIR technique. It cannot be linked to isotope-ratio mass spectrometry so samples must be analysed separately for ^{15}N. Additional details (and references) about using the methodology are provided in Unkovich *et al.* (2008).

10.6 Accounting for below-ground N

Total plant (crop) biomass and N have usually been determined from measurements of shoot biomass, assuming that N in the roots represents only a small fraction (5–15%) of the total plant N, and that shoot N provides a reasonable approximation to total plant N. However, that assumption has been proven incorrect; below-ground N associated with, or derived from, roots can represent 30–50% of the total plant N of both legumes and cereals (*e.g.* Russell and Fillery 1996; McNeill *et al.* 1997; Rochester *et al.* 1998; Unkovich and Pate 2000; Khan *et al.* 2002). Total inputs of fixed N could therefore be 50–100% greater than those determined from measurements based only on the shoots.

There is no single value for below-ground N as a fraction of total plant N; published estimates reflect the influence of species, soil and climate. To account for below-ground N when calculating total plant or crop N and N$_2$ fixation, we

suggest multiplying shoot N by 2.0 for chickpea (assumes 50% of plant N is below ground), 1.5 for soybean (assumes 33% below-ground N) and 1.4 for the remainder of the grain legumes (assumes 30% below-ground N). With the pasture/fodder legumes, multiply shoot N by 2.0 for lucerne and 1.4 for the remainder. Use a factor of 1.4 with cereal crops. We believe that the errors associated with these approximations are far less than those incurred by ignoring below-ground N or by using values for physically-recovered roots.

10.7 Nitrogen difference method

With this method, total N accumulated by N_2-fixing plants is compared with that of neighbouring non-N_2-fixing plants, with the difference in the two assumed to be due to N_2 fixation. Nitrogen difference is a simple, low-cost method usable when facilities for only DM determination and %N analysis are available. Thus:

N_2 fixed = (legume plant N) – (non-N_2-fixing control plant N)

Where plant N is derived from plant DM and %N:

$$\text{Plant N} = \frac{(\text{plant DM}) \times (\%\text{N})}{100}$$

This method assumes that the N_2-fixing plants assimilate the same amount of soil mineral N as the neighbouring non-N_2-fixing plants. In infertile soils with little mineral N, the method can be highly accurate. It may be less useful in soils with moderate to high levels of mineral N because differences between N_2-fixing and non-N_2-fixing plants in root morphology and rooting depth can result in different capacities to capture the soil N (Chalk 1998). The method has little value for on-farm surveys where discrete areas of appropriate non-N_2-fixing plants may not be present.

A successful application of the N-difference method is shown in Table 10.3. Pigeonpea genotypes of different maturities were grown on a sandy clay-loam in Zimbabwe (Mapfumo *et al*. 1999) with maize plants of matching maturities as the non-N_2-fixing references. As expected, shoot N increased with increasing growth duration for both the N_2-fixing pigeonpea and the non-N_2-fixing maize; the estimated shoot N fixed also increased with time.

Table 10.3 Estimations of N_2 fixation by five genotypes of pigeonpea grown on a sandy clay-loam in Zimbabwe using the N difference method with maize of matching maturity as the non-N_2-fixing controls (Mapfumo *et al*. 1999).

Maturity of pigeonpea	Shoot N pigeonpea (kg/ha)	Shoot N non-N_2-fixing reference (kg/ha)	Shoot N fixed (kg/ha)
Short	9–43	8	1–35
Medium	145	54	91
Long	172–183	71	101–112

Unfortunately, it is not always this straightforward. N$_2$ fixation by seven tropical forage legumes in Brazil was estimated (data for three legumes shown in Table 10.4). Total N accumulation by the legumes ranged from 102 to 117 kg N/ha, and for the three grass species used as non-N$_2$-fixing reference plants 33–56 kg N/ha. Two grass reference species (*Brachiaria brizantha* and *Panicum maximum*) provided very similar estimates of N$_2$ fixation while the estimates using the grass that accumulated the most total N (*Brachiaria arrecta*) were around 20 kg/ha lower. It is not clear which reference plant provided the most correct estimate; additional information such as growth duration of the different species would be needed to determine this.

This method for quantifying N$_2$ fixation is less popular now and has been largely replaced by ^{15}N and ureide methods. However, the method remains appropriate and can provide simple, accurate estimates of legume N$_2$ fixation for little cost and without the need to access sophisticated analytical facilities.

Table 10.4 Estimations of N$_2$ fixation by three legumes using the N difference method with three grass species as the non-N$_2$-fixing controls (from Unkovich *et al*. 2008; data from Viera-Vargas *et al*. 1995b)

	Plant	Total shoot N (kg/ha)	Legume N$_2$ fixation estimated using three different reference plants (kg N/ha)		
			Brachiaria brizantha	*Panicum maximum*	*Brachiaria arrecta*
Legumes	*Centrosema* (hybrid)	107	69	74	52
	Galactia striata	117	79	84	62
	Desmodium ovalifolium	102	64	69	47
Grasses	*Brachiaria brizantha*	38			
	Panicum maximum	33			
	Brachiaria arrecta	56			

10.8 ^{15}N dilution (and incorporation) methods

Of the two stable N isotopes, ^{14}N and ^{15}N, the lighter isotope ^{14}N is naturally much more abundant (>99.6% of the N in the atmosphere). The isotopic abundance of the minor isotope (^{15}N) is expressed as a percentage of the total N present, atom% ^{15}N, or as atom% ^{15}N excess (Table 10.5):

$$\text{atom\% } ^{15}\text{N} = \left(\frac{^{15}\text{N}}{^{15}\text{N} + {^{14}}\text{N}} \right) \times 100$$

$$\text{atom\% } ^{15}\text{N excess} = \text{atom\% } ^{15}\text{N} - 0.3663$$

Atmospheric N$_2$ has a constant abundance of just 0.3663 atom% ^{15}N (Table 10.5; Mariotti 1983). Only small natural variations around this value occur in other N pools (*e.g.* soil N) within the biosphere (Högberg 1997). Natural variations in ^{15}N

abundance are usually expressed in terms of δ units, which are the parts per thousand (‰) deviation relative to the international standard of atmospheric N_2, *i.e.* 0.3663 atom% ¹⁵N (Table 10.5). The δ units are calculated from atom% ¹⁵N values:

$$\delta^{15}N\ (‰) = \left(\frac{\text{Sample atom \%}^{15}N - 0.3663}{0.3663} \right) \times 1000$$

The natural abundance of atmospheric N_2 by definition has a $\delta^{15}N$ of 0‰ (Table 10.5). Values for plants and soils can either be positive (more ¹⁵N than in atmospheric N_2), or negative (less ¹⁵N than in atmospheric N_2).

To illustrate natural abundance calculations, consider a plant sample with 0.36784 atom% ¹⁵N. It will have a $\delta^{15}N$ value of + 4.2‰:

$$\delta^{15}N = \left(\frac{0.36784 - 0.3663}{0.3663} \right) \times 1000 = +4.2(‰)$$

¹⁵N was first used to detect N_2 fixation by bacteria in the 1940s (Burris *et al.* 1942); the unavailability of ¹⁵N-enriched materials and mass spectrometers to analyse the samples severely restricted use of ¹⁵N methods. Since the 1970s, mass spectrometers and isotopes have been more generally available, facilitating greater use of ¹⁵N methodologies.

Experimental protocols involve: (i) ¹⁵N_2 incorporation—namely labelling the N_2 gas in the atmosphere surrounding the N_2-fixing plants (Warembourg *et al.* 1982) and measuring incorporation of ¹⁵N by the plants; and (ii) ¹⁵N isotope dilution—growing the plants in ¹⁵N-enriched soil or other medium and calculating the extent of dilution of ¹⁵N in the plants by atmospheric (fixed) ¹⁴N (McAuliffe *et al.* 1958; Chalk 1985). A later variation of ¹⁵N isotope dilution, known as the natural ¹⁵N abundance method, utilised the natural ¹⁵N enrichment of soils, thereby avoiding the need to add ¹⁵N-enriched materials (Shearer and Kohl 1986).

Table 10.5 Terms associated with ¹⁵N stable isotope methods

Term	Definition
Atom% ¹⁵N	Abundance of ¹⁵N atoms as a percentage of the total $\left(\frac{^{15}N}{^{14}N + ^{15}N} \right) \times 100$
Natural abundance	Atom% ¹⁵N naturally present in materials
¹⁵N abundance of atmospheric N_2	0.3663 atom% ¹⁵N
$\delta^{15}N$ (‰)	Sample natural abundance expressed as parts per thousand relative to atmospheric N_2 $1000 \times \left(\frac{\text{sample atom\% }^{15}N - 0.3663}{0.3663} \right)$
$\delta^{15}N$ of atmospheric N_2	0‰
¹⁵N-enriched nitrogen	Nitrogen with an atom% ¹⁵N greater than atmospheric N_2
Atom% ¹⁵N excess	A measure of a sample's ¹⁵N content above the atmospheric N_2: sample atom% ¹⁵N – 0.3663
Labelled nitrogen	Material generated with a specific ¹⁵N enrichment
%Ndfa	The percentage of plant N derived from atmospheric N_2

^{15}N$_2$ incorporation is the most direct measure of N$_2$ fixation but is limited to short experimental periods in a laboratory or growth chamber.

^{15}N isotope dilution with artificially enriched soil was, until a few years ago, used widely to quantify %Ndfa and hence N$_2$ fixation of legumes (Chalk and Ladha 1999). In recent years, the natural abundance method has gained prominence for work in both experimental plots and in farmers' fields.

^{15}N isotope dilution provides an estimate of the plant's dependence on N$_2$ fixation (%Ndfa) as follows:

$$\%\text{Ndfa} = 100 \times \frac{x - y}{x - \text{'}B\text{'}}$$

Where:

x = ^{15}N content of the non-N$_2$-fixing reference plants (surrogate for plant-available soil N)

y = ^{15}N content of the N$_2$-fixing legume

'B' = a measure of the ^{15}N content of the target legume fully dependent on N$_2$ fixation for growth. This is generally assumed to be zero in ^{15}N enrichment studies but needs to be considered when using ^{15}N natural abundance (see below).

The estimate of %Ndfa is then combined with total legume N to calculate plant N$_2$ fixed. If the aim is to estimate the total amount of N fixed by a legume crop in a season, the crop should be sampled for biomass and %N at the time of peak biomass (see Section 10.4):

$$\text{Amount of N}_2 \text{ fixed} = \frac{(\text{legume N}) \times (\%\text{Ndfa})}{100}$$

Where legume N is calculated as legume DM × (%N)/100 (see above). Note that legume N should include the N in the soil derived from the plant, *i.e.* the N in roots, nodules and the soil around the roots. Accounting for below-ground N is discussed in Section 10.6.

Advantages of the method are that: (1) it provides a time-integrated estimate of %Ndfa for a period of growth; (2) amounts of N$_2$ fixed can be estimated from a single analysis of the ^{15}N contents of the N$_2$-fixing legume and the non-N$_2$-fixing reference combined with a measure of legume biomass and %N; and (3) the ^{15}N natural abundance variation of the method allows N$_2$ fixation to be monitored almost anywhere if both N$_2$-fixing and non-N$_2$-fixing plants are present, *e.g.* in farmers' fields.

The limitations of the method are: (1) that the ^{15}N composition of plant-available soil N can change with soil depth and with time during the growing season, particularly where ^{15}N-enriched materials have been applied to soil (Witty 1983; Chalk 1985); (2) that the efficacy of the non-N$_2$-fixing reference plant to provide an accurate measure of the isotopic composition of plant-available soil N can be compromised by differences in the rooting depths and patterns of N uptake of the

N$_2$-fixing and reference plants; (3) the high cost of ^{15}N-enriched materials (when used); and (4) that the ^{15}N abundance of plant-available soil N can be either too low and/or too variable for the methodology.

10.8.1 Natural ^{15}N abundance

The non-N$_2$-fixing reference for this method can be an unnodulated or non-N$_2$-fixing legume or a non-legume growing in the vicinity. The equation for estimating %Ndfa using the natural ^{15}N abundance variation of the N isotope dilution method is as follows:

$$\%\text{Ndfa} = \frac{\delta^{15}\text{N of reference plant} - \delta^{15}\text{N of N}_2\text{-fixing legume}}{\delta^{15}\text{N of reference plant} - \delta^{15}\text{N of N}_2} \times 100$$

In a hypothetical example, if the δ^{15}N of the legume was +4.2‰, the δ^{15}N of the non-N$_2$-fixing reference was +6.2‰ and the 'B' value was 0.0‰, then %Ndfa is estimated to be 32%:

$$\%\text{Ndfa} = \frac{6.2 - 4.2}{6.2 - 0.0} \times \frac{100}{1} = \frac{2.0}{6.2} \times 100 = 32\%$$

As stated above, B values are almost never 0.0‰, generally ranging between –1.0 and –2.0‰. Table 10.6 presents average B values for a range of crop and fodder/tree legumes; full data sets and a fuller discussion can be found in the Appendix section of Unkovich *et al.* (2008).

Table 10.6   Average B values for a number of tropical and subtropical crop, forage and shrub legumes (source: Unkovich *et al.* 2008)

Tropical and subtropical crop legumes	B value Shoot δ^{15}N (‰)	Tropical and subtropical forage, shrub and tree legumes	B value Shoot δ^{15}N (‰)
Vigna angularis	–0.91	*Centrosema*	–1.65
Vigna mungo	–1.75	*Desmodium*	–1.14
Phaseolus vulgaris	–2.16	*Macrotyloma axillare*	–1.83
Vigna unguiculata	–1.61	*Macroptilium atropurpureum*	–2.35
Vigna radiata	–2.05	*Calopogonium*	–0.95
Arachis hypogaea	–0.88	*Crotalaria*	–1.08
Cajanus cajan	–1.12	*Dolichos lablab*	–1.09
Vigna umbellata	–0.91	*Mucuna pruriens*	–1.82
Glycine max	–1.83	*Pueraria*	–1.22
Psophocarpus tetragonolobus	–1.54	*Calliandra*	–0.90

With soybean, more substantial ^{15}N:^{14}N discrimination may be associated with N$_2$ fixation when the plants are nodulated by certain strains of *Bradyrhizobium elkanii* (see Unkovich *et al.* 2008) resulting in shoot B values as low as –4.5‰. However, without evidence to the contrary, the average for soybean in Table 10.6 of –1.83 ‰ is a reasonable value.

The choice of non-N_2-fixing reference plant(s) to provide an estimate of the ^{15}N enrichment of the soil is critical. The impact of the reference on the calculations of %Ndfa is generally most important when the %Ndfa of the legume is low (<30%) or where the $\delta^{15}N$ of the reference is less than about 4‰. Unkovich *et al.* (2008) strongly recommend the use of more than one reference species, *e.g.* a broad-leaf (dicotyledonous) non-legume plus a cereal or grass. If the $\delta^{15}N$ values differ amongst reference species, %Ndfa should be calculated for each species to show the range and to derive a mean %Ndfa.

Adjei-Nsiah *et al.* (2008) used this approach to quantify N_2 fixation for five cultivars of cowpea in the forest/savannah transitional zone of Ghana (Table 10.7). The average $\delta^{15}N$ values for the non-N_2-fixing references ranged between 3.38 and 6.78‰; for cowpea, $\delta^{15}N$ values ranged between –0.21 to 1.34‰.

Table 10.7 Ranges and average %Ndfa estimates for cowpea genotypes in the forest/savannah transitional zone of Ghana (source: Adjei-Nsiah *et al*. 2008)

Cultivar	Range of %Ndfa estimates	Average %Ndfa
Adom	64–79	75
Asontem	63–78	73
Ayiyi	46–68	61
IT810D-1010	60–77	71
Legion prolific	68–81	77

The ranges of %Ndfa estimates are shown in the second column of Table 10.7; the average %Ndfa estimates, those most likely to be used in further analysis of the data are shown in the third column.

Additional details of the natural ^{15}N abundance method, particularly related to experimental design and working in farmers' fields, are to be found in Chapter 8 of Unkovich *et al.* (2008). Plant sampling protocols are considered in Section 10.4. Most $\delta^{15}N$ values in agricultural systems range from –2‰ to +12‰ (Letolle 1980); values less than –3‰ or greater than +15‰ should be carefully examined with re-analysis usually warranted.

10.8.2 ^{15}N enrichment

The ^{15}N enrichment methodology was most widely used in the 1970s to 1990s, before improvements in mass spectrometry in the 1980s led to the development of the ^{15}N natural abundance technique. Where the $\delta^{15}N$ of plant-available N is <2‰ and the $\delta^{15}N$ methodology should not be used, or the researcher cannot access high-precision mass spectrometers ($\delta^{15}N \pm 0.3‰, \pm 0.0001$ atom% ^{15}N), plant-available soil N can be artificially enriched with ^{15}N-labelled materials.

Legume and non-N_2-fixing reference plants are then grown in soil receiving the same ^{15}N-labelled material, usually fertiliser. Shoots of both sets of plants are

analysed for ^{15}N, and the percent of N derived from the atmosphere (%Ndfa) by the legume is calculated (McAuliffe *et al.* 1958) as:

$$\%\text{Ndfa} = \left(1 - \frac{\text{atom\% }^{15}\text{N excess N}_2\text{ fixing plant}}{\text{atom\% }^{15}\text{N excess reference plant}}\right) \times 100$$

The principal assumption is that the ^{15}N enrichment of the non-N$_2$-fixing reference plants accurately reflects the ^{15}N enrichment of soil N taken up by the legume. The ^{15}N enrichment of the soil N therefore needs to be relatively constant over time and space, and the time course and depth of soil N uptake by the reference and N$_2$-fixing plants identical.

Because materials labelled with ^{15}N are expensive, the amount of ^{15}N used is minimised. There is no single correct method for the addition of ^{15}N to label plant-available N in the soil. The most common, however, is to add ^{15}N-labelled inorganic salts (*e.g.* $(NH_4)_2SO_4$, NH_4Cl, KNO_3, NH_4NO_3) or ^{15}N urea fertiliser as a liquid using a watering can, by spraying or injecting into soil or as a solid by broadcasting, banding or mixing with soil. Some possible sources of stable isotope-labelled materials are given in Appendix 8 of Unkovich *et al.* (2008).

Following addition of $^{15}NH_4^+$ or $^{15}NO_3^-$ to soil, the mineralisation of N from unlabelled soil organic N causes the ^{15}N enrichment of the plant-available soil N pool to decline. Resulting differences in the ^{15}N enrichment of the N taken up from the soil by N$_2$-fixing and reference plants may undermine the principal assumption of this method.

Further, the added ^{15}N is not distributed evenly with regard to depth when applied to the soil surface. Thus, the ^{15}N enrichment of soil mineral N can change with both time and depth during plant growth. Practices to reduce the impact of uneven distribution of ^{15}N in space and in time include:

▶ regular additions of ^{15}N-labelled inorganic N to the soil (Viera-Vargas *et al.* 1995a)

▶ addition of soluble C to immobilise mineral N (Giller and Witty 1987; Boddey *et al.* 1995)

▶ the use of ^{15}N-labelled plant residues to provide mineralised N of a more constant value (Watanabe *et al.* 1990)

▶ using residual ^{15}N fertiliser carried over in soil from a previous experiment (McNeill *et al.* 1998)

▶ the use of a series of different reference plants to capture variations in ^{15}N enrichments in space and time (Viera-Vargas *et al.* 1995a)

▶ repeated sampling and analysis of extractable soil N over time (Chalk 1996)

▶ combinations of the above.

All authors highlight the fact that using non-N_2-fixing reference plants to estimate legume ^{15}N uptake from the soil is the principal weakness of the isotope dilution methodology. However, access to a yield-independent and time-integrated estimate of %Ndfa makes it worthwhile to persist with this potentially powerful technique.

The next question is how much ^{15}N to add? The total amount of N applied in the ^{15}N-labelled material should be small enough that it does not influence N_2 fixation, *i.e.* <5 kg N/ha). It is also much less expensive per gram of ^{15}N to buy 10 atom% ^{15}N than 99 atom% ^{15}N; only a few situations would justify enrichments of >10 atom% ^{15}N excess.

The areas of crop to be labelled with ^{15}N (microplots) are typically only 1–2 m² per replicate, and usually much smaller than the areas sampled for crop DM. Steel boxes are often placed into the soil around the ^{15}N-labelled area to reduce lateral movement and runoff of ^{15}N and to prevent the scavenging of unlabelled N by plant roots (Sanchez *et al.* 1987). Separate plant biomass samples for DM estimation are usually taken from the plot areas surrounding the ^{15}N-labelled microplots, with plant ^{15}N and %N determined from samplings within them.

The non-N_2-fixing reference plants should be as similar as possible to the legume in growth habit, duration *etc*. Because it is very difficult to find a single species that fulfils all of the above requirements, we recommend using more than one reference species in any given experiment.

A typical set of data from a ^{15}N isotope dilution experiment is given in Table 10.8 with %Ndfa for the 128-day-old plants calculated as follows:

$$\%Ndfa = \left(1 - \frac{0.0681}{0.3189}\right) \times 100 = 78.6$$

Note that %Ndfa would be calculated for each replicate, to allow for statistical analysis of the data.

Table 10.8 ^{15}N enrichments of shoots of lupin and wheat sampled from ^{15}N-labelled microplots and estimates of %Ndfa at 123 and 193 days after sowing (data of Evans *et al.* 1987)

Species	Days after sowing	atom% ¹⁵N	atom% ¹⁵N excess	Lupin %Ndfa
Lupin	128	0.4344	0.0681	79
Wheat		0.6852	0.3189	
Lupin	193	0.4112	0.0449	85
Wheat		0.6561	0.2898	

The enriched and natural abundance variations of the ^{15}N isotope dilution method are arguably the benchmark for quantifying N_2 fixation by nodulated legumes. Use of centralised laboratories, *e.g.* University of California, Davis (http://stableisotopefacility.ucdavis.edu/) for analysing ^{15}N (and %N) through

fee-for-service or collaboration provides a means for all researchers to use these techniques.

10.9 Ureide (N solute) method

The basis of the ureide method is the export by many agronomically-important tropical legumes (*e.g.* soybean, common bean, in the tribes Phaseoleae and Desmodieae within the Papilionoideae subfamily) of allantoin and allantoic acid (collectively known as ureides) as the products of N_2 fixation from their nodules to the shoots (Figure 10.2).
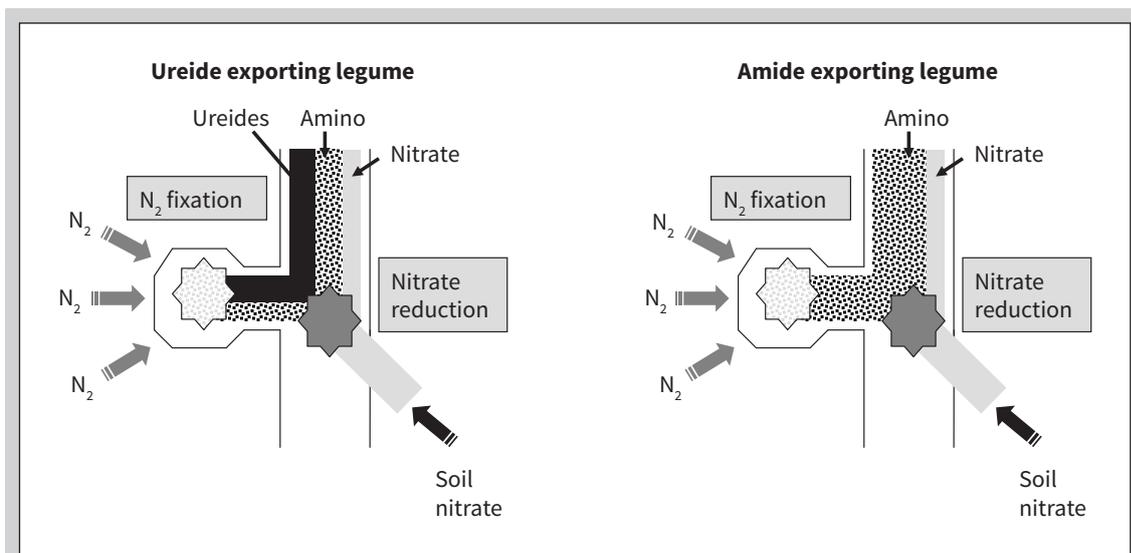
Figure 10.2   Assimilation and export of N from the nodulated roots of ureide- and amide-producing legumes. The ureide species have three major groups of N solutes in the xylem stream: ureides, amino compounds and nitrate, while the amide legumes have only two: amino compounds and nitrate.

Other legumes produce and export the amides, asparagine and glutamine, from their nodules. Nitrogen is also taken up from the soil by the legume roots, principally as nitrate. Once inside the plant, the N solutes derived from soil mineral N are transported in the xylem as free nitrate or, after reduction and/or ammonium metabolism in the root, as organic products (principally asparagine and glutamine).
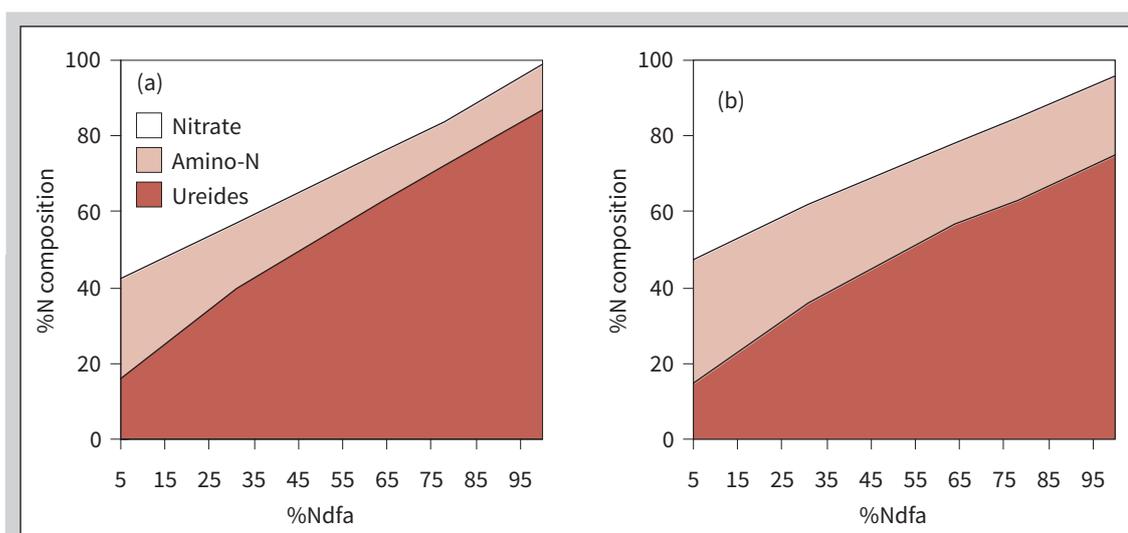
Figure 10.3   Changes in the composition of N solutes in: (A) root-bleeding and (B) vacuum-extracted xylem sap of nodulated soybean, supplied with different concentrations of ^{15}N-labelled nitrate to generate different values of %Ndfa (data from Herridge and Peoples 1990).

With the ureide legumes, the ratio of ureide N to total N in xylem sap or stem segments is highly correlated with %Ndfa (Figure 10.3). Quantifying % ureide-N provides a means of estimating %Ndfa. Although not applicable to all legumes, or to other N_2-fixing associations, the technique has been widely used with both experimental and non-experimental (farmer) crops.

The relationships between %ureide-N in xylem sap or stem segments and %Ndfa need to be specified for each legume species. Since the technique was established about 30 years ago, many experiments have defined legume species as either ureide (Table 10.9) or amide exporters and calibrated particular species.

The % ureide-N in xylem sap is calculated as:

$$\% \text{ ureide-N} = \frac{400a}{4a + b + c}$$

where a is the molar concentration of ureides (ureides contain four N atoms per molecule), b is the molar concentration of nitrate-N and c is the molar concentration of α-amino-N (Herridge 1984).

% ureide-N in extracts of whole stems or stem segments is calculated as:

$$\% \text{ ureide-N} = \frac{400a}{4a + b}$$

where a and b are, respectively, the molar concentrations of ureides and nitrate. α- Amino-N is not included in this equation, principally because the relationship between % ureide-N and %Ndfa was sufficiently robust without it (Herridge and Peoples 1990).

Table 10.9 Some of the legumes that transport the ureides, allantoin and allantoic acid, as the dominant products of N$_2$ fixation in xylem sap (source: Unkovich *et al*. 2008).

Grain legumes	Forage and tree legumes
Soybean (*Glycine max*)	*Calopogonium caeruleum*
Pigeon pea (*Cajanus cajan*)	*Centrosema pubescens*
Cowpea (*Vigna unguiculata*)	*Codariocalyx gyroides*
Mung bean (*V. radiata*)	*Desmodium ovalifolium*.
Black gram (*V. mungo*)	*D. rensonii*
Adzuki bean (*V. angularis*)	Siratro (*Macroptilium atropurpureum*)
Rice bean (*V. umbellata*)	Kudzu (*Pueraria phaseoloides*)
Bambara groundnut (*V. subterranea*)	*Hardenbergia* spp.
Common bean (*Phaseolus vulgaris*)	Hyacinth bean (*Lablab purpureus*)
Lima bean (*P. lunatus*)	
Runner bean (*P. coccineus*)	
Winged bean (*Psophocarpus tetragonolobus*)	
Guar (*Cyamopsis tetragonoloba*)	
Kersting's groundnut (*Macrotyloma geocarpum*)	

Relationships between %ureide-N (y) and %Ndfa (x) have now been published for a number of species of crop, forage and shrub legumes (see Unkovich *et al*. 2008). To estimate %Ndfa, the %ureide-N values are inserted into the calibration equations for the particular species. For example, for soybean xylem sap these are:

$y = 0.64x + 7.7$ (for vacuum-extracted sap during vegetative growth and flowering)

$y = 0.64x + 15.9$ (for vacuum-extracted sap during pod-fill)

$y = 0.83x + 4.8$ (for root-bleeding sap during vegetative growth and flowering)

$y = 0.67x + 21.3$ (for root-bleeding sap during pod-fill)

The %Ndfa value, when combined with measures of legume total N, can then be used to calculate amounts of N fixed during a period of growth (*e.g.* Herridge *et al*. 1990).

10.9.1 Sampling of legumes for N solutes

Samples can be collected from legumes for N solute analysis as vacuum-extracted xylem sap (Herridge 1984, Herridge *et al*. 1988), root-bleeding xylem sap (Norhayati *et al*. 1988) or aqueous extracts from stem segments or petioles (Herridge and Peoples 1990). Procedures for the three methods are described below. Irrespective of the method used, it is advisable to sample the plants between 0900 and 1600 hours to avoid error due to diurnal variations in relative ureide-N (*e.g.* Herridge *et al*. 1988; 1996).

10.9.1.1 Vacuum-extracted sap

With most species, sufficient (0.1–0.3 mL/plant) xylem sap can be recovered from each whole stem or stem section of field-grown crop legumes for complete N-solute analysis. Thus, the method may also be used as a non-destructive assay on individual plants (Herridge *et al.* 1988).

Equipment requirements

Items needed for vacuum-extraction of xylem sap are:

▶ sharp secateurs or pruning shears

▶ syringe needles (19 or 20 gauge)

▶ silicon- or latex-rubber tubing of a range of internal diameters (3–15 mm)

▶ fittings or adaptors (*e.g.* disposable micropipette tip cut to size)

▶ 5 mL Vacutainers

▶ a vacuum source, which may be a hand-held vacuum pump (*e.g.* Nalgene, Sybron Corp., Rochester, New York, USA, as depicted in Figure 10.4), a foot pump or a laboratory vacuum pump powered by a petrol-run generator or a car battery (*e.g.* a Waters/Millipore DOA-V130-BN vacuum pump with a Kawasaki GA1000A portable generator).
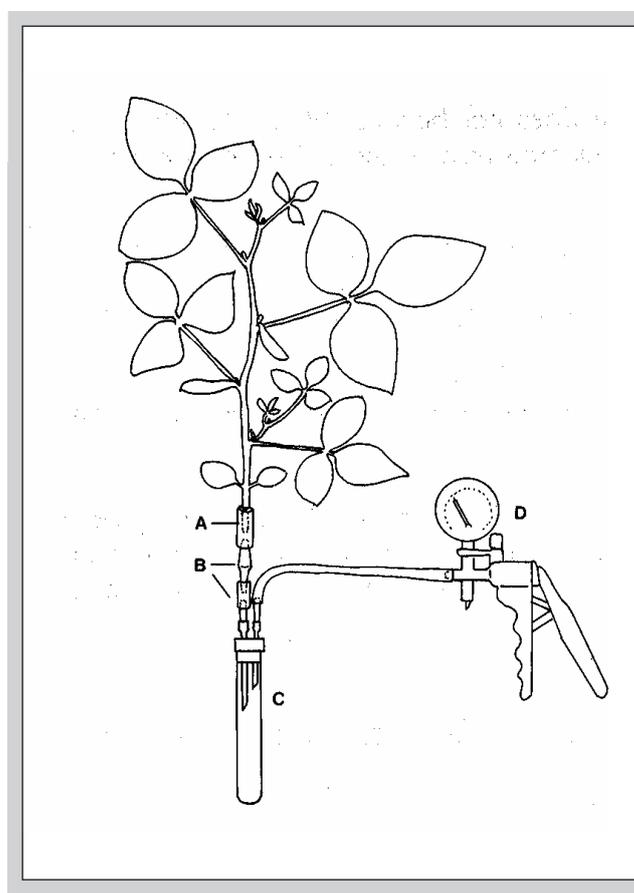
Figure 10.4 Apparatus for vacuum-extraction of xylem sap using a hand-held vacuum pump. The base of a freshly-detached stem is placed into an appropriately-sized silicon rubber tubing sleeve (A) and fitted onto a syringe needle (using an adaptor (B) if necessary). The syringe needle is then inserted through the rubber stopper of a Vacutainer (C) connected to a vacuum pump (D) via a second syringe needle connection (source: Unkovich *et al.* 2008).

The advantage of the laboratory vacuum pump is that a manifold can provide more than one vacuum line for several operators to simultaneously sample sap from different plants. Photographs of a foot pump and powered vacuum pump plus battery are provided in Unkovich *et al.* (2008).

Procedure

1. For crop legumes, use secateurs to cut a stem >3 mm in diameter close to ground level. For tree or shrub legumes, 1.0 m long stems or canes are cut from the main plant and used for sap sampling (Herridge *et al.* 1996).

2. The detached stem is immediately inserted into a sleeve of silicon- or latex-rubber tubing with an internal diameter slightly smaller than the stem (Figure 10.4), and fitted onto a syringe needle using an adaptor of appropriate size.

3. The needle is then pushed through the rubber stopper of a 5 mL Vacutainer that has been linked to a vacuum pump via another syringe needle connection and a flexible plastic-tubing line. *NB:* the base of the two syringe needles must not be level or sap may be sucked directly into the vacuum pump line rather than collecting in the Vacutainer.

4. A vacuum (60–70 kPa) is applied and 3–4 cm segments of the stem are then cut with secateurs successively from the top to the bottom of the shoot. Entry of air at the cut surface displaces the xylem sap from the base of the stem to be collected within the Vacutainer. Vacuum extraction should commence immediately after detachment of stems from the root. A time delay of more than five minutes can introduce errors because of changes in concentrations of N solutes (see Herridge *et al.* 1988). The technique will not work if the crop is infested with pests, such as stem borers, since a vacuum on the stem cannot be maintained. Avoid the needle becoming blocked with debris by clearing it frequently and changing needles periodically.

5. Sap samples should be kept chilled on ice until frozen at –15°C for long-term storage or, if ice is unavailable, stabilised immediately after extraction by adding an approximately equal volume of ethanol to the sap collected in the Vacutainer.

10.9.1.2 Root-bleeding sap

1. The shoot is cut below the first node close to ground level with secateurs or a very sharp blade.

2. A sleeve of silicon- or latex-rubber tubing, 2–4 cm long with an internal diameter slightly smaller than the stem, is placed over the exposed root stump (Figure 10.5).

3. The sap exuding under root pressure can easily be collected from within the tubing sleeve using a Pasteur pipette or syringe. We recommend that root stumps be allowed to exude for no more than 20 to 30 minutes. The accumulated sap

should be collected about every 10 minutes, placed in a sealable tube or vial (*e.g.* Vacutainer®, Becton Dickinson, Rutherford, New Jersey, USA) and kept on ice to minimise potential decomposition or metabolism.

4. Sap samples should be kept chilled on ice until frozen at –15°C for long-term storage or, if ice is unavailable, stabilised immediately after collection by mixing with an equal volume of ethanol in the collection tube.
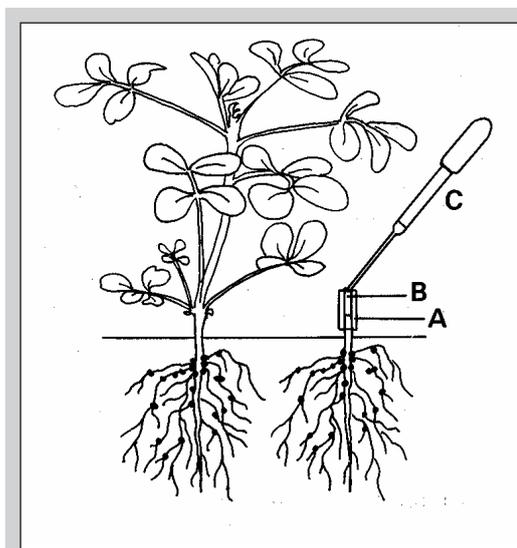
Figure 10.5 Sampling of root-bleeding xylem sap. A length of silicon rubber tubing (A) is placed over the root stump following decapitation of the shoot. The sap (B) collects in the tubing to be drawn out of the tubing with a Pasteur pipette or syringe (source: Unkovich *et al*. 2008)

10.9.1.3 Stems and stem segments

The stem, because it is mainly involved in transport of N solutes, is the most suitable plant part for N-solute extraction and analysis. Leaves are not suitable because they rapidly metabolise incoming N-compounds and tissue N-solutes can vary with changes in plant metabolism unrelated to N_2 fixation. Relative ureide N in stems is insensitive to diurnal fluctuations and unchanged by storage at 20–30°C for up to 24 hours after harvest before being oven dried.

The advantages of using dried and ground stems or stem segments are ease of sampling and the 24-hour stability of the samples. This may also be the only means of field sampling small plants or legumes that do not readily yield vacuum-extracted or root-bleeding xylem sap. The disadvantages are the additional steps in drying and grinding the stem segments and in solute extraction.

Procedure

1. Stems and stem segments are harvested and leaves removed.

2. Samples are placed in clearly labelled bags and dried at 65–80 °C in a forced-air oven for two days.

3. Tissue is ground to pass through a 60-mesh (1.0 mm) screen and stored in a dry place until extraction.

4. Subsamples (0.5 g) of dried and ground material are weighed and transferred to 100 mL beakers or Erlenmeyer flasks.

5. Distilled water (25 mL) is added to each subsample, which is then boiled for one to two minutes. An electric frying pan half-filled with sand can be used.

6. Extracts are filtered while hot through 15 cm filter paper (Whatman No. 40) in a funnel into a 50 mL volumetric flask. The residue is washed onto the filter and rinsed with a little distilled water.

7. When contents of flask are cool, the volume is made up to 50 mL with distilled water.

8. The extract can be stored indefinitely in a freezer in small vials or flasks until analysis of N solutes.

10.9.2 Analysis of xylem sap and aqueous stem extracts

The following items are required for analysis of xylem sap or extracts:

► weighing balances (accurate to 0.1 and 1 mg)

► test tube racks and glass test tubes to match (*e.g.* 85 × 15 mm)

► micropipettes and tips, and/or dispensers to cover 2–20 μL, 50–200 μL, 0.2–1 mL, 1–5 mL ranges

► vortex mixer

► boiling-water bath

► cold-water ice bath (*e.g.* ice in a foam box), or refrigerated water bath

► spectrophotometer or colorimeter.

10.9.2.1 Ureide assay (Young and Conway 1942)

Reagent preparation

a. Sodium hydroxide (0.5 N NaOH)

   Add 20 g NaOH to 1 L distilled H_2O and pour into **Dispenser 1**.

b. Hydrochloric acid (0.65 N HCl)

   Add 6.5 mL concentrated HCl to 100 mL distilled H_2O.

c. Phenylhydrazine hydrochloride (0.33% (w/v))

   Add 0.33 g phenylhydrazine hydrochloride to 100 mL distilled H_2O.

   Take great care with the phenylhydrazine, as it is toxic. Make fresh daily.

d. The 0.65 N HCl and the phenylhydrazine are now mixed together; total volume 200 mL.

(Pour the HCl and phenylhydrazine mix into **Dispenser 2**.)

e. Potassium ferricyanide ($K_3Fe(CN)_6$ – 1.67% (w/v))

Add 1.67 g to 100 mL distilled H_2O. Make fresh daily.

Caution: $K_3Fe(CN)_6$ is highly toxic.

f. Concentrated hydrochloric acid (10N HCl)

Decant 400 mL concentrated HCl from the bottle.

g. The concentrated HCl and 1.67% $K_3Fe(CN)_6$ are mixed together; total volume 500 mL, and placed in a freezer (if possible) or a fridge/ice bath. (Pour the concentrated HCl and $K_3Fe(CN)_6$ mix into **Dispenser 3**; leave in fridge.) Make fresh daily.

h. Ureide (allantoin) standard—1 mM

Add 39.53 mg allantoin to 250 mL distilled H_2O.

i. Distilled H_2O (**Dispenser 4**)

The 1 mM ureide standard (h) is used to make the following concentrations for a standard curve determination:

Concentration (mM)	1.0 mM allantoin (mL)	Water (mL)
0.00	0.0	100.0
0.01	1.0	99.0
0.02	2.0	98.0
0.04	4.0	96.0
0.10	10.0	90.0

Typical standard curve for ureides
Range 0–0.25 μmol

$y = 5.259x + 0.0454$
$R^2 = 0.9928$

O.D. (525 nm)

μmol ureide
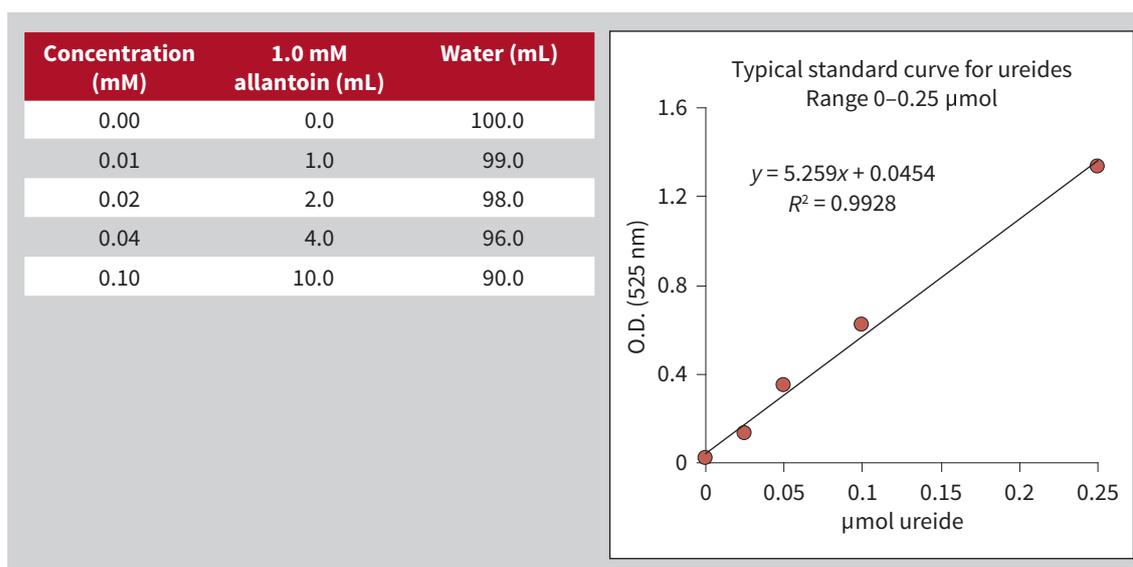
Analysis

1. A standard curve covering the range 0–0.10 mM allantoin should be constructed periodically as part of the laboratory's quality control. Add 2.5 mL of each of the five concentrations to duplicate test tubes corresponding to 0, 0.025, 0.05, 0.1 and 0.25 μmol ureide.

2. Add 0.5 mL 0.5 N sodium hydroxide (NaOH) using Dispenser 1 sufficiently forcefully that additional mixing is unnecessary.

3. Place rack of tubes in a boiling water bath for 10 minutes. Make sure the level of the boiling water is above the contents of the tubes.

4. Remove from water bath and place on bench.

5. Add 1.0 mL HCl/phenylhydrazine mix using Dispenser 2. Again, the dispenser should be used in such a way that additional mixing is unnecessary.

6. Place rack of tubes in the boiling water bath for exactly two minutes. Make sure the boiling water is higher than the contents of the tubes.

7. Remove from boiling water bath and immediately plunge rack of tubes into an ice bath (plastic tub containing ice is okay) and leave for 15 minutes.

8. Remove rack of tubes from the ice bath and add 2.5 mL cold HCl/$K_3Fe(CN)_6$ using Dispenser 3. Thorough mixing of the contents of the test tubes and the $K_3Fe(CN)_6$ is vital for uniform development of colour, and can be achieved with careful use of the dispenser. The red colour that develops with high ureide concentrations should be uniform; layered colour indicates inadequate mixing.

9. Leave on the bench for 10 minutes and read the O.D. at 525 nm on a spectrophotometer. The optical densities must be read as quickly as possible because the colour will fade after a further 15 minutes.

The xylem sap samples together with internal allantoin standards and water blanks are best analysed in a batch. A convenient batch size is 26 tubes, consisting of:

3 water blanks	2.5 mL
3 internal standards (0.04 mM ureide)	2.5 mL
20 sap samples	0.1 mL + 2.4 mL distilled H_2O (1:25 dilution)
or	
20 stem extracts	0.5 mL + 2.0 mL distilled H_2O (1:5 dilution)

10.9.2.2 Amino-N (ninhydrin method) (Yemm and Cocking (1955) as adapted by Herridge 1984)

Reagent preparation

a. Ninhydrin reagent

Add 4.79 g (Merck) ninhydrin plus 167 mg ascorbic acid dissolved in 16 mL distilled H_2O to 500 mL methoxyethanol slowly, without aerating

the methoxyethanol. Store in a dark bottle in the fridge (pour into **Dispenser 1**). Can be stored for up to two weeks.

b. Citrate buffer

Add 67.2 g citric acid plus 25.6 g NaOH to 400 mL distilled H_2O (pour into **Dispenser 2**).

c. Ethanol (60% v/v)

Add 300 mL absolute ethanol to 200 mL distilled H_2O (pour into **Dispenser 3**).

d. 1 mM Asparagine (Asn)/1 mM glutamine (Gln) standard

Add 66 mg Asn and 73 mg Gln to 500 mL distilled H_2O.

e. Distilled H_2O (fill **Dispenser 4**)

The 2 mM standard is now used to make the following concentrations for a standard curve determination:

Concentration (mM)	2.0 mM amino-N (mL)	Water (mL)
0.00	0.0	100.0
0.10	5.0	95.0
0.20	10.0	90.0
0.40	20.0	80.0
1.00	50.0	50.0

Typical standard curve for amino-N Range 0–0.20 nmol

$y = 0.1014x + 0.0634$
$R^2 = 0.9936$
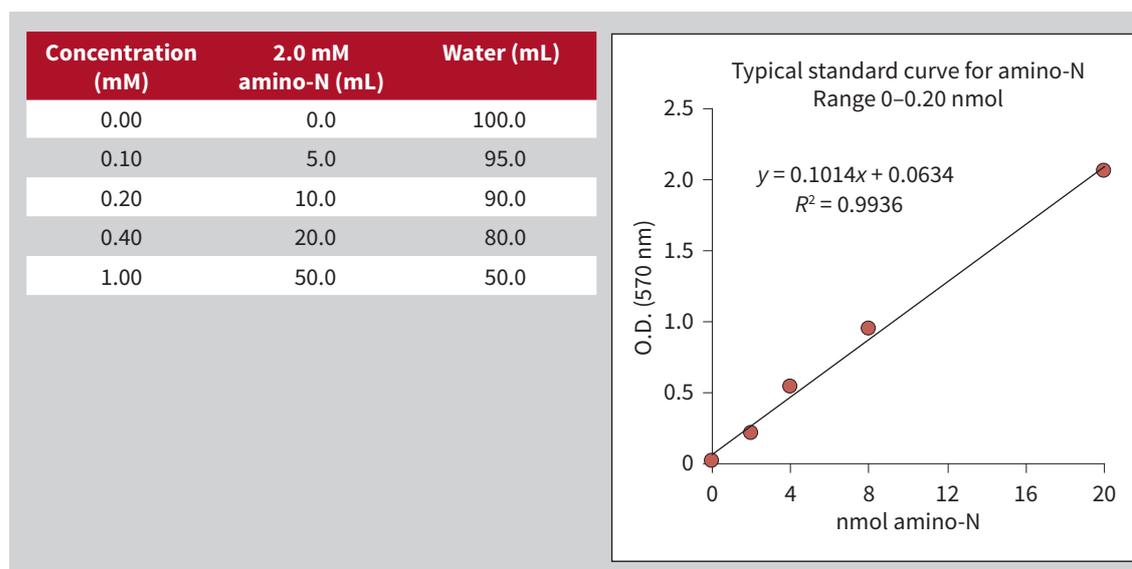
Analysis

1. A standard curve covering the range 0–1.0 mM should be constructed periodically as part of the laboratory's quality control. Add 1.0 mL of each of the five concentrations to duplicate test tubes.

2. Using Dispenser 1 add 0.5 mL citrate buffer in such a way that additional mixing is unnecessary.

3. Using Dispenser 2 add 1.2 mL ninhydrin reagent in such a way that additional mixing is unnecessary.

4. Place in a boiling water bath for 15 minutes.

5. Remove from water bath and place on bench.

6. Using Dispenser 3, add 3.0 mL 60% ethanol in such a way that additional mixing is unnecessary. The contents of the test tubes and the EtOH must be thoroughly mixed for uniform colour development. The blue colour should be uniform; uneven colour development in the test tubes indicates inadequate mixing.

7. Read O.D. at 570 nm on a spectrophotometer.

The xylem sap samples together with internal amino standards and water blanks are best analysed in a batch. A convenient batch size is 26 tubes, consisting of:

3 water blanks	1.0 mL
3 internal standards (0.20 mM)	1.0 mL
20 sap samples	0.1 mL + 0.9 mL H_2O -(1:10 dilution)

10.9.2.3 Salicylic acid method for nitrate determination (Cataldo *et al*. 1975)

This method is suitable for all legume xylem sap samples tested to date except pigeon pea (colour interference). Other recommended methods include Cu-hydrazine reduction (Kamphake *et al*. 1967) and an automated flow injection technique (Alves *et al*. 2000).

Reagent preparation

a. Salicylic acid in concentrated sulfuric acid (H_2SO_4) (5% w/v).

Add 5 g salicylic acid to 100 mL concentrated H_2SO_4 (beaker). Leave for 24 hours before use.

b. Sodium hydroxide (2M NaOH)

Add 40 g NaOH to 500 mL distilled H_2O (Dispenser 1).

c. Nitrate standard (25 mM)

Add 632 mg KNO_3 to 250 mL distilled H_2O.

The 25 mM standard is now used to make the following concentrations for a standard curve determination:

Concentration (mM)	25 mM KNO$_3$ (mL)	Water (mL)
0.00	0.0	100.0
1.25	5.0	95.0
2.50	10.0	90.0
5.00	20.0	80.0
10.0	40.0	60.0
15.0	60.0	40.0

Typical standard curve for nitrate
Range 0–0.75 µmol

$y = 1.8649x + 0.0257$
$R^2 = 0.9949$

O.D. (410 nm)

µmol nitrate
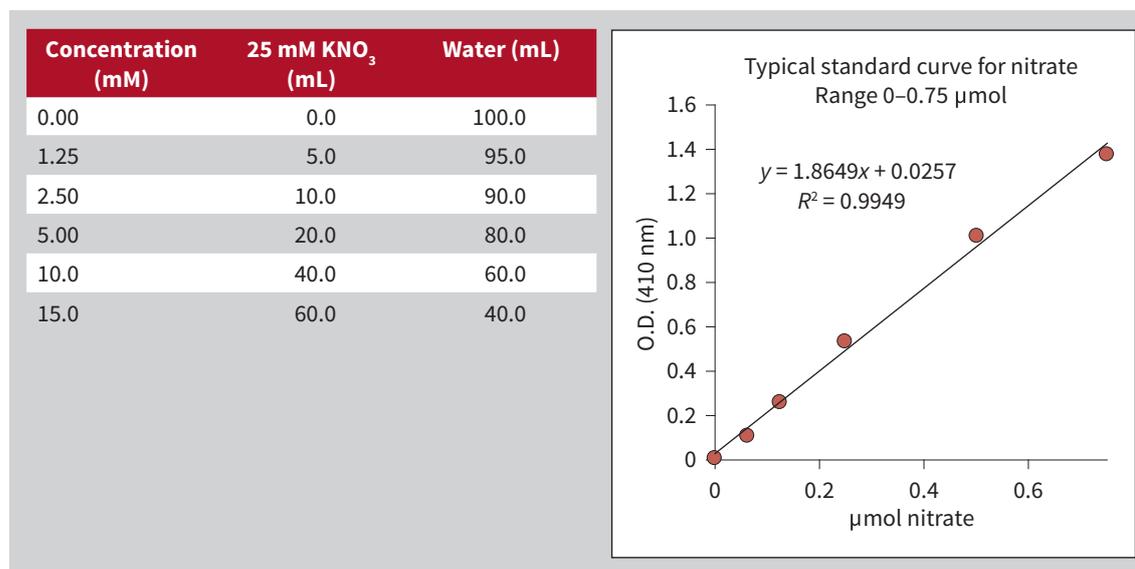
Analysis

1. A standard curve describing the O.D. response to increasing concentrations of nitrate should be constructed within the range 0–15.0 mM nitrate. Pipette 0.05 mL of each of the six concentrations into duplicate test tubes.

2. Pipette 0.20 mL salicylic/sulfuric acid into the tubes. Mix and leave on the bench for 20 minutes. Make sure that the solution in the tube is clear. Mix further if cloudy.

3. Add 4.75 mL 2 M NaOH using Dispenser 1. The dispenser should be used in such a way that additional mixing is unnecessary.

4. Leave on the bench for 10 minutes and read O.D. at 410 nm on a spectrophotometer.

A white precipitate will sometimes form with the addition of salicylic acid. It is very important that the test tubes are shaken well to dissolve this precipitate. It forms when the salicylic acid reagent is freshly made up and often when it is cold. It is therefore best to make it up the day before analysis and make sure it is at room temperature.

The xylem sap samples together with internal amino standards and water blanks are best analysed in a batch. A convenient batch size is 26 tubes, consisting of:

3 water blanks	0.05 mL
3 internal standards (2.5 mM)	0.05 mL
20 sap samples or stem extracts	0.05 mL

10.9.3 Calculating N solute concentrations, %ureide-N and %Ndfa

10.9.3.1 Calculating concentrations of ureides, nitrate and amino-N

For the purposes of this exercise, the hypothetical sample for analysis is xylem sap vacuum extracted from soybean harvested during early pod-fill, *i.e.* R3. The sample was analysed using the methods described above, with the following results.

Analysis	Sample O.D.	Dilution	Internal standard		Sample conc.* (mM)
			Conc. (mM)	O.D.	
Ureide	0.50	×25	0.04	0.57	0.88
Amino	0.85	×10	0.20	0.46	3.69
Nitrate	0.14	×1	2.50	0.24	1.46

* sample conc. = standard conc. × (O.D. sample/O.D. standard) × dilution
  Ureide = 0.04 × (0.50/0.57) × 25 = 0.88 mM
  Amino = 0.2 × (0.85/0.46) × 10 = 3.69 mM
  Nitrate = 2.5 × (0.14/0.24) × 1 = 1.46 mM

10.9.3.2 Calculating %ureide-N and %Ndfa

Now %ureide-N can be calculated as follows:

$$\% \text{ Uride-N} = \left(\frac{4 \times \text{ureide}}{4 \times \text{ureide} + \text{nitrate} + \text{amino-N}} \right) \times 100$$

$$= \left(\frac{4 \times 0.88}{4 \times 0.88 + 1.46 + 3.69} \right) \times 100$$

$$= 41\%$$

The standard curve relating %ureide-N to %Ndfa for sap vacuum extracted from soybean during pod-fill is:

  %ureide-N = (0.64 × %Ndfa) + 15.9

Thus: %Ndfa = 1.56 × (%ureide-N − 15.9)

  = 1.56 (41 − 15.9)

  = 39%

10.9.4 Sampling frequency and other factors to consider

If the experiment aims to compare the N_2-fixing activity of treatments, one or two samplings of xylem sap or stem segments will be sufficient. If, however, the objective is to quantify N_2 fixation for the full growth cycle of the crop, multiple, *i.e.* six to eight, samplings at regular intervals from mid vegetative to late reproductive stages are recommended. Seasonal profiles of %Ndfa are then calculated from those of %ureide-N and used to partition accumulated crop N, estimated from repeated biomass samplings, between fixed N and soil-derived N (see Herridge *et al.* 1990, and a worked example in Unkovich *et al.* 2008).

The ureide method would have more appeal if the number of samplings could be reduced to only one or two. The ^{15}N methods integrate all N$_2$ fixation activity from the onset of growth until the time of sampling, so total N fixed can be estimated by sampling only once at the time of maximum biomass N. A single sampling of xylem sap or stem segments for N-solute analysis would not necessarily coincide with the sampling for maximum biomass N and ^{15}N, but would be done when the point-of-time, ureide-determined %Ndfa had a similar value to the integrative, ^{15}N-determined %Ndfa.

Two studies in Australia (Herridge and Peoples 2002) on soybean, cowpea, mung bean and black gram showed %Ndfa could be estimated with reasonable accuracy from a single determination of %ureide-N during early pod-fill. These estimates were very similar to the ^{15}N-determined %Ndfa values, assessed at the time of maximum biomass N (late pod-fill).

Other factors to consider when using the method are presented in Table 10.10.

Table 10.10 Factors to consider when using the ureide technique for quantifying legume N$_2$ fixation (source: Unkovich *et al*. 2008).

Variable	Comments
Plant species	Relationships between %ureide-N and %Ndfa are similar, but not identical, amongst ureide-exporters. It is therefore recommended that each species under study is calibrated.
Cultivar/genotype	Relationships between %ureide-N and %Ndfa appear to be unaffected by cultivar/genotype within a species.
Strain of rhizobia	Conflicting reports on effects of rhizobial strain on relationships between %ureide-N and %Ndfa. No strain effect for pigeon pea and soybean, except in Brazil. Brazilian data also suggest a strain effect with common bean.
Plant age	Different calibrated relationships between %ureide-N and %Ndfa may need to be used for vegetative and reproductive stages of development (Herridge and Peoples 1990; Hansen *et al*. 1993).
N stress, including drought, and senescence	Relationships between %ureide-N and %Ndfa appear to be invalid when plants are under severe N stress or are in senescence since ureides may also be synthesised from degradation products of nucleic acids. Indicated by xylem N-solute concentrations of <1–2 mM.
Ureides not associated with N$_2$ fixation	High levels of ureides in vacuum-extracted xylem sap of *Gliricidia sepium* were not associated with N$_2$ fixation (Herridge *et al*. 1996). Need to firmly establish the link between %ureide-N and N$_2$ fixation.
Sampling of vacuum-extracted sap (VES)	Sampling best between 0900 and 1600 hours, because of diurnal effects (see Herridge *et al*. 1988; Peoples *et al*. 1989; Herridge *et al*. 1996); %ureide-N for VES unaffected by source or strength of vacuum. A time delay of >5 minutes between sampling and extraction of xylem sap progressively increases %ureide-N levels (Herridge *et al*. 1988; Peoples *et al*. 1989) creating an artefact. For non-destructive sampling of single soybean plants for xylem sap, best results in terms of volume of sap collected and separation of treatments are at late flowering, using the shoot detached at internode 5 (Herridge *et al*. 1988).
Storage of xylem sap	Xylem sap is stable at 25°C for at least 14 days when diluted 1:1 in ethanol. Undiluted sap stable at 25°C for just one day. Freeze for long-term storage.
Errors in analysis of xylem sap, such as colour interference	False readings when analysing xylem sap for ureides may occur with certain species (*e.g. Sesbania grandiflora*; Herridge *et al*. 1996).

In conclusion, the ureide method is relatively simple, accurate and versatile, and can be applied in glasshouse and field experiments, or used in farmers' fields, to assess N_2 fixation by ureide-exporting legumes. Many samples can be collected and analysed in a single day; plants can be sampled non-destructively from the top of the stem, or from lateral branches, with the base left intact to continue to grow (Herridge *et al.* 1988). The resulting estimates of N_2 fixation (%Ndfa and total N_2 fixed) for field-grown legumes are similar to those using more expensive techniques.

10.10 Nitrogen balance method

Nitrogen balance compares the total N of a plant–soil system on two separate occasions, with increases in N attributed to N_2 fixation only after other possible inputs and outputs of N have been quantified. The list of potential inputs and outputs is daunting, making this method one of last resort.

Inputs include: fertiliser N; manures and other sources of organic N; N in irrigation water; wet deposition (mineral N in rainfall); dry deposition (mineral and organic N in dust); runon; lateral subsoil flow and N_2 fixation (which includes N_2 fixation by associative and free-living bacteria as well as from the legume-rhizobia symbiosis).

Outputs include: the N in harvested crop and animal products; gaseous N emissions (NH_3 via volatilisation, N_2O via nitrification and NO_x and N_2 via denitrification); soil erosion, runoff and leaching.

N_2 fixation will be underestimated or overestimated if N outputs or inputs are not accurately measured. An accurate N balance therefore requires measurements of as many potential N inputs and outputs as possible. The time frame is generally several years because of the need to quantify incremental changes in the N content of the soil against a large background (Giller and Merckx 2003).

The methodology is technically challenging, requiring substantial commitments of labour for long periods. Errors in quantifying the N fluxes, and inaccuracies in sampling and analysing soil for changes in total N and bulk density, can introduce substantial errors into the final estimates of N_2 fixation (Chalk 1998).

Nitrogen balance was more commonly used in the past (*e.g.* Vallis 1973; Firth *et al.* 1973; Wetselaar *et al.* 1973) but has now been essentially replaced by other methods.

10.11 Acetylene reduction method (assaying nitrogenase activity)

The enzyme nitrogenase, universally responsible for biological N_2 fixation, can also reduce acetylene (C_2H_2) to ethylene (C_2H_4), both of which are readily quantified using gas chromatography (Hardy *et al*. 1968). Although the C_2H_2 reduction assay is a sensitive measure of nitrogenase activity at a point in time, and can be very useful for detecting N_2 fixation activity of, for example, bacterial cultures, it is less useful for quantifying N_2 fixation by legumes.

Acetylene reduction remains a valuable tool, such as when assessing the impacts on N_2 fixation of mutational studies with rhizobia or assessing N_2 fixation in free living cultures (McComb *et al*. 1975). Methods to undertake an assay are given in Chapter 5.

10.12 Measuring N_2 fixation at the farm and beyond

If estimates of N_2 fixation at farm, farming system, regional or national scale are required, the area of legumes at the particular scale needs to be quantified. This can be done by a walking field survey. Areas can be measured using a hand-held GPS (geographic positioning system) if the fields are large enough (>0.5 ha) in relation to the precision of the GPS (commonly ±5 m). Another useful tool is to use GoogleEarth to create a geo-referenced image of the study area in a geographical information system. This can be used to prepare a field map in advance of a field survey to allow accurate measurement of field areas. Remote sensing can be used to map areas of different crops, at small scales (less than 1 km²) using sensors lifted using octocopters, kites or balloons, and at larger scales using unmanned aerial vehicles (UAVs or drones). If remote sensing methods are used at large scales, detailed ground-truthing is needed for accurate results.

Once legume fields are identified, they can be sampled as described above. The number of fields that need to be sampled depends on the measured differences in N yield and N_2 fixation, and statistical advice is probably needed to develop an optimal sampling strategy. Ideally, surveys need to be conducted several times during the year, or over several years, to gain insights into the frequency with which legumes are grown on each field. If this is not always possible, farmers are often willing to share their records or questionnaires can be used.

Studies at regional or global level usually depend on statistical records. The accuracy of estimates of N_2 fixation made with production records depends on the reliability of the available data used for scaling up. Herridge *et al*. (2008) estimated N_2 fixation inputs at global scale and discuss the accuracy and pitfalls of such estimates.

10.13 Conclusions

No method for assessing N_2 fixation is perfect. Therefore other lines of investigation should be included that can provide insights into treatment effects, such as assessment of nodulation and soil mineral N levels. Although all methods have specific limitations and sources of error (some more than others), the N difference, [15]N (isotope dilution and natural abundance) and ureide methods are arguably the best available. The choice of method needs to be made after consideration of the question being addressed. Confidence in the answer can be enhanced if two independent methods are used.

All of the methods depend on quantifying legume biomass N to calculate the amount of N_2 fixed, so none are truly independent. Although much attention is paid to accurately estimating %Ndfa, an accurate measurement of the total amount of N accumulated in the legume is of equal importance and deserves care and attention to achieve the best results.

Finally, legumes are often grown in farming systems to benefit other cash crops, such as cereals or canola. A discussion of how to estimate the N benefit to rotational crops, and the concept of 'free N farming' is provided in Chapter 8.

10.14 References

Alves B.F.R., Zotareli L., Resende A.S., Polidoro J.C., Urquiaga S. and Boddey R.M. 2000. Rapid and sensitive determination of nitrate in plant tissue using flow injection analysis. Communications in Soil Science and Plant Analysis 31, 2739–2750.

Adjei-Nsiah S., Kuyper T.W., Leeuwis C., Abekoe M., Cobbinah J., Sakyi-Dawson O. and Giller K.E. 2008. Farmers' agronomic and social evaluation of productivity, yield and N_2-fixation in different cowpea varieties and their subsequent residual effects on a succeeding maize crop. Nutrient Cycling in Agroecosystems 80, 199–209.

Boddey R.M., de Oliveira O., Alves B. and Urquiaga S. 1995. Field application of the ^{15}N isotope dilution technique for the reliable quantification of plant-associated biological nitrogen fixation. Fertilizer Research 42, 77–87.

Burris R.H., Eppling F.J., Wahlin H.B. and Wilson P.W. 1942. Studies of biological nitrogen fixation with isotopic nitrogen. Soil Science Society America Proceedings 7, 258–262.

Cataldo D.A., Haroon M., Schrader L.E. and Youngs V.L. 1975. Rapid colorimetric determination of nitrate in plant tissue by nitration of salicylic acid. Communications in Soil Science and Plant Analysis 6, 71–80.

Chalk P.M. 1985. Review: estimation of N_2-fixation by isotope dilution: an appraisal of techniques involving ^{15}N enrichment and their application. Soil Biology and Biochemistry 17, 389–410.

Chalk P.M. 1996. Estimation of N_2 fixation by ^{15}N isotope dilution: the *A*-value approach. Soil Biology and Biochemistry 28, 1123–1130.

Chalk P.M. 1998. Dynamics of biologically fixed N in legume–cereal rotations: a review. Australian Journal of Agricultural Research 49, 303–316.

Chalk P.M. and Ladha J.K. 1999. Estimation of legume symbiotic dependence: an evaluation based on ^{15}N dilution. Soil Biology and Biochemistry 31, 1901–1917.

Danso S.K.A., Hardarson G. and Zapata F. 1993. Misconceptions and practical problems in the use of ^{15}N soil enrichment techniques for estimating N_2 fixation. Plant and Soil 152, 25–52.

Evans J., Turner G.L., O'Conner G.E. and Bergersen F.J. 1987. Nitrogen fixation and accretion of soil nitrogen by field-grown lupins (*Lupinus angustifolius*). Field Crops Research 16, 309–322.

Firth P., Thitipoca H., Suthipradit S., Wetselaar R. and Beech D.F. 1973. Nitrogen balance studies in the central plain of Thailand. Soil Biology and Biochemistry 5, 41–46.

Giller K.E. 2001. Nitrogen fixation in tropical cropping systems: second edition. CAB International: Wallingford.

Giller K.E. and Merckx R. 2003. Exploring the boundaries of N_2-fixation in non-legumes: An hypothetical and experimental framework. Symbiosis 35, 3–17.

Giller K.E. and Witty J.F. 1987. Immobilized ^{15}N-fertilizer sources improve the accuracy of field estimates of N_2-fixation by isotope-dilution. Soil Biology and Biochemistry 19, 459–463.

Hansen A.P., Rerkasem B., Lordkaew S. and Martin P. 1993. Xylem-solute technique to measure N_2 fixation by *Phaseolus vulgaris* L.: calibration and sources of error. Plant and Soil 150, 223–231.

Hardy R.W.F., Burns R.C. and Holsten R.D. 1973. Application of the acetylene–ethylene assay for measurement of nitrogen fixation. Soil Biology and Biochemistry 5, 47–81.

Hardy R.W.F., Holsten R.D., Jackson E.K. and Burns R.C. 1968. The acetylene–ethylene assay for N_2 fixation: laboratory and field evaluation. Plant Physiology 43, 1185–1207.

Herridge D.F. 1984. Effects of nitrate and plant development on the abundance of nitrogenous solutes in root-bleeding and vacuum extracted exudates of soybean. Crop Science 25, 173–179.

Herridge D.F., Bergersen F.J. and Peoples M.B. 1990. Measurement of nitrogen fixation by soybean in the field using the ureide and natural ^{15}N abundance methods. Plant Physiology 93, 708–716.

Herridge D.F., O'Connell P. and Donnelly K. 1988. The xylem ureide assay of nitrogen fixation: sampling procedures and sources of error. Journal of Experimental Botany 39, 12–22.

Herridge D.F., Palmer B., Nurhayati D.P. and Peoples M.B. 1996. Evaluation of the xylem ureide method for measuring N_2 fixation in six tree legume species. Soil Biology and Biochemistry 28, 281–289.

Herridge D.F. and Peoples M.B. 1990. Ureide assay for measuring nitrogen fixation by nodulated soybean calibrated by ^{15}N methods. Plant Physiology 93, 495–503.

Herridge D.F. and Peoples M.B. 2002. Timing of xylem sampling for ureide analysis of nitrogen fixation. Plant and Soil 238, 57–67.

Herridge D., Peoples M. and Boddey R. 2008. Global inputs of biological nitrogen fixation in agricultural systems. Plant and Soil 311, 1–18.

Högberg P. 1997. Tansley Review No. 95 ^{15}N natural abundance in soil-plant systems. New Phytologist 137, 179–203.

Hunt P., Burnham K. and Matheny T. 1987. Precision and bias of various soybean dry matter sampling techniques. Agronomy Journal 79, 425–428.

Kamphake L.J., Hannah S.A. and Cohen J.M. 1967. Automated analysis for nitrate by hydrazine reduction. Water Research 1, 205–216.

Khan D.F., Peoples M.B., Chalk P.M. and Herridge D.F. 2002. Quantifying below ground nitrogen of legumes: a comparison of ^{15}N and non-isotopic methods. Plant and Soil 239, 277–289.

Kipe-Nolt J.A. and Giller K.E. 1993. A field evaluation using the ^{15}N isotope dilution method of lines of *Phaseolus vulgaris* L. bred for increased nitrogen fixation. Plant and Soil 152, 107–114.

Letolle R. 1980. Nitrogen-15 in the natural environment. Pp. 407–433 in 'Handbook of environmental isotope geochemistry', ed. by P. Fritz and J.C. Fontes. Elsevier: Amsterdam.

Mapfumo P., Giller K.E., Mpepereki S. and Mafongoya P.L. 1999. Dinitrogen fixation by pigeonpea of different maturity types on granitic sandy soils in Zimbabwe. Symbiosis 27, 305–318.

Mariotti A. 1983. Atmospheric nitrogen is a reliable standard for ^{15}N natural abundance measurements. Nature 303, 685–687.

McAuliffe C., Chamblee D.S., Uribe-Arango H. and Woodhouse W.W. 1958. Influence of inorganic nitrogen on nitrogen fixation by legumes as revealed by N[15]. Agronomy Journal 50, 334–337.

McComb J.A., Elliot J. and Dilworth M.J. 1975. Acetylene reduction by *Rhizobium* in pure culture. Nature (London) 256, 409–410.

McNeill A.M., Pilbeam C.J., Harris H.C. and Swift R.S. 1998. Use of residual fertiliser ^{15}N in soil for isotope dilution estimates of N_2 fixation by grain legumes. Australian Journal of Agricultural Research 49, 821–828.

McNeill A.M., Zhu C. and Fillery I.R.F. 1997. Use of *in situ* ^{15}N-labelling to estimate the total belowground nitrogen of pasture legumes in intact soil-plant systems. Australian Journal of Agricultural Research 48, 295–304.

Minchin F.R., Witty J. and Sheehy J.E. 1983. A major error in the acetylene reduction assay: decreases in nodular nitrogenase activity under assay conditions. Journal of Experimental Botany 34, 641–649.

Minchin F.R., Sheehy J.E. and Witty J.F. 1986. Further errors in the acetylene reduction assay: effects of plant disturbance. Journal of Experimental Botany 37, 1581–1591.

Norhayati M., Noor S.M., Chong K., Faizah A.W., Herridge D.F., Peoples M.B. and Bergersen F.J. 1988. Adaptation of methods for evaluating N_2 fixation in food legumes and legume cover crops. Plant and Soil 108, 143–150.

Peoples M.B., Boddey R.M. and Herridge D.F. 2002. Quantification of nitrogen fixation. Pp. 357–389 in 'Nitrogen fixation at the millenium', ed. by G.J. Leigh. Elsevier Science: Amsterdam.

Peoples M.B., Unkovich M.J. and Herridge D.F. 2009. Measuring symbiotic nitrogen fixation by legumes. Pp. 125–170 in 'Nitrogen Fixation in Crop Production', ed. by D.W. Emerich and H.B. Krishnan. Agronomy Monograph 52, American Society of Agronomy, Crop Science Society of America and Soil Science Society of America.

Peoples M.B., Faizah A.W., Rerkasem B. and Herridge D.F. 1989. Methods for Evaluating Nitrogen Fixation by Nodulated Legumes in the Field. ACIAR: Canberra.

Rochester I.J., Peoples M.B., Constable G.A. and Gault R.R. 1998. Faba beans and other legumes add nitrogen to irrigated cotton cropping systems. Australian Journal of Experimental Agriculture 38, 253–260.

Russell C.A. and Fillery I.R.P. 1996. *In situ* labelling of lupin below-ground biomass N. Australian Journal of Agricultural Research 47, 1035–1046.

Sanchez C., Blackmer A., Horton R. and Timmons D. 1987. Assessment of errors associated with plot size and lateral movement of nitrogen-15 when studying fertilizer recovery under field conditions. Soil Science 144, 344–351.

Shearer G. and Kohl D.H. 1986. N_2-Fixation in field settings: estimations based on natural ^{15}N abundance. Australian Journal of Plant Physiology 13, 699–756.

Swan A., Peoples M., Gault R., Crews T., Khan D.F., Urquiaga S., Alves B., Boddey R.M. and Forrester R. 2003. Evaluating sources of error when quantifying N_2 fixation in commercial legume crops. Pp 1–4 in '11th Australian Agronomy Conference'. Geelong: Australian Society of Agronomy.

Unkovich M., Herridge D., Peoples M., Cadisch G., Boddey B., Giller K., Alves B. and Chalk P. 2008. Measuring plant-associated N_2 fixation in agricultural systems. ACIAR: Canberra.

Unkovich M.J. and Pate J.S. 2000. An appraisal of recent field measurements of symbiotic N_2-fixation by annual legumes. Field Crops Research 65, 211–228.

Unkovich M.J., Pate J.S. and Sanford P. 1993. Preparation of plant samples for high precision nitrogen isotope ratio analysis. Communications in Soil Science and Plant Analysis 24, 2093–2106.

Vallis I. 1973. Sampling for soil nitrogen changes in large areas of grazed pasture. Communications in Soil Science and Plant Analysis 4, 163–170.

Vessey K.J. 1994. Measurement of nitrogenase activity in legume root nodules: in defense of the acetylene reduction assay. Plant and Soil 158, 151–162.

Viera-Vargas M.S., Deoliveira O.C., Souto C.M., Cadisch G., Urquiaga S. and Boddey R.M. 1995a. Use of different ^{15}N labeling techniques to quantify the contribution of biological N_2 fixation to legumes. Soil Biology & Biochemistry 27, 1185–1192.

Viera-Vargas M.S., Souto C.M., Urquiaga S. and Boddey R.M. 1995b. Quantification of the contribution of N_2 fixation to tropical forage legumes and transfer to associated grass. Soil Biology and Biochemistry 27, 1193–1200.

Warembourg F.R., Montange D. and Bardin R. 1982. The simultaneous use of $^{14}CO_2$ and $^{15}N_2$ labelling techniques to study the carbon and nitrogen economy of legumes grown under natural conditions. Physiologia Plantarum 56, 46–55.

Watanabe I., Chiu C. and Yoshida T. 1990. Estimation of N_2 fixation in soybean and cowpea by using soil residual ^{15}N. Soil Science and Plant Nutrition 36, 375–381.

Wetselaar R., Jakobsen P. and Chaplin G. 1973. Nitrogen balance in crop systems in tropical Australia. Soil Biology and Biochemistry 5, 35–40.

Williams P.C., Norris K.H., Gerkhe C.W. and Bernstein K. 1983. Comparison of near infra-red methods for measuring protein and moisture in wheat. Cereal Foods World 150, 149–152.

Witty J.F. 1983. Estimating N_2-fixation in the field using ^{15}N-labelled fertilizer: some problems and solutions. Soil Biology and Biochemistry 15, 631–639.

Witty J.F. and Minchin F.R. 1988. Measurement of nitrogen fixation by the acetylene reduction assay; myths and mysteries. Pp. 331–344 in 'Nitrogen Fixation by Legumes in Mediterranean Agriculture', ed. by D.P. Beck and L.A. Materon. Martinus Nijhoff: Dordrecht.

Yemm E.W. and Cocking E.C. 1955. The determination of amino acids with ninhydrin. Analyst 80, 209–213.

Young E.G. and Conway C.F. 1942. On the estimation of allantoin by the Rimini-Schryver reaction. Journal of Biological Chemistry 142, 839–853.

CHAPTER 11
Fundamental molecular techniques for rhizobia

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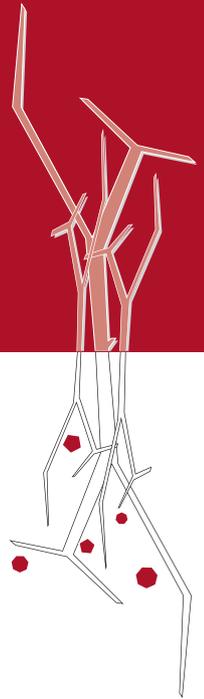
11.1 Introduction to nucleic acid purification

Working with DNA is now a fundamental skill in working with rhizobia. It is necessary for typing strains using PCR methods and for sequencing activities applied to understanding genomes; their structure, how they function, and their taxonomic position.

Nucleic acid purification is the separation of nucleic acids from proteins, cell wall debris and polysaccharide after lysis of cells. For rhizobia, we provide here a number of commonly used methods for the extraction of genomic and plasmid DNA. Methods for extraction of total RNA are presented in Chapter 13. The CTAB method (Protocol 11.1.1) has been used extensively for extraction of total genomic DNA for DNA sequencing while Protocol 11.1.2 gives higher yields but generally with slightly lower purity. Plasmid DNA can be differentially displayed using Protocol 11.2.1 for determination of replicon number. This method allows localisation of genes to replicons, confirmation of genome assemblies and identification of genetic changes. The plasmids can subsequently be purified from low melting point gels using GELase (Epicentre, http://www.epibio.com/item.asp?id=297). Protocol 11.2.2 presents a method to recover introduced plasmids from rhizobia (*i.e.* complementing plasmids) for transformation into *Escherichia coli* prior to restriction analysis. Protocol 11.2.3 provides an alternative method to the GELase procedure for purifying plasmids but has not been tested as extensively.

11.1.1 Protocol for bacterial genomic DNA isolation using CTAB

This CTAB protocol comes from the DOE Joint Genome Institute Standard Operating Procedures (http://my.jgi.doe.gov/general/protocols.html) and has been successfully used by the authors of this chapter for the isolation of genomic DNA from 130 rhizobial strains to date.

11.1.1.1 Materials and reagents

Materials and reagents are listed in Table 11.1

Table 11.1 Materials and reagents for isolation of genomic DNA

Disposables	Vendor	Stock number
1.5 mL microcentrifuge tube	Eppendorf	22 36 320-4
50 mL Nalgene Oak Ridge polypropylene centrifuge tube	VWR	21010-568
10 mL pipette	Falcon	357551
1 mL pipette tips	MBP	3781

Reagents:	Vendor	Stock number
CTAB (cetyltrimethylammonium bromide)	Sigma	H-6269
NaCl	Sigma	S-3014
TE buffer (10 mM Tris; 1 mM EDTA, pH 8.0)	Ambion	9858
Lysozyme	Sigma	L-6876
Proteinase K	Qiagen	19131
5M NaCl	Ambion	9759
10% (w/v) SDS (Sodium dodecyl sulfate)	Sigma	L-4522
Chloroform	Sigma	C-2432
iso-Amyl alcohol	Sigma	I-9392
Phenol	Sigma	P-4557
Isopropanol	VWR	PX-1835-14
Ethanol	AAPER	
RNAse A (100 mg mL^{-1})	Qiagen	19101

Equipment

► Hot plate

► 250 mL glass beaker

► Magnetic stirring bar

► Thermometer

► Automatic pipette dispenser

► Sorvall 500 Plus centrifuge (DuPont, Newtown, CT)

► 65°C water bath

► 37°C incubator

11.1.1.2 Reagent/stock preparation

CTAB/NaCl (cetyltrimethylammonium bromide)

Dissolve 4.1 g NaCl in 80 mL of water and slowly add 10 g CTAB while heating (~65°C) and stirring. This takes more than three hours to dissolve CTAB. Adjust final volume to 100 mL and sterilise by filtering or autoclaving.

Procedure

Cell preparation and extraction techniques

Notes

▶ In step 1 below, do not use too many bacterial cells (OD_{600nm} of not more than 1 is recommended) or DNA will not separate well from the protein.

▶ Most of the time, inverting several times is sufficient to mix well. Avoid vigorous shaking which will shear the DNA.

▶ Use any standard protocol for DNA precipitation (Ausubel *et al.* 2013).

Total volume ....**1.5 mL   30 mL**

1. Grow cells in broth and pellet for five minutes or scrape from plate.

2. Transfer bacterial suspension to the appropriate centrifuge tube.

3. Pellet cells for five minutes.

4. Discard the supernatant.

5. Re-suspend cells in TE buffer (10 mM Tris; 1mM EDTA; pH 8.0) to an $OD_{600nm} \sim 1.0$.

6. Transfer given amount of cell suspension to a clean centrifuge tube.................**740 µL   14.8mL**

7. Add lysozyme (conc. 100 mg mL^{-1}). Mix well. ....**20 µL   400 µL**

   This step is necessary for hard-to-lyse bacteria.

8. Incubate for five minutes at room temperature.

9. Add 10% (w/v) SDS. Mix well. ....**40 µL   800 µL**

10. Add Proteinase K (10 mg mL^{-1}). Mix well..**8 µL   160 µL**

11. Incubate for one hour at 37°C

12. Add 5 M NaCl. Mix well. ....**100 µL   2 mL**

13. Add CTAB/NaCl (heated to 65°C). Mix well. ....**100 µL   2 mL**

14. Incubate at 65°C for 10 minutes.

15. Add chloroform:isoamyl alcohol (24:1). Mix well..**0.5 mL   10 mL**

16. Spin at maximum speed for 10 minutes at room temperature.

17. Transfer aqueous phase to clean microfuge tube (should not be viscous).

18. Add phenol:chloroform:isoamyl alcohol (25:24:1).
    Mix well. ....**0.5 mL   10 mL**

19. Spin at maximum speed for 10 minutes at room temperature.

20. Transfer aqueous phase and add 0.6 volumes (vol.) of isopropanol (−20°C).

    ▷ E.g. if 400 μL of aqueous phase is transferred, add 240 μL of isopropanol

21. Incubate at room temp for 30 minutes.

22. Spin at maximum speed for 15 minutes.

23. Wash pellet with 70% (v/v) ethanol, spin at maximum speed for five minutes.

24. Discard the supernatant and let pellet dry for five to 10 minutes at room temperature.

25. Re-suspend in TE buffer plus RNAse (99 μL TE + 1 μl RNAse (10 mg mL^{-1}).... **20 μL   400 μL**

26. Transfer to sterile micro-centrifuge tubes.

27. Incubate at 37°C for 20 minutes.

28. Run 1 μL in a 1% (w/v) agarose gel with concentration standards (Figure 11.1).

Figure 11.1 Gel electrophoresis of genomic DNA extracted from rhizobia using Protocol 11.1.1 described above from: *Mesorhizobium sp.* WSM3224 (Lane 1); *Rhizobium leguminosarum* bv *trifolii* SRDI565LMG23256 (Lane 2); *R. leguminosarum* bv *trifolii* SRDI943 (Lane 3); and Lambda *Hind*III cut DNA marker (Lane 4).
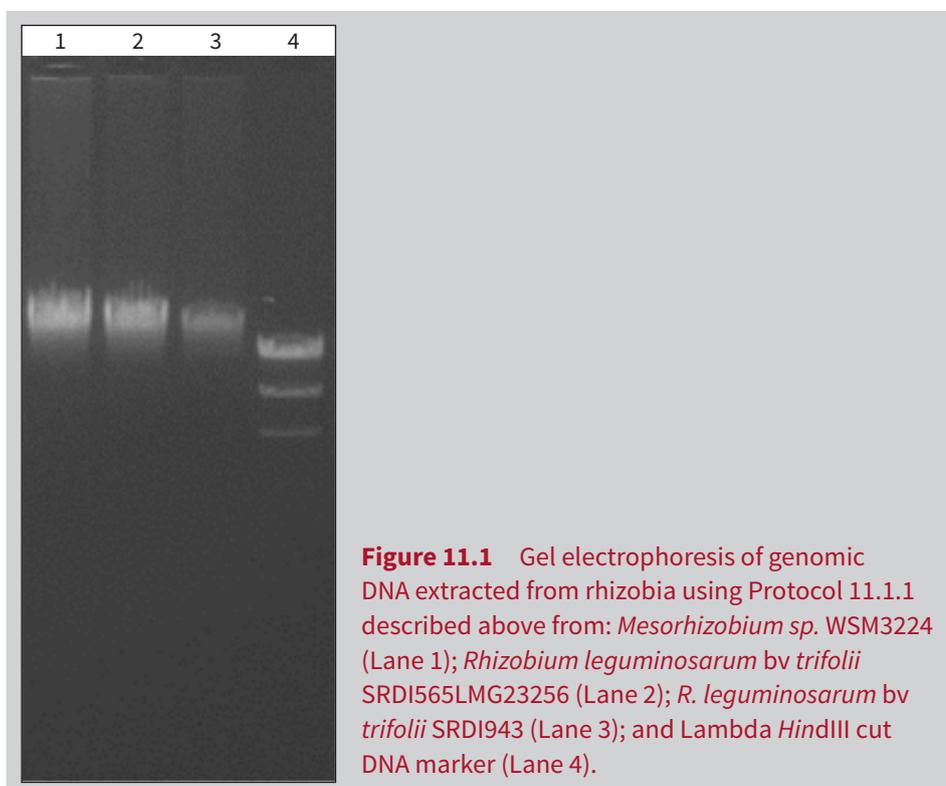
11.1.2 Protocol for genomic DNA preparation using phenol-chloroform

11.1.2.1 Precautionary measures

► All pipette tips should be filter barrier tips.

► Benches should be scrupulously clean (wipe with 70% (v/v) ethanol before use) and gloves must be worn at all times to prevent contamination.

► Containers should be disposable plastic ware, and glassware needs to be soaked in 0.1 N HCl and then 0.1 N NaOH prior to rinsing with RODI (reverse-osmosis, deionised) water.

► Genomic DNA should be prepared using pipette tips with the tip end cut off to prevent shearing of the DNA.

General preparation

TY broth

► Tryptone   5 g

► Yeast-extract   3 g

► $CaCl_2.2H_2O$ 0.75 g (reduced from original recipe to avoid precipitation)

► Distilled H_2O to 1 L

► Adjust pH to neutral

Procedure

1. Inoculate a 10 mL TY broth in a 50 mL conical flask with a loopful of cells and grow to late log phase (for root nodule bacteria, it usually requires two to three days at 28°C). Subculture the cells if necessary to ensure the cells are in the log phase.

2. Transfer the log phase culture into a centrifuge tube.

3. Pellet cells at 7,500 *g* for five minutes at room temperature.

4. Remove the supernatant (last drops can be removed using a pipette).

5. Re-suspend pellet in 1 mL TES buffer (30 mM Tris pH 8.0, 50 mM NaCl, 5 mM EDTA) by vortexing for one to two minutes.

6. Repeat steps 3 and 4.

7. Re-suspend cell pellet in 1 mL TES buffer.

8. Add 0.2 mL lysozyme solution (10 mg mL^{-1} in TES buffer).

9. Incubate at 37°C for 30 minutes.

10. Add 0.15 mL 10% (w/v) SDS and 60 µl of proteinase K (6 mg mL^{-1} in TES).

11. Incubate at 45°C for one hour.

12. Add another aliquot of 60 µl proteinase K and incubate at 55°C for one hour.

13. Extract DNA twice using an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1).

14. Add 0.1 volume of 3 M sodium acetate (pH 5.2) to the extracted DNA.

15. Add an equal volume of isopropanol and mix gently.

16. Centrifuge at room temperature at 7,500 *g* for 10 minutes.

17. Remove supernatant carefully without losing the pellet.

18. Add 0.5 mL of 70% (v/v) ethanol, close the lid and invert the tube carefully a few times.

19. Centrifuge at 7,500 *g* at room temperature for five minutes and remove supernatant carefully without losing the pellet.

20. Invert tubes on a sterile paper towel and drain the last drops of liquid.

21. Leave the tubes to air dry for 15 to 20 minutes.

There is no need to vacuum dry DNA; it makes resuspension of the DNA difficult.

22. Re-suspend pellet in sterile RODI water.

23. Treat with RNAse (final concentration 20 µg mL^{-1}) for one hour at 37°C to remove RNA.

11.1.3 Protocol for nucleic acid extraction from soil

This method is modified from Griffiths *et al.* (2000).

General preparation

► 5% CTAB/phosphate buffer (120 mM, pH 8)

  ▷ mix equal volumes of 10% (w/v) CTAB in 0.7 M NaCl and 240 mM potassium phosphate buffer (pH 8.0) and autoclave

► phenol:chloroform:isoamyl alcohol (pH 8.0) (25:24:1)

Danger—never autoclave

► chloroform:isoamyl alcohol (24:1)

Danger—never autoclave

▶ 30% (w/v) PEG (Polyethylene glycol) purchased from Fluka, SIGMA)

Don't autoclave PEG

▶ 70% (v/v) ethanol. Keep in the freezer.

No need to autoclave

▶ Molecular grade, sterile water

▶ 100 mL of 240 mM potassium phosphate buffer (pH 8)

  ▷ 1 M K_2HPO_4 : 22.56 mL

  ▷ 1 M KH_2PO_4 : 1.44 mL

  ▷ Dilute to 100 mL

▶ Elution Buffer (EB)

  ▷ 10 mM Tris-Cl, pH 8.5

▶ Cell homogenisation/lysis equipment (*i.e.* Fast-Prep beadbeater)

▶ Bead tubes

▶ 0.1 mm zirconia/silica beads, 0.5 mm glass beads (fill this bead mixture up to 20% of the tube's volume and add one of a 2 mm glass ball).

Procedure

1. Weigh out 1 g of soil.

2. Add 0.5 mL CTAB buffer and 0.5 mL phenol:chloroform:isoamyl alcohol (25:24:1) into ready-made 2 mL bead tubes, tighten lids and keep on ice.

3. Place tubes in Fast-Prep beadbeater and lyse cells for 30 seconds at speed 5.5 (repeat three times with a five minute break in between lysing treatments).

4. Centrifuge at full speed (16,000 g) for five minutes at 4°C.

5. Extract the top aqueous layer and transfer to a new microfuge tube.

6. Add 0.5 mL of chloroform:isoamyl alcohol (24:1) and vortex for five seconds to form an emulsion.

7. Centrifuge at full speed (16, 000 g) for five minutes (4°C).

8. Extract the top layer again and precipitate nucleic acids by adding 1 mL of PEG solution. Mix well.

9. Leave samples for one to two hours at room temperature.

10. Centrifuge at 16,000 g for 10 minutes (room temperature).

11. Pour off supernatant and wash pellet with 70% (v/v) ethanol (200 μL). Vortex, centrifuge for 10 minutes (16, 000 g) and discard alcohol.

12. Repeat step 11, removing all traces of liquid.

13. Re-suspend pellet in 50 μL of EB buffer.

Removal of humic acids from DNA samples

One-step™ PCR inhibitor removal kit (ZYMO RESEARCH cat. No D6030). The protocol is provided here.

1. Remove the cap and snap off the base of the supplied filter tubes. Place them into collection tube (provided).

2. Spin down at 8,000 g for exactly three minutes.

3. Place the filter into 1.5 mL microfuge tube and add 100 to 200 μL of DNA sample.

4. Spin down at 8,000 g for exactly one minute. The eluate should be free of humic acid.

11.2 Plasmid purification

11.2.1 Protocol for plasmid profiling using a modified Eckhardt procedure

The original method (Eckhardt 1978) has been subsequently modified (Priefer, 1984; Hynes *et al.*, 1985; and Hynes and McGregor, 1990).

Recipes (stocks and media)

The components of TBE buffer and HP media are given in Tables 11.2 and 11.3.

Table 11.2 10× TBE buffer

Component	Quantity
Tris	108 g L^{-1}
Boric acid	55 g L^{-1}
EDTA (0.5 M, pH 8.0)	40 mL L^{-1}

Make up to 1 L with RODI water, pH to 8.0 with HCl, autoclave.

1× TBE buffer

► Dilute from 10× TBE buffer using RODI water. Also maintain a stock of sterile TBE buffer for preparing the agarose gels, Sarcosyl and lysis solution.

10% SDS in 1× TBE buffer (200 mL)

▶ Dissolve 20 g of SDS in 160 mL of pre-warmed 1× TBE buffer.

▶ Adjust to pH 8.0.

▶ Make up to 200 mL with 1× TBE buffer.

▶ Autoclave.

NB: if the SDS precipitates, re-dissolve it at 50°C in a water bath.

0.3% (w/v) Sarcosyl in 1× TBE buffer

▶ Dissolve 0.15 g Sarcosyl (*n*-lauroylsarcosine) in 50 mL of 1× sterile TBE buffer.

▶ Chill at 4°C for at least one hour prior to use.

E1 Solution—10% (w/v) sucrose in 1× TBE buffer + 10 µg mL⁻¹ RNase

▶ Dissolve 1 g sucrose in 10 mL 1× sterile TBE buffer.

▶ Store at 4°C.

Lysozyme Stock Solution 100×

▶ 100 mg mL^{-1} lysozyme dissolved in 1× TBE

▶ Store at –20°C.

Lysis Solution (100 µg mL⁻¹ lysozyme in E1 Solution)

▶ Prepare fresh on the day.

▶ Mix 986 µL of E1 solution with 4 µL RNAase stock solution (10 mg mL^{-1}).

▶ Add 10 µL of 100× lysozyme stock solution .

Table 11.3 HP media

Component	Quantity
Peptone	4.0 g L^{-1}
Yeast extract	0.5 g L^{-1}
Tryptone	0.5 g L^{-1}
$CaCl_2.2H_2O$	0.2 g L^{-1}
$MgSO_4.7H_2O$	0.2 g L^{-1}

Procedure

Step 1

Inoculate strains into prepared 5 mL broths (add antibiotics if selection pressure is required) and grow at 28°C with shaking at 200 rpm.

NB: selection of the broth media depends on which genera of root nodule bacteria (RNB) are used. *Rhizobium leguminosarum* lyses well when grown in TY while *Sinorhizobium medicae* lyses better when grown in TY with half the normal $CaCl_2$ or in HP media (when using HP media, omit the antibiotics). *Agrobacterium sp.* may be grown in LB. Grow overnight (28°C, 200 rpm) to an OD_{600nm} of 0.3. (OD is important; try not to grow over 0.3). It is best to do a range of subcultures to ensure the correct OD; *i.e.* subculture 2, 5, 10 and 20 µL into 5 mL broths. Faster growing strains can be further subcultured.

Step 2

Prepare and pour the 0.75% (w/v) agarose plus 1% (w/v) SDS gel:

1. Add 7.5 g agarose to 900 mL 1× TBE (pH 8.0) buffer and microwave (stirring occasionally) until clear.

2. Cool the agarose to 55°C, pour 90 mL into a conical flask and add 10 mL of 10% (w/v) SDS solution. Mix by gentle swirling and pour into the gel tray.

This quantity is sufficient for a 15 cm × 15 cm tray; the gel should be as thin as possible (3 to 4 mm) to give the best results.

3. When the gel is set, place in the gel tank and cover with 1× TBE buffer.

Preparation of the cultures

1. Set the centrifuge to 4°C (or place centrifuge in a refrigerated room).

2. Pipette 200 µL of culture ($OD_{600nm} \leq 0.3$—this is the best cell density but it can be up to 0.5) into a 1.5 mL micro-centrifuge tube and place on ice.

You can vary the volume of culture used in this step according to the OD_{600nm} i.e. 300 µL at OD 0.2. Additionally, a series of aliquots i.e. 200, 400, and 800 µL may be used. Exopolysaccharide may be removed by pelleting cells and re-suspending them in sterile RODI water.

3. Add 1.0 mL of cold 0.3% (w/v) Sarcosyl and mix by inversion twice.

4. Place on ice for 10 minutes.

5. Prepare the lysis solution and place on ice.

6. Centrifuge the Sarcosyl-culture mix (4°C, 16,000 rpm; five minutes).

7. Carefully pour off the supernatant and place the tube back on ice. Using a P200 pipette, carefully remove the remaining supernatant, leaving the pellet as dry as possible.

8. Place the tubes in a rack at room temperature, add 20 µL of lysis solution and re-suspend the pellet by carefully pipetting up and down twice. Avoid creating bubbles in the solution.

9. Immediately load 20 µL of sample into the gel well. The sample should be evenly re-suspended and slightly cloudy. The loading dye can be added to one well as a marker.

10. When loading of all samples is complete, initiate electrophoresis at 5 to 10 V for 15 to 30 minutes (maximum) until the samples have lysed and are clear. Then run the gel electrophoresis at 70 to 80 V overnight. LEAVE THE GEL TANK LID OFF to avoid over-heating the gel tank. DANGER: Indicate with appropriate signage that the lid off means exposed live wires. Alternatively, run in the cold room or in an air-conditioned laboratory leaving the gel tank lid on.

11. After staining with ethidium bromide (for 30 minutes to one hour), de-stain in sterile RODI water for 30 minutes (longer de-stain times may improve the resolution of the bands; see Figure 11.2).

Figure 11.2 Plasmid profile of five *Rhizobium leguminosarum* strains compared with control *R. l.* bv. *viciae* VF39 (lane 1) showing bands of sizes 900, 700, 500, 400, 220 and 150 kb (top to bottom).
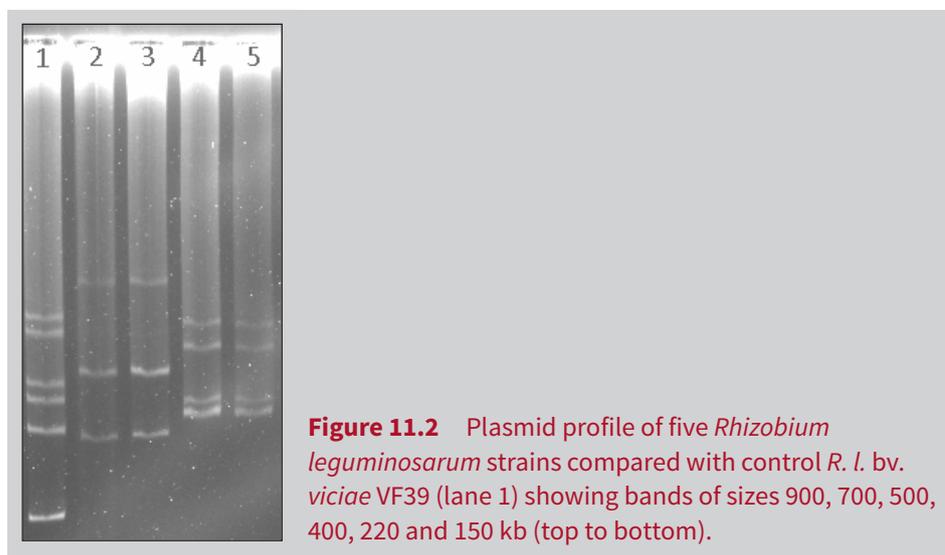
11.2.2 Protocol for plasmid preparation by alkaline lysis

This alkaline lysis method is recommended for rapid extraction and purification of small (<50 kb) plasmids that have been introduced into rhizobia (*i.e.* via conjugation; see Protocol 11.2.1). The following protocol (Ausubel *et al.* 2013*)* was adapted specifically for the isolation of rhizobial megaplasmids. It is important to mention that although plasmids may be isolated from RNB using this method, the profile after separation by gel electrophoresis will appear smeared and the authors have noted inhibition of restriction enzyme digest reactions. Plasmids prepared from RNB using this method will need to be transformed back into *E. coli* prior to extraction (using the original version of this protocol) and restriction enzyme digestion.

General preparation

► Prepare Solution I from standard stocks in batches of approximately 100 mL; sterilise by autoclaving.

► Prepare Solution II fresh using sterile distilled H_2O and use at room temperature. Warm solution II if a precipitate is observed.

► Store Solution III at 4°C and use at 4°C.

► 100% (v/v) ethanol at room temperature.

► 70% (v/v) ethanol at room temperature.

► Molecular grade H_2O.

Recipes

Alkaline Lysis Solution I

► 25 mM Tris-Cl (pH 8.0)

► 10 mM EDTA (pH 8.0)

Alkaline Lysis Solution II

► 0.2 N NaOH (freshly diluted from a 10 N stock)

► 1% (w/v) SDS (diluted on the day of use from a 10% (v/v) SDS stock)

Alkaline Lysis Solution III

The resulting solution is 3 M with respect to potassium and 5 M with respect to acetate. Prepare 100 mL total volume.

► 5 M potassium acetate, pH 4.8   60.0 mL

► glacial acetic acid   11.5 mL

► molecular grade H_2O 28.5 mL

Procedure

1. Inoculate 2 mL of TY containing the appropriate antibiotic with a single colony of bacteria. Incubate the culture overnight at 28°C with vigorous shaking.

2. Pour 1.5 mL of the culture into a micro-centrifuge tube. Centrifuge at maximum speed for 30 seconds in a micro-centrifuge at room temperature. Store the unused portion of the original culture at 4°C.

3. Remove the medium by aspiration, leaving the bacterial pellet as dry as possible.

4. Re-suspend the bacterial pellet in 100 μL of Alkaline Lysis Solution I at room temperature by vigorous vortexing.

5. Add 200 μL of freshly prepared Alkaline Lysis Solution II at room temperature to each bacterial suspension. Close the tube tightly and mix the contents by inverting the tube rapidly several times. Do not vortex and rapidly proceed to Step 6.

6. Add 150 μL of ice-cold Alkaline Lysis Solution III. Close the tube and disperse Alkaline Lysis Solution III through the viscous bacterial lysate by inverting the tube several times. Transfer tube to –20°C for 15 minutes to promote precipitation of SDS.

7. Centrifuge the bacterial lysate at maximum speed for five minutes at room temperature in a micro-centrifuge. Transfer the supernatant to a fresh tube and warm to >15°C to solubilise any remaining SDS.

8. Precipitate nucleic acids from the supernatant by adding two volumes of ethanol at room temperature (cold ethanol is best avoided as this promotes precipitation of residual SDS). Mix the solution by inverting several times.

9. Collect the precipitated nucleic acids by centrifugation at maximum speed for five minutes at room temperature in a micro-centrifuge.

10. Remove the supernatant by gentle aspiration as described in Step 3 above. Stand the tube in an inverted position on a paper towel to allow all of the fluid to drain away. Use a Kimwipe or disposable pipette tip to remove any drops of fluid adhering to the walls of the tube.

11. Add 1 mL of room temperature 70% (v/v) ethanol to the pellet and invert the closed tube several times. Recover the DNA by centrifugation at maximum speed for two minutes at room temperature in a micro-centrifuge.

12. Remove all of the supernatant by gentle aspiration as described in Step 3. Take care with this step as the pellet sometimes does not adhere tightly to the tube.

13. Remove any beads of ethanol that form on the sides of the tube. Store the open tube at room temperature until the ethanol has evaporated and no fluid is visible in the tube (five to 10 minutes).

    NB: residual ethanol in the prepared DNA will cause the DNA (visualised with loading dye) to rise out of the agarose gel well when setting up for gel electrophoresis.

14. Dissolve the nucleic acids in 50 μL of sterile H_2O. Gently mix by repetitive pipetting. Warm solution to 55°C for 10 minutes.

15. Store the DNA solution at –20°C.

11.2.3 Protocol for plasmid preparation by cesium chloride

General preparation

► Cesium chloride (CsCl) preparation.

  ▷ Dissolve 11 g CsCl in 5 mL TE buffer, pH 8.0.

  ▷ Because CsCl will not dissolve at low temperatures, a water bath may be required. All solutions and equipment must be maintained at >20°C during the procedure to ensure CsCl remains in solution.

► Ethidium bromide (EtBr) is a carcinogen and appropriate protective wear (gloves, lab coat and glasses) should be worn at all times during this procedure. Consult the MSDS for EtBr prior to use.

► Preparing salt-saturated isopropanol (flammable solution, avoid contact with open flame).

  ▷ Prepare 500 mL of a 5 M NaCl solution (dissolve the NaCl in TE, pH 8.0). Autoclave in a 1 L Schott bottle.

  ▷ Add 500 mL isopropanol to the solution above, mix and allow the phases to separate.

  ▷ On the day of use, dispense the top layer of the salt-saturated isopropanol into 30 mL aliquots (1 aliquot per DNA sample).

► Prepare the bent needle for the isopropanol extraction steps; the 90° bend helps to maintain phase separation.

  ▷ To bend the needle, hold the needle firmly with a sterile implement either side (*i.e.* flame-sterilised pliers).

  ▷ Pass the needle through a flame to bend it into a loop.

  ▷ Avoid touching the needle tip to minimise DNA contamination.

► TE buffer.

  ▷ 10 mM Tris and 1 mM EDTA, pH 8.0.

► TY broth (see Protocol 11.1.2).

Procedure

1. Grow overnight culture of bacteria in 400 mL TY broth.

2. Spin cultures in sterile 500 mL tubes.

3. Remove all growth media; use vacuum if required.

4. Re-suspend pellet in 30 mL TE buffer ensuring there are no lumps.

5. Prepare lysate using genomic method in 15 mL quick-seal (Beckman) centrifuge tubes.

Steps 6–13 must be carried out in dim light to prevent UV/EtBr interaction that can damage the DNA.

6. Add 5 mL of CsCl solution to the cleared lysate in the quick seal tube. Take up 0.2 mL pre-warmed EtBr (10 mg mL^{-1}) into a syringe with needle attached and dispense into the quick-seal centrifuge tubes containing the DNA/cesium chloride solution.

7. Inject the DNA/EtBr solution and mix thoroughly but gently.

There must be no bubbles in the gradient tubes.

8. Seal the tubes with the heat sealer and place in a fixed angle rotor which has been pre-warmed to 28°C.

9. Centrifuge at 45,000 g for 48 hours to enhance separation of the forms of DNA.

10. Remove the desired DNA from the gradient by drawing off the band (intensely stained red) with a 20 mL syringe (Figure 11.3), beveled edge of needle uppermost. (*NB:* supercoiled DNA will migrate faster and be located at the bottom of the gradient.)

The procedure is easier if a needle is inserted in the top of the tube to allow air in, and a piece of tape is applied to the side of the tube at the point where the second needle is inserted to prevent blocking up the needle (Figure 11.3).
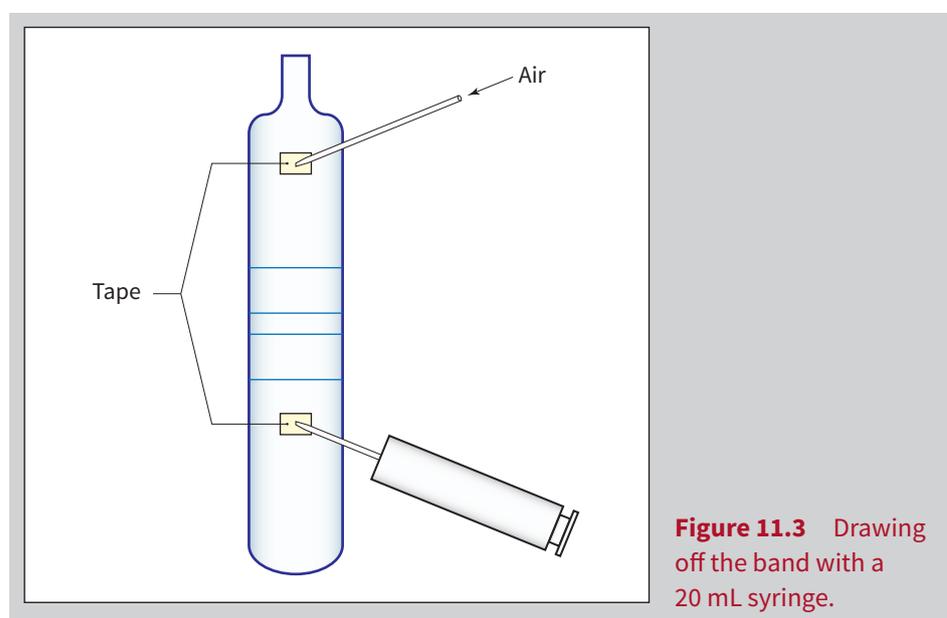
Figure 11.3 Drawing off the band with a 20 mL syringe.

11. Replace the straight needle with a needle pre-bent at 90° to allow ease of isopropanol uptake. Using the syringe containing the DNA, take up an equal volume of salt-saturated isopropanol. Mix gently and allow phases to separate while keeping the syringe upright. Expel the top phase with the syringe upright.

Steps 11 and 12 must be carried out in dim light to prevent UV/EtBr interaction that can damage the DNA.

12. Repeat the above step six times, or as required, until the ethidium bromide is no longer visible.

13. Add the washed DNA to sterile 50 mL tubes then add two volumes of sterile water and six volumes of 100% (v/v) EtOH (volumes are proportional to the original volume of DNA solution).

14. Spin for 10 minutes at maximum speed in a micro-centrifuge at 25°C. Dry pellet under vacuum.

15. Re-suspend pellet in 0.45 mL TE buffer and transfer solution into a micro-centrifuge tube.

16. Add 50 μL of 3 M Na acetate (pH 5.2) then 1 mL of 100% EtOH; mix well.

17. Spin for 10 minutes at 10, 000 *g.*

18. Wash pellet with 70% EtOH, then dry under vacuum.

19. Re-suspend pellet in 0.5 mL (high copy number) or 0.1 mL (low copy number) TE buffer, pH 8.0 (adjust volume according to pellet size).

20. Determine the concentration using the Dot method (Ausubel *et al.* 2013).

11.3 PCR techniques

Polymerase Chain Reaction (PCR) is a common molecular biology technique used to amplify DNA from a starting template (*i.e.* cells, genomic DNA or plasmids). Methods for cell preparation are provided in 11.3.1 and can be used in both single and multi-amplicon generation (11.3.2 and 11.3.3 respectively). Genomic DNA preparations (Protocol 11.1.1 and 11.1.2) may be used in PCR when problems arise from the use of cell templates. The amplified product, known as the amplicon, can then be used for a variety of purposes, including sequencing, cloning, probe design. Multi-amplicons can be used for fingerprinting RNB.

11.3.1 Protocol for preparation of cell template

A. Basic cell preparation

This procedure is modified from Schneider and deBruijn (1996).

► Prepare a pure rhizobial culture by dilution streaking on to ½ LA or TY agar plates.

► Incubate at 28°C until growth is visible on final dilution streak.

► Re-suspend a loopful of culture in 1 mL of 0.89% (w/v) saline.

1. Centrifuge the culture at 10,000 *g* for 30 seconds and remove the supernatant.

   NB: cultures producing large amounts of EPS may need to be washed with 0.89% (w/v) saline for a second time.

2. Re-suspend the cell pellet in sterile (DNA-free) water to an OD_{600nm} of 6.0 (*NB:* an OD_{600nm} of 10 has been used reproducibly for *Rhizobium leguminosarum*).

3. Store at 4°C for up until one month or use immediately in the PCR reaction below.

NB: it is important that fresh cultures of rhizobia, free of contaminants, are used to prepare the template.

B. Cell preparation using PEG

Method (Chomczynski and Rymaszewski 2006).

Reagents

1. Alkaline PEG reagent preparation

   a. Combine 60 g PEG 200 (Sigma-Aldrich or equivalent) with 0.93 mL 2 M KOH and 39 mL water. If desired, NaOH can substitute for KOH in the reagent. *Note that PEG 200 is measured by mass rather than volume because of the viscosity of the liquid.*

   b. Confirm that the pH is 13.3 to 13.5. Due to storage, some batches of PEG 200 have an acidic rather than neutral pH. In this case, add an additional amount of alkali to reach the target pH range.

2. Scoop a loopful of a RNB colony containing your gene of interest into 500 μL of sterile molecular- grade water in a sterile microfuge tube.

3. Spin down at 6000 rpm for four minutes.

4. Leave 10 μL of water in the microfuge tube.

5. Add 100 μL of alkaline PEG reagent and mix well.

6. Leave at room temperature for 15 minutes.

7. Take 1.0 to 1.5 μL of the mixture and add into 20 μL of PCR reaction.

11.3.2 Protocol for single amplicon PCR

Targeted single amplicons can be generated and sequenced for the purposes of typing and taxonomic analysis (see Protocol 12.7.1 Building phylogenetic trees). A selection of commonly used primers for single amplicon generation is provided in Tables 11.4 and 11.5. Products used for phylogenetic analysis should be fully

sequenced on both DNA strands for the largest fragment possible. Cells can be prepared as described in Protocol 11.3.1A but if the desired amplicon cannot be generated, an alternative cell preparation method is provided in Protocol 11.3.1.B.

Table 11.4 Oligonucleotide primers used for PCR amplification of specific loci (chromosomal and pSYM localised) from rhizobia

Target gene	Primer Name	Primer sequence (5'-3')*	Reference
nodC	*nodC*-251F	AYGTHGTYGAYGACGGTTC	(Laguerre *et al*. 2001)
	nodCI-1160R	CGYGACAGCCANTCKCTATTG	(Laguerre *et al*. 2001)
nodA	*nodA*F	TGCRGTGGAARNTRNNCTGGGAAA	(Haukka *et al*. 1998)
	*nodA*R	GNCCGTCRTCRAAWGTCARGTA	(Haukka *et al*. 1998)
nifH	*nifH*1	AAGTGCGTGGAGTCCGGTGG	(Eardly *et al*. 1992)
	*nifH*2	GTTCGGCAAGCATCTGCTCG	(Eardly *et al*. 1992)
dnaK	DnaK1468F	AAG GAGCAGCAGATCCGCATCCA	(Stepkowski *et al*. 2003)
	DnaK1772R	GTACATGGCCTCGCCGAGCTTCA	(Stepkowski *et al*. 2003)
recA	*recA*F	ATCGAGCGGTCGTTCGGCAAGGG	(Gaunt *et al*. 2001)
	*recA*R	TTGCGCAGCGCCTGGCTCAT	(Gaunt *et al*. 2001)
16s rRNA[1]	16S rRNA Universal forward (fD1)	AGAGTTTGATCCTGGCTCAG	(Weisburg *et al*. 1991)
	16S rRNA Universal reverse (rP3)	ACGGATACCTTGTTACGACTT	(Weisburg *et al*. 1991)
	FGPS1490	TGCGGCTGGATCACCTCCTT	(Navarro *et al*. 1992)
	FGPS6	GGAGAGTTAGATCTTGGCTCAG	(Laguerre *et al*. 1996)
	FGPS132'	CCGGGTTTCCCCATTCGG	(Ponsonnet and Nesme 1994)
gyrB	gyrB343F	TTCGACCAGAAYTCCTAYAAGG	(Martens *et al*. 2008)
	gyrB1043	AGCTTGTCCTTSGTCTGCG	(Martens *et al*. 2008)
rpoB	rpoB83F	CCTSATCGAGGTTCACAGAAGGC	(Martens *et al*. 2008)
	rpoB1061R	AGCGTGTTGCGGATATAGGCG	(Martens *et al*. 2008)
atpD	*atpD*F	ATCGGCGAGCCGGTCGACGA	(Gaunt *et al*. 2001)
	*atpD*R	GCCGACACTTCCGAACCNGCCTG	(Gaunt *et al*. 2001)
glnA	*glnA532F* (GSI-1)	AAG GGC GGC TAY TTC CCG GT	(Turner and Young, 2000)
	*glnA*1124R (GSI-2)	GTC GAG ACC GGC CAT CAG CA	(Turner and Young, 2000)
dnaJ	*dnaJ*F	CAGATCGAGGTSACCTTCGAC	(Alexandre *et al*. 2008)
	*dnaJ*R	CGTCRYCATMGAGATCGGCAC	(Alexandre *et al*. 2008)

* Abbreviations: H = Adenine, Cytosine or Thymine; I = Inosine; N= Adenine, Cytosine, Guanine or Thymine; Y = Cytosine or Thymine.

[1] A single primer pair needs to be selected from the collection provided here

Table 11.5 Common reference genes selected for qRT-PCR amplification from prokaryotic RNA

Gene	Bact.*	Forward Primer (5'-3')	Reverse Primer (5'-3')	Reference
Recombinase A (*recA*)	*Pa*	GGTGAGCTGGTTGATCTGGG	GCATTCGCTTTACCCTGACC	Takle *et al*. (2007)
Malate dehydrogenase (mdh)	*Ec*	CTGCGTAACATCCAGGACACTAACG	CGACGGTTGGGGTATAAATAACAGG	Wang *et al*. (2009)
Malate dehydrogenase (mdh)	*Rlv*	GCGATGACCTTCTCGGCATCA	CATGGCGTCGAGCGGATTG	Karunakaran *et al*. (2009)
Sigma factor σ54, (*rpoN*)	*Pp*	GTTAAGGCTTTGCACCAG	GATTTCATCGACCTGCTC	Chang *et al*. (2009)
sigma factor σ70 (*rpoD*)	*Pp*	CGATGGAAATCACCAGAC	GCTGATCGACCTTGAGAC	Chang *et al*. (2009)
16S ribosomal RNA (16s rRNA)	*Pp*	CCGTGTCTCAGTTCCAGT	TGAGCCTAGGTCGGATTA	Chang *et al*. (2009)
Cation transporting ATPase (*atkA*)	*Pp*	GTAGTCGGCAAAGGTCTG	CAACTTCTGGGTCGACAT	Chang *et al*. (2009)

* Pa, *Pectobacterium atrosepticum*; Ec, *Escherichia coli*; Rlv, *Rhizobium leguminosarum* bv. *viciae*; Pp, *Pseudomonas putida*

The products of those reactions can be purified from the reaction components (*i.e.* salts, nucleotides and enzymes) or from agarose gel using either a commercially available silica-column based kit or a non-commercial method (Ausubel *et al*. 2013). A number of the latter methods have been used by the current authors, including isolation from low melting temperature agarose gels followed by phenol extraction or purification of DNA fragments using a Sephacryl S-300 column. However, commercially available kits are selected when rapid sample processing is required. The factors to consider before purchasing these kits include both the DNA yield/recovery, the size of the DNA fragment and the elution buffer volume and type (for example Tris-EDTA buffer may interfere with downstream reactions such as ligations). The authors frequently use both the Wizard® SV Gel and PCR Clean-Up System (Promega, USA) and the QIAquick Gel Extraction Kit (QIAGEN, Germany). If the final concentration of your purified DNA sample is low, then a simple ethanol-salt precipitation of that DNA followed by re-suspension in a smaller volume is an easy solution. Alternatively, the MinElute PCR purification kit (Qiagen) enables re-suspension of the purified DNA in a small volume.

11.3.3 Protocol for multi-amplicon profiling (fingerprinting)

Identification and phylogenetic analysis of rhizobia can be performed using PCR and gel electrophoresis techniques. Oligonucleotide primers (Table 11.6) targeting naturally occurring interspersed repetitive DNA elements enable amplification of multiple DNA fragments of different sizes simultaneously. The unique banding pattern (DNA fingerprint) is visualised using gel electrophoresis to type rhizobia. Primer pairs have been designed from the repetitive extragenic palindromic (REP), enterobacterial repetitive intergenic consensus (ERIC) (Versalovic

et al. 1991) and BOX sequences. The complete protocols for preparation of cell templates and repetitive-PCR (see Table 11.7 for PCR reaction conditions) are provided here, and have frequently been used by the authors for the genera *Bradyrhizobium*, *Mesorhizobium*, *Rhizobium* and *Sinorhizobium*. Information and protocols for additional repetitive-PCR types are available from http://www.msu.edu/~debruijn/ (Rademaker and De Bruijn 1997).

Table 11.6   Oligonucleotide primers used for PCR fingerprint profiling of rhizobia

Primer	Sequence (5'-3')*	Reference
ERIC 1R	ATGTAAGCTCCTGGGGATTCAC	Versalovic *et al.* (1991)
ERIC 2	AAGTAAGTGACTGGGGTGAGCG	Versalovic *et al.* (1991)
REP1R-I	IIIICGICGICATCIGGC	Versalovic *et al.* (1991)
REP2-I	ICGICTTATCIGGCCTAC	Versalovic *et al.* (1991)
BOXAIR	CTACGGCAAGGCGACGCTGACG	Versalovic *et al.* (1994)
RPO1	AATTTTCAAGCGTCGTGCCA	Richardson *et al.* (1995)
PucFor	GTAAAACGACGGCCAGT	Laguerre *et al.* (1996)

* Abbreviations: I = Inosine.

Table 11.7   PCR reactions for ERIC, BOX and rep

Reagent (stock concentration)	Volume (µL) for 1 reaction
Template cells (OD$_{600nm}$ 6.0)	1
Gitschier buffer (5×) (See Table 11.8)	5
Bovine Serum Albumin (BSA at 20 mg mL^{-1})	0.2
Dimethyl sulfoxide (100% DMSO)	2.5
Primer 1 (50 µM)	1
Primer 2 (50 µM)*	1*
dNTP mix (25 mM)	1.25
Taq Polymerase (5 U/µL)	0.4
DNase-free water	to 25

*   Not relevant for BOX, RPO1, pUCF as they are the only primer required.

NB: the authors use *Taq* Polymerase (Invitrogen).

Use DMSO at room temperature and mix stock if precipitates form.

Use filter-tips when preparing the PCR mixture and all stock solutions.

PCR Cycle

Temperature (°C)	Time	Cycles
95	7 minutes	1
94	1 minutes	
52*	1 minutes	35
65	8 minutes	
65	16 minutes	1

*   53°C for BOX, 40°C for REP

NB: store the reaction tubes at −20°C until ready to visualise by gel electrophoresis.

11.3.4 Gel electrophoresis

1. Prepare a 1.5% (w/v) agarose gel in TAE buffer (0.75 g of molecular-grade agarose in 50 mL of 1× TAE buffer).

    Melt mixture in a microwave or water bath until clear. Cool to 50ºC and add SYBR- safe DNA gel stain (Invitrogen; use at a final concentration of 1×) or Ethidium Bromide (final concentration of 0.5 μg mL⁻¹) and mix by swirling. Pour the gel into a gel tray, slot in the appropriate size comb and allow to set.

2. Mix loading buffer to 1× final concentration with PCR amplified sample.

3. Place the set gel inside the gel tank filled with 1× TAE buffer.

4. Load PCR-dye mix prepared in Step 2 into a well. Also load a commercial 1 kb DNA ladder with size ranges from 250 to 10,000 bp in a well.

5. Connect the electrodes to the power source and run the gel at 80 V (time is dependent on gel size but approximately three to six hours; reproducible results require this to be standardised between runs).

11.3.5 Analysis

Unique banding patterns (*e.g.* Figure 11.4) can be obtained by these repetitive-PCR methods to assist with rhizobia typing. Banding patterns are analysed by determination of amplicon size relative to a 1 kb ladder. The presence of a control (mother-culture-isolate) is necessary to validate your samples. Reproducibility may be enhanced by performing duplicate PCR reactions in independent cycling runs. The authors also use the *Phoretix*™ 1D Advanced software analysis package (Nonlinear Dynamics, UK) for analysis of banding patterns from uploaded gel images. Alternative software packages such as AMBIS and GelCompar are mentioned at http://www.msu.edu/~debruijn/ (Rademaker and De Bruijn 1997).
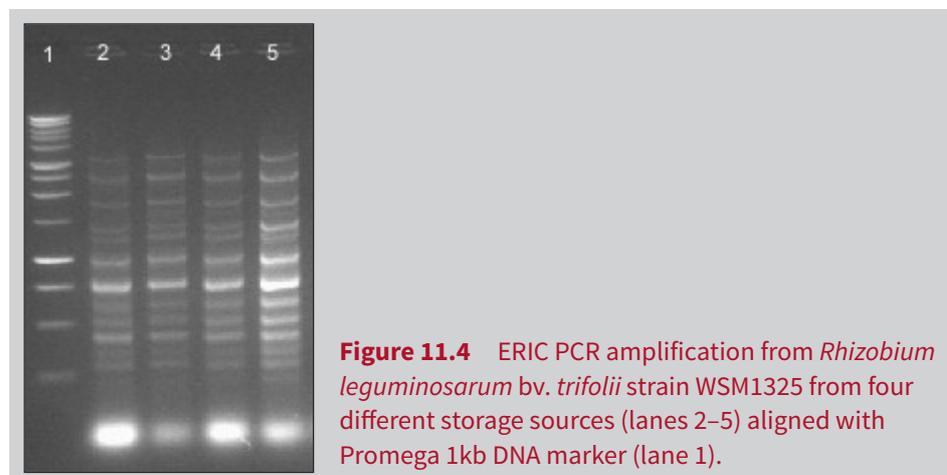
Figure 11.4   ERIC PCR amplification from *Rhizobium leguminosarum* bv. *trifolii* strain WSM1325 from four different storage sources (lanes 2–5) aligned with Promega 1kb DNA marker (lane 1).

Reagent stock preparation

Table 11.8 5× Gitschier Buffer

Reagent	Volume (mL)
Tris (1 M, pH 8.8)	6.7
$(NH_4)_2SO_4$ (1 M)	1.66
0.005 M EDTA (pH 8.8)	0.13
$MgCl_2$ (1 M)	0.67
2-Mercaptoethanol (14.4 M)	0.21
DNase-free water	10.63
Total Volume	20

NB: prepare 5× Gitschier buffer and store 1 mL aliquots at –20°C.

6× Loading Dye: 0.4% (w/v) bromophenol blue in 50% (v/v) glycerol.

TAE Buffer (50×): 242 g Tris base, 57.1 mL glacial acetic acid, 100 mL of 0.5 M EDTA (pH 8.0), distilled water to 1 L final volume.

11.4 References

Alexandre A., Laranjo M., Young J.P. and Oliveira S. 2008. *dnaJ* is a useful phylogenetic marker for alphaproteobacteria. International Journal of Systematic and Evolutionary Biology 58, 2839–2849.

Ausubel F.M., Brent R., Kingston R.E., Moore D.D., Seidman J.G., Smith J.A. and Struhl K. 2013. Current Protocols in Molecular Biology. Wiley and Sons.

Chang C., Damiani I., Puppo A. and Frendo P. 2009. Redox changes during the legume-*Rhizobium* symbiosis. Molecular Plant 2, 370–377.

Chomczynski P. and Rymaszewski M. 2006. Alkaline polyethylene glycol-based method for direct PCR from bacteria, eukaryotic tissue samples, and whole blood. BioTechniques 40, 454–458.

Eardly B.D., Young J.P. and Selander R.K. 1992. Phylogenetic position of *Rhizobium* sp. strain Or 191, a symbiont of both *Medicago sativa* and *Phaseolus vulgaris*, based on partial sequences of the 16S rRNA and *nifH* genes. Applied and Environmental Microbiology 58, 1809–1815.

Eckhardt T. 1978. A rapid method for the identification of plasmid deoxyribonucleic acid in bacteria. Plasmid 1, 584–588.

Gaunt M.W., Turner S.L., Rigottier-Gois L., Lloyd-Macgilp S.A. and Young J.P. 2001. Phylogenies of *atpD* and *recA* support the small subunit rRNA-based classification of rhizobia. International Journal of Systematic and Evolutionary Biology 51, 2037–2048.

Griffiths R.I., Whiteley A.S., O'Donnell A.G. and Bailey M.J. 2000. Rapid method for coextraction of DNA and RNA from natural environments for analysis of ribosomal DNA- and rRNA-based microbial community composition. Applied and Environmental Microbiology 66, 5488–5491.

Haukka K., Lindstrom K. and Young J.P. 1998. Three phylogenetic groups of *nodA* and *nifH* genes in *Sinorhizobium* and *Mesorhizobium* isolates from leguminous trees growing in Africa and Latin America. Applied and Environmental Microbiology 64, 419–426.

Hynes M.F. and McGregor N.F. 1990. Two plasmids other than the nodulation plasmid are necessary for formation of nitrogen-fixing nodules by *Rhizobium leguminosarum*. Molecular Microbiology 4, 567–574.

Hynes M.F. Simon R. and Pühler A. 1985. The development of plasmid-free strains of *Agrobacterium tumefaciens* by using incompatibility with a *Rhizobium meliloti* plasmid to eliminate pAtC58. Plasmid 13, 99–105.

Karunakaran R., Ramachandran V.K., Seaman J.C., East A.K., Mouhsine B., Mauchline T.H., Prell J., Skeffington A. and Poole P.S. 2009. Transcriptomic analysis of *Rhizobium leguminosarum* biovar

viciae in symbiosis with host plants *Pisum sativum* and *Vicia cracca*. Journal of Bacteriology 191, 4002–4014.

Laguerre G., Mavingui P., Allard M.R., Charnay M.P., Louvrier P., Mazurier S.I., Rigottier-Gois L. and Amarger N. 1996. Typing of rhizobia by PCR DNA fingerprinting and PCR-restriction fragment length polymorphism analysis of chromosomal and symbiotic gene regions: application to *Rhizobium leguminosarum* and its different biovars. Applied and Environmental Microbiology 62, 2029–2036.

Laguerre G. Nour S.M., Macheret V., Sanjuan J., Drouin P. and Amarger N. 2001. Classification of rhizobia based on *nodC* and *nifH* gene analysis reveal a close phylogenetic relationship among *Phaseolus vulgaris* symbionts. Microbiology 147, 981–993.

Martens M., Dawyndt P., Coopman R., Gillis M., De Vos P. and Willems A. 2008. Advantages of multilocus sequence analysis for taxonomic studies: a case study using 10 housekeeping genes in the genus *Ensifer* (including former *Sinorhizobium*). International Journal of Systematic and Evolutionary Biology 58, 200–214.

Navarro E., Simonet P., Normand P. and Bardin R. 1992. Characterization of natural populations of *Nitrobacter* spp. using PCR/RFLP analysis of the ribosomal intergenic spacer. Archives of Microbiology 157, 107–115.

Ponsonnet C., Nesme X. 1994. Identification of *Agrobacterium* strains by PCR-RFLP analysis of pTi and chromosomal regions. Archives of Microbiology 161, 300–309.

Priefer U.B. 1984. *Isolation of Plasmid DNA* Springer-Verlag: Berlin.

Rademaker J.L.W., andDe Bruijn F.J. 1997. Characterization and classification of microbes by REP-PCR genomic fingerprinting and computer-assisted pattern analysis. Pp 151–171 in 'DNA Markers: Protocols, applications and overviews', ed. by G. Caetano-Anolles and P.M. Gresshoff. John Wiley: New York.

Richardson A.E., Viccars L.A., Watson J.M. and Gibson A.H. 1995. Differentiation of *Rhizobium* strains using the polymerase chain reaction with random and directed primers. Soil Biology and Biochemistry 27, 515–524.

Schneider M. and deBruijn F.J. 1996. Rep-PCR mediated genomic fingerprinting of rhizobia and computer-assisted phylogenetic pattern analysis. World Journal of Microbiology and Biotechnology 12, 163–174.

Stepkowski T., Czaplinska M., Miedzinska K. and Moulin L. 2003. The variable part of the *dnaK* gene as an alternative marker for phylogenetic studies of rhizobia and related alpha Proteobacteria. Systematic and Applied Biology 26, 483–494.

Takle G.W., Toth I.K. and Brurberg M.B. 2007. Evaluation of reference genes for real-time RT-PCR expression studies in the plant pathogen *Pectobacterium atrosepticum*. BMC Plant Biology 7, 50.

Turner S.L. and Young J.P. 2000. The glutamine synthetases of rhizobia: phylogenetics and evolutionary implications. Molecular Biology and Evolution 17, 309–319.

Versalovic J., Koeuth T., Lupski J.R. 1991. Distribution of repetitive DNA sequences in eubacteria and application to fingerprinting of bacterial genomes. Nucleic Acids Research 19, 6823–6831.

Versalovic J., Schneider M., de Bruijn F.J. and Lupski J.R. 1994. Genomic fingerprinting of bacteria using repetitive sequence based PCR (rep-PCR). Methods Molecular and Cell Biology 5, 25–40.

Wang M., Jacoby G.A., Mills D.M. and Hooper D.C. 2009. SOS regulation of *qnrB* expression. Antimicrobial Agents and Chemotherapy 53, 821–823.

Weisburg W.G., Barns S.M., Pelletier D.A. and Lane D.J. 1991. 16S ribosomal DNA amplification for phylogenetic study. Journal of Bacteriology 173, 697–703.

Specialised genetic techniques for rhizobia

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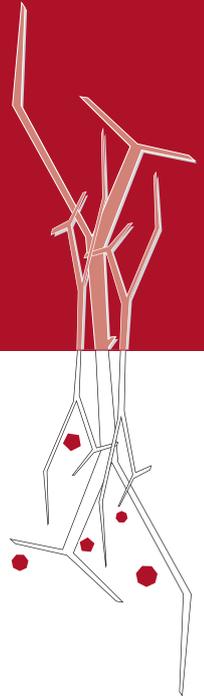
12.1 Introduction

The aim of this chapter is to provide a selection of specialised genetic techniques which are widely used to interrogate gene functions in root nodule bacteria (RNB). The reader is referred to reviews of the applicability of these techniques to understand biological processes occurring in RNB (*i.e.* Long 1989; Stanley and Cervantes 1991). The techniques presented here cover the ability to randomly mutate genes, select mutants of interest based on their phenotype and identify the gene affected, verify that the gene mutation caused the observed phenotypic defect, examine expression of a target gene, perform genome structural and functional studies and conduct comparative analyses with other RNB.

12.2 Transposon mutagenesis

Mutations can be introduced into RNB by the use of chemical, physical and biological mutagens. Transposon mutagenesis is the most widely used technique for introducing random mutations in RNB. Alternative methods for targeting specific genes have been described elsewhere (Kokotek and Lotz 1991; Quandt and Hynes 1993; Selbitschka *et al.* 1993). Random mutations can be introduced by transfer of a mobilisable, suicidal plasmid carrying a trans-positionally active mobile element (transposon, mini-transposon or plasposon). A plasmid carrying the mobile element is conjugally transferred from an RP4 integrant of *Escherichia coli*, such as BW20767 (Metcalf *et al.* 1996) or S17.1 (Simon 1984). Alternatively, transfer can be mediated by a helper *E. coli* strain, such as MT616 (Finan *et al.* 1986) or HB101 (pRK2013) (Figurski and Helinski 1979) to provide RP4 transfer functions

to a non-RP4 integrant. A range of transposons, or derivatives, are available and include: Tn*5* and antibiotic-resistant derivatives (De Vos *et al.* 1986; Quandt *et al.* 2004; Selvaraj and Iyer, 1983; Simon 1984); mTn*5* constructs (de Lorenzo *et al.* 1990; Herrero *et al.* 1990; Pobigaylo *et al.* 2006; Reeve *et al.* 1999; Wilson *et al.* 1995); mTn*10* constructs (Herrero *et al.* 1990); and plasposons (Dennis and Zylstra, 1998). An appropriate choice of mutagen will be dependent on the antibiotic sensitivity of the rhizobial strain. The presence of a promoterless reporter in the mutagen enables gene expression to be monitored at the site of insertion, while a promoter driven reporter in a mutagen can permit cell localisation, which is particularly useful for nodule occupancy or infection studies. Reporters that have been used include: *cat* (Kim *et al.* 1986); *celB* (Sessitsch *et al.* 1996); *gusA* (Reeve *et al.* 1999); *lacZ* (de Lorenzo *et al.* 1990; Herrero *et al.* 1990); *gfp* (Xi *et al.* 1999); and derivatives, *lux* (Legocki *et al.* 1986), *nptII* (Simon *et al.* 1989)and *phoA* (de Lorenzo *et al.* 1990; Muller 2004).

Many of the constructs in current use are based on the pUT plasmid delivery system. This plasmid is stable only in hosts synthesising PIR proteins, such as *E. coli* strain BW20767 (chromosomal *gusA:pir*), and not in root nodule bacteria devoid of *pir*. In this plasmid delivery system, the *tnpA* gene is located on the plasmid adjacent to the minitransposon effectively 'disarming' the transposon and preventing further transposition. To illustrate this process (Figure 12.1), an approach is provided to mutagenise *Sinorhizobium medicae* WSM419 with mTn*5*-GNm. The mini-transposon mTn*5*-GNm contains a kanamycin resistance (*nptII*) and a promoterless *gusA* gene (Reeve *et al.* 1999). This strain is naturally resistant to chloramphenicol.

When the suicide plasmid is transferred conjugally from the donor to recipient *S. medicae*, the plasmid pCRS487 cannot replicate in the new background and kanamycin-resistant transconjugants arise from the transposition of the minitransposon into the rhizobial genome. Insertion of mTn*5* will inactivate the target gene, and if in the correct orientation, the *gusA* gene will be expressed under the inactivated gene's promoter. GUS expression can be monitored as described in Protocol 12.5.

Transconjugants are selected on a medium containing kanamycin and chloramphenicol, preventing growth of the donor *E. coli* and recipient parent strain *S. medicae* WSM419. Kanamycin-resistant *Sinorhizobium* transconjugants are insertion mutants. The phenotype of the mutated cells can then be examined by replica plating colonies onto particular types of selective media.
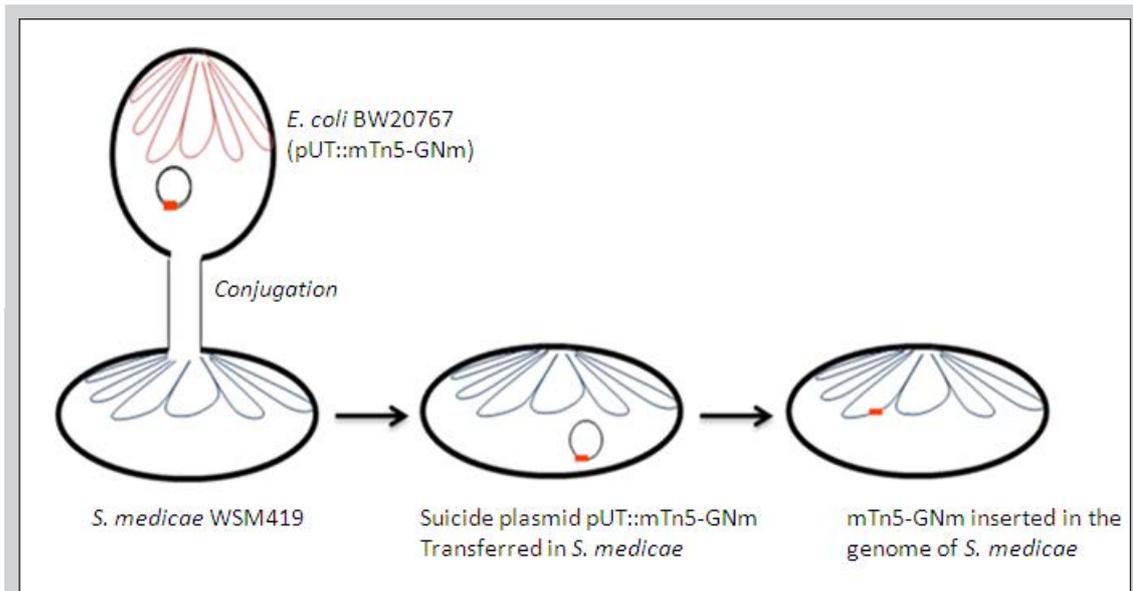
Figure 12.1   A schematic for minitransposon mutagenesis of *S. medicae*

12.2.1 Frequency of isolation of mutants

The genome of *S. medicae* WSM419 contains 6,518 protein coding genes, and if a single gene is involved in the expression of a characteristic, one could expect, on average, to have to screen 6,518 kanamycin-resistant colonies in order to be sure of isolating a mutation in a particular gene. In reality, this number can be higher due to the presence of hotspots for insertion.

12.2.2 Protocol for transposon mutagenesis

The overall strategy for isolating minitransposon-induced mutants involves:

1. transfer of mTn*5* into rhizobial cells by conjugation

2. selection of rhizobial transconjugants containing mTn*5* insertions

3. selection of the desired mutant displaying the expected phenotype.

Strains

▶ *E. coli* BW20767 (pCRS487) Ap^R, Km^R.

▶ *S. medicae* WSM419 Cm^R, Nx^R.

Recipe

TY broth:

▶ Tryptone 5 g

▶ Yeast extract 3 g

▶ $CaCl_2.2H_2O$ 0.75 g (reduced from original recipe to avoid precipitation)

▶ Distilled H_2O to 1 L

▶ Adjust pH to neutral

▶ For plates: add 1.5 % (w/v) agar and autoclave.

Procedure

Day 1

1. Inoculate one loopful of the *Sinorhizobium* culture (chloramphenicol resistant) into 5 mL TY broth containing chloramphenicol (20 µg mL^{-1}). Place the inoculated broth on a gyratory shaker set to 200 rpm at 28ºC.

Day 3

2. Inoculate *E. coli* culture BW20767 (pUT::mTn5-GNm) into 5 mL LB broth (containing ampicillin 100 µg mL^{-1} and kanamycin 100 µg mL^{-1}) and incubate on a gyratory shaker set to 200 rpm at 37°C.

Day 4

3. Subculture *E. coli* 1:50 into LB broth containing the same antibiotics and place the culture on a 37ºC shaker. Allow it to grow for three to four hours.

4. Centrifuge 5 mL of the 4 d *Sinorhizobium* culture and the subcultured *E. coli* culture. Discard the supernatant and re-suspend pellets in 250 µL of TY broth.

5. Spot 50 µL of each parent separately onto TY plates to act later as controls. Combine *E. coli* and *Sinorhizobium* cultures, mix and spot four aliquots of 100 µL onto TY plates, divided into quadrants. Leave until dry and incubate the TY plates at 28ºC.

Day 5

6. Re-suspend each spot (mating mixture) into 1 mL sterile saline containing 15% (v/v) glycerol.

7. Prepare 10^{-1} to 10^{-8} dilutions of a mating mixture and spread 100 µL from neat to 10^{-3} dilutions onto TY plates containing chloramphenicol and kanamycin, and 100 µL from 10^{-5} to 10^{-8} on TY plates containing just chloramphenicol. In

addition, spread neat to 10^{-3} dilutions of each control onto TY plates containing chloramphenicol and kanamycin. Incubate the plates at 28ºC.

Day 8

8. Perform counts on plates containing between 30 and 300 colonies. These data can be used to calculate the efficiency of transposition but are only necessary if the transposition frequency in your strain needs to be determined.

9. From the counts obtained, spread the appropriate volumes to obtain the desired number of mutants that need to be screened, and incubate for three to four days at 28°C.

Days 11 to 13

10. Replica plate kanamycin-resistant *Sinorhizobium* transconjugants onto selective media. For example, mutations in genes expressed in acidic conditions can be identified by replica patching mutants on minimal media containing X-Glc (a substrate for β-glucuronidase) which has been buffered to pH 7 and 5.7. The random nature of transposition can be verified by analysing auxotrophic phenotypes in your strain (Selvaraj and Iyer 1983).

Day 14

Observe plates for growth and also for colour development. Plates can be stored at 4°C until required. The plates will enable you to select the following.

a. Mutants of *Sinorhizobium* lacking one of the proteins required to grow at low pH. These mutants will grow on the pH 7 plate but will not grow on the low pH plate.

b. Reporter gene fusions with low pH up-regulated or low pH down-regulated genes. Transcription of the target gene will control the expression of the inserted promoterless *gusA* gene. Expression of the *gusA* gene will produce β-glucuronidase enzyme that converts X-Glc to the blue-coloured product. Thus, mutants containing a reporter gene fusion with a low pH up-regulated gene will produce colonies with deeper blue colour at pH 5.7 than at pH 7.0. Reporter gene fusions in a low pH down-regulated gene will show the reverse pattern of colour on low and neutral pH plates.

12.2.3 Identification of the mutated gene

Once mutants are isolated by phenotype testing, the affected gene in the mutant can be identified by DNA cloning and sequencing. The presence of an antibiotic marker in the biological mutagen facilitates cloning of the transposon and associated rhizobial flanking sequence. Genomic DNA can be isolated from the mutant, as described in Protocol 11.1 (Chapter 11). The selection of a restriction enzyme that does not cut inside the mutagen is particularly useful for this purpose.

Restriction enzyme-cut genomic DNA can be ligated with a suitable vector (such as pUC18) and transformants selected on growth media supplemented with antibiotics that select for the cloning vector and the mutagen. For example, the enzyme *Eco*RI (or *Hin*dIII) can be used to cleave out mTn5-GNm and associated rhizobial DNA from mutant DNA; it can then be ligated into *Eco*RI (or *Hin*dIII)-cut pUC18 DNA. In this case, transformants can be selected on LB plates supplemented with ampicillin and kanamycin.

The plasmid DNA of desired transformants can be purified by conventional methods and the DNA flanking the transposon sequenced using primers designed at the ends of the transposon. For example, the primers WIL3 and TAC-105F can be used to sequence out from the ends of mTn5-GNm (Reeve *et al.* 1999). The gene affected can be identified by searching DNA databases (Protocol 12.7) with the sequence generated.

12.3 Protocol for transduction

Generalised transduction is one method that can be used to determine whether a specific tagged mutation (*i.e.* antibiotic resistance marker on a transposon) is the cause of a noted phenotype. The process requires the presence of a suitable generalised transducing phage for the RNB host since the process is very specific. Prior to the commencement of the transduction protocol, the phage is passaged twice through the wild-type host followed by the preparation of a mutant lysate at high titre. The mutant lysate is irradiated and used to infect the wild-type to reintroduce the mutation. The phenotype of transduced cells can then be compared to the expected phenotype for the mutation of interest.

An example of transduction is provided using the generalised transducing phage RL38 on the host *Rhizobium leguminosarum* bv. *viciae*. A host-specific transducing phage is required for these procedures to be useful.

12.3.1 Preparation of the wild-type phage lysate

1. Grow culture of wild-type RNB strain overnight in 5 mL TY broth (see recipe in Section 12.2.2).

2. Subculture 0.5 mL into 5 mL and grow until $OD_{600nm} = 0.3$.

    This gives approximately 3×10^8 cells mL^{-1}.

3. Infect bacteria with RL38 phage at a ratio of 0.5:1 (phage:cell).

    While this ratio is ideal, it can be less. The method works successfully if the ratio drops to 1:10 phage:cells.

4. Incubate overnight at 28°C with shaking.

5. Spin cells for 15 minutes at full speed in a microcentrifuge and take supernatant into fresh sterile glass tubes. Add a drop of chloroform, mix and store at 4°C.

   THIS IS THE PRIMARY LYSATE

6. Repeat the procedure (Steps 1 to 5) by infecting wild-type cells with the primary lysate generated in Step 5. The titre can be established as detailed below.

   This step overcomes the restriction mechanism of the host bacteria and produces a secondary lysate.

Determine the phage titre to calculate yields and volumes to be used for infection cells. Yields vary from between about 10^4 to 10^8 pfu mL^{-1}.

12.3.2 Improving phage titre

To obtain higher yields of phage, the lysates can be collected from agar plates using the following procedure.

1. Mix the lysate and bacteria with 5 mL 42°C top agar (0.7% (w/v) TY agar) in Falcon 2057 tubes and pour onto pre-warmed TY plates.

2. Incubate at 28°C for three to four days.

3. Elute phage with 3 mL TY broth (see recipe in Section12.2.2); leave for two hours.

4. Remove elution buffer to fresh sterile glass tubes, add a drop of chloroform and store at 4°C.

12.3.3 Preparation of the mutant phage lysate

1. Grow overnight culture of mutant strain at 28°C in 5 mL TY broth.

2. Subculture 0.5 mL into 5 mL and grow until OD$_{600nm}$ = 0.3.

   This gives approximately 3×10^8 cells mL^{-1}.

3. Infect bacteria with RL38 phage at a ratio of 0.5:1 (phage:cell).

   This is an ideal ratio but it can also be less; the method works successfully if the ratio drops to 1:10 phage:cells

4. Incubate overnight at 28°C with shaking.

5. Spin cells for 15 minutes at full speed in a microcentrifuge and remove the supernatant to fresh sterile glass tubes. Add a drop of chloroform and store at 4°C.

   There is no need to obtain a secondary lysate from mutant stock.

Determine the phage titre to calculate yields. Yields needs to be at least 10^8 plaque-forming units per mL (pfu mL^{-1}).

To improve yields of phage, the mutant lysate can be collected from agar plates using the procedure 'Improving phage titre' (Section 12.3.2).

12.3.4 Calculation of phage titre

1. Add 100 μL of neat and various dilutions (10^{-2} to 10^{-8}) of lysate to Falcon 2057 tubes containing 100 μL of log-phase wild-type bacteria (OD$_{600nm}$=0.3).

2. Incubate at 28°C for between 15 minutes and three hours.

3. Add 5 mL 42°C top agar (0.7% (w/v) TY agar) and pour onto pre-warmed TY plates.

4. Incubate at 28°C for three to four days.

5. Select plates with between 50 to 300 plaques and count. Back calculate titre using the following formula.

   Number of plaques × dilution factor × 10 (converts to mL) = number of pfu mL^{-1} of lysate

   E.g. If 0.1 mL of 10^{-4} dilution gives 20 plaques then titre is:

   $20 \times 10^4 \times 10 = 2 \times 10^6$ pfu mL^{-1}

12.3.5 Phage irradiation

1. Add phage lysate to a Petri plate placed on a shaking platform. A good volume is 5 mL. Set the shaker to gentle shaking. UV irradiate the suspension with the Petri lid off until the titre has dropped 1000-fold. Establish titre using the method 'Calculation of phage titre'. (Section 12.3.4).

   Optimal irradiation is 2 mJ/m^2/s.

12.3.6 Transduction

1. Grow overnight culture of wild-type bacteria in 5 mL TY broth.

2. Subculture 0.5 mL into 5 mL and grow until OD$_{600nm}$=0.3 (approximately 3×10^8 cells mL^{-1}).

3. Infect 0.1 mL bacteria with 1, 10 and 100 μL irradiated RL38 mutant phage lysate (see above for irradiation protocol).

   Washing 0.1 mL of bacterial cells in 0.9% (w/v) saline and then re-suspending in 0.1 mL of TY prior to infection may aid in optimising the infection cycle for highly mucoid strains. However, problems with infection have also been encountered after performing this step.

4. Incubate at 28°C for between 15 minutes and three hours.

5. Spin mixture, wash in 0.9% (w/v) saline and re-suspend in 0.23 mL of 0.9% (w/v) saline.

   This step removes phage that have not attached and injected their DNA into the bacteria. It also removes calcium, preventing any further infection. Selection is for infected cells, preferably those infected with non-lysing phage damaged by irradiation.

6. Spread 1, 10 and 100 μL aliquots of the mixture onto minimal media plates containing no more than the absolute required amount of calcium for cell growth.

   E.g. 0.25 mM $CaCl_2$ in JMM minimal medium works well. Add the appropriate selective agent for the marker to be transduced. For example, if checking for Tn5 transduction, add kanamycin (an appropriate level for RNB would be 100 μg mL^{-1}).

7. Check kanamycin-resistant colonies on selective media for co-transduction of the mutant phenotype. Ensure the cells grow at the expected rate; occasionally abortive transduction may yield slow-growing colonies. Perform Southern hybridisation or PCR to confirm position of mutation.

12.4 Protocol for conjugation

Conjugation, also known as mating, is used by the authors to transfer plasmids from *E. coli* into rhizobial strains. A variety of plasmids can be mobilised into rhizobia; a list of commonly used vectors is provided in Table 12.1. Biparental mating is the transfer of DNA from one *E. coli* parent to one rhizobial strain, relying upon the availability of a *mob* (mobilisable) gene present on the plasmid of transfer. The authors recommend the use of the *E. coli* donor BW20767 for conjugative transfer of R6K *oriV* and RP4 *oriT* plasmids (Metcalf *et al*. 1996). Triparental mating involves the transfer of DNA from one *E. coli* parent to one rhizobial strain using an additional *E. coli* strain containing a helper plasmid, such as pRK2013 which has a ColE1 replicon (Figurski *et al*. 1979). Triparental mating would be used in the circumstance where the donor *E. coli* does not carry the *tra* genes conferring transfer of the plasmid. Either of the two methods can be used to mobilise a plasmid into a rhizobial strain for the purpose of complementation (restoration of a wild-type phenotype), gene expression studies and mutant construction. Conjugation can also be used to mobilise a plasmid from a rhizobial strain back into *E. coli* using a triparental mating involving a helper *E. coli* strain. Two methods for mating are described (filter and plate methods) and preference for their use is at the discretion of the researcher.

Table 12.1   Common mobilisable plasmids and their useful features

Plasmid	Replicon type	Antibiotic marker[1]	Application	Features[2,4]	Reference
pBBR series	pBBR1	Varies (Cm or Km or Tc or Ap or Gm)	Complementation	BHR, blue-white selection	(Kovach *et al*. 1995; Kovach *et al*. 1994)
pJP2series [3]	IncP	Tc	Expression and cloning	BHR, autofluorescent genes and some carry *gusA*	(Karunakaran *et al*. 2005)
pOT series [3]	pBBR1	Gm	Expression and cloning	BHR, autofluorescent genes and some carry *lacZ*	(Karunakaran *et al*. 2005)
pFUS1	IncP	Tc	Expression	Promoterless *gusA*, BHR	(Reeve *et al*. 1999)
pFUS1P	IncP	Tc	Expression	Promoterless *gusA*, BHR, includes *par* stability loci	(Yost *et al*. 2004)
pMP220	IncP	Tc	Expression	Promoterless *lacZ*, BHR	(Spaink *et al*. 1987)
pWR220	IncP	Tc	Complementation	BHR	(Reeve *et al*. 1999)
pUFR009	IncQ	Km	Complementation	BHR	(DeFeyter *et al*. 1990)
pUFR042	IncW	Gm, Nm	Cloning and Complementation	BHR, blue white screening, phase packaging site (cos)	(Defeyter and Gabriel 1991)
pUFR047	IncW	Ap, Gm	Cloning and complementation	BHR, blue white screening, *lacZ*	(De Feyter *et al*. 1993)
pSUP series	ColE1 or p15A	Varied	Homologous recombination or transposon mutagenesis	NHR and varied features dependent on construct	(Simon *et al*. 1986)
pWS233	p15A	Gm, Tc	Homologous recombination	NHR, sucrose sensitive (*sacB*), mobilisable	(Selbitschka *et al*. 1993)
pJQ200	P15A	Gm	Homologous recombination	NHR, sucrose sensitivity (*sacB*), blue-white selection	(Quandt and Hynes 1993)
pUT series	R6K	Ap	Transposon mutagenesis	NHR in *pir*-strain	(de Lorenzo *et al*. 1990); (Herrero *et al*. 1990); (Reeve *et al*. 1999)

[1]   Ap: ampicillin; Cm: chloramphenicol; Gm: gentamycin; Km: kanamycin; Tc: tetracycline.
[2]   BHR: broad-host range; NHR: narrow host-range (suicidal vectors are unable to replicate in rhizobia).
[3]   Deposited in addgene (www.addgene.com)
[4]   Reporter fusions can be assayed in multiple copy (BHR plasmid-borne) in different genetic backgrounds or in single copy as integrants (NHR delivered).

12.4.1 General preparation

▶ This protocol relies on the use of the recipient rhizobial strain at stationary phase and the donor *E. coli* strain at log phase.

  ▷ rhizobium at stationary phase.

  ▷ *E. coli* at log phase after subculture from overnight growth.

  ▷ Pre-warm 0.89% (w/v) saline (sterile) and TY plates (see recipe in Protocol 12.2.2) to 28°C for optimum efficiency.

▶ Day 1: inoculate 10 mL TY culture with rhizobium (recipient) and grow at the appropriate temperature until it reaches saturation (*e.g.* three-day growth of *Sinorhizobium medicae* WSM419).

▶ Day 3: inoculate 10 mL LB culture with *E. coli* (donor carrying plasmid of interest) maintaining selection for the plasmid with the appropriate antibiotics.

▶ Day 4: subculture *E. coli* (1 in 50 dilution) and grow (approximately three hours) without antibiotics.

▶ Day 4: prepare TY plates.

▶ Recommended ratio for biparental matings:

  ▷ log-phase *E. coli* RP4 integrant (donor) and stationary-phase rhizobium at 1:1 ratio.

▶ Recommended ratios for triparental matings:

  ▷ log-phase *E. coli* (donor):log-phase *E. coli* (helper plasmid):stationary-phase rhizobium (recipient) 1:1:1 ratio. Alternatively, stationary-phase *E. coli* can be mixed with cultures of rhizobium at a ratio of 1:5.

12.4.2 Procedure

Protocol 1: Plate mating

1. Separately centrifuge 5 mL of each culture at maximum speed for one minute and re-suspend pellet in 200 μL of 0.89% (w/v) saline (1:25).

2. Combine the donor and recipient (and helper) cultures together and tap gently to mix.

3. Dot 100 μL amounts (mating spots) of the prepared mixed culture onto a pre-warmed TY plate (no antibiotics) and incubate overnight at 28°C.

4. *NB:* additional mating spots may be made on the same plate well separated from each other.

Protocol 2: Filter mating

1. Pass 1 mL *E. coli* culture through a 0.45 μm filter then pass 1 mL rhizobium culture through (this washes away any residual antibiotics).

2. Place filter cell-side up on pre-warmed TY plate, incubate overnight at 28°C.

Protocol continued for Protocols 1 or 2

3. Re-suspend one mating in saline containing glycerol (850 μL 0.89% (w/v) saline with 150 μL of 80% (v/v) glycerol).

    Cells from Protocol 1 can be scraped off a plate while the entire filter from Protocol 2 can be immersed in the re-suspension tube.

4. At this stage, the mixture can be stored at 4°C or –80°C.

5. Plate mating mixture (100, 10 and 1 μL aliquots) onto desired selective media using marker on host and selecting for plasmid or transposon.

6. Plate controls:

    i.   rhizobium on selective media

    ii. rhizobium dilutions on TY to determine the rate of conjugation

    iii. *E. coli* on selective media.

12.5 Reporter assays

The measurement of reporter gene expression enables expression of native RNB genes to be determined quantitatively or qualitatively under the conditions assayed. Reporters can be mobilised by conjugation (see Protocol 12.4) into rhizobia on various vector backbones (see Table 12.1). There are many protocols to reveal the level of expression of a reporter and this will ultimately depend on the type of reporter gene chosen and the relative ability to permeabilise the cells (to ensure the rapid onset of enzyme activity in the presence of the appropriate substrate). This protocol has been widely used to determine *gusA*, *phoA* or *lacZ* expression. Section 12.5.1 describes three different methods for cell permeabilisation/lysis. The most appropriate method to be used will be determined by the degree of permeabilisation/lysis, which is dependent on the cell type. The authors suggest the use of Method 1 (toluene method) in the first instance. This method is highly reproducible and results in efficient permeabilisation of a wide range of rhizobial strains. However, it suffers from the following disadvantages: (i) toluene is toxic; (ii) the process speed is reduced by the need for vigorous vortexing of samples after toluene addition; and (iii) an additional 30 minutes incubation is required to remove toluene. An alternative is to use either Method 2 or Method 3 listed in Section 12.5.1. These latter two methods are amenable to high throughput but may not lyse/permeabilise all cell types efficiently. The method should be

optimised for each cell type to ensure that there is no significant lag in enzyme activity resulting from incompletely permeabilised/lysed cells. A procedure is provided for both low (cuvette) and high throughput (microtitre plate).

12.5.1 Quantitative method

Growing the cells

1. Prepare a starter culture by growing cells in 5 mL media supplemented with appropriate antibiotics.

2. Add culture to final choice of media (containing antibiotic if required; do not add tetracycline at low pH) so that after 16 to 20 hours, growth OD_{595nm} reaches 0.5 to 0.8 (growth phase of the cells is critical for reproducible results).

3. Harvest cells the next day by centrifugation and adjust OD_{595nm} of cell suspension to 2.0 using normal saline (0.89% w/v NaCl) for Method 1 and 2 OR 100 mM Tris (pH 8.0) for Method 3.

12.5.1.1 Cell permeabilisation

Reporter enzyme buffers

▶ **GusA assay buffer:** 50 mM Na phosphate buffer (60 mM Na_2HPO_4 + 40 mM NaH_2PO_4; pH 7.0); 50 mM dithiothreitol; 1 mM EDTA.

▶ **PhoA assay buffer:** 100 mM Tris pH 8.0 (pH adjusted using HCl).

▶ **LacZ assay buffer:** 50 mM Na phosphate buffer (60 mM Na_2HPO_4 + 40 mM NaH_2PO_4; pH 7.0), 10 mM KCl and 1 mM $MgSO_4$, 50 mM β-mercaptoethanol (2.7 mL L^{-1}).

Method 1 (toluene method)

1. Dispense 200 μL of each re-suspended culture at $OD_{595nm}= 2$ into a microcentrifuge tube.

   Perform this step in triplicate. The volume used will depend on the activity of the fusion and may have to be determined empirically.

2. Add 800 μL of the appropriate reporter enzyme buffer to each microcentrifuge tube.

3. Add two drops toluene (addition of two drops has no effect on GUS activity) to each tube and **VORTEX vigorously** for a standardised period of time (*i.e.* ten seconds/sample).

   This is the critical step to ensure that the rate is linear from time zero. Shaking samples on a microtitre plate shaker is not vigorous enough.

4. Place microfuge tubes at 37°C for 30 minutes with the lids open.

   Evaporates **toxic** toluene. Perform in a fume hood.

Method 2 (lysozyme and SDS method)

1. Dispense 200 μL of each re-suspended culture at OD_{595nm}=2 into a microcentrifuge tube.

   Perform this step in triplicate. The volume used will depend on the activity of the fusion and may have to be determined empirically.

2. Add 690 μL of the appropriate reporter buffer to each microfuge tube.

3. To each tube, add 100 μL of lysozyme (5 mg mL^{-1} in 10 mM phosphate buffer at pH 7.8) giving a final concentration of 0.5 mg mL^{-1}. Invert several times and leave for five minutes at room temperature. Add 15 μL of 0.05M EDTA, pH 8.0. Invert several times and leave for 15 minutes.

4. Add 10 μL of 1% (w/v) SDS (*i.e.* final concentration 0.01% (w/v)). Invert several times.

5. Equilibrate samples at 30ºC for at least five minutes.

Method 3 (chloroform and SDS method)

1. Dispense 200 μL of each re-suspended culture at OD_{595nm}= 2 into a microcentrifuge tube.

   Perform this step in triplicate. The volume used will depend on the activity of the fusion and may have to be determined empirically.

2. Add 800 μL of the appropriate reporter enzyme buffer to each microfuge tube.

3. Add 20 μL of chloroform and 10 μL of 10% (w/v) SDS and vortex.

12.5.1.2 Substrate addition to start the reaction

Reagents required:

▶ GusA substrate: *p*-Nitrophenyl β-D-glucuronide (PNPG) 35 mg mL^{-1} prepared in sterile RODI water

▶ LacZ substrate: *o*-Nitrophenyl β-D-galactoside (ONPG) 35 mg mL^{-1} prepared in sterile RODI water

▶ PhoA substrate: *p*-Nitrophenylphosphate (PNPP) 35 mg mL^{-1} prepared in 100 mM Tris (pH 8.0).

Assay method in microtitre plates

1. Equilibrate samples at assay temperature (28°C or 37°C) for five minutes.

   Both temperatures have been used in the literature. The enzymes: *PhoA*, *GusA* and *LacZ* from transcriptional fusions are stable at 37°C. However, translational fusions to rhizobial proteins may not necessarily be stable at 37°C.

2. Transfer 200 μL from each microfuge tube into a well of a microtitre plate.

   If possible, consider placing the samples in order so that cultures expressing the least GUS are positioned in the beginning on plate.

3. Add 5 μL substrate using a multichannel pipette.

4. Position plate in the microplate reader and start reading at 405 nm. Read samples every two minutes with prior shaking over a total of 1.5 hours.

Assay method in cuvettes

1. Equilibrate samples at assay temperature (28 °C or 37°C) for five minutes.

   Both temperatures have been used in the literature. The enzymes: alkaline phosphatase, β- glucuronidase and β-glactosidase from transcriptional fusions are stable at 37°C. However, translational fusions to rhizobial proteins may not necessarily be stable at 37°C.

2. Add 10 μL of substrate (stagger time of addition for each sample) and monitor colour development.

3. When the samples begin to turn yellow, transfer 200 μL of the reaction mixture to a new tube and stop the reaction by adding 800 μL of 125 mM K_2HPO_4 for *PhoA* assays or 800 μL of 0.4 M Na_2CO_3 for *lacZ* and *gusA* assays. Record the assay time for each sample.

Measurement of assay culture OD

1. Add 40 μL of normalised culture (OD_{595nm} =2.0, cells from 12.5.1. section 3) to 160 μL saline into the well of a microtitre plate. Add 200 μL saline into 2 wells as a control.

   Perform samples at least in triplicate.

2. Position plate in reader and start OD protocol using wavelength set to 595 nm.

   A 600 nm filter could be used as a substitute.

3. Finally, subtract OD_{595nm} reading of saline from OD_{595nm} readings of cultures determined in the microtitre plate reader. Multiply these readings by five to provide the cell OD_{595nm} reading.

NB: OD_{595nm} of cells (from Section 12.5.1, Step 3) can be measured in cuvettes using standard spectrophotometric technique.

Calculating PNP or ONP molar extinction coefficient (E_M)

Generate a standard curve by making two-fold serial dilutions from a stock of 1.0 mM *p*-nitrophenol or *o*-nitrophenol prepared in reporter assay buffer. For the microtitre method, add 205 μL of each dilution to microtitre wells and read absorbances. For the cuvette method, add 200 μL of each dilution and 800 μL of 125 mM K_2HPO_4 for PhoA assay or 800 μL of 0.40 M Na_2CO_3 for LacZ or GusA assays. Do a linear regression of OD_{405nm} versus concentration (mol L^{-1}). The slope of the line is the molar extinction coefficient. Absorbance is linear with concentration up to an OD_{405nm} of approximately 2.0.

NB: the range of molar extinction coefficients will vary depending on the conditions used. For this reason, we recommend determining it empirically according to the method above. The molar extinction coefficients for PNP determined in a cuvette method at pH 7.0 and pH 9.0 falls in the ranges of 4,800 to 5,820 (mean= 5,165 RSD 8.4%) and 14,790–18,180 (16,640, RSD 8.4%) M^{-1} cm^{-1} respectively (http://web.viu.ca/krogh/chem331/PNP extinctions.PDF).

Calculation of specific activity of enzymes in microtitre plate method

Export data from microplate reader software into Excel spreadsheet and use the following calculation to determine specific activity.

$$\text{Enzyme activity} = \frac{\text{slope} \times \text{total volume in microtitre well (μL)} \times 10^6}{E_M \times \text{volume of cells in microtitre well (μL)}}$$

$$i.e. \quad \text{Enzyme activity} = \frac{\text{slope} \times 200 \times 10^6}{E_M \times 40}$$

Enzyme activity is expressed as nmol PNP or ONP min^{-1} mL^{-1}

$$\text{Specific enzyme activity} = \frac{\text{enzyme activity}}{\text{cell } OD_{595nm}}$$

Calculation of specific activity of enzymes in cuvette method

$$\text{Enzyme activity} = \frac{OD_{405nm} \times \text{total assay volume in μL} \times 10^6}{E_M \times \text{volume of cells in assay reaction in μL} \times \text{assay time (minutes)}}$$

$$i.e. \quad \text{Enzyme activity} = \frac{OD_{405nm} \times 1000 \times 10^6}{E_M \times 40 \times \text{assay time (minutes)}}$$

Enzyme activity is expressed as nmol PNP or ONP min^{-1} mL^{-1}

$$\text{Specific enzyme activity} = \frac{\text{enzyme activity}}{\text{cell } OD_{595nm}}$$

12.5.2 Qualitative determination

Reporter genes provide a useful tool to determine if a particular gene is differentially expressed under specific conditions. Qualitative determination on agar plates can reveal if a gene is up- or down-regulated. Solid growth media are supplemented with a water-insoluble chromogen which is converted into a coloured product by the activity of the reporter enzyme. The production of colour is proportional to the amount of reporter enzyme produced. The insoluble nature of the pigment prevents it from spreading across the plate, maintaining it in the vicinity of the colony. Commonly used chromogens include X-Gal, X-Glc and X-Phosphate for reporter genes *lacZ, gusA* and *phoA*, respectively. In the presence of the reporter enzyme, an appropriate substrate is cleaved, releasing X (5-bromo-4-chloro-3-indole) which is a blue-coloured compound. The application of this qualitative method using the *gusA* reporter has been described previously in Protocol 12.1, Step 10.

12.5.2.1 Protocol for genomic libraries

The creation of genomic libraries from RNB can be particularly important for a number of reasons, including complementation, sequencing and expression studies. This section provides a detailed protocol for the construction of a DNA library but does not discuss the many types of vectors that can be chosen for this purpose. The reader is referred to Table 12.1 for a table of vectors which may be appropriate for the construction of a particular DNA library better suited to your specific experiment. The protocol provided here illustrates one specific example for the construction of a library suitable for functional studies, and which can be sequenced for genome compilation efforts. The vector pTH1522 (Figure 12.2) was used in this example. Vector pTH1522 is a mobilisable plasmid that is unable to replicate in RNB but which can be converted to a replicative cointegrant when required to enable expression to be monitored in different genetic backgrounds (Cowie *et al.* 2006). This protocol has been successfully used to generate libraries of *S. medicae* WSM419 (Reeve *et al.* 2010a) and *R. leguminosarum* bv. *trifolii* strains WSM1325 (Reeve *et al.* 2010b) and WSM2304 (Reeve *et al.* 2010c) for genome sequence efforts.

Integration reporter vector: pTH1522

- *oriV* – ColE1
- Mobilizable
- XhoI cloning site
- Gentamicin resistance
- GFP gene and *lacZ* in one direction
- RFP gene and *gusA* in the other direction
- FRT and attP (ΦC31) sites
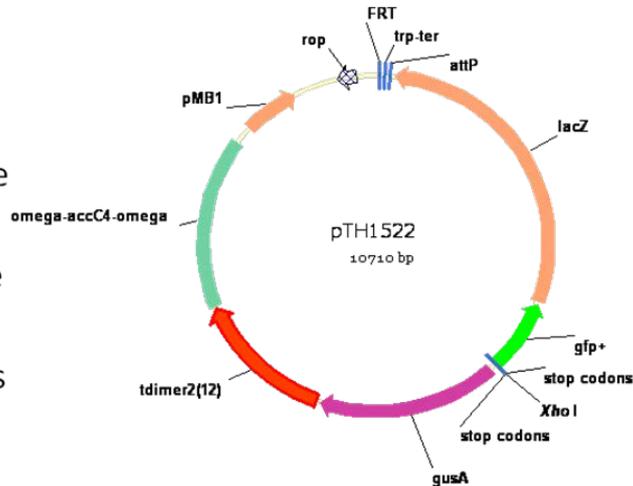
Cointegrate Vector
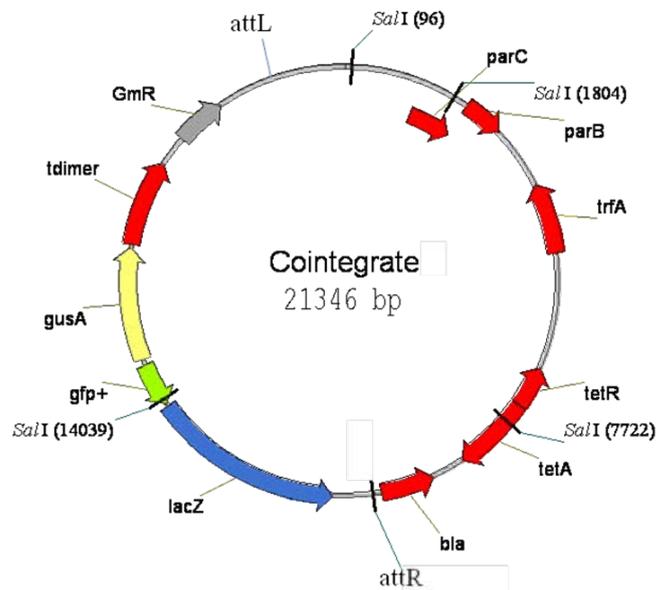
Figure 12.2   The features of the vector pTH1522 and its replicative cointegrate

Precautionary measures

► All pipette tips should be filter barrier tips.

► Benches should be scrupulously clean (wiped with 70% (v/v) ethanol before use) and gloves must be worn at all times to prevent contamination.

► Containers should be disposable plastic ware. If glassware is used, it needs to be soaked in 0.1 N HCl and then 0.1 N NaOH prior to rinsing with sterile RODI water.

► Genomic DNA should be prepared using wide-bore pipette tips to prevent shearing.

Procedure

a. Vector preparation

1. CsCl purified pTH1522 (50 μg) should be digested to completion with *Xho*I (typically 5 U μg^{-1} DNA for two hours). The *Xho*I digest should then be heat inactivated at 65°C for 15 minutes.

2. The cut plasmid should then be purified using a Qiagen minelute column and eluted in sterile, molecular-grade water.

3. The purified cut plasmid should then be half end-filled using dTTP and dCTP, and the Klenow fragment of DNA polymerase (1 U μg^{-1} DNA) in 1× polymerase buffer. The reaction should be incubated at 37°C for 30 minutes. Following this, the polymerase should then be heat inactivated by incubating the mixture at 75°C for 15 minutes. The cut half end-filled plasmid can then be purified on a Qiagen minelute column and eluted with sterile, molecular-grade water.

4. The *Xho*I half end-filled plasmid needs to be dephosphorylated using Antarctic shrimp alkaline phosphatase (SAP or equivalent) (0.5 U μg^{-1}) in 1× phosphatase buffer at 37°C for 20 minutes. The SAP should be heat inactivated by incubating the reaction at 65°C for 5 minutes. The dephosphorylated plasmid should be purified using a Qiagen minelute column and eluted in sterile, molecular-grade water.

5. The purified dephosphorylated *Xho*I digested half end-filled DNA should then be incubated overnight at room temperature in 1× T4 DNA ligase buffer containing T4 DNA ligase (3 U μg^{-1}). The reaction mixture is then electrophoresed on a 1% (w/v) agarose gel and the 10.5 kb linear non-religated pTH1522 excised from the gel and purified using a Qiagen minelute column. The DNA should be eluted in water.

b. Genomic DNA preparation

1. DNA can be extracted from a three-day-old culture of RNB using a suitable genomic DNA isolation method, such as the CTAB method (Protocol 11.1.1, Chapter 11). Choose the appropriate method for your strain to obtain high molecular weight DNA that provides intact DNA (no smearing on a gel after electrophoresis).

2. Re-suspend the genomic DNA in sterile, molecular-grade water and treat with RNASe (10 μg mL^{-1}). The genomic DNA concentration can then be estimated by UV spectrophotometry ensuring a 260nm/280nm absorbance ratio of 1.7 to 2.0.

c. Preparation of insert DNA

1. Partially digest RNB genomic DNA with *Sau*3AI (at 0.05 U μg^{-1}) in a 50 μL reaction and terminate by the addition of 1 μL 0.5 M EDTA and gel loading buffer. A sample needs to be electrophoresed and checked on a gel for partial digestion. The approximately 2 kb fragments can then be excised from the gel and purified using a Qiagen minelute column and then eluted in sterile, molecular-grade water. (Expected yield: 100 ng from 150 μg genomic DNA.)

2. Half end-fill the 2 kb purified genomic fragments using dGTP and dATP and the Klenow fragment of DNA polymerase (1 U μg^{-1} DNA) in 1× polymerase buffer by incubating the mixture at 37°C for 30 minutes. The polymerase should be heat inactivated by incubating the mixture at 75°C for 15 minutes. The DNA fragments can then be purified using a Qiagen minelute column and eluted in sterile, molecular-grade water.

3. Incubate the half end-filled 2 kb genomic partial *Sau*3AI DNA fragments overnight at room temperature in 1× T4 DNA ligase buffer in the presence of T4 DNA ligase. Electrophorese the ligation mixture on a 1% (w/v) agarose gel and excise the 2 kb linear genomic DNA fragments from the gel. Purify the DNA from the gel using a Qiagen minelute column and re-suspend in sterile, molecular-grade water. (Expected yield: 100 ng from 150 μg genomic DNA.)

d. Ligation

The dephosphorylated *Xho*I digested, half end-filled, religated, purified 10.5 kb linear pTH1522 should be ligated to partial *Sau*3AI half end-filled religated purified 2 kb linear genomic DNA fragments overnight at room temperature. The ligation reaction should contain 90 ng pTH1522 and 10 ng insert. Appropriate controls need to be included, such as religation of vector only and a control containing the religation of insert.

e. Transformation

Dilute the ligated DNA (100 ng in 20 μL) in sterile, molecular-grade water (1:3) and transform 1 μL into 40 μL electrocompetent DH10B. (A suitable supplier of eletrocompetent DH10B is Invitrogen, which supplies cells with an electroporation efficiency of 1×10^{10} cfu μg^{-1} DNA.) The efficiency of the competent cells can be calculated at the time of the experiment but should be at least 1×10^{8} cfu μg^{-1} of a vector such as pGEM7Zf(+) DNA. A typical number of transformants obtained would be in the order of 100 gentamicin resistant (10 μg mL^{-1} in AM3 media containing X-Gal at 50 μg mL^{-1}) transformants per 250 μL of transformation mixture spread (Figure 12.3). There should be no growth of the same number of DH10B cells on AM3 containing gentamicin (10 μg mL^{-1}).
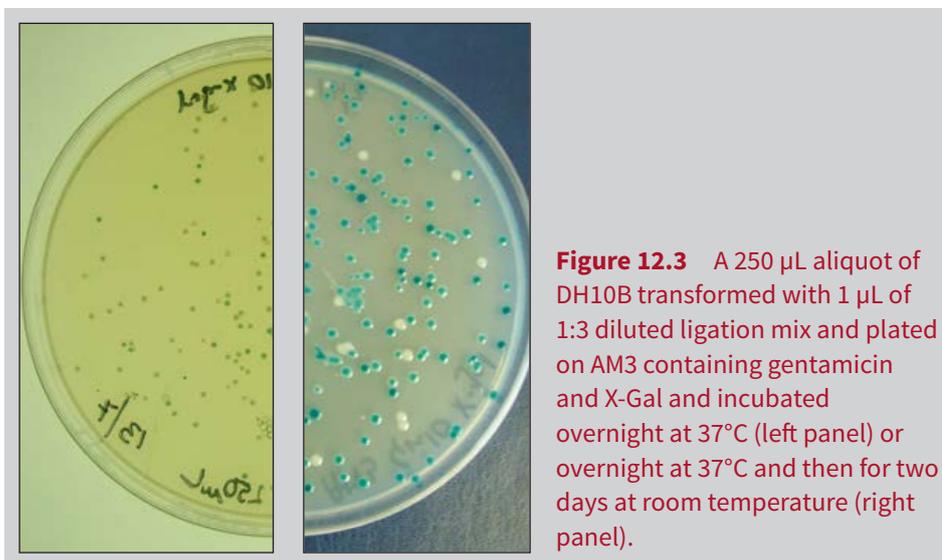
Figure 12.3 A 250 μL aliquot of DH10B transformed with 1 μL of 1:3 diluted ligation mix and plated on AM3 containing gentamicin and X-Gal and incubated overnight at 37°C (left panel) or overnight at 37°C and then for two days at room temperature (right panel).

NB: blue DH10B colonies contain the inserts and this feature is specific to the construction of recombinants in pTH1522.

Colour distinction is significantly enhanced by leaving the cells at room temperature until the desired size is obtained and then placing the plates at 4°C.

f. Insert verification

The presence or absence of an insert in pTH1522 should be verified by PCR analysis using the following primers:

▷ gfplac3970*F   5'-TTAggACAACTCCAgTgAAAAgTTC-3'

▷ gusrfp-4106R   5'-ATAAgggACTCCTCATTAAgATAAC-3'.

The PCR reaction mixture of 25 μL should contain:

▷ 1 μL of the cell suspension

▷ 0.5 μL of 5 U mL^{-1} *Taq* DNA polymerase (Invitrogen Life Technologies)

▷ 0.5 μL of 50 μM gfplac3970*F primer

▷ 0.5 μL of 50 μM gusrfp-4106R primer

▷ 5 μL of 5× polymerisation buffer[1]

▷ 15 μL of sterile, molecular-grade water

▷ 2.5 μL of 10 mM $MgCl_2$ (Promega Corp.).

Cycling conditions:

▷ four minutes at 95°C;

▷ 30 cycles of:

    – 30 seconds at 96°C

    – 15 seconds at 58°C

    – two minutes at 70°C;

▷ final hold at 15°C.

[1] 67 mM Tris.HCl, pH 8.8 at 25°C; 16.6 mM $(NH_4)_2SO_4$, 0.45% (w/v) Triton X-100, 0.2 mg mL^{-1} gelatin, 0.2 mM dNTP's (Fisher Biotech, Australia).

Amplified PCR-products should be visualised after electrophoresis on a 1% (w/v) agarose gel following staining with ethidium bromide (0.5 μg mL^{-1}). The bands can be visualised after staining using a UV transilluminator connected to a digital camera. Gel images can be stored using appropriate software, such as Quantity One (BIORAD). An appropriate DNA ladder should be used as a reference. One suitable marker is the 1 kb Promega DNA ladder.

A range of white and blue colonies are chosen and the recombinant rate can then be calculated. Typically, around 50 colonies are chosen. The following gel (Figure 12.4) shows the PCR product profile for five white and 44 blue colonies selected from AM3 containing gentamicin (10 μg mL^{-1}) and X-Gal (50 μg mL^{-1}).
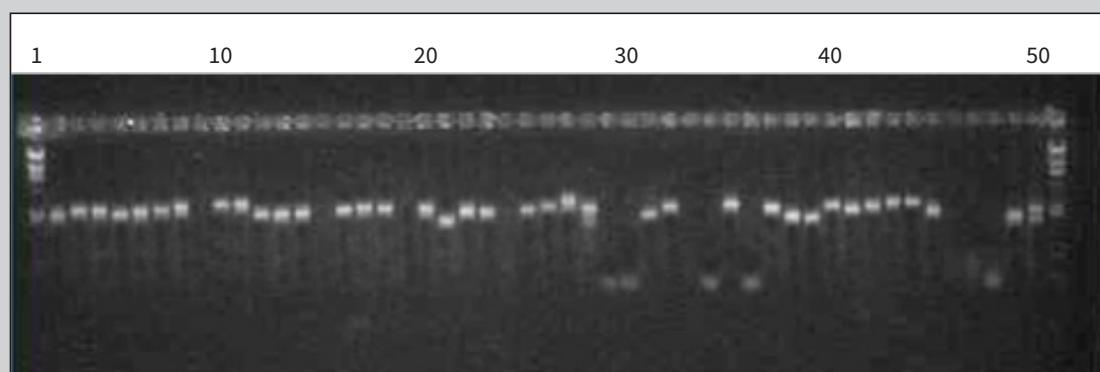
Figure 12.4 Analysis of *E. coli* DH10B white (lanes 9, 15, 19, 24, 33, 46, 47) and blue (lanes 2–8, 10–14, 16–18, 20–23, 25–32, 34–45, 49–50) transformants from a pTH1522 library of *S. medicae* WSM419. Lane 48 contains the 100 bp amplification product from pTH1522. Lanes 1 and 51 are DNA size ladders.

NB: in this example, the percentage of clones that contained an insert was approximately 92%. As expected, the white colonies did not contain an insert and are typically deletion derivatives of the parent plasmid.

g. Potential library size

A greater than 10-fold coverage library can easily be obtained from the ligated mixture. The fold representation can be calculated from the following:

number of transformants in total × [insert size (kb)/genome size (kb)]

12.6 Protocol for nucleic acid sequencing

DNA sequencing involves determining the sequential order of the nucleotide bases (adenine, guanine, cytosine and thymine). Variations of the original chain-termination method (Sanger method) using radioactively- or fluorescently- labelled dideoxynucleotide triphosphates (ddNTPs) for detection in automated sequencing machines are the most popular choices of low-throughput DNA sequencing methods. DNA sequencers now use capillary electrophoresis for size separation, detection and recording of dye fluorescence. (Each of the four ddNTP terminators is labelled with a fluorescent dye [Smith *et al*. 1986]). These automated sequencers (*e.g.* Genetic Analyzer, Applied Biosystems, Life Technologies Corp., US) can sequence up to 384 DNA samples in a batch, with software programs accurately calling approximately 700–900 bp prior to the deterioration of quality of base calling. Due to this limitation, two priming sites in opposite orientations (one in 5'-3' and one in 3'-5') are initially selected, and upon analysis of the sequencing results, another two priming sites in opposite orientation are again selected to achieve accurate double-stranded DNA sequence information of a prokaryotic gene. This approach is, for example, recommended prior to submitting a 16s rRNA gene sequence to Genbank (NCBI) which is a public sequence database. Developments in high-throughput sequencing technologies now produce thousands or millions of sequences at once, enabling researchers to sequence whole plasmids and genomes. The major sequencing centres and their sequencing platforms are provided in Table 12.2.

There are three major international sequence databases that are publicly available: GenBank in the U.S. (http://www.ncbi.nlm.nih.gov/); EMBL in Europe (http://www.ebi.ac.uk/); and DDBJ in Japan (http://www.ddbj.nig.ac.jp/). Each day, all newly submitted sequences are exchanged between the three databases. Every scientific publication in which bacterial strains are identified or described requires the author to deposit the gene sequences in one of these three public databases. Therefore, the databases contain the gene sequence of nearly all sequenced bacterial species and enable researchers to compare their own sequences with those in the databases for identification. For example, the nucleic acid sequence of your gene may be submitted to the National Centre for Biotechnology Information (NCBI) using the web-based submission tool, BankIt, or the off-line submission tool, Sequin.

Information comparing the two options is provided online: http://www.ncbi.nlm.nih.gov/WebSub/?tool=genbank. Submission of sequences to NCBI relies on the researcher providing both metadata and evidence of sequence verification; these requirements should be considered prior to initiating a sequencing project (http://www.ncbi.nlm.nih.gov/WebSub/html/requirements.html).

Table 12.2 Major sequencing centres used by the authors for either routine sequencing of DNA fragments, sequencing of large inserts, plasmids or whole genomes using a shotgun approach or high-throughput sequencing of whole genomes and/or transcriptomes using next generation sequencing technologies

Sequencing centre	Location	Access information at	Sequencing platforms available as of May 2013
Joint Genome Institute	California, USA	http://www.jgi.doe.gov/	Roche 454, Illumina GAII and PacBio
Macrogen	Seoul, Korea	http://www.macrogen.com/eng/sequencing/sequence_main.jsp	ABI 3730XL and ABI3700
Australian Genome Research Facility (AGRF)	Australia (multiple locations)	http://www.agrf.org.au/	AB 3730XL, Roche GS FLX and Illumina GAII
BGI Genomics	Beijing, China	http://en.genomics.cn/navigation/index.action	Illumina HiSeq, AB SOLiD and Ion Torrent

12.7 Protocol for bioinformatic tools

Comparative genomic tools enable researchers to interrogate the genome of newly sequenced RNB by comparison with genomes of other organisms. Here we have provided a brief synopsis of two genomic tools used by the authors which are available for free online.

12.7.1 Joint Genome Institute (JGI)

The Integrated Microbial Genomes–Genome Encyclopedia of Bacteria and Archaea Genomes (IMG/GEBA) serves as the preliminary release of genomes from the United States Department of Energy GEBA project as soon as they are submitted to Genbank. The number of genomes available is constantly growing but as of August 2015 there were 26,888 bacterial genomes available with 934 *Rhizobiales* genomes.

Access at http://img.jgi.doe.gov/cgi-bin/geba/main.cgi

IMG Manual available at http://img.jgi.doe.gov/geba/doc/userGuide.pdf

Tools

► Neighbourhood matching/genome comparisons.

► Basic Local Alignment Search Tool (BLAST), whereby a nucleotide or amino acid sequence is submitted and returns sequences with regions of similarity.

▶ Gene search—finds genes using a variety of search criteria. (The gene neighbourhood display is very useful for visualising homologs.)

▶ Function alignment—search for COG, KOG or Pfam.

▶ Genome statistics—*i.e.* number of genes, number of paralogs, number of plasmids, size *etc.*

▶ Chromosome maps—circular view of plasmids coloured according to COG function.

▶ Synteny view—compares scaffolds (VISTA), plasmids or whole genomes (Artemis or Dot Plot) based on nucleotide or amino acid identity (Figure 12.5).
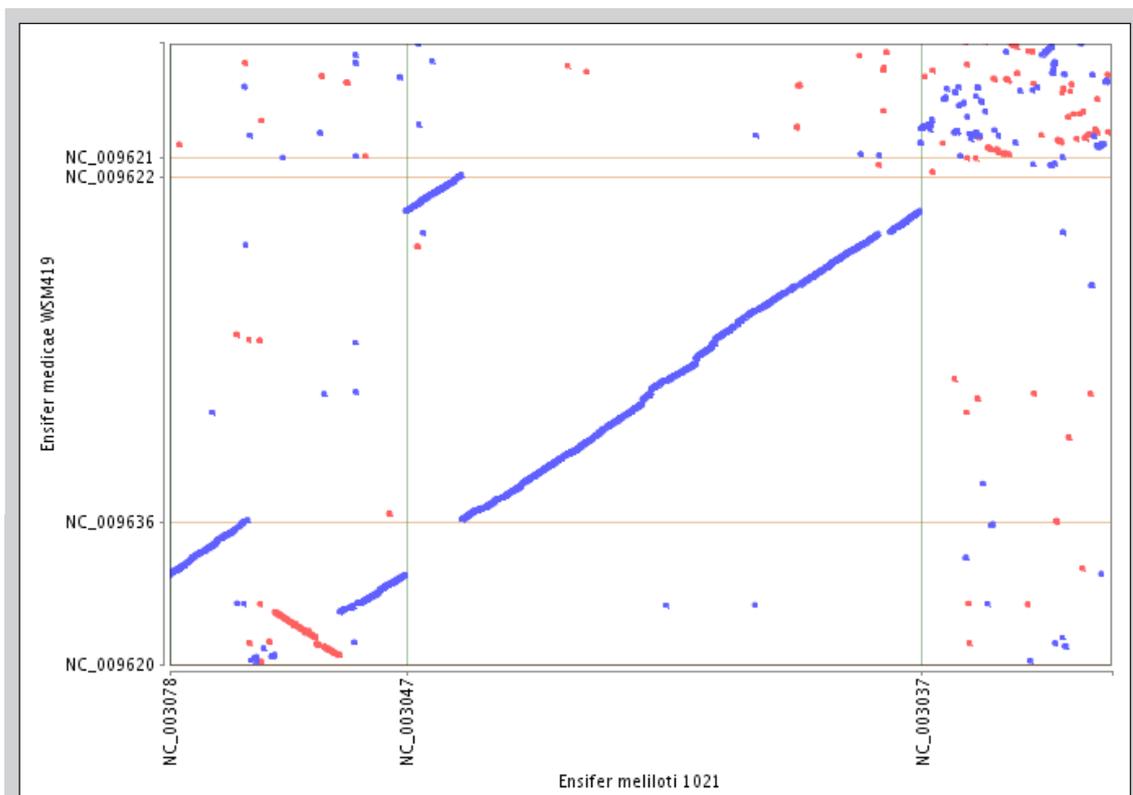
Figure 12.5   Synteny between the nucleotide genome sequences of *Ensifer (Sinorhizobium) meliloti* strain 1021 and *Ensifer (Sinorhizobium) medicae* strain WSM419 using Dot Plot analysis (IMG, JGI).

▶ Abundance profile—visual display of the abundance of all protein families (COGs or Pfams) or functional families (enzymes).

▶ Functional profile—the number of genes in an organism within a functional group and link to associated genes.

▶ Genome clustering—genomes can be clustered based on protein/functional family.

▶ COG category statistics—statistics for genomes by COGs (cluster of orthologous genes) in tabular format, pie chart or bar graph.

► KEGG category statistics—statistics for genomes by KEGG (Kyoto Encyclopedia of Genes and Genomes) category and link to KEGG pathway maps.

NB: the KEGG database can be accessed directly from http://www.genome.jp/kegg/. Useful search tools include:

► KEGG pathway—search for a metabolic pathway map and view all contributing enzymes via E.C. (Enzyme Commission) number.

► KEGG orthology (KO)—known functions of genes and proteins (using experimental evidence) are organised in the KO database

► KEGG organisms—each KO entry can be taxonomically mapped, enabling identification of the presence/absence of gene orthologs.

12.7.2 NCBI access at http://www.ncbi.nlm.nih.gov/

Access Microbial Genomes at http://www.ncbi.nlm.nih.gov/genomes/lproks.cgi; 8,055 bacterial genomes are available with 2,258 proteobacteria and 688 alpha-proteobacteria.

Tools

► BLAST. Key criteria used to select BLAST type are given in Table 12.3.

Table 12.3   Key criteria used to select BLAST type

Query	Genome	BLAST
nucleic acid	nucleic acid	BLASTN or TBLASTX
Protein	nucleic acid	TBLASTN
nucleic acid	protein	BLASTX
Protein	protein	BLASTP

► NCBI access to BLAST with Microbial Genomes http://www.ncbi.nlm.nih.gov/sutils/genom_table.cgi

► NCBI access to concise microbial protein BLAST. (Proteins have been clustered at the genus level.) http://www.ncbi.nlm.nih.gov/genomes/prokhits.cgi

► CD Tree—analyse conserved domain data (CDD).

► COBALT—protein multiple alignment.

► Cn3D—viewing 3D protein structures.

► Gene Plot—pairwise comparison of two prokaryotic genomes displaying pairs of protein homologs.

► Genetic Codes—genetic code for organism.

Bacterial genetic code below accessed at: http://www.ncbi.nlm.nih.gov/Taxonomy/taxonomyhome.html/index.cgi?chapter=tgencodes#SG11. Initiation of translation is most efficient at AUG but also occurs at GUG and UUG in bacteria.

TTT F Phe	TCT S Ser	TAT Y Tyr	TGT C Cys
TTC F Phe	TCC S Ser	TAC Y Tyr	TGC C Cys
TTA L Leu	TCA S Ser	TAA * Ter	TGA * Ter
TTG L Leu	TCG S Ser	TAG * Ter	TGG W Trp
CTT L Leu	CCT P Pro	CAT H His	CGT R Arg
CTC L Leu	CCC P Pro	CAC H His	CGC R Arg
CTA L Leu	CCA P Pro	CAA Q Gln	CGA R Arg
CTG L Leu	CCG P Pro	CAG Q Gln	CGG R Arg
ATT I Ile	ACT T Thr	AAT N Asn	AGT S Ser
ATC I Ile	ACC T Thr	AAC N Asn	AGC S Ser
ATA I Ile	ACA T Thr	AAA K Lys	AGA R Arg
ATG M Met	ACG T Thr	AAG K Lys	AGG R Arg
GTT V Val	GCT A Ala	GAT D Asp	GGT G Gly
GTC V Val	GCC A Ala	GAC D Asp	GGC G Gly
GTA V Val	GCA A Ala	GAA E Glu	GGA G Gly
GTG V Val	GCG A Ala	GAG E Glu	GGG G Gly

▶ Genome ProtMap—maps each protein from a Cluster of Orthologous Genes (COG) back to its genome.

▶ Map Viewer—view and search an organism's complete genome and display maps.

12.8 Protocol for phylogenetic trees

Phylogenetic analysis was once considered too difficult and therefore not widely utilised by molecular and biochemical biologists. This changed with the arrival of DNA sequencing and accessible software programs (Hall 2011). Building phylogenetic trees is now a four-step process. First, you need to identify and acquire the sequences to include in the tree. Second, you need to align the sequences and estimate the tree by one of the given methods. Finally, you draw the tree and edit according to the journal's requirements.

Several free software programs exist to build phylogenetic trees. Here we discuss in detail how to use MEGA (http://www.megasoftware.net/) but alternative software can be PHYML (http://www.atgc-montpellier.fr/phyml/) or MrBayes (http://mrbayes.sourceforge.net/index.php). A short overview of the most commonly used phylogenetic tree types is given below.

▶ Neighbor-Joining (NJ) is currently the most widely used tree type of the distance methods. It produces a single, strictly bifurcate tree, which means that each internal node has exactly two branches descending from it. In distance methods, the fraction of sites that differ between two sequences in a multiple

alignment are expressed as the distances between them. Another example of a distance method is UPGMA (Unweighted Pair-Group Method with Arithmetic Mean). Due to the fact that the tree derived from this method is ultrametric (all taxa are equally distant from the root) it is advisable not to use this for publications.

▶ Maximum Likelihood (ML) is a method that searches for the tree that makes the data most likely. It uses the log-likelihood to compare the various models of nucleotide substitution for a particular dataset. The ML program seeks the tree with the largest log-likelihood. ML trees are gaining popularity since they are based on more solid calculations, and the latest software makes them more easily accessible.

For further assistance with phylogenetic trees and more background theory, visit: http://www.sinauer.com/hall/3e/

12.8.1 Steps to build a phylogenetic tree in MEGA

In the MEGA main window go to Align and choose Do BLAST search (Figure 12.6). A new window opens (Figure 12.7) where you can paste your sequence in the sequence box or upload a file. Click the BLAST button and a summary window will appear (Figures 12.8 and 12.9).
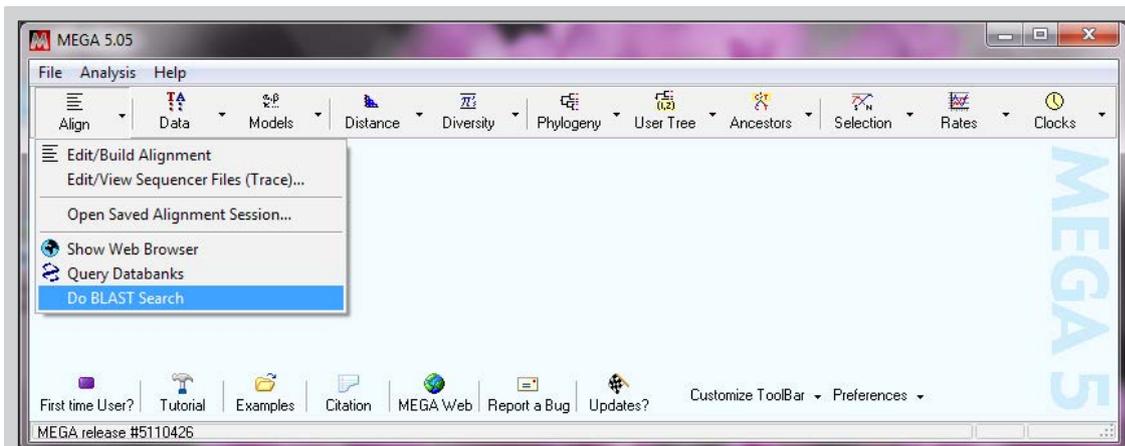
Figure 12.6 Undertake a BLAST search
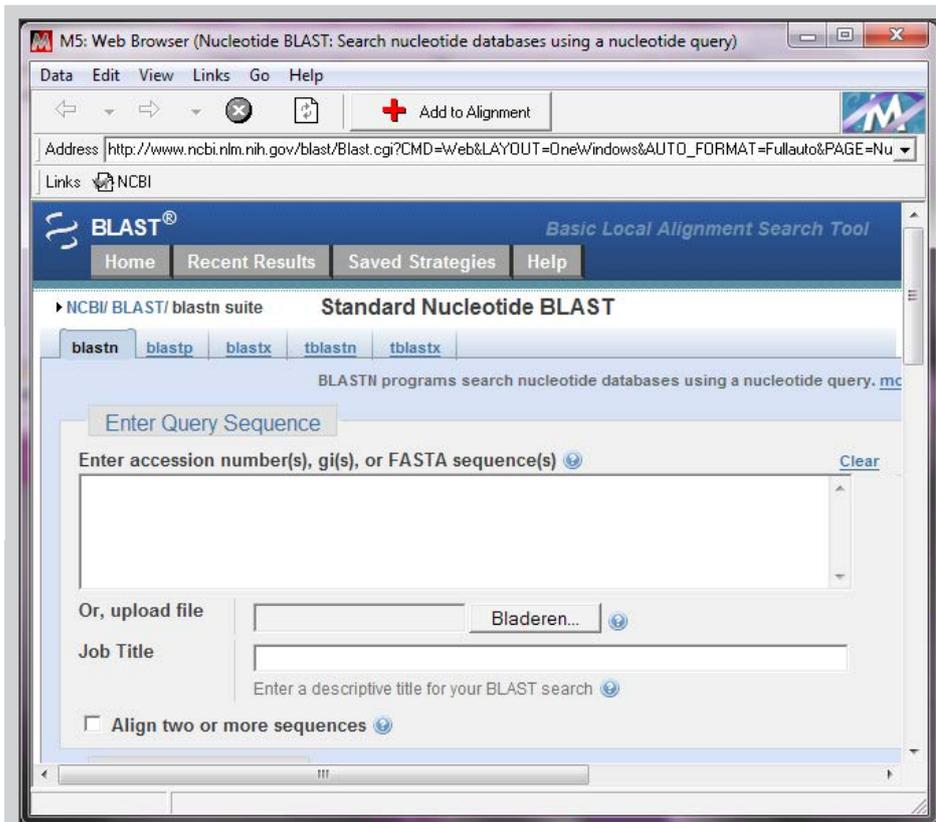
Figure 12.7 NCBI Standard Nucleotide BLAST showing where to add query sequence
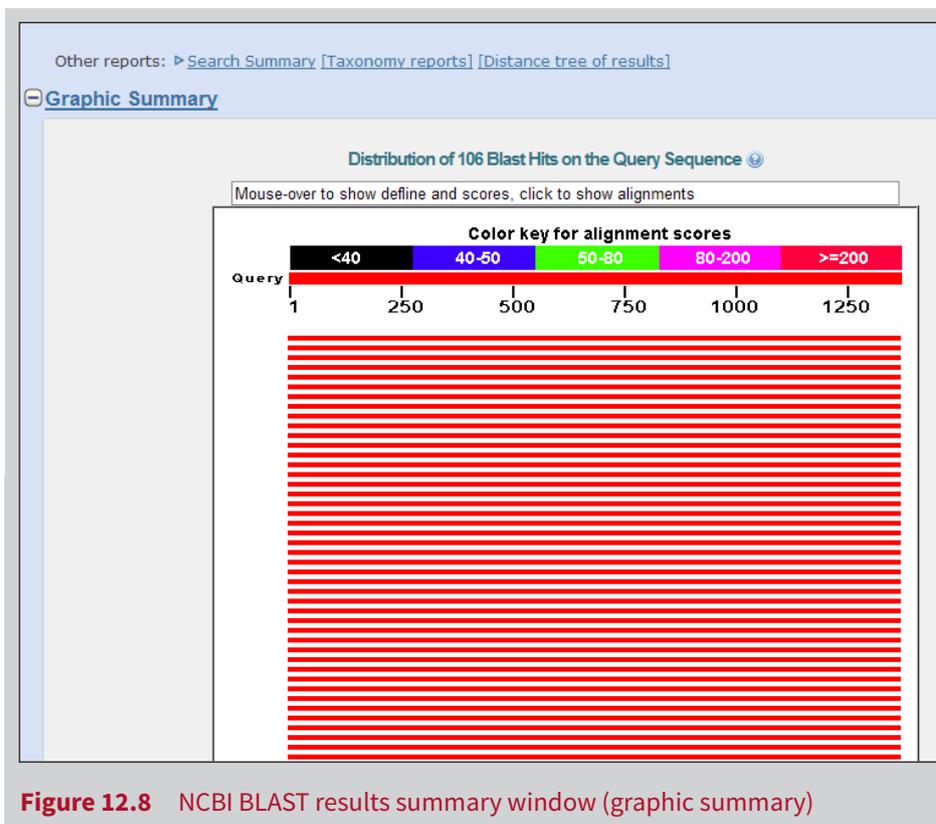
Figure 12.8 NCBI BLAST results summary window (graphic summary)

Accession	Description	Max score	Total score	Query coverage	E value	Max ident	Links
HQ670653.1	Rhizobium sp. CCGE 510 16S ribosomal RNA gene, partial sequence	2516	2516	100%	0.0	100%	
EU730598.2	Rhizobium leguminosarum bv. viciae strain BIHB 1185 16S ribosomal RNA g	2516	2516	100%	0.0	100%	
EU730596.2	Rhizobium leguminosarum bv. viciae strain BIHB 1209 16S ribosomal RNA g	2516	2516	100%	0.0	100%	
EU730594.2	Rhizobium leguminosarum bv. viciae strain BIHB 1167 16S ribosomal RNA g	2516	2516	100%	0.0	100%	
FJ593639.1	Rhizobium leguminosarum bv. trifolii strain Len-4 (2) 16S ribosomal RNA ge	2516	2516	100%	0.0	100%	
GQ863505.1	Rhizobium leguminosarum strain LPB0205 16S ribosomal RNA gene, partial	2516	2516	100%	0.0	100%	
GQ863504.1	Rhizobium sp. LCS0411 16S ribosomal RNA gene, partial sequence	2516	2516	100%	0.0	100%	
GQ863503.1	Rhizobium leguminosarum strain LCS0407 16S ribosomal RNA gene, partial	2516	2516	100%	0.0	100%	
GQ863502.1	Rhizobium sp. LCS0401 16S ribosomal RNA gene, partial sequence	2516	2516	100%	0.0	100%	
GQ863501.1	Rhizobium sp. LCS0303 16S ribosomal RNA gene, partial sequence	2516	2516	100%	0.0	100%	
GQ863500.1	Rhizobium sp. RPVR32 16S ribosomal RNA gene, partial sequence	2516	2516	100%	0.0	100%	
GQ863499.1	Rhizobium sp. RPVR24 16S ribosomal RNA gene, partial sequence	2516	2516	100%	0.0	100%	
GQ863498.1	Rhizobium sp. RPVR04 16S ribosomal RNA gene, partial sequence	2516	2516	100%	0.0	100%	
GQ863495.1	Rhizobium leguminosarum strain BAPVT02 16S ribosomal RNA gene, partial	2516	2516	100%	0.0	100%	
CP001622.1	Rhizobium leguminosarum bv. trifolii WSM1325, complete genome	2516	7548	100%	0.0	100%	
FJ748555.1	Rhizobium sp. HTP05 16S ribosomal RNA gene, partial sequence	2516	2516	100%	0.0	100%	
FJ748554.1	Rhizobium sp. HTP01 16S ribosomal RNA gene, partial sequence >gb|HQ7:	2516	2516	100%	0.0	100%	
CP001191.1	Rhizobium leguminosarum bv. trifolii WSM2304, complete genome	2516	7548	100%	0.0	100%	
FJ025096.1	Rhizobium leguminosarum strain SEMIA 2083 16S ribosomal RNA gene, par	2516	2516	100%	0.0	100%	
FJ025095.1	Rhizobium leguminosarum strain SEMIA 2050 16S ribosomal RNA gene, par	2516	2516	100%	0.0	100%	
FJ025093.1	Rhizobium leguminosarum strain SEMIA 3026 16S ribosomal RNA gene, par	2516	2516	100%	0.0	100%	
FJ025090.1	Rhizobium leguminosarum strain SEMIA 235 16S ribosomal RNA gene, parti	2516	2516	100%	0.0	100%	
FJ025088.1	Rhizobium leguminosarum strain SEMIA 265 16S ribosomal RNA gene, parti	2516	2516	100%	0.0	100%	
FJ025086.1	Rhizobium leguminosarum strain SEMIA 2002 16S ribosomal RNA gene, par	2516	2516	100%	0.0	100%	
AY196964.1	Rhizobium leguminosarum bv. trifolii strain ARPV02 16S ribosomal RNA gen	2516	2516	100%	0.0	100%	

Figure 12.9   NCBI BLAST summary results window (results summary)

After checking the results, select the sequences you want to import into MEGA by clicking the **Accession link** and click the **Add to alignment** button (Figure 12.10). In this way, you can select sequences one by one. The program will ask you how to name your entry (Figure 12.11); ensure that you add the accession number under the sequence label. The accession number is the only part that makes your entry unique and traceable. If you already have the required sequences in a FASTA file you just open this file in the MEGA program (→ MEGA main window → File → Open a file/session and select the correct file).

Figure 12.10   NCBI example of gene sequence information
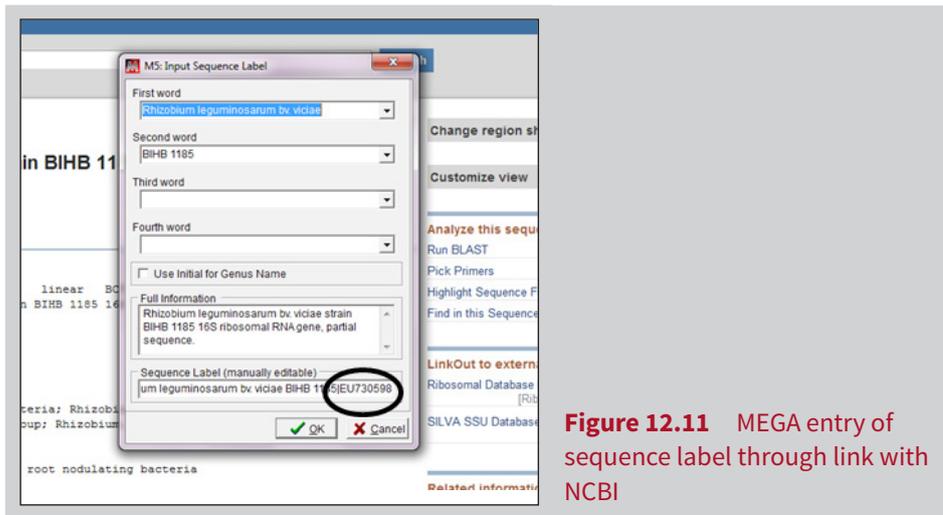
Figure 12.11 MEGA entry of sequence label through link with NCBI

To build a tree, we also want to include reference sequences (sequences published in the IJSEM journal or on the approved list in IJSEM, see http://ijs.sgmjournals. org/). An easier way to access reference sequences is to visit StrainInfo (http://www.straininfo.net/). Type in the genus and the browser gives you a list of all currently described species. An alternative is the List of Prokaryotic names with standing in Nomenclature (http://www.bacterio.cict.fr/). This site also provides currently described species and the accession numbers as given in the new species description paper. After adding the appropriate sequences to MEGA we can start to align them. Save the sequence list as a .mas file (Figure 12.12). Go to data and click on save session.

Figure 12.12 Saving reference and query sequences in MEGA as a .mas file

The next step is to align your sequences; a process designed to introduce gaps into the sequences to shift the bases back to their corresponding homologous positions. A phylogenetic tree is only as good as the quality of the alignment, which makes this step crucial for building trees. Two algorithms are available in MEGA, including ClustalW and Muscle. Here we will choose align by ClustalW (Figure 12.13), although Muscle is just as powerful. A dialogue box will appear and remind you that nothing is selected, click OK to select all sequences. A ClustalW parameters window will appear, click OK and the program will start aligning your sequences (Figure 12.14).
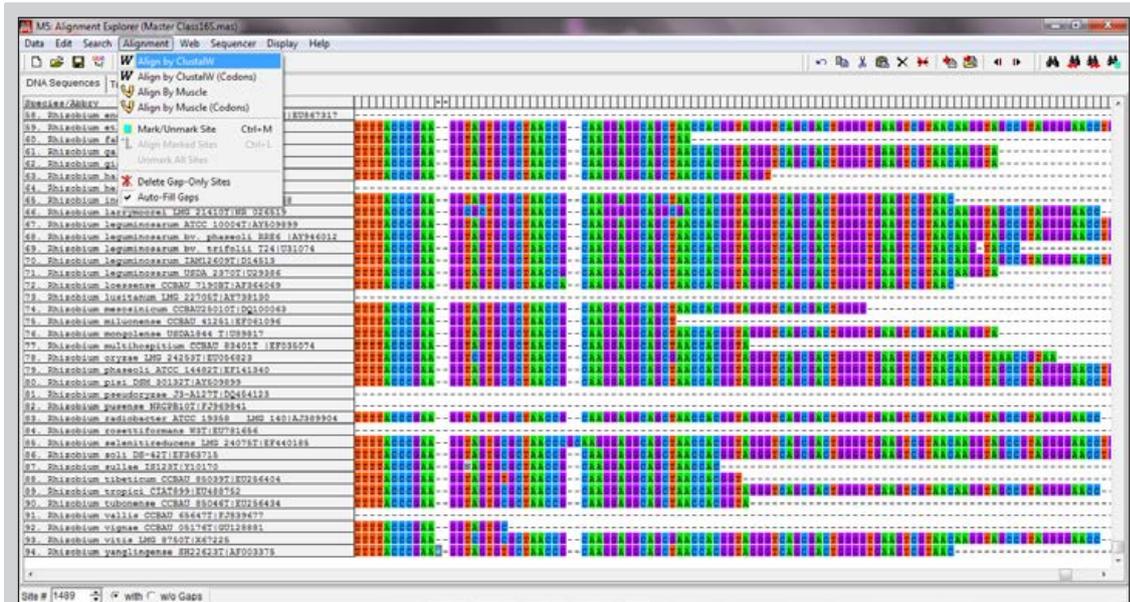
Figure 12.13   ClustalW alignment in MEGA
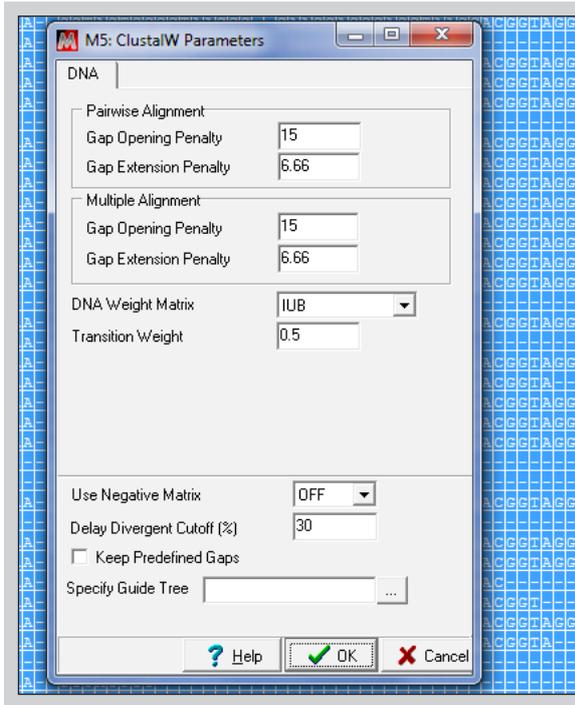
Figure 12.14   ClustalW Parameters in MEGA

The default values are optimal for aligning DNA sequences. When the program is finished calculating the alignment, you have to check whether the sequences are aligned correctly and delete those that are too short (Figure 12.15). Subsequently, the beginning and the end of the alignment must be trimmed (deleted) so that the tree is based on the actual similarity of the sequences (Figure 12.16). When you are satisfied with the alignment, you need to export it as a .meg file as shown in Figure 12.17.
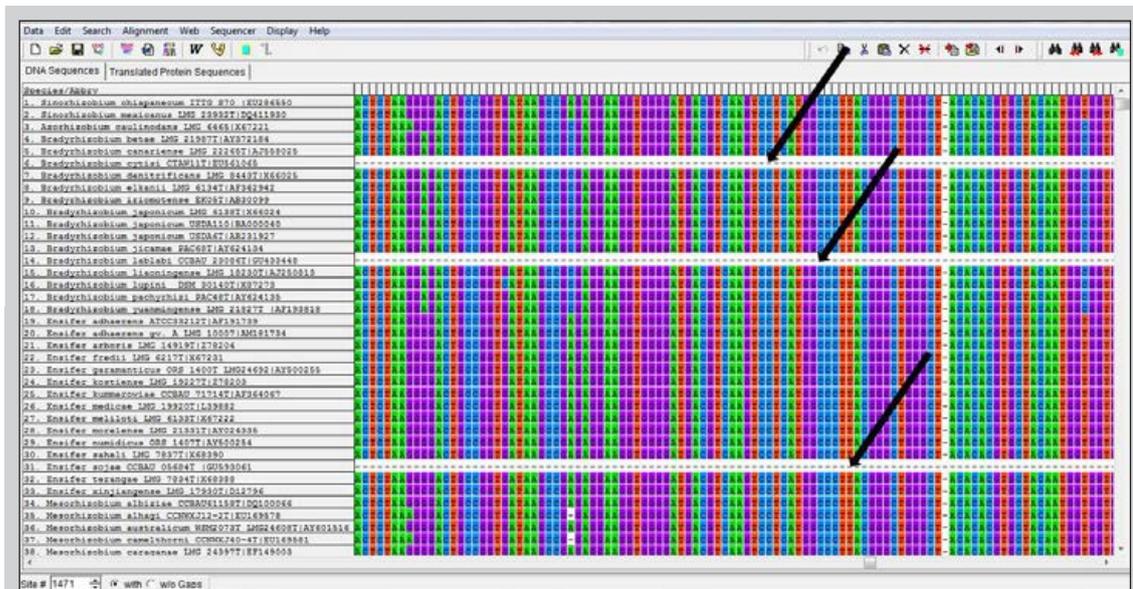
Figure 12.15   Arrows indicate short sequences in MEGA after ClustalW alignment
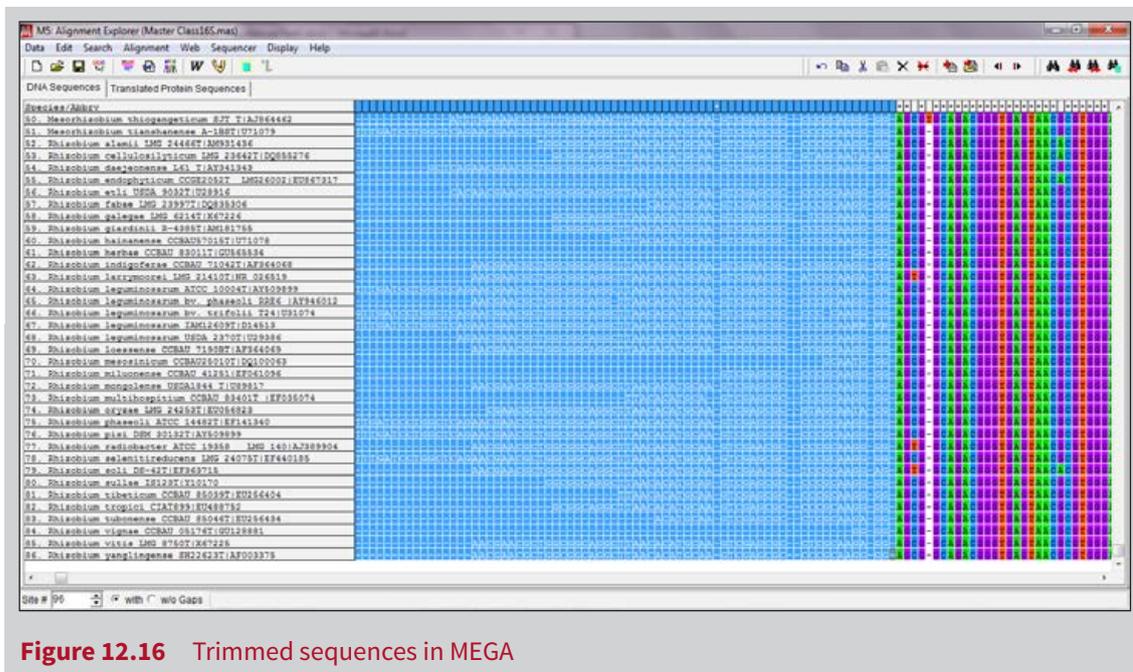
Figure 12.16   Trimmed sequences in MEGA
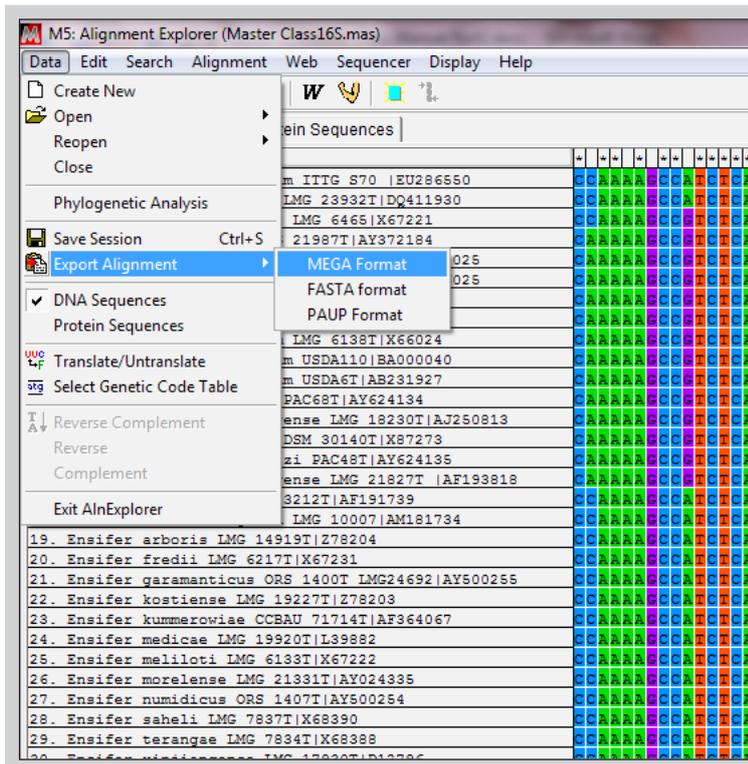
Figure 12.17 Export alignment as a .meg file

To build the actual tree, you have to go back to the home window of MEGA (Figure 12.18). Here you can choose between several trees; currently Neighbor-joining and Maximum Likelihood are the most frequently used methods. The Maximum Likelihood method is discussed in Section 12.8. When clicking on the construct ML tree button, you will have to search for your .meg file. An analysis window opens and here you can define if you want bootstrap analysis, the number of replications (which is mostly 500 or 1,000), which model to use (generally General Time Reversal model is used), gaps treatment (mostly complete deletion) and ML heuristic method (mostly CNI). The characteristics given in Figure 12.19 work well for general ML trees; more information about these characteristics is available (Hall 2011).
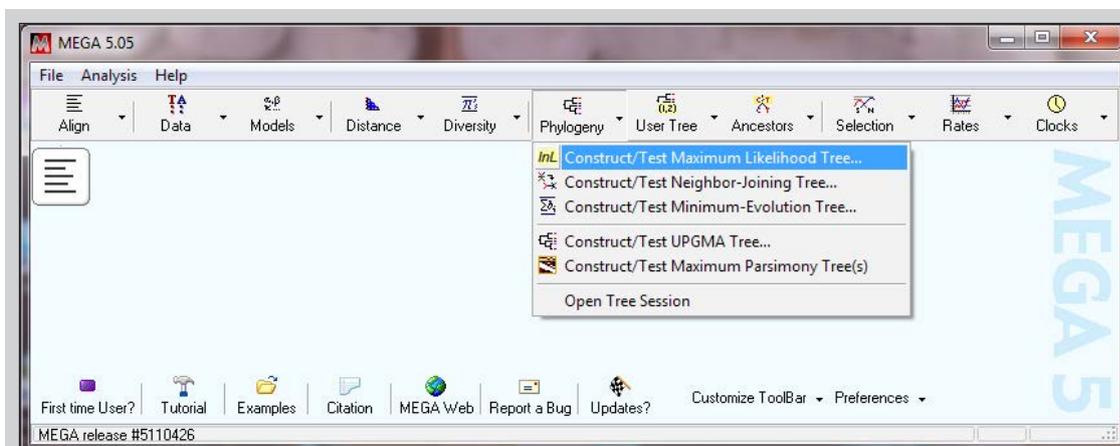
Figure 12.18 Constructing a phylogenetic tree using Maximum Likelihood
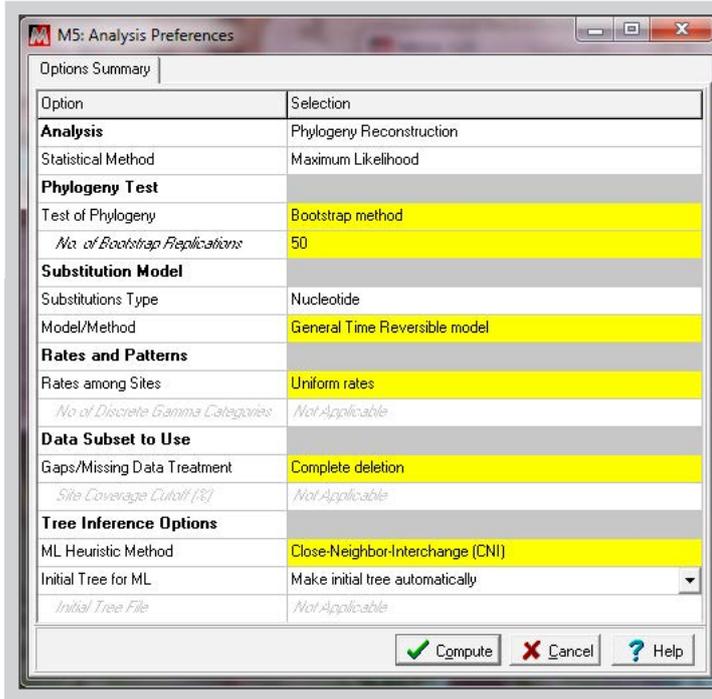
Figure 12.19 MEGA analysis preferences for constructing a phylogenetic tree

Select **calculate** to begin building the tree. When the program is finished, a new window will open showing your ML tree. To set the root, go to **Subtree** and select the **root** button, then select the preferred root branch (Figure 12.20). When you are satisfied with the tree, you can export it as a pdf, click Image and save as a **pdf** file (Figure 12.21). Additionally, you can use graphic analysis software (*i.e.* Adobe Photoshop or Adobe Illustrator) to conform your tree to the journal's specific requirements.
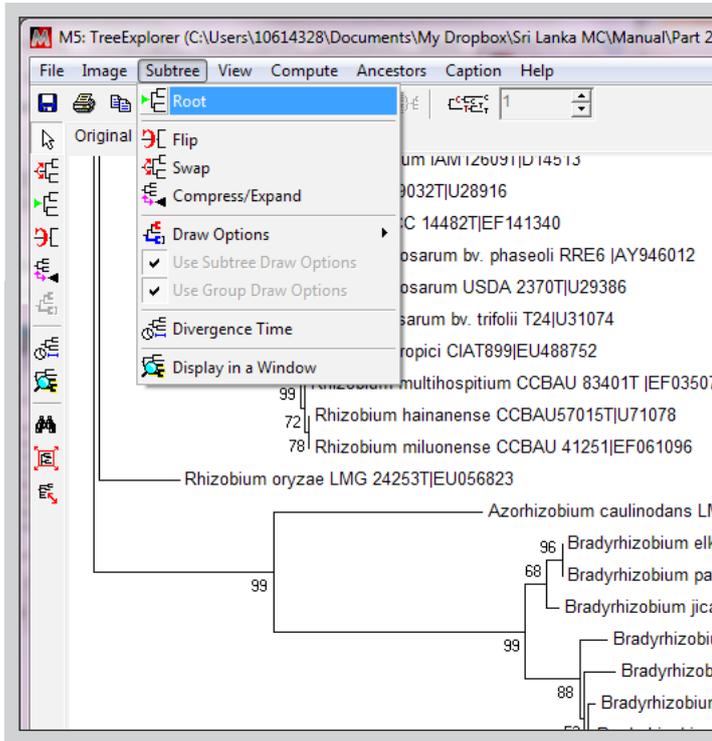
Figure 12.20 Setting the 'root' in MEGA TreeExplorer
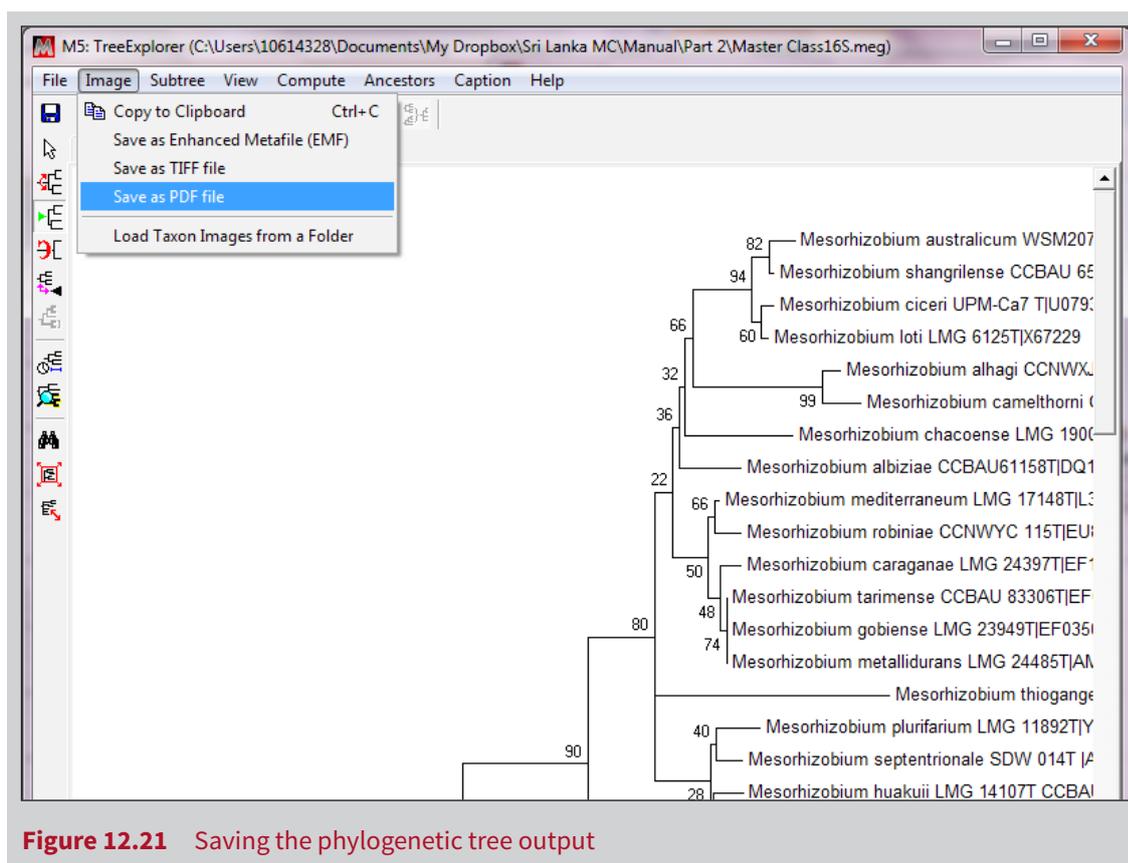
Figure 12.21 Saving the phylogenetic tree output

12.9 References

Cowie A., Cheng J., Sibley C.D., Fong Y., Zaheer R., Patten C.L., Morton R.M., Golding G.B. and Finan T.M. 2006. An integrated approach to functional genomics: construction of a novel reporter gene fusion library for *Sinorhizobium meliloti*. Applied and Environmental Microbiology 72, 7156–7167.

De Feyter R., Yang Y. and Gabriel D.W. 1993. Gene-for-genes interactions between cotton R genes and *Xanthomonas campestris* pv. *malvacearum avr* genes. Molecular Plant Microbe Interactions 6, 225–237.

de Lorenzo V., Herrero M., Jakubzik U. and Timmis K.N. 1990. Mini-Tn5 transposon derivatives for insertion mutagenesis, promoter probing, and chromosomal insertion of cloned DNA in gram-negative eubacteria. Journal of Bacteriology 172, 6568–6572.

De Vos G.F., Walker G.C. and Signer E.R. 1986. Genetic manipulations in *Rhizobium meliloti* utilizing two new transposon Tn5 derivatives. Molecular and General Genetics 204, 485–491.

Defeyter R. and Gabriel D. 1991. At least six avirulence genes Are clustered on a 90-Kilobase plasmid in *Xanthomonas campestris* pv. *malvacearum*. Molecular Plant Microbe Interactions 4, 423–432.

DeFeyter R., Kado C.I. and Gabriel D.W. 1990. Small, stable shuttle vectors for use in *Xanthomonas*. Gene 88, 65–72.

Dennis J.J. and Zylstra G.J. 1998. Plasposons: modular self-cloning minitransposon derivatives for rapid genetic analysis of gram-negative bacterial genomes. Applied and Environmental Microbiology 64, 2710–2715.

Figurski D.H. and Helinski D.R. 1979. Replication of an origin-containing derivative of plasmid RK2 dependent on a plasmid function provided *in trans*. Proceedings of the National Academy of Sciences USA 76, 1648–1652.

Figurski D.H., Meyer R.J. and Helinski D.R. 1979. Suppression of Co1E1 replication properties by the Inc P-1 plasmid RK2 in hybrid plasmids constructed *in vitro*. Journal of Molecular Biology 133, 295–318.

Finan T.M., Kunkel B., De Vos G.F. and Signer E.R. 1986. Second symbiotic megaplasmid in *Rhizobium meliloti* carrying exopolysaccharide and thiamine synthesis genes. Journal of Bacteriology 167, 66–72.

Hall B.G. 2011. Phylogenetic trees made easy: A how-to manual. Sinauer Associates Inc.

Herrero M., de Lorenzo V. and Timmis K.N. 1990. Transposon vectors containing non-antibiotic resistance selection markers for cloning and stable chromosomal insertion of foreign genes in gram-negative bacteria. Journal of Bacteriology 172, 6557–6567.

Karunakaran R., Mauchline T.H., Hosie A.H. and Poole P.S. 2005. A family of promoter probe vectors incorporating autofluorescent and chromogenic reporter proteins for studying gene expression in Gram-negative bacteria. Microbiology 151, 3249–3256.

Kim C.H., Helinski D.R. and Ditta G. 1986. Overlapping transcription of the *nifA* regulatory gene in *Rhizobium meliloti*. Gene 50, 141–148.

Kokotek W. and Lotz W. 1991. Construction of a mobilizable cloning vector for site-directed mutagenesis of gram-negative bacteria: application to *Rhizobium leguminosarum*. Gene 98, 7–13.

Kovach M.E., Elzer P.H., Hill D.S., Robertson G.T., Farris M.A., Roop R.M. 2nd and Peterson K.M. 1995. Four new derivatives of the broad-host-range cloning vector pBBR1MCS, carrying different antibiotic-resistance cassettes. Gene 166, 175–176.

Kovach M.E., Phillips R.W., Elzer P.H., Roop R.M. 2nd and Peterson K.M. 1994. pBBR1MCS: a broad-host-range cloning vector. Biotechniques 16, 800–802.

Legocki R.P., Legocki M., Baldwin T.O. and Szalay A.A. 1986. Bioluminescence in soybean root nodules: Demonstration of a general approach to assay gene expression in vivo by using bacterial luciferase. Proceedings of the National Academy of Sciences USA 83, 9080–9084.

Long S.R. 1989. *Rhizobium* genetics. Annual Reviews in Genetics 23, 483–506.

Metcalf W.W., Jiang W., Daniels L.L., Kim S.K., Haldimann A. and Wanner B.L. 1996. Conditionally replicative and conjugative plasmids carrying *lacZ* alpha for cloning, mutagenesis, and allele replacement in bacteria. Plasmid 35, 1–13.

Muller P. 2004. Use of the multipurpose transposon Tn KPK2 for the mutational analysis of chromosomal regions upstream and downstream of the *sipF* gene in *Bradyrhizobium japonicum*. Molecular Genetics and Genomics 271, 359–366.

Pobigaylo N., Wetter D., Szymczak S., Schiller U., Kurtz S., Meyer F., Nattkemper T.W. and Becker A. 2006. Construction of a large signature-tagged mini-Tn*5* transposon library and its application to mutagenesis of *Sinorhizobium meliloti*. Applied and Environmental Microbiology 72, 4329–4337.

Quandt J., Clark R.G., Venter A.P., Clark S.R., Twelker S. and Hynes M.F. 2004. Modified RP4 and Tn*5*-Mob derivatives for facilitated manipulation of large plasmids in Gram-negative bacteria. Plasmid 52, 1–12.

Quandt J. and Hynes M.F. 1993. Versatile suicide vectors which allow direct selection for gene replacement in gram-negative bacteria. Gene 127, 15–21.

Reeve W., Chain P., O'Hara G., Ardley J., Nandesena K., Brau L., Tiwari R., Malfatti S., Kiss H., Lapidus A., Copeland A., Nolan M., Land M., Hauser L., Chang Y.J., Ivanova N., Mavromatis K., Markowitz V., Kyrpides N., Gollagher M., Yates R., Dilworth M. and Howieson J. 2010a. Complete genome sequence of the *Medicago* microsymbiont *Ensifer* (*Sinorhizobium*) *medicae* strain WSM419. Standards in Genomic Sciences 2, 77–86.

Reeve W., O'Hara G., Chain P., Ardley J., Brau L., Nandesena K., Tiwari R., Copeland A., Nolan M., Han C., Brettin T., Land M., Ovchinikova G., Ivanova N., Mavromatis K., Markowitz V., Kyrpides N., Melino V., Denton M., Yates R. and Howieson J. 2010b. Complete genome sequence of *Rhizobium leguminosarum* bv. *trifolii* strain WSM1325, an effective microsymbiont of annual Mediterranean clovers. Standards in Genomic Sciences 2, 347–356.

Reeve W., O'Hara G., Chain P., Ardley J., Brau L., Nandesena K., Tiwari R., Malfatti S., Kiss H., Lapidus A., Copeland A., Nolan M., Land M., Ivanova N., Mavromatis K., Markowitz V., Kyrpides N., Melino V., Denton M., Yates R. and Howieson J. 2010c. Complete genome sequence of *Rhizobium leguminosarum* bv *trifolii* strain WSM2304, an effective microsymbiont of the South American clover *Trifolium polymorphum*. Standards in Genomic Sciences 2, 66–76.

Reeve W.G., Tiwari R.P., Worsley P.S., Dilworth M.J., Glenn A.R. and Howieson J.G. 1999. Constructs for insertional mutagenesis, transcriptional signal localization and gene regulation studies in root nodule and other bacteria. Microbiology 145, 1307–1316.

Selbitschka W., Niemann S. and Pühler A. 1993. Construction of gene replacement vectors for Gram- bacteria using a genetically modified *sacRB* gene as a positive selection marker. Applied Microbiology and Biotechnology 38, 615–618.

Selvaraj G. and Iyer V.N. 1983. Suicide plasmid vehicles for insertion mutagenesis in *Rhizobium meliloti* and related bacteria. Journal of Bacteriology 156, 1292–1300.

Sessitsch A., Wilson K.J., Akkermans A.D. and de Vos W.M. 1996. Simultaneous detection of different *Rhizobium* strains marked with either the *Escherichia coli gusA* gene or the *Pyrococcus furiosus celB* gene. Applied and Environmental Microbiology 62, 4191–4194.

Simon R. 1984. High frequency mobilization of gram-negative bacterial replicons by the in vitro constructed Tn*5*-Mob transposon. Molecular and General Genetics 196, 413–420.

Simon R., O'Connell M., Labes M. and Puhler A. 1986. Plasmid vectors for the genetic analysis and manipulation of rhizobia and other gram-negative bacteria. Methods in Enzymology 118, 640–659.

Simon R., Quandt J. and Klipp W. 1989. New derivatives of transposon Tn*5* suitable for mobilization of replicons, generation of operon fusions and induction of genes in gram-negative bacteria. Gene 80, 161–169.

Smith L.M., Sanders J.Z., Kaiser R.J., Hughes P, Dodd C., Connell C.R., Heiner C., Kent S.B. and Hood L.E. 1986. Fluorescence detection in automated DNA sequence analysis. Nature 321, 674–679.

Spaink H.P., Okker R.J.H., Wijffelman C.A., Pees E., Lugtenberg B.J.J. 1987. Promoters in the nodulation region of the *Rhizobium-leguminosarum* Sym Plasmid Prl1jI. Plant Molecular Biology 9, 27–39.

Stanley J. and Cervantes E. 1991. Biology and genetics of the broad host range *Rhizobium* NGR234. *Journal of* Applied Bacteriology 70, 9–19.

Wilson K.J., Sessitsch A., Corbo J.C., Giller K.E., Akkermans A.D. and Jefferson R.A. 1995. beta-Glucuronidase (GUS) transposons for ecological and genetic studies of rhizobia and other gram-negative bacteria. Microbiology 141, 1691–1705.

Xi C., Lambrecht M., Vanderleyden J. and Michiels J. 1999. Bi-functional *gfp-* and *gusA*-containing mini-Tn*5* transposon derivatives for combined gene expression and bacterial localization studies. Journal of Microbiological Methods 35, 85–92.

Yost C.K., Del Bel K.L., Quandt J. and Hynes M.F. 2004. *Rhizobium leguminosarum* methyl-accepting chemotaxis protein genes are down-regulated in the pea nodule. Archives of Microbiology 182, 505–513.

Methods for isolation of RNA from rhizobia

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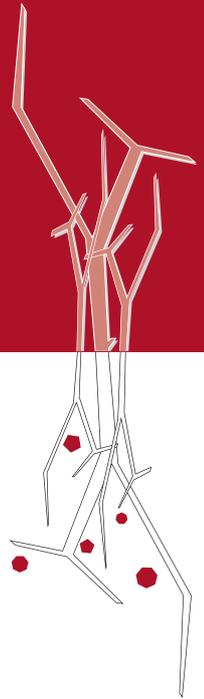
13.1 Introduction

This chapter provides methods for the isolation of RNA from rhizobia under a variety of growth conditions. There are many ways in which this RNA can be used, including Q-RT-PCR, microarray analysis and for RNA sequencing (RNAseq). We do not provide detailed protocols for these downstream applications as these are procedure specific and methods are changing very rapidly. However, isolation of high-quality RNA is key for all applications, representing the transcriptome of living cells that in many cases will be actively growing. This may be central in choosing to isolate RNA as well as, or instead of DNA, which may come from living or dead cells. Consider for example whether amplification of the 16S rRNA gene from a complex microbial community (*e.g.* soil or rhizosphere) will give the same community profile when DNA or cDNA derived from RNA is used as the target nucleic acid. Amplification from DNA will give the community of live and dead bacteria while cDNA will highlight the living community. However, many of the RNA procedures are prohibitively expensive. Ask whether you need to use RNA-based procedures or if DNA-based procedures will suffice. Further, procedures that work routinely in the best equipped molecular biology laboratories may be difficult to establish when setting up a laboratory from scratch. In spite of these caveats, the power of modern RNA-based methods, which can lead to a metatranscriptome analysis of the rhizosphere or soil, is remarkable. Choose wisely and consider your hypothesis carefully.

13.2 Isolation of total and small RNA (sRNA) from free-living rhizobia

In this first protocol, a commercial RNA isolation kit is used to produce very high-quality RNA suitable for almost all downstream applications. It has been adapted for use with rhizobia but should work with most bacteria and should be used as your first choice. However, some rhizobia are very resistant to isolation of RNA and in the following protocol (13.2) an alternative hot SDS procedure is given.

13.2.1 General preparation for RNA isolation

▶ All Gilson tips should be filter barrier tips guaranteed to be RNA free. Pipettes should be wiped with RNA Zap before working and laid on paper towel.

▶ Benches should be scrupulously clean (wipe with 70% ethanol before use) and gloves must be worn at all times; you are one of the main sources of RNAases.

▶ As a rule, avoid making solutions; wherever possible buy commercial reagent solutions guaranteed to be RNAase free.

▶ If it is unavoidable to weigh-out a chemical then spatulas must be dry-oven baked or wiped with RNA Zap to remove RNAases before use. Make sure the chemical is weighed into an RNAase-free container (*e.g.* Falcon tube) and add RNAase-free water (*e.g.* Sigma water cat. no. (catalogue number) W4502).

▶ Containers should be disposable plastic ware (such as Falcon tubes) but if equipment or glassware needs to be used then soak it in 0.1 N NaOH with 1 mM EDTA before washing with RNAase-free water (*e.g.* Sigma water cat. no. W4502). Use sterile Falcon tubes to measure out large volumes and dispose of them after each use.

▶ Use Amber Eppendorf tubes that are supplied sterile and guaranteed RNAase free (*e.g.* Eppendorf 0030 120 191).

▶ The recipes for RNAase inhibitor solution and how to prepare Fast Prep lysing tubes can be found in Appendices 13.10.1 and 13.10.2.

13.2.2 Isolation of total and small RNA (sRNA) from free-living cells using Qiagen RNeasy Plus Micro kit (Cat No: 74034)

Reagent preparation

▶ Add 10 μL 2-mercaptoethanol per 1 mL Buffer RLT Plus (from kit). Dispense in a fume hood. Buffer RLT Plus is stable at room temperature (15–25°C) for one month after addition of 2-mercaptoethanol.

▶ Buffer RLT Plus may form a precipitate upon storage. If necessary, re-dissolve by warming and then place at room temperature.

▶ Buffer RLT Plus and Buffer RW1 contain a guanidine salt and are therefore not compatible with disinfecting reagents containing bleach.

▶ Perform all steps of the procedure at room temperature. During the procedure, work quickly.

▶ Perform all centrifugation steps at 20–25°C in a standard microcentrifuge.

▶ Ensure that the centrifuge does not cool below 20°C.

▶ Prepare 10 mM Tris-Cl pH 8.0 (500 µL for each RNA preparation) from 1M Tris-Cl pH 8.0 stock (Sigma RNAase free cat. no. T2694) by diluting with Sigma (cat. no. W4502) RNAase-free water. Prepare the dilution in a sterile Falcon tube.

Procedure

1. Grow rhizobia according to your normal laboratory procedure. While the precise conditions will depend on the experiment, it is important to conduct preliminary growth experiments. For microarray and RNAseq experiments, it is quite important to harvest batch-cultured bacteria early in exponential growth phase to avoid inconsistent gene expression and shut-down of ribosomal protein synthesis.

2. Add 24 mL of RNAase inhibitor to a 50 mL Sorval SS34 centrifuge tube. (The Sorval tube must have been cleaned and autoclaved from a batch of tubes kept for RNA work, but the tubes will not be RNAase-free so the addition of RNAase inhibitor is essential to inactivate RNAases).

3. Add 12 mL of cells of 0.4–0.6 OD (600 nm), vortex for five seconds and incubate for five minutes at room temperature.

4. Harvest the cells at 10,000 rpm at 4°C for 10 minutes in the Sorval SS34 rotor.

5. Decant the supernatant and carefully dry the tube on a filter paper.

6. Add 250 µL of 10 mM Tris-HCl pH 8.0 and re-suspend the bacteria.

7. Add the re-suspended cells to 350 µL of pre-cooled RLT Plus buffer (with 2-mercaptoethanol) in the FastPrep Tube (either use commercial FastPrep lysing matrix B or prepare your own tubes as described above).

8. Lyse the cells in Fast Prep instrument (MP Bio 29525 Fountain Parkway Solon, OH 44139 United States) at speed 6.5 for 30 seconds. If a Fast Prep is not available, cells can be lysed with lysozyme at Step 6 modified as follows: add 250 µL of 10 mM Tris-HCl pH 8.0 containing 400 µg/mL lysozyme. Incubate at room temperature for 15 minutes. Add 350 µL of pre-cooled RLT Plus buffer (with

2-mercaptoethanol) and proceed to Step 9. However, RNA yields are not as good with the lysozyme procedure.

9. Incubate the tube on ice for three minutes. Steps 10–19 that follow are common for all RNA isolation procedures.

10. Spin tubes for three minutes at 13000 rpm in a microfuge at 4°C. Carefully remove the supernatant by pipetting and transfer 600 μL to a Qiagen gDNA Eliminator spin column placed in a 2 mL collection tube.

11. Spin for 30 seconds at 10,000 rpm in a microfuge. Transfer the flow-through from the 2 mL collection tube to a sterile 15 mL Falcon tube. Add any remaining solution from Step 10 to the gDNA eliminator column and centrifuge again. Add the flow-through to the 15 mL Falcon tube and discard the gDNA eliminator column. Measure the total flow-through volume.

12. Add one volume (approximately 1500 μL) of 70% (v/v) ethanol to the flow-through and mix well by pipetting. Transfer up to 700 μL of the sample, including any precipitate that may have formed, to an RNeasy spin column placed in a 2 mL collection tube. (Columns should be stored in the fridge).

13. Close the lid gently and centrifuge for 15 seconds at 10,000 rpm. Discard the flow-through. Repeat this step, adding 700 μL of sample from Step 12 until the entire sample has been added to the column.

14. Add 700 μL Buffer RW1 to the RNeasy spin column. Close the lid gently and centrifuge for 15 seconds at 10,000 rpm in a microfuge to wash the spin column membrane. Discard the flow-through.

15. Add 500 μL Buffer RPE to the RNeasy spin column. Close the lid gently and centrifuge for 15 seconds at 10,000 rpm in a microfuge to wash the spin column membrane. Discard the flow-through.

16. Add 500 μL Buffer 80% Ethanol to the RNeasy spin column. Close the lid gently and centrifuge for two minutes at 10,000 rpm in a microfuge to wash the spin column membrane. Discard the collection tube.

17. Place the column in a new collection tube (provided in the Qiagen kit), open the lid and centrifuge at 10,000 rpm in a microfuge for five minutes.

18. Place the RNeasy spin column in a new 1.5 mL collection tube.

19. Add 35 μL RNase-free water directly to the spin column membrane, Close the lid gently and centrifuge for one minute at 10,000 rpm to elute the RNA.

13.2.3 Measuring quantity and integrity of RNA

The RNA prepared above is suitable for use in a variety of applications, including Q-RT-PCR, microarray analysis or RNAseq. However, before being used for any downstream application, it must be quantified and its integrity checked. To determine the concentration and purity of RNA, obtain A_{260} and A_{280} readings in

0.1× TE Buffer on a spectrophotometer. This should only be attempted for samples with predicted sufficient yield. For example, for an RNA sample (400–500 μL) placed in a 1 mL cuvette (1 cm path length), a concentration of approximately 4 μg/mL is typically required to obtain A_{260} readings of 0.1 or higher. It is strongly recommended that RNA is quantified using the Bio-Rad Experion, Agilent Bioanalyzer or equivalent system. These systems can also be used to estimate yield from quantities as small as 10 ng. An additional reason for using these systems is that they will show whether the RNA is intact or degraded (Figure 13.1 A, B).

Use the following equation to calculate RNA concentration:

Concentration (μg/mL) = A_{260} × DF (dilution factor) × 40.

Calculate the A_{260}/A_{280} ratio to determine RNA purity. A ratio of 2.0–2.3 is most desirable and indicates high-quality RNA with little contaminating protein.
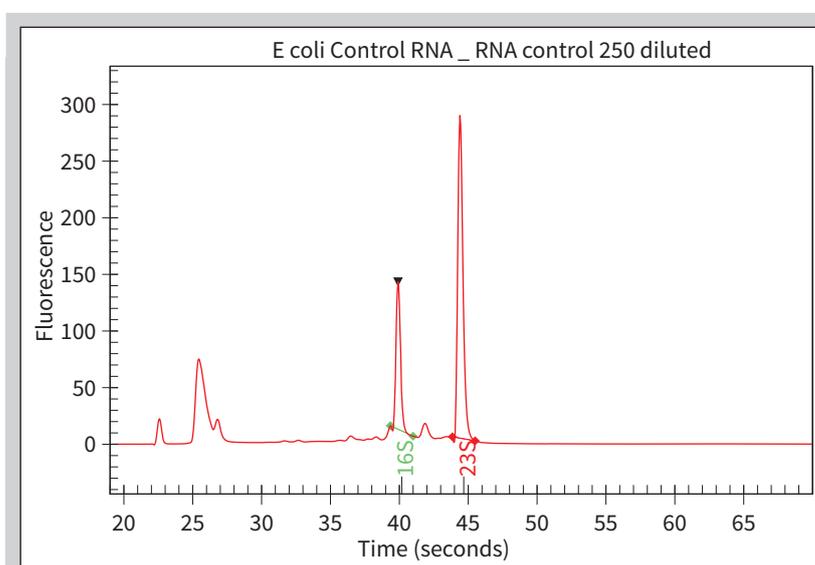
Figure 13.1 A An Experion profile of high-quality RNA from *E. coli.* Note the very sharp 23S and 16S rRNA peaks. Broadening of these peaks indicates RNA degradation.
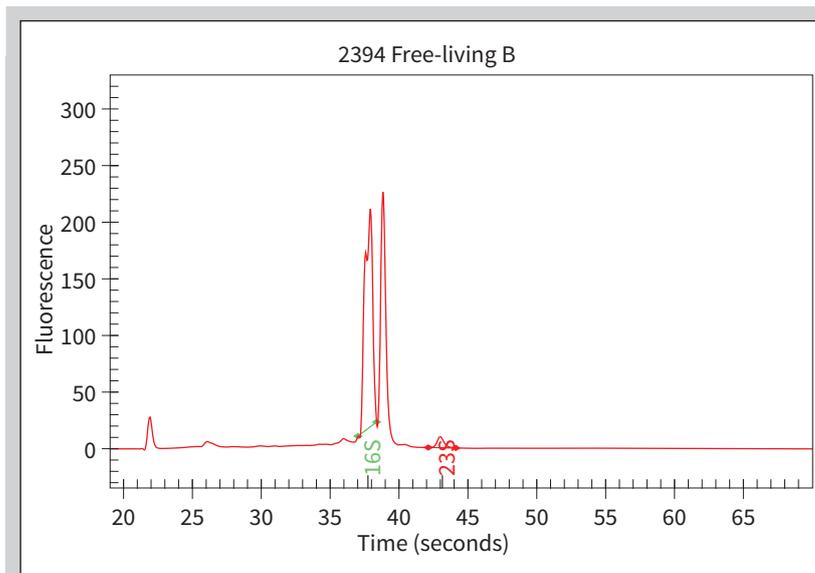
Figure 13.1 B An Experion profile of high-quality RNA from *R. leguminosarum.* Note the very small 23S rRNA peak. Many, but not all, rhizobia have an RNAase processing site in the middle of the 23S peak. Notice there is a new peak to the left of the 16S peak. It is actually a doublet of two 1.3 kb peaks formed by processing of 23S rRNA.

13.3 Protocol for isolation of total and small RNA (sRNA) from the rhizosphere of legumes

This protocol describes how to set up a rhizosphere colonisation experiment for small- and large-seeded legumes. It was designed so that microarray or RNAseq experiments could be conducted on bacteria recovered from the rhizosphere after a suitable incubation. However, similar procedures can be used to score bacterial colonisation by culturing bacteria rather than isolating their RNA. Which protocol to use will depend on the precise legume and inoculation. The large-seeded legume protocol is slightly simpler, but either procedure should work for most legumes if preliminary growth trials are conducted. If RNA recovery is insufficient, grow the plant for longer, either before or after bacterial inoculation, to enable a stronger root system to develop. In these protocols, various legumes are grown in fine or medium grade vermiculite. The appropriate grade for a particular legume should be tested in a growth experiment. While vermiculite is used, there is no fundamental reason why other media, such as sand, perlite or leca could not be used, although this needs to be tested; *i.e.* use the best sterile growth medium for the legume to be grown. However, to isolate bacterial RNA from the rhizosphere of plants grown in soil, use soil isolation procedures such as the commercial Mo Bio Power Soil, which works well.

13.3.1 General preparation

▶ Ensure seeds are germinable, surface sterilised and sort them for quality as described in Chapter 5, Section 5.8.

▶ Prepare a nitrogen-free rooting solution, as described in Appendix 13.10.3 or in Chapter 5, Section 5.7.

13.3.2 Procedure for large-seeded legumes such as pea

1. Add 10 mL of rooting solution to 25 mL vermiculite (fine grade, do not pack tightly) in a 50 mL Falcon tube, cap loosely and autoclave.

2. Sow surface sterilised seeds 1–2 cm below the vermiculite and incubate in the growth room for seven days (keep the tube open). The caps can be left off, leaving the Falcon tubes open, but holes can be drilled in the lid to allow the shoot to grow (see small-seeded legumes). The advantage of capping is that it reduces contamination with other bacteria.

3. Streak rhizobial strains on TY agar slopes four days after seed germination.

4. On the seventh day after seed germination, re-suspend the slope inoculated with a strain of rhizobia in 5 mL sterile water and wash the cells three times in sterile water, recovering cells each time by centrifuging at 4,000 rpm for 10 minutes. You may wish to wash in your standard sterile minimal medium

rather than water, *e.g.* JMM (Chapter 3) but omitting nitrogen and carbon sources from the minimal medium.

5. Measure the OD_{600} and adjust the cell count to 10^8 cells mL^{-1} (Chapter 6). *Rhizobium leguminosarum* has approximately 10^9 cells/mL at an OD_{600} of 1. This should be validated for each laboratory spectrophotometer and strain.

6. Inoculate each seeding with 1 mL containing 10^8 cells. It should be noted that 10^8 bacteria is a large inoculum. Inocula as low as 10^3 bacteria can produce excellent microarray results when peas are harvested seven days post inoculation. However, low inocula will not yield sufficient RNA for successful experiments if plants are harvested after a short time interval (*e.g.* one day) or if there is a weak root system. It can also be very challenging to maintain sterility in long-term plant growth experiments, and if a low inoculum is used, your organism may be overgrown by contaminants.

7. The standard incubation time of seven days produces excellent results for pea. However, peas have been harvested at one and three days with excellent results. The only real limit to the bacterial inoculation and plant growth time is whether sufficient RNA can be isolated from rhizosphere bacteria.

13.3.3 Procedure for small-seeded legumes such as clover

1. After surface sterilisation (Chapter 5) place seeds on water agar and wrap plate in foil. After the seeds have fully imbibed (approximately three hours) invert the plates.

2. Leave at room temperature in the dark for one to two days, checking for root development daily.

3. Place plates at 4°C to slow growth if required.

4. Wash medium-size (grade 3) vermiculite, then oven dry at 60°C overnight.

5. Drill a central hole into the white cap of a McCartney vial (see photo in Figure 13.2).

6. Add nitrogen-free rooting solution to wet the vermiculite in a bucket.

7. Fill McCartney vials (use acid-washed, rinsed and dried vials only) to the very top with the medium-wet vermiculite. While McCartney or Universal bottles (name depends on local usage) were used, it should be possible to use other sterile containers such as Falcon tubes,

8. Place the caps with the central hole on to the vials. Excess solution should come out of the holes in the cap.

9. Place vials in a rack and into an autoclave bag to sterilise on a fluid cycle (121°C for 15 minutes).

10. Store vials within the closed autoclaved bags in the fridge to cool.

11. Starter nitrogen (4 mL per vial of 200 mg/L NH_4NO_3) may need to be added for some small-seeded legumes, but is not required for *T. polymorphum* or *T. purpureum* or usually for large-seeded legumes.

12. Make a hole in the vermiculite through the cap hole using a sterile toothpick.

13. Place pre-germinated seeds so that the top of the seed is just sitting above cap level.

14. Place KimCare wipes over the vials and dampen with sterile water.

15. Place tray of vials in the autoclave bag and transfer to growth room for two to three days.

16. Remove vials from bag and remove tissues gently. Seed coats should be removed by this process but any remaining seed coats should be removed with sterile tweezers.

17. After five days of growth in the growth room (seven days post-plate germination) select uniform seedlings and inoculate seedlings with 1 mL of a culture of rhizobia via the hole in the cap. Typically 10^8 cells will be inoculated (see large-seeded legumes above) but this can be varied depending on the experiment.

18. Vials will need to be watered once in a three-week period (maximum 30 mL water).

19. A Schott bottle with auto dispenser attached with hose and needle will assist with large-scale watering (sterilise needle with ethanol and flame before each watering). Alternatively, on a smaller scale, a sterile pipette tip can be used for each vial.

20. Seedlings are typically grown for seven days in a growth room at 22°C with a 16 hour light/eight hour dark cycle before isolation of rhizosphere bacteria. However, the precise timing depends on the experiment. Growth may need to be extended for slow-growing legumes.
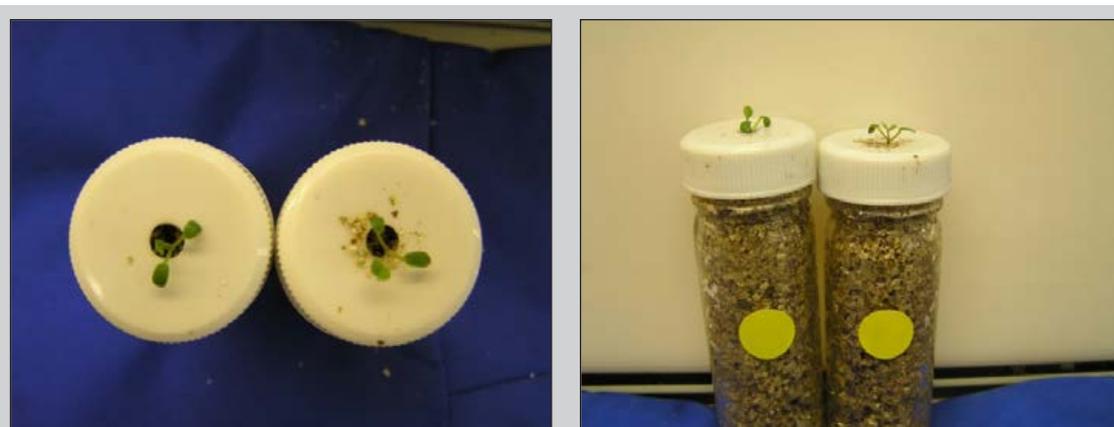
Figure 13.2   Germinated *Trifolium polymorphum* seedlings in the vial system

13.3.4 RNA isolation from rhizosphere bacteria using Qiagen RNeasy PLUS Micro kit

General preparation

▶ Prepare 10 mM Tris-Cl pH 8.0 from 1 M Tris-Cl pH 8.0 (Sigma) stock. (Dilute 100 µL of 1M Tris to 10 mL of sterile water).

▶ Aliquot the required amount of RLT Plus buffer (Qiagen RNeasy PLUS Micro kit) cat. no. 74034 (500 µl for three plants) and add 10 µL 2-mercaptoethanol (2-ME) (Sigma) to 1 mL of RLT Plus buffer.

▶ Add 500 µL RLT Plus-2-ME buffer into the Fast Prep Lysing tube containing FastPrep matrix B (Thermo) and incubate in ice. Alternatively, you can prepare your own FastPrep lysing tubes as described in Appendix 13.10.2.

Procedure

1. Autoclave muslin cloth funnels and water. Treat funnels with RNase ZAP (Ambion) before use.

2. Cut and discard the shoot. Add 6 mL of sterile water and 12 mL of RNase inhibitor to the Falcon tube containing roots and vermiculite.

3. Vortex immediately for five minutes.

4. Pool the vortexed solution from three plants, *e.g.* pea (for small plants this can be increased to six to eight) and immediately filter the mix through four layers of muslin cloth. A note of WARNING: Miracloth is often used in place of muslin but we found that it clogs with vermiculite fines, preventing bacteria entering the filtrate and resulting in failure to recover significant RNA.

5. Spin the filtrate at 1,000 rpm, 4°C for one minute to remove any plant debris and heavy vermiculite.

6. Transfer the supernatant to a 50 mL centrifuge tube and spin at 10,000 rpm, 4°C for 10 minutes (Sorvall centrifuge—SS-34 head) to pellet the bacteria. You will also see vermiculite sticking to the bottom of the tube with the bacteria but this is not of concern.

7. Decant the supernatant and dry the tube on a filter paper.

8. Add 1,000 µL of 10 mM Tris-Cl pH 8.0, re-suspend (it will look like mud) and load everything into a Fast Prep lysing tube (either use commercial FastPrep lysing matrix B or prepare your own tubes as described in Appendix 13.10.2).

9. Lyse vermiculite-containing cells in the FastPrep (Thermo) at level 6.5 for 30 seconds. Place the tubes onto ice for one minute. Lyse again using the Fast Prep at level 6.5 for 30 seconds.

10. Incubate tubes on ice for three minutes.

11. Go to Step 10 of Protocol 13.2.2 (isolation of RNA from free-living *Rhizobium*).

13.4 Protocol for isolation of total and small RNA (sRNA) from bacteroids of rhizobia

The legume nodules are picked into liquid nitrogen, ground and bacteroids isolated by a simple differential spin. More complex procedures for isolation of bacteroids may increase their purity but may slow down the isolation procedure. Speed is important once the nodules are removed from liquid nitrogen and ground because changes in the amount of bacteroid RNA could occur. If RNA degradation is a problem, you should consider using two-parts RNAase inhibitor per part of isolation buffer for grinding of nodules (Step 3 below).

General preparation

► Prepare 10 mM Tris-Cl pH 8.0 from 1 M Tris-Cl pH 8.0 (Sigma) stock.

► Aliquot the required amount of RLT buffer (Qiagen RNeasy Plus micro kit) (700 μL for each plant) and add 10 μL 2-mercaptoethanol (Sigma) for 1 mL of RLT buffer.

► Add the 700 μL RLT-2-ME buffer to a FastPrep tube containing Lysing matrix B (Thermo) or prepare your own tubes, as described above, and incubate in ice.

► Sterile 15 mL centrifuge tubes.

► Autoclaved mortar and pestle.

► Prepare Isolation Buffer (10 mM phosphate buffer pH 7.4, 300 mM sucrose and 2 mM $MgCl_2$). (See Table 13.1).

► Phosphate buffer (Maniatis-A 1.5): Take 80.2 μL of 1 M K_2HPO_4 and 19.8 μL of 1 M KH_2PO_4 and make up to 10 mL with sterile distilled water to get phosphate buffer pH 7.4.

Table 13.1 Isolation buffer

Components	Amount (10 mL)	Amount (50 mL)
1M K_2HPO_4	80.2 μL	401 μL
1M KH_2PO_4	19.8 μL	99 μL
Sucrose	1.03 g	5.15 g
$MgCl_2 . 7(H_2O)$	4.0 mg	20 mg
Make the volume to	10 mL	50 mL

Filter sterilise the isolation buffer through a 0.22 μm filter and store at room temperature.

Procedure

1. Pick nodules (1.0 g) into liquid nitrogen.

2. Filter the nodules using a tea strainer and weigh the nodules (do this quickly).

3. Macerate 1.0 g nodules in a mortar with the pestle with 5 mL of isolation buffer. Macerate with an up and down motion; do not macerate with a circular motion as it shears the genomic DNA.

4. Add 1 mL of isolation buffer if the macerated mix is too thick.

5. Transfer the mix to a 15 mL Falcon tube.

6. Spin at 1,000 rpm for five minutes (to remove plant debris).

7. Carefully transfer the supernatant to a 1.5 mL microfuge tube.

8. Spin at 6,000 rpm for five minutes and discard the supernatant.

9. The pellet contains the bacteroids.

10. Re-suspend the pellet in 250 µL of 10 mM Tris-Cl pH 8.0. Go straight to Step 7 of the RNA isolation procedure for free-living bacteria (Protocol 13.2.2). It is important to go immediately to Step 7 because in this step the bacteroids are added to RLTplus buffer which prevents RNA degradation.

13.5 Protocol for preparation of DNA-free RNA for use in Q-RT-PCR by treatment with Ambion Turbo DNA free

You should now have very pure intact RNA which is suitable for Q-RT-PCR. There are many variations of Q-RT-PCR so you should consult the manuals of your Q-PCR machine and molecular biology suppliers of standard kits. However, it is crucial to remove all traces of genomic DNA (gDNA) and Ambion's Turbo DNAase is particularly effective.

Procedure

1. Set up a reaction in a 0.5 mL RNAase-free tube with the additions from Table 13.2.

Table 13.2   Components required to prepare DNA-free RNA

Components	Volume
Total RNA with contaminating gDNA	1–44 µL
RNase-free water	0–43 µL
0.1 vol of 10 × Turbo DNase buffer	5 µL
Turbo DNase (2U/µL)	1 µL
Final volume	50 µL

2. Incubate at 37°C for 60 minutes.

3. Add 0.1 volume of DNAase inactivation reagent.

4. Incubate for two minutes at room temperature with occasional mixing.

5. Spin at 11,000 rpm for two minutes at room temperature.

6. Transfer the supernatant to a new tube without disturbing the DNAase inactivation reagent.

13.6 Protocol for SenseAmp—linear RNA amplification

This procedure enables small quantities of RNA to be highly amplified by incorporating a T7 promoter into the RNA. The final product is a sense strand RNA that can be used directly to produce fluorescently-labelled cDNA for microarray analysis. In this book, it is used for the production of single-stranded antisense cDNA that is chemically labelled with cy dyes for use in microarrays. The protocol is based on the commercial kit SenseAmp, available from Genisphere. This kit is used because it produces sense stranded RNA which can be used directly for reverse transcription to produce cDNA for use in microarrays. Ambion produce a similar kit which can be used to produce amplified RNA (aRNA) although it is in the antisense orientation. Ultimately, which kit you choose to use will depend on your final application.

Reagent preparation

▶ Thaw Vials 1, 2, 6, 8, 10, 11 and 12 as provided in the Genisphere kit at room temperature, vortex and briefly microfuge.

▶ Thaw Vials 3, 4, 5, 7, 9, and 13 on ice, briefly microfuge if necessary and keep on ice at all times. Do not vortex.

▶ Reverse transcribe up to 0.25 μg of total RNA using the provided random primer (Vial 2) in a total volume of 20 μL.

▶ The random primer should be used at 2× by mass to the input total RNA (for example, use 2 μL of Random Primer (0.5 μg) per 0.25 μg of total RNA).

Procedure

13.6.1 First strand cDNA synthesis with random primers

1. For each RNA sample, prepare the following RNA/primer mix in a tube on ice: 1–9 μL total RNA (do not exceed 0.25 μg).

2. Add 2 μl Random 9mer RT primer (Vial 2); use RT primer at 2× by mass of RNA.

3. Add Nuclease Free Water (Vial 10) to a volume of 11 μL.

4. Heat to 80°C for 10 minutes. Keep on ice immediately for two minutes. Briefly microfuge and return to ice.

5. For each reaction, prepare a Master Mix in a separate tube on ice:

    ▷ 4 μL 5× First Strand Buffer

    ▷ 2 μL 0.1 M DTT

    ▷ 1 μL Superase-In (Vial 4)

    ▷ 1 μL dNTP Mix (Vial 3)

    ▷ 1 μL Superscript II (Invitrogen)

    ▷ 9 μL total volume.

6. Combine the Master Mix and the RNA/primer mix for a volume of 20 μL. Mix gently and microfuge. Incubate at 42°C for two hours. Microfuge briefly after incubation.

7. Add 80 μL of 1× TE buffer for a final volume of 100 μL.

13.6.2 Purification of cDNA

Purify the 100 μL of cDNA produced above using the Qiagen MinElute PCR Purification Kit (Catalog # 28006) as follows.

1. Add 500 μL Buffer PB to the 100 μl cDNA sample and mix.

2. Apply the cDNA mixture to the MinElute column and centrifuge for one minute at 13,000 rpm in a microcentrifuge.

3. Discard the flow-through. Place the MinElute column into the same collection tube.

4. Add 750 μL Buffer PE to the MinElute column and centrifuge for one minute.

5. Discard the flow-through. Place the MinElute column back into the same collection tube.

6. Add 500 μL 80% ethanol to the MinElute column and centrifuge for two minutes.

7. Discard the flow-through. Place the MinElute column back into the same collection tube.

8. Open the column caps and place in a microfuge with the cap opposite the direction of the rotation of the rotor to avoid breaking the cap off. Centrifuge for five minutes.

9. Place the MinElute column into a clean, labelled 1.5 mL microfuge tube.

10. To elute cDNA, add 10 µL Buffer EB to the center of the column membrane. Incubate at room temperature for two minutes. Centrifuge for two minutes. Discard the column and save the 10 µL eluted cDNA. If the eluted cDNA is less than 10 µL, increase the volume to 10 µL with Nuclease Free Water (Vial 10).

13.6.3 Tailing of first strand cDNA

Caution: do not exceed 2 µg equivalent of dT-primed total RNA (or 0.25 µg random- or random/dT-primed total RNA) for the tailing reaction.

1. Heat purified cDNA (10 µL) to 80°C for 10 minutes. Add immediately to ice for one to two minutes. Briefly microfuge and return to ice.

2. For each reaction, prepare a Master Mix in a separate tube on ice:

    ▷ 2 µL 10× Reaction Buffer (Vial 6)

    ▷ 2 µL Nuclease Free Water (Vial 10)

    ▷ 4 µL 10mM dTTP (Vial 5)

    ▷ 2 µL TdT Enzyme (Vial 7)

    ▷ 10 µL Total volume

3. Combine the Master Mix and the cDNA for a volume of 20 µL. Mix gently and microfuge.

4. Incubate in a 37°C heat block for three minutes. Do not exceed three minutes.

5. Stop the reaction by heating to 80°C for 10 minutes. Briefly microfuge and cool to room temperature for one to two minutes.

13.6.4 T7 Promoter synthesis

NB: when amplifying less than 10 ng total RNA, or when performing two rounds of SenseAmp amplification, diluting the T7 Template Oligo (Vial 8) in the first amplification round may help to reduce T7 amplification artefacts commonly associated with amplification of very small sample sizes and with multiple rounds of amplification.

1. For amplification of total RNA samples greater than 10 ng, add 2 µL of T7 Template Oligo (Vial 8) to the tailed cDNA for a volume of 22 µL. Briefly vortex and microfuge.

2. Incubate at 37°C for 10 minutes to anneal the strands.

3. To each reaction, add the following components for a volume of 25 µL:

    ▷ 1 µL 10× Reaction Buffer (Vial 6)

    ▷ 1 µL dNTP mix (Vial 3)

    ▷ 1µL Klenow Enzyme (Vial 9).

4. Mix gently and microfuge. Incubate at room temperature for 30 minutes.

5. Stop the reaction by heating to 65°C for 10 minutes. Place on ice.

6. Option 1 (recommended): proceed to the in vitro transcription reaction using half (12.5 µL) of the promoter-modified cDNA. Save the remaining modified cDNA at −20°C for future use or for use in a parallel amplification reaction.

   Option 2: The entire 25 µL of promoter-modified cDNA may be used in the *in vitro* transcription reaction but it must be purified first. Add 75 µL of 1× TE Buffer for a final volume of 100 µL and purify using the MinElute PCR Purification Kit as directed in the cDNA purification step. Add Nuclease Free Water (Vial 10) to adjust the volume to 11.5 µL. Add 1 µL of the T7 Template Oligo (Vial 8) to the purified cDNA for a volume of 12.5 µL.

13.6.5 In vitro transcription

1. Incubate the 12.5 µL of cDNA at 37°C for 10 minutes to re-anneal the strands.

2. Thaw the T7 Nucleotide Mix (Vial 11) and 10× T7 Reaction Buffer (Vial 12) at room temperature and keep at room temperature until use. Thoroughly vortex the 10× T7 Reaction Buffer (Vial 12) to avoid precipitation of buffer components.

3. For each reaction, add the following components at room temperature, for a final volume of 25 µL:

   ▷ 8.0 µL T7 Nucleotide Mix (Vial 11)

   ▷ 2.5 µL 10× T7 Reaction Buffer (Vial 12)

   ▷ 2.0 µL T7 Enzyme Mix (Vial 13).

4. Mix gently and microfuge. Incubate in a thermocycler (with heated lid) at 37°C for four to 16 hours. Or, place the reaction in a 37°C heat block for five minutes and then transfer to a 37°C air hybridisation oven for four to 16 hours. It is essential to avoid evaporation and condensation of the reaction during this step.

13.6.6 Purification of senseRNA

Purify senseRNA using the RNeasy Kit (Qiagen cat. no. 74106) following Qiagen's protocol for RNA Cleanup.

1. Add 75 µL of RNAase-free water to the reaction mix.

2. Add 350 µL of RLT buffer (+2-ME) and mix well by pipetting.

3. Add 250 µL of 95% (v/v) ethanol and mix well by pipetting. Immediately add to the purification column.

4. Spin the column at 12,000 rpm for 30 seconds.

5. Add 500 μL of RPE buffer and spin at 12,000 rpm for 15 seconds; repeat the step twice.

6. Discard the collection tube and place the column in a new collection tube, and spin at 12,000 rpm for two minutes.

7. Add 50 μL of RNAase-free water, incubate at room temperature for two minutes and spin at 12,000 rpm for one minute.

8. Add the eluted 50 μL RNA to the column, incubate for two minutes and spin at 12,000 rpm for one minute for maximum recovery of senseRNA.

9. Quantitate the RNA as described above. An OD_{260}/OD_{280} ratio of 2.0–2.3 is most desirable. Higher ratios may indicate that an excessive poly (A) tail was generated during the amplification reaction.

13.7 Protocol for indirect labelling of first–strand cDNA synthesis using GE healthcare Cyscribe Post-Labelling Kit

This protocol describes how to reverse transcribe RNA into cDNA for microarray or other experiments that require a DNA template. The protocol uses a secondary coupling procedure where RNA is reverse transcribed to cDNA with the use of aminoallyl UTP to which either cy3 or cy5 can be subsequently chemically coupled. Thus, it is optimally adapted for microarray use. It is included here because cy labelling of DNA is still an important technique, even though the use of microarrays is now waning as people switch to RNAseq.

Reagent preparation

▶ Cyscribe Post-Labelling kit with Cyscribe GFX purification kit—GE Healthcare Cat No RPN 5660X.

▶ Cyscribe GFX purification kit Cat No 27-9606-02 (optional).

▶ 2.5 M NaOH (1 g/10 mL sterile Milli-Q water; filter sterilise with 0.45 μm filter).

▶ 2M HEPES (4.77 g/10 mL sterile Milli-Q Water; filter sterilise with 0.45 μm filter).

▶ 0.1 M Na_2CO_3 (Sigma S7795) 0.53 g/50 mL sterile Milli-Q water.

▶ 0.1 M sodium bicarbonate pH 9.0 (Sigma S6297): 4.2 g in 500 mL sterile Milli-Q water; adjust to pH 9.0 with 0.1 M Na_2CO_3 (25–30 mL). Filter sterilise and dispense into aliquots and store at –20°C for up to two months.

▶ Ethanol absolute (Sigma-Aldrich 32221) diluted to 80% (v/v) ethanol.

▶ 4 M Hydroxylamine hydrochloride Aldrich 15941-7 (2.78 g in 100 mL sterile Milli-Q Water). **Make fresh every time.**

▶ Heating block set at 65°C, 70°C, and water bath at 42°C.

▶ Microcentrifuge.

▶ RNAase-free filter tips.

▶ Spectrophotometer.

▶ Microcuvettes (Brand UV-cuvettes) Cat No Fischer SUPC CXA-205-020F.

▶ Nitrile Gloves.

▶ *NB:* prepare a 100 mM aa-dUTP solution as follows: dissolve 1 mg of aa-dUTP in 17 μL DEPC-treated water (Sigma RNAase Free Water) and 0.68 μL 1 M NaOH. Measure pH with a drop on a pH filter paper and immediately adjust to pH 7.0 using 0.1–0.2 μL of 1M NaOH. Store aa-(dUTP) solutions at −20°C.

Table 13.3 Composition of the mix (50X)

Components	Volume	Final Concentration
100 mM dATP	10.0 μL	25 mM
100 mM dGTP	10.0 μL	25 mM
100 mM dCTP	10.0 μL	25 mM
100 mM dTTP	2.0 μL	5 mM
aa-dUTP	8.0 μL	20 mM

Procedure

13.7.1 Primer annealing

1. Add the following components to a 1.5 mL microcentrifuge tube on ice. The RNA may need to be concentrated in a Speed Vac. Wipe out machine with RNAZap and set temperature to 37°C. To reduce 16.5 μL to 11.5 μL will take about five minutes; to reduce 22 μL to 11.5 μL will take about 15 minutes. Do not dry the sample. Reduce volume to slightly less than 11.5 μL then make up to 11.5 μL with sterile water.

Total RNA (10–15 μg)	11.5 μL
Random Nanomers (from Cyscribe kit)	1 μL

Mix gently by pipetting up and down.

2. Incubate the reaction at 70°C for five minutes.

3. Cool reaction at RT for 10 minutes to allow the primers and mRNA template to anneal.

4. Spin in a microfuge for 10 seconds to collect material.

13.7.2 Extension reactions

1. Place the cooled annealing reaction on ice and add the following components, making sure to add the enzyme last.

5× Cyscript Buffer	4 µL
0.1M DTT	2 µL
50× aa-UTP/Nucleotide mix (see Table 13.3)	0.5 µL
Cyscript RT	1.0 µL

2. Mix by stirring with the pipette tip (Cyscript is sensitive to mechanical disruption) and spin in a microfuge for 10 seconds to collect material.

3. Incubate the reaction at 42°C in a water bath for 5 hours.

13.7.3 Degradation of mRNA

1. Add 2 µL 2.5 M NaOH to each cDNA reaction.

2. Mix reaction by vortexing and spin for 15 seconds.

3. Incubate reaction at 37°C for 15 minutes.

4. Add 10 µL 2 M HEPES (free acid used to neutralise the NaOH added in Step 1) to each cDNA reaction.

5. Mix reaction by vortexing and spin for 15 seconds.

The cDNA reaction is ready for purification or can be stored at –20°C.

13.7.4 Purification of cDNA with Cyscribe GFX purification kit

1. For every cDNA reaction to be purified, place one Cyscribe GFX column into a clean collection tube. Add 500 µL of capture buffer to each Cyscribe GFX column.

2. Briefly spin down the cDNA reaction and add into Capture Buffer, pipetting up and down five times.

3. Centrifuge the column at 13,000 rpm in a microfuge for 30 seconds.

4. Discard the flow-through.

5. Add 600 µL of 80% (v/v) ethanol to each column and centrifuge the column at 13,000 rpm in a microfuge for 30 seconds.

6. Repeat Step 5 another two times.

7. Centrifuge the column at 13,000 rpm for 10 seconds to remove the residual wash buffer. Discard the collection tube.

8. Transfer the column into a new microfuge tube and add 60 µL 0.1 M sodium bicarbonate (pH 9.0) to the top of the glass fibre matrix.

9. Incubate at room temperature for one to five minutes.

10. Centrifuge the column at 13,000 rpm for one minute to collect the purified cDNA.

13.7.5 Labelling of amino-allyl modified cDNA with cy dyes

▶ Turn the lights off for all subsequent steps

1. Add (60 μL) amino-allyl modified cDNA (in 0.1 M sodium bicarbonate pH 9.0) directly into an aliquot of cyDye NHS ester (from the Cyscribe kit) and re-suspend NHS ester by pipetting several times.

2. Spin down at 13,000 rpm for one minute to collect sample at the bottom.

3. Incubate in the dark overnight (preferably in a cupboard).

4. Next day, add 15 μL 4 M hydroxylamine to each coupling reaction;

5. Mix by pipetting up and down and **INCUBATE IN DARK** at room temperature for 15 minutes.

13.7.6 Purification of Cy Dye–Labelled DNA with Cyscribe GFX purification kit

▶ Keep the Elution Buffer at 65°C.

1. For every cDNA reaction to be purified, place one Cyscribe GFX column into a clean collection tube. Add 500 μL of capture buffer to each Cyscribe GFX column.

2. Microfuge pulse the cDNA reaction (from Step 5 above) and add into Capture Buffer, pipetting up and down five times (work quickly, capture buffer will degrade cy dyes).

3. Centrifuge the column at 13,000 rpm for 30 seconds (on the membrane, Cy3 will look pink and cy5 blue). Discard the flow-through.

4. Add 600 μL of wash buffer to each column and centrifuge the column at 13,000 rpm for 30 seconds.

5. Repeat Step 4 another two times.

6. Centrifuge the column at 13,000 rpm for 10 seconds to remove the residual wash buffer. Discard the collection tube.

7. Transfer the column into a new microfuge tube and add 70 μL elution buffer (65°C) to the top of the glass fibre matrix.

8. Incubate at RT for one to five minutes.

9. Centrifuge the column at 13,000 rpm for one minute to collect the purified cDNA.

Measure the absorbance of the labelled cDNA (all 70 μL) on a spectrophotometer using a microcuvette (100 μL volume). Use the following formulae:

$$\text{pmole cy3} = \frac{OD_{550} \times \text{volume (μL)} \times 10^3}{150}$$

$$\text{pmole cy5} = \frac{OD_{650} \times \text{volume (μL)} \times 10^3}{250}$$

$$\text{pmole cDNA} = \frac{OD_{260} \times \text{volume (μL)} \times 37 \text{ ng/mL} \times 1000 \text{ pg/ng}}{324.5 \text{ pg/pmole}}$$

Calculate the Nucleotide/Dye ratio and required volume for x pmole of both Cy5 and Cy3 labelled cDNA.

Nucleotide/ Dye ratio = pmole cDNA/pmole Cy Dye

(Example values are provided in the Excel sheet below. If you have an electronic version of this document, you can enter your data after double clicking on the sheet.)

Abs/Dye	A260	A550	A650
Cy5	0.354	0.029	0.22
Cy3	0.277	0.107	0.019
Volume cy 5(μL)	65		
Volume cy 3(μL)	68		
pmol cy5	57.2		
pmol cy3	48.51		
pmol cDNA cy5	2624		
pmol cDNA cy3	2148		
pmol cy5/pmol cDNA	45.87		
pmol cy3/pmol cDNA	44.28		

http://www.corning.com/lifesciences/technical_information/techdocs/calculator/index.asp

13.8 Protocol for Rubicon WTA amplification of RNA to produce double-stranded cDNA (SIGMA CAT NO WTA2)

This protocol describes how to convert RNA to cDNA and amplify this by a very simple two-step process (Rubicon WTA amplification). It can be used instead of the more complex linear T7 amplification of RNA described in Protocol 13.6 and has the added advantage of producing double-stranded cDNA. The following Protocol (13.9) describes how to produce cy-labelled DNA from any double-stranded

DNA. Protocols 13.8 and 13.9 can therefore be used instead of Protocols 13.6 and 13.7 to produce cy-labelled cDNA.

Before starting the Rubicon WTA procedure, RNA should be treated with Turbo DNAase (Protocol 13.5).

Procedure

Part A: Library synthesis reaction

1. Thaw the library synthesis buffer, library synthesis solution, library synthesis enzyme and nuclease-free water.

   Thoroughly mix the library synthesis buffer and the library synthesis solution. Dissolve any precipitate in these solutions by briefly heating at 37°C with mixing.

2. To at least 25 ng of total RNA, add 2.5 μL library synthesis solution and make up to 16.6 μL with nuclease-free water.

3. Mix and incubate in a thermocycler programmed for 70°C for five minutes then cool to 18°C.

4. To the cooled-primed RNA immediately add the following:
   a. 2.5 μL library synthesis buffer
   b. 3.9 μL water
   c. 2 μL of library synthesis enzyme.

5. Incubate in a thermal cycler using the following parameters (one cycle):
   ▷ 18°C for 10 minutes
   ▷ 25°C for 10 minutes
   ▷ 37°C for 30 minutes
   ▷ 42°C for 10 minutes
   ▷ 70°C for 20 minutes
   ▷ Cool to 4°C.

6. Mix and centrifuge in a microfuge.

Part B: Amplification reaction

1. Thaw the amplification mix and 10 mM dNTP mix.

2. Prepare the following master mix:

   ▷ 301 μL of nuclease-free water

   ▷ 37.5 μL Amplification Mix

   ▷ 7.5 μL WTA dNTP mix

▷ 3.75 µL Amplification enzyme.

3. Add the entire library synthesis reaction mix from Step 6 of Part A (25 µL) to the master mix solution from Step 2 Part B and mix.

4. Divide the sample from Step 3 into five 75 µL reactions and incubate in a thermal cycler using the following parameters:

▷ 94°C for two minutes

▷ 17 cycles × (94°C for 30 seconds, 70°C for five minutes)

▷ Cool to 4°C.

5. After cycling is complete, maintain the reaction at 4°C or store at −20°C.

6. Pool the samples (approx. 375 µL) and purify using Qiagen PCR purification kit (cat no 28104).

7. Elute in 100 µL of 50°C preheated 10 mM Tris-EDTA (pH 8.0) or EB Buffer.

8. Quantify using Nanodrop or Agilent Bioanalyser.

13.9 Protocol for direct labelling of double-stranded cDNA made by Rubicon WTA kit

This protocol is a very simple way of producing cy-labelled DNA that can be used for microarrays or to produce biotinylated double-stranded DNA. It can be used for any double-stranded DNA including genomic DNA or cDNA. It is particularly easy to use after Rubicon amplification of RNA to produce double-stranded DNA. Thus, by using Protocols 13.8 and 13.9 together it is possible to use ng quantities of environmental RNA to produce µg quantities of cDNA labelled with various labels, including fluorescent tags and biotin.

1. In a sterile amber microfuge tube, add 10 µL of ds cDNA (2 µg of ds cDNA) isolated using the Rubicon WTA kit (Sigma Cat Number WTA2) and make up the volume to 18 µL with Sigma ultra-pure water (molecular biology reagent, Cat: W4502).

2. Add 2 µl of random primer (3 µg/µL) (*e.g.* Invitrogen SKU# 48190-011). Heat at 95°C for five minutes and then put on ice for five minutes.

3. On ice, add:

a. 3 µL of 10× Klenow buffer (Fermentas)

b. 5 µL of 10× dNTP mix (10× dNTP mix: 1.2 mM each dATP, dGTP, dTTP; 0.6 mM dCTP; 10 mM Tris pH 8.0; 1 mM EDTA. Do **NOT** use the dNTP mix from the kit).

c. 2 μL of Cy5 dCTP or Cy3 dCTP (1 mM stock, GE Healthcare Lifesciences, Cat: PA55321).

d. 2 μL of Klenow enzyme (Fermentas);

4. The total reaction volume is 30 μL.

5. Spin briefly and incubate the reaction mixture at 37°C overnight protected from light.

6. Use a Qia-quick PCR purification kit (Qiagen Cat: 28104) to remove unincorporated/quenched Cy dyes. Elute twice using 30 μL of Sigma water to maximise recovery.

13.10 Appendices

13.10.1 RNAase inhibitor composition

The final composition of RNAase inhibitor is:

► 20 mM EDTA disodium salt dihydrate(EDTA Sigma cat no E5134)

► 25 mM trisodium citrate dihydrate (Fisher cat no S/3320/53)

► 70 g/100 mL ammonium sulfate (Sigma cat no A4418).

To make RNAase inhibitor, first prepare stock solutions of EDTA and sodium citrate as follows:

► 0.5 M EDTA pH 8.0 (dissolve 18.61 g EDTA disodium salt dihydrate/100 mL and adjust the pH to 8.0 with NaOH while stirring)

► 1 M sodium citrate (dissolve 29.4 g trisodium citrate dihydrate/100 mL).

To prepare the final solution, add the following to a beaker:

► 93.5 mL sterile water

► 4 mL 0.5 M EDTA

► 2.5 mL 1 M sodium citrate

► 70 g ammonium sulfate.

Stir the reagents on a heated stirring block until the salt completely dissolves. Allow the solution to cool, with constant stirring. Adjust the pH to 5.2 with 1 M H_2SO_4 Transfer to a screw-capped bottle and store at room temperature or at 4°C.

RNAase inhibitor is used at twice the volume of samples to be stabilised.

13.10.2 FastPrep Lysing tubes

Materials required

▶ 0.1 mm Silica Beads (Biospec Products Inc. cat no: 1107910z).

▶ 0.1 mm Glass Beads (Biospec Products Inc. cat no: 1109101).

▶ Micro tubes 2.0 mL (Sarstedt cat no: 72.694.005) or equivalent.

Preparation procedure

1. Wash the silica beads and glass beads separately in RNAase-free water and oven dry them.

2. Add 300 mg of washed silica beads and 100 mg of washed glass beads to micro-tubes.

3. Loosely cap the micro-tubes and autoclave before use. These steps cannot be guaranteed to produce RNAase-free tubes and lysing matrix. All cell disruption procedures should therefore be done with either RNA extraction buffer (*e.g.* RLT buffer) or RNAase inhibitor present to prevent RNA degradation.

13.10.3 A nitrogen-free rooting solution

Chemical	Stocks	Stock conc.	Volume for 1L	Final conc.
$CaCl_2.2H_2O$	73.51 g/500 mL	1 M	1 mL	1 mM
KCl	3.73 g/500 mL	100 mM	1 mL	100 µM
$MgSO_4.7H_2O$	98.59 g/500 mL	800 mM	1 mL	800 µM
Fe EDTA	1.84 g/500 mL	10 mM	1 mL	10 µM
H_3BO_3	2.16 g/100 mL	350 mM	0.1 mL	35 µM
$MnCl_2.4H_2O$	1.78 g/100 mL	90 mM	0.1 mL	9 µM
$ZnCl_2$	0.109 g/100 mL	8 mM	0.1 mL	0.8 µM
$Na_2MoO_4.2H_2O$	0.121 g/100 mL	5 mM	0.1 mL	0.5 µM
$CuSO_4.5H_2O$	0.075 g/100 mL	3 mM	0.1 mL	0.3 µM
KH_2PO_4	25 g/1 L	18 mM	20 mL	368 µM
Na_2HPO_4	28.4 g/1 L	20 mM	20 mL	400 µM

Add each chemical in turn to 900 mL of water and make up to 1 L. It is essential that the phosphates are added last otherwise they may precipitate and not dissolve.

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