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Efficient nutrient use in rice production in Vietnam achieved using inoculant biofertilisers

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Efficient nutrient use in rice production in Vietnam achieved using inoculant biofertilisers

Proceedings of a project (SMCN/2002/073) workshop held in Hanoi, Vietnam, 12–13 October 2007

Editors: I.R. Kennedy, A.T.M.A. Choudhury, M.L. Kecskés and M.T. Rose

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Cover: Planting rice at Dai Mo in 2004

Foreword

It is almost a decade since the Australian Centre for International Research (ACIAR) sponsored a small project to assess the significance of a biofertiliser called BioGro. This was a multistrain microbial product formulated in the early 1990s by Professor Nguyen Thanh Hien of the Hanoi University of Science. Professor Hien, whose early education was in China, obtained her doctorate in microbiology in Russia and gained research experience at the International Rice Research Institute (IRRI), the University of Dundee and in Wageningen, the Netherlands. A suggestion by Dr Peter Stewart of the Australian National University to the director of the Sydney University Nitrogen Fixation Centre (SUNFix) in 1998 that BioGro might have some efficacy in improving yields of rice—and allowing farmers to economise on inputs of chemical fertilisers—seemed worth investigating.

By 2000, despite initial scepticism, preliminary evidence had been obtained from field and laboratory trials that lent merit to the claim, and a possible scientific basis for these observations was beginning to emerge. The beneficial effects on plant growth of some soil micro-organisms, for example nitrogen (N_2)-fixing species like *Azospirillum,* had been recognised since the 1960s in Rothamsted by M.E. Brown. A complex of beneficial processes catalysed by micro-organisms, including phytohormone production leading to accelerated root growth and various means of nitrogen (N) and phosphorus (P) nutrient mobilisation, could be demonstrated in laboratory and greenhouse tests.

A grant from the Rural Industries Research and Development Corporation (RIRDC) helped bring Professor Hien to an international symposium in Sydney in late 2000 hosted by SUNFix, and allowed publication of a book in 2002 entitled 'Biofertilisers in action'. This RIRDC publication summarised the possible basis for such beneficial effects in the growth of rice.

A 2-year AusAID – Capacity-building for Agriculture and Rural Development (CARD) project allowed additional field experiments to be carried out. The research data, published in 2003, showed that the hypothesis that BioGro indeed had significant benefits for the growth and yield of rice was a robust one. Even a sceptical mind such as that of Dr Rodney Roughley, former director of the Australian Inoculants Research and Control Service responsible for quality control of *Rhizobium* cultures used for legume nodulation, was won over. He became a research consultant for the field experiments in Vietnam and then co-author of a 2003 paper in the journal Symbiosis. He recognised that this use of new inoculants occupied a place in scientific debate similar to that of the inoculation of legumes with rhizobia a century earlier. Both systems had good prospects for benefiting crops, but both required a long period of research before their potential could be consistently realised and applied with confidence by farmers.

These proceedings provide a summary of the research outputs of a much larger ACIAR project that commenced in 2004. It is remarkable that the Vietnamese

researchers in the project were able to complete more than 20 major field experiments studying BioGro. This is an illustration of Vietnam's strong comparative advantage due to its many highly competent research scientists. Apart from its technological content, this volume also contains five chapters emphasising economic or commercial assessments of the application of BioGro.

Readers are left to draw their own conclusions about the significance of the project's outputs. There is still room to debate; however, there seem to be very good reasons to conclude that prospects for the benefits mentioned above are now tangible. In the past decade the prices of N and P fertilisers have more than trebled, with few if any prospects that this trend can be reversed. As a consequence, it may be justifiable to expect major benefits from biofertilisers like BioGro for smallholder rice farmers in Vietnam and other developing countries in the immediate future rather than much later.

Peter Core
Chief Executive Officer
ACIAR

Contents

List of abbreviations

ACIAR	Australian Centre for International Agricultural Research
BG	BioGro
BGA	blue-green algae
BINA	Bangladesh Institute of Nuclear Agriculture
BNF	biological nitrogen fixation
BRRI	Bangladesh Rice Research Institute
C	carbon
CARD	Capacity-building for Agriculture and Rural Development
CEC	cation exchange capacity
cfu	colony forming unit
ELISA	enzyme-linked immunosorbent assay
FBB	fungal–bacterial biofilm
FRB	fungal–rhizobial biofilm
FYM	farmyard manure
HUS	Hanoi University of Science
IAS	Institute of Agricultural Sciences (of Southern Vietnam)
ISF	Institute of Soils and Fertilizers
MARD	Ministry of Agriculture and Rural Development
MDI	Mekong Delta (Development Research) Institute
MNb	modified nutrient broth
MPN	most probable number
N	nitrogen
N_2	atmospheric nitrogen
NA	nutrient agar
NIBGE	National Institute of Biotechnology and Genetic Engineering
NIR	near infrared reflectance
P	phosphorus
PBS	phosphate buffer saline
PCR	polymerase chain reaction
PGP	plant growth promoting
PGPR	plant growth promoting rhizobacteria
PPB	phosphate peptone buffer
QC	quality control
RCB	randomised complete block
RIRDC	Rural Industries Research and Development Corporation
SUNFix	Sydney University Nitrogen Fixation Centre
TP	thermophosphate
VAAS	Vietnam Academy of Agricultural Science
VASI	Vietnam Agricultural Science Institute
w/w	weight to weight

Overview of project objectives and experimental outcomes

Ivan R. Kennedy[1]

Abstract

The principal objectives of the ACIAR project SMCN/2002/073 'Efficient nutrient use in rice production in Vietnam achieved using inoculant biofertilisers' were to:

- conduct field trials in Vietnam and Australia designed to optimise, extend and promote biofertiliser technology on rice farms for increased profit, and to extend this technology to the south of Vietnam
- design and evaluate a set of simple field tests for quality control of biofertiliser products aimed at ensuring their effectiveness under typical paddy rice field conditions
- investigate the economic and commercial feasibility of inoculant biofertiliser production in Vietnam.

It will become obvious from examining these proceedings that these objectives have been largely achieved. The many well-executed field experiments conducted in Vietnam and their results show that agronomic research with inoculant biofertilisers is now well established and further improvement of this agricultural technology is highly feasible. The two invited contributions in this volume from Sri Lanka (Seneviratne et al. 2008) and Bangladesh (Sattar et al. 2008) strengthen our conclusion of the significant benefits that can be obtained from this technology. The scientific basis for application of inoculants such as BioGro for field cultivation of rice that allows more efficient utilisation of nitrogen (N) fertiliser now has a firm foundation.

Introduction

This project aimed to test the hypothesis that the application of inoculant biofertilisers is a reliable technology for rice farmers, and to prove their efficacy throughout Vietnam. It also aimed to foster the extension of such application more widely by studying its economic, social and environmental benefits and how these could best be implemented.

The meaning of the term biofertiliser is still in a state of flux. It has a degree of uncertainty and some would say the term is misleading and should not be used. It certainly does not mean organic fertiliser, such as decaying manures that release substantial amounts of nutrients. The response curve by crops such as rice to organic fertilisers such as manures is similar to that of inorganic fertilisers, with a generally increasing crop response with rate of application because of the limited rate of release of mineral N.

By contrast, inoculant biofertilisers contain only traces of fertiliser nutrients. They should be regarded as primary *catalysts* that have the property of making available chemical nutrients in the soil environment that would not otherwise be mobilised for use by plants. These micro-organisms must be *catalytic* since they are normally applied at a rate of less than 10^{12} microbial cells per ha, amounting to only 1 g or 1 cm^3 of living organic material. However, they are often applied in a much larger weight of carrier, such as peat or soil with a high organic content; even so, the nutrient content of the carrier materials is usually similar to that of the soil into which they are placed. Therefore, the beneficial effects of inoculation cannot be attributed to nutri-

[1] SUNFix Centre for Nitrogen Fixation, Faculty of Agriculture, Food and Natural Resources, University of Sydney, NSW 2006, Australia

ents in the inoculant materials, as has been verified by chemical analysis or controls using carrier material without added microbes.

Consistent with this, Professor Hien's work (Nguyen et al. 2003) shows that the response curve to the inoculant biofertiliser BioGro rapidly reaches a maximum value, after which the response does not increase with higher inoculation rates, as established in previous work in Vietnam. This suggests that the ability of the inoculated micro-organisms to multiply in the rhizosphere of rice plants can compensate to some extent for the actual numbers of microbial cells supplied at inoculation. While the total 'dose' of cells applied on carrier cannot be more than 1 g, during the following process of colonisation of the rice rhizosphere over several days, the microbial strains in BioGro could multiply by several orders of magnitude. Once a dose is reached that can provide sufficient numbers to ensure ecological competence in colonising the rhizosphere, higher rates of inoculation make little difference to the final populations—indeed, these will be limited by the availability of carbon (C) and other substrates.

It appears that the catalytic role of these plant growth promoting (PGP) micro-organisms is actually multifunctional and cumulative (Dobbelaere et al. 2003; Bashan et al. 2004; Kennedy et al. 2004). Before this project commenced, the PGP function was well recognised, primarily as a result of phytohormonal effects on plants that lead to more robust growth of root systems. Atmospheric nitrogen (N_2)-fixing diazotrophs were often associated with the PGP effects, which were hypothesised (Balandreau 2002; Kennedy et al. 2004) to be favoured by excretion of C compounds from plants into the rhizosphere, raising the C:N ratio of this environment. Nutrient mobilisation of N and phosphorus (P) was then favoured by excretion of chelating substances. Thus, the PGP function inherently involves a suite of functions that can improve the growth of plants, and these effects are integrated in such a way that no single factor can be attributed as the major cause (Figure 1).

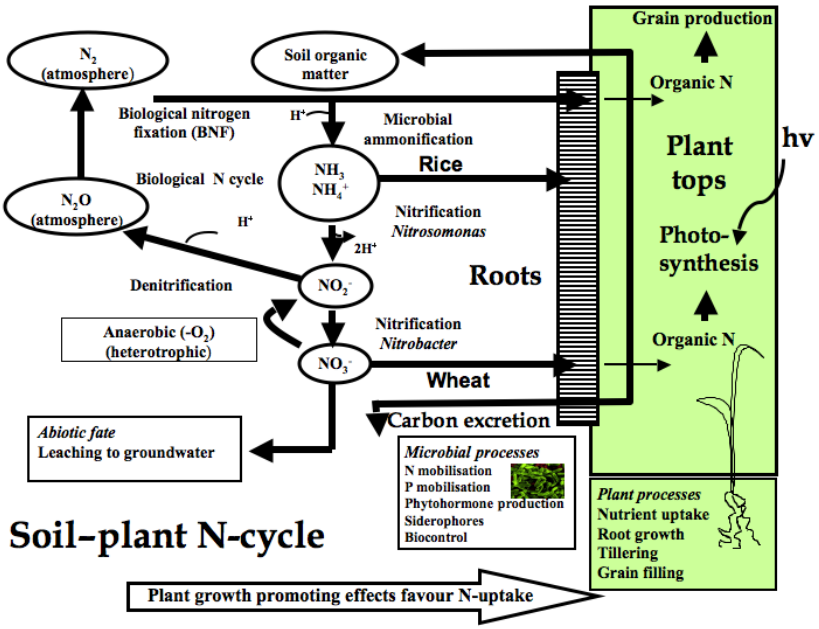

Figure 1. The biofertiliser hypothesis for the soil–plant nitrogen (N) cycle: More efficient N utilisation and plant growth promotion by biofertiliser micro-organisms occurs as a mutually beneficial but complex process. The process involves primary excretion of organic carbon from the plant roots, leading to microbial processes of N fixation, N mobilisation, phosphorus mobilisation, phytohormone production, siderophore production aiding nutrient uptake, and biocontrol of pathogens.

It is also important to note, as carefully explained by Balandreau (2002), that the species of inoculant microbes introduced in such a biofertiliser should represent strains that are already likely to be present and are well adapted to the habitat of the rice root zone. Increasing their numbers by inoculation directly into the rhizosphere has the advantage of ensuring early colonisation and increasing the probability that the beneficial effects of these strains can be exerted on plants.

Objectives

Despite the rational basis that exists for PGP function discussed above, there was still reason to doubt, when this project began, that this complex of effects could be the basis of an effective technology on rice farms.

There were originally four stated objectives of the project proposal:

- to conduct field trials in Vietnam and Australia designed to optimise, extend and promote biofertiliser technology on rice farms for increased profit, and to extend this technology to the south of Vietnam
- to design and evaluate a set of simple field tests for quality control of biofertiliser products aimed at ensuring their effectiveness under typical paddy rice field conditions
- to investigate the economic and commercial feasibility of inoculant biofertiliser production in Vietnam
- to conduct laboratory and field research to reveal the mechanisms of the plant growth promoting rhizobacteria (PGPR) biofertiliser effect.

In preliminary discussions with ACIAR, the first three of these objectives were given greater weight in the use of project resources, given their focus on gaining benefits for farmers. The research strategy employed has involved a series of about 25 field experiments and farmer demonstration trials using the commercial biofertiliser, BioGro. This field work was very well conducted by personnel of the Hanoi University of Science, the Institute of Agricultural Sciences (IAS) of Southern Vietnam, the Institute of Soils and Fertilizers (ISF at the Vietnam Academy of Agricultural Science (VAAS)) in Hanoi, the Mekong Delta Development Research Institute (MDI) at Can Tho University and the University of Sydney. The very low coefficients of variation obtained in the field experiments conducted by the IAS attest to the high quality of this field work.

In addition, a substantial amount of laboratory work associated with objectives 2 and 4 has been completed, related to quality control for the manufacture and application of BioGro and some research on the mechanisms. The award of an AusAID Youth

Scientist and farmer cooperate during yield harvesting at Professor Hien's trial at Dai Mo in 2004.

Ambassadorship to Michael T. Rose of the University of Sydney, who worked for almost a year in Hanoi and Ho Chi Minh City with members of the ACIAR project team, enabled more progress in this part of the project than would otherwise have been possible.

Project outputs

Despite the development of biofertiliser technology involving challenging research, the results obtained in this project have been very positive. Selected results are described in other chapters of this volume and the remaining datasets will be available to researchers on the project's Final Report CD-ROM. Among the findings of the project are the following:

- New information (Nguyen 2008) has been obtained regarding the effects of factors such as the rate of biofertiliser application, the best combination of bacterial strains and rice cultivars, and the need for extra inoculations for a particular rice crop or continued inoculations at the same field site for successive crops. BioGro now consists of a more reliable and safer combination of strains compared to the set used when the project started. Evidence has been obtained that each crop requires its own inoculation to obtain the maximum effect in terms of yield, although there is some evidence of a residual effect.
- A number of trials conducted by the VAAS have shown that rice crops grown with BioGro do not differ in nutritional or consumer preference (cooking and eating) quality from those grown by traditional methods (Pham et al. 2008a, b). However, some anecdotal evidence suggests that rice inoculated with BioGro and grown in the Mekong Delta was brighter in colour when harvested and therefore more valuable in the market.
- Clear demonstrations have been obtained from field experiments that BioGro can reduce the need for fertiliser-N application as urea by at least 50 kg N/ha (Phan and Tran 2008; Phan et al. 2008). Together with actual yield increases observed with BioGro on many occasions when compared to normal farmer fertiliser practice, it has been possible to show clear economic benefits from the application of the biofertiliser technology. Field experiments in Australia have been less convincing (Kecskés et al. 2008a), with the Vietnamese strains in BioGro suggesting poor adaptation to Australian soils; however, progress has been strongly impeded by the drought.
- Farmer demonstration trials have been successfully conducted using BioGro in the Mekong Delta by the MDI and in Nam Dinh province in the Red River Delta by the VAAS (Tran et al. 2008a, b; Pham et al. 2008a, b). These trials have confirmed the efficacy of BioGro in farmers' hands, adding weight to similar results obtained in the Red River Delta near Hanoi in an earlier project (Nguyen et al. 2003).
- Progress has been made in developing simple nutritional and immunoblotting tests for quality control (QC) of biofertiliser products (Kecskés et al. 2008b; Deaker et al. 2008). These are based on the use of selective media and immunological tests. The methods are summarised in a QC manual to be made available in English and Vietnamese with the final report.
- Some important lessons have been learnt regarding the most effective models for commercial production of BioGro (Marsh 2008b; Marsh and Nguyen 2008). These will allow recommendations to be made for ongoing activity in this area that should help improve the likelihood of commercial success.
- The efficacy and economic benefits from applying the bacterial strains contained in BioGro for plant growth promotion and rice yield increase have been confirmed in most of the field experiments performed (Marsh 2008b). This confirmation is supported, in many cases, by analysis of variance of the data at the P=0.05 level of significance and, in other cases, by consistent trends in the data.
- Regarding the mechanism of action of BioGro, clear evidence has been obtained from field and greenhouse experiments using ^{15}N-urea that the inoculant micro-organisms improve the efficiency of N uptake (Phan et al. 2008). This is partly as a result of improved rates of root growth from phytohormonal effects, but there may also be some role for biological N fixation. There may also be benefits regarding mobilisation of P (Rose et al. 2008), but these effects were less striking than those for N in the field experiments conducted in southern Vietnam in Tay Ninh province.

BioGro is not unique. Similar products for rice and wheat have been developed in Pakistan by Dr Kauser Malik's team in Faisalabad through the National Institute of Biotechnology and Genetic

Engineering (NIBGE), and are also reported for Bangladesh in this volume (Sattar et al. 2008). This points to the general significance of the kinds of micro-organisms involved in BioGro.

However, the integration of technology with commercial and extension aspects in one study, as in this project, is probably unique. These advances reported in biofertiliser theory and practice clearly justify commercial application of validated biofertilisers such as BioGro in Vietnam, but many outstanding questions regarding their application remain to be answered. Nevertheless, the various outputs of this ACIAR project have provided a strong basis for ongoing research and development.

Acknowledgments

Together with all my colleagues in Vietnam and Australia, I am grateful to ACIAR for their support since 1999. Tony Fisher (in particular), Ian Willett, Christian Roth and Gamini Keerthisinghe have all contributed amply from their professional experience as program managers, as have other staff in Canberra (particularly Helen Laughlin) and the Australian embassy in Hanoi (Misha Coleman, Geoff Morris). A productive and memorable discussion on a day trip to Nam Dinh from Hanoi with Dr Bo of the Ministry of Agriculture and Rural Development (MARD) and Dr Toan, arranged by Misha Coleman, comes to mind. I am particularly grateful to all my younger colleagues whose contributions appear in these proceedings. I would also like to pay tribute to Dr Rodney Roughley, Honorary Research Associate in SUNFix, who has provided quality control for all our activities since they began in Vietnam.

References

Balandreau J. 2002. The spermosphere model to select for plant growth promoting bacteria. In 'Biofertilisers in action', ed. by I.R. Kennedy and A.T.M.A. Choudhury. Rural Industries Research and Development Corporation: Barton ACT.

Bashan Y., Holguin G. and de-Bashan L.E. 2004. *Azospirillum*–plant relationships: physiological, molecular, agricultural and environmental advances (1997–2003). Canadian Journal of Microbiology 50, 521–577.

Deaker R., Mijajlovic G. and Casteriano A. 2008. Estimating the most probable number of bacteria in multistrain biofertiliser inoculants using a multiple-tube fermentation test. These proceedings.

Professor Hien drying rice from field experiments at Dai Mo at the University of Science in 2004

Dobbelaere S., Vanderleyden J. and Okon Y. 2003. Plant growth-promoting effects of diazotrophs in the rhizosphere. Critical Reviews in Plant Science 22, 107–149.

Kecskés M.L., Choudhury A.T.M.A., Casteriano A.V., Deaker R., Roughley R.J., Lewin L., Ford R. and Kennedy I.R. 2008a. Effects of bacterial inoculant biofertilisers on growth, yield and nutrition of rice. These proceedings.

Kecskés M.L., Rose M., Tran Thi Kim Cuc, Nguyen Kim Oanh, Michel E., Lauby B., Rakotondrainibe M., Casteriano A., Palágyi A., Krishnen G. and Kennedy I.R. 2008b. Identification and quality control of BioGro inoculant biofertiliser strains. These proceedings.

Kennedy I.R., Choudhury A.T.M.A. and Kecskés M.L. 2004. Non-symbiotic bacterial diazotrophs in crop-farming systems: can their potential for plant growth promotion be better exploited? Soil Biology and Biochemistry 36, 1229–1244.

Marsh S. 2008a. Economically optimal N-fertiliser rates with BioGro. These proceedings.

Marsh S. 2008b. BioGro and farm-level benefits. These proceedings.

Marsh S. and Nguyen Thanh Hien. 2008. Models of commercial biofertiliser production: Experiences with BioGro. These proceedings.

Nguyen Thanh Hien. 2008. The product BioGro and improvements in its performance. These proceedings.

Nguyen Thanh Hien, Deaker R., Kennedy I.R. and Roughley R.J. 2003. The positive yield response of field-grown rice to inoculation with a multi-strain biofertiliser in the Hanoi area, Vietnam. Symbiosis 35, 231–245.

Pham Van Toan, Vu Thuy Nga and Luong Huu Thanh. 2008a. Adoption and regulation of biofertiliser technology for rice production. These proceedings.

Pham Van Toan, Vu Thuy Nga and Nguyen Van Thanh. 2008b. Evaluation of varietal difference and N–P–K fertiliser combinations on the effectiveness of BioGro in rice cultivation and on rice quality. These proceedings.

Phan Thi Cong and Tran Dang Dung. 2008. Interaction effects of BioGro with nitrogen and phosphorus on grain yield and nutrient uptake of rice in light-textured soils of southern Vietnam. These proceedings.

Phan Thi Cong, Luong Thu Tra, Tran Dang Dung and Tran Thanh Ha Vy. 2008. Effects of BioGro strain *Pseudomonas fluorescens* (1N) on dry matter production and nitrogen uptake of rice: A ^{15}N tracer study. These proceedings.

Rose M.T., Tran Minh Hien, Tran Thi Kim Cuc, Duc Hoang and Phan Thi Cong. 2008. Phosphorus mobilisation by biofertiliser strains. These proceedings.

Sattar M.A., Rahman M.F., Das D.K. and Choudhury A.T.M.A. 2008. Prospects of using *Azotobacter*, *Azospirillum* and cyanobacteria as supplements of urea nitrogen for rice production in Bangladesh. These proceedings.

Seneviratne G., Kecskés M.L. and Kennedy I.R. 2008. Biofilmed biofertilisers: Novel inoculants for efficient nutrient use in plants. These proceedings.

Tran Thanh Be. 2008a. Challenge of the 'Three Reductions' program in Vietnam: The potential role of inoculant biofertiliser technology. These proceedings.

Tran Thanh Be, Pham Thi Phan and Marsh S. 2008b. Farmer extension trials using BioGro for rice production in the Mekong Delta. These proceedings.

The product BioGro and improvements in its performance

Nguyen Thanh Hien[1]

Abstract

Our research was focused on isolation and selection of microbial strains that can fix atmospheric nitrogen (N_2), solubilise phosphate, stimulate plant growth and break down soil organic matter. Criteria for selection of strains have been developed. The biofertiliser BioGro now consists, as a commercial product, of four strains: a nitrogen (N)-fixing bacterium *Pseudomonas fluorescens*, a phosphorus (P)-solubilising yeast and two other strains (*Bacillus amyloliquefaciens* and *Bacillus subtilis*) that can potentially break down cellulose, protein and starch. All four strains promote plant growth by stimulating root growth. BioGro can be produced in local communities providing there is technical backup in the supply of starter cultures and quality control of the final product. This paper assesses the consistent positive effect of BioGro on grain yield and some agronomic parameters, the optimum rates and times for its application, and the need for continued inoculation of crops grown on the same site.

Introduction

Vietnam is an agricultural rice-growing country, with about 7 million hectares of land in annual rice production. At least 400,000 tonnes of urea-N, much of it imported, may be applied to this acreage per rice crop. The price of urea is increasing, particularly in the last 2 years. To meet the increasing demand for food, both industrial N production and biological nitrogen fixation (BNF) will be needed.

It is essential to maintain a balance between the needs of plant nutrition and soil fertility. Soil microbes play an important role in maintaining this balance through their role in BNF, phosphorus (P) solubilisation and plant growth promotion through stimulation of root growth, and by releasing nutrients from soil organic matter. Their role can be further exploited.

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Advantages of BNF include lower emission costs, reduced production of greenhouse gases and less nitrate contamination of groundwater, which is more in tune with the development of sustainable agriculture. P-solubilising microbes could help mobilise P and other soil nutrients that plants may have difficulty in accessing at an adequate rate. Experimental results at the University of Sydney demonstrated that P-solubilising bacteria can increase desorption of the adsorbed P in the soil (Ahmed et al. 2008). The synergistic effect of N_2-fixing strains and P solubilisers in the rhizosphere of crop plants and mangroves (Rojas et al. 2001) has been clearly demonstrated.

Yields of crop plants could be improved by plant growth promoting rhizobacteria (PGPR), including those which: fix N; stimulate plant growth by producing auxins, cytokinins, gibberellins and ethylene; solubilise P; increase nutrient uptake; enhance stress resistance; and exercise biocontrol of plant pathogens (Dobbelaere et al. 2001).

The application of microbial strains that perform some of the above functions as inoculant bioferti-

lisers could substitute for more expensive chemical fertilisers if produced using local labour. This could become a significant factor in the alleviation of poverty in poor farming communities.

The work reported in this paper concentrated on making available to farmers a product that could both reduce their dependence on chemical fertilisers and increase the yield of rice. The optimum rates and times of application and the persistence of any effects after inoculation were also investigated.

Materials and methods

Biofertiliser

Before 2005, BioGro contained three strains of bacteria, namely 1N, 4P and 3C, selected from rice rhizospheres in the Hanoi area of Vietnam. Since 2005, a combination of four strains has been used: 1N, HY, B9 and E19. 1N (*Pseudomonas fluorescens*) was selected for its ability to grow on N-free media and to reduce acetylene to ethylene, which is an indicator of potential N_2 fixation. HY is an unidentified plant growth promoting (PGP) yeast and was selected for its ability to also solubilise insoluble mineral P in an agar medium. The two other strains, B9 (*Bacillus subtilis)* and E19 (*Bacillus amyloliquefaciens)*, were selected for their ability to break down protein, cellulose and starch.

Each of the four organisms was grown in separate broth cultures, separately added to the carrier formulation (which consisted of a mixture of 75% peat (clay soil), 24% aqueous broth culture of microbes and 1% sugar) and incubated for 48 hours at 30 ˚C. These four carriers with separate microbes were then mixed before use in equal proportions and then constituted as the BioGro components of these experiments, and stored under cool conditions for up to 1 month.

Biofertiliser was applied in the nursery by mixing with seeds at sowing at 15% of the rate to be used in the plots to which they were to be transplanted. Biofertiliser was applied to the field plots by spreading the carrier mixture evenly by hand directly to the soil. To control plots (uninoculated), carrier only was added at a rate equivalent to 270 kg/ha.

Microbial culture

Storage media for biofertiliser strains were as follows:

1. HY in 10 g/L glucose; 5.0 g/L $Ca_3(PO_4)_2$; 0.2 g/L KCl; 0.5 g/L $(NH_4)_2SO_4$; 0.01 g/L $MnSO_4$; 0.01 g/L $FeSO_4$; 0.01 g/L $MgSO_4.7H_2O$; 6.5 g/L yeast extract; 2.0 g/L agar, all to 1 L H_2O, pH 7.0
2. 1N was grown on agar containing 30 g/L Tryptic Soy Broth; 15 g/L agar in 1 L H_2O, pH 7.0
3. B9 and E19 were grown on agar containing 10 g/L peptone; 3 g/L tryptone; 3 g/L NaCl; 15 g/L agar in 1 L H_2O, pH 7.0.

Fermentation media were as follows:

1. HY as for storage but lacking agar
2. 1N in 5.0 g/L peptone; 15 mL fish sauce in 1 L H_2O
3. B9 and E19 were grown in media containing 10 g/L peptone; 20 mL fish sauce in 1 L H_2O, pH 7.0.

Media for plate counting were prepared as follows:

1. HY contained 0.5 g/L $(NH_4)_2SO_4$; 0.2 g/L NaCl; 10.0 g/L glucose; 5.0 g/L $Ca_3(PO_4)_2$; 1 mL of trace element solution in 1 L H_2O. The trace element solution contained 5.0 g/L H_3BO_3; 5.0 g/L $(NH_4)_2MoO_4$; 0.15 g/L $AlCl_3$; 0.2 g/L $ZnSO_4$ per l L.
2. 1N was grown on agar containing 10 g/L glucose; 3.0 g/L peptone; 2.0 g/L yeast extract; 10 mL trace element solution; 16 g/L agar in 1 L H_2O. The trace element solution contained 5.0 g/L $(NH_4)_2SO_4$; 1.0 g/L $MgSO_4$; 1.0 g/L NaCl; 2.0 g/L KH_2PO_4; 1.0 g/L $CaCl_2$ in 1 L H_2O.
3. B9 and E19 were grown in media containing 0.5 g/L K_2HPO_4; 0.2 g/L $MgSO_4.7H_2O$; 0.01 g/L $FeSO_4.7H_2O$; 0.01 g/L $CaCl_2$; 10 g/L casein; 15 g/L agar in 1 L H_2O.

Tests for P solubilisation were conducted on solid media containing precipitated $Ca_3(PO_4)_2$ agar plates. P solubilisation was determined by the production of zones of clearing around isolated colonies. Similarly, assessment of breakdown of organic substance was conducted on solid media containing casein by counting the production of zones of clearing around isolated colonies.

Seedling production

Seedlings of the short-duration rice variety Khang Dan were sown in the field in traditional nursery bays to assist the farmers in planting three seedlings per hill uniformly. Seedlings were grown for 26–28 days for the spring crop before transplanting (Figure 1).

Figure 1. Harvesting seedlings for transplanting

Sites and experimental design

Field trials were established in the Hanoi area, Vietnam, in the spring of 2005, 2006 and 2007 in alluvial soils of the Red River at Dai Mo village, where the soil pH varied between 5.19 and 5.58 ($CaCl_2$).

Two types of experiments were established. The field experiments called trials 1 and 2 were conducted in randomised complete block design replicated four times (Figure 2). Plot sizes were about 40 m^2 and were protected from cross-contamination from adjoining plots by sinking asbestos roof tiles 20–30 cm into the soil. Blanket fertiliser doses of KCl (muriate of potash) at 56 kg/ha before flowering, thermophosphate (TP) at 200 kg/ha and urea at 55 kg/ha were broadcast and spread evenly using rakes at transplanting. Rice seedlings were planted at a spacing of 21 cm × 14 cm.

Trial 1 was established in the spring of 2005 to investigate the effect of inoculation with BioGro on the number of panicles per hill, the number of fertile seeds per panicle, the weight of 1,000 seeds and grain yield. There were two treatments: one with BioGro and a control without BioGro. BioGro was applied in two splits: at 40 kg/ha during seed sowing and at 200 kg/ha during transplanting.

Trial 2 was established in the spring of 2006 to investigate the effect of inoculation of three successive rice crops grown in the same plots compared with a single inoculation. Fertiliser was applied at the same rates as in trial 1. The treatments were:

1. no BioGro applied
2. inoculated with BioGro in the first crop only
3. inoculated with BioGro in the first and second crops, but not in the third crop
4. inoculated with BioGro in all three crops.

Trials 3 and 4 were non-replicated experiments conducted in farmer fields (six farmers for trial 3 and three farmers for trial 4). Fields were divided into two plots—one to receive biofertiliser at 4.5 times the normal fertiliser input and the other to receive the normal fertiliser input but with no added biofertiliser. Plot sizes varied between 91 m^2 and 210 m^2. Rice seedlings were transplanted at the same spacings as for trials 1 and 2.

Figure 2. Division of experimental plots using fibre-board, as employed in trials 1 and 2

Trial 3 was established in spring 2006 on plots on six farms in the Hanoi area to investigate the effect of the rate of BioGro applied on grain yield and yield components. Four rates of BioGro (0, 50, 100 and 200 kg/ha) were applied at transplanting. Treatment plots received 200 kg/ha of BioGro (farmers 1 and 2), 100 kg/ha of BioGro (farmers 3 and 4) and 50 kg/ha of BioGro (farmers 5 and 6). Control (0 kg/ha of BioGro) was included at the farmers' plots.

Trial 4 was established in spring 2007 to evaluate the effects of higher amounts of BioGro and the timing of the application on yield and yield components of rice. The experiment was conducted on three farms in Dai Mo village near Hanoi. An uninoculated control using carrier only was also established on each farm.

On farm 1, two 150 m^2 plots were used, one for the uninoculated control and the other for rice seedlings treated with BioGro at 38 kg/ha in the nursery area. In farm 2, two 120 m^2 plots were used, one for the uninoculated control treatment and the other for rice treated at the seedling stage with 38 kg/ha and at transplanting with BioGro at 278 kg/ha. In farm 3, two 203 m^2 plots were used, one for the uninoculated control treatment and the other for rice treated at the seedling stage and at transplanting as in farm 2, plus 139 kg/ha BioGro at 1 month after transplanting.

Harvesting

At the harvest of trials 1 and 2, the number of panicles in five hills and the number of fertile and non-fertile seeds per panicle in five panicles were counted. Samples of 1,000 seeds and grain yield of 45 hills (1,323 m^2) were weighed and the results converted to t/ha. The grain yields of all trials were adjusted to 14% moisture content.

Statistical analysis

All data were analysed by ANOVA at University of Sydney using GenStat version 7 (Payne et al. 2003).

Results

Effect of BioGro on rice yield

Spring 2005

This field experiment was conducted to compare BioGro with a control (without BioGro) supplied with 55 kg urea and 200 kg TP/ha. Inoculation with BioGro with the same rate of urea increased grain yield significantly over the control ($P<0.05$) (Table 1), confirming its beneficial effect on rice crops. The yield increase was the result of significantly more panicles per hill and a larger number of fertile seeds per panicle. The weight of 1,000 seeds and the number of non-fertile seeds were unaffected by the application of BioGro.

Effect of successive inoculation

Spring 2005

Field experiments were conducted for three successive crops, beginning in spring 2005, using BioGro at the same site and rate to determine whether repeated inoculation would further increase grain yield or affect its components.

Spring 2006

In the third season (spring 2006), application of BioGro for three consecutive seasons increased grain yield significantly over both uninoculated rice and rice inoculated in the first season only at 10% level of probability, confirming its beneficial effect on rice crops (Table 2). When inoculated with BioGro for two seasons, the yield of rice was 0.57 t/ha less than when applied for three consecutive seasons, but the

Table 1. Effect of BioGro on grain yield of rice and some agronomic parameters in the spring crop 2005

Treatment	Grain yield (t/ha)	Number of panicles/hill	Number of fertile seeds/ panicle	1,000 seeds weight (g)	Number of non-fertile seeds/panicle
Control (without BioGro)	7.39	5.90	188.90	19.00	17.4
BioGro	7.96[a]	7.20 [a]	222.00 [b]	19.03	21.3
F probability	0.018	0.023	0.074	0.649	0.196
LSD (0.05)	0.38	0.96	–	–	–
LSD (0.10)	–		28.83	–	–
CV (%)	5.1	16.8	15.5	0.7	42.8

[a] and [b] indicate significantly higher yield over control at 5% and 10% probability levels, respectively.

yields were not significantly different (P=0.10). However, inoculation in two seasons increased yield by 1.30 t/ha compared with a single inoculation in the first season. Application of BioGro in all three seasons increased the number of panicles per hill significantly over all other treatments at 5% level of probability.

Table 2. Effect of inoculating rice with BioGro for one, two or three seasons on yield and panicle formation in the third season, spring 2006

BioGro inoculation program	Grain yield (t/ha)	Number of panicles/ hill
Uninoculated	5.99	6.25
Inoculated in season 1 only	5.45	6.40
Inoculated in seasons 1 & 2	6.75	6.70
Inoculated in seasons 1, 2 & 3	7.32	7.90
F probability	0.054	0.013
LSD (0.05)	–	0.95
LSD (0.10)	1.10	0.77
CV (%)	9.5	24.8

Rate of BioGro inoculation

Farmer field tests in spring 2006

BioGro applied at 50, 100 or 200 kg/ha increased grain yield significantly compared with uninoculated rice at the 5% probability level (Table 3). Increasing the amount of BioGro from 50 to 100 or 200 kg/ha did not further increase yield. There was no consistent effect of BioGro on the number of fertile seeds per panicle although the highest amount applied (200 kg/ha) significantly increased the number of fertile seeds per panicle over all other treatments.

Nursery versus field inoculation

Farmer field tests in spring 2007

On farm 1, where a single application of BioGro at 38 kg/ha was used in the nursery only, grain yield increased by 9% (Table 4). On farm 2, where a total of 316 kg/ha was applied in a split application, the increase was 80%. When three applications of BioGro were used on farm 3, the increase was 39%. Grain yield obtained by two or three applications were almost similar. However, the variation in percentage increase over control was due to variation in the yields in control plots between the farms. This indicates the effect of soil fertility on the effectiveness of biofertiliser application.

When averaged over all plots, inoculation increased the number of panicles per square metre from 240 to 331 and the number of fertile seeds per panicle from 76 to 150. Neither of these parameters appeared to be influenced by the third application of 139 kg/ha at 28 days after transplanting the seedlings.

BioGro's PGP effect

Roots of rice seedlings sampled within the nursery area in the spring crop of 2007 indicated the PGP effects. Figures 3, 4 and 5 show the better root development of the inoculated seedlings compared with the uninoculated ones. This effect was translated into increased yield (Table 4), and data are presented in the text for the number of panicles per square metre and fertile seeds per panicle.

Discussion

The data from previous field trials and farmers' tests (Nguyen et al. 2003) and from other authors in these proceedings show that BioGro consistently

Table 3. Effect of the amount of BioGro applied on grain yield, number of panicles per hill and number of fertile seeds per panicle, spring 2006

Amount of BioGro applied (kg/ha)	Grain yield (t/ha)	Number of panicles/hill	Number of fertile seeds/panicle
0	5.45	6.80	187.5
50	6.91	6.40	190.1
100	6.83	8.60	173.9
200	6.25	8.40	253.8
F value	0.010	0.005	0.012
LSD (0.05)	0.72	1.24	38.37
CV (%)	3.73	18.49	21.26

increased grain yield, reduced the need for fertiliser-N and -P and farmyard manure, and increased farmers' profit as a result of cost savings and yield increase. The trials have been conducted in different soils from the north to the south of Vietnam with similar results. The increases in yield could be the result of BNF, P solubilisation and PGP effects.

Our criteria for strain selection are:

- The strains should be the most abundant of their kind in the soil.
- They should have high activity for a particular desirable character, e.g. N fixation, P-solubilising activity. All strains should have a PGP effect.
- If possible, they should be fast-growing.
- They must be non-pathogenic to plants and animals.
- They should be reselected periodically to ensure their characteristics are unchanged.

The effectiveness of BioGro depends on the quality of biofertiliser product (Kennedy and Roughley 2002). A negative response in a trial in 2000 emphasised the importance of quality control of the biofertiliser to ensure reliable results. The quality of biofertiliser depends on the number of cells per gram of carrier and the characteristics of each strain. Details of appropriate checks and standards have been published elsewhere in these proceedings (Deaker et al. 2008; Kecskés et al. 2008).

Table 4. Grain yield of rice on three farms when uninoculated or following inoculation with BioGro at different times, spring 2007

Farmer/inoculation	Total amount of BioGro (kg/ha)	Grain yield (t/ha)		Increase in yield with inoculation (t/ha)	Percentage increase
		Uninoculated	Inoculated		
Farm 1: inoculated once in nursery	38	4.35	4.75	+ 0.4	9
Farm 2: inoculated twice—in nursery and at transplanting	316	3.46	6.24	+ 2.78	80
Farm 3: inoculated three times—in nursery, in field at transplanting and 28 days later	455	4.44	6.17	+ 1.73	39

Figure 3. Seedlings at 12 days of age with one application of BioGro (right)

All four strains in BioGro have a PGP effect (i.e. stimulation of root development). Roots become thicker and longer in the seedling stage. Root development and nutrient uptake are key factors in expressing the beneficial effects of PGP (Mia et al. 2002), which could contribute to yield increase (Table 4) and a 50% reduction in applied fertiliser.

All rates of BioGro (50, 100 and 200 kg/ha) applied at transplanting increased grain yield significantly over the control ($P<0.05$). This means that a rate of 50 kg/ha, which is approximately the same as the number of root nodule bacteria applied for soybean (5×10^{11} cfu/ha) in Australian legume inoculants, could be applied in the future. This would further reduce the input costs for farmers.

Because of limitations in replication and variation between the sites, it is difficult to draw definite conclusions from the pilot trial comparing timing of BioGro

Figure 4. Rice at tillering stage inoculated once at seedling stage (right)

Figure 5. Rice at harvest inoculated once at seedling stage (right)

applications and the amount applied. However, it is clear that inoculation had a beneficial effect at all sites as a result of producing more panicles and more fertile seeds per panicle. The size of this effect was dependent on the amount of BioGro applied and the potential for the site to produce rice grain without inoculation. BioGro produced the largest response when the uninoculated rice yielded least. Further detailed examination of the rates and timing of BioGro applications are warranted. An interim recommendation would be for two applications to be made in the early stages for a total application of 100 kg/ha.

Despite some evidence for a residual effect of BioGro inoculation on a later crop, repeated inoculation of the same field in each season was shown to be necessary if the maximum benefit from inoculation by BioGro was to be achieved. Because of the limited nature of the data, based on one site only, more detailed studies are required to determine the general applicability of this finding. Because these strains have been isolated from soils where they were a significant component of the soil microflora, and because there is a relatively short interval between successive rice crops in many areas, it would be reasonable to expect that they would survive in sufficient numbers to have a biological effect on the next crop. The persistence of BioGro in crops fed after rain would be worthy of further investigation, as would the effect on numbers of different break crops or fallow over time.

Data of Phan and Tran 2008 in these proceedings showed that application of BioGro significantly increased total N and P uptakes. Increase in N uptake might be due to increase in fertiliser-N uptake, BNF or increase in soil-N uptake due to a thicker and longer root system. The fertiliser-N uptake can be quantified by using 15N-enriched urea (Choudhury and Khanif 2001, 2004). The %BNF contribution can be measured by the 15N isotope dilution technique (Fried and Middelboe 1977; Kurdali et al. 2007). Malik et al. (2002) reported that two species of the genus *Pseudomonas* (*P. stutzeri* K1 and *Pseudomonas* 96-51) fixed N2 at 19.5% Ndfa and 3.0% Ndfa, respectively, while rice seedlings inoculated with these organisms resulted in significantly increased rice grain yields over the control. As BioGro contained 1N (*Pseudomonas fluorescens*), there might be some BNF contribution that led to increase in total N uptake by rice. This, as well as the effect of BioGro on fertiliser-N uptake by rice plants, can be quantified by using the 15N tracer technique.

Conclusion

These results confirm our earlier data on the effectiveness of applying BioGro to rice (Nguyen et al. 2003). The effectiveness of BioGro could be a result of PGP, N fixation and P solubilisation. In the future, ^{15}N-based studies are recommended to quantify the fertiliser-N uptake as well as the possible

Traditional winnowing of the grain at Dai Mo near the temple site

BNF contribution of BioGro. The data indicate that, for best results, BioGro must be applied for each consecutive rice crop and that the yield may increase further with continued applications of BioGro. However, an additional inoculation of soil did not improve the yield of the current rice crop. On the contrary, reduced rates of application to current recommendation may be possible if quality control measures confirm that high numbers of the BioGro strains are present in the biofertiliser product.

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References

Ahmed M.F., Kennedy I.R., Choudhury A.T.M.A., Kecskés M.L. and Deaker R. 2008. Phosphorus adsorption in some Australian soils and influence of bacteria on the desorption of phosphorus. Communications in Soil Science and Plant Analysis 39, 1269–1294.

Choudhury A.T.M.A. and Khanif Y.M. 2001. Evaluation of effects of nitrogen and magnesium fertilization on rice yield and fertiliser nitrogen efficiency using ^{15}N tracer technique. Journal of Plant Nutrition 24, 855–871.

Choudhury A.T.M.A. and Khanif Y.M. 2004. Effects of nitrogen and copper fertilization on rice yield and fertiliser nitrogen efficiency: A ^{15}N tracer study. Pakistan Journal of Scientific and Industrial Research 47, 50–55.

Deaker R., Mijajlovic G. and Casteriano A. 2008. Estimating the most probable number of bacteria in multistrain biofertiliser inoculants using a multiple-tube fermentation test. These proceedings.

Dobbelaere S., Croonenborghs A., Thys A., Ptacek D., Vanderleyden J., Dutto P., Labandera-Gonzalez C., Caballero-Mellado J., Aguirre J.F., Kapulnik Y., Brener S., Burdman S., Kadouri D., Sarig S. and Okon Y. 2001. Responses of agronomically important crops to inoculation with *Azospirillum*. Australian Journal of Plant Physiology 28, 871–879.

Fried M. and Middelboe V. 1977. Measurement of amount of nitrogen fixed by a legume crop. Plant and Soil 47, 713–715.

Kecskés M.L., Rose M., Tran Thi Kim Cuc, Nguyen Kim Oanh, Michel E., Lauby B., Rakotondrainibe M., Casteriano A., Palágyi A., Krishnen G. and Kennedy I.R. 2008. Identification and quality control of BioGro inoculant biofertiliser strains. These proceedings.

Kennedy I.R. and Roughley R.J. 2002. The inoculant biofertiliser phenomenon and its potential to increase yield and reduce costs of crop production: the need for quality control. Pp. 4–10 in 'Biofertilisers in action', ed. by I.R. Kennedy and A.T.M.A. Choudhury. Rural Industries Research and Development Corporation: Canberra.

Kurdali F., Al-Ain F., Al-Shammaa M. and Razzouk A.K. 2007. Performance of sorghum grown on a salt affected soil manured with dhaincha plant residues using a ^{15}N isotopic dilution technique. Journal of Plant Nutrition 30, 1605–1621.

Malik K.A., Mirza M.S., Hassan U., Mehnaz S., Rasul G., Haurat J., Bally R. and Normand P. 2002. The role of plant-associated beneficial bacteria in rice-wheat cropping system. Pp. 73–83 in 'Biofertilisers in action', ed. by I.R. Kennedy and A.T.M.A. Choudhury. Rural Industries Research and Development Corporation: Canberra.

Mia M.A.B., Shamsuddin Z.H., Zakaria W. and Marziah M. 2002. Root stimulation and nutrient accumulation of hydroponically grown tissue-cultured banana plantlets inoculated with rhizobacteria at lower level of nitrogen fertilisation. Pp. 64–72 in 'Biofertilisers in action', ed. by I.R. Kennedy and A.T.M.A. Choudhury. Rural Industries Research and Development Corporation, Canberra.

Nguyen Thanh Hien, Deaker R., Kennedy I.R. and Roughley R.J. 2003. The positive yield response of field-grown rice to inoculation with a multi-strain biofertiliser in the Hanoi area, Vietnam. Symbiosis 35, 231–245.

Payne R., Murray D., Harding S., Baird D., Soutar D. and Lane P. 2003. GenStat for Windows, 7th edn. VSN International: Oxford, United Kingdom.

Phan Thi Cong and Tran Dang Dung 2008. Interaction effects of BioGro with nitrogen and phosphorus on grain yield and nutrient uptake of rice in light-textured soils of southern Vietnam. These proceedings.

Rojas A., Holguin G., Glick B.R. and Bashan Y. 2001. Synergism between *Phyllobacterium* sp. (N_2-fixer) and *Bacillus licheniformis* (P-solubilizer) both from a semi-arid mangrove rhizosphere. FEMS Microbiology Ecology 35, 181–187.

Interaction effects of BioGro with nitrogen and phosphorus on grain yield and nutrient uptake of rice in light-textured soils of southern Vietnam

Phan Thi Cong and Tran Dang Dung[1]

Abstract

Three field experiments were conducted to evaluate the effects of the multistrain inoculant biofertiliser BioGro (two formulations: BioGro1 and BioGro2) on yield and nutrient uptake of rice under variable rates of nitrogen (N) and phosphorus (P) fertilisers. The first experiment near Cu Chi was conducted during the first rainy season in 2004 using BioGro1 with four N rates (0, 40, 80 and 120 kg N/ha) with the test rice variety LVN 95-20. The second experiment was conducted with Trau Nam rice during the first and second rainy seasons in 2006 using BioGro2 with five N rates (0, 30, 60, 90 and 120 kg N/ha). The third experiment, also with Trau Nam rice, was conducted during the first and second rainy seasons in 2006 using BioGro2 with four P rates (0, 10, 30 and 60 kg P_2O_5/ha).

The estimated grain yield response of LVN 95-20 rice to added N was quadratic in nature both with and without BioGro1. BioGro1 outyielded the control at all rates of added N. Estimated grain yield response of Trau Nam rice to added N was also quadratic in nature in both seasons. BioGro2 outyielded the control, with a potential to save 43 kg N/ha as well as an increased grain yield of 270 kg/ha in two consecutive seasons; in general, when BioGro2 was applied, the same yield was achieved with about 50 kg urea-N/ha less than the approximately 100 kg urea-N/ha required to achieve maximum yield in the absence of BioGro. In contrast to the first season, BioGro gave consistent yield increases at all rates of N in the second season, suggesting a seasonal influence on the effectiveness of BioGro. The effect of BioGro2 on P uptake was not significant in this experiment in the first season but was significant in the second season. These results provide convincing evidence of the capacity of BioGro to improve the efficiency of use of both N and P by rice.

Introduction

Both N and P are essential macronutrient elements for plants. Because of acute N and P deficiencies in most light-textured rice soils, these elements are supplemented by the application of the chemical fertilisers urea and fused magnesium phosphate, respectively. But the reality is that substantial portions of applied N and P fertilisers are lost due to different mechanisms that cause environmental pollution problems (Sharpley et al. 2001; Choudhury and Kennedy 2005; Choudhury et al. 2007). These problems cannot be alleviated completely. However, plant growth promoting (PGP) micro-organisms can enhance plants' capacity to absorb nutrients like N and P, resulting in increased yield (Yanni et al. 1997; Biswas et al. 2000; Choudhury and Kennedy 2004; Kennedy et al. 2004).

P-solubilising bacteria can also mobilise fixed and adsorbed P for plant uptake (Ahmed et al. 2008). When inoculant biofertilisers containing PGP microbes are used in rice culture, rice plants are able to use fertiliser-N and -P more efficiently, resulting

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in decreased environmental pollution problems caused by N and P losses. Previous field experimental results in the northern part of Vietnam near Hanoi indicated that the multistrain biofertiliser BioGro1 increased rice grain yield and N uptake significantly (Nguyen et al. 2002, 2003). However, the bulk of Vietnam's rice crop is produced in the south, where farm size is generally larger. Therefore, it is necessary to evaluate the effectiveness of BioGro in the south. With this in mind, field experiments were conducted to evaluate the effects of BioGro with variable rates of fertiliser-N and -P on yield and the N and P nutrition of rice, and to test the hypothesis that PGP strains can significantly improve the efficiency of nutrient use by rice.

For more details on the experimental methods and results regarding the improved efficiency of N use with BioGro, see our recently accepted publication in the European Journal of Soil Biology (Phan et al., in press).

Materials and methods

The effect of BioGro on rice grown in southern Vietnam was investigated in three experiments. BioGro1, including three microbial strains, 1N, 3C and 4P, was used in experiment 1 at Cu Chi district, Ho Chi Minh City, in 2004. BioGro2, a combination of 1N, HY, B9 and E19, was used in experiments 2 and 3 at Chau Thanh district, Tay Ninh province, in 2006.

Experiment 1 included three factors. Factor A comprised two levels of BioGro1 inoculant: without and with 40 kg/ha added to the seeds in the nursery bed plus 200 kg/ha added to the field at transplanting. Factor B comprised two levels of farmyard manure: without and with 5 t/ha local farmyard manure. Factor C was the N rate: 0, 40, 80 and 120 kg N/ha. Phosphorus and K were added at 60 kg P_2O_5/ha as fused magnesium phosphate and 60 kg K_2O/ha as KCl, respectively. A split-split plot design was used where BioGro1 was put in the main plots to reduce contamination from plot to plot.

The effect of BioGro2 biofertiliser on nutrient uptake and grain yield of rice was investigated on a grey degraded soil of Thanh Dien village, Chau Thanh district, Tay Ninh province, southern Vietnam. This soil was a loamy sand with pH 5.31, 1.5% organic matter content, 4.08 $cmol_c$/kg cation exchange capacity and 0.11 $cmol_c$/kg exchangeable K. Field experiments were carried out in two consecutive seasons—the first and the second rainy seasons 2006. Different application rates of N and P were used to investigate the response of the rice crop and the interactions between biofertiliser and nutrients. Trau Nam, a local variety with duration of 110 days, was used as the test crop.

Experiments 2 and 3 included two factors: Factor A was the biofertiliser rate while factor B comprised the N or P rates. BioGro2 included four microorganisms, 1N, HY, B9 and E19. Two levels of BioGro2 inoculant were used: without and with 40 kg/ha added to the seeds in the nursery bed plus 200 kg/ha added to the field at transplanting. Nitrogen was added at five rates (0, 30, 60, 90 and 120 kg N/ha) in the second experiment. Phosphorus and K were added at 30 kg P_2O_5/ha as fused magnesium phosphate and 60 kg K_2O/ha as KCl. In the third experiment, four P rates (0, 10, 30 and 60 kg P_2O_5/ha) from fused magnesium phosphate were used to investigate the interaction between biofertiliser and P. Nitrogen and K were added at 90 kg N/ha as urea and 60 kg K_2O/ha as KCl.

A split-plot design was used for experiments 2 and 3, where factor A (BioGro2) was set at the main plots and factor B (N or P rates) was assigned in the subplots. All treatments were repeated four times. Unit subplot size was 5.1 m × 3.9 m = 19.89 m^2. Planting space was 15 cm × 15 cm.

All data were analysed at the University of Sydney using ANOVA with the statistical program GenStat version 7 (Payne et al. 2003), and grain yield response data were interpreted using differential calculus (Gomez and Gomez 1984).

Results and discussion

Effect of BioGro1, farmyard manure and N rate on yield of rice (experiment 1)

BioGro1 increased grain yield significantly at 10% probability level while the effect of farmyard manure on grain yield was not significant. Grain yield responded highly significantly (P<0.001) with N rate. The estimated grain yield response due to N fertilisation was quadratic in nature both with and without BioGro1 (Figure 1). BioGro1 outyielded the control at all rates of added N.

Effects of BioGro2 and N rate on yield and nutrition of rice (experiment 2)

First rainy season

In the first rainy season the application of BioGro2 increased grain yield by 0.10 t/ha at 10% probability level. Grain yield increased significantly (P<0.05) due to N fertilisation up to 90 kg N/ha. Beyond this N rate, there was no significant increase in grain yield. Estimated grain yield response due to N fertilisation was quadratic in nature both with and without BioGro2 (Figure 2). The N rates for maximum grain yields with and without BioGro2 were obtained by differentiating the quadratic N response equation following the method of Gomez and Gomez (1984). The N rate for maximum grain yield with BioGro2 was 103 kg N/ha and 143 kg N/ha without BioGro2. The maximum estimated grain yields were 3.21 and 3.18 t/ha with and without BioGro2, respectively. This information indicates that application of BioGro2 has the potential to save 40 kg N/ha with an additional yield of 30 kg/ha in the experimental site of Chau Thanh district, Tay Ninh Province, southern Vietnam, in the first rainy season.

Total N uptake increased significantly (P<0.05) due to both BioGro2 and N rates up to 90 kg N/ha (Table 1). Total P uptake was not significantly different when BioGro2 was added but N fertilisation increased P uptake significantly (P<0.05) up to 60 kg N/ha.

Second rainy season

In the second rainy season the effect of BioGro2 on grain yield improved compared with the first season and was statistically significant (P<0.05). Grain yield increased 0.24 t/ha when BioGro2 was added. Grain yield increased significantly (P<0.05) due to N fertilisation and was highest at 90 kg N/ha. Beyond this N rate, there was a reduction in grain yield although the difference was not statistically significant.

Estimated grain yield response due to N fertilisation was quadratic in nature both with and without BioGro2 (Figure 3). At all N rates, grain yield was higher when BioGro2 was added. The N rate for maximum grain yield with BioGro2 was 94 kg N/ha and 97 kg N/ha without BioGro2. The maximum estimated grain yields were 3.49 t/ha and 3.25 t/ha

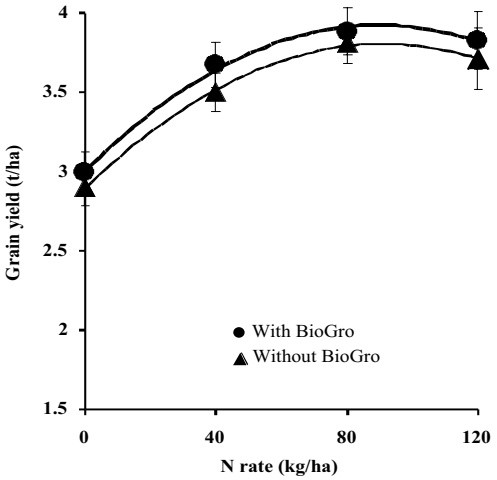

Figure 1. Estimated grain yield response of LVN 95-20 rice to added N with and without BioGro1, Cu Chi District, Ho Chi Minh City, Vietnam, first rainy season 2004
$y = 3.0004 + 0.0206x - 0.0001x^2$ (with BioGro), $r^2 = 0.9957$
$y = 2.8923 + 0.02x - 0.0001x^2$ (without BioGro), $r^2 = 0.9987$

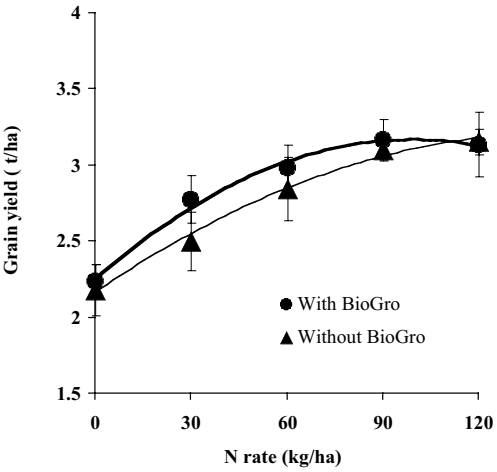

Figure 2. Estimated grain yield response of Trau Nam rice to added N with and without BioGro2, Chau Thanh District, Tay Ninh province, Vietnam, first rainy season 2006
$y = 2.2541 + 0.0185x - 0.00009x^2$ (with BioGro), $r^2 = 0.991$ (P<0.01)
$y = 2.1611 + 0.0143x - 0.00005x^2$ (without BioGro), $r^2 = 0.9915$ (P<0.05)

with and without BioGro2, respectively. This information indicates that maximum yield from application of BioGro2 has the potential to save only 3 kg N/ha with an additional rice yield of 240 kg/ha in the experimental site of Chau Thanh district, southern Vietnam, in the second rainy season. The combined results from the two seasons show that application of BioGro2 saved 43 kg N/ha with an additional rice yield of 270 kg/ha. More importantly, the maximum yield with urea was achieved with about half as much added urea when BioGro2 was applied. This indicates the important role of BioGro2 in managing rice production on this soil type.

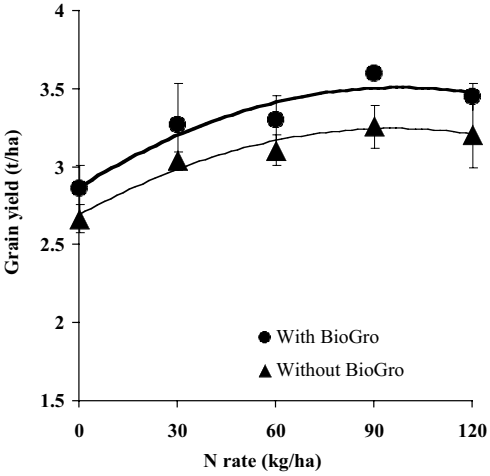

Figure 3. Estimated grain yield response of Trau Nam rice to added N with and without BioGro2, Chau Thanh district, Vietnam, second rainy season 2006
$y = 2.8726 + 0.0131x - 0.00007x^2$ (with BioGro), $r^2 = 0.911$ at $P<0.05$
$y = 2.6894 + 0.0116x - 0.00006x^2$ (without BioGro), $r^2 = 0.9668$ at $P<0.05$

Total N uptake increased significantly ($P<0.05$) due to both BioGro2 and N rates up to 90 kg N/ha (Table 2). Total P uptake increased significantly due to both BioGro2 and N rates up to 90 kg N/ha.

Effects of BioGro2 and P rate on yield and nutrition of rice (experiment 3)

First rainy season

In the first rainy season the application of BioGro2 slightly increased grain yield by 0.08 t/ha although the difference was not statistically significant. The failure of BioGro2 to increase grain yield significantly might be due to the high N rate (90 kg N/ha) used in this experiment. Phosphorus application increased grain yield significantly ($P<0.05$) up to 60 kg P_2O_5/ha. This P response could be due to the low soil fertility status of the experimental field. The soil has a light texture where the P buffering capacity is definitely low. Estimated grain yield response to added P was quadratic in nature both with and without BioGro2 (Figure 4).

Application of BioGro2 increased total N and P uptakes slightly although the differences were not statistically significant (Table 3). Total N and P uptakes increased significantly ($P<0.05$) due to P rates up to 60 kg P_2O_5/ha.

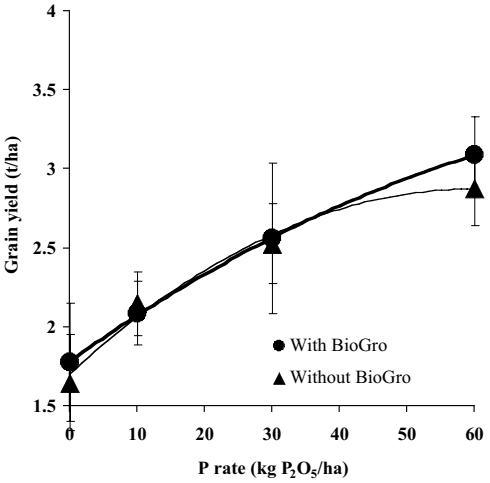

Figure 4. Estimated grain yield response of Trau Nam rice to added P with and without BioGro2, Chau Thanh district, Tay Ninh province, Vietnam, first rainy season 2006
$y = 1.7876 + 0.0303x - 0.0001x^2$ (with BioGro), $r^2 = 0.9997$
$y = 1.7004 + 0.0392x - 0.0003x^2$ (without BioGro), $r^2 = 0.9839$

Second rainy season

In the second rainy season the application of BioGro2 increased grain yield significantly ($P<0.05$) although the same N rate (90 kg N/ha) was used. This indicates a positive effect of continuous application of biofertiliser. Phosphorus application also increased grain yield significantly ($P<0.05$) up to 60 kg P_2O_5/ha. At any P rate, BioGro2 outyielded the control.

Table 1. Effects of BioGro2 and N rates on total N and P uptakes by Trau Nam rice, Chau Thanh District, Tay Ninh province, southern Vietnam, first rainy season 2006

N rate (kg/ha)	BioGro2		Mean
	Without	With	
	Total N uptake (kg/ha)		
0	34.16	38.76	36.46
30	41.22	48.63	44.93
60	50.82	50.86	50.84
90	56.10	56.67	56.39
120	56.78	58.21	57.50
Mean	47.82	50.63	
	Total P uptake (kg/ha)		
0	6.38	6.78	6.58
30	7.54	8.65	8.10
60	9.00	9.34	9.17
90	9.24	9.43	9.34
120	9.75	9.52	9.64
Mean	8.38	8.74	

Notes:
Interaction effect of BioGro2 and N rate was not significant on both parameters.
Effect of BioGro2 was significant on total N uptake at F probability level of 0.021 with LSD (0.05) value of 1.994. Effect of BioGro2 was not significant on total P uptake.
Effect of N rate was significant at F probability level of <0.001 on both parameters with LSD (0.05) values of 3.815 and 0.5845 for total N and P uptakes, respectively.

Table 2. Effects of BioGro2 and N rates on total N and P uptakes by Trau Nam rice, Chau Thanh District, Tay Ninh province, southern Vietnam, second rainy season 2006

N rate (kg/ha)	BioGro2		Mean
	Without	With	
	Total N uptake (kg/ha)		
0	39.60	47.85	43.73
30	47.30	59.22	53.26
60	54.07	61.33	57.70
90	60.85	72.60	66.73
120	62.35	68.87	65.61
Mean	52.83	61.97	
	Total P uptake (kg/ha)		
0	8.69	9.30	9.00
30	9.59	10.43	10.01
60	10.60	10.52	10.56
90	10.41	11.88	11.15
120	10.26	10.51	10.39
Mean	9.91	10.53	

Notes:
Interaction effect of BioGro2 and N rate was not significant on both parameters.
Effect of BioGro2 was significant on total N and P uptakes with F probability levels of 0.021 and 0.037, respectively, with LSD (0.05) values of 6.53 and 0.55 for total N and P uptakes, respectively.
Effect of N rate was significant on both parameters at F probability level of <0.001 with LSD (0.05) values of 3.69 and 0.85 for total N and P uptakes, respectively.

Table 3. Effects of BioGro2 and P rates on total N and P uptakes by Trau Nam rice, Chau Thanh district, Tay Ninh province, southern Vietnam, first rainy season 2006

P rate (kg P_2O_5/ha)	BioGro2		Mean
	Without	With	
	Total N uptake (kg/ha)		
0	34.80	39.12	36.96
10	43.99	44.96	44.48
30	49.58	51.93	50.76
60	56.51	59.53	58.02
Mean	46.22	48.89	
	Total P uptake (kg/ha)		
0	5.76	6.69	6.23
10	7.50	8.22	7.86
30	9.02	9.20	9.11
60	10.28	11.01	10.65
Mean	8.14	8.78	

Notes:
Single effect of BioGro2 as well as its interaction effect with P rate was not significant on both parameters.
Effect of P rate was significant on both parameters at F probability levels of <0.001 with LSD (0.05) values of 4.701 and 1.056 for total N and P uptakes, respectively.

Table 4. Effects of BioGro2 and P rates on total N and P uptakes by Trau Nam rice, Chau Thanh district, Tay Ninh province, southern Vietnam, second rainy season 2006

P rate (kg P_2O_5/ha)	BioGro2		Mean
	Without	With	
	Total N uptake (kg/ha)		
0	47.21	47.86	47.54
10	57.19	57.23	57.21
30	59.99	64.13	62.06
60	66.06	69.20	67.63
Mean	57.61	59.61	
	Total P uptake (kg/ha)		
0	6.20	6.97	6.59
10	8.85	8.42	8.64
30	9.25	10.80	10.03
60	11.83	12.39	12.11
Mean	9.03	9.65	

Notes:
Single effect of BioGro2 as well as its interaction effect with P rate was not significant on both parameters.
Effect of P rate was significant on both parameters at F probability levels of <0.001 with LSD (0.05) values of 2.59 and 1.03 for total N and P uptakes, respectively.

Estimated grain yield response to added P was quadratic in nature both with and without BioGro2 (Figure 5).

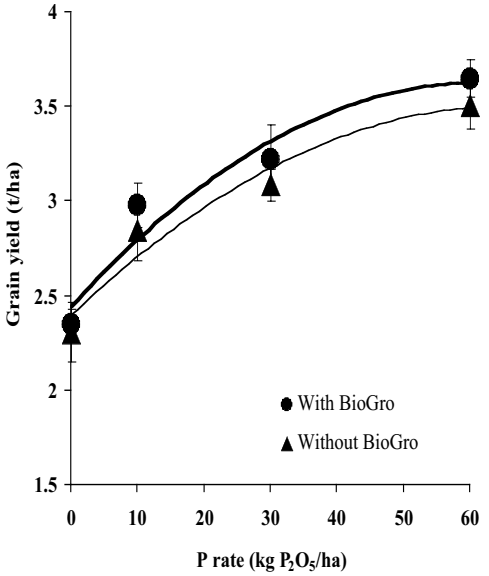

Figure 5. Estimated grain yield response of Trau Nam rice to added P with and without BioGro2, Chau Thanh district, Tay Ninh province, Vietnam, second rainy season 2006
$y = 2.4492 + 0.0383x - 0.0003x^2$ (with BioGro), $r^2 = 0.9412$
$y = 2.3918 + 0.0336x - 0.0003x^2$ (without BioGro), $r^2 = 0.953$

Application of BioGro2 increased total N and P uptakes slightly although the differences were not statistically significant (Table 4). Phosphorus application up to 60 kg P_2O_5/ha increased total N and P uptakes significantly (P<0.05).

Conclusion

The effect of BioGro1 on grain yield was statistically significant at 10% probability level after one application on a light-textured soil at Cu Chi district. BioGro2 also had positive effects on grain yield as well as N and P uptakes of rice on another light-textured soil at Chau Thanh district in two consecutive seasons. Furthermore, the effectiveness of BioGro2 was higher in the second season compared with the first, indicating a positive cumulative effect of biofertiliser application in rice cultivation. However, given the higher yields in the second rainy season, when there are fewer cloudy days and perhaps more photosynthesis, this effect may indicate greater effectiveness of the inoculated micro-organisms in this season. The amount of N fertiliser needed was substantially reduced when BioGro2 was added. Phosphorus fertilisation increased grain yield and the response was quadratic in nature in both seasons with and without BioGro2. In the second season BioGro2 outyielded the control at all P rates, which is also possibly a response to greater photosynthesis or a cumulative effect of biofertiliser application.

Acknowledgments

The assistance of many local farmers in the field experiments at Cu Chi and Tay Ninh is acknowledged. Many other Institute of Agricultural Sciences (IAS) staff also contributed to the project.

References

Ahmed M.F., Kennedy I.R., Choudhury A.T.M.A., Kecskés M.L. and Deaker R. 2008. Phosphorus adsorption in some Australian soils and influence of bacteria on the desorption of phosphorus. Communications in Soil Science and Plant Analysis 39, 1269–1294.

Biswas J.C., Ladha J.K. and Dazzo F.B. 2000. Rhizobia inoculation improves nutrient uptake and growth of lowland rice. Soil Science Society of America Journal 64, 1644–1650.

Choudhury A.T.M.A. and Kennedy I.R. 2004. Prospects and potentials for systems of biological nitrogen fixation in sustainable rice production. Biology and Fertility of Soils 39, 219–227.

Choudhury A.T.M.A. and Kennedy I.R. 2005. Nitrogen fertiliser losses from rice soils and control of environmental pollution problems. Communications in Soil Science and Plant Analysis 36, 1625–1639.

Choudhury A.T.M.A., Kennedy I.R., Ahmed M.F. and Kecskés M.L. 2007. Phosphorus fertilization for rice and control of environmental pollution problems. Pakistan Journal of Biological Sciences 10, 2098–2105.

Gomez K.A. and Gomez A.A. 1984. Statistical procedures for agricultural research, 2nd edn. John Wiley and Sons: New York.

Kennedy I.R., Choudhury A.T.M.A. and Kecskés M.L. 2004. Non-symbiotic bacterial diazotrophs in crop-farming systems: can their potential for plant growth promotion be better exploited? Soil Biology and Biochemistry 36, 1229–1244.

Nguyen Thanh Hien, Deaker R., Kennedy I.R. and Roughley R.J. 2003. The positive yield response of field-grown rice to inoculation with a multi-strain biofertiliser in the Hanoi area, Vietnam. Symbiosis 35, 231–245.

Nguyen Thanh Hien, Kennedy I.R. and Roughley R.J. 2002. The response of field-grown rice to inoculation with a multi-strain biofertiliser in the Hanoi district, Vietnam. Pp. 37–44 in 'Biofertilisers in action', ed. by I.R. Kennedy and A.T.M.A. Choudhury. Rural Industries Research and Development Corporation: Canberra.

Payne R., Murray D., Harding S., Baird D., Soutar D. and Lane P. 2003. GenStat for Windows, 7th edn. VSN International: Oxford, United Kingdom.

Phan Thi Cong, Tran Dang Dung, Tran Minh Hien, Nguyen Thanh Hien, Choudhury, Abu T.M.A., Kecskés, M.L. and Kennedy, I.R. Inoculant plant growth-promoting microorganisms enhance utilisation of urea-N and grain yield of paddy rice in southern Vietnam. European Journal of Soil Biology, in press.

Sharpley A.N., McDowell R.W. and Kleinman P.J.A. 2001. Phosphorus losses from land to water: integrating agricultural and environmental management. Plant and Soil 237, 287–307.

Yanni Y.G., Rizk R.Y., Corich V., Squartini A., Ninke K., Philip-Hollingsworth S., Orgambide G., de Bruijn F., Stoltzfus J., Buckley D., Schmidt T.M., Mateos P.F., Ladha J.K. and Dazzo F.B. 1997. Natural endophytic association between *Rhizobium leguminosarum* bv. *trifolii* and rice and assessment of its potential to promote rice growth. Plant and Soil 194, 99–114.

Farmers transplanting rice inoculated with BioGro at Dr Cong's field experiment in Tay Ninh province in 2005. Photo by Phan Thi Cong

Evaluation of varietal difference and N–P–K fertiliser combinations on the effectiveness of BioGro in rice cultivation and on rice quality

Pham Van Toan[1], Vu Thuy Nga[1] and Luong Huu Thanh[1]

Abstract

Field experiments to statistically evaluate the effects of BioGro and common rice varieties on rice yield were conducted during the spring and summer seasons of 2006 at Thanh Tri in Hanoi province, Vietnam. BioGro in combination with 50% nitrogen (N) and phosphorus (P) fertiliser gave similar grain yields as those obtained with application of 100% N and P fertiliser without BioGro, indicating the beneficial effect of BioGro in reducing fertiliser inputs. The individual effects of both factors were significant ($P<0.001$); however, the interaction effect of BioGro and rice variety was absent in the spring crop but significant in the summer crop, indicating possible seasonal impacts on the effectiveness of BioGro. The beneficial effect of BioGro was observed with four common rice varieties but not with two other varieties.

In Nam Dinh province at Hai Hau, the response to BioGro was evaluated of two high-priced, quality sticky rices, LT3 and Tam Xoan, that were grown mainly with farmyard manure. Different combinations of N, P and K fertilisers were tested with and without BioGro to evaluate their benefits and the effectiveness of BioGro in reducing fertiliser inputs. The experiment was conducted in four consecutive seasons (spring 2005, summer 2005, spring 2006 and summer 2006). A statistically significant beneficial effect of BioGro in reducing N and P fertiliser requirements to 50% was observed in only one season (summer 2005). From these results, it is recommended that the N fertiliser rate be reduced to a maximum 90 kg N/ha in the experimental site of Nam Dinh province for these high-quality rices.

When comparing 100% N and P fertiliser treatments with 50% treatments with BioGro in harvested common rices, no difference was observed in quality parameters such as the percentages of unhusked grain, amylose and protein. However, a combination of BioGro and farmyard manure improved the glutinous grade and other quality parameters of Tam Xoan, a trademarked rice.

Introduction

Vietnam is predominantly an agricultural country, in which land used for agriculture is expanding at a rate of about 235,200 ha/year. The demand for fertilisers is high and has been increasing year by year. At present, the chemical fertiliser factories in Vietnam can meet 40–50% of N-fertiliser and 80% of P-fertiliser demands, with the government balancing any shortage by importing. To improve this situation, Vietnam seeks to stimulate the research, production and utilisation of biofertilisers.

Various biofertilisers have been developed in Vietnam, with research on N-fixing and P-solubilising micro-organisms commencing many years ago. The results of various studies have indicated that N-fixing inoculants can reduce N application by 30–60 kg N/ha/year and increase crop yields by 5–25%. P-solubilising inoculants and rock phosphate can replace 30–50% of mineral P fertiliser without significant changes in crop yields. Inoculants are pro-

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duced from single strains as well as from a mixed culture of N-fixing, P-solubilising and plant growth promoting micro-organisms for different crops in different ecosystems (Pham and Ha 2004). However, their production and application for rice is erratic.

BioGro1 is one of the multistrain inoculant biofertilisers developed in Vietnam. Field experiments in Vietnam previously showed that BioGro1 increased rice yield and N uptake significantly (Nguyen et al. 2002, 2003). Another formulation named BioGro2 has more recently been developed at the Hanoi University of Science, replacing 3C identified as *Citrobacter freundii* and 4P as *Klebsiella pneumoniae* with a soil yeast (HY) and two *Bacillus* spp. Under the ACIAR project SMCN/2002/073, the Vietnam Academy of Agricultural Science (VAAS) has carried out numerous experiments to evaluate the effects of BioGro2 on growth and rice yield. This paper discusses the salient findings of those experiments, which illustrate the beneficial effects of BioGro2 in rice production and indicate the need to refine the research strategy in the future.

Materials and methods

Analysis

Soil properties

Soil pH, total NPK, available P, cation exchange capacity (CEC) and bulk density were determined by standard procedures at the VAAS soils laboratory. Microbiological tests were also carried out using standard laboratory procedures such as N-free media for diazotrophs.

Rice quality

Percentages of amylose and protein of rice grain were determined by standard methods at VAAS laboratories after dehusking. Percentage of dehusked grain, stickiness, softness and glutinous grade were also estimated by standard techniques.

Field experiments

Field experiments at Thanh Tri, Hanoi

The effect of BioGro on the need for fertiliser-N and -P for the growth of common rice varieties including hybrids was examined in these experiments. The experiments were initiated in spring 2006 and repeated in the following season (summer 2006) to evaluate the beneficial effects of BioGro2 on six rice varieties: three common varieties (KD18, AYT01 and VD8) and three quality varieties (LT2, HT1 and BT7).

Experimental design

Experiments were laid out in a split-plot design with treatments in the main plots and varieties in the subplots. The unit subplot was 10 m^2. Three replications were used. In both seasons the spacing for transplanting was 15 cm × 15 cm. Seed sowing, transplanting and harvesting dates were 20 January, 22 February and 22 May 2006, respectively, for the spring season and 20 June, 12 July and 3 October 2006, respectively, for the summer season.

Treatments

The three treatments used in the experiment were:

- T1 (control): 100% NPK fertiliser + farmyard manure (FYM)
- T2: BioGro + 50% N and P fertiliser + 100% K fertiliser + FYM
- T3: BioGro + 30% N and P fertiliser + 100% K fertiliser + FYM.

N fertiliser (urea) rates for spring and summer seasons were 260 kg/ha and 220 kg/ha, respectively. In both seasons 450 kg/ha triple superphosphate (TSP), 180 kg/ha muriate of potash (KCl), 10 t/ha FYM and 283 kg/ha BioGro were used.

Fertiliser applications

Full amounts of FYM and P fertiliser were applied at transplanting time in both seasons. Nitrogen and potassium (K) were applied in two splits—50% at transplanting and 50% at active tillering stage—in both seasons.

BioGro application

BioGro was applied in two splits—83 kg/ha during seed sowing, by mixing with seeds, and 200 kg/ha on experimental plots during transplanting. The formulation of BioGro was 1N + HY + B9 + E19 in the ratio 1:1:1:1. Culture codes are as follows: 1N is *Pseudomonas fluorescens*; B9 is *Bacillus subtilis* and E19 is *Bacillus amyloliquefaciens*. B9 and E19 can break down cellulose, protein and starch.

Intercultural operations and harvesting

Necessary intercultural operations, including weeding and pest control, were carried out during the growing period as and when required. Rice plants were harvested at maturity and grain yield

was recorded. The grain yield data were adjusted at 14% moisture content.

Statistical analysis

All data were analysed with two-way ANOVA at the University of Sydney using the statistical software GenStat version 7 (Payne et al. 2003).

Field experiment at Hai Hau, Nam Dinh

Experimental design

The experiment was laid out in a randomised complete block design with three replications. The unit plot size was 20 m^2 and the spacing for transplanting was 15 cm × 15 cm. The seasonal sowing, transplanting and harvesting schedule is provided in Table 1.

Treatments

The experiment was initiated in spring 2005 and repeated in the following three consecutive seasons (summer 2005, spring 2006 and summer 2006) to evaluate different combinations of chemical fertilisers with BioGro2 on the high-quality rice variety LT3 in spring, and Tam Xoan in summer, seasons. The treatments used in the experiment were as follows:

- T1: farmyard manure (FYM)
- T2: 25% N and P fertiliser + 100% K fertiliser + FYM
- T3: 50% N and P fertiliser + 100% K fertiliser + FYM
- T4: 75% N and P fertiliser + 100% K fertiliser + FYM
- T5 (control): 100% N and P and K fertiliser + FYM
- T6: BioGro + 25% N and P fertiliser + 100% K + FYM
- T7: BioGro + 50% N and P fertiliser + 100% K + FYM
- T8: BioGro + 75% N and P fertiliser + 100% K + FYM
- T9: BioGro + FYM

The fertiliser rates applied were: 120 kg/ha urea for N; 75 kg/ha of P_2O_5 for P and 60 kg/ha of K_2O for K. The FYM rate was 8 t/ha and the BioGro rates were 417 kg/ha and 278 kg/ha in summer and spring seasons, respectively.

Fertiliser applications

Full amounts of FYM and P fertilisers were applied at transplanting time in all the seasons trialled. N and K were applied in two splits—50% at transplanting and 50% during the active tillering stage.

BioGro application

The formulation of BioGro2 has been described above. BioGro2 was applied in two splits—78 kg/ha during seed sowing, by mixing with seeds, for both spring and summer; and 200 kg/ha and 339 kg/ha on experimental plots during transplanting in spring and summer, respectively.

Intercultural operations and harvesting

Necessary intercultural operations, including weeding and pest control, were carried out during the growing period as and when required. Rice plants were harvested at maturity and grain yield was recorded. The grain yield data were adjusted at 14% moisture content.

Statistical analyses

All data were analysed at the University of Sydney using the procedure mentioned above.

Results and discussion

The full dataset collected for all experiments conducted by the VAAS is available on the CD-ROM prepared with the final report of this ACIAR project. A selection of the data collected during the field experiments is reported here.

Table 1. Seasonal sowing, transplanting and harvesting schedule for Hai Hau field experiment

Season	Sowing date	Transplanting date	Harvesting date
Spring 2005	19 January 2005	20 February 2005	26 June 2005
Summer 2005	12 June 2005	21 July 2005	21 November 2005
Spring 2006	3 January 2006	3 February 2006	18 June 2006
Summer 2006	13 June 2006	22 July 2006	19 November 2006

Field experiment at Thanh Tri, Hanoi

Soil properties

No differences were observed at harvest in general soil properties (pH, total NPK, available P, CEC and the density of N_2-fixing or P-solubilising micro-organisms) between plots treated with BioGro and control plots. This indicates that any effects of BioGro are localised, without measurable effects on the bulk soil.

Rice yields

In spring 2006 BioGro with 50% N and P fertiliser produced similar yields with all rice varieties as obtained with 100% N and P fertiliser without BioGro, indicating that BioGro has the potential to save 50% N and P fertiliser in this season. However, a further reduction in N and P fertiliser (30%) even with BioGro, reduced grain yield significantly. The interaction between varieties and treatments was not statistically significant. However, individual effects of variety and treatment were significant ($P<0.001$, Table 2). There were significant differences among the varieties in regard to grain production. The highest grain yield (6.48 t/ha) was obtained with the VD8 variety, followed by KD18 (6.05 t/ha), and the lowest yield (4.89 t/ha) was observed in the LT2 variety.

In summer 2006 the interaction between treatments and varieties was also significant ($P<0.001$, Table 3). While individual treatment effects were not significant in the LT2 and AYT01 varieties, they were in the other four varieties. For the HT1 variety, BioGro with 30% N and P fertiliser gave similar yields as those obtained with 100% N and P fertiliser without BioGro; while in the BT7 variety, reduced rates of N and P fertiliser (either 50% or 30%) with BioGro produced significantly lower grain yields compared with 100% N and P fertiliser applications. For the KD18 variety, BioGro with 50% N and P fertiliser also gave similar grain yields as obtained with 100% N and P fertiliser, but a further reduction in fertiliser (30%) decreased the grain yield significantly.

The most interesting finding is that, with the VD8 variety, BioGro with 50% N and P fertiliser produced significantly higher grain yields than applications of 100% N and P fertiliser. With the same variety, BioGro with 30% N and P fertiliser produced significantly less grain compared with application of 100% N and P fertiliser. Varietal differences in grain production varied among the treatments. For example, KD18 and VD8 were statistically similar in T1 and T3, while VD8 produced significantly higher grain yields than KD18 in T2.

The results indicate some seasonal impact on the effectiveness of BioGro in different varieties—the interaction of treatments and varieties was not significant in spring 2006 whereas it was significant in the following season (summer 2006). The intensity of solar energy, which is a seasonal attribute, might influence the activity of micro-organisms as well as the response of different rice varieties to N uptake. This should be verified by correlating solar radiation data with experimental findings for both seasons.

Table 2. Grain yields (t/ha) of rice varieties as affected by BioGro in combination with chemical fertilisers, Thanh Tri, Hanoi, spring 2006

Variety	Treatment [a]			Mean [b]
	T1 (control) 100% NP + FYM	T2 50% NP + FYM + BioGro	T3 30% NP + FYM + BioGro	
LT2	5.23	5.17	4.27	4.89 d
HT1	5.47	5.57	4.73	5.26 c
BT7	5.30	5.27	4.20	4.92 d
KD18	6.33	6.33	5.50	6.05 b
VD8	6.73	6.93	5.77	6.48 a
AYT01	5.70	5.77	5.00	5.49 c
Mean [b]	5.79 a	5.84 a	4.91 b	

[a] as described in Materials and Methods section

[b] Means followed by the same letter in a row or column are not significantly different for the respective rows or columns ($P>0.05$, two-way ANOVA). The interaction between treatment and variety was not significant. Individual effects of variety and treatments were significant at F probability of <0.001. The LSD (0.05) values for the treatment and variety were 0.245 and 0.295, respectively. CV was 5.5%.

Field experiment at Hai Hau, Nam Dinh

In the first season (spring 2005) BioGro with any combination of N and P fertiliser failed statistically to produce similar yields as obtained with 100% N and P fertiliser without BioGro. In the second season (summer 2005) BioGro with 50% N and P fertiliser produced similar yields as those obtained with 75% and 100% N and P fertiliser without BioGro (Table 4). In the following two seasons of 2006 this beneficial effect was not observed at a significant level (P>0.05). However, treatments with BioGro showed a general trend of producing higher yields than with 25%, 50% or 75% N and P fertiliser as for recommended farmer practice. Fully fertilised treatments and 75% N and P fertiliser without BioGro produced similar yields in the three seasons (summer 2005, spring 2006 and summer 2006). Therefore, a reduction in the N fertiliser rate to a maximum of 90 kg/ha is recommended.

Effects of BioGro on rice quality

When comparing 100% N and P fertiliser treatments and 50% treatments with BioGro for hybrid and high-quality common rices, no differences were observed in quality parameters, such as the percentages of unhusked grain, amylose and protein, at Thanh Tri in 2005 (Table 5). Similar results were obtained in 2006. However, BioGro with 75% N and P and FYM improved the glutinous and sticky grade of Tam Xoan, a trademarked rice, when compared to 100% and 75% NPK with FYM alone. No significant differences were observed in quality parameters of the LT3 variety grown at Nam Dinh in 2005 when BioGro was applied.

Table 3. Grain yields (t/ha) of rice varieties as affected by BioGro in combination with chemical fertilisers, Thanh Tri, Hanoi, summer 2006

Variety	Treatment[a]		
	T1 (control) 100% NP + FYM	T2 50% NP + BioGro + FYM	T3 30% NP + BioGro + FYM
LT2	3.79 c A	3.73 d A	3.70 c A
HT1	4.42 b AB	4.53 c A	4.19 b B
BT7	3.91 c A	3.57 d B	3.57 c B
KD18	5.57 a A	5.53 b A	4.68 ab B
VD8	5.60 a B	5.95 a A	4.84 a C
AYT01	4.28 b A	4.27 c A	4.39 b A

[a] Values followed by a common capital letter in a row or a common small letter in a column are not significantly different (P>0.05, two-way ANOVA). Treatment–variety interaction was significant at F probability levels of <0.001 with LSD (0.05) value of 0.29 and CV of 3.9%.

Table 4. Effects of BioGro and chemical fertilisers on grain yield of rice, Hai Hau, Nam Dinh 2005–06

Treatment	Grain yield (t/ha)			
	LT3, spring 2005	Tam Xoan, summer 2005	LT3, spring 2006	Tam Xoan, summer 2006
FYM	3.16 [a]	2.20 [a]	2.51 [a]	2.27 [a]
25% N and P + 100% K + FYM	3.44 [a]	2.33 [a]	2.74 [a]	2.35 [a]
50% N and P + 100% K + FYM	3.75 [a]	2.47 [a]	3.12 [a]	2.42 [a]
75% N and P + 100% K + FYM	4.15 [a]	2.90	3.79	2.67
100% NPK + FYM (control)	4.63	2.78	4.13	2.73
BioGro + 25% N and P + 100% K + FYM	3.65 [a]	2.50 [a]	2.88 [a]	2.30 [a]
BioGro + 50% N and P + 100% K + FYM	3.73 [a]	2.78	3.25 [a]	2.51 [a]
BioGro + 75% N and P + 100% K + FYM	4.03 [a]	2.93	3.97	2.74
BioGro + FYM	3.04 [a]	2.18 [a]	2.16 [a]	2.16 [a]
F probability	<0.001	<0.001	<0.001	<0.001
LSD (0.05)	0.26	0.21	0.428	0.16
CV (%)	4.0	4.7	7.8	3.7

[a] significantly different compared to control (P<0.05, two-way ANOVA)

Table 5. Effect of BioGro on quality parameters of high-quality and hybrid rices at Thanh Tri, 2005

Parameter	Control	BioGro	Control	BioGro	Control	BioGro
Quality variety rice	LT2	LT2	BT7	BT7	HT1	HT1
% unhusked seed	62.1	62.0	60.3	61.6	48.8	47.6
% amylose	11.8	11.7	12.5	11.9	14.7	15.0
% protein	8.5	8.8	8.7	8.6	7.7	8.0
Common variety rice	AYT01	AYT01	BM 9820	BM 9820	KD18	KD18
% unhusked seed	51.3	54.3	49.3	57.4	55.8	63.9
% amylose	11.7	12.8	25.1	26.0	24.1	26.6
% protein	7.3	7.5	6.9	7.8	7.9	7.9

Conclusions

In general, BioGro had the potential to save 50% N and P fertilisers in the spring season. During the summer season the beneficial effect of BioGro was observed in four varieties (HT1, BT7, KD18 and VD8) but not in the other two hybrid varieties (LT2 and AYT01). The most interesting finding is that, for the VD8 variety, application of BioGro with 50% N and P fertiliser produced significantly higher grain yield than application of 100% N and P fertiliser. Varietal differences in grain production also varied among the treatments. For example, the KD18 and VD8 varieties gave similar results in T1 and T3, and the VD8 variety produced significantly higher amounts of grain than KD18 in T2.

The beneficial effects of BioGro were less obvious with the higher quality, lower yielding, rices LT3 and Tam Xoan in Nam Dinh province. There was a tendency for the beneficial effects of BioGro to be most obvious in the summer season.

References

Nguyen Thanh Hien, Deaker R., Kennedy I.R. and Roughley R.J. 2003. The positive yield response of field-grown rice to inoculation with a multi-strain biofertiliser in the Hanoi area, Vietnam. Symbiosis 35, 231–245.

Nguyen Thanh Hien, Kennedy I.R. and Roughley R.J. 2002. The response of field-grown rice to inoculation with a multi-strain biofertiliser in the Hanoi district, Vietnam. Pp. 37–44 in 'Biofertilisers in action', ed by I.R. Kennedy and A.T.M.A. Choudhury. Rural Industries Research and Development Corporation: Canberra.

Payne R., Murray D., Harding S., Baird D., Soutar D. and Lane P. 2003. GenStat for Windows, 7th edn. VSN International: Oxford, United Kingdom.

Pham Van Toan and Ha Dinh Tuan. 2004. The role of biological nitrogen fixation in increasing crop production and soil fertility in Vietnam. Pp. 311–317 in 'Symbiotic nitrogen fixation prospects for enhanced application in tropical agriculture', ed. by R. Serraj. Oxford & IBH: New Delhi, India.

Farmer extension trials using BioGro for rice production in the Mekong Delta

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Abstract

Farmer trials in three regions of the Mekong Delta were used to assess the economic effectiveness of using BioGro as a part-replacement of chemical nitrogen (N) fertiliser for rice production. Nine split-plot trials, comparing the BioGro treatment with farmer practice, were conducted in each of two seasons—the dry season of 2005–06 and the wet season of 2006. In addition, two larger scale trials were conducted at research stations in Can Tho and Soc Trang in the dry season of 2006–07. The chemical N fertiliser application in the BioGro treatment was, in all cases, cut by at least 50% compared to farmer practice. Results showed that, for both seasons, there was no statistical difference in agronomic characteristics (e.g. plant height, % filled grain) between the two treatments; and yields were almost equivalent despite the much lower application of N fertiliser in the BioGro treatment. Financial analysis showed that the use of BioGro resulted in better economic effectiveness of rice production because of equivalent yields and the reduced costs of fertilisers and pesticides used in the BioGro treatment.

Introduction

More effective extension of new techniques and farming approaches often involves researchers working closely with farmers on their own farms. This is because information from on-farm trials is perceived by farmers to be more reliable and relevant than trials and experiments on research stations or locations away from the farmers' own areas (Marra et al. 2003; Abadi Ghadim et al. 2005). When trying to assess whether to adopt BioGro as a part-replacement of chemical N, it is likely that farmers will be more influenced by information from on-farm trials that 'requires less investment to seek out, analyse and integrate into existing farm-specific knowledge (and) reduces the overall information-seeking and learning costs associated with this adoption decision' (Llewellyn 2007).

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A disadvantage from the research perspective is that on-farm trial results are not generally statistically analysed, as the plots are not usually replicated and treatments are not controlled in the same rigorous way as in an experimental situation. However, the advantages of involving farmers in field trials for extension purposes are considerable as farmers learn more readily from other farmers (van den Ban and Hawkins 1996). With regard to the argument about the need for relevance versus rigour (e.g. Carberry 2001), it is likely that, in a case such as this where there was already experimental data showing the benefits of BioGro, it is more important to make the information as relevant as possible for farmers.

Furthermore, adoption of innovations is positively influenced by farmers being able to trial the innovation and observe the outcome (Rogers 1995). Pannell (1999) believes that 'trialability' is perhaps the most important factor influencing more rapid uptake of new technologies, as it enables farmers to assess the relevance and benefit of a new practice to their individual situation. In this paper we discuss

the results and insights gained from on-farm trials conducted by farmers in the Mekong Delta.

Materials and methods

Farmer extension trials in the Mekong Delta have examined the economic effectiveness of using BioGro in rice production in the region after positive results in other regions of Vietnam within the ACIAR project SMCN/2002/073. A series of two-treatment (BioGro and non-BioGro) non-replicated on-farm trials using 500 m^2 plots were conducted in three different agroecological subzones in the Mekong Delta—acidic saline (Soc Trang province), alluvial with three rice crops per year (Vinh Long) and flood prone (An Giang) (Figure 1). Nine extension trials were conducted in the dry season of 2005–06, and another nine at the same plots in the 2006 wet season. In the dry season of 2006–07 there were two larger scale trials at research stations in Soc Trang (1.5 ha) and Can Tho (0.5 ha). The rice varieties used varied by site and season depending on farmers' preferences.

The BioGro treatment is the use of BioGro at rates indicated in Table 1 plus chemical fertiliser applied at rates of 45 kg N/ha, 40 kg P_2O_5/ha and 30 kg K_2O/ha. In two seasons BioGro was also applied as a spray to the crop leaves at 15 days after sowing and 7 days before harvesting, as well as being applied with the seed. The non-BioGro treatment is the farmer practice of chemical fertilisers applied at various rates by site and season, as summarised in Table 1. The chemical N fertiliser application in the BioGro treatment was, in all cases, cut by at least 50% compared to farmer practice.

Results and discussion

Growth and yields of rice crops in the on-farm trails

Summary findings for three seasons are presented in Table 2. Results from the first trials in the dry season of 2005–06 showed that growth, yield components and yields of the rice crop vary by site due to different fertiliser applications. With much higher levels of applied N, the canopy of the rice crop in the non-BioGro plot was a darker green than that of the BioGro treatment. Between the two treatments, however, there was no statistically significant differ-

Figure 1. Field sites in the Mekong Delta included An Giang, Vinh Long and Soc Trang

ence in other agronomic traits at any site. In the second season (wet season of 2006) the benefits of BioGro were similar at the same sites but, again, the gaps in yield components and yields from the two treatments were not statistically significant. In the larger scale trials in the dry season of 2006–07, where BioGro-spray was not used and the N application rate in the non-BioGro plot was 90 kg/ha, the gaps in growth and yield were less than those in previous trials. It is worth noting that yields were almost equivalent despite the much lower application of chemical N fertiliser, and that high N application rates are commonly practiced for rice cultivation throughout Vietnam.

Economic effectiveness

Financial analysis showed that the use of BioGro resulted in better economic effectiveness of rice production (Table 3). Due to equivalent yields, there was not much difference in gross returns resulting from BioGro and non-BioGro (farmer) treatments. Differences in net returns between the two treatments result from the reduced costs of fertilisers used in the BioGro treatment. Also, it was found that less pesticide was used in the BioGro treatment plots because of fewer observations of damage to these plots from insects and diseases. The difference in net return was particularly high in the second season (VND[3]2.29 million/ha), where the trials were repeated in the same plots with high rates of chemical N fertiliser used in the non-BioGro plots. The production cost per kilogram of rice in BioGro plots was lower than that of non-BioGro treatments, as much as VND409/kg less for rice produced in the wet season of 2006. The benefit:cost ratios of BioGro when used in rice production for these on-farm trials in the Mekong Delta are around 30% and 100% higher in the 2006–07 dry season and the 2006 wet season, respectively, than those from chemical fertiliser application.

[3] Vietnamese currency (dong)

Table 1. Average treatments by season

Season	Treatment	BioGro mixed with seed (kg/ha)	BioGro sprayed (L/ha)	$N–P_2O_5–K_2O$ broadcast (kg/ha)
Dry season 2005–06	BioGro	300	1.5	45–40–30
	Farmer	–	–	130–33–50
Wet season 2006	BioGro	300	1.5	45–40–30
	Farmer	–	–	150–30–33
Dry season 2006–07	BioGro	200	–	45–30–30
	Farmer	–	–	90–60–60

Table 2. Percentage differences in agronomic traits, yield components and yields of rice crop of BioGro plots compared with non-BioGro (farmer) plots for on-farm trials averaged over the trial sites in different seasons

Criteria	Dry season 2005–06	Wet season 2006	Dry season 2006–07
Root length (cm)	>2%	>7%	<3%
Plant height (cm)	<1%	<3%	<1%
Leaf colour index	<17%	<21%	<6%
Tiller number/m^2	<14%	<3%	> <0%
Panicle number/m^2	<5%	>6%	>2%
% filled grain	>5%	>5%	> <0%
Filled grains/panicle	<4%	>8%	>1%
1,000-grain weight (g)	>1%	>3%	>1%
Grain yield (t/ha)	<1%	>4%	<1%

Note: > = more by, < = less by, > < = more or less the same at; all differences apart from the leaf colour index were NOT statistically significant.

If the results are examined in a site-specific way, it can be seen that the increases in net profit are consistent across the three different sites and in the two different seasons (Figure 2). In all cases, except for the wet season in Vinh Long, the net return from the BioGro plot was greater than for the farmer practice plot. Sometimes the difference was considerable; for example, for the 2006 wet season in An Giang the average of the three BioGro plots had a net return that was double that of the three farmer practice plots. Further data on the site-specific results of these on-farm trials are given in Tran et al. (2006, 2007) and Nguyen et al. (2007).

Farmers' opinions

Seven field days and workshops were held, focusing on the field sites and involving participation of 250 local farmers and extension and technical staff. Farmers said that using BioGro resulted in less disease, stronger rice stems, brighter and cleaner grains, better yields, less costs and more benefits. However, they also complained that BioGro fertiliser was bulky and heavy, difficult to mix with other chemicals (such as pesticide and fertilisers as these products can be harmful to the micro-organisms in BioGro) and was not available locally. Overall, they

Table 3. Effectiveness of BioGro used in rice production averaged over the trial sites in different seasons (million VND/ha [a] unless otherwise indicated).

Criteria	Dry season 2005–06		Wet season 2006		Dry season 2006–07	
	BioGro	Farmer	BioGro	Farmer	BioGro	Farmer
Cost	5.67	7.34	5.37	7.24	4.07	4.93
Gross return	13.26	13.41	12.08	11.66	13.92	14.13
Net return	7.59	6.07	6.71	4.42	9.85	9.20
Difference	+ 1.52		+ 2.29		+ 0.65	
Benefit:cost ratio	1.34	0.83	1.25	0.61	2.42	1.86
Production cost (VND/kg [b])	1,028	1,320	1,054	1,463	875	1,047
Difference (VND/kg [b])	–292		–409		–172	

[a] A$1 is approximately equivalent to VND16,500 (June 2008)
[b] kg of paddy rice

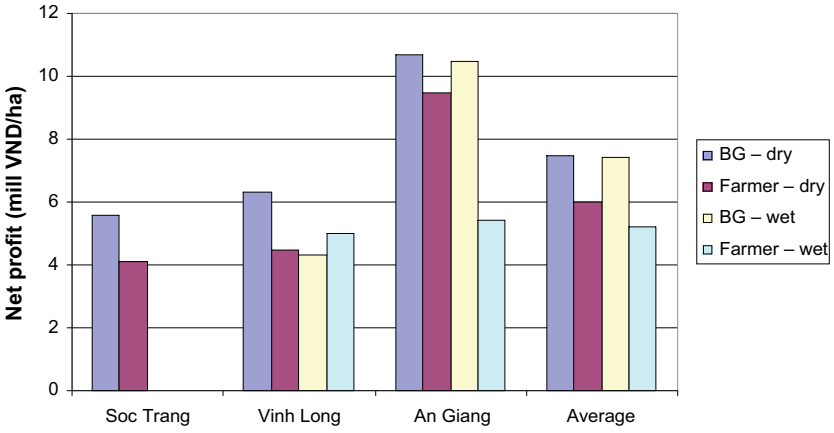

Figure 2. Net return (million VND/ha) in BioGro (BG) and non-BioGro (Farmer) plots in Soc Trang, Vinh Long and An Giang in the dry season 2005–06 and wet season 2006

said that they would really like to use BioGro for rice production if it was available. These benefits and inconveniences are consistent with those reported by farmers elsewhere in Vietnam (Barrett and Marsh 2002).

Mekong Delta farm visit during project review in October 2007

Conclusions and suggestions

Supplementing a part of chemical N fertiliser with BioGro, as in a series of on-farm trials in the Mekong Delta during the three seasons of 2005–07, has not yet given significant increases in growth and yield of rice crops compared to farmer practice. However, roughly equivalent yields were obtained with much lower N fertiliser application rates. Overall, the use of BioGro fertiliser in rice production resulted in remarkably lower costs and hence higher profits for farmers. Local rice farmers are likely to use BioGro for their production if it is available in the region.

To enhance the effective use of BioGro fertiliser in rice production in the Mekong Delta, it will be necessary to improve the quality of BioGro and conduct more region-wide demonstrations. Improvements that could be achieved with more research and trials include lower BioGro application rates (to address the issue of BioGro being perceived as bulky and heavy) and better knowledge on the role of BioGro sprays.

References

Abadi Ghadim A.K., Pannell D.J. and Burton M.P. 2005. Risk, uncertainty and learning in adoption of a crop innovation. Agricultural Economics 33, 1–9.

Barrett G. and Marsh S. 2002. An economic analysis of inoculant biofertiliser production and use in Vietnam. Pp. 102–111 in 'Biofertilisers in action', ed. by I.R. Kennedy and A.T.M.A. Choudhury. Rural Industries Research and Development Corporation: Canberra.

Carberry P.S. 2001. Are science rigour and industry relevance both achievable in participatory action research? The Regional Institute Ltd. At: <http://regional.org.au/au/asa/2001/plenary/5/carberry.htm>.

Llewellyn R.S. 2007. Information quality and effectiveness for more rapid adoption decisions by farmers. Field Crops Research 104, 148–156.

Marra M.C., Pannell D.J. and Abadi Ghadim A. 2003. The economics of risk, uncertainty and learning in the adoption of new agricultural technologies: where are we on the learning curve? Agricultural Systems 75, 215–234.

Nguyen Thanh Tam, Tran Thanh Be and Pham Thi Phan 2007. Effect of 'BioGro' on rice growth and yield in the Mekong Delta. Pp. 555–559 in 'Proceedings of the Third National Science and Technology Conference for Young Researchers of Universities of Agriculture, Forestry and Aquaculture in Vietnam', 24–25 March 2007. Ministry of Education and Training and Hue University of Agriculture and Forestry, Vietnam.

Pannell D.J. 1999. Social and economic challenges in the development of complex farming systems. Agroforestry Systems 45, 393–405.

Rogers E.M. 1995. Diffusion of innovations, 4th edn. Collier Macmillan: London.

Tran Thanh Be, Pham Thi Phan, Nguyen Thanh Tam, Le Re Son and Tran Van Con 2006. Effect of BioGro on rice production and cost in the Mekong Delta. Internal project report of 2005–06 seasons, September 2006, Can Tho University, Vietnam, 10 pp. (unpublished).

Tran Thanh Be, Pham Thi Phan, Nguyen Kim Chung and Nguyen Thanh Tam 2007. Effect of BioGro fertiliser on growth and yield of rice in the Mekong Delta, dry season of 2006–07. Internal project report, July 2007, Can Tho University, Vietnam, 7 pp. (unpublished).

van den Ban A.W. and Hawkins H.S. 1996. Agricultural extension, Blackwell Science Ltd: Oxford.

BioGro and farm-level benefits

Sally Marsh[1]

Abstract

There is concern that inorganic fertilisers are overused in the production of irrigated rice in South-East Asia. When used in the production of paddy rice, results from the ACIAR project SMCN/2002/073 have shown that an inoculant biofertiliser, BioGro, can maintain or increase crop yield with the use of less chemical fertiliser. This potentially translates into an increase in net income per hectare for farmers. Additionally, the use of BioGro provides potential long-term ecological benefits associated with decreased use of chemical fertilisers and pesticides. However, farmers generally respond to individual economic incentives (private benefits) rather than less certain and less tangible environmental outcomes (public benefits). Hence, the size of the on-farm economic benefit from using BioGro is important.

Introduction

Inorganic fertilisers are widely used in intensive rice cultivation. Pingali et al. (1998) report that in the 1990s rates of nitrogen (N) fertiliser application in South-East Asia for irrigated rice systems ranged from 80 to 150 kg/ha, with chemical fertilisers typically accounting for a 20–25% of total production costs. Pandey (1999, p. 103) notes that 'appropriate crop management technologies to increase the efficient use of inputs and to reduce production cost of rice are needed to increase the supply and keep the price of rice at a level affordable to low-income consumers'. Inoculant biofertiliser technology aims to improve nutrient use and enable farmers to use less inorganic fertiliser inputs.

The expected benefits from using the inoculant biofertiliser BioGro to reduce the amount of inorganic N applied to rice are twofold. First, there are farm-level private benefits resulting from reduced fertiliser costs (and often reduced pesticide costs) and possible increased yields. Second, there are public benefits to society from a decreased use of chemical fertilisers, which can have negative environmental impacts. In this ACIAR project the farm-level benefits from using BioGro (i.e. enhanced nutrient uptake by rice) have been investigated and quantified. Experimental data have been used to estimate the decrease in application rate of chemical fertiliser that could be achieved by using BioGro. The environmental impacts from decreased chemical fertiliser use have not been quantified.

In this chapter the overall results from the experiments conducted during the ACIAR project are reviewed and discussed in the context of farm-level benefits. Potential environmental benefits are also outlined.

Results from field experiments

Results from field experiments conducted by four different institutions in the north and south of Vietnam during the course of the project show that, on the whole, the application of BioGro increased rice production (Table 1). Although not all results are significant, most results are favourable to the application of BioGro. The trials were conducted to examine different aspects of the response to BioGro, but of particular interest to economics at the farm level are field

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experiments where 50% of the standard nitrogen and phosphorus (NP) application is replaced with BioGro and compared to an application of 100% NP. In a number of trials the yields obtained from these two treatments are statistically similar.

These results from a range of sites and rice varieties show that, by using BioGro, N and P fertiliser applications can be reduced by 50% and crop yields at least maintained. The trial results have been consistent under very different management conditions: transplanted rice seedlings in the Red River Delta in northern Vietnam and broadcast pre-germinated rice seed in the Mekong Delta in southern Vietnam. Fertiliser and, often, pesticide costs are reduced. Farmers consistently report stronger plants with less lodging, less pests and diseases, and healthier (softer) soil. They also report some inconveniences, mainly that BioGro is a bulky product compared to chemical fertilisers. The size of the economic benefit to farmers varies between sites (and seasons) and it is well known that private economic benefit is a major determinant of uptake by farmers (Feder and Umali 1993; Rogers 1995).

Better results have been achieved in farmer field trials in the Mekong Delta than in the north of Vietnam. Field experiments conducted by the Mekong Delta Research Development Institute (MDI) have produced the best economic results at the farm level. In general, yields with 50% NP + BioGro are at least maintained compared to 100% NP, but cost savings calculated by MDI staff can be substantial—in the range of VND[2] 1–2 million/ha (see Tran et al. 2008 in these proceedings). Specific examples from various sites (trial numbers in

[2] Vietnamese currency (dong)

Table 1. Summary of BioGro field experiment results

Trial [a]	Season	Field experiments [b]	Result
IAS 1	Summer 04	BG, FYM and N levels on rice	BG + NS
IAS 2	Rainy 06	BG and N levels on rice	BG ++ Sig 10%
IAS 2A	Aut–wint 06	BG and N levels on rice	BG +++ Sig 5%
IAS 3	Rainy 06	BG and P levels on rice	BG + NS
IAS 3A	Aut–wint 06	BG and P levels on rice	BG +++ Sig 5%
HUS 1	Summer 04	Different biofertilisers on rice	BG ++ Sig 10%
HUS 1A	Spring 05	Different biofertilisers on rice	BG + NS
HUS 2A	Spring 05	BG on rice (repeated inoculation)	BG +++ Sig 5%
HUS 2B	Summer 05	BG on rice (repeated inoculation)	BG +++ Sig 5%
HUS 2C	Spring 06	BG on rice (repeated inoculation)	BG ++ Sig 10%
HUS 2D	Summer 06	BG on rice (repeated inoculation)	BG ++ Sig 10%
HUS 3	Spring 06	Rate of BG application	BG +++ Sig 5%
HUS 3A	Summer 06	Rate of BG application	BG +/– NS
HUS 4	Spring 07	Timing of BG application	BG +++ Sig 5%
VASI 1	Summer 04	BG on different rice varieties	BG +/– NS
VASI 2	Summer 04	BG, FYM and NP levels on rice	BG +/– NS
VASI 3	Spring 05	BG on different rice varieties	BG +/– NS
VASI 4	Spring 05	BG, FYM and NP levels on rice	BG +/– NS
VASI 5	Summer 05	BG on different rice varieties	50% NP + BG = 100% NP
VASI 6	Summer 05	BG, FYM and NP levels on rice	50% NP + BG = 100% NP
VASI 7	Spring 06	BG, FYM and NP levels on rice	50% NP + BG = 100% NP
VASI 8	Spring 06	BG, FYM and NP levels on rice	BG + NS
VASI 9	Summer 06	BG on different rice varieties	BG +++ Sig 1%
VASI 10	Summer 06	BG, FYM and NP levels on rice	BG + NS
MDI 1	Dry 05–06	+/– BG on rice (3 sites)	30% N + BG = 100% N
MDI 2	Wet 06	+/– BG on rice varieties (3 sites)	BG ++ Sig (2 sites) BG – – Sig (1 site)
MDI 3	Dry 06–07	+/– BG on rice (2 sites)	BG ++ Sig (2 sites)

[a] IAS = Institute of Agricultural Science in southern Vietnam; HUS = Hanoi University of Science in northern Vietnam; VASI = Vietnam Agricultural Science Institute in northern Vietnam; MDI = Mekong Delta Development Research Institute in southern Vietnam

[b] BG = BioGro; FYM = farmyard manure; N = nitrogen; P = phosphorus

brackets) showing the range of farm-level benefits in VND/ha are:

- Soc Trang, dry season 2005–06: VND1.6 million (MDI 1)
- Soc Trang, dry season 2006–07: VND0.37 million (MDI 3)
- Vinh Long, wet season 2006: VND0.8 million (MDI 2)
- An Giang, wet season 2006: VND2.1 million (MDI 2).

At the Vietnam Agricultural Science Institute (VASI) in northern Vietnam, the effect of BioGro on quality rices, which command a higher market price, was explored over a number of seasons. Results from earlier trials were not significant but a field experiment at VASI for the summer 2006 crop produced significant results. The economic results, calculated by VASI staff, at the farm level represent a considerable percentage increase in net income per hectare (Table 2). Yields were slightly higher for one variety (HT1) and slightly lower for the other two varieties (BT7 and LT2), and the increase in net income per hectare ranged from 19% to 33%. The cost savings of VND942,500/ha for NP fertiliser are partially eroded by additional costs for BioGro (VND339,720/ha) and labour (VND35,000/ha), leaving a cost saving of VND567,780/ha, or 3.5% when labour is costed. However, in Vietnam the opportunity cost of rural labour can be low (Hung et al. 2007) so there is some justification for only considering the cash costs. If labour is excluded from the calculations, the total costs without BioGro reduce to VND5,987,000/ha. The cost saving of VND567,780/ha then represents a considerable cost reduction of 9.5%.

Field trials conducted by the BioGro production factories Bai Bang and Dat Viet in the north of Vietnam have also shown good results at the farm level. For example, in a field trial conducted by Dat Viet company in Thanh Hoa province for the spring 2005 crop, the plot with 50% nitrogen, phosphorus and potassium (NPK) fertiliser + BioGro yielded 286 kg/sao[3], compared to 263 kg/sao in the plot with 100% NPK and no BioGro. Cost reductions for the BioGro plot totalled VND22,550/sao (VND451,000/ha), and the 23 kg/sao yield increase achieved on the BioGro plot translates into an increase of VND73,150/sao (VND1,463,000/ha) at a rice price of VND2,200/kg. The cost reduction is in the range of 13% and the yield increase around 9%. Although both cost reductions and yield increases were substantial, yield increases delivered a greater benefit to farmers (more than three times that of the cost reductions). This is consistent with results from previous field trials using BioGro, which also indicated the financial advantages to farmers of yield increases compared to cost reductions (Barrett and Marsh 2002).

[3] A sao in northern Vietnam is equal to 360 m^2.

Table 2. Economic results of field experiment at VASI: BioGro and NPK on quality rices (BT7, HT1 and LT2), summer 2006

	100% NPK			50% NP, 100% K + BioGro		
Costs/Income	BT7	HT1	LT2	BT7	HT1	LT2
FYM	2,500,000	2,500,000	2,500,000	2,500,000	2,500,000	2,500,000
BioGro				339,720	339,720	339,720
NPK	2,605,000	2,605,000	2,605,000	1,662,500	1,662,500	1,662,500
Plant protection	432,000	432,000	432,000	432,000	432,000	432,000
Seed	450,000	450,000	450,000	450,000	450,000	450,000
Labour	10,395,000	10,395,000	10,395,000	10,430,000	10,430,000	10,430,000
Total cost (VND/ha)	16,382,000	16,382,000	16,382,000	15,814,220	15,814,220	15,814,220
Dry yield (t/ha)	5.3	5.47	5.23	5.27	5.57	5.17
Gross income (VND/ha)	18,550,000	19,145,000	18,305,000	18,445,000	19,495,000	18,095,000
Net income (NI) (VND/ha)	2,168,000	2,763,000	1,923,000	2,630,780	3,680,780	2,280,780
Increase in NI (VND/ha)				462,780	917,780	357,780
% increase in NI				21	33	19

Labour cost: VND30,000/day
Price of quality rice at the market: VND3,500/kg
Price of fertiliser: urea (N) VND5,200/kg; potassium (K) VND4,800/kg; superphosphate (P) VND1,500/kg

The research conducted during this ACIAR project indicates that the farm-level benefits of BioGro are consistent but generally moderate, and mostly result from cost savings (reduced fertiliser and pesticide costs). In cases like this, experience would indicate that adoption will be slow (Pannell 1999). The environmental benefits are potentially considerable (see the following section) but farmers do not generally respond to public environmental benefits without incentives (Pannell et al. 2006). In such situations, where private benefits are moderate to low but public benefits exist, there can be a role for government support through extension and perhaps financial incentives.

In a country like Vietnam, where land holdings are small but there are many farmers, small increases in profit for individuals translate into large amounts when considered on a nation-wide basis. Assuming a very conservative profit increase from cost savings of VND500,000/ha by using BioGro over a total of 7 million hectares of rice grown nation-wide per year, and that 5% of this area uses BioGro, the profit increase for farmers nation-wide would total VND175 billion (approximately US$11 million). Assuming a moderate profit increase of VND1 million/ha and an adoption rate of 10% over the 7 million hectares of rice, the profit increase for farmers nation-wide would be VND700 billion (around US$44 million).

There is one further observation to make about the size of economic benefits. It became apparent during the project that the size of the economic benefit per hectare was viewed differently by farmers in northern compared to southern Vietnam. On-farm benefits of VND500,000/ha to VND1 million/ha in northern Vietnam were viewed by farmers as not sufficient to encourage them to use BioGro, whereas a similar level of benefit in southern Vietnam was regarded in a much more positive light. It seems that these different perceptions may be related to farm size.

Farms in the Red River Delta in Vietnam are generally smaller and more fragmented than in the Mekong Delta. For example, Marsh et al. (2006) report an average farm size of 5,310 m^2 for farms surveyed in Dan Phuong district in Ha Tay province in the Red River Delta. Based on the above benefit per hectare, if a farmer with 5,310 m^2 of land planted all this area to rice using BioGro, the total on-farm benefit would range from VND265,500 to VND531,000. This relatively small amount may not be sufficient for these small farmers to invest time and effort in the learning required to use the new technology. Pandey (1999) also notes that, for small

Effect of inoculation with BioGro on rice plants in (a) a laboratory test and (b) a field test in Tay Ninh province in 2006. In each case, the plants on the left are the control, and those on the right the BioGro-treated plants. Note the greater root mass and healthier appearance and whiteness of the BioGro-treated rice plants from the field test.
Photos by Nguyen Thanh Hien

farmers, the efforts in acquiring and learning to use improved nutrient management technologies may not be economically justifiable. In the Mekong Delta farms are more likely to be nearer to 1 hectare in size and be in one or two parcels of land. For example, Marsh et al. (2006) report an average farm size of 15,943 m^2 for farms surveyed in O Mon district in the Mekong Delta. Total on-farm benefits for farmers with this much land, based on the earlier per hectare benefit, would range from VND797,150 to VND1,595,300, which represents a more substantial benefit for these farmers.

Public environmental benefits of BioGro

Pandey (1999) states that there is a need to change from agricultural systems encouraging high input use to systems focused on achieving increased input-use efficiency. The extensive use of biofertilisers has the potential to better recycle the current nutrients contained in the soil and water of agricultural ecosystems and to reduce the negative impacts of chemical fertilisers on ecosystems (Kennedy and Nguyen 1999).

Widespread adoption of biofertiliser technology has the potential to alleviate problems associated with chemical fertiliser use, leading to benefits for the community of a cleaner agricultural environment. Potential environmental benefits from the use of BioGro are:

- reduction in the use of inorganic fertilisers, resulting in reduction in denitrification and other production of greenhouse gases; reduction in nitrate toxicity in the groundwater by minimising leaching loss; and lowered pollution of waterways by reduction in the growth of algae and other harmful microbes capable of toxin production
- reduction in the use of pesticides, resulting in environmental and health benefits.

This project has not quantified these public benefits in economic terms but they could potentially be substantial. The project has, however, identified the potential reduction in chemical N use that could be achieved by more efficient nutrient use with BioGro. For example, in field experiments conducted by the Institute of Agricultural Science (IAS) in southern Vietnam for the 2006 summer–autumn rice crop, economically optimal N application rates are always lower if N is applied with BioGro at all prices of urea and rice (see Marsh 2008 in these proceedings). Furthermore, net income is usually higher with these lower optimal N application rates. For the 2006 autumn–winter crop, economically optimal N application rates with BioGro were similar (but always slightly higher) to those without BioGro but yields and net income were considerably higher. Again, these results are an indication of more efficient nutrient use with BioGro.

As for on-farm benefits, small reductions in the use of inorganic N on small individual land holdings translate into a large reduction when considered on a nation-wide basis. Assuming a very conservative reduction in inorganic N use of 25 kg/ha by using BioGro over a total of 7 million hectares of rice grown nation-wide per year, and that 5% of this area uses BioGro, the N saved would be 8,750 t or 19,022 t of urea. Assuming a moderate reduction in inorganic N use of 50 kg/ha and an adoption rate of 10% on the 7 million hectares of rice, N saved would be 35,000 t or 76,087 t of urea.

Conclusion

The farm-level benefits of BioGro are consistent but generally moderate, and mostly result from cost savings (reduced fertiliser and pesticide costs). Consistent results from a range of sites and rice varieties show that, by using BioGro, N and P fertiliser use can be reduced by 50% and crop yields at least maintained. However, the size of the economic benefit will be the primary determinant of uptake by farmers and this varies between sites and seasons. It seems likely that the technology will be more applicable in the south of Vietnam where farm sizes are larger and, hence, total on-farm benefits more substantial.

The research work has also identified the potential reduction in chemical N use that could be achieved by more efficient nutrient use with BioGro. Reduced inorganic fertiliser use has potentially substantial benefits to society, and ways of achieving this are of interest worldwide.

References

Barrett G. and Marsh S. 2002. An economic analysis of inoculant biofertiliser production and use in Vietnam. Pp. 102–111. In 'Biofertilisers in action', ed. by I.R. Kennedy and A.T.M.A. Choudhury. Rural Industries Research and Development Corporation Publication No. 02/086: Barton, ACT, Australia.

Feder G. and Umali D.L. 1993. The adoption of agricultural innovations: A review. Technological Forecasting and Social Change 43, 215–239.

Hung Pham Van, MacAulay T.G. and Marsh S.P. 2007. The economics of land fragmentation in the north of Vietnam. Australian Journal of Agricultural and Resource Economics 51, 195–211.

Kennedy I.R. and Nguyen Thanh Hien 1999. Biofertiliser inoculant technology for the growth of rice in Vietnam: Developing technical infrastructure for quality assurance and village production for farmers. Project proposal for Capacity Building for Agriculture and Rural Development (CARD) Program. Australian Agency for International Development: Canberra.

Marsh S.P. 2008. Economically optimal nitrogen fertiliser rates with BioGro. These proceedings.

Marsh S.P., Hung Pham Van, Dac N.T. and MacAulay T.G. 2006. Farm size change and the market for agricultural land use rights in Vietnam since 1993. In 'Agricultural development and land policy in Vietnam', ed. by S.P. Marsh, T.G. MacAulay and Hung Pham Van. ACIAR Monograph No. 123, 85–108.

Pandey S. 1999. Adoption of nutrient management technologies for rice production: economic and institutional constraints and opportunities. Nutrient Cycling in Agroecosystems 53, 103–111.

Pannell D.J. 1999. Social and economic challenges in the development of complex farming systems. Agroforestry Systems 45, 393–405.

Pannell D.J., Marshall G.R., Barr N., Curtis A., Vanclay F. and Wilkinson R. 2006. Understanding and promoting adoption of conservation practices by rural landholders. Australian Journal of Experimental Agriculture 46(11), 1407–1424.

Pingali P.L., Hossain M., Pandey S. and Price L.L. 1998. Economics of nutrient management in Asian rice systems: Towards increasing knowledge intensity. Field Crops Research 56, 157–176.

Rogers E.M. 1995. Diffusion of innovations, 4th edn. Collier Macmillan: London.

Tran Thanh Be, Pham Thi Phan and Marsh, S. 2008. Farmer extension trials using BioGro for rice production in the Mekong Delta. These proceedings.

Effects of bacterial inoculant biofertilisers on growth, yield and nutrition of rice

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Abstract

A greenhouse experiment and two field experiments were established in New South Wales to evaluate the effects of inoculation of rice with bacterial strains from a Vietnamese inoculant biofertiliser (BioGro) and several other plant growth promoting bacteria, including azospirilla and a rice endophyte (*Rhizobium leguminosarum* bv. *trifolii* R4), on the growth, yield and nutrient content of rice. In the greenhouse trial bacterial inoculations increased shoot and root weights of rice plants significantly over controls. In the field experiments, particularly with *Rhizobium leguminosarum,* similar effects including significant differences in nitrogen (N) uptake in vegetative matter were observed at the panicle initiation (PI) stage. However, these effects were not significant as grain yield at harvest, and it is concluded that the much longer period of growth for Australian rice may allow compensation between treatments. Reinoculation of plants at the PI stage and application of N fertiliser in at least two splits (2/3 at final land preparation and 1/3 at PI stage) and at lower application rates are recommended for future field experiments. The possible use of ^{15}N-labelled fertiliser is also recommended to quantify the effects of bacterial inoculants on the recovery of fertiliser-N.

Introduction

Application of nitrogen (N) fertiliser is essential to maintain growth and yield of rice due to acute N deficiency in rice soils. However, a substantial portion of the applied fertiliser-N is lost as a result of ammonia volatilisation, denitrification and leaching, causing environmental pollution (Choudhury and Kennedy 2005). Recovery of fertiliser-N in rice culture is very low, generally around 30–40% and, in some cases, even lower (Choudhury and Khanif 2001, 2004). Inoculation with bacterial biofertilisers may reduce the application of fertiliser-N by increasing N uptake by plants (Choudhury and Kennedy 2004; Kennedy et al. 2004). Vigorous seedling growth is important for the successful establishment of rice and other crops.

Both single and multistrain biofertilisers are used to inoculate rice seedlings. Previous investigations in several countries have shown that rice seedling growth was enhanced following inoculation with plant growth promoting micro-organisms, leading to increased grain and straw yields and enhanced efficiency of fertiliser-N use (Biswas et al. 2000a, b; Yanni et al. 1997; Mirza et al. 2000; Malik et al. 2002).

Field studies in Vietnam (Nguyen et al. 2002, 2003) showed that BioGro, containing three bacterial strains (1N, 3C & 4P) isolated from Vietnamese rice cropping soils, increased rice yields signifi-

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cantly. The first strain, *Pseudomonas fluorescens* (1N) has the ability to reduce C_2H_2 to C_2H_4, indicating its potential for N_2 fixation. The second strain, *Citrobacter freundii* (3C), apparently produces extra-cellular compounds that inhibit the growth of some other rhizosphere organisms. The third strain, *Klebsiella pneumoniae* (4P), also a diazotroph, can solubilise precipitated $Ca_3(PO_4)_2$ in an agar medium. One greenhouse and two field experiments were conducted to evaluate the effects of bacterial inoculant biofertilisers on growth, yield and nutrition of rice.

Materials and methods

Greenhouse experiment

Preparation of bacterial cultures

All bacterial strains were obtained from the SUNFix Centre for Nitrogen Fixation culture collection and grown for 24 hours at 25 ˚C in 200 mL of modified nutrient broth (MNb). While being shaken at 1,500 rpm, the number of colony forming units (cfu) was counted by plating a serial dilution from each individual culture broth. Finally, individual cultures were either mixed or applied separately as follows: T1 = control (sterile MNb); T2 = 1N plus 3C and 4P (10:1:10); T3 = 1N:4P (1:1); T4 = *Azospirillum lipoferum* 596. The description of the treatments is presented in Table 1.

Growing rice seedlings

Rice seeds of variety Amaroo were soaked in water for 24 hours prior to being immersed for another hour in bacterial suspensions prepared as per treatment described above. Plastic pots filled with 850 g of sand were moistened with 100 mL of water, allowing for six replicates of each treatment plus controls. The pots were completely randomised in the greenhouse, where the temperature fluctuated between 20 ˚C to 30 ˚C for the duration of the trial. Four rice seeds were transferred to each pot and allowed to germinate. Germination was completed in 8 days. The seedlings were allowed to grow for a further 4 weeks in flooded conditions by adding 100 mL of potable Sydney water to each pot on alternate days, and were harvested 31 days after germination. Root and shoot lengths were measured. Roots and shoots were separated and placed in paper bags and dried at 70 ˚C for 4 days. Shoot and root weights were recorded.

Field experiment at Yanco Agricultural Research Institute

Soil analysis

The experiment was conducted at Yanco Agricultural Research Institute, New South Wales, Australia. Prior to commencement, soil samples were collected from the experimental plots at a depth of 0–15 cm. The samples were air dried, ground and passed through a 2-mm sieve. The processed soil was analysed for pH; organic matter content; particle size; cation exchange capacity (CEC); total N; available P; and exchangeable K, Ca and Mg. Soil pH (soil:water ratio 1:5) was measured by a glass electrode (Peech 1965). Organic matter was analysed by the potassium dichromate and H_2SO_4 digestion method (Walkley and Black 1934). Particle size was analysed by the hydrometer method (Black 1965). CEC and exchangeable K, Mg and Ca were determined by ammonium acetate extraction (Schollenberger and Simon 1945). Total N was determined by the LECO combustion method (Sweeney and Rexroad 1987) and available P by the NH_4F–HCl extraction method (Bray and Kurtz 1945). The soil analytical results are presented in Table 2. As the soil was deficient in N, the application of fertiliser-N was essential to meet the rice crop's demands.

Table 1. Treatment plan for greenhouse trial, greenhouse experiment at the University of Sydney, October–November 2004

Treatment	Strain	Ratio
T1 (control)	No strain: sterile modified nutrient broth only	n.a.
T2 (BioGro)	1N (*Pseudomonas fluorescens*), 3C (*Citrobacter freundii*) and 4P (*Klebsiella pneumoniae*)	10:1:10
T3 (modified BioGro)	1N and 4P	1:1
T4 (*Azospirillum*)	*Azospirillum lipoferum* 596	–

n.a. = not applicable

Experimental design, basal fertilisers and inoculation of rice seeds

The experiment was laid out in a randomised complete block (RCB) design with four replications. Unit plot size was 3 m × 10 m. Description of the treatments is presented in Table 3. The recommended rate of P fertiliser for the area (20 kg P/ha from triple superphosphate) was applied as blanket to all treatments. Nitrogen fertiliser was applied as urea as described in the treatments (Table 3). Both N and P were applied at final land preparation.

Table 2. Properties of the initial soil, field experiment at Yanco, November 2004 to April 2005

Soil property	
pH	6.35
Organic matter (%)	3.2
Particle size analysis	
% sand	63.26
% silt	7.35
% clay	29.39
Textural class	Sandy clay loam
Cation exchange capacity (cmol/kg)	12.64
Total N (%)	0.10
Available P (mg/kg)	18.06
Exchangeable K (cmol/kg)	0.62
Exchangeable Ca (cmol/kg)	7.15
Exchangeable Mg (cmol/kg)	4.01

Pre-inoculated peat was applied to the experimental plots according to the treatments presented in Table 3 one day before sowing the rice seeds. A short-duration rice variety, Jarrah, was used as the test crop. Pre-soaked seeds of Jarrah were sown by broadcasting at a rate of 140 kg/ha.

Sampling and plant analyses

Plants were sampled at panicle initiation (PI) stage (59 days after seed sowing) and at maturity (130 days after seed sowing). At PI stage, rice plant biomass, tiller number and nutrient (N, P, K, S and Mg) uptakes were measured. At maturity, grain and straw yields, plant height, yield components (panicle number, number of filled grains per panicle and 1,000-grain weight) and N uptake by rice plants were measured. At PI stage, an area of 1 m^2 was sampled from each plot. At maturity the sampling area for grain yield varied among the plots. However, the minimum area of sampling was 21.2 m^2 per plot. Chemical analysis of plant tissue for nutrient (N, P, K, S and Mg) contents was conducted using near infrared reflectance (NIR) spectroscopy (Batten 1998; Batten et al. 1991).

Field experiment at Jerilderie Rice Research Institute

Soil analysis and experimental design

The experiment was conducted at Jerilderie Rice Research Institute, New South Wales, Australia. The soil properties of the experimental plots were measured and determined as described above for the trial at Yanco Agricultural Institute, and the results are presented in Table 4. The experiment was conducted in an RCB design using six replications with a 2.5 m x 15 m unit plot size. A description of the treatments is presented in Table 5.

Inoculation of rice and fertilisation

The experimental plots were treated with pre-inoculated peat 1 day prior to sowing rice seeds. A long-duration rice variety, Amaroo, was used for this trial. Seeds were pre-soaked and sown at a rate

Table 3. Description of treatments used in trial, field experiment at Yanco, November 2004 to April 2005

Treatment designation	Treatment	Description
T1	Control	Peat containing sterile modified nutrient broth + 50 kg N/ha as urea
T2	Recommended N rate	Peat without broth + 150 kg N/ha as urea
T3	BioGro	Vietnamese bacterial strains (a 10:1:10 mixture of *Pseudomonas fluorescens*, *Citrobacter freundii* and *Klebsiella pneumoniae*) + 50 kg N/ha as urea
T4	*Azospirillum* and *Herbaspirillum*	A 1:1:1 mixture of *Azospirillum brasilense* Sp7-S, *A. lipoferum* 687 and *Herbaspirillum seropedicae* + 50 kg N/ha as urea
T5	*Rhizobium*	*Rhizobium leguminosarum* bv. *trifolii* R4 + 50 kg N/ha as urea

of 140 kg/ha. Phosphorus (20 kg P/ha from monoammonium phosphate) was applied to all plots and fertiliser-N (as urea) was applied as per treatment (Table 5). All of the N and P were applied at final land preparation.

Table 4. Properties of the initial soil, field experiment 2005–06, Jerilderie

Soil properties	
Texture	Clay
pH (water)	5.5
Organic matter (%)	3.1
CEC (meq/100 g)	16.7
EC (dS/m)	0.09
NO_3-N (ppm)	7.1
NH_4-N (ppm)	<1.0
Available P (ppm)	5
Exchangeable K (me/100 g)	1.12
Exchangeable Ca (me/100 g)	6.37
Exchangeable Mg (me/100 g)	7.59
Available S (ppm)	12
Available B (ppm)	0.7
Available Cu (ppm)	1.7
Available Fe (ppm)	133
Available Mn (ppm)	31.8
Available Zn (ppm)	0.5

Sampling times

Samplings were conducted at PI stage (91 days after sowing) and at maturity (154 days after sowing). At the PI stage, rice plant biomass and N uptake were measured. Chemical analysis of plant tissue for N content was conducted using NIR spectroscopy (Batten 1998; Batten et al. 1991). At maturity, grain and straw yields were recorded.

Statistical analyses

All the data were analysed by two-way ANOVA using the GenStat program version 7 (Payne et al. 2003).

Results

Greenhouse experiment

Bacterial counts

The number of cfu per mL of culture broth for starter inoculant was determined using the dilution plating method. The average starter cultures were 4.8×10^8, 1.2×10^9, 1.2×10^9 and 3.3×10^7 for 1N, 3C, 4P and *Azospirillum*, respectively. The cfu per treatment per pot ranged from 6.6×10^7 (*Azospirillum*, T4) to 1.2×10^9 (4P, T2 and T3).

Shoot and root growth

While shoot and root lengths were not affected by the inoculations, shoot weight increased significantly (P<0.05, Table 6). There was no significant difference between the bacterial treatments, with all except one Vietnamese multistrain (1N, 3C and 4P) showing significantly increased root weight compared to the control. Root weight per seedling ranged from 19.6 mg to 35.8 mg.

Table 5. Description of the treatments used in the trial, field experiment at Jerilderie, October 2005 to April 2006

Treatment designation	Treatment	Description
T1	N0	N control
T2	N50	50 kg N/ha: 9.1 kg N/ha as monoammonium phosphate + 40.9 kg N/ha as urea
T3	N100	100 kg N/ha: 9.1 kg N/ha as monoammonium phosphate + 90.9 kg N/ha as urea
T4	N50 + BioGro [a]	T2 + 200 kg/ha of BioGro [a] (1N:3C:4P = 10:1:10) inoculum in peat
T5	N50 + *Citrobacter freundii*	T2 + 200 kg/ha of *Citrobacter freundii* inoculum in peat
T6	N50 + *Rhizobium* low rate	T2 + 47.5 kg/ha of *Rhizobium leguminosarum* bv. *trifolii* R4 inoculum in peat
T7	N50 + *Rhizobium* high rate	T2 + 237.3 kg/ha of *Rhizobium leguminosarum* bv. *trifolii* R4 inoculum in peat

[a] 1N, 3C and 4P = *Pseudomonas fluorescens*, *Citrobacter freundii* and *Klebsiella pneumoniae*, respectively.

Field experiment at Yanco Agricultural Research Institute

Plant biomass, tiller and height

Treatment effects were not significant on dry plant biomass (t/ha) at the PI stage. However, inoculation with *Rhizobium* and BioGro outyielded the control by 0.61 t/ha and 0.48 t/ha, respectively (Table 7). Tillering of the rice plants was not significantly affected by the treatments although there were increases in tiller number per square metre due to inoculation with *Rhizobium* and BioGro (Table 7). Correlation between tiller number and plant biomass was high ($r = 0.94$). Recommended fertiliser-N rate increased plant height at maturity significantly ($P<0.05$) compared with the bacterial treatments (Table 7).

Yield components, grain and straw yields

Treatments did not affect any of the yield components (panicle number, number of filled grains, 1,000-grain weight); however, estimated grain yield was increased notably ($P<0.1$) by the recommended N rate over all other treatments except the *Rhizobium* treatment (Table 7). This significant increase was attributed to a higher number of filled grains per panicle in this treatment. There was no significant treatment effect on grain and straw yields, total biomass and harvest index (Table 8). Grain yield increased by 1.07, 0.36 and 0.32 t/ha over the control in the recommended N rate, *Rhizobium* and BioGro-treated plots, respectively, but these differences were not statistically significant.

Table 6. Effects of bacterial inoculant biofertilisers on shoot and root growth of rice seedlings, greenhouse experiment at the University of Sydney, October–November 2004

Treatment	Shoot weight/ seedling (mg)	Root weight/ seedling (mg)	Shoot length (cm)	Root length (cm)
Control	12.5 b	19.6 c	12.5	20.1
BioGro	19.6 a	23.9 bc	12.6	19.9
Modified BioGro ratio	22.9 a	35.8 a	12.1	19.3
Azospirillum	19.3 a	32.1 ab	12.6	20.7
F value	0.027	0.029	0.670	0.461
LSD (0.05)	6.64	11.41	–	–
CV (%)	29.7	34.0	6.6	7.0

Values followed by a common letter in a column are not significantly different by LSD (least significant difference) at $P = 0.05$

Table 7. Effects of chemical- and biofertilisers on some agronomic parameters; and estimated grain yield of Jarrah rice, field experiment at Yanco, November 2004 to April 2005

Treatment	Plant biomass (t/ha) at panicle initiation stage	Tiller no./m^2 at panicle initiation stage	Plant height (cm) at maturity	Panicle no./m^2 at maturity	No. of filled grains/ panicle at maturity	1,000-grain weight (g)	Estimated grain yield (t/ha)
Control	2.38	613	73.46	580.0	52.4	22.81	6.38
Recommended N rate	2.28	609	76.26	536.5	69.3	22.65	9.28
BioGro	2.86	642	68.74	546.5	53.3	23.40	6.57
Azospirillum and *Herbaspirillum*	1.78	504	68.94	521.0	46.2	22.98	5.58
Rhizobium	2.99	737	70.20	490.0	60.5	23.42	8.20
F probability	0.103	0.273	0.041	0.373	0.14	0.67	0.083
LSD (0.05)	–	–	5.23	–	–	–	–
LSD (0.10)	–	–	–	–	–	–	2.21
CV (%)	22.75	18.68	4.63	9.50	20.90	3.52	24.27

Nitrogen content and uptake

The N content as a percentage of the whole plant (mixture of grain and straw) at harvest was significantly lower in all treatments compared with the fully fertilised treatment (Table 9). Total N uptake (kg/ha) was significantly higher in the fully fertilised treatment compared to all other treatments. This was attributed to significantly higher N content in plant tissue in this treatment. Total N uptake for the production of 1 t of rough rice ranged from 12.05 to 18.72 kg/ha.

Nitrogen content (%) in plant tissue at PI stage was significantly higher in the fully fertilised treatment compared to other treatments ($P<0.05$, Table 8) but other treatments showed similar values. The N uptake (kg/ha) at PI stage was also similar in the fully fertilised and *Rhizobium* inoculation treatments (Table 10). In the control as well as in the *Azospirillum* + *Herbaspirillum*-treated plants, the N uptake values were significantly lower compared to the fully fertilised and *Rhizobium*-inoculation treatments. Although the N content in plant tissues was significantly lower in the *Rhizobium*-treated plants compared to the fully fertilised ones, higher plant biomass due to *Rhizobium* inoculation contributed to similar amounts of N uptake as in fully fertilised plants.

Other nutrient contents and uptake at PI stage

None of the treatments significantly affected the P, K and Mg contents (%) in plant tissue at PI stage. However, the recommended N rate increased the S content (%) compared with BioGro-treated plants, but this difference did not reach great significance ($P<0.1$, Table 10). There was an indication that *Rhizobium* inoculation increased S and Mg uptakes significantly over the control and *Azospirillum* + *Herbaspirillum*-treated plants. BioGro and fully fertilised treatments increased S and Mg uptakes compared to *Azospirillum* + *Herbaspirillum*-treated plants but, again, this difference was not very significant ($P<0.1$).

Table 8. Effects of chemical- and biofertilisers on grain and straw yields, total biomass and harvest index of Jarrah rice, field experiment at Yanco, November 2004 to April 2005

Treatment	Grain yield (t/ha)	Straw yield (t/ha)	Total biomass (t/ha)	Harvest index (%)
Control	6.10	5.69	11.79	51.73
Recommended N rate	7.17	6.50	13.67	52.45
BioGro	6.42	5.83	12.25	52.41
Azospirillum and *Herbaspirillum*	5.57	5.48	11.05	50.41
Rhizobium	6.46	5.71	12.17	53.08
F probability	0.185	0.576	0.310	0.699
CV (%)	13.28	15.06	13.36	4.05

Table 9. Effects of chemical- and biofertilisers on total N content of and uptake by Jarrah rice; and internal N efficiency, field experiment at Yanco, November 2004 to April 2005

Treatment	N content (%) in whole plant at maturity	N uptake (kg/ha) by whole plant at maturity	Total N uptake (kg/ha) per t of rough rice production	Internal N efficiency (kg grain / kg absorbed N)
Control	0.62	75.3	12.05	83.5
Recommended N rate	0.97	131.6	18.72	54.2
BioGro	0.64	80.0	12.27	83.1
Azospirillum and *Herbaspirillum*	0.62	69.6	12.35	88.2
Rhizobium	0.65	81.0	12.23	84.0
F probability	0.009	<0.001	0.020	0.087
LSD (0.05)	0.19	20.99	4.54	–
LSD (0.10)	–	–	–	21.03
CV (%)	17.17	15.10	19.00	21.03

Field experiment at Jerilderie Rice Research Institute

Plant biomass and N uptake at PI stage

Dry plant biomass increased significantly ($P<0.05$) over the control (N0) in all treatments except T5 (N50 + *Citrobacter freundii*) (Table 11). Addition of a higher N rate (100 kg N/ha) also increased plant biomass over N50 (but $P<0.1$). Bacterial inoculants did not increase plant biomass significantly over N50 although there was an increase of 0.49 t/ha with the *Rhizobium* high rate. N content (%) in plant tissue increased significantly ($P<0.05$) over the control (N0) by the addition of 100 kg N/ha alone. All treatments except N50 increased N uptake (kg/ha) significantly over the control ($P<0.05$). *Rhizobium* inoculant applied at 237 kg/ha with 50 kg N/ha increased N uptake (kg/ha) significantly over N50. *Rhizobium* low rate + N50 increased N uptake over N50 alone but the difference failed to be really significant ($P<0.05$).

Grain and straw yields

Grain yield increased significantly over the control (N0) due to application of 50 kg N/ha (Table 12). Addition of a higher N rate or inoculation with bacteria did not increase grain yield over N50 at all. Rather, bacterial inoculation treatments decreased grain yield from N50, although such differences were not statistically significant.

Discussion

In the field experiment at Yanco a high rate of N (150 kg/ha) was proposed for the long-duration (160 days growth) variety Amaroo in two splits; however, the short-duration (140 days growth) variety Jarrah was later used due to a late planting

Table 10. Effects of chemical- and biofertilisers on N, P, K, S and Mg contents of and uptake by Jarrah rice at panicle initiation stage, field experiment at Yanco, November 2004 to April 2005

Treatment	Nutrient content (%)					Nutrient uptake (kg/ha)				
	N	P	K	S	Mg	N	P	K	S	Mg
Control	2.29	0.29	1.71	0.20	0.21	50.29	7.16	41.56	4.50	4.77
Recommended N rate	2.95	0.29	1.55	0.23	0.24	67.33	6.91	35.37	5.27	5.37
BioGro	2.01	0.29	1.83	0.19	0.21	56.19	8.30	51.95	5.30	5.90
Azospirillum and *Herbaspirillum*	2.33	0.28	1.72	0.20	0.21	41.21	5.03	30.27	3.58	3.85
Rhizobium	2.27	0.26	1.51	0.20	0.22	67.92	8.13	46.28	5.92	6.59
F probability	0.03	0.84	0.32	0.09	0.50	0.09	0.103	0.078	0.09	0.08
LSD (0.05)	0.53	–	–	–	–	–	–	–	–	–
LSD (0.10)	–	–	–	0.03	–	16.19	–	12.54	1.25	1.37
CV (%)	14.22	11.77	12.15	10.07	11.09	22.83	22.16	24.16	20.32	20.76

Table 11. Effects of N fertiliser and bacterial inoculant biofertiliser treatments on biomass and N uptake of Amaroo rice at panicle initiation stage, field experiment at Jerilderie, 2005–06

Treatment	Dry plant biomass (t/ha)	N content (%)	N uptake (kg/ha)
N0	1.83	1.51	25.3
N50	2.66	1.61	37.3
N100	3.37	2.14	65.9
N50 + BioGro	2.77	1.78	44.8
N50 + *Citrobacter freundii*	2.56	1.74	41.1
N50 + *Rhizobium* low rate	2.99	1.84	52.1
N50 + *Rhizobium* high rate	3.15	1.81	55.8
F value	0.019	0.028	<0.001
LSD (0.05)	0.82	0.34	14.85
LSD (0.10)	0.69	0.28	12.34
CV (%)	25.3	16.3	27.4

schedule but N fertiliser rates were not reduced. No control (without N) treatment could be included so it was not possible to calculate agronomic efficiency of N (kg grain per kg added N) and apparent recovery (%) of added N. Actual fertiliser-N recovery could be calculated if ^{15}N-labelled N fertiliser was applied. The ^{15}N tracer technique is widely used as the precise method to quantify fertiliser-N actual recovery (Cao et al. 1984; Choudhury et al. 2002).

Rhizobium inoculation clearly increased N uptake (kg/ha) at PI stage over the control (visibly to the human eye as extra greenness) (Table 10). This significant effect of *Rhizobium* on N uptake had disappeared at maturity stage (Table 9) as grain yield. Nevertheless, *Rhizobium* inoculation increased plant biomass by 0.61 t/ha over the control at PI stage (Table 7) while increasing grain yield by 0.36 t/ha (Table 8). These results indicate that the positive effects of *Rhizobium* inoculation decreased at the later growth stages of the rice plants. This might be a result of the longer growth duration of rice plants in Australia compared to Vietnam. Reinoculation of bacteria at PI stage and application of N fertiliser in at least two splits (2/3 at final land preparation and 1/3 at PI stage) are recommended for the next field experiments, along with lower N rates.

Table 12. Effects of N fertiliser and bacterial inoculant biofertiliser treatments on grain and straw yields, and total biomass of Amaroo rice, field experiment at Jerilderie, 2005–06

Treatment	Grain yield (t/ha)	Straw yield (t/ha)	Total biomass (t/ha)
N0	4.20	4.00	8.20
N50	7.28	6.33	13.61
N100	7.08	6.54	13.62
N50 + BioGro	6.27	6.11	12.38
N50 + *Citrobacter freundii*	6.56	5.68	12.24
N50 + *Rhizobium* low rate	6.57	6.11	12.68
N50 + *Rhizobium* high rate	6.78	5.95	12.73
F value	0.001	0.002	<0.001
LSD (0.05)	1.30	1.09	2.26
LSD (0.10)	1.08	0.90	1.87
CV (%)	15.6	14.3	14.2

Australian field trial at harvest conducted at the Australian Rice Research Institute, Jerilderie, New South Wales

The use of ^{15}N-labelled N fertiliser is also recommended to quantify the effects of bacterial inoculants on fertiliser-N actual recovery.

In all treatments except the fully fertilised one, the estimated amount of N removed per tonne of rough rice production was low (Table 9). Generally, estimated N removal for the production of 1 t of rough rice is 16–17 kg (Sahrawat 2000; Choudhury and Kennedy 2005). The lower amount of N removal in this study indicates that N estimation by NIR was not accurate. In the original program it was proposed to analyse grain and straw separately for total N, but analysis of the whole plant was finally done without separating grain and straw. This could not estimate the total N uptake accurately. Moreover, it was not possible to calculate the N harvest index, i.e. the percentage of N uptake by grain compared to total N uptake by the whole plant. Separate analysis of grain and straw for total N content is recommended for future studies.

In the field experiment at Jerilderie the grain yield response due to added N was, at 50 kg N/ha, significant. Addition of higher N rates did not increase grain yield over N50. Lower N rates (25 and 50 kg N/ha) are proposed for future experiments on this site. Bacterial inoculants can be used at a rate of 25 kg N/ha.

Conclusion

The experiments showed that bacterial inoculation increased shoot and root weights significantly in the greenhouse. Similar effects were noticed in the field experiments at PI stage although the effects disappeared at the harvesting stage. Reinoculation of bacteria at PI stage, and application of N fertiliser in at least two splits (2/3 at final land preparation and 1/3 at PI stage) are recommended for the next field experiments, along with lower N rates. The use of ^{15}N-labelled N fertiliser is also recommended to quantify the effects of bacterial inoculants on fertiliser-N actual recovery.

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References

Batten G.D. 1998. Plant analysis using near infrared reflectance spectroscopy: The potential and the limitations. Australian Journal of Experimental Agriculture 38, 697–706.

Batten G.D., Blakeney A.B., Glennie-Holmes M.G., Henry R.J., McCaffery A.C., Bacon P.E. and Heenan D.P. 1991. Rapid determination of shoot nitrogen status in rice using infrared reflectance spectroscopy. Journal of the Science of Food and Agriculture 54, 191–197.

Biswas J.C., Ladha J.K. and Dazzo F.B. 2000a. Rhizobia inoculation improves nutrient uptake and growth of lowland rice. Soil Science Society of America Journal 64, 1644–1650.

Biswas J.C., Ladha J.K., Dazzo F.B., Yanni Y.G. and Rolfe B.G. 2000b. Rhizobial inoculation influences seedling vigour and yield of rice. Agronomy Journal 92, 880–886.

Black C.A. (ed.) 1965. Methods of soil analysis, Parts I and II. American Society of Agronomy: Madison, Wisconsin.

Bray R.H. and Kurtz L.T. 1945. Determination of total, organic and available forms of phosphorus in soils. Soil Science 59, 39–45.

Cao Z.H., De Datta S.K. and Fillery I.R.P. 1984. Effect of placement methods on floodwater properties and recovery of applied nitrogen (^{15}N-labeled urea) in wetland rice. Soil Science Society of America Journal 48, 196–203.

Choudhury A.T.M.A. and Kennedy I.R. 2004. Prospects and potentials for systems of biological nitrogen fixation in sustainable rice production. Biology and Fertility of Soils 39, 219–227.

Choudhury A.T.M.A. and Kennedy I.R. 2005. Nitrogen fertiliser losses from rice soils and control of environmental pollution problems. Communications in Soil Science and Plant Analysis 36, 1625–1639.

Choudhury A.T.M.A. and Khanif Y.M. 2001. Evaluation of effects of nitrogen and magnesium fertilization on rice yield and fertiliser nitrogen efficiency using ^{15}N tracer technique. Journal of Plant Nutrition 24, 855–871.

Choudhury A.T.M.A. and Khanif Y.M. 2004. Effects of nitrogen and copper fertilization on rice yield and fertiliser nitrogen efficiency: a ^{15}N tracer study. Pakistan Journal of Scientific and Industrial Research 47, 50–55.

Choudhury A.T.M.A., Khanif Y.M., Aminuddin H. and Zakaria W. 2002. Effects of copper and magnesium fertilization on rice yield and nitrogen use efficiency: a ^{15}N tracer study. In 'Proceedings of the 17th World Congress of Soil Science, 14–21 August 2002, Bangkok, Thailand, CD Transactions', ed. by I. Kheoruenromne. Symposium No. 50, Paper No. 226, 1–10.

Kennedy I.R., Choudhury A.T.M.A. and Kecskés M.L. 2004. Non-symbiotic bacterial diazotrophs in crop-farming systems: Can their potential for plant growth

promotion be better exploited? Soil Biology and Biochemistry 36, 1229–1244.

Malik K.A., Mirza M.S., Hassan U., Mehnaz S., Rasul G., Haurat J., Bally R. and Normand P. 2002. The role of plant-associated beneficial bacteria in rice-wheat cropping system. Pp. 73–83 in 'Biofertilisers in action', ed. by I.R. Kennedy and A.T.M.A. Choudhury. Rural Industries Research and Development Corporation: Canberra.

Mirza M.S., Rasul G., Mehnaz S., Ladha J.K., So R.B., Ali S. and Malik K.A. 2000. Beneficial effects of inoculated nitrogen-fixing bacteria on rice. Pp. 191–204 in 'The quest for nitrogen fixation in rice', ed by J.K. Ladha and P.M. Reddy. International Rice Research Institute: Los Baños, The Philippines.

Nguyen Thanh Hien, Deaker R., Kennedy I.R. and Roughley R.J. 2003. The positive yield response of field-grown rice to inoculation with a multi-strain biofertiliser in the Hanoi area, Vietnam. Symbiosis 35, 231–245.

Nguyen Thanh Hien, Kennedy I.R. and Roughley R.J. 2002. The response of field-grown rice to inoculation with a multi-strain biofertiliser in the Hanoi district, Vietnam. Pp. 37–44 in 'Biofertilisers in action', ed by I.R. Kennedy and A.T.M.A. Choudhury. Rural Industries Research and Development Corporation: Canberra.

Payne R., Murray D., Harding S., Baird D., Soutar D. and Lane P. 2003. GenStat for Windows, 7th edn. VSN International: Oxford, United Kingdom.

Peech M. 1965. Hydrogen ion activity. Pp. 914–926 in 'Methods of soil analysis, Part 2', ed. by C.A. Black. American Society of Agronomy: Madison, Wisconsin.

Sahrawat K.L. 2000. Macro- and micronutrients removed by upland and lowland rice cultivars in West Africa. Communications in Soil science and Plant Analysis 31, 717–723.

Schollenberger C.J. and Simon R.H. 1945. Determination of exchange capacity and exchangeable bases in soil-ammonium acetate method. Soil Science 59, 13–23.

Sweeney R.A. and Rexroad P.R. 1987. Comparison of LECO FP-228 'Nitrogen Determinator' with AOAC copper catalyst Kjeldahl method for crude protein. Journal of Association of Official Analytical Chemists 70, 1028–1030.

Walkley A. and Black I.A. 1934. An examination of Degtjaref method for determining soil organic matter and a proposed modification of the chromic acid titration method. Soil Science 37, 29–38.

Yanni Y.G., Rizk R.Y., Corich V., Squartini A., Ninke K., Philip-Hollingsworth S., Orgambide G., de Bruijn F., Stoltzfus J., Buckley D., Schmidt T.M., Mateos P.F., Ladha J.K. and Dazzo F.B. 1997. Natural endophytic association between *Rhizobium leguminosarum* bv. *trifolii* and rice and assessment of its potential to promote rice growth. Plant and Soil 194, 99–114.

Prospects of using *Azotobacter*, *Azospirillum* and cyanobacteria as supplements of urea nitrogen for rice production in Bangladesh

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Abstract

The responses to inoculation with (i) *Azotobacter* and *Azospirillum* of boro rice under five levels of nitrogen (N) fertiliser (0, 60, 80, 100 and 120 kg N/ha from urea) and (ii) blue-green algae (BGA) of transplanted aman rice under four levels of N fertiliser (0, 20, 30 and 40 kg N/ha from urea) were examined in two field experiments in Bangladesh. Maximum grain yield (8.43 t/ha) was recorded with *Azospirillum* at 100 kg N/ha, 1.34 t/ha higher than the yield obtained with 120 kg N/ha applied alone, suggesting the benefit of reducing N application by 20 kg/ha with extra yield. Similarly, *Azotobacter* showed the potential to save 20 kg N/ha with additional yield of 1.24 t/ha. Mixed cultures (*Azospirillum* + *Azotobacter*) were statistically similar to individual cultures at all the N rates, showing that single species inoculation suffices to increase grain yield and suggesting a common mechanism of the effect. In the case of aman rice, grain yield increased significantly from N fertilisation at 20 kg N/ha without inoculation, but increasing N rates above this rate only gave a non-significant trend in increasing grain yield. With inoculation treatments, grain yield responses to added N were significant up to 40 kg N/ha, indicating that BGA increases the rice plants' capacity to use more fertiliser-N for grain production. The grain yield obtained by the local isolates at 30 kg N/ha were higher than the yield obtained at 40 kg N/ha without inoculation, indicating the prospect of reducing urea by 25%, although the yields were lower than those obtained at 40 kg N/ha with these inoculants.

Introduction

Rice is the main cereal food for the people of Bangladesh. It occupies about 77% of the total cropped area, supplies 92% of cereal food requirements, 75% of calorie intake and 55% of the protein in the average daily diet of the people (Bhuiyan et al. 2004). It is grown all year round in three distinct rice-growing seasons called *aus* (summer), *aman* (rainy autumn) and *boro* (winter–spring) on a total of 11.25 million hectares, with 1.13, 5.78 and 4.34 million hectares cultivated in aus, aman and boro, respectively (DAE 2006).

Although the country is now nearer to self-sufficiency in cereal food production, it will not be able to ensure food security for an ever-increasing population, as the requirement will double in the next 25 years while the natural resource base will shrink. To keep pace with population growth and the shrinking land resource base, total production of food crops will have to be increased by 60–70% within that period (Islam and Haq 1999; Alam 2007). Increase in total yield through horizontal expansion (i.e. using more land for production) is

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not possible, so vertical increase (yield/unit area) is the only option. In this regard the Bangladesh Rice Research Institute (BRRI) and Bangladesh Institute of Nuclear Agriculture (BINA) are developing modern rice varieties that have high yield potential. But these high-yielding varieties need high amounts of N for biomass production (Choudhury and Bhuiyan 1991; Choudhury et al. 1997). As most of the rice soils of Bangladesh are deficient in N, fertiliser-N applications are necessary to meet the rice crops' nutrition demands.

The high cost of chemical nitrogenous fertilisers and the low purchasing power of most of the farmers restricts its use in proper amounts, hampering crop production. Besides, a substantial amount of the urea-N is lost through different mechanisms including ammonia volatilisation, denitrification and leaching losses, causing environmental pollution problems (Ponnamperuma 1972; De Datta and Buresh 1989; Choudhury and Kennedy 2005). Hence, the efficiency of added urea-N is very low, often only 30-40% and, in some cases, even lower (De Datta 1978; Choudhury and Khanif 2001, 2004).

The utilisation of biological nitrogen fixation (BNF) technology can decrease the use of urea-N, prevent the depletion of soil organic matter and reduce environmental pollution to a considerable extent (Jeyabal and Kuppuswamy 2001; Choudhury and Kennedy 2004; Kennedy et al. 2004). Yield increases in rice due to inoculation of *Azospirillum* and *Azotobacter* are reported to be in the 5–60% range (Kumar and Balasubramanian 1986; Sattar 1991; Balandreau 2002), and due to cyanobacteria (BGA) 2.2–44.0% (Bhuiya et al. 1984; Begum et al. 1990).

Different BNF systems including the use of plant growth promoting bacteria and BGA are in use on a limited scale in Bangladesh agriculture. Before large-scale extension of BNF systems at the farm level, further research is needed to determine their N supplement potentials. With this in mind, two field experiments were conducted to evaluate the effects of *Azospirillum* and *Azotobacter* on boro rice, and BGA on transplanted aman rice, under various levels of N fertiliser as urea.

Materials and methods

Experiment with *Azospirillum* and *Azotobacter* on boro rice

Site and soil

The experiment was conducted at the Agronomy Experimental Farm of Bangladesh Agricultural University, Mymensingh, Bangladesh (with soil of the Sonatala series under the agroecological zone of Old Brahmaputra Floodplain) at a mean elevation of 18.0 m above sea level. The initial soil was, texturally, a silty loam with pH 6.8, organic matter 1.42%, total N 0.13%, available phosphorus (P) 23.48 mg/kg and cation exchange capacity (CEC) 7.5 cmol/kg. Analysis of the soil was conducted following standard procedures (Black 1965). Basal doses of triple superphosphate (26 kg P/ha), muriate of potash (60 kg K/ha), gypsum (13 kg S/ha) and zinc sulfate (3.58 kg Zn/ha) were applied during final land preparation 1 day before transplanting rice seedlings.

Inoculants and inoculation procedure

There were four inoculation treatments—uninoculated, *Azotobacter, Azospirillum* and a mixed culture of *Azotobacter* and *Azospirillum.* The *Azospirillum* and *Azotobacter* cultures, obtained from the Soil Microbiology Laboratory of the Bangladesh Institute of Nuclear Agriculture (BINA), were grown in Okon's broth and Jensen's broth, respectively, for 5 days under aerated conditions. The mixed culture was prepared by mixing equal quantities of each of the culture broths with a concentration of 10^9 cells/mL. Roots of 43-day-old seedlings were dipped in respective culture solutions (10 mL of the inoculant broth with 1 L of sterilised water) for 1 hour before transplanting.

N levels

The response of the rice (variety BRRI dhan29, a high-yielding variety developed by BRRI) to inoculation was examined against five levels of N (0, 60, 80, 100 and 120 kg N/ha) applied as urea in three splits (at 15, 45 and 55 days after transplanting).

Experimental design

The experiment was laid out in a split-plot design with four replications, assigning N levels in the main plots and inoculations in the subplots at random. The size of each subplot was 5 m × 4 m.

Data collection and analysis

Data on grain and straw yields were recorded at maturity. Grain yield was adjusted at 14% moisture content while straw yield was recorded on an oven-dry basis. Chemical analysis for N in grain and straw was conducted using the micro-Kjeldahl procedure (Yoshida et al. 1976). The analysis of variance for different parameters was done following the computer package MSTAT. Mean differences among the treatments, in the case of significant F values, were compared using Duncan's multiple range test (DMRT) as mentioned by Gomez and Gomez (1984).

Experiment with cyanobacteria on transplanted aman rice

Site and soil

The experiment was conducted at the Soil Science Experimental Farm of BINA, Mymensingh, Bangladesh (with soil of the Sonatala series under the agro-ecological zone of Old Brahmaputra Floodplain). The initial soil was a silty loam in texture with organic matter 1.71%, pH 6.5, total N 0.11%, available P 20 mg/kg and CEC 7.5 cmol/kg. Analysis of the soil was conducted following standard procedures (Black 1965). Basal doses of P (18 kg P/ha) as triple superphosphate and K (15 kg K/ha) as muriate of potash were applied during final land preparation.

Inoculants and application procedure

There were five inoculation treatments—uninoculated, BGA local 1, BGA local 2, BGA exotic and a mixed culture of all the BGA inoculants. Two locally isolated strains of cyanobacteria (*Anabaena* sp.), designated as BGA local 1 and BGA local 2 and obtained from BINA, Bangladesh; and one exotic strain of *Nostoc* sp. obtained from the Microbiology Division of the Indian Agricultural Research Institute, New Delhi, India, were grown separately in Fog's N-free medium, followed by preparation of a straw-based BGA biofertiliser. The mixed culture inoculant was prepared by mixing equal quantities of all the BGA inoculants. The inoculants were applied at 1 kg/ha 10 days after transplanting the rice seedlings.

N levels

The response of a low-input rice (variety Binashail developed by BINA through an irradiation technique) to inoculants was investigated against four levels of N (0, 20, 30 and 40 kg N/ha) as urea. Urea was applied in two equal splits at 15 and 35 days after transplanting.

Experimental design

The design of the experiment was split-plot, assigning N levels in the main plots and inoculation treatments in the subplots with three replications. The unit subplot size was 3.5 m × 4 m.

Data collection and chemical analysis of samples

Grain and straw yields were recorded at maturity. Grain yield was adjusted at 14% moisture content while straw yield was recorded on an oven-dry basis. Nitrogen contents of the grain samples were analysed following the same procedure used for the experiment on boro rice above. All data were analysed statistically and the means were compared by DMRT as above.

Results and discussion

Experiment with *Azospirillum* and *Azotobacter* on boro rice

Interaction effects of inoculation and N rate were significant on grain and straw yields (Table 1). Grain yield increased significantly over the uninoculated control due to inoculations at 60, 80 and 100 kg N/ha but there was no significant effect of inoculations at 0 and 120 kg N/ha. There was no significant difference among the three inoculants. Grain yield increased significantly due to N fertilisation up to 120 kg N/ha without inoculation and the N response was significant up to 100 kg N/ha with inoculants, but beyond this N rate there were significant decreases in yield. Similar results were observed for straw yield. Agronomic efficiency (kg grain / added N) was higher with inoculations compared to the uninoculated control at 60, 80 and 100 kg N/ha, but the opposite result was obtained at 120 kg N/ha due to decrease in yield (Table 2).

The interaction effect of inoculation and N rate was not significant on total N uptake; however, the individual effects of both factors were significant (Table 3). All three inoculants were similar and significantly higher amounts of total N uptake over the uninoculated control were recorded due to inoculations. Total N uptake increased significantly due to N fertilisation up to 100 kg N/ha. The increases in total N uptake due to inoculations and N rates were due to increased grain and straw yields.

A N response of rice crops in this soil was expected as the soil was deficient in N, with a total N content below the critical deficiency level of 0.20%. Inoculation treatments with 100 kg N/ha gave additional grain yields of 1.06, 1.24 and 1.34 t/ha over 120 kg N/ha without inoculation, demonstrating the potential to save 20 kg N/ha with additional yield. The mixed culture was not better than the single cultures, indicating that single cultures are sufficient to supplement urea-N as well as giving extra yield. Several authors reported the beneficial effects of *Azospirillum* inoculation in increasing rice yield (Jayaraman and Ramiah 1986; Islam et al. 2005; Fakir et al. 2007).

Investigations showed that *Azospirillum* inoculation increased rice yield significantly by 1.6–10.5 g per plant (32–81% increase) in greenhouse conditions (Mirza et al. 2000; Malik et al. 2002). However, under field conditions the estimated yield increase was approximately 1.8 t/ha (22% increase), as reported by Balandreau 2002. Yanni and El-Fattah (1999) found that rice yields in field trials increased by 0.4–0.9 t/ha (7–20% increase) due to *Azotobacter* application.

Experiment with cyanobacteria on transplanted aman rice

Interaction effects of BGA inoculations and N rates were significant on grain and straw yields as well as N uptake by grain (Table 4). Without inoculation, grain yield increased significantly due to N fertilisation at 20 kg N/ha; however, there was no further significant increase in yield due to higher N rates, although the trend was positive. On the other hand, grain yield increase due to N fertilisation was significant up to 40 kg N/ha with all four inoculants, indicating the beneficial effects of BGA on rice plants' capacity to use fertiliser-N in grain production. A similar trend was noticed for straw yield. At

Table 1. Effects of N rates and inoculations (*Azotobacter* and *Azospirillum*) on grain and straw yields of boro rice (variety BRRI dhan29)

Inoculation	N rate (kg/ha)				
	0	60	80	100	120
	Grain yield (t/ha)				
Uninoculated (control)	2.00 a E	4.80 b D	5.33 b C	6.96 b B	7.09 a A
Azotobacter	2.20 a D	5.34 a C	6.53 a B	8.33 a A	7.01 a B
Azospirillum	2.18 a D	5.45 a C	6.57 a B	8.43 a A	6.79 a B
Azotobacter + *Azospirillum*	2.35 a D	5.52 a C	6.36 a B	8.15 a A	6.55 a B
	Straw yield (t/ha)				
Uninoculated (control)	2.46 b E	5.30 b D	5.79 b C	7.51 b B	7.71 a A
Azotobacter	2.84 ab E	5.88 a D	7.10 a C	8.74 a A	7.94 a B
Azospirillum	2.64 ab D	6.14 a C	7.23 a B	8.99 a A	7.78 a B
Azotobacter + *Azospirillum*	3.05 a E	6.11 a D	6.91 a C	8.79 a A	7.64 a B

Interaction effects of inoculation and N rate for both parameters were significant.
Within a parameter, values followed by a common small letter in a column or a common capital letter in a row are not significantly different by Duncan's Multiple Range Test (DMRT) at 5% probability level.

Table 2. Effects of N rates and inoculations (*Azotobacter* and *Azospirillum*) on agronomic efficiency of added N in boro rice (variety BRRI dhan29)

Inoculation	N rate (kg/ha)			
	60	80	100	120
	Agronomic efficiency (kg grain / kg added N) [a]			
Uninoculated (control)	46.57	41.63	49.60	44.42
Azotobacter	52.33	54.13	61.30	40.08
Azospirillum	54.50	54.88	62.50	38.42
Azotobacter + *Azospirillum*	52.83	50.13	58.00	35.00

[a] Agronomic efficiency = (grain yield in N treated plot – grain yield in N control plot)/N rate (kg/ha)

20 kg N/ha all inoculants gave statistically similar grain yields to that of the uninoculated control, while at other N rates BGA local 1 and BGA mixed gave significantly higher grain yields over the control. BGA local 2 gave significantly higher grain yield over the control at 30 and 40 kg N/ha, while BGA exotic was similar to the control at all the N rates. This indicates that the exotic strain could not adapt to the local environment. Nitrogen uptake by grain increased significantly due to N fertilisation at 20 kg N/ha, with further significant increases at 40 kg N/ha without inoculation and at 30 and 40 kg N/ha with inoculants.

A N response of rice crops in this soil was expected as the soil was deficient in N, with a total N content below the critical deficiency level of 0.20%. Grain yields obtained at 30 kg N/ha with BGA mixed, BGA local 1 and BGA local 2 were 0.62, 0.44 and 0.17 t/ha higher, respectively, than the yield obtained at 40 kg N/ha without inoculation. But BGA exotic did not show this benefit. These results showed that inoculation with BGA mixed can save

Table 3. Effects of N rates and inoculations (*Azotobacter* and *Azospirillum*) on total N uptake (kg/ha) by boro rice (variety BRRI dhan29)

Inoculation	N rate (kg/ha)					Mean
	0	60	80	100	120	
	Total N uptake (kg/ha)					
Uninoculated (control)	24.89	77.03	97.66	130.76	126.78	91.36 b
Azotobacter	32.50	94.91	121.10	135.43	133.91	103.57 a
Azospirillum	32.41	101.34	118.34	138.23	128.14	103.69 a
Azotobacter + Azospirillum	32.78	97.39	114.10	134.73	125.71	100.94 a
Mean	30.64 C	92.67 B	112.80 B	134.79 A	128.56 A	

Interaction effect of inoculation and N rate was not significant; however, the individual effects were significant.
Within a parameter, values followed by a common small letter in a column or a common capital letter in a row are not significantly different by DMRT at 5% probability level.

Table 4. Effects of N rates and cyanobacteria (BGA) inoculations on grain and straw yields, and grain N uptake of transplanted aman rice (variety Binashail)

BGA inoculation	N rate (kg/ha)			
	0	20	30	40
	Grain yield (t/ha)			
Uninoculated	2.70 b B	3.10 a A	3.22 d A	3.45 c A
BGA local 1	3.07 a C	3.29 a C	3.89 b B	4.40 ab A
BGA local 2	3.03 ab C	3.19 a C	3.62 bc B	4.28 ab A
BGA exotic	2.97 ab C	3.17 a BC	3.39 cd B	4.05 bc A
BGA mixed	3.18 a C	3.25 a C	4.07 a B	4.52 a A
	Straw yield (t/ha)			
Uninoculated	3.78 b B	4.42 a A	4.50 c A	4.73 d A
BGA local 1	4.20 ab C	4.54 a C	5.46 ab B	6.06 ab A
BGA local 2	4.20 ab C	4.50 a BC	4.97 bc B	5.85 bc A
BGA exotic	4.06 ab C	4.43 a BC	4.74 c B	5.37 c A
BGA mixed	4.45 a C	4.73 a C	5.88 a B	6.48 a A
	N uptake by grain (kg/ha)			
Uninoculated	24.78 c C	31.26 b B	35.99 c B	41.67 d A
BGA local 1	32.78 ab C	33.49 b C	48.54 ab B	57.55 ab A
BGA local 2	31.14 ab C	35.34 ab C	44.45 b B	53.84 bc A
BGA exotic	29.93 b C	32.90 b C	40.61 bc B	49.73 c A
BGA mixed	35.23 a C	39.26 a C	52.42 a B	60.43 a A

Interaction effects of BGA inoculation and N rates were significant for all three parameters.
Within a parameter, values followed by a common small letter in a column or a common capital letter in a row are not significantly different by DMRT at 5% probability level.

10 kg N/ha with additional grain yield of 0.62 t/ha. As the individual effect of BGA exotic was not significant at all, further research is needed by excluding it from the mixed culture. At 40 kg N/ha, additional grain yields over the control were 1.07, 0.95 and 0.83 t/ha with BGA mixed, BGA local 1 and BGA local 2, respectively. These three inoculation treatments were statistically similar at this N rate, while at 30 kg N/ha the mixed culture gave significantly higher grain yield over the single cultures.

There are numerous reports on the beneficial use of cyanobacteria in increasing the growth and yield of rice (Ladha and Reddy 1995; Kannaiyan et al. 1997; Kennedy and Islam 2001). However, cyanobacteria cannot meet all the N requirement of rice, and failure of algalisation in the presence of N fertiliser has been claimed by some workers (Watanabe 1973; Sankaran 1975, 1977). Bhuiya et al. (1984) reported an increase in rice yield of up to 44% at 40 kg N/ha due to algal inoculation, while the increase was only 32% at 60 kg N/ha. A significantly higher increase in grain yield due to algalisation under pot culture was reported by Begum et al. (1990) in both boro and aman crops.

The efficiency of cyanobacteria in increasing rice yield also varies depending on soil type. The findings of several field experiments conducted on different types of soil showed that cyanobacteria supplemented 25–35% of urea-N for rice crop in acid, saline and red soils, while its effect was less in calcareous and neutral soils (Hashem 2001). Cyanobacteria can play a major role in improving soil environment in addition to N fixation. They have the capacity to reclaim soil salinity (Hashem et al. 1995; Uma and Kannaiyan 1999), improve the organic matter content and water-holding capacity of soil, and reduce soil erosion. They can benefit rice plants by producing growth-promoting substances, and by increasing the availability of P through excretion of organic acids (Roger and Kulasooriya 1980).

Conclusion

The agronomic efficiency of added N and total N uptake by rice plants were both increased due to inoculation treatments. This shows greater efficiency of utilisation of added fertiliser-N in the intermediate range of urea applications. Single BGA local isolates have the potential to increase grain yield by 0.83–0.95 t/ha over uninoculated controls at 40 kg N/ha. At the same N rate BGA mixed culture outyielded the control by 1.07 t/ha. The individual effect of BGA exotic was not significant, so it is recommended that it be excluded from the mixed culture to allow quantification of the actual benefit of mixing the two local isolates. The grain yields obtained by the local isolates at 30 kg N/ha were higher than the yield obtained at 40 kg N/ha without inoculation, indicating the potential of reducing added fertiliser-N by 10 kg N/ha, although the yields were lower than those obtained at 40 kg N/ha with these inoculants.

References

Alam M.N. 2007. Food security: Bangladesh perspective. Paper presented at World Food Day Symposium at Bangladesh Agricultural Research Council, Dhaka, Bangladesh, 16 October 2007.

Balandreau J. 2002. The spermosphere model to select for plant growth promoting rhizobacteria. Pp. 55–63 in 'Biofertilisers in action', ed. by I.R. Kennedy and A.T.M.A. Choudhury. Rural Industries Research and Development Corporation: Canberra.

Begum Z.N.T, Mandal R., Akhter F. and Das N.C. 1990. Residual effect of cyanobacteria on the growth and yields of rice. Pp. 379–381 in 'Perspectives in phycology', ed. by V.N. Rajarao. Today and Tomorrow's Printers & Publishers: New Delhi, India.

Bhuiya Z.H., Islam A.K.M.N., Hashem M.A., Begum Z.N.T. and Rahman M.M. 1984. Effect of cyanobacteria as biofertilizer on rice. Bangladesh Journal of Agriculture 9, 47–51.

Bhuiyan N.I., Paul D.N.R. and Jabbar M.A. 2004. Feeding the extra millions by 2025: Challenges for rice research and extension in Bangladesh. Pp. 1–24 in 'Proceedings of the workshop on modern rice cultivation in Bangladesh' ed. by M.M. Haque, A.R. Gomosta and M.R. Mandal. Bangladesh Rice Research Institute: Gazipur, Bangladesh.

Black C.A. 1965. Methods of soil analysis, Parts I and II. American Society of Agronomy: Madison, Wisconsin.

Choudhury A.T.M.A. and Bhuiyan N.I. 1991. Yield and nitrogen nutrition of modern rice as affected by nitrogen fertilization under irrigated culture. Bangladesh Rice Journal 2, 122–127.

Choudhury A.T.M.A. and Kennedy I.R. 2004. Prospects and potentials for systems of biological nitrogen fixation in sustainable rice production. Biology and Fertility of Soils 39, 219–227.

Choudhury A.T.M.A. and Kennedy I.R. 2005. Nitrogen fertiliser losses from rice soils and control of environmental pollution problems. Communications in Soil Science and Plant Analysis 36, 1625–1639.

Choudhury A.T.M.A and Khanif Y.M. 2001. Evaluation of the effects of nitrogen and magnesium fertilizer on rice yield and fertilizer nitrogen efficiency using ^{15}N tracer technique. Journal of Plant Nutrition 24, 855–871.

Choudhury A.T.M.A. and Khanif Y.M. 2004. Effects of nitrogen and copper fertilization on rice yield and fertiliser nitrogen efficiency: A ^{15}N tracer study. Pakistan Journal of Scientific and Industrial Research 47, 50–55.

Choudhury A.T.M.A., Zaman S.K. and Bhuiyan N.I. 1997. Nitrogen response behavior of modern rice varieties with different agronomic traits. Thai Journal of Agricultural Science 30, 185–193.

DAE 2006. Krishi Diary 2007. Agriculture Information Service, Department of Agricultural Extension, Government of the People's Republic of Bangladesh: Dhaka.

De Datta S.K. 1978. Fertilizer management for efficient use in wetland rice soils. Pp. 671–701 in 'Soils and rice', ed. by F.N. Ponnamperuma. International Rice Research Institute: Los Baños, the Philippines.

De Datta S.K. and Buresh R.J. 1989. Integrated nitrogen management in irrigated rice. Advances in Soil Science 10, 143–169.

Fakir M.A.I., Hasan S.M.R. and Sattar M.A. 2007. Growth and yield of rice as influenced by *Azotobacter* and *Azospirillum* inoculation in presence and absence of urea-N. Journal of the Bangladesh Society for Agricultural Science and Technology 4, 247–250.

Gomez K.A. and Gomez A.A. 1984. Statistical procedures for agricultural research, 2nd edn. John Wiley and Sons: New York, USA.

Hashem M.A. 2001. Problems and prospects of cyanobacterial biofertilizer for rice cultivation. Australian Journal of Plant Physiology 28, 881–888.

Hashem M.A., Islam M.R. and Banu N.A. 1995. Nitrogen fixation and salinity reduction by blue-green algae. Bangladesh Journal of Agricultural Science 22, 251–256.

Islam M.F. and Haq M.F. 1999. Integrated nutrient management with inorganic fertilizers and green manure in some dominant cropping patterns. Pp. 55–72 in 'Proceedings of the national workshop on integrated nutrient management for crop production and soil fertility', ed. by M.F. Islam, D.A. Choudhury, G.M. Panaullah, M.U. Miah, J. Rijpma and M.F. Haque. Bangladesh Agricultural Research Institute: Gazipur, Bangladesh.

Islam M.Z., Sumera Y., Malik K.A., Sattar M.A. and Hafeez F.Y. 2005. Potentials of PGPR to increase rice production in Bangladesh. Pp. 87–96 in 'Proceedings of International Seminar on Rice Crop, 2–3 October 2005', ed. by M. Akhtar and M. Ahmad. Rice Research Institute: Kala Shah Kaku, Pakistan.

Jayaraman S. and Ramiah. 1986. Effect of seedling root dipping of *Azospirillum* in rice. Madraj Agriculture Journal 73, 226–229.

Jeyabal A. and Kuppuswamy G. 2001. Recycling of organic wastes for the production of vermicompost and its response in rice legume cropping system and soil fertility. European Journal of Agronomy 15, 153–170.

Kannaiyan S., Aruna S.J., Kumari S.M.P. and Hall D.O. 1997. Immobilized cyanobacteria as biofertilizer for rice crop. Journal of Applied Phycology l9, 167–174.

Kennedy I.R., Choudhury A.T.M.A. and Kecskés M.L. 2004. Non-symbiotic bacterial diazotrophs in crop-farming systems: can their potential for plant growth promotion be better exploited? Soil Biology and Biochemistry 36, 1229–1244.

Kennedy I.R. and Islam N. 2001. The current and potential contribution of asymbiotic nitrogen fixation to nitrogen requirements on farms: a review. Australian Journal of Experimental Agriculture 41, 447–457.

Kumar K. and Balasubramanian B. 1986. Field response of rice to *Azospirillum* biofertilizer. Current Research 15, 74–76.

Ladha J.K. and Reddy P.M. 1995. Extension of nitrogen fixation to rice: Necessity and possibilities. GeoJournal 35, 363–372.

Malik K.A., Mirza M.S., Hassan U., Mehnaz S., Rasul G., Haurat J., Bally R. and Normand P. 2002. The role of plant-associated beneficial bacteria in rice-wheat cropping system. Pp. 73–83 in 'Biofertilisers in action', ed. by I.R. Kennedy and A.T.M.A. Choudhury. Rural Industries Research and Development Corporation: Canberra.

Mirza M.S., Rasul G., Mehnaz S., Ladha J.K., So R.B., Ali S. and Malik K.A. 2000. Beneficial effects of inoculated nitrogen-fixing bacteria on rice. Pp. 191–204 in 'The quest for nitrogen fixation in rice', ed. by J.K. Ladha and P.M. Reddy. International Rice Research Institute: Los Baños, the Philippines.

Ponnamperuma F.N. 1972. The chemistry of submerged soils. Advances in Agronomy 24, 29–96.

Roger P.A. and Kulasooriya S.A. 1980. Blue-green algae and rice. International Rice Research Institute: Los Baños, the Philippines.

Sankaran S. 1975. Cyanobacteria-role in rice culture. Farmer and Parliament 10, 15–16.

Sankaran S. 1977. Cyanobacteria-role in rice culture. Farmer and Parliament 12, 11–12.

Sattar M.A. 1991. Rice based BNF research in Bangladesh: Problems and prospects. Pp. 211–218 in 'Biological nitrogen fixation associated with rice production', ed. by S.K. Dutta and C. Sloger. Oxford & IBH Publishing Company Ltd: New Delhi, India.

Uma D. and Kannaiyan S. 1999. Studies on salt stress on growth, ammonia excretion and nitrogen fixation by the

cyanobacterial mutant *Anabaena variabilis* –NTG-2T-SK-DU. Journal of Microbial World 1, 9–18.

Watanabe A. 1973. On the inoculation of paddy fields in the Pacific area with nitrogen fixing blue-green algae. Soil Biology and Biochemistry 5, 161–162.

Yanni Y.G. and El-Fattah F.K.A. 1999. Towards integrated biofertilization management with free living and associative dinitrogen fixers for enhancing rice performance in the Nile delta. Symbiosis 27, 319–331.

Yoshida S., Forno D.A., Cock J.H. and Gomez K.A. 1976. Laboratory manual for physiological studies of rice, 3rd edn. International Rice Research Institute: Los Baños, the Philippines.

Phosphorus mobilisation by biofertiliser strains

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Abstract

Two microbial biofertiliser strains (*Klebsiella pneumoniae* [4P] and an unidentified yeast [HY]) isolated from rice rhizosphere soil in Vietnam were characterised for their ability to solubilise inorganic phosphates. Both strains were able to increase the amount of soluble tricalcium phosphate (TCP) in unbuffered liquid culture by approximately 300% and 200%, respectively, but were unable to solubilise aluminium phosphate (AlP). Both strains reduced the pH of the growth medium when grown with TCP or AlP as a sole phosphate source. When TCP was provided, the initial pH of the culture medium was 6.5 and strain 4P reduced the pH to a greater extent than HY; however, when AlP was provided, the initial pH was 5.0 and strain 4P quickly died out as the medium pH dropped below 4, whereas strain HY survived and was apparently resistant to aluminium toxicity. The pH of buffered medium was also reduced by both strains but to a lesser extent. This resulted in a lower concentration of soluble phosphorus (P) than in unbuffered solution, yet the soluble P concentration was still significantly greater than in sterile control flasks. In both buffered and unbuffered media, fumarate was the main organic anion excreted by both strains. Since knowledge regarding the role of fumarate is sparse, further study is recommended, including the field application of these strains.

Introduction

Phosphorus (P) is an essential requirement for high yields of rice (5–6 t/ha) in tropical Asia, with an average recommended P application of 20–25 kg/ha for each crop (Dobermann et al. 1996). Rice crops require about 3–4 kg/ha for the production of one tonne of rough rice including straw (reviewed in Choudhury et al. 2007). However, most countries rely on P imports to combat low indigenous resources (Cook et al. 1990). Much of the P applied to soils as fertiliser can become fixed into forms unavailable to the plant (Choudhury et al. 2007), leading to agronomic and economic inefficiency. Altogether, P fertiliser management is complex, requiring knowledge of: the supply of other nutrients (N, K, S) to the crop; the effective P supply from indigenous soil resources; the overall P balance, accounting for fertiliser application, crop P export and stubble recycling; and the processes that govern the availability of P in a particular soil (Dobermann et al. 1996). Inefficient use of P by rice crops can result in P losses from rice fields to water bodies, causing environmental pollution problems through eutrophication (Sharpley et al. 2001).

In order to overcome these inefficiencies, microbial inoculants are now being investigated worldwide for their potential to mobilise unavailable P, increase the ability for plant P uptake and increase yields (Zahir et al. 2004). A number of different micro-organisms have been isolated and characterised for their P-solubilising ability from locations around the world, including Australia (Harris et al. 2006), South America (Pérez et al. 2007), the

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Middle East (Alikhani et al. 2006) and Asia (Chen et al. 2006). Phosphorus can be mobilised from soils or low-grade rock fertiliser by rhizosphere micro-organisms via a number of mechanisms, including acidification to mobilise P from acid-soluble soils and fertilisers, release of organic anions to mobilise hydrous oxide-bound P, and release of enzymes (phosphatases) to mobilise organic P (Trolove et al. 2003). Because each of these fractions varies in amount and form, soil type will have a major influence on the effectiveness of biofertiliser strains selected on the basis of P mobilisation. Thus, determination of the mechanism of P mobilisation may help predict the effectiveness of biofertiliser strains in certain soils.

Both BioGro formulations investigated in ACIAR project SMCN/2002/073 contained one strain known to dissolve TCP ($Ca_3(PO_4)_2$) on solid media. The first formulation contained *Klebsiella pneumoniae*, 4P, and the second formulation contained an unidentified yeast strain, HY. This paper describes a number of experiments that were conducted to compare the P-mobilisation capacity of each strain and identify their mechanisms of action. In light of the results, their P-mobilising potential is discussed with reference to other isolates described elsewhere, and recommendations on their use for rice production in Vietnam are given.

Materials and methods

Micro-organisms

The isolation, identification and maintenance of strains 4P and HY are described in a previous chapter of these proceedings (Kecskés et al. 2008). Both strains were revived on nutrient agar plates from glycerol stock. Before each experiment, strains were subcultured in nutrient broth for 48 hours at 30 ˚C on a rotary shaker set to 100 rpm. Broth cultures were centrifuged at 5,000 ×g for 15 minutes, and the cell pellet was washed twice with sterile 0.15% NaCl to provide inoculum for each experiment.

Growth media

Modified Pikovskaya medium was used for all experiments unless otherwise stated, containing (g/L in distilled water) glucose 10.0, $(NH_4)_2SO_4$ 0.5, NaCl 0.2, $MgSO_4.7H_2O$ 0.1, KCl 0.2, yeast extract 0.1, $MnSO_4$ trace, $FeSO_4.7H_2O$ trace. The pH was adjusted to 6.8 for all experiments before autoclaving. Phosphate (PO_4^{3-}) was added at 3.0 g/L as either $Ca_3(PO_4)_2$ (TCP) or $AlPO_4$ (Al-P).

Experimental set-up and conditions

Three different experiments were performed. For each experiment treatments were inoculated with a similar biomass of cells (rather than actual number of cells) by adjusting saline inoculum suspensions to the same optical density at 600 nm. This was done because of the large difference in size between yeast and bacteria cells. All experiments were conducted with triplicate sample flasks for each time sample, with each flask containing 25 mL of liquid growth media. Flasks were incubated aerobically with shaking (100 rpm) for 10 days at 30 ˚C.

Effect of phosphorus and carbon source

In the first and second experiments P mobilisation by 4P and HY from two different P sources, TCP and Al-P, was determined in unbuffered liquid media. In the first experiment 10.0 g/L of glucose was supplied as a carbon (C) source, while in the second experiment the strains were given 5.0 g/L of fructose, 4.5 g/L saccharose and 0.5 g/L glucose as a C source, representing the main sugars exuded by rice roots (Lin and You 1989). Colony forming units (cfu), pH and soluble P were measured at each sampling time. Each parameter was subject to Student's t-test to compare means at different time periods throughout the experiment.

Effect of buffer

In the third experiment the solubilisation of $Ca_3(PO_4)_2$ by both 4P and HY was compared in unbuffered liquid media and media buffered with 20 mM 3-(N-morpholino)propanesulfonic acid (MOPS) buffer, with the mixed C source as above. Colony forming units, pH, soluble P and organic acid were measured at each sampling time. Each parameter was subject to Student's t-test to compare means at different time periods throughout the experiment.

Analytical methods

At each sample time 100 µL of culture suspension was taken and serially diluted for plate counting of cfu. The remainder of the sample was centrifuged at 5000 ×g for 15 minutes and the pH, soluble P and organic acid in the supernatant were analysed. The pH was measured with a PHM210 meter (Radiom-

eter Analytical, France). Soluble P was measured using the ascorbic acid method (Olsen et al. 1954). Briefly, 5 mL of samples and analytical standards of KH_2PO_4 were dispensed individually into glass test tubes. To each tube 1 mL molybdate reagent was added, containing 8.0 g ammonium molybdate, 0.2 g antimony potassium tartrate and 7.0 g ascorbic acid per litre of 1M H_2SO_4.

For organic acid analysis 20 mL of supernatant was acidified to pH 2.5 with concentrated phosphoric acid, and extracted three times with 5 mL ethyl acetate. The combined extracts were evaporated under a stream of N, resuspended in 2 mL nanopure H_2O and filtered (0.45 µm) for analysis by high-performance liquid chromatography (HPLC). Organic acids were separated using a Phenomenex C_{18} column (4.6 mm × 250 mm × 5 µm) with a mobile phase of 10 mM KH_2PO_4:CH_3OH (95:5), pH 2.7, at a flow rate of 0.8 mL/minute. Acids were detected at 220 nm using a Gilson 118 UV-Vis detector and samples were compared to analytical standards of butyric, citric, fumaric, formic, α-ketogluconic, gluconic, maleic, malonic, oxalic, propionic, succinic and tartaric acids.

Results

Effect of inorganic P source and organic C source

In all treatments the growth of both strains caused a decrease in the solution pH by up to 2.0 units (Figure 1). Treatments containing Al-P as the P source had a lower initial pH than TCP flasks but this did not affect the production of acid by either strain. However, the production of acid in treatments containing Al-P limited the growth of strain 4P and reduced cell numbers substantially after 1 day. The type of C source did not affect the growth rate of either strain, nor the production of acid (P>0.05). Although cell numbers of 4P were higher than those of HY in all treatments, the actual biomass was similar because of the large cell size of HY yeast.

Throughout all experiments the mobilisation of inorganic P over 10 days could not be reliably described by any available models with our ancillary data. Consequently, only maximum amounts of solubilised P could be statistically compared, rather than rates of mobilisation.

Both strains were able to significantly mobilise TCP compared to sterile controls (P<0.001 for both), but not Al-P (Table 1). No significant difference was observed in the extent of solubilisation of TCP between treatments growing on glucose and treatments supplied with mixed sugars as a C source (P>0.05). Overall, the maximum amount of TCP solubilised by 4P was significantly greater than that solubilised by HY (P<0.001)—approximately 300% of the soluble phosphate in sterile controls compared to 200% effected by HY yeast.

Effect of buffer

The presence or absence of buffer did not affect the growth of either strain, but unbuffered solution pH was lowered to a greater extent than buffered solution in both 4P- and HY-treated flasks (Figure 2). This difference was less pronounced with 4P.

Again, the rate of P solubilisation did not show a consistent trend in either buffered or unbuffered flasks, with an initial soluble phosphate spike followed by a reduction and return to higher soluble P levels (Figure 3). Overall, the addition of buffer reduced P solubilisation by both strains but less so with 4P.

Table 1. Solubilisation of inorganic phosphates by HY and 4P strains with different carbon sources.

P source	Carbon source [a]	Sterile control PO_4^{3-} (mg/L)	HY		4P	
			PO_4^{3-} (mg/L)	% of control	PO_4^{3-} (mg/L)	% of control
$Ca_3(PO_4)_2$	G	46 (6)	85 (20)	186	150 (25)	328
	G+F+S	31 (3)	63 (5)	192	99 (6)	300
$AlPO_4$	G	13 (1)	8 (4)	56 [b]	23 (8)	172 [b]
	G+F+S	17 (5)	18 (3)	86	21 (3)	97

Numbers in brackets represent ±95% confidence levels of triplicate measurements.
[a] Carbon source was either 10 g/L glucose (G) or 10 g/L mixed sugars (G+F+S).
[b] These flasks showed visible contamination and are not reliable.

The major organic acid produced by both strains had the same HPLC retention time as fumaric acid (Figure 4). Maximum concentrations of 517 µmol/L and 477 µmol/L were produced by 4P and HY, respectively. Chromatograms from 4P cultures also showed the presence of another acid that could not be identified, as well as small amounts of citrate, malate and succinate.

Discussion

Both HY and 4P were capable of solubilising TCP but not Al-P. This is not surprising as, in most cases, TCP is more easily solubilised by acid-producing micro-organisms compared to iron or aluminium phosphates (Whitelaw et al. 1999; Pérez et al. 2007) due to its higher solubility at low pH. Similarly, it appears that acidification was the main mechanism of solubilisation of both BioGro strains, with 4P more capable of solubilising TCP than HY because of a greater reduction in pH (Figure 2). This is in agreement with a number of papers that identified proton concentration, rather than organic anion chelation, as the main mechanism of TCP solubilisation (Illmer and Schinner 1995; Lin et al. 2006). Interestingly, although neither strain could solubilise Al-P, HY was able to continue excreting protons and maintain high cell numbers at a pH lower than 4, while the survival of 4P was dramatically reduced (Figure 1). The change of BioGro formulation from

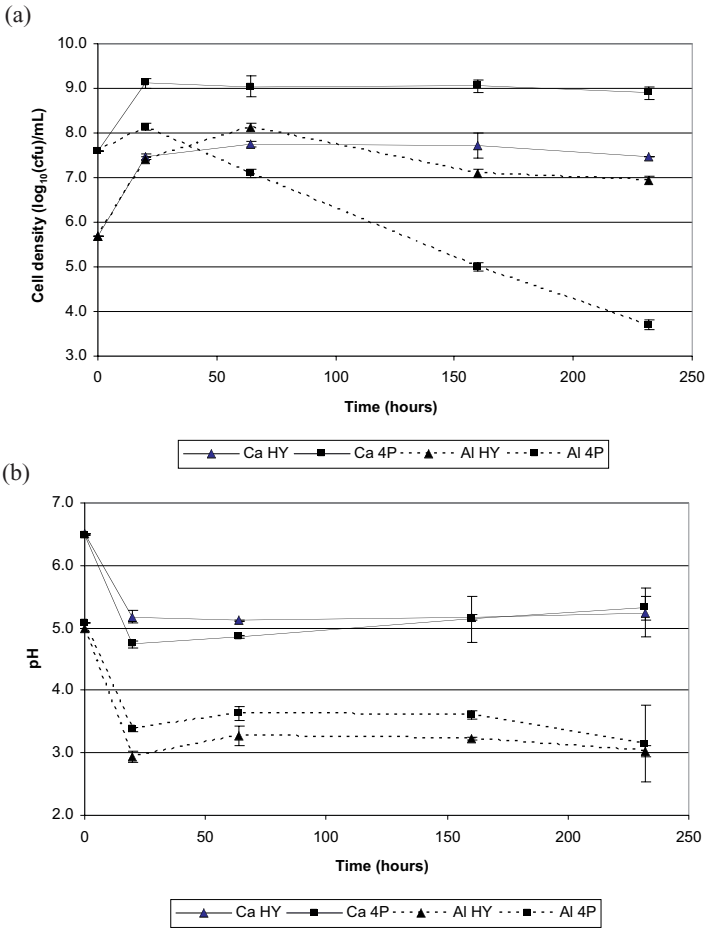

Figure 1. Cell density (a) and pH (b) of flasks treated with $AlPO_4$ and $Ca_3(PO_4)_2$. Error bars represent 95% confidence intervals of triplicate measurements.

4P to HY is therefore likely to be beneficial for its application in low pH environments.

Buffered growth media suppressed acidification and P solubilisation in both strains, supporting the view that acidification was the main P-solubilisation mechanism. The importance of buffering in limiting P solubilisation has been demonstrated previously (Gyaneshwar et al. 1998; Ahuja et al. 2007) and is very important when considering the effectiveness of these strains in highly buffered soils. However, it must also be noted that some P-solubilising micro-organisms, such as *Penicillium bilaiae*, can produce enough acid to overcome high buffering capacity to solubilise more P than in unbuffered culture solutions (Takeda and Knight 2006). Our experiment also confirmed the variability of the P concentration in supernatant over time and the lack of a predictable trend in P solubilisation (Figure 3b). Such an effect has been witnessed in a number of experiments similar to ours (Illmer and Schinner 1995; Gyaneshwar et al. 1999). It highlights the possible influence of P uptake and release, induction and repression of P-solubilising enzyme systems (Babenko et al. 1984), and phosphate precipitation (Illmer and Schinner 1995) on soluble P concentration in the culture solution.

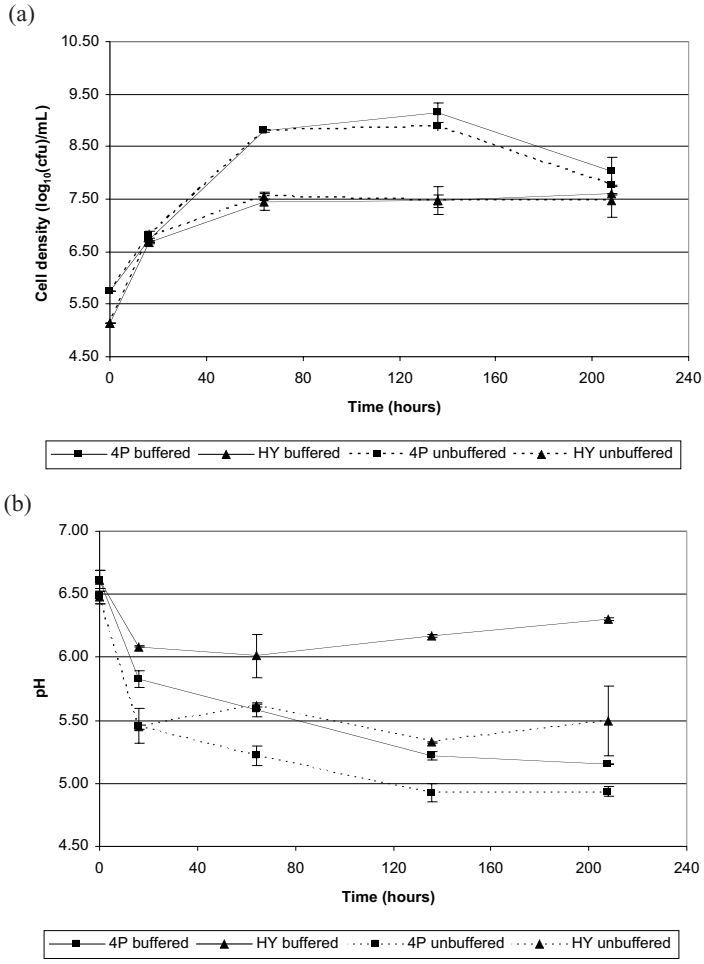

Figure 2. Cell density (a) and pH (b) of buffered and unbuffered $Ca_3(PO_4)_2$ broths. Error bars represent 95% confidence intervals of triplicate measurements.

Both HY and 4P strains were capable of growth on sucrose without any impact on their ability to produce acid or solubilise TCP. This will be critical for their survival and usefulness if they are to be used as inoculants for rice, whose main sugar exudates are sucrose and fructose (Lin and You 1989). Other investigations have found an effect of C source on P solubilisation by micro-organisms, including increased Ca-P and Al-P solubilisation by *Aspergillus niger* growing on mannitol or maltose (Barroso et al. 2006). However, growth on simple sugars will provide substrates for the citric acid cycle metabolism that is responsible for producing commonly found, P-solubilising, low molecular weight organic anions.

Interestingly, both BioGro strains excreted up to 0.5 mM of fumarate as the main organic anion. Fumarate is not normally associated with P solubilisation as compared with citric, oxalic and gluconic acids (Trolove et al. 2003). Indeed, most TCP-solubilising isolates that have been characterised in the literature produce high amounts of gluconic acid, for example *Rahnella aquatilis* (>15 mM) (Kim et al. 1997); *Penicillium radicum* (>30 mM) (Whitelaw et al. 1999); *Burkholderia cepacia* CC-Al74 (>15 mM) (Lin et al. 2006); *Pantoea* spp., *Burkholderia* sp. MMB127 and *Ralstonia* sp. MMB075 (Pérez et al. 2007). A number of other P solubilisers produce high amounts of citric acid and oxalic acid, which are suggested to be more effective at dissolving iron and alu-

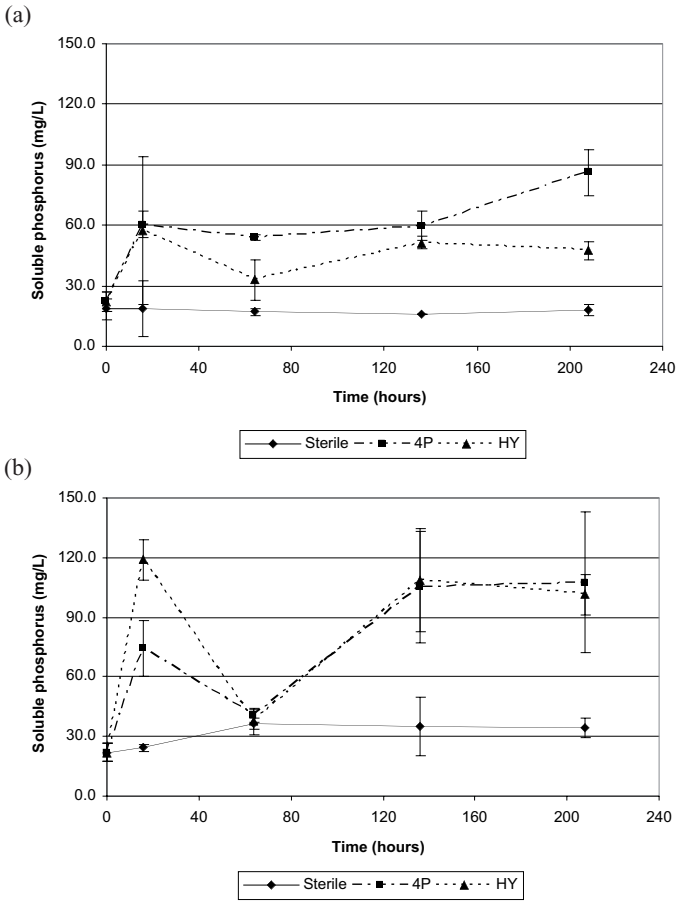

Figure 3. Phosphorus solubilisation in buffered (a) and unbuffered (b) $Ca_3(PO_4)_2$ broths inoculated with HY or 4P. Error bars represent 95% confidence intervals of triplicate measurements.

minium phosphates than monocarboxylic acids (e.g. gluconic) because they can chelate polyionic metallic ions. These isolates include the fungi *Aspergillus niger* (Chuang et al. 2007) and *Penicillium bilaiae* (Takeda and Knight 2006).

Fumaric acid is a dicarboxylic acid, which may explain the success of both strains in mobilising P from a di-cation (Ca^{2+}) but not the tri-cation Al^{3+}. The low C content of fumarate (as compared to citrate and other organic anions) could suggest an

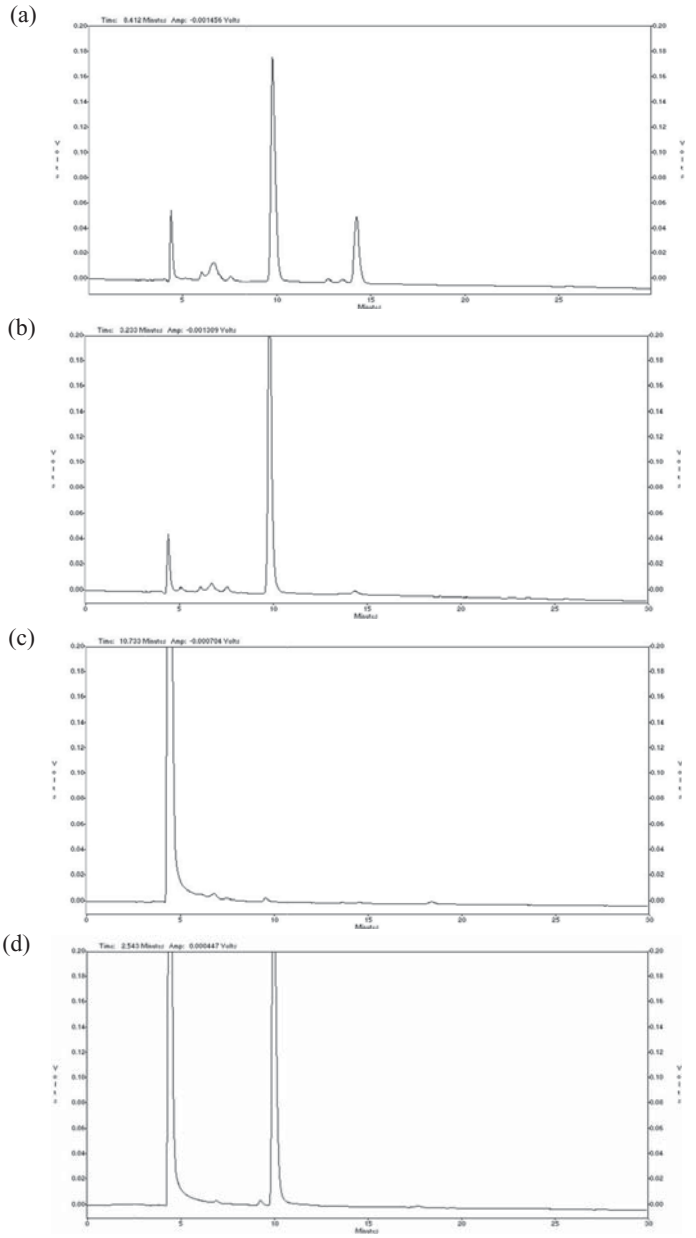

Figure 4. HPLC chromatograms of organic acid extract from broth cultures of 4P (a), HY (b), sterile control (c) and a fumaric acid standard (100 mM) (d).

energy efficiency or C conservation strategy. Another possibility may be the resistance of fumarate to catabolism by other micro-organisms, enabling it to have a longer lasting solubilising effect. Finally, fumarate could also potentially act as a pathogen inhibitor, a synergistic plant growth promotion (PGP) mechanism that has been noted in numerous other P-solubilising micro-organisms (Vassilev et al. 2006). Le Bayon et al. (2006) recently found that fumarate was the major organic acid excreted by lupins growing under P-deficient conditions, and they also highlighted a lack of knowledge on the role of fumarate in P solubilisation. More research is clearly needed in this area.

Conclusions and recommendations

These results suggest that strain HY may not be as effective as strain 4P in mobilising soil P, at least in buffered systems. However, more research is necessary to determine the survival of HY in soil along with its potential to supply any mobilised P to plant roots. Both strains are unlikely to be successful in P supply to plants in soils comprised mainly of metal (e.g. Al or Fe) hydroxides, but are more likely to demonstrate PGP with calcium or magnesium phosphate fertilisers. Pot trials are recommended to confirm this.

Acknowledgments

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References

Ahuja A., Ghosh S.B. and D'Souza S.F. 2007. Isolation of a starch utilizing phosphate solubilizing fungus on buffered medium and its characterization. Bioresource Technology 98, 3408–3411.

Alikhani H.A., Saleh-Rastin N. and Antoun H. 2006. Phosphate solubilization activity of rhizobia native to Iranian soils. Plant and Soil 287, 35–41.

Babenko Y.S., Tyrygina G.I., Grigorev E.F., Dolgikh L.M. and Borisova, T.I. 1984. Biological activity and physiological–biochemical properties of phosphate-dissolving bacteria. Microbiology 53, 427–433.

Barroso C.B., Pereira G.T. and Nahas E. 2006. Solubilization of CaHPO(4) and AlPO(4) by *Aspergillus niger* in culture media with different carbon and nitrogen sources. Brazilian Journal of Microbiology 37, 434–438.

Chen Y.P., Rekha P.D., Arun A.B., Shen F.T., Lai W.A. and Young C.C. 2006. Phosphate solubilizing bacteria from subtropical soil and their tricalcium phosphate solubilizing abilities. Applied Soil Ecology 34, 33–41.

Choudhury A.T.M.A., Kennedy I.R., Ahmed M.F. and Kecskés M.L. 2007. Phosphorus fertilization for rice and control of environmental pollution problems. Pakistan Journal of Biological Sciences 10, 2098–2105.

Chuang C.C., Kuo Y.L., Chao C.C. and Chao W.L. 2007. Solubilization of inorganic phosphates and plant growth promotion by *Aspergillus niger*. Biology and Fertility of Soils 43, 575–584.

Cook P.J., Banerjee D.M. and Southgate P.N. 1990. The phosphorus resources of Asia and Oceania: Phosphorus requirements for sustainable agriculture in Asia and Oceania. International Rice Research Institute: Manila, the Philippines.

Dobermann A., Cassman K.G., Cruz P.C.S., Adviento M.A.A. and Pampolino M.F. 1996. Fertilizer inputs, nutrient balance and soil nutrient supplying power in intensive, irrigated rice systems. III. Phosphorus. Nutrient Cycling in Agroecosystems 46, 111–125.

Gyaneshwar P., Kumar G.N. and Parekh L.J. 1998. Effect of buffering on the phosphate-solubilizing ability of micro-organisms. World Journal of Microbiology and Biotechnology 14, 669–673.

Gyaneshwar P., Parekh L.J., Archana G., Poole P.S., Collins M.D., Hutson, R.A. and Kumar G.N. 1999. Involvement of a phosphate starvation inducible glucose dehydrogenase in soil phosphate solubilization by *Enterobacter asburiae*. FEMS Microbiology Letters 171, 223–229.

Harris J.N., New P.B. and Martin P.M. 2006. Laboratory tests can predict beneficial effects of phosphate-solubilising bacteria on plants. Soil Biology and Biochemistry 38, 1521–1526.

Illmer P. and Schinner F. 1995. Solubilization of inorganic calcium phosphates-solubilization mechanisms. Soil Biology and Biochemistry 27, 257–263.

Kecskés M.L., Choudhury A.T.M.A., Casteriano A.V., Deaker R., Roughley R.J., Lewin L., Ford R. and Kennedy I.R. 2008. Effects of bacterial inoculant biofertilisers on growth, yield and nutrition of rice. These proceedings.

Kim K.Y., Jordan D. and Krishnan H.B. 1997. *Rahnella aquatilis*, a bacterium isolated from soybean rhizosphere, can solubilize hydroxyapatite. FEMS Microbiology Letters 153, 273–277.

Le Bayon R.C., Weisskopf L., Martinoia E., Jansa J., Frossard E., Keller F., Follmi K.B. and Gobat J.M. 2006. Soil phosphorus uptake by continuously cropped *Lupinus albus*: A new microcosm design. Plant and Soil 283, 309–321.

Lin M. and You C. 1989. Root exudates of rice (*Oryza sativa* L.) and its interaction with *Alcaligenes faecalis*. Sinica 6, 6–12.

Lin T.F., Huang H.I., Shen F.T. and Young C.C. 2006. The protons of gluconic acid are the major factor responsible for the dissolution of tricalcium phosphate by *Burkholderia cepacia* CC-A174. Bioresource Technology 97, 957–960.

Olsen S.R., Cole C.V., Watanabe F.S. and Dean L.A. 1954. Estimation of available phosphorus in soils by extraction with sodium bicarbonate. In: US Department of Agriculture Circular 939. U.S. Government Printing Office: Washington DC.

Pérez E., Sulbaran M., Ball M.M. and Yarzabal L.A. 2007. Isolation and characterization of mineral phosphate-solubilizing bacteria naturally colonizing a limonitic crust in the south-eastern Venezuelan region. Soil Biology and Biochemistry 39, 2905–2914.

Sharpley A.N., McDowell R.W. and Kleinman P.J.A. 2001. Phosphorus loss from land to water: integrating agricultural and environmental management. Plant and Soil 237, 287–307.

Takeda M. and Knight J.D. 2006. Enhanced solubilization of rock phosphate by *Penicillium bilaiae* in pH-buffered solution culture. Canadian Journal of Microbiology 52, 1121–1129.

Trolove S.N., Hedley M.J., Kirk G.J.D., Bolan N.S. and Loganathan P. 2003. Progress in selected areas of rhizosphere research on P acquisition. Australian Journal of Soil Research 41, 471–499.

Vassilev N., Vassileva M. and Nikolaeva I. 2006. Simultaneous P-solubilizing and biocontrol activity of micro-organisms: potentials and future trends. Applied Microbiology and Biotechnology 71, 137–144.

Whitelaw M.A., Harden T.J. and Helyar K.R. 1999. Phosphate solubilisation in solution culture by the soil fungus *Penicillium radicum*. Soil Biology and Biochemistry 31, 655–665.

Zahir Z.A., Arshad M. and Frankenberger W.T. 2004. Plant growth promoting rhizobacteria: Applications and perspectives in agriculture. Advances in Agronomy 81, 97–168.

Effects of BioGro strain *Pseudomonas fluorescens* (1N) on dry matter production and nitrogen uptake of rice: A ^{15}N tracer study

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Abstract

The effect of inoculating *Pseudomonas fluorescens* (1N) bacteria on rice was evaluated in a greenhouse using ^{15}N-labelled urea and variable nitrogen (N) fertiliser levels. At 20 days after sowing (DAS), inoculation halved the fertiliser-N uptake compared to the control, which lacked bacteria. There was a significant increase in dry weight of the rice plants but no difference in total N uptake. The biofertiliser strain clearly enabled rice to access other sources of N, such as N_2 fixation or soil organic N. The decreased proportion in fertiliser-N uptake at 45 DAS resulting from inoculation was much less but the increase in total N uptake was even more significant. The results showed beneficial effects of inoculation with this organism on dry matter production and N accumulation in rice plants from both fertiliser and non-fertiliser. The results are consistent with the hypothesis that BioGro improves the efficiency of N uptake from soil.

Introduction

Investigations have shown that the multistrain inoculant biofertiliser BioGro has the potential to increase grain and straw yields as well as nitrogen (N) uptake of rice (Nguyen et al. 2002, 2003). BioGro contains 1N (*Pseudomonas fluorescens*) bacteria, and other strains of the genus *Pseudomonas* have been identified as N fixers in greenhouse experiments using the ^{15}N isotope dilution technique in Pakistan (Mirza et al. 2000; Malik et al. 2002). Therefore, the strain 1N might contribute in biological nitrogen fixation (BNF) and fertiliser-N uptake as well as in growth promotion. The fertiliser-N uptake can be estimated precisely from the ^{15}N atom excess data by using ^{15}N-labelled N fertiliser (Panda et al. 1995; Choudhury and Khanif 2004). The BNF contribution can be estimated by the ^{15}N isotope dilution technique using non-fixing reference plants (Malik et al. 2002; Ladha and Reddy 2003; Rodrigues et al. 2008). With this in mind, a preliminary experiment was conducted to evaluate the effect of 1N bacteria on dry matter production and fertiliser-N uptake of rice plants. As no non-fixing reference was used in this experiment, the estimation of BNF was beyond the objective of this study. The possible BNF contribution of 1N bacteria will be evaluated in future experiments.

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Materials and methods

Experimental

A greenhouse experiment was conducted to evaluate the inoculation effect on 1N (*Pseudomonas fluorescens*) bacteria on dry matter production and

fertiliser-N uptake of rice plants. A grey degraded soil, collected from Chau Thanh district, Tay Ninh province of southern Vietnam, was used for this experiment. Four kilograms of soil were used per pot, and four replicates were used per treatment. Phosphorus and potassium were added to all pots as fused magnesium phosphate (P_2O_5) and K_2O as muriate of potash (KCl), respectively, at a rate equivalent to 60 kg/ha of each fertiliser.

In the first set N was added as ^{15}N-urea (4.634% ^{15}N atom excess) at two rates equivalent to 0 and 20 kg N/ha. Twenty-five pre-germinated seeds, inoculated with 1N bacteria at 10^6 colony forming units (cfu) per gram, were placed in each pot, which is equivalent to 100 kg seed sown per hectare. There were two inoculation treatments—with 1N and without 1N. Seedlings were harvested at 20 days after sowing (DAS) by cutting rice plants at ground level.

In the second set, N was added from the same source at three rates equivalent to 0, 20 and 40 kg N/ha. Inoculated pre-germinated seeds were placed in the pots and five seedlings were left in each pot. There were two inoculation treatments—with and without 1N (10^8 cfu/g). Seedlings were harvested at 45 DAS by cutting rice plants at ground level.

Chemical analysis

Plant samples were cut at ground level, air and oven dried at 70 °C, and ground. Total N contents of the plant samples were determined by the micro-Kjeldahl procedure (Yoshida et al. 1976), followed by estimation of ^{15}N abundance by emission spectrometry (Hauck 1982) at the Nuclear Centre of Ho Chi Minh City using emission spectrometer NOISE-7. The per cent ^{15}N atom excess (AE) was calculated by subtracting the natural abundance of ^{15}N (0.3663) from the abundance data of plant samples (Axmann and Zapata 1990; Panda et al. 1995).

The calculations for estimating recovery in the plant from ^{15}N-labelled fertiliser were made according to procedures described by Axmann and Zapata (1990). The percentage of N derived from fertiliser (NdfF) was calculated using equation (1).

The following computations were done as described by Choudhury and Khanif (2001):

1. Total N uptake (mg/pot) = total N in plant samples (%) × plant dry matter weight (g) × 1,000 / 100
2. Fertiliser N uptake by rice plants = NdfF (%) × total N uptake by rice plants / 100
3. Non-fertiliser N uptake = total N uptake – fertiliser N uptake.

Statistical analysis

All data were analysed at the University of Sydney using the GenStat version 7 (Payne et al. 2003). Treatments were compared by a two-way ANOVA. Effects were considered significant whenever $P<0.05$.

Results and discussion

At 20 DAS the interaction effect of inoculation and applied N rate was not significant. However, inoculation individually increased plant dry weight significantly (Table 1). On the other hand, ^{15}N AE (%), NdfF (%) and fertiliser-N uptake were decreased significantly ($P<0.05$) as a result of inoculation (Table 2). These results were attributed to the increase in non-fertiliser-N uptake from some source dependent on inoculation. The effect of N rate was significant on dry weight, total N content (%) and total N uptake (Table 1). Inoculation decreased fertiliser-N uptake significantly, while non-fertiliser-N uptake increased from inoculation (Table 2) although the difference was not significant; however, it contributed to the total N uptake, which was not affected significantly due to inoculation (Table 1). The increase in non-fertiliser-N uptake due to inoculation might be due to BNF. This can be estimated in future studies by using a non-fixing reference crop. These results indicated the benefit on growth of using inoculating 1N bacteria as it significantly increased plant dry matter at 20 DAS.

At 45 DAS the interaction effect of N rates and inoculation was significant ($P<0.05$) on plant dry weight and total N content (%), and between weight and non-fertiliser-N uptake (Tables 3 and 4). With no applied N, inoculation increased dry matter yield significantly, while at the other two N rates the effect of inoculation was not significant. N fertilisation increased dry matter yield significantly with

$$\text{NdfF (\%)} = \frac{^{15}\text{N atom excess \% in plant sample}}{^{15}\text{N atom excess \% in labelled fertiliser}} \times 100 \qquad (1)$$

increasing N rates without inoculation, while the effect of N rate was significant only at N rates of 40 kg/ha with inoculation. Total N uptake increased significantly with inoculation and with N rates. Nitrogen fertilisation increased ^{15}N AE (%), NdfF (%) and fertiliser-N uptake significantly. Inoculation increased fertiliser-N uptake significantly but its effect was not significant on ^{15}N AE (%) and NdfF (%). This was a result of increased fertiliser-N uptake in inoculated plants.

The interaction effect of N rates and inoculation was significant on non-fertiliser-N uptake. Inoculation increased non-fertiliser-N uptake significantly at both N rates, while the N fertiliser effect was variable between inoculation treatments. The effect of N rate was not significant on non-fertiliser-N uptake without inoculation, but increased it significantly with inoculation. The possible reason might be the increase in root mass due to 1N inoculation, which increased the rice plants' capacity to absorb more soil N with increase in fertiliser-N application rate. Increases in root mass of rice seedlings were observed resulting from inoculation in a greenhouse experiment at the University of Sydney (Kecskés et al. 2008), and similar results are reported by Nguyen (2008), in these proceedings.

These results indicate the positive effects of 1N inoculation in increasing plant biomass, and N uptake from both fertiliser and non-fertiliser sources at 45 DAS. Although this experiment was not continued to maturity stage, evidence from other field experiments showed the beneficial effect of BioGro inoculation on grain and straw yields as well as N and P uptakes at maturity (Phan and Tran 2008). An increase in N uptake might be due to increased fertiliser-N uptake, BNF or increased soil-N uptake due to thicker and longer root systems. The results of this experiment demonstrate the beneficial effect of 1N inoculation in increasing fertiliser-N uptake by rice plants.

This study clearly demonstrates the significant effect on the growth of rice of the plant growth promoting organism, the 1N strain of *Pseudomonas*

Table 1. Effects of inoculation of 1N (*Pseudomonas fluorescens*) bacteria and fertiliser-N rates on plant dry weight, total N content and uptake of rice seedlings at 20 days after sowing

Inoculation	Fertiliser N rate (kg N/ha)		Mean
	0	20	
Dry weight (g/pot)			
Without 1N	8.73	9.02	8.88 b
With 1N	9.02	9.24	9.13 a
Mean	8.88 B	9.13 A	
Total N content (%)			
Without 1N	3.19	3.66	3.43 a
With 1N	3.24	3.57	3.41 a
Mean	3.22 B	3.62 A	
Total N uptake (mg/pot)			
Without 1N	278.4	330.3	304.4 a
With 1N	292.6	330.0	311.3 a
Mean	285.5 B	330.2 A	

Interaction effect of inoculation and N rate was not significant among the three parameters.
Within a parameter, values followed by different capital letters in a row or different small letters in a column are significantly (P<0.05) different by least significant difference (LSD).

Table 2. Effects of inoculation of 1N bacteria on ^{15}N atom excess (AE), %N derived from fertiliser (NdfF), and fertiliser-N and non-fertiliser-N uptakes of rice seedlings at 20 days after sowing

Inoculation	^{15}N atom excess (%)	NdfF (%)	Fertiliser-N uptake (mg/pot)	Non-fertiliser-N uptake (mg/pot)
Without 1N	0.84 a	18.01 a	59.2 a	271.0 a
With 1N	0.47 b	10.14 b	33.4 b	296.5 a

Within a parameter, values followed by different letters in a column are significantly (P<0.05) different by LSD.

fluorescens, isolated by Professor Nguyen Thanh Hien near Hanoi as a N_2-fixing organism (Nguyen 2008). The use of labelled urea has indicated that the bacterium allows the plant to access significant (more than 40 mg per plant) alternative sources of N to urea after 3 weeks. Once a more extensive root system has developed, aided by inoculation as discussed above, the plant's access to fertiliser-N is also enhanced. These results are consistent with the biofertiliser hypothesis indicated in the first chapter of these proceedings (Kennedy 2008).

Table 3. Effects of inoculation of 1N bacteria and fertiliser N rates on plant dry weight, total N content and uptake of rice seedlings at 45 days after sowing

Inoculation	Fertiliser N rate (kg/ha)			Mean
	0	20	40	
Dry weight (g/pot)				
Without 1N	19.24 b C	22.43 a B	24.71 a A	
With 1N	21.79 a B	22.55 a B	24.55 a A	
Total N content (%)				
Without 1N	1.51 a A	1.48 b A	1.51 b A	
With 1N	1.44 b C	1.74 a B	1.95 a A	
Total N uptake (mg/pot)				
Without 1N	290.1	331.3	371.8	331.1 b
With 1N	314.2	392.3	479.2	395.2 a
Mean	302.2 C	361.8 B	425.5 A	

Interaction effect of inoculation and N rate was significant (P<0.05) on dry weight and total N content but not significant on total N uptake.
Within a parameter, values followed by different capital letters in a row or different small letters in a column are significantly (P<0.05) different by LSD.

Table 4. Effects of inoculation of 1N bacteria and fertiliser N rates on ^{15}N AE, NdfF (%), and fertiliser-N and non-fertiliser-N uptakes of rice seedlings at 45 days after sowing

Inoculation	Fertiliser N rate (kg/ha)		Mean
	20	40	
^{15}N AE (%)			
Without 1N	0.55	1.14	0.85 a
With 1N	0.54	1.09	0.82 a
Mean	0.55 B	1.12 A	
NdfF (%)			
Without 1N	11.86	24.51	18.19 a
With 1N	11.69	23.53	17.61 a
Mean	11.78 B	24.02 A	
Fertiliser-N uptake (mg/pot)			
Without 1N	39.3	91.1	65.2 b
With 1N	45.9	112.7	79.3 a
Mean	42.6 B	101.9 A	
Non-fertiliser-N uptake (mg/pot)			
Without 1N	292.0 b A	280.7 b A	
With 1N	346.4 a B	366.4 a A	

Interaction effect of inoculation and N rate was significant only on non-fertiliser-N uptake.
Within a parameter, values followed by different capital letters in a row or different small letters in a column are significantly (P<0.05) different by LSD.

Ms Tra with her ^{15}N emission spectrometer in Ho Chi Minh City

Conclusion

Inoculation with 1N bacteria increased dry matter yield as well as N uptake of rice plants from both fertiliser and non-fertiliser sources at 45 DAS. It would be of interest to estimate N_2 fixation using the ^{15}N techniques with a non-fixing reference crop, allowing the crop to grow to maturity stage to evaluate the possible BNF contribution of *Pseudomonas fluorescens.* However, the majority of the N uptake by rice plants at 20 and 45 DAS is not from the urea fertiliser, particularly at 20 DAS.

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References

Axmann H. and Zapata F. 1990. Stable and radioactive isotopes. In 'Use of nuclear techniques in studies of soil–plant relationships'. Training course series No. 2. International Atomic Energy Agency: Vienna.

Choudhury A.T.M.A. and Khanif Y.M. 2001. Evaluation of effects of nitrogen and magnesium fertilization on rice yield and fertiliser nitrogen efficiency using ^{15}N tracer technique. Journal of Plant Nutrition 24, 855–871.

Choudhury A.T.M.A. and Khanif Y.M. 2004. Effects of nitrogen and copper fertilization on rice yield and fertiliser nitrogen efficiency: A ^{15}N tracer study. Pakistan Journal of Scientific and Industrial Research 47, 50–55.

Hauck, R.D. 1982. Nitrogen-isotope-ratio analysis. Pp. 735–779 in 'Methods of soil analysis', Part 2, 2nd edn., ed. by A.L. Page, R.H. Miller and D.R. Keeny. American Society of Agronomy: Madison, Wisconsin.

Kecskés M.L., Choudhury A.T.M.A., Casteriano A.V., Deaker R., Roughley R.J., Lewin L., Ford R. and Kennedy I.R. 2008. Effects of bacterial inoculant biofertilisers on growth, yield and nutrition of rice. These proceedings.

Kennedy, I.R. 2008. Overview of research on efficient nutrient use in rice production using inoculant biofertilisers. These proceedings.

Ladha J.K. and Reddy P.M. 2003. Nitrogen fixation in rice systems: State of knowledge and future prospects. Plant and Soil 252, 151–167.

Malik K.A., Mirza M.S., Hassan U., Mehnaz S., Rasul G., Haurat J., Bally R. and Normand P. 2002. The role of plant-associated beneficial bacteria in rice-wheat cropping system. Pp. 73–83 in 'Biofertilisers in action', ed. by I.R. Kennedy and A.T.M.A. Choudhury. Rural Industries Research and Development Corporation: Canberra.

Mirza M.S., Rasul G., Mehnaz S., Ladha J.K., So R.B., Ali S. and Malik K.A. 2000. Beneficial effects of inoculated nitrogen-fixing bacteria on rice. Pp. 191–204 in 'The quest for nitrogen fixation in rice', ed. by J.K. Ladha and P.M. Reddy. International Rice Research Institute: Los Baños, the Philippines.

Nguyen Thanh Hien 2008. The product BioGro and improvements in its performance. These proceedings.

Nguyen Thanh Hien, Deaker R., Kennedy I.R. and Roughley R.J. 2003. The positive yield response of field-grown rice to inoculation with a multi-strain biofertiliser in the Hanoi area, Vietnam. Symbiosis 35, 231–245.

Nguyen Thanh Hien, Kennedy I.R. and Roughley R.J. 2002. The response of field-grown rice to inoculation with a multi-strain biofertiliser in the Hanoi district, Vietnam. Pp. 37–44 in 'Biofertilisers in action', ed. by I.R. Kennedy and A.T.M.A. Choudhury. Rural Industries Research and Development Corporation: Canberra.

Panda M.M., Mosier A.R, Mohanty S.K, Chakravorti S.P, Chalam A.B. and Reddy M.D. 1995. Nitrogen utilization by lowland rice as affected by fertilization with urea and green manure. Fertilizer Research 40, 215–223.

Payne R., Murray D., Harding S., Baird D., Soutar D. and Lane P. 2003. GenStat for Windows, 7th edn. VSN International: Oxford, United Kingdom.

Phan Thi Cong and Tran Dang Dung 2008. Interaction effects of BioGro with nitrogen and phosphorus on grain yield and nutrient uptake of rice in light-textured soils of southern Vietnam. These proceedings.

Rodrigues E.P., Rodrigues L.S., de Oliveira A.L.M., Baldani V.L.D., Teixeira K.R.D.S., Urquiaga S. and Reis V.M. 2008. *Azospirillum amazonense* inoculation: Effects on growth, yield and N_2 fixation of rice (*Oryza sativa* L.). Plant and Soil 302, 249–261.

Yoshida S., Forno D.A., Cock J.H. and Gomez K.A. 1976. Laboratory manual for physiological studies of rice, 3rd edn. International Rice Research Institute: Los Baños, the Philippines.

Models of commercial biofertiliser production: Experiences with BioGro

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Abstract

The commercial production of a microbial fertiliser such as BioGro presents a number of challenges. The applicability of various production models (village/communal, entrepreneurial) in different circumstances was investigated, with the aim of providing guidelines for the establishment of these new enterprises. Insights from supply chain management and transaction costs theory were used to understand the potential difficulties that could face commercial producers of BioGro. A sustained demand for BioGro (especially on rice) is still to be developed and this has created difficulties for commercial production. Less than 50 per cent of production businesses have survived for more than 2 years. Successful factories are private businesses that produce BioGro as a specialist product and actively promote their product. Extension and farmer training are needed but, currently, producers have limited capacity to invest in training for farmers, and experts in the technology are limited.

Introduction

The production of BioGro and its adoption by farmers are closely linked. For commercial production to be profitable and sustainable, the product needs to be profitable and practical for farmers to use. This chapter focuses on the economic and commercial feasibility of inoculant biofertiliser production in Vietnam.

The commercial production of a microbial fertiliser such as BioGro presents a number of challenges:

- The microbial starter must be able to be produced in an abundant and durable form and supplied reliably to producers in regional areas.
- The microbes must have genetic stability; persistence in a range of soil types and conditions; the ability to undertake the expected microbial actions (e.g. N fixation, P solubilisation); and a limited ability to survive or spread in nature so as to ensure the need for continuing sales.
- The product must be able to be patented so that development costs can be recovered.

In this chapter factors that could be expected to influence the production of biofertilisers are briefly discussed, issues and experiences with the production of BioGro in Vietnam are outlined, and key areas that need to be addressed for success are identified.

Factors expected to influence biofertiliser production

To assess the factors that influence successful commercial production, insights from two theoretical frameworks for examining BioGro production were used: supply chain management and transaction costs theory.

Supply chain management

A supply chain is the network of organisations involved through upstream and downstream link-

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ages in the different processes and activities that produce value in the form of products and services in the hands of ultimate consumers (Christopher 1998). A further definition by Chopra and Meindl (2001) describes a supply chain as a process consisting of all the stages involved, directly or indirectly, in fulfilling a customer request, not only including the manufacturer and supplier but also the transporters, warehouses, retailers and customers themselves. An illustrative example of a manufacturing company's supply chain is given in Figure 1.

Modern supply chain management is defined as a process for designing, developing, optimising and managing the internal and external components of the supply system, including material supply, transformation of materials, and distribution of finished products or services to customers that is consistent with overall objectives and strategies (Spekman et al. 1998).

Some key areas (Grimsdell 1996) that have been identified for an efficient supply chain in fresh produce between suppliers and customers involve:

- the scale of operations
- strategic alliances
- production flexibility
- continuity of supply
- quality control
- communications.

While biofertiliser is not strictly fresh produce, it shares some characteristics of fresh produce, namely perishability and non-visible quality aspects.

The need for 'strategic alliances' and 'communications' suggests that the relationship elements of supply chains are important. Essential components of supply chain management are seen to exist in elements of cooperation (i.e. the exchange of essential information and engagement of suppliers/customers in longer term contracts) and coordination (i.e. specified workflow and information exchange to facilitate transactions between supply chain partners). Key indicators that contribute to effective supply chain relationships have been identified as:

- trust—the belief that one's supply chain partner(s) will act in a consistent manner and do what they say they will do, resulting in the willingness of partners to forgo opportunistic behaviour (Spekman et al. 1998).
- commitment—the belief that the trading partners are willing to devote energy to sustaining the relationship (Dion et al. cited in Spekman et al. 1998).
- satisfaction—the evaluation by trading partners of a predetermined expectation of performance and the actual performance as a result of the transaction (Homburg and Giering 2001). Satisfaction includes both the economic and non-economic aspects of the exchange, and will impact on the morale of supply chain participants and their willingness to participate in collaborative activities (Geystens et al. 1999).

A first step in applying this framework to biofertiliser production is to identify the supply chain that

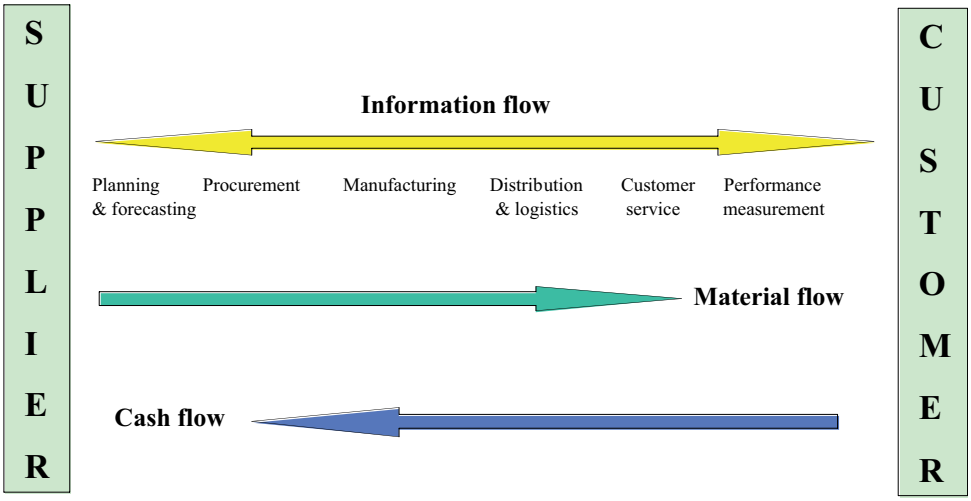

Figure 1. An illustration of a manufacturing company's supply chain (from Spekman et al. 1998)

exists, followed by close investigation of the relationships, processes and transactions involved in the supply chain, and assessment against the key indicators.

Transaction costs analysis

Transaction costs economic theory has the premise that the more efficient transactions will be those that minimise transaction costs. Transaction costs are simply the costs, both direct and indirect, that occur during a transaction. They may occur beforehand, e.g. the expenditure of time and resources identifying suitable trading partners or gathering price information; during a transaction, e.g. paying agent or middlemen fees, or direct costs associated with making the transaction; or following a transaction, e.g. costs associated with ensuring that the pre-agreed terms of the transaction are adhered to. Vertical integration, for example, has been one way that firms have sought to minimise transaction costs.

The relationships between generic product features and transaction characteristics can be categorised as shown in Table 1. These transaction characteristics will influence the extent of transaction costs, and can be used to influence decisions about the way the product can best be marketed so as to minimise the transaction costs. Generally speaking, if transactions are characterised by uncertainty, frequency or complexity, transaction costs will be increased. Biofertiliser has a number of product characteristics such as perishability, variability and invisible quality that add to uncertainty and complexity, and these can be expected to substantially contribute to transaction costs.

Other factors

Other factors that influence the production and use of biofertilisers in Vietnam and other countries is that the product faces substantial competition from larger chemical companies who can offer farmers good deals (e.g. input loans with payment after harvest) and on-farm services (e.g. agronomic advice). These chemical products are also often extensively promoted in the mass media. A further issue exists in developing countries where low-paid extension workers often promote and sell chemical inputs to supplement their incomes (Davidson and Ahmad 2003).

BioGro production in Vietnam

Overview

The production of BioGro grew steadily between 2000 and 2004, then stabilised at around 1,700 t/year (Figure 2) for 3 years before falling to just over 800 t in 2007. There is a two-stage supply chain: the BioGro laboratory supplies inoculant starter to factories which produce BioGro and supply to either distributors or farmers (Figure 3). One kilogram of inoculant starter sold by the BioGro laboratory will produce 100 kg of BioGro, and 1 t of BioGro will treat 5 ha of rice fields at 200 kg/ha. However, very little of the current production is being used for rice, with most being used on perennial crops such as coffee, rubber and pepper.

Production models

During the project more than 10 different factories have been in production, one for the entire period and others commencing and ceasing production. There have been two basic production models—factories that are commune based (Model 1) and factories owned by commercial private companies (Model 2). These can be broken down into factories that only manufacture and are not directly involved in marketing (Model 2A) and those that manufacture and sell the product to farmers. This final category can be further distinguished between companies that sell only BioGro (Model 2B) and those that sell many products (Model 2C). Figure 4 shows the current (and past) producers within specific production model categories. Model 2B is the most common—factories operating as a private commercial business that produce and market only BioGro.

Model 1A: Commune-based production—farmers produce and sell to local farmers

Commune-based factories, run by local farmers and selling the product locally, were initially envisaged as a suitable and appropriate way for BioGro to be produced and sold (Barrett and Marsh 2002). However, all commune-based factories operating in this way have ceased production. Those that have continued, the Ba Vi and Thanh Hoa factories, have changed their operational structure. The core problem that faced commune-based production was a lack of business acumen, resulting in difficulties with marketing in general and collecting monies owed by farmers in particular.

Table 1. Generic model of the relationship between product characteristics, drivers and transaction characteristics (from Hobbs and Young 2000)

	Transaction characteristics						
	Uncertainty for buyer: quality	Uncertainty for buyer: reliable supply (timeliness and quantity)	Uncertainty for buyer and seller: price	Uncertainty for seller: finding a buyer	Frequency of transaction	Relationship-specific investment	Complexity of transaction: (variety of outcomes)
Product characteristics:							
Perishability	X	X		X	X		X
Product differentiation	X	X	X	X		X	X
Quality variability and visibility		X	X	X			X
Quality variability and invisibility	X	X	X				X
New characteristics of importance to consumers	X	Sometimes	X	X		X	X
Regulatory drivers:							
Liability	X			X		Sometimes	X
Traceability				X		X	X
Technology drivers:							
Company-specific technology						X	Sometimes

The Ba Vi factory in Ha Tay province was the first to produce BioGro in Vietnam. A grant in 1999 from the Global Fund for the Environment for a 3-year project entitled 'Improvement of soil fertility for poor soils in Ba Vi' provided the seed money for this factory. The first batch of 20 t of biofertiliser was produced in 2000 and the project finished in 2002. At this time the local commune took over management of the factory and 11 people from the commune formed a cooperative to produce BioGro. However, within 2 years the cooperative faced marketing and financial difficulties, and a family from the commune took over the management. This arrangement also ran into marketing difficulties and in 2006 Vietnam Agricultural Organic Company took over the distribution and sale of the product. The Ba Vi factory is the longest continuously operating BioGro factory and has had a variable production per year ranging from 15 t in 2006 to 650 t in 2004, and averaging 190 t between 2000 and 2006.

Thanh Hoa factory started in 2002 as a commune-based operation, producing for local farmers, with the assistance of a non-government organisation (NGO) which helped with the business operations. This operation was small but reportedly successful (Le Phuong 2006); however, when the NGO withdrew support, the business transferred to private ownership (Dat Viet Company) in 2005 because the 'farmers could produce but not sell'.

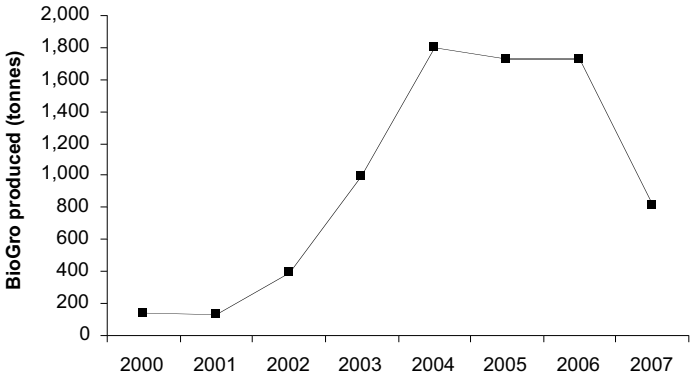

Figure 2. Production of BioGro, 2000–07

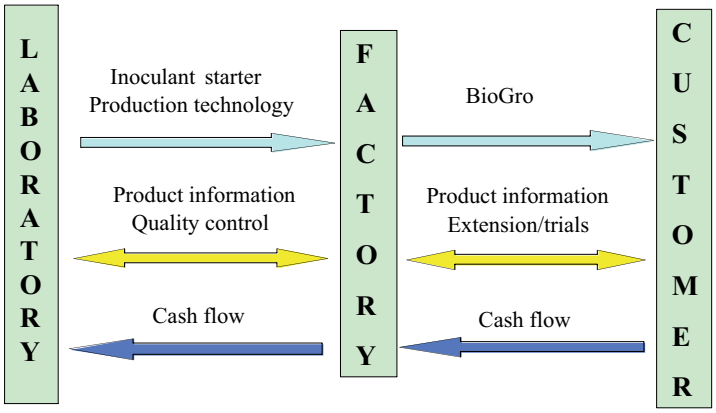

Figure 3. The two-stage BioGro supply chain

Model 1B: Commune-based production—farmers produce only and sell to company

As discussed earlier, the Ba Vi factory now operates according to this model. The commune-based producer said that he faced challenges because of delayed payments from purchasers, and that he 'can only produce but not distribute or sell'.

Model 2A: Production by private company—produces and sells to government contracts

The Hoa Lac factory in Ha Tay province, operating since 2001, is the only factory operating according to this model. Some product is sold to farmers but production mainly occurs specifically to meet government contracts. This producer has contacts within government and does not conduct any extension or trials with the product. The factory has produced consistently between 2002 and 2006 with a production average of 280 t/year during that time. In 2007 it appeared that contracts previously available would not continue and there was no production during this year.

Model 2B: Production by private company—produces and sells only BioGro to farmers

Factories operating according to this model have tended to go in and out of production for a number of reasons, but all are related to marketing and management issues. Three factories currently remain in operation: Dat Viet factory (who took over the Thanh Hoa factory) and factories in Pleiku and Nha Trang. These latter two have been in production for less than 2 years. Dat Viet factory has been in production for 3 years, producing consistently around 350 t of BioGro per year. The owners of the Dat Viet factory are young and enthusiastic and understand their product well. They have links through the business structure of their company to farmers with land in excess of 2,000 ha, land which previously belonged to two state-owned enterprises that are now equitised. The company produces to orders from these large farms, organises training for farmers on how to use BioGro and works with local extension agents to run trials and demonstrations. The company supplies farmers via a distribution

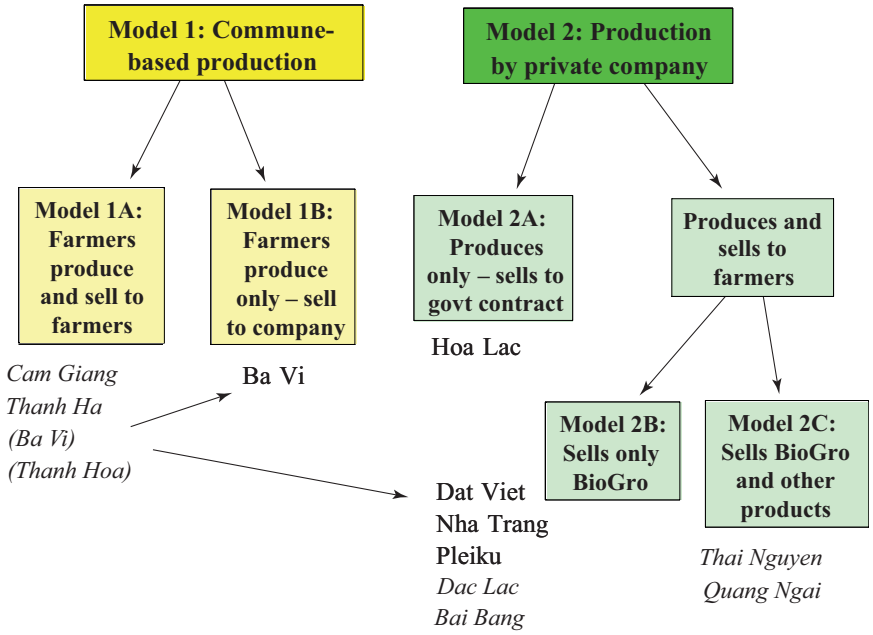

Figure 4. Production models for BioGro manufacture and sale showing current and past factories by 'production model' (factories with names in *italics* have ceased production)

agency which is responsible for collecting payments for the product.

When the Bai Bang factory in Phu Tho province commenced operation, it was thought that it could prove to be a successful model; however, it is no longer in operation. The factory operated for 2 years and produced 550 t of BioGro per year. Production was linked to waste material from a paper factory being used to produce compost which was, in turn, the carrier medium for the BioGro instead of peat. Two staff of the laboratory worked at Bai Bang for 6 months to train 10 staff of the company how to produce compost and BioGro. However, the company failed to understand that they would need to conduct trials and promote the product. The chairman of the company said that the product was 'perfect' and people should come to the company to demand the product. Training for farmers using BioGro was not sufficient, and within 2 years the company was having difficulty in marketing such a large quantity of BioGro and production ceased.

A BioGro mixer installed in the Hoa Lac factory in Ha Tay province

Model 2C: Production by private company—produces and sells BioGro and other products to farmers

No factories remain operating according to this model. The Thai Nguyen factory ran for 3 years, producing over 300 t at its peak in 2004. The factory ran into difficulties because, despite the best efforts of the laboratory, the producer didn't understand the product well and advocated its use in inappropriate ways (e.g. as a complete replacement for chemical fertiliser) in an effort to develop the market. Trials were conducted by the producer over too large an area and were not adequately supervised. Following these failures, farmers stopped buying the product and production ceased.

Issues facing BioGro production

Experiences producing BioGro in Vietnam since 2000 indicate that the key factor affecting production appears to be the lack of a consistent market and hence the need for the producer to engage in time-consuming and costly market promotion activities. Marketing activities are necessary for production of any product but, for small commune-based or even small private companies producing BioGro, they have proved difficult to accommodate. Another factor affecting production is that the perishability of the product increases production risk, and also increases transaction costs because of the need to produce to orders.

The factories that have survived are operated as commercial private businesses. They manufacture only BioGro as a specialist product that they sell either to distributors who market the product (Ba Vi and Hoa Lac factories) or direct to farmers (Dat Viet, Nha Trang and Dac Lac factories). The scale of operations of these successful factories varies but is generally in excess of 200 t/year. Of these factories, Ba Vi has been operating for 7 years, Hoa Lac for 6 years, Dat Viet for 3 years, Dac Lac for 2 years and Nha Trang for 1 year only. The sustainable production of BioGro has proved to be a difficult and challenging task.

Notwithstanding, there are a number of supply chain factors that have been addressed well:

- The transfer of technology to the factories and protection of the product for the patent holder does not appear to be a problem. Selection of factory owners has relied heavily on building personal relationships between the laboratory and

the factory, a common situation for products where the technology is specific to a company (Hobbs and Young 2000).
- Systems for quality control for the produced BioGro product are in place.
- Supply and transport of the inoculant starter has not been a production issue.
- The product is well branded but, despite this, issues with product differentiation still exist. Many other products labelled as 'biofertiliser' are in the market place in Vietnam but some of these are only chemical fertilisers mixed with organic material.
- The product is continually being developed and promoted in new ways and for new uses: for example, combined with wetting agents for use on perennial tree crops (produced by the Dac Lac factory) or formulated as a spray for use particularly on horticulture crops and tea.

The link between profitable production and on-farm demand for BioGro is an important challenge. Biofertiliser technology in general is knowledge intensive, with significant differences compared to farmers' current understanding about fertiliser use. For example, farmers tend to overuse chemical fertilisers with BioGro (Barrett and Marsh 2002), compromising the activity of the inoculant biofertiliser, and crops fertilised with BioGro tend to have lighter leaf colour than crops fertilised only with chemical nitrogen (Tran 2008; Tran et al. 2008). BioGro could also offer farming systems benefits through improved soil quality and decreased need for pesticides. Extension and farmer training are needed, but producers currently have limited capacity to invest in training for farmers, and experts in the technology are limited. A further challenge is to secure the interest of the currently uninvolved government extension service, and work towards obtaining government support for farmer training.

Conclusions

Difficulties that continue to challenge BioGro production are clear. There is a need for a sustained demand for the product, sufficient to overcome difficulties related to the perishability of the product. Associated with this need for sustained demand is the expense and time incurred by BioGro producers to run trials and demonstrations to promote the product. This is not straightforward as benefits from BioGro (e.g. improvements in soil quality) are sometimes difficult to assess by farmers, and many compounding factors (e.g. water availability, overall crop management) can

Packaging BioGro in the Bai Bang factory in Phu Tho province in 2006

influence yield. In such a case, where assessing the results from trialling an innovation is not straightforward, it is known that farmer learning and adoption is generally slow (Pannell et al 2006).

BioGro also faces competition from many other 'biofertilisers', and work will need to continue to distinguish the product from others. Furthermore, BioGro faces competition from chemical fertilisers that are sold by heavily resourced companies able to offer farmers delayed payments. At present, with the exception of the 'three reductions three gains' program in southern Vietnam (Tran 2008), there is a lack of institutional support in the country for the environmental benefits to be gained from lower chemical fertiliser use.

A sustained demand for BioGro (especially on rice) is still to be developed. This creates difficulties for production. Successful factories are private businesses that produce BioGro as a specialist product and actively promote it. Successful factory size would seem at this stage to be variable but tending to be relatively small. Economies of size will definitely be apparent in a developed market but, at this stage, in an underdeveloped market a large factory has more difficulty in selling all they are able to produce. A good working relationship between the BioGro laboratory and the factories has been advantageous in maintaining product quality and product information/promotion, as well as opportunities presented by new product uses. Further efforts to minimise supply chain transaction costs, for example by increasing the shelf life of the product and improving product efficacy and quality, would be advantageous.

References

Barrett G. and Marsh S. 2002. An economic analysis of inoculant biofertiliser production and use in Vietnam. Pp. 102–111 in 'Biofertilisers in action', ed. by I.R. Kennedy and A.T.M.A. Choudhury. Rural Industries Research and Development Corporation Publication No. 02/086: Barton, ACT.

Chopra S. and Meindl P. 2001. Supply chain management: Strategy, planning and operation. Prentice Hall: New Jersey.

Christopher M. 1998. Logistics and supply chain management: Strategies for reducing costs and improving services. Pitman: London.

Davidson A.P. and Ahmad M. 2003. Privatization and the crisis of agricultural extension: The case of Pakistan. Ashgate Publishing Ltd.: Hants England and Burlington USA.

The product BioGro ready to use

Geystens I., Steenkamp J.B. and Kumar N. 1999. A meta-analysis of satisfaction in marketing channel relationships. Journal of Marketing Research 36, 223–238.

Grimsdell K. 1996. The supply chain for fresh vegetables: What it takes to make it work. Supply Chain Management 1(1), 11–14.

Hobbs J.E. and Young L.M. 2000. Closer vertical co-ordination in agri-food supply chains: A conceptual framework and some preliminary evidence. Supply Chain Management 5(3), 131–142.

Homburg C. and Giering A. 2001. Personal characteristics as moderators of the relationship between customer satisfaction and loyalty: An empirical analysis. Psychology and Marketing 18, 43–66.

Le Phuong. 2006. Introducing sustainable biofertiliser use to farmers of Vinh Tan commune, Thanh Hoa province, Vietnam. A research project for Master of Science in Administration with an emphasis on International Development. Andrews University: MI, USA.

Pannell D.J., Marshall G.R., Barr N., Curtis A., Vanclay F. and Wilkinson R. 2006. Understanding and promoting adoption of conservation practices by rural landholders. Australian Journal of Experimental Agriculture 46(11), 1407–1424.

Spekman R.E., Kamauff Jr J.W. and Myhr N. 1998. An empirical investigation into supply chain management: a perspective on partnerships. Supply Chain Management 3(2), 53–67.

Tran Thanh Be. 2008. Challenge of the 'Three Reductions' program in Vietnam: The potential role of inoculant biofertiliser technology. These proceedings.

Tran Thanh Be, Pham Thi Phan, and Marsh, S. 2008. Farmer extension trials using BioGro for rice production in the Mekong Delta. These proceedings.

Adoption and regulation of biofertiliser technology for rice production

Pham Van Toan, Vu Thuy Nga and Luong Huu Thanh[1]

Abstract

An extension study to evaluate the response on farms of a special quality rice (LT3) to BioGro application was carried out in Nam Dinh province. A combination of BioGro, farmyard manure and 75% of normally recommended rates of nitrogen (N) and phosphorus (P) fertilisers was used. The study showed that the use of BioGro can allow reduction of 25% of the NP requirement without a significant difference in rice yield. In combination with 75% of the normally required NP, BioGro produced an extra average profit of VND1,095,602/ha for the six farmers involved in the study. Evaluation of farmers' responses to BioGro technology indicated that the use of such products requires some degree of regulation regarding quality control. Information must be supplied to farmers on whether the product contains chemical fertiliser in addition to biological agents, or whether it is mainly biological, as in the case of the microbial strains in BioGro. The application of biofertilisers such as BioGro has great potential to allow Vietnam to produce rice more economically and in an environmentally friendly way. However, according to the responses to farmer questionnaires, there is uncertainty regarding positive field results because of a reluctance to reduce fertiliser to less than 75% of normal practice. This, the small size of landholdings and the easy availability of chemical fertilisers, are all factors that could limit the application of BioGro.

Introduction

Earlier field experimental results indicated the beneficial effects of BioGro in rice cultivation in Vietnam (Nguyen et al. 2002, 2003). With this in mind, a study was undertaken to evaluate the adoption issues for BioGro at the farm level, and to identify problems and possible solutions. The beneficial effects of BioGro cannot be utilised if the technology is not adopted by farmers.

Materials and methods

Based on the result of previous experiments to evaluate the response of special quality rice to BioGro, a combination of BioGro with farmyard manure (FYM) and 75% of required nitrogen (N) and phosphorus (P) fertilisers was used for LT3 rice in farmer extension trials in Nam Dinh province in spring 2007. The purpose of the trials, conducted by the Vietnam Academy of Agricultural Science (VAAS), was to examine the economic effectiveness of using BioGro in special rice production in the province, and evaluate farmer responses to this technology. A series of two-treatment (BioGro plus 75% of required NP plus FYM; and non-BioGro plus FYM plus 100% of required NP), non-replication, on-farm trials were conducted on six farmer plots with a total area of 1.008 ha (Table 1). Seed sowing, transplanting and harvesting dates in 2007 were 19 January, 7 February and 3 June, respectively. The fertiliser rates (kg/ha) were 120 N, 75 P_2O_5, 60 K_2O, 280 BioGro and 4,200 FYM, respectively. Transplanting spacing was 15 cm × 15 cm.

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Results

Rice yield

The results of a field trial are presented in Table 1. The grain yield of LT3 was variable from farmer to farmer, but no statistically significant difference was observed between controls using farmer practice compared with BioGro with 25% reduction in NP. This result is identical with the results of small-scale experiments carried out in previous seasons, and confirmed again that BioGro application can save 25% of required NP fertiliser without changing the grain yield of special rice varieties.

Economic effectiveness

Cost analysis (Table 2) showed that BioGro application reduced the production cost in comparison to farmer practice due to the reduction of 25% of required NP fertiliser. On average, farmers can save VND195,602/ha on production costs when they use BioGro. The farm income difference achieved by using BioGro was variable among farmers (Table 3) but, on average, was an additional VND900,000/ha. The combined results of cost and income analysis (Table 4) showed that BioGro can realise an increased profit of around VND1 million/ha for each farmer.

Effect of BioGro on rice seedlings in Nam Dinh province (left—control; right—plus BioGro)

Table 1. Effect of BioGro on the grain yield of LT3 rice variety on six different farms used in this study

No. of farm	Name of farmer	Area (sao [a])	Grain yield (t/ha)	
			Control	BioGro
1	Tran Nguyen Soai	7	4.4	4.4
2	Tran Van Toan	4	3.9	4.6
3	Tran Van Toai	5	4.9	4.7
4	Tran Trong Phieu	4	4.3	5.1
5	Nguyen Van Kham	4	4.2	4.2
6	Tran Van Thach	4	4.3	4.2
			Mean grain yield	
			4.33	4.53

[a] 1 sao = 360 m^2

Table 2. Effect of BioGro on production cost of LT3 rice

No. of farm	Control (VND/ha)	BioGro (VND/ha)	Reduction in cost (VND/ha)
1	12,177,778	11,976,389	201,389
2	11,900,000	11,768,056	131,944
3	12,177,778	11,976,389	201,389
4	12,455,556	12,184,722	270,833
5	12,177,778	11,976,389	201,389
6	12,038,889	11,872,222	166,667
Mean	12,154,630	11,959,028	195,602

Discussion

Using BioGro can reduce the NP fertiliser requirement by 25% without significant difference in the level of rice yield. Use of BioGro in combination with 75% of the normally required NP can increase the average farm profit by VND1,095,602/ha. The results obtained with this quality rice are not as impressive as results from using BioGro elsewhere (Nguyen et al. 2002, 2003). Nevertheless, there are strong indications from the trial conducted by VAAS that BioGro can produce considerable savings through the reduced use of chemical fertilisers such as urea.

Table 3. Effect of BioGro application on farm income

No. of farm	Output of farmer (VND/ha)		Income difference (VND/ha)
	Farmer practice	BioGro	
1	19,800,000	19,800,000	0
2	17,550,000	20,700,000	3,150,000
3	22,050,000	21,150,000	–900,000
4	19,350,000	22,950,000	3,600,000
5	18,900,000	18,900,000	0
6	19,350,000	18,900,000	–450,000
Mean	19,500,000	20,400,000	900,000

Table 4. Benefit of BioGro in LT3 rice production

No. of farm	Costs (VND/ha)	Income difference (VND/ha)	Profit increase (VND/ha)
1	–201,389	0	201,389
2	–131,944	3,150,000	3,281,944
3	–201,389	–900,000	–698,611
4	–270,833	3,600,000	3,870,833
5	–201,389	0	201,389
6	–166,667	–450,000	–283,333
Mean	–195,602	900,000	1,095,602

Table 5. Regulatory issues and solutions

Issue	Solution
Variable composition (of microbial strains)	Standardise microbial strains for BioGro production
Biosafety of microbial strains (e.g. *Klebsiella pneumoniae*)	Clarify the safety of micro-organisms to human and animal health. This organism has been excluded from the BioGro2 formulation
Quality control of end product	Develop procedures for quality control of the end product
Shelf life of each micro-organism in BioGro and relationship between micro-organisms in BioGro	Conduct more research in survival and biological activity of micro-organisms in different organic materials and multistrain conditions

Table 6. Recommended application issues and solutions

Issue	Solution
Application time, rate and method	Unify and simplify the application procedure for BioGro
Recommendation on the rate of N and P reduction	This should be based on scientific background and acceptable experimental data

The legal definition of a biofertiliser in Vietnam has been set out in legislation. According to the Vietnam Standard (TCVN 6169-1996), biofertiliser is defined as a product containing a selected living micro-organism with a density that meets the requirement of the promulgated standard. Through the living activity of inoculated micro-organisms, nutrients like N, P, K and S can be available for plants, or biological substances can be produced that contribute to increasing plant yield or improving the quality of agricultural products. Biofertiliser should be safe to humans, animals and the environment.

Some issues that arose from the application of biofertilisers such as BioGro in this project are provided in Table 5. For regulatory processes, it is an advantage that microbial strains have a standard composition, and that methods of quality control such as selective media are standardised for identification or other tests. However, commercial producers will need to have some flexibility in strain composition so that improvements can be made if any issues of risk related to human or environmental health arise. The withdrawal of *Klebsiella pneumoniae* (see Table 5) is consistent with such prudent management. Approval may need to be sought for the replacement of strains in commercial products.

Conclusion

This study indicates that BioGro is economically profitable and may be adopted by farmers if regulatory issues such as biosafety of the microbial strains and quality control of the end products are ensured. The application procedure for BioGro should be unified and simplified based on the research findings. Recommended rates of chemical (e.g. N, P) fertilisers should be location specific and based on field experimental results. The acceptance of biofertilisers not only depends on successful demonstrations of their effectiveness but also on the quality of information provided regarding their application in response to the issues shown in Table 6.

From farmer questionnaires, it is unlikely that there would be a high adoption rate of biofertiliser technology in Nam Dinh province. There is uncertainty regarding positive field results because of a reluctance to reduce fertiliser to less than 75% of normal practice. This, the small size of landholdings and the easy availability of chemical fertilisers (in 2007) are all factors that could limit the application of BioGro.

Professor Hien framed by BioGro-treated seedlings (left) compared with a control (right) at Nam Dinh in 2004

References

Nguyen Thanh Hien, Kennedy I.R. and Roughley R.J. 2002. The response of field-grown rice to inoculation with a multi-strain biofertiliser in the Hanoi district, Vietnam. Pp. 37–44 in 'Biofertilisers in action', ed. by I.R. Kennedy and A.T.M.A. Choudhury. Rural Industries Research and Development Corporation: Barton, ACT.

Nguyen Thanh Hien, Deaker R., Kennedy I.R. and Roughley R.J. 2003. The positive yield response of field-grown rice to inoculation with a multi-strain biofertiliser in the Hanoi area, Vietnam. Symbiosis 35, 231–245.

Challenge of the 'Three Reductions' program in Vietnam: The potential role of inoculant biofertiliser technology

Tran Thanh Be[1]

Abstract

High rates of fertiliser and pesticide application are commonly used on rice in the Mekong Delta. Since 2002 Vietnam has promoted the concept of 'three reductions' for 'three gains' (3R3G) in rice production. The concept, developed by the International Rice Research Institute and promoted by a range of international and government agencies, requires the use of 'less seed, less pesticides and less fertiliser' for 'increased income, lowered exposure to pesticides and environmental benefits'. The program has had some success in reducing seeding rates, but has made fewer advances in reducing farmers' use of fertiliser. Biofertiliser technology, using the product BioGro, has been used successfully in both northern and southern Vietnam to replace 50% of chemical nitrogen (N) fertiliser on rice crops while maintaining yield. There is great potential for further work with farmers to integrate this technology into the 3R3G program to help achieve reduced fertiliser use. However, work is also needed to establish commercially viable production of BioGro in the Mekong region to enable continued research of the product under farm conditions, and to extend the technology to farmers.

Introduction

In the Mekong Delta of Vietnam farmers grow two or three rice crops per year using high rates of seeds (200–300 kg/ha), N fertiliser (150–300 kg/ha) and pesticides. Farmers have believed that these practices will lead to higher yields and profits (Nguyen et al. 2004; IRRI 2005). However, research has shown that, generally, it is possible for farmers to halve their seeding rates and reduce nitrogenous fertiliser and pesticide applications by 30% and 50%, respectively, and still achieve higher income (IRRI 2005).

Since 2002 Vietnam has promoted the concept of 'three reductions' for 'three gains' (3R3G or Ba Giam, Ba Tang) in rice production. Developed by the International Rice Research Institute (IRRI) and promoted by a range of international and government agencies (Huelgas et al. 2008), this concept requires the use of 'less seed, less pesticides and less fertiliser' for 'increased income, lowered exposure to pesticides and environmental benefits'. The program is an appeal to both economic principles and environmental good sense.

However, experience since the program began has only partly met expectations in the Mekong Delta (Table 1). Seeding rate and pesticide use have both been significantly reduced but fertiliser use has only declined moderately, and even increased in the case of potassium (K), although yield has been maintained. Similarly, a study by Huelgas et al. (2008) found that the component of the 3R3G program more readily adopted by farmers has been reduction in seeding rates rather than reduction in fertiliser use.

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The potential role of biofertiliser technology in 3R3G

The experience gained from the farmer extension trials for BioGro in the Mekong Delta conducted in this project (Tran et al. 2008) has demonstrated how biofertilisers could contribute to these reductions, particularly to the third goal of less fertiliser but also to reduced pesticide applications. Indeed, using less N and phosphorus (P) fertiliser is central to the idea of a biofertiliser based on beneficial micro-organisms, as higher rates of chemical fertilisers inhibit the effectiveness of the micro-organisms. The results of these farmer trials have shown that leaf colour index of BioGro-treated rice plants consistently shows a significant difference from rice crops grown with ample urea-N without BioGro. Crops grown with BioGro are a lighter green, and farmers associate this with both greater robustness of the rice stems and leaves and apparent resistance to pests, which sometimes allows a reduction in costs for pest control.

An economic analysis of the benefit:cost ratio of applying BioGro with reduced chemical N fertiliser showed a benefit from the application of BioGro in farmer trials in northern Vietnam (Barrett and Marsh 2002). Furthermore, analysis by the Institute of Agricultural Sciences (IAS) in southern Vietnam of experimental data from experiments conducted in Tay Ninh province has shown how BioGro can have commercial significance for farmers (Phan and Tran 2008; Marsh 2008).

The extension of biofertiliser technology to farmers has both technical and economic aspects. Any commercial product must be backed up by continuing research and quality control related to its performance. Trials such as those conducted by the Mekong Delta Development Research Institute (MDI) and the National Institute for Soils and Fertilisers in northern Vietnam to establish whether particular rice varieties respond differently to BioGro also need to be conducted at farmers' fields. The research and technical infrastructure must be available to support a good-quality biological product with a good shelf life, and achieve the economic results needed for successful production and use.

Farmers who have participated and observed the field trials in the Mekong Delta say they are willing to use BioGro in rice production. Through their own observations, measurements from several on-farm trials during 2005–07 and discussions at workshops, they have observed that the use of BioGro results in:

- less disease and lodging
- stronger stems, and brighter and cleaner grains
- better grain yields
- less costs and more benefits.

However, success in future extension of biofertiliser technology will be dependent on the following factors:

- capital and expert advice to set up commercially viable production facilities for biofertiliser products; in collaboration with the owner of the BioGro technology and the IAS in southern Vietnam, the MDI is investigating the possibility of establishing production in the Mekong area
- reliable biofertiliser products with good quality control, to help obtain economic benefits as frequently as possible
- clear demonstrations to farmers that good-quality biofertilisers like BioGro will give them significant and consistent economic benefits
- research at the farm level to show the best conditions for reduction in fertiliser inputs and obtain the maximum benefits for yield.

Table 1. Seed, fertiliser and pesticide application rates, and yield for standard farmer practice compared with the 3R3G program for rice production in the Mekong Delta

Criteria	Farmer	3R3G	% comparison
Seed rate (kg/ha)	197.5	117.8	–40.4
N fertiliser (kg N/ha)	95.4	83.4	–12.6
P fertiliser (kg P_2O_5/ha)	55.2	46.4	–15.9
K fertiliser (kg K_2O/ha)	36.0	40.0	+11.1
Insecticide (sprays/season)	1.7	0.4	–76.5
Fungicide (sprays/season)	2.7	2.0	–25.9
Yield (t/ha)	6.3	6.5	+3.2

(Source: Nguyen 2004)

The analysis conducted during the ACIAR project of the different models so far used for commercial production of BioGro is also highly relevant to success (Marsh and Nguyen 2008). It appears that commercial production by private companies, well supported by government or university research institutions, will have the best chance of being sustainable.

Conclusion

Huelgas et al. (2008) note that yield-increasing and cost-saving technologies are important to increase food supply and farm household incomes, and assist in addressing food security and poverty. They note that there is increasing interest in technologies that can reduce the use of agrochemicals but still maintain yield. The 3R3G management package is a knowledge-based technology that falls into this category. Biofertiliser technology, based on the product BioGro, has been used successfully in both northern and southern Vietnam to replace 50% of chemical N fertiliser on rice crops while maintaining yield. Given the constraints of BioGro production outlined earlier, there is a great potential for further work with farmers to integrate this technology into the 3R3G program to help achieve reduced fertiliser use.

References

Barrett G. and Marsh S. 2002. An economic analysis of inoculant biofertiliser production and use in Vietnam. Pp. 102–111 in 'Biofertilisers in action', ed. by I.R. Kennedy and A.T.M.A. Choudhury. Rural Industries Research and Development Corporation: Barton, ACT.

Huelgas Z.M., Templeton D. and Castanar P. 2008. Three reductions, three gains (3R3G) technology in south Vietnam: Searching for evidence of economic impact. In '52nd Annual Conference of the Australian Agricultural and Resource Economics Society', Canberra, 5–8 February 2008.

International Rice Research Institute (IRRI). 2005. Three reductions three gains: Program to improve environment and livelihood of millions of rice farmers in Vietnam. At: <http://www.irri.org/media/achievements/pdfs/Three%20Reductions%20Three%20Gains.pdf>.

Marsh S. 2008. Economically optimal nitrogen fertiliser rates with BioGro. These proceedings.

Marsh S. and Nguyen Thanh Hien 2008. Models of commercial biofertiliser production: Experiences with BioGro. These proceedings.

Nguyen Huu Huan. 2004. Scaling out resource management in the Mekong Delta: The case of 'three reductions, three gains'. Pp. 208–214 in 'Proceedings of the Mekong Rice Conference', ed. by D.W. Puckridge, B.C. Buu, K.L. Heong, T.P. Tuong and N.V. Bo: Ho Chi Minh City, Vietnam.

Nguyen Huu Huan, Thiet Le Van Thiet, Ho Van Chien and Heong K.L. 2004. Farmers' participatory evaluation of reducing pesticides, fertilizers and seed rates in rice farming in the Mekong Delta, Vietnam. Crop Protection 24, 457–464.

Phan Thi Cong and Tran Dang Dung 2008. Interaction effects of BioGro with nitrogen and phosphorus on grain yield and nutrient uptake of rice in light-textured soils of southern Vietnam. These proceedings.

Tran Thanh Be, Pham Thi Phan and Marsh S. 2008. Farmer extension trials using BioGro for rice production in the Mekong Delta. These proceedings.

Economically optimal nitrogen fertiliser rates with BioGro

Sally Marsh[1]

Abstract

The yield response curves generated from experimental data over two seasons for nitrogen (N) levels on rice, with and without BioGro, were used to calculate optimal N use. In line with the economic theories of marginal value and opportunity cost, optimal N use is dependent on the prices of the output (rice) and the N inputs. In this case N can be made available through urea-N and with BioGro. Different production function responses to urea-N and urea-N plus BioGro were obtained in the two seasons. Therefore, the relationship between the optimal N rates with and without BioGro are different for the two seasons for changing prices of rice and urea. Analysis of these experimental N response data has shown that the use of BioGro results in more efficient nutrient use in rice, and applied N levels needed for optimal yield are lower (or roughly equivalent) with changing input/output prices.

Introduction

The value of nitrogen (N) supplied by BioGro rests on two fundamental economic concepts—marginal value and opportunity cost. Marginal value, or diminishing returns, can be illustrated by a typical production function for N (Figure 1). As more N is applied, the increase in yield diminishes for each extra kilogram of N until maximum yield is reached, when extra N produces no increase in yield. However, the maximum yield is only the economically optimal yield if N comes at no cost. Optimal yield can be calculated by maximising the difference between the cost of N and the benefits from the crop (Schilizzi and Pannell 2001). When the cost of N is high it is a mistake to strive for maximum yield. The correct amount of N to be provided is N* which produces Y*, the economically optimal yield (Figure 1).

This reasoning is based on the concept of marginal value applied to a given input (N) and a given output (rice). The concept of opportunity cost expands the framework to account for other possible inputs and outputs. In this case N can be supplied by either chemical fertilisers or BioGro. Money spent on chemical N might be more economically spent on BioGro.

In this chapter the data generated by the Institute of Agricultural Sciences (IAS) in southern Vietnam showing the yield response to levels of N with and without BioGro for two different seasons (Phan and Tran 2008) is used to illustrate economically optimal levels of N application with and without BioGro. Using the production functions estimated from the data, an optimisation model was built in Excel and solved using Solver to find optimal N application rates at different prices of urea and rice.

Results

Summer–autumn rice crop 2006

The estimated production functions for this crop using the IAS experimental results are shown in Figure 2. In this experiment the use of BioGro has

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shifted the production function to the left, resulting in higher yields at lower N rates. The maximum yields with and without BioGro are roughly equivalent but the maximum yield with BioGro is reached at a lower N application rate than without BioGro.

These production functions were used to calculate the optimal application rates of N, and associated optimal yields, with and without BioGro at different urea prices with the rice price set at VND[2]4,000/kg (Figure 3). When this ACIAR project (SMCN/2002/073) commenced in 2003, the urea price was around VND2,600/kg but it is currently (June 2008) in excess of VND8,000/kg ($A0.50/kg; $A1 = VND16,500). Given the prevailing global economic conditions, which are driving the prices of petroleum-based products higher, the sensitivity analysis was conducted for urea prices ranging from VND6,000/kg to VND11,000/kg.

The optimal N rates, 102 kg/ha without BioGro and 80 kg/ha with BioGro (with urea price at VND7,500/kg and rice price at VND4,000/kg), are below the levels of N application required to reach the maximum yield (Figure 3). Optimal N rates with BioGro are lower at all prices of urea used in the analysis, but the difference between the optimal N rates with and without BioGro decreases as the price

[2] Vietnamese currency (dong)

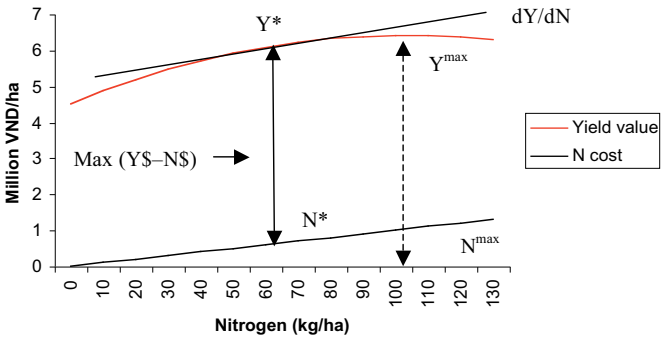

Figure 1. The economically optimal levels of nitrogen application and yield achievement Y^{max}

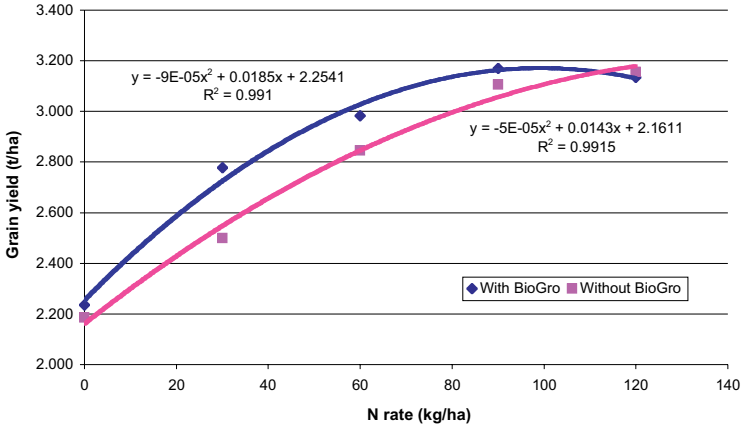

Figure 2. Estimated grain yield response of Trau Nam rice to added nitrogen with and without BioGro, Tay Ninh province, southern Vietnam, first rainy season 2006

of urea increases. For example, at a urea price of VND7,500/kg, 22 kg less N can be applied for optimal yield using BioGro; but at a urea price of VND9,000/kg, 18.5 kg less N can be applied. The optimal yield obtained from optimal N applied with BioGro is always higher even though the rate of N applied is lower; furthermore, the difference in yield increases as the price of urea increases.

The substantial difference in yield (e.g. 58 kg/ha at urea price of VND7,500/kg) does not translate directly into a corresponding increase in net income because of the relative prices of urea and BioGro. The same data for changing urea prices are shown in Figure 4, but with net income instead of yield shown on the secondary y-axis. In this example net income only takes into account the costs of N and BioGro. Optimal N rates are the same as in Figure 3 (i.e. less when BioGro is used), and net income when N is applied with BioGro also becomes increasingly higher compared to net income with urea application

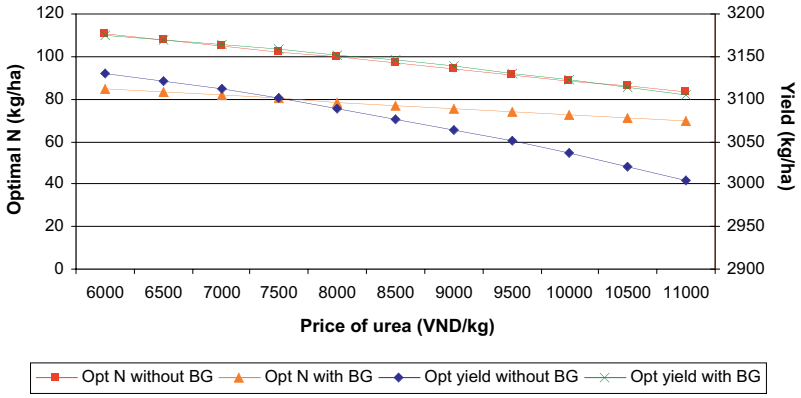

Figure 3. Optimal nitrogen application rates and yield with and without BioGro at urea prices ranging from VND6,000/kg to VND11,000/kg (rice price at VND4,000/kg, BioGro price at VND2,000/kg and applied N rate at 200 kg/ha) for experimental data from summer–autumn rice crop 2006

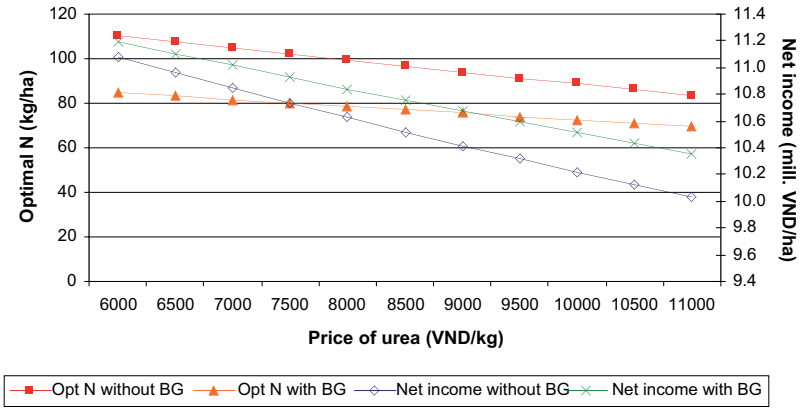

Figure 4. Net income and optimal nitrogen application rates with and without BioGro at different prices of urea (rice price at VND4,000/kg, BioGro price at VND2,000/kg and applied N rate at 200 kg/ha) for experimental data from summer–autumn rice crop 2006

only, as the urea price increases. For example, the difference in net income at a urea price of VND9,000/kg is VND259,000/ha; and VND193,000/ha when the urea price is VND7,500/kg.

Rice prices in Vietnam are currently (June 2008) much higher than VND4,000/kg. Following rapid rises in grain prices in 2008, the price of paddy rice in Vietnam is around VND6,000–8,000/kg. Figure 5 shows the effect of increasing rice prices on optimal levels of N application and net income, for rice prices ranging from VND2,000/kg to VND12,000/kg and with the urea price set at VND7,500/kg. As rice prices increase, the optimal level of N application increases but at a decreasing rate. The difference in optimal N application with and without BioGro increases as the rice price increases. For example, at a rice price of VND4,000/kg, 22 kg less N can be applied per hectare with BioGro, and net income

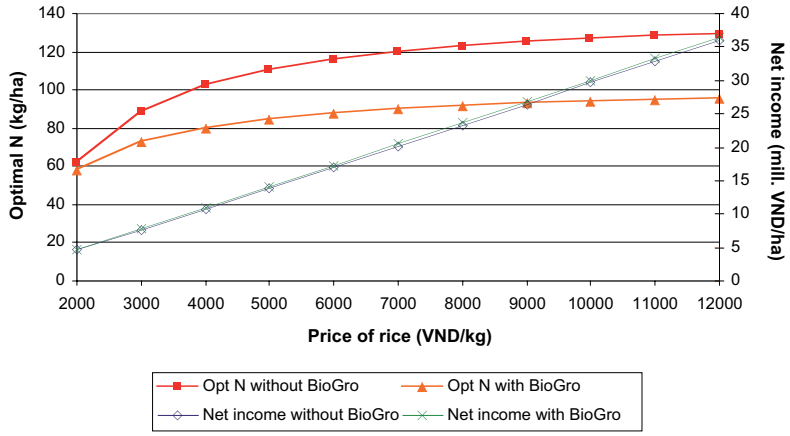

Figure 5. Net income and optimal nitrogen application rates with and without BioGro for rice prices between VND2,000/kg and VND12,000/kg (urea price at VND7,500/kg, BioGro price at VND2,000/kg and applied N rate at 200 kg/ha) for experimental data from summer–autumn rice crop 2006

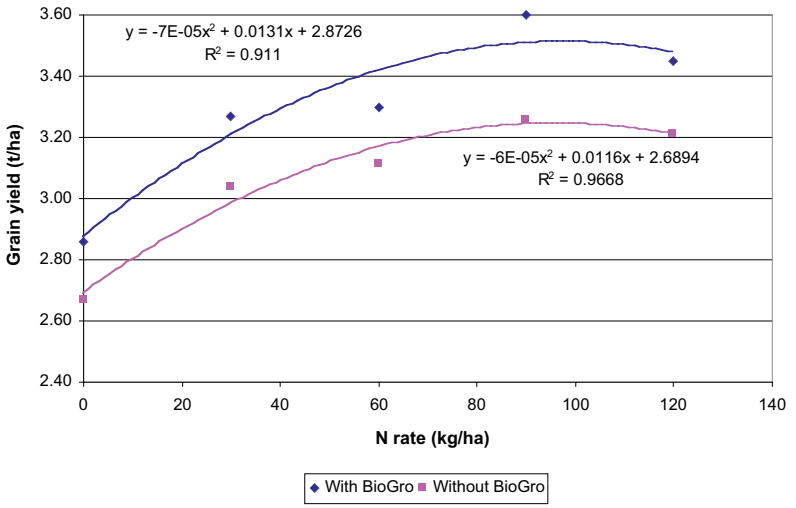

Figure 6. Estimated grain yield response of Trau Nam rice to added nitrogen with and without BioGro, Chau Thanh district, Tay Ninh province, southern Vietnam, second rainy season 2006

increases by VND193,000/ha, than without BioGro. At a rice price of VND6,000/kg, 28 kg less N is used and net income increases by VND285,000/ha compared to without BioGro. At a rice price of VND8,000/kg, 31 kg less N is used and net income increases by VND352,000/ha. Environmental impacts would provide further uncosted benefits.

Autumn–winter rice crop 2006

The estimated production functions for this crop using the IAS experimental results are shown in Figure 6. In this experiment there has been a different response to BioGro than in the summer–autumn crop. With BioGro the production function has shifted uniformly upwards, resulting in higher yields at all N application rates and a higher maximum yield than without BioGro.

As before, these production functions were used to calculate the optimal application rates of N, and associated optimal yields, with and without BioGro at different urea prices (Figure 7). The optimal N rates, 63.5 kg/ha without BioGro and 64.5 kg/ha with BioGro (with urea price at VND7,500/kg and rice price at VND4,000/kg), are well below the levels of N application required to reach the maximum yield (Figure 7). In this case and differing from the previous example, the optimal N rates with BioGro are slightly higher at all but the lowest price (VND6,000/kg) of urea, and the difference between the optimal N rates with and without BioGro increases slightly as the price of urea increases. At all prices of urea the optimal yield obtained from optimal N applied with BioGro is much higher than without BioGro, increasing by 233 kg/ha when the urea price is VND6,000/kg, 237 kg/ha when the urea price is VND7,500/kg and 241 kg/ha when the urea price is VND9,000/kg.

The same data are shown in Figure 8, but with the secondary y-axis showing net income instead of yield. As before, net income only takes into account the cost of N and BioGro. Optimal N rates are the same as in Figure 7 and, for these yield response curves, net income is always around VND500,000/ha higher for N applied with BioGro at all prices of urea.

Figure 9 shows the effect of increasing rice prices on optimal levels of N application and net income. As before, as rice prices increase, the optimal level of N application increases but at a decreasing rate. The optimal N application with BioGro is higher than without BioGro until a rice price of VND5,000/kg, but then becomes less than without BioGro at higher rice prices. The net income difference for optimal N applied with BioGro increases markedly as the rice price increases. At a rice price of VND2,000/kg, 5.8 kg less N can be applied per hectare without BioGro, but the net income with BioGro is

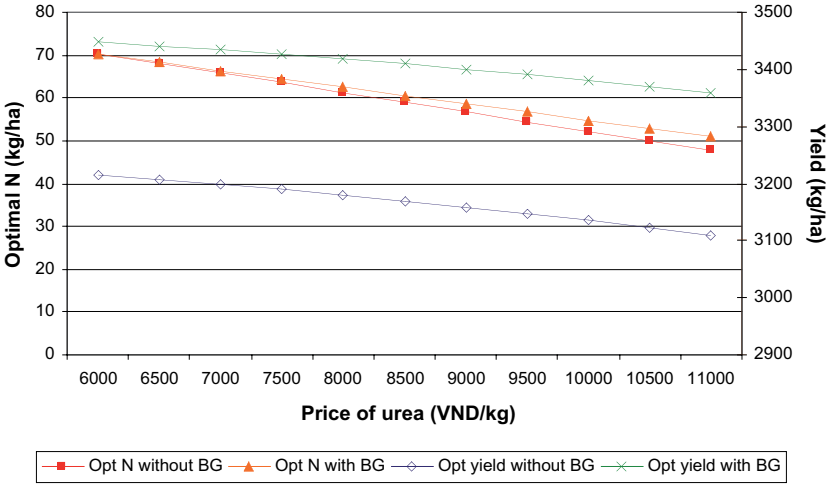

Figure 7. Optimal nitrogen application rates and yield with and without BioGro at urea prices ranging from VND6,000/kg to VND11,000/kg (rice price at VND4,000/kg, BioGro price at VND2,000/kg and applied N rate at 200 kg/ha) for experimental data from autumn–winter rice crop 2006

VND38,000/ha higher. However, an increase of VND521,000/ha in net income is achieved using optimal N with BioGro at a rice price of VND4,000/kg. At a rice price of VND8,000/kg, the increase in net income is VND1.46 million/ha.

Discussion

These calculations are based on production functions derived from IAS experimental results for different levels of N application, with and without BioGro, to rice in the 2006 summer–autumn and autumn–winter seasons. Differently shaped production functions were obtained for these two seasons, and the relationship between the optimal N rates with and without BioGro are hence different for these two crops. Similar N responses, that is a response function shift to the left (a N-saving effect) and a response function shift upwards (a yield boost effect), are also known to occur in crops following crop legume rotations (D.P. Pannell, pers. comm.). Considering responses to different sources of N is complex because artificial and biological N are interdependent so that the level of one affects the value of the other (Pannell and Falconer 1988). These authors describe how they valued the combination of N-saving effect and yield boost effect that characterises responses in cereal crops following legume crops. As in the situation discussed in this chapter, the yield boost component of the response in cereal crops following legume crops gives higher income increases than the N-saving component.

Nitrogen required for maximum versus optimal yield

Similar over both seasons is the large difference between N needed for maximum yield compared to optimal yield, both with and without BioGro. For the IAS summer–autumn crop data the N needed for *maximum* yield without BioGro is approximately 120 kg/ha, whereas for *optimal* yield (with rice price at VND4,000/kg and urea price at VND7,500/kg) it is only 102 kg/ha, i.e. 18 kg/ha less. For *maximum* yield with BioGro, the N needed is approximately 90 kg/ha, whereas for *optimal* yield (with rice price at VND4,000/kg, urea price at VND7,500/kg and BioGro price at VND2,000/kg) it is only 80 kg/ha, i.e. 10 kg/ha less. For the IAS autumn–winter crop data the N needed for *maximum* yield without BioGro is approximately 90 kg/ha, whereas for *optimal* yield (with prices as before) it is only 64 kg/ha, i.e. 26 kg/ha less. The N needed for *maximum* yield with BioGro is higher at approximately 95 kg/ha, and for *optimal* yield (with prices as before) it is 65 kg/ha, i.e. 30 kg/ha less.

Unless the cost of N is zero, maximum yield will not be the optimal yield. The optimal use of N will

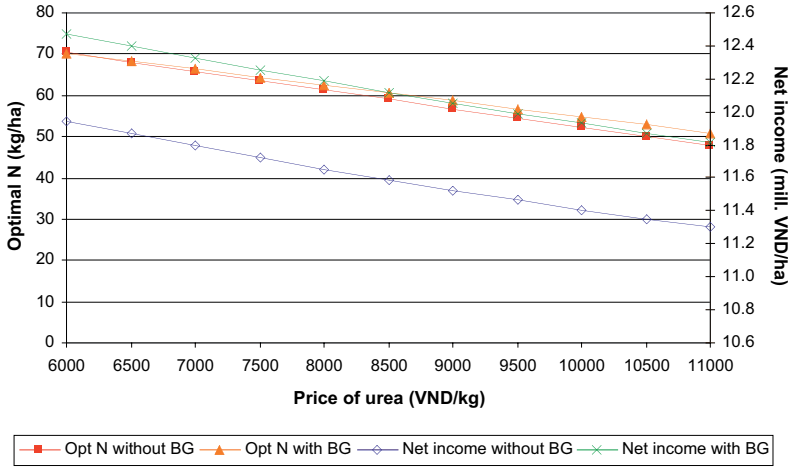

Figure 8. Net income and optimal nitrogen application rates with and without BioGro at different prices of urea (rice at VND4,000/kg, BioGro at VND2,000/kg and applied N rate at 200 kg/ha) for experimental data from autumn–winter rice crop 2006.

depend on the prices of both rice and N. Even without BioGro, considerable N is saved if it is applied at the optimal rate, taking account of the prices of both the output and the inputs. For the summer–autumn crop, considerable further N savings could be achieved if BioGro is used. However, this was less so for the autumn–winter crop because of the differing production responses in the two seasons.

Summer–autumn rice crop 2006

For these data, if farmers use optimal N with BioGro when the price of urea is VND7,500/kg:

- inorganic N used is reduced, compared to without BioGro, by 22 kg/ha if the rice price is VND4,000/kg
- yield increases by 58 kg/ha if the rice price is VND4,000/kg
- net income with BioGro is VND193,000/ha more than without BioGro if the rice price is VND4,000/kg
- net income with BioGro is VND352,000/ha more than without BioGro if the rice price is VND8,000/kg.

Rising prices for urea reduce the N saved (at optimum levels) but increase the net income difference with BioGro. For example, if the price of urea is VND9,000/kg:

- inorganic N used with BioGro, compared to without BioGro, is reduced by 18.5 kg/ha
- yield increases by 74 kg/ha
- net income increases by VND259,000/ha if the rice price is VND4,000/kg.

In both these scenarios the farm-level benefits are not high but BioGro allows more efficient nutrient use, especially at higher rice prices (see Figure 5). If the prices of both urea and rice are very high, for example urea at VND12,000/kg and rice at VND10,000/kg, the farm-level benefits become considerable at VND710,000/ha. These prices for urea and rice seem quite possible at current trends.

Generally speaking, for the data from the summer–autumn crop, economically optimal N application rates are always lower if it is applied with BioGro at all prices of urea and rice. The nature of the production response has caused a consistent and substantial N-saving effect when BioGro is used with inorganic N. As the price of rice increases, the difference in optimal N rates favours the application of BioGro to achieve lower optimal N application rates. As the price of urea increases, optimal N rates with BioGro increase relative to without BioGro, resulting in comparatively higher yields and net incomes. Higher urea prices would also favour the adoption of BioGro for higher yields and net incomes—a yield boost effect.

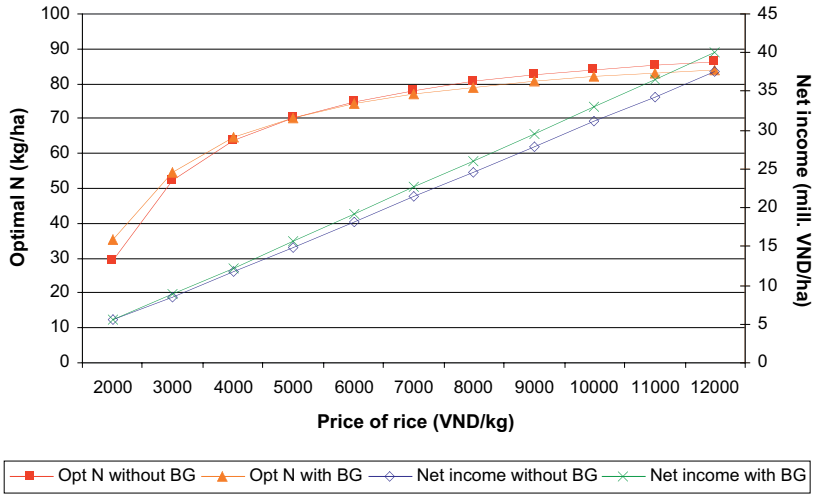

Figure 9. Net income and optimal nitrogen application rates with and without BioGro for rice prices between VND2,000/kg and VND12,000/kg (urea price at VND7,500/kg, BioGro price at VND2,000/kg and applied N rate at 200 kg/ha) for experimental data from autumn–winter rice crop 2006

Autumn–winter crop 2006

For these data, if farmers use optimal N with BioGro when the price of urea is VND7,500/kg:

- inorganic N used, compared to without BioGro, is increased by 1 kg/ha if the rice price is VND4,000/kg
- yield increases by 237 kg/ha if the rice price is VND4,000/kg
- net income increases by VND531,000/ha if the rice price is VND4,000/kg
- net income increases by VND1.46 million/ha if the rice price is VND8,000/kg.

As urea become more expensive, the comparative increase in net income reduces slightly and the optimal N used with BioGro increases relative to optimal N without BioGro. For example, if the price of urea is VND9,000/kg:

- inorganic N used is increased by 2 kg/ha
- yield increases by 241 kg/ha
- net income increases by VND527,000/ha.

For these response curves, farm-level benefits are much higher, but BioGro encourages slightly higher use of inorganic N in order to achieve higher yields. However, at rice prices higher than VND5,000/kg, optimal N application with BioGro becomes slightly less than without BioGro. Generally speaking, for the data from the autumn–winter crop, there are no or minimal savings in N used with BioGro for all prices of urea and rice. However, there are higher yields and net incomes achieved at all prices of urea and rice. The response to BioGro application has been mainly a yield boost effect.

The two different production responses to levels of N with and without BioGro are responsible for these differing results. The optimal use of N and the farm-level benefits that result will depend on the nature of the BioGro response, and it appears that this will vary. As mentioned earlier, similar variation in N response curves has been noted in cereal crops grown after legume crops, specifically lupins, in Western Australia. Ongoing trial work and/or demonstrations will be important to further explore the nature of the BioGro response in different seasons and at different sites. Site-specific (e.g. different soil types) and varietal responses may be important. However, whatever the combination of N-saving effect and yield boost effect, both responses to BioGro either increase net income or reduce N use, and often achieve both of these effects together.

Conclusion

Analysis of these experimental N response data has shown that the use of BioGro can result in more efficient nutrient use in rice, and applied N levels needed for optimal yield are lower (or roughly equivalent) with changing input/output prices. The BioGro response has demonstrated both 'N-saving' and 'yield boost' effects. Reduced inorganic fertiliser use has potentially substantial benefits to society, and ways of achieving this are of interest worldwide.

However, consistent with results from farmer field experiments reported in earlier chapters in these proceedings, the increase in net income achieved from both cost savings and higher yields obtained with optimal N application rates with BioGro are not high on a per hectare basis if rice prices are VND4,000/kg or lower. This is particularly the case if the response to BioGro is mainly a N-saving effect, as for the summer–autumn crop data in 2006. However, at higher rice and urea prices, the net income benefits increase, particularly if the response to BioGro is mainly a yield boost effect, as for the autumn–winter crop data in 2006. In this case net income benefits are greater than VND500,000/ha for urea prices above VND6,000/kg, and increase substantially when rice prices rise above VND4,000/kg. Based on the analysis of these data, higher rice and urea prices will favour the use of BioGro on rice crops in Vietnam for substantial N-saving and net income increases.

Acknowledgments

The author acknowledges helpful discussions with David Pannell about the responses to and economics of nitrogen application to crops.

References

Pannell D.P. and Falconer D.A. 1988. The relative contributions to profit of fixed and applied nitrogen in a crop–livestock farm system. Agricultural Systems 26, 1–17.

Phan Thi Cong and Tran Dang Dung 2008. Interaction effects of BioGro with nitrogen and phosphorus on grain yield and nutrient uptake of rice in light-textured soils of Southern Vietnam. These proceedings.

Schilizzi S. and Pannell D.J. 2001. The economics of nitrogen fixation. Agronomie 21, 527–537.

Estimating the most probable number of bacteria in multistrain biofertiliser inoculants using a multiple-tube fermentation test

Rosalind Deaker, Geraldine Mijajlovic and Andrea Casteriano[1]

Abstract

Quality control for multistrain inoculants is difficult as there is a lack of methods to easily and accurately determine the number of viable cells of particular species from a species-rich environment. A multiple-tube carbohydrate fermentation method was found to be accurate for determining the number of viable cells of individual biofertiliser strains in a mixed culture prepared in sterile peat. The most probable number of viable cells using this method compared well with plate counts for pure cultures, and results could be obtained within 24 hours depending on the growth rate of the organism. This method requires a similar level of skill, labour and cost as general microbiological culturing techniques, making it particularly accessible to quality control laboratories in developing countries.

Introduction

It is well established that many bacterial species can have growth promoting effects on plants (reviewed by Kennedy and Islam (2001)). Nguyen et al. (2003) described a beneficial effect on rice development and yield of inoculating with a number of bacterial isolates from rice rhizospheres in Vietnam. This effect was observed in two out of three field trials using no or reduced quantities of fertiliser (farmyard manure or urea and superphosphate). The bacterial isolates were effective when applied in a combination consisting of *Pseudomonas* spp. (1N and 2N), *Klebsiella pneumoniae* (4P) and *Citrobacter freundii* (3C). Each species was observed to perform specific metabolic functions in laboratory cultures that could be of benefit to plant growth, as previously described in these proceedings.

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There is a great deal of evidence that successful inoculation of legumes depends upon the delivery of high numbers of viable rhizobia to the rhizosphere. Legume yield in field trials can be related to high inoculum potentials when introduced into competitive (Ireland and Vincent 1968) and non-competitive environments (Hume and Blair 1992; Roughley et al. 1993). In other studies increasing the inoculum potential of 3C, when applied on its own, enhanced root development of wheat grown in sterile sand, indicating that plant growth promotion by this strain is dependent on inoculum potential (Mijajlovic 2007). This indicates the advantage of high numbers for rapid colonisation of the rhizosphere, maximising the physiological effect on plants.

Many micro-organisms are capable of colonising the rhizosphere of plants, resulting in complex bacterial communities. The rhizosphere community structure is affected by soil conditions and the amount and composition of root exudates. The plant can exert a highly selective effect on the community structure (Marschner et al. 2004). However, the

association between non-leguminous plants and bacteria is much less specific than the legume symbiosis, and it is therefore possible to assume that competition between organisms in the rhizosphere would have a much larger effect on the success of inoculation. Results of field trials using biofertilisers are often variable and survival of the organisms in the rhizosphere is difficult to measure. For maximum potential benefit, it is essential that high numbers of viable bacteria are present in the inoculant at the time of application.

Counting viable cells in inoculants containing single biofertiliser strains is relatively straightforward but, when strains are combined or introduced into non-sterile carriers, counting individual strains becomes more difficult and there is a need to distinguish between strains. Several researchers are looking to methods such as real-time polymerase chain reaction (PCR) to quantify strain-specific regions of microbial DNA in diverse communities (Ben-dov 2007; Hierro et al. 2007; Shannon et al. 2007). However, capital investment and operating costs for molecular techniques can be high and therefore inaccessible to many researchers and quality control laboratories, particularly in developing countries. DNA-based techniques have clear advantages for detecting and measuring non-culturable organisms or organisms in communities that may be overgrown by dominant species. (For an interesting debate on cultivation of micro-organisms versus non-cultivation for studies in microbial ecology, see Ritz 2007 and Nichols 2007.) However, most organisms produced as inoculants are first grown in culture and may therefore be well suited to classical culture techniques. The question of whether cultured organisms are metabolically similar in vitro and in vivo will not be discussed in this paper but is an important consideration for the action of biofertilisers.

Selective or differential media that allow growth or detection of one particular organism within an ecological group are useful for the isolation and enumeration of micro-organisms and, in several cases, have been improved to allow the isolation of previously non-culturable organisms (reviewed by Nichols (2007)). These media have been used in most-probable-number (MPN) multiple-tube fermentation approaches using published MPN tables or software to estimate numbers of specific physiological groups in an environment (Reith et al. 2002; Smith and Macfarlane 1998). The accuracy of the multiple-tube fermentation method for counting organisms should be tested by calibration with other methods or through measures of internal coherency (Scott and Porter 1986). The MPN is an estimate of numbers based on probability, the presumption that cells are evenly dispersed and that one cell will produce a positive response in a tube. The probability of the number of organisms in a known volume is based on Poisson theory, whereby the probability of having a negative tube is equal to $e^{-\nu\delta}$ (where ν is the volume and δ is the density of cells (Scott and Porter 1986).

This paper describes the use of carbohydrate fermentation media to determine the MPN of the biofertiliser strains 3C and 4P in pure and mixed peat cultures.

Materials and methods

Carbohydrate utilisation and media for MPN

The utilisation pattern of 49 carbohydrates was determined for the four biofertiliser strains 1N, 2N, 3C and 4P (described in Nguyen et al. (2003)) and a fifth strain *Enterobacter* sp. (5P, also isolated by Professor Nguyen Thanh Hien from rice rhizospheres in Vietnam) using the API 50 CH test (bioMérieux, France). Strains were suspended in phenol red media and transferred to API strips containing carbohydrates according to manufacturer's instructions. After overnight incubation at 30 ˚C, positive results for carbohydrate fermentation and acid production were detected by a change in colour of the indicator from red to yellow. The patterns of carbohydrate utilisation for each of the five strains were compared, and carbohydrates that were fermented by single strains were used to prepare media for application of the MPN technique. Stock solutions of individual carbohydrates were prepared, filter sterilised and added to a final concentration of 0.5% to the sterile phenol red media [phosphate buffer 1L (K_2HPO_4 1.21 g/L, KH_2PO_4 0.34 g/L); $(NH_4)_2SO_4$ 2 g/L; yeast extract 0.5 g/L; tryptone 1 g/L; phenol red 0.18 g/L; trace elements 10 mL (H_3BO_3 2.86 g, $MnSO_4.4H_2O$ 2.03 g, $ZnSO_4.7H_2O$ 0.22 g, $CuSO_4.5H_2O$ 0.08 g, $NaMoO_4.2H_2O$ 0.14 g)].

MPN in single and multistrain peat cultures

Preparation of peat cultures containing one, two and three biofertiliser strains

Biofertiliser strains 3C, 1N, 4P were shaken overnight in nutrient broth at 30 ˚C. The number of cells per mL of broth was determined by serially diluting to 10^{-7} in sterile phosphate peptone buffer (PPB—K_2HPO_4 1.21 g/L, KH_2PO_4 0.34 g/L, peptone 1 g/L) and spreading 10^{-5}–10^{-7} dilutions on nutrient agar. Plates were inverted and incubated at 30 ˚C until colonies were visible. The gravimetric moisture content of gamma-sterilised peat (50 g dry peat per packet, supplied by Gary Bullard, Bio-Care Technology Pty Ltd) was measured, adjusted to 20% (w/w dry weight) with sterile water to avoid heat of wetting at inoculation (described by Roughley and Vincent (1967)) and allowed to equilibrate overnight. Bacterial cultures were then injected into peat by adding 30 mL broth culture for single-strain, 15 mL for double-strain and 10 mL for triple-strain inoculants.

MPN in peat culture and calibration with plate counts

The MPN of 3C and 4P was estimated using the multiple-tube fermentation method (in single and multistrain inoculants) and spread plate counting on nutrient agar (for single-strain inoculants). Moist peat cultures (10 g or 1 g) were suspended in 90 mL PPB and shaken on a wrist-action shaker for 15 minutes. Tenfold serial dilutions were made by transferring 0.1 mL suspension to 0.9 mL PPB in Eppendorf tubes and repeating until a dilution of 10^{-8} was reached. Triplicate Eppendorf tubes containing fermentation media (0.9 mL) were inoculated with aliquots of dilutions (0.1 mL) from 10^{-3} to 10^{-8}. Dilutions from inoculants containing 3C were inoculated into the fermentation medium supplemented with L-sorbose, and those containing 4P were inoculated into the medium supplemented with α-methyl-D-glucoside. When both strains were present, dilutions were inoculated into both fermentation media. Numbers of viable cells in inoculants containing only one strain were also determined by viable plate count by spreading 0.1 mL from dilutions 10^{-5} to 10^{-7} onto the surface of nutrient agar and counting individual colonies after growth at 30 ˚C.

Results

Fermentation of carbohydrates

Fermentation of carbohydrates was detected by a colour change from red to yellow in the phenol red carbohydrate media, indicating acid production. Only 3C and 4P fermented unique carbohydrates that could distinguish them from the other three strains (Table 1). The *Pseudomonas* spp., 1N and 2N, fermented fewer carbohydrates than the other species but were not identical in their fermentation patterns. All carbohydrates fermented by the *Pseudomonas* spp. were fermented by at least one of the other biofertiliser strains. Fermentation of inuline and melezitose was unique to *Enterobacter* sp. (5P) but the results were difficult to judge as colour change was weak.

Calibration of MPN with spread plate counts

The number of viable cells of 3C and 4P in peat cultures containing the individual strains were counted using both the spread plate and multiple-tube fermentation methods (Table 2). The numbers detected by both methods were in close agreement and an analysis of variance indicated no significant difference between means calculated using each method (P=0.853).

MPN of 3C and 4P in multistrain peat cultures

The multiple-tube fermentation method was used to determine the number of 3C and 4P in multistrain peat cultures with one, two and three strains. The results are presented in Figure 1 as the mean $\log_{10}$ number recovered after 14 days of incubation at 30 ˚C and after further storage for 28 days at 15 ˚C (42 days after inoculation). Despite a difference in the number of cells injected for single and multi-strain cultures, there was no significant difference in the total numbers of 3C in each culture after 14 days (P=0.801). However, the mean number of 3C was slightly lower when all three strains were combined. The recovery of 4P per gram of peat culture was more dependent on the number of strains present. The number of 4P decreased slightly when one other strain was present, and there was a significant decrease when all three strains were present (P=0.023).

Table 1. Fermentation of carbohydrates by biofertiliser strains

Strain	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25
1N	+	-	-	-	-	+	-	-	-	+	+	+	+	-	-	-	-	+	-	-	-	-	-	-	-
2N	+	-	-	-	-	-	-	-	-	-	+	+	-	-	-	-	-	+	-	-	-	-	-	-	+
3C	+	-	+	+	+	+	-	-	-	+	+	+	+	+	+	-	-	+	+	-	-	+	-	+	-
4P	+	-	-	+	+	+	-	+	-	+	+	+	+	-	+	-	+	+	+	-	+	+	+	+	+
5P	+	-	-	+	+	+	-	-	-	+	+	+	+	-	+	-	+	+	-	-	-	+	i	i	-

Strain	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48	49
1N	-	-	-	-	+	+	+	-	-	-	-	-	-	-	-	-	-	+	-	+	-	-	-	-
2N	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
3C	-	-	+	+	+	+	+	-	-	+	+	-	+	+	-	-	-	-	+	-	-	+	+	+
4P	+	+	+	+	+	+	+	-	-	+	-	+	-	+	+	-	-	+	+	+	-	-	-	-
5P	+	+	+	+	+	+	+	w	w	-	-	-	w	+	-	-	-	+	-	+	-	+	-	+

A positive result indicates fermentation of carbohydrate resulting in a decrease in pH and a colour change from red to yellow; a negative result indicates that there was no change in colour; and 'w' indicates a weakly positive result where colour change was only slight

Carbohydrates

1. glycerol	11. D-glucose	21. α-methyl-D-glucoside	31. saccharose	41. D-lyxose
2. erithritol	12. D-fructose	22. N-acetyl glucosamine	32. trehalose	42. D-tagatose
3. D-arabinose	13. D-mannose	23. amygdaline	33. inuline	43. D-fucose
4. L-arabinose	14. L-sorbose	24. arbutine	34. melezitose	44. L-fucose
5. ribose	15. rhamnose	25. esculin	35. D-raffinose	45. D-arabitol
6. D-xylose	16. dulcitol	26. salicine	36. amidon	46. L-arabitol
7. L-xylose	17. inositol	27. cellobiose	37. glycogene	47. gluconate
8. adonitol	18. mannitol	28. maltose	38. xylitol	48. 2-ceto-gluconate
9. β-methyl-xyloside	19. sorbitol	29. lactose	39. β-gentiobiose	49. 5-ceto-gluconate
10. galactose	20. α-methyl-D-mannoside	30. melibiose	40. D-turanose	

Table 2. Comparison of MPN method with spread plate counts [a]

BioGro strain	Method	Mean $\log_{10}$ cfu	Standard error
3C	Spread plate	9.01	0.30
	MPN	9.12	0.25
4P	Spread plate	9.54	0.02
	MPN	9.57	0.20

[a] Counts (cfu) are means of three replicates after $\log_{10}$ transformation. There was no effect of method on number counted for each strain (P=0.853); MPN = most probable number.

A repeated measures analysis (REML) indicated no significant change at the 5% level in the number of cells present after storage for a further 28 days at 15 ˚C (Chi square = 0.655 for 3C, 0.093 for 4P). While there was slight variation in the means, the overall mean $\log_{10}$ number of 3C after 14 days was 8.99 and after 42 days was 8.78. The number of 4P recovered from peat cultures at both sampling times was slightly higher than the numbers of 3C. At 14 days the $\log_{10}$ mean number was 9.12 and at 42 days it was 9.04. The number of 4P in the triple-strain combination had increased from the significantly lower number at 14 days. For both 3C and 4P, numbers were generally higher when strains had been combined with 1N than with each other or in triple-strain combinations.

The percentage of cells of 3C and 4P recovered after 14 days' incubation at 30 ˚C was calculated by dividing the measured number by the theoretical number inoculated at injection and multiplying by 100 (Table 3). In all cases the number of 3C increased from the initial number inoculated into peat culture. Although not significant, the growth of 3C after 14 days was higher when the inoculum was diluted except when all three strains were combined. Only 38.1% of the 14-day numbers were recovered from pure culture of 3C after further storage for 28 days at 15 ˚C. Initial growth of 3C in peat was highest when combined with 4P (743% of initial inoculum number) but no further growth occurred between 14 and 42 days, and numbers had declined significantly to 30.3% of the 14-day count (P=0.049). Growth of 3C only continued between 14 and 42 days when combined with 1N or in the presence of both 1N and 4P.

Growth of 4P in peat culture after 14 days' incubation at 30 ˚C was highest when combined with 1N and in pure culture. When 4P was combined with both 3C and 1N in the triple-strain inoculant, numbers were initially reduced but further growth occurred between 14 and 42 days at 4 ˚C. No further growth of 4P occurred between 14 and 42 days in the double-strain inoculants, and numbers declined in the pure culture.

Discussion

Efficacy of the multiple-tube fermentation method for quality control

This paper describes a multiple-tube fermentation method whereby acid production by fermentation of

Table 3. Percentage growth of 3C and 4P BioGro strains at 14 and 42 days after injection into peat individually and in combination with other strains

Measured strain	Combined strains	Percentage of cells remaining			
		14 days after inoculation (mean of initial inoculum size)	Standard error	42 days after inoculation (mean of 14-day count)	Standard error
3C	None	231.0	87.1	38.1	7.4
	1N	411.0	121.0	141.0	29.7
	4P	743.0	362.0	30.3	10.2
	1N + 4P	278.0	147.0	141.0	69.1
4P	None	284.0	140.0	57.8	26.3
	1N	297.0	116.0	116.0	52.4
	3C	200.0	73.7	95.5	48.3
	1N + 3C	64.8	11.2	271.0	90.8

Values at 14 days calculated as percentage of cells remaining from theoretical number at injection (from broth counts). Values at 42 days (14 days at 30 ˚C and 28 days at 4 ˚C) calculated as percentage of 14-day cell numbers.

carbohydrates in media allows differentiation between different species of bacteria combined in multistrain inoculants. The method was calibrated with plate counts using single-strain inoculants. There was strong agreement between the methods, indicating that the multiple-tube fermentation method is a useful technique for the enumeration of specific strains of bacteria from peat cultures. However, the two methods were not compared for multistrain cultures because of the difficulty associated with distinguishing between colonies of each strain.

Accuracy using the multiple-tube fermentation method is determined by two principles, summarised by Cochran (1950)—the suspension must be well homogenised so that cells are randomly distributed, and the presence of an individual cell should produce a positive result. These principles were confirmed by Brockwell (1963), who found a close agreement between plate counts of rhizobia and the MPN using the plant infection test, when suspensions were agitated thoroughly to encourage even distribution of cells in solution. Brockwell (1963)

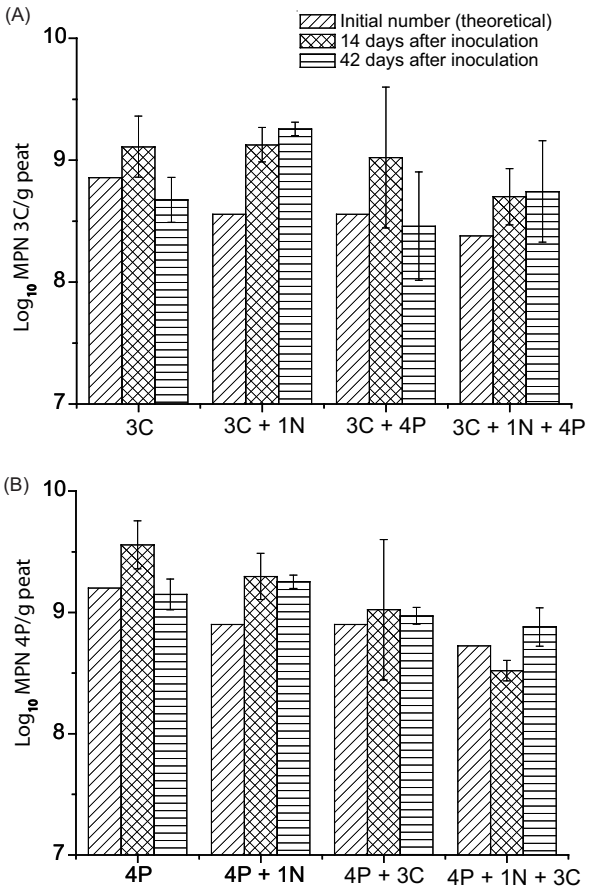


Figure 1. MPN of 3C (A) using phenol red media containing L-sorbose and 4P (B) using phenol red media containing α-methyl-D-glucoside from single and mixed peat cultures 14 days and 42 days after inoculation. Cultures were incubated at 30 ˚C for the first 14 days and then stored at 4 ˚C from day 15 to day 42. Error bars represent standard error of the mean of three replicates.

also found that cell numbers were underestimated when plants were grown in vermiculite compared with agar because of the discontinuity of the medium restricting growth of the organisms in the rhizosphere. Hence, one cell was not always able to produce a positive result. The results reported in this paper suggest close agreement between plate counts and MPN estimates using the multiple-tube method, indicating that errors due to poor distribution of cells or restriction of growth were not different between the two methods. It is, however, expected that the volume of both the inoculum and the carbohydrate media would be critical to the accuracy of this test.

The accuracy of the multiple-tube fermentation method increases with an increase in the number of tubes inoculated at each dilution level or by reducing the base of the dilution ratio. The inaccuracy of the method is often reflected in large confidence limits. Where calibration with another counting method is difficult, as in the case of multistrain inoculants, tests of internal coherency may be used as an indicator of accuracy (Scott and Porter 1986). In our experiments there was no difference in the number of dilution levels between all positive and all negative tubes when the technique was applied to pure or mixed cultures. This indicates that fermentation by the measured strain was not affected by the presence of other strains. However, the mean number of dilution levels in all cases was low, ranging from 1.33 to 2.33, possibly decreasing the sensitivity of internal coherency tests and resulting in large confidence limits. The accuracy of the MPN determination in this case may be improved by decreasing the base of the dilution ratio. There were no unusual codes detected, although this may be better determined with inoculation of more tubes at each dilution level.

Carbohydrate fermentation in media for the detection of cells was effective for the strains measured in these experiments as the positive result was very clear for 3C after 24 hours and only a few tubes were weakly positive for 4P. The weakly positive tubes all became strongly positive after 48 hours. Devriese and Van de Kerckhove (1980) found that the strength of the carbohydrate fermentation by *Staphylococcus* strains varied according to the media used. If alkali production from peptone or yeast extract is greater than acid production from carbohydrate, the acid reaction is not as clear. The authors also found that fermentation by staphylococci in phenol red media was weakly positive in carbohydrates glycerol, melezitose, ribose and turanose. Xylitol and salicin gave variable results with one strain. In our experiments fermentation by 5P was weakly positive when incubated with inuline, melezitose and xylitol. Positive reactions may be difficult to determine if they are weak and may be a result of alkali production. As the carbohydrates inuline and melezitose may be useful to estimate the MPN of 5P in combination with the other biofertiliser strains, reference tubes inoculated with bacteria but without carbohydrate would be useful in determining acid production. In the case of 4P, weakly positive tubes occurred only where inoculum potential was low, and positive results occurred after further growth.

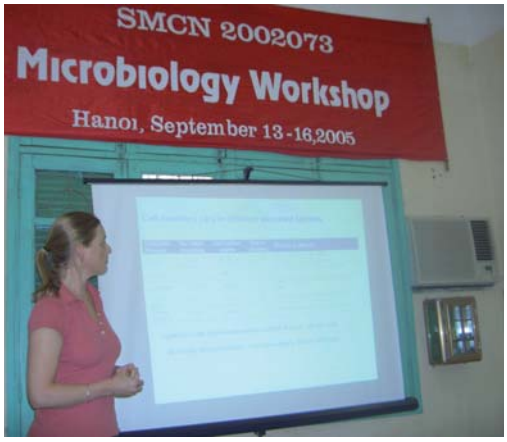
This method is useful when organisms can be distinguished by their carbohydrate utilisation. Simple fermentation media for fastidious species with oxidative fermentation (e.g. *Pseudomonas*) may be difficult to find, and other physiological properties may need to be detected to quantify cells. A modification combining multiple-tube fermentation with immunosorbent-based assays is currently being developed to improve specificity of the method.

Growth and survival in multistrain peat cultures

Growth of biofertiliser strains in peat cultures was dependent on the strain, strain combination and initial inoculum size. Despite differences in the initial inoculum size, there were no significant differences between total numbers of each measured strain after incubation in pure culture or when combined with other strains (with the exception of the numbers of 4P in the triple-strain inoculant after 14 days). The mean number of cells per gram of peat for each strain was close to 1×10^9, which is the minimum standard for peat-based legume inoculants at manufacture (Deaker et al. 2004); it is consistent with numbers of 4P determined by Nguyen et al. 2003 and 100-fold higher than the estimated number of 3C. These results suggest that it would be possible to prepare sterile peat-based multistrain biofertiliser inoculants using these biofertiliser strains mixed at the time of production rather than separating peat cultures until use. However, the ratio of application of each strain would not be the same as that used by Nguyen et al. 2003. It is also not known what minimum number of bacteria per seed/plant is required to have a positive effect on growth. It is assumed that the number of biofertiliser bacteria required for a positive effect will be higher than the number of rhizobia required for effective nodulation because of the non-symbi-

otic and probably less selective nature of the biofertiliser–plant associations.

The calculation of percentage recovery at each time allows measurement of the rate of growth of each strain in each culture condition. The growth rate varied with treatment but did not always reflect initial inoculum size or potential antagonism as expected. Both strains in pure culture grew in the first 14 days to two to three times the initial population size, and then decreased between 14 and 42 days to the original size. A similar pattern occurred with both strains when 3C and 4P were combined. When 1N was included either individually with each strain or in combination with both strains, the numbers of 3C and 4P continued to increase, indicating that 1N enhances growth of these strains. As the population of 1N in the peat cultures was not determined, it is not possible to know whether 1N provided a direct or indirect source of energy for additional growth of 3C and 4P. It may be that 1N modified conditions that better supported growth and persistence of 3C and 4P. While these results were not significant, there is consistency in the general pattern of growth of both strains when 1N was included, and the usefulness of 1N to enhance growth of biofertiliser strains in culture and in the rhizosphere should be further investigated.



Dr Rosalind Deaker provides instruction in simple tests for strain identification at the Hanoi microbiology workshop.

Measurement of growth rate indicated some antagonism between strains. In the presence of other strains, the initial growth rate of 4P may have been reduced but the growth rate of 3C was not. The initial rate of growth in multistrain cultures was more related to a lower inoculum size except when all three strains were combined. The results suggest that 3C may be a more competitive strain than 4P, which may be an advantage in growth in non-sterile peat and colonisation of the rhizosphere.

A workshop participant learns quality control procedures, Hanoi Unversity of Science, September 2005.

Conclusions

The multiple-tube fermentation method for estimating the MPN of different strains of bacteria may be useful for quality control of multistrain biofertiliser inoculants. The use of carbohydrate fermentation as a means of detecting specific strains is effective where fermentation of a carbohydrate is unique to a strain within a group. Estimation of the MPN using carbohydrate fermentation media in a multiple-tube test compares well with plate-counting techniques. The number of cells of biofertiliser strains 3C and 4P after growth in peat, either individually or in combination with other strains, was similar and close to the minimum standard of rhizobial numbers in legume inoculants. While there may be some antagonism between strains during growth, final numbers are generally similar. Inclusion of some strains (e.g. 1N) may enhance growth, which could have commercial significance. The results suggest that multistrain inoculants may be successfully produced at the point of manufacture, effectively reducing the volume of inoculant when compared to individually cultured strains. Multi-

strain inoculants would be economically advantageous by reducing shipping costs and application rates.

Acknowledgments

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References

Ben-dov E., Brenner A. and Kushmaro A. 2007. Quantification of sulfate-reducing bacteria in industrial wastewater by real-time polymerase chain reaction (PCR) using *dsrA* and *apsA* genes. Microbial Ecology 54, 439–451.

Brockwell J. 1963. Accuracy of a plant-infection technique for counting populations of *Rhizobium trifolii*. Applied Microbiology 11, 377–383.

Cochran W.G. 1950. Estimation of bacterial densities by means of the 'most probable number'. Biometrics 6, 105–116.

Deaker R., Roughley R.J. and Kennedy I.R. 2004. Legume seed inoculation technology – a review. Soil Biology and Biochemistry 36, 1275–1288.

Devriese L.A. and Van de Kerckhove A. 1980. Carbohydrate dissimilation tests in the identification of staphylococci. Antonie van Leeuwenhoek 46, 65–72.

Hierro N., Esteve-Zarzoso B., Mas A. and Guillamón J.M. 2007. Monitoring of *Saccharomyces* and *Hanseniaspora* populations during alcoholic fermentation by real-time quantitative PCR. FEMS Yeast Research 7, 1340–1349.

Hume D.J. and Blair D.H. 1992. Effect of numbers or *Bradyrhizobium japonicum* applied in commercial inoculants on soybean seed yield in Ontario. Canadian Journal of Microbiology 38, 588–593.

Ireland J.A. and Vincent J.M. 1968. A quantitative study of competition for nodule formation. Proceedings of the 9th International Congress of Soil Science. Transactions 2, 85–93.

Kennedy I.R. and Islam N. 2001. The current and potential contribution of asymbiotic nitrogen fixation to nitrogen requirements on farms: a review. Australian Journal of Experimental Agriculture 41, 447–457.

Marschner P., Crowley D.E. and Yang C.H. 2004. Development of specific rhizosphere bacterial communities in relation to plant species, nutrition and soil type. Plant and Soil 261, 199–208.

Mijajlovic G., Deaker R. and Kennedy I.R. 2007. Method for quantifying beneficial bacteria in the rhizosphere of crop plants. Proceedings Rhizosphere 2: Montpellier, France.

Nguyen Thanh Hien, Deaker R., Kennedy I.R. and Roughley R.J. 2003. The positive yield response of field-grown rice to inoculation with a multi-strain biofertiliser in the Hanoi area, Vietnam. Symbiosis 35, 231–245.

Nichols D. 2007. Cultivation gives context to the microbial ecologist. FEMS Microbiology Ecology 60, 351–357.

Reith F., Drake H.L. and Küsel K. 2002. Anaerobic activities of bacteria and fungi in moderately acidic conifer and deciduous leaf litter. FEMS Microbiology Ecology 41, 27–35.

Ritz K. 2007. The plate debate: Cultivable communities have no utility in contemporary environmental microbial ecology. FEMS Microbiology 60, 358–362.

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.J., Gemell L.G., Thompson J.A. and Brockwell J. 1993. The number of *Bradyrhizobium* sp. (*lupinus*) applied to seed and its effect on rhizosphere colonization, nodulation and yield of lupin. Soil Biology and Biochemistry 25, 1453–1458.

Scott J.M. and Porter F.E. 1986. An analysis of the accuracy of a plant infection technique for counting rhizobia. Soil Biology and Biochemistry 18, 355–362.

Shannon K.E., Lee D-Y., Trevors J.T. and Beaudette L.A. 2007. Application of real-time quantitative PCR for the detection of selected bacterial pathogens during municipal wastewater treatment. Science of the Total Environment 382, 121–129.

Smith E.A. and Macfarlane G.T. 1998. Enumeration of amino acid fermenting bacteria in the human large intestine: effects of pH and starch on peptide metabolism and dissimilation of amino acids. FEMS Microbiology Ecology 25, 355–368.

Identification and quality control of BioGro inoculant biofertiliser strains

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Abstract

In the characterisation of potential biofertiliser inoculant strains for quality control and regulatory purposes, identification is the first step. Strains of BioGro inoculant biofertiliser developed at the Hanoi University of Science were identified at the University of Sydney using a number of different techniques, including morphological, biochemical and genetic methods. Subsequently, the identity of the strains was also confirmed at the laboratories of the German Culture Collection of Microbes (DSMZ), Braunschweig, Germany. The strains comprising BioGro 1 were identified as *Pseudomonas fluorescens* (1N), *Citrobacter freundii* (3C) and *Klebsiella pneumoniae* (4P), whereas BioGro 2 consisted of *P. fluorescens* (1N), *Bacillus subtilis* (B9), *B. amyloliquefaciens* (E19) and an as-yet-unidentified yeast (HY). Polyclonal rabbit antibodies were produced for all strains in Ho Chi Minh City, Vietnam, and further characterised for sensitivity and specificity to individual biofertiliser strains. The antibodies raised against *C. freundii* (3C) were subsequently used to optimise a colony immunoblotting protocol, which was demonstrated as a valid technique for the quantification of this strain in pure cultures and sand with low numbers of indigenous micro-organisms. The colony immunoblotting method described in this paper is ready to be used as a quality control tool at the inoculant laboratory and factory level, where mixed microbial cultures need to be monitored. Initial trials using a DNA colony blotting method also showed promise as a viable option for following population dynamics of inoculant biofertiliser strains in soil.

Introduction

The application of beneficial microbes to enhance plant growth while reducing chemical fertiliser inputs has been growing over recent decades (Zahir et al. 2004). Quality control, which starts with their identification and requires feedback points throughout the production process, is necessary to ensure the successful commercialisation of biofertilisers worldwide. A lack of quality control has the potential to disrupt both production and marketing of inoculant biofertilisers. Technical difficulties related to microbe identification and quantification and the absence of regulations for biofertiliser production companies are major hurdles that need to be overcome. The purpose of this paper is to describe the identification and characterisation of microbial

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strains of BioGro, a successful inoculant biofertiliser in Vietnam, and the progress made in developing suitable methods for strain enumeration and quality control.

Material and methods

Strain identification

Strains were grown using nutrient agar (NA) or broth-modified NA media for 24–48 hours using methods described in the Bergey's manual. Semi-selective King's B growth medium was used to help preliminary identification of fluorescent pseudomonads. Specific amplification of the partial 16S rDNA analysis was carried out by crude extraction of bacterial DNA by heat denaturation at 100 ºC for 10 minutes, followed by centrifugal separation at 15,000 rpm for 30 seconds. Supernatant was used in subsequent polymerase chain reaction (PCR) cycling carried out as follows with the universal 16S rDNA primer pair—initial denaturation at 94 ºC for 2 minutes; 38 cycles of denaturation at 94 ºC for 30 seconds, annealing at 62 ºC for 30 seconds and extension at 72 ºC for 2 minutes; then final extension at 72 ºC for 5 minutes.

Antisera production

Polyclonal antibodies against all strains were raised in New Zealand White rabbits at the Post Harvest Technology Institute, Vietnam, with the exception of *C. freundii*, which was developed at the University of Sydney, Australia. Immunisation of the rabbits was performed using heat-killed cells of each strain, with two rabbits for each strain. The immunogen was diluted in 0.9% saline and emulsified in Freund's complete (first immunisation) or incomplete (subsequent immunisation) adjuvant. After an initial injection, a booster injection was given monthly for 3 months. A small amount of blood from each rabbit was collected to check for specific antibody production. A non-competitive, solid-phase enzyme immunoassay with an immobilised antigen was applied. The specific antibody of rabbits was detected with goat anti-rabbit immunoglobulin (IgG)-HRP conjugate. IgGs were purified from antiserum using protein A-Sepharose affinity chromatography provided by Sigma.

Enyzme-linked immunosorbent assay (ELISA) and immunoblotting reagents

Concentrated antibody stock (as prepared above) was stored in sealed plastic vials at –20 ºC. Working antibody solutions were prepared at concentrations as needed by diluting concentrated stock with phosphate buffer saline (PBS). PBS contained 8.7×10^{-3} M Na_2HPO_4, 1.7×10^{-3} M NaH_2PO_4 and 0.15 M NaCl, adjusted to pH 7.4. Washing solution contained 2 g/L Tween 20 in PBS. Blocking solution contained 50 g/L skim milk powder in washing solution. Horseradish peroxidise-conjugated swine anti-rabbit antibodies (HRP-conjugate) were purchased from Dako Australia Pty Ltd, stored at 4 °C and diluted to specified volumes with nanopure H_2O for working stock as necessary. HRP-substrate contained 2.5 g/L ß-cyclodextrin and 50 µL/L 30% H_2O_2 in 0.1 M sodium acetate, adjusted to pH 5 with acetic acid. Chromogen contained 10 mg/mL 3,3',5,5'-tetramethylbenzidine (TMB) in dimethyl sulfoxide, and was sealed and stored in an amber glass vial at room temperature.

ELISA characterisation of antibodies

Strains were grown aerobically for 24 hours in NA at 30 °C with shaking. The microbial cells were then harvested by centrifugation at 5,000 ×g for 20 minutes, washed twice in sterile 0.15% NaCl and resuspended in 0.05 M sodium carbonate buffer, pH 9.6. Subsequently, the absorbance values of the cell suspensions were measured at 600 nm and each suspension adjusted to the same level representing 10^8 cfu/mL as estimated by a set of McFarland $BaCl_2$ standards. Serial dilutions were made in carbonate buffer, and 150 µL of each was pipetted in 96-well plates in triplicate. After 2 hours' incubation at 37 °C, the plates were emptied and blocked with 150 µL of blocking solution for 1 hour at 37 °C, followed by emptying and plate washing twice with washing solution.

Antisera, diluted 1 in 2,000, were added to the wells (150 µL), incubated for 1 hour at 37 °C, and emptied and washed twice with washing solution. HRP-conjugate was diluted 1 in 1,000 in blocking solution, added to the wells (150 µL), incubated for 1.5 hours at 37 °C, and emptied and washed twice with washing solution and once with PBS. Colour was developed by the addition of 100 µL of freshly prepared substrate:chromogen mix and incubated at 37 °C for 20 minutes. Finally, colour development was stopped by the addition of 50 µL 1M H_2SO_4,

and the plates were read at 450 nm on a 96-well plate reader.

Immunoblotting method optimisation

Immunoblotting was carried out by following the method of Duez et al. (2000), and the method was optimised thereafter. Briefly, pure culture bacterial colonies were grown for 24 hours on NA plates as mock colonies from toothpick smears, or serially diluted colonies from broth cultures. The initial procedure involved taking colony blots by applying nylon membranes (5 cm diameter, Roche, Sydney, Australia) to plates for 30 minutes. The membranes were then removed from the plate and the blot was heat fixed at 80 °C for 30 minutes. After cooling, the membranes were incubated in freshly prepared blocking solution for 1 hour on a rocking shaker, and were then transferred into antibody solution (10 µg/mL) without drying, and incubated with shaking for 60 minutes at room temperature. Following washing three times in blocking solution for 5 minutes each time, the membranes were incubated in HRP-conjugate solution (0.75 µL/mL of commercial stock in blocking solution) for 1 hour. Three more 5-minute wash steps followed, twice with washing solution and once with PBS, before colour was developed by incubation with HRP-substrate:chromogen (95:5) mix solution for 5 minutes. Finally, the background was decoloured with 1M H_2SO_4 and the membranes were rinsed with distilled water.

This procedure was optimised in a number of experiments by varying solution concentrations and incubation times, to increase sensitivity and reduce reagent volumes and reaction times. Parameters were optimised individually on triplicates by visually assessing the colour intensity and blot resolution.

Colony hybridisation

Colony hybridisation was performed using commercially available chemicals. DNA oligonucleotide probes were synthesised at GeneWorks, Adelaide, South Australia. Labelling of probes was prepared by using Roche Diagnostics' second generation DIG oligonucleotide 3'-end labelling kit.

Inoculant recovery experiment

Plastic pots of 6-inch diameter were filled with sterilised sand (autoclaved for 30 minutes at 121 ºC) or non-sterile sand and placed inside a glasshouse. Each pot was planted with five pre-germinated wheat seeds (variety 'Dollarbird') using sterile water to moisten the soil. Two days later seedlings were inoculated by infiltrating the root zones with 5 mL of a 24-hour liquid nutrient culture of 3C containing 10^8 cfu/mL. The suspension was prepared by picking a colony of 3C from NA into a conical flask containing 250 mL of sterilised modified nutrient broth. The wheat was grown for 6 weeks before an attempt was made to recover and estimate the density of surviving inoculum. Approximately 1 g of rhizosphere soil adhering to roots was collected, weighed and suspended in 100 mL sterile water. The suspension was shaken using an orbital shaker (Ika Labortechnik, Denmark) at 200 rotations per minute, followed by dilution plating and subsequent incubation at 32 °C for 24 hours. Colonies were then identified and, at the same time, counted using the immunoblotting technique described above.

In another experiment a non-sterile sandy loam soil (pH 5.36, organic matter 5.1%, sand 60%, silt 26%, clay 14%) from Camden, New South Wales, Australia, was potted without plants and infiltrated with 3C (5 mL of 10^8 cfu/mL). Each pot was monitored weekly for 5 weeks in the greenhouse and, again, immunoblotting was used to follow the fate of the *C. freundii*.

Results

The identity of BioGro strains

Preliminary identification of the microbial strains in both BioGro 1 and BioGro 2 confirmed the presence of three Gram-negative bacteria (1N, 3C and 4P), two Gram-positive bacteria (B9 and E19) and a yeast species (HY). Initial biochemical studies of 3C and 4P indicated that they belong to the family Enterobacteriaceae, while 1N was classified as a fluorescent pseudomonad. Further biochemical studies of bacterial strains using commercial API® tests (bioMérieux) combined with sequencing of 16S rDNA gave good agreement of strain identity (Table 1). Independent studies by the German Collection of Micro-organisms and Cell Cultures (DSMZ), Braunschweig, Germany, or the University of

Western Sydney confirmed our results. DSMZ is currently carrying out identification for HY, the preliminary unidentified yeast in BioGro 2.

Antisera production and characterisation

Antisera were successfully produced for all strains. Initial ELISA testing showed varying sensitivities and specificities for each antiserum; Figure 1 is given as an example. From these results, one antiserum was selected for each strain for further characterisation. All antisera showed limited cross-reactivity with the other biofertiliser strains tested with the exception of both antisera raised against the *Bacillus* strains, which were cross-reactive with cells of each other (Figure 2).

Optimisation of immunoblotting conditions

The establishment of the immunoblotting procedure was based on the work of Duez et al. (2000). After optimisation the original fixation time and antibody concentration were reduced, the concentration and incubation of the chromogen were increased, and the incubation time with the antibody was left unchanged (Figure 3; Table 2). The optimised method was then used for identification of 3C in inoculant recovery experiments.

Table 1. Identity of BioGro biofertiliser strains

Strain	Biochemical and 16S rDNA analysis (University of Sydney)	Independent confirmation
4P	*Klebsiella pneumoniae*	*Klebsiella pneumoniae* [a]
3C	*Citrobacter freundii*	*Citrobacter freundii* [b]
1N	*Pseudomonas fluorescens*	*Pseudomonas fluorescens* [b]
E19	*Bacillus licheniformis*	*Bacillus amyloliquefaciens* [a]
B9	*Bacillus subtilis*	*Bacillus subtilis* [a]
HY	Yeast [c]	

[a] DSMZ German Collection of Micro-organisms and Cell Cultures
[b] University of Western Sydney
[c] No confirmation to species level available

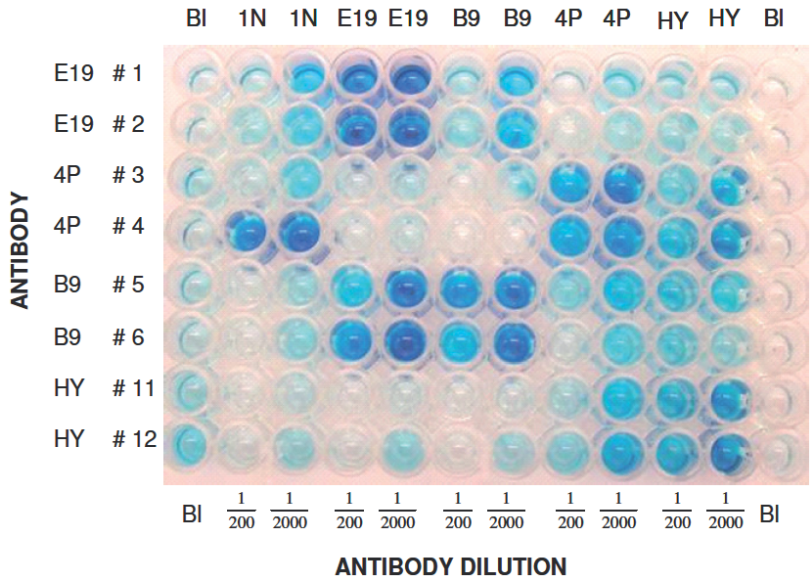

Figure 1. Preliminary antisera screening. Dark blue indicates a positive binding reaction between the antibodies and cells.

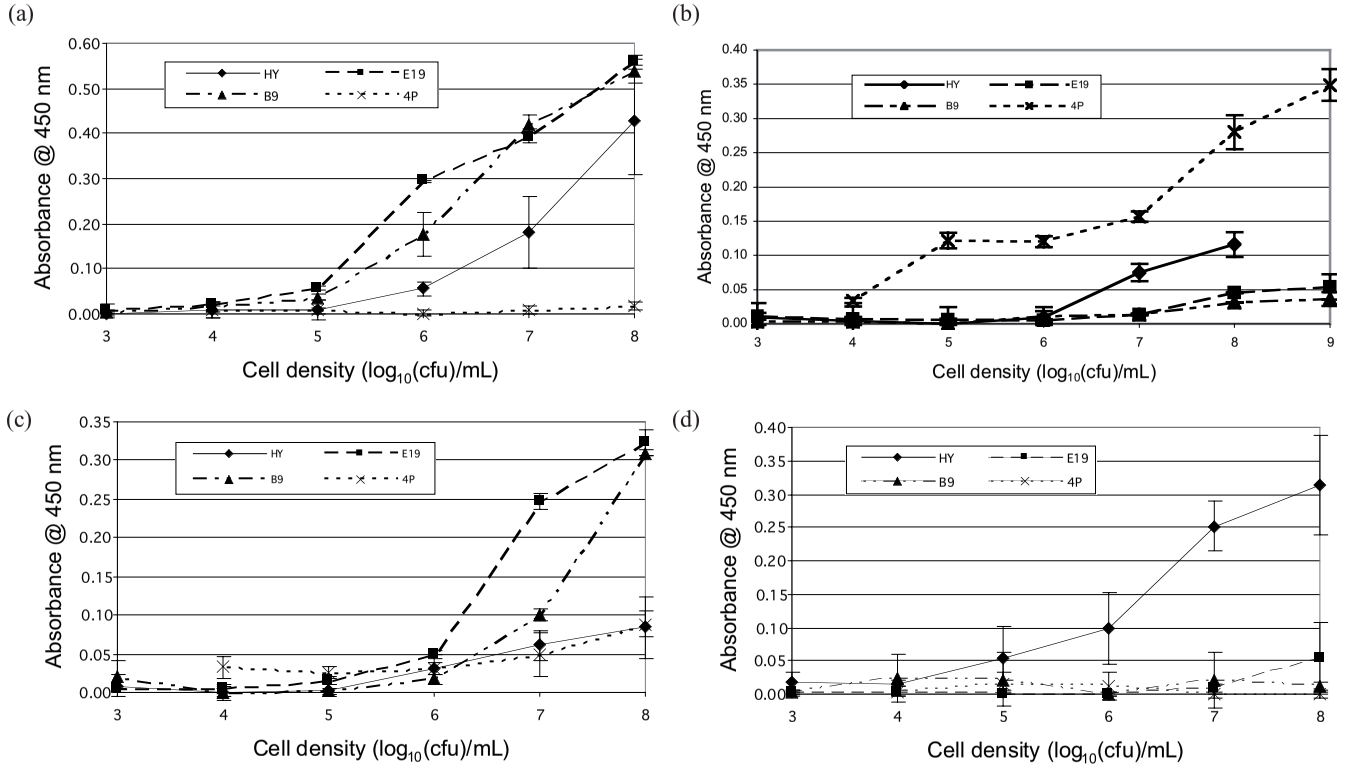

Figure 2. Sensitivity and specificity of antisera from rabbit #2 injected with E19 (a), rabbit #3 injected with 4P (b), rabbit #5 injected with B9 (c) and rabbit #11 injected with HY (d)

The final step of the immunoblotting procedure was also modified to enhance the uniformity of decolourisation and colony blot visualisation. This involved using a perfume spray bottle (Sydney Essential Oil Co Pty Ltd, catalogue number PGAB73-000, 018CPWM) to apply H_2SO_4 to the membrane, providing much finer droplet sizes and even coverage, thus avoiding smearing of the chromogen and giving greater resolution of blots (Figure 3).

Monitoring inoculant in biofertiliser carriers and soil by immunoblotting

Both sterile and non-sterile sand samples contained a low background microbial population when counted on NA dilution plates of sand samples. Immunoblotting performed on inoculated samples after 5 weeks of seedling growth was effective for counting and identification of surviving *C. freundii*

Table 2. Optimisation of the immunoblotting protocol

Parameter	Original protocol	Improved protocol
Fixation time	30 minutes	15 minutes
Antibody incubation	1 hour at room temperature	1 hour at room temperature or more at 4 °C
Antibody (IgG) concentration	10 µg/mL	5 µg/mL
HRP incubation	1 hour	1 hour or more
Chromogen concentration	3%	5%
Chromogen incubation	5 minutes	≤ 5 minutes

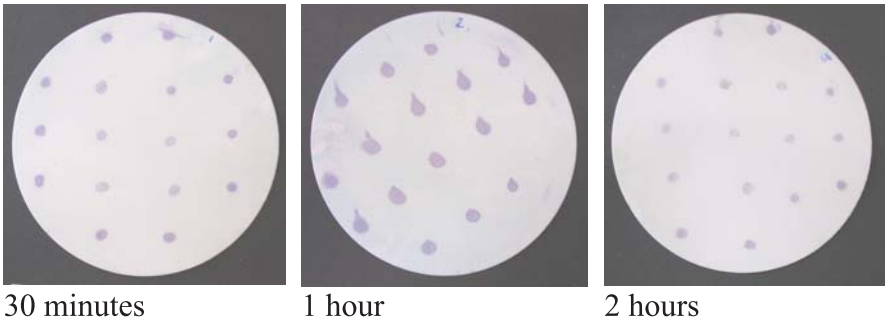

Figure 3. Comparison of the effect of different incubation times (0.5, 1 and 2 hours) on immunoblots of *C. freundii* using anti-*C. freundii* polyclonal antibody

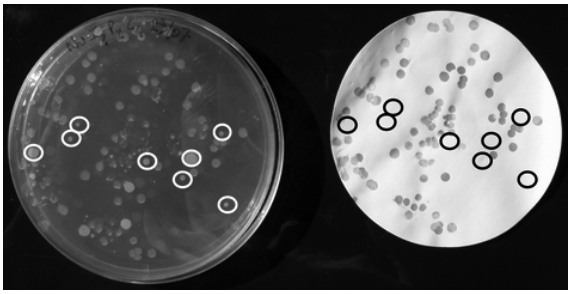

Figure 4. Dilution plate (left) and subsequent immunoblot (right) carried out with anti-*C. freundii* antibody from a soil sample inoculated by *C. freundii* (dark dots). Empty white circles mark some of the strain colonies of the background population shown to be missing by the empty black circles on the immunoblot.

(Figure 4). Our results also indicated that, at higher to equal inoculant fertiliser:background-population ratios, it was possible to determine the identity and number of strains within a few hours. We have successfully applied the technique to counting mixed cultures in peat, as used for commercial Australian rhizobial cultures, and a more detailed study for such inoculant biofertilisers will be reported later.

In soil treatments changes both in total microbes and *C. freundii* populations were observed over the 5-week period (Figure 5). The background concentration of indigenous micro-organisms increased directly after inoculation of *C. freundii* culture but declined uniformly after the second sample taken at 7 days. In contrast, the concentration of *C. freundii* did not follow a consistent trend, with an increase in concentration until week 2 to nearly 10^8 cfu/g soil, followed by a decline to approximately 2×10^6 cfu/g, where it remained stable for the next week. The densities of *C. freundii* and the background microbial population were only significantly different after 4 weeks, when *C. freundii* numbers were less.

Discussion

The microbial species making up both BioGro products belong to genera that have been shown to promote plant growth elsewhere. Representative strains from *Pseudomonas*, *Bacillus*, *Klebsiella* and *Citrobacter* have been isolated that can fix nitrogen in association with plants (Dobbelaere et al. 2003; Dong et al. 2003; Xie et al. 2003), while numerous *Pseudomonas, Bacillus* and *Klebsiella* isolates can also mobilise inorganic phosphates (Toro et al. 1997; Chung et al. 2005; Hoberg et al. 2005; Alikhani et al. 2006; Young et al. 2006). Moreover, both the pseudomonads (including *P. fluorescens*) and bacilli (including *B. subtilis* and *B. amyloliquefaciens*) have been well studied for their ability to produce various antibiotic and antiparasitic compounds that can decrease pathogen densities and favour plant growth, in particular polyketides by the former (Bender et al. 1999) and lipopeptides by the latter (Gardener and Driks 2004; Koumoutsi et al. 2004). Both bacterial groups can also induce systemic resistance in crop plants (Kloepper et al. 2004). Together, it is an encouraging result that the group of organisms selected for inclusion in the BioGro product by Professor Hien represent common plant growth promoting micro-organisms that have been isolated and studied worldwide. This partly validates the isolation procedure used by the Vietnamese scientists and provides substantial background knowledge for further development.

It is also encouraging that the polyclonal antisera raised against all strains were effective in their binding sensitivity and, at minimum, were genus specific. All antisera had threshold sensitivities of

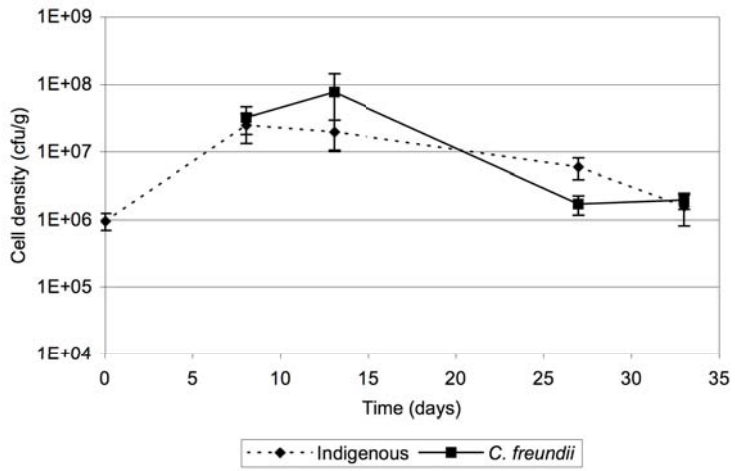

Figure 5. The population of *C. freundii* in the rhizosphere of wheat grown in soil pots compared with indigenous micro-organisms. Error bars represent 95% confidence levels ($n \geq 3$).

approximately 10^6 cfu/mL, with the exception of one antiserum raised against B9 (#5), which was only sensitive to B9 at densities of 10^7 cfu/mL. Some highly sensitive polyclonal antibodies have been raised that can quantify cell densities of specific micro-organisms in environmental samples to as low as 10^4 cfu/mL or 10^5 cfu/mL in an ELISA format (Brigmon et al. 1998; Vagelas et al. 2007); however, thresholds of 10^6 cfu/mL will limit the usefulness of our antisera in such applications. Nevertheless, this sensitivity is sufficient for immunoblotting methods in which samples are first grown on plates overnight to produce colonies of concentrated cells.

More problematic are the cross-reactions observed between the *Bacillus* antibodies and cells. Cross-reactions may occur for polyclonal antibodies raised against whole cells rather than specific epitopes (e.g., see Brigmon et al. (1998)), and they will hinder the use of immuno-methods for the quality control of peat biofertilisers after mixing different *Bacillus* cultures. Cross-reactivity can be reduced by the adsorption of non-specific antibodies onto cross-reacting cells (e.g., see Jin et al. (2003)), but the sensitivity of the purified specific antibodies will need to be verified as sufficient. An alternative is to use the raw polyclonal antibodies as a first-stage, genus-specific selection followed by a secondary, species-differential test such as colony morphology, nutritional selection or DNA oligoprobing. Potential methods like these are currently being evaluated at the SUNFix Centre, University of Sydney.

Meanwhile, for the remaining biofertiliser strains, immunoblotting using the polyclonal antibodies described above is a promising method for their enumeration and quality control, as demonstrated by the experiments using *C. freundii* (3C). These rhizosphere inoculation experiments also highlighted the potential for further applications of this method in more complex environmental media and demonstrated the good survival of 3C in the rice rhizosphere.

Importantly, optimisation of the immunoblotting protocol achieved a reduction in the time and volume of reagents necessary for successful visualisation and counting. This is crucial for the success of such methods, where high cost can prevent their uptake and application in developing countries. Research is already being undertaken to further reduce the cost of the immunoblotting method, in particular by finding more economic alternatives to the relatively expensive nylon membranes.

Conclusions

The micro-organisms comprising BioGro fertiliser were identified as *Klebsiella pneumoniae* (strain 4P), *Citrobacter freundii* (strain 3C), *Pseudomonas fluorescens* (strain 1N), *Bacillus subtilis* (strain B9), *Bacillus amyloliquefaciens* (strain E19) and a yeast (strain HY). All strains belong to genera recognised as plant growth promoters. Sensitive polyclonal antibodies were raised to all strains and were demonstrated to be effective for use in strain quantification by an immunoblotting method. The present technique can already be applied as a quality control method at the factory level, where mixed cultures need to be monitored as individual strains.

Acknowledgments

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References

Alikhani H.A., Saleh-Rastin N. and Antoun H. 2006. Phosphate solubilisation activity of rhizobia native to Iranian soils. Plant and Soil 287, 35–41.

Bender C.L., Rangaswamy V. and Loper J. 1999. Polyketide production by plant-associated pseudomonads. Annual Review of Phytopathology 37, 175–196.

Brigmon R.L., Franck M.M., Bray J.S., Scott D.F., Lanclos K.D. and Fliermans C.B. 1998. Direct immunofluorescence and enzyme-linked immunosorbent assays for evaluating organic contaminant degrading bacteria. Journal of Microbiological Methods 32, 1–10.

Chung H., Park M., Madhaiyan M., Seshadri S., Song J., Cho H. and Sa T. 2005. Isolation and characterization of phosphate solubilizing bacteria from the rhizosphere of crop plants of Korea. Soil Biology and Biochemistry 37, 1970–1974.

Dobbelaere S., Vanderleyden J. and Okon Y. 2003. Plant growth-promoting effects of diazotrophs in the rhizosphere. Critical Reviews in Plant Sciences 22, 107–149.

Dong Y.M., Iniguez A.L. and Triplett E.W. 2003. Quantitative assessments of the host range and strain specificity of endophytic colonization by *Klebsiella pneumoniae* 342. Plant and Soil 257, 49–59.

Duez H., Pelletier C., Cools S., Aissi E., Cayuela C., Gavini F., Bouquelet S., Neut, C. and Mengaud J. 2000. A colony immunoblotting method for quantitative detection of a *Bifidobacterium animalis* probiotic strain in human faeces. Journal of Applied Microbiology 88, 1019–1027.

Gardener B.B.M. and Driks, A. 2004. Overview of the nature and application of biocontrol microbes: *Bacillus* spp. Phytopathology 94, 1244–1244.

Hoberg E., Marschner P. and Lieberei R. 2005. Organic acid exudation and pH changes by *Gordonia* sp. and *Pseudomonas fluorescens* grown with P adsorbed to goethite. Microbiological Research 160, 177–187.

Jin J.F., Yuan L.D., Liu L., Zhao Z.J. and Xie W. 2003. Preparation and characterization of polyclonal antibodies against ARL-1 protein. World Journal of Gastroenterology 9, 1455–1459.

Kloepper J.W., Ryu C.M. and Zhang S.A. 2004. Induced systemic resistance and promotion of plant growth by *Bacillus* spp. Phytopathology 94, 1259–1266.

Koumoutsi A., Chen X.H., Henne A., Liesegang H., Hitzeroth G., Franke P., Vater J. and Borriss R. 2004. Structural and functional characterization of gene clusters directing nonribosomal synthesis of bioactive cyclic lipopeptides in *Bacillus amyloliquefaciens* strain FZB42. Journal of Bacteriology 186, 1084–1096.

Toro M., Azcon R. and Barea J.M. 1997. Improvement of arbuscular mycorrhiza development by inoculation of soil with phosphate-solubilizing rhizobacteria to improve rock phosphate bioavailability (P-32) and nutrient cycling. Applied and Environmental Microbiology 63, 4408–4412.

Vagelas I.K., Pembroke B., Gowen S.R. and Davies K.G. 2007. The control of root-knot nematodes (*Meloidogyne* spp.) by *Pseudomonas oryzihabitans* and its immunological detection on tomato roots. Nematology 9, 363–370.

Xie G.H., Cai M.Y. and Tao G.C. and Steinberger Y. 2003. Cultivable heterotrophic N-2-fixing bacterial diversity in rice fields in the Yangtze River Plain. Biology and Fertility of Soils 37, 29–38.

Young C.C., Rekha P., Lai W.A. and Arun A.B. 2006. Encapsulation of plant growth-promoting bacteria in alginate beads enriched with humic acid. Biotechnology and Bioengineering 95, 76–83.

Zahir Z.A., Arshad M. and Frankenberger W.T. 2004. Plant growth promoting rhizobacteria: Applications and perspectives in agriculture. Advances in Agronomy 81, 97–168.

Biofilmed biofertilisers: Novel inoculants for efficient nutrient use in plants

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Abstract

Microbial communities attached to surfaces, or biofilms, are found in many environments, including the soil. This chapter describes the potential applications of developed biofilms as biofertilisers in crop production. Formation of fungal–bacterial biofilms (FBBs) by bacterial colonisation on biotic fungal surfaces gives the biofilms enhanced metabolic activities compared to monocultures. Incorporation of a nitrogen (N_2)-fixing rhizobial strain to FBBs to form fungal–rhizobial biofilms (FRBs) has been shown to improve potential biofilm applications in N-deficient settings and in the production of biofilmed inocula for biofertilisers and biocontrol in plants. A developed biofilmed inoculant of the FRB significantly increased N_2 fixation in soybean by ca. 30% compared to a conventional inoculant of rhizobium alone (monoculture inocula). The FBB and FRB increased biomass in early growth of rice by ca. 25% compared to the conventional inocula. Root colonisation of wheat by FRBs forming 'pseudonodules' was also observed recently. The FRBs increased N and phosphorus (P) availabilities when inoculated directly to the soil. They also improved P biosolubilisation from rock phosphate. The FBBs of beneficial endophytes produced higher acidity and plant growth promoting hormones than their mono- or mixed cultures with no biofilm formation. This indicates that the highest microbial effect may not be achieved by plant inoculation with the conventional inocula of effective microbes, but only by biofilmed inocula.

Introduction

Certain microbes can attach to biotic or abiotic surfaces and differentiate to form complex, multicellular communities called biofilms. A biofilm consists of microbial cells (algal, fungal, bacterial and/or other microbial) plus an extracellular biopolymer (known as an extracellular polymeric substance (EPS)) produced by the cells which provides structure and protection to the community. These communities can be found in medical, industrial and natural environments. They can also be engineered in vitro for various biotechnological applications (Seneviratne 2003; Seneviratne et al. 2007). Microbes undergo profound changes during their transition from planktonic (free swimming) organisms to cells that are part of a complex, surface-attached biofilm. Genes and regulatory circuits important for initial cell–surface interactions, biofilm maturation, and the return of biofilm microorganisms to a planktonic mode of growth have been identified (O'Toole et al. 2000). Biofilms have a unique pattern of gene expression that is different from their non-biofilm-forming stages (Vilain and Brözel 2006).

The distinctiveness of the action of beneficial biofilms developed in vitro has already shown potential for bringing numerous favourable effects to microbial biotechnological applications (Seneviratne et al. 2007). Their use in agricultural and environmental

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settings, enzyme technology, drug discovery studies and green energy research are being investigated. In this chapter the adoption of biofilms as biofertilisers is discussed with the aid of preliminary demonstrations which showed their promising applications as 'next generation biofertilisers' in the future.

Microbial biofilms in the soil

There are three major types of biofilms that can occur in the soil—bacterial (including *Actinomycetes*), fungal and FBBs. The bacterial and fungal biofilms are formed on abiotic surfaces in the soil. In FBBs fungi act as the biotic surface to which the bacteria adhere (Figure 1). In the case of non-filamentous fungi, both bacteria and fungi can act as the biotic surface. Formation of FBBs by bacterial colonisation on biotic fungal surfaces gives the biofilm enhanced metabolic activities compared to monocultures, and perhaps also to multispecies bacterial or fungal biofilms on abiotic surfaces (Seneviratne et al. 2007). Such microbial associations between bacteria and mycorrhizal fungi have been observed to occur naturally in the soil (Artursson and Jansson 2003), promoting mycorrhizal symbiosis (Frey-Klett et al. 2007). Biofilms attached to the plant roots of some crops help in the cycling of nutrients as well as the biocontrol of pests and diseases, resulting in improved agricultural productivity (Seneviratne 2003). However, because the density of biofilms in the soil is not adequate to give maximum beneficial effects, biofilms developed in vitro should be applied as inocula to increase plant growth.

Biofilmed biofertilisers

With the first in-vitro development and observation of interactions between common non-mycorrhizal soil fungi and rhizobia, forming FRBs (Seneviratne and Jayasinghearachchi 2003), a series of studies was conducted to demonstrate their potential applications for various purposes. It was observed that FRBs fixed nitrogen (N_2) biologically, as revealed by nitrogenase activity and N accumulation, in contrast to when a rhizobial strain was grown as a monoculture (Jayasinghearachchi and Seneviratne 2004a). The rhizobial strain used here was *Bradyrhizobium elkanii* SEMIA 5019, a soybean-nodulating strain with a high N_2-fixing capability.

Application of a developed biofilmed inoculant of the FRB can significantly increase N_2 fixation in soybean (by ca. 30%) compared to a rhizobium-only (conventional) inoculant (Jayasinghearachchi and Seneviratne 2004b). Another recent study showed that the contribution of developed biofilmed inocula in enhanced release of organic acids and plant growth promoting (PGP) substances led to a ca. 25% increase in plant dry weight in early growth of rice compared to conventional monocultured inocula (M.L.M.A.W. Weerasekara, unpublished). Co-inoculation of PGP rhizobacteria and arbuscular mycorrhizal fungi in rainfed wheat fields produced the highest protein contents of grains compared to their monocultures (Roesti et al. 2006). Initial observations of FRB formation on wheat roots by a biofilmed inoculant were made recently (Figure 2; G. Seneviratne et al., unpublished).

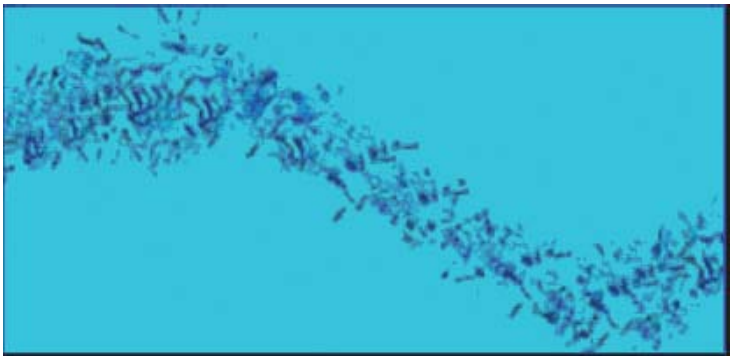

Figure 1. A phase-contrast microscopic view of a fungal filament attached by bacterial cells forming a fungal-bacterial biofilm (FBB). Magnification: 2,000×.

FRBs may act as 'pseudonodules', fixing N_2 biologically on the roots of non-legumes. In a review by Bashan (1998) on microbial inocula in agriculture, mixed inoculation with arbuscular-mycorrhizal fungi and diazotrophic bacteria has been reported to generate synergistic interactions. Possible consequences of this synergism include significant increase in growth and in the P content of the plants, enhanced mycorrhizal infection and an improvement in the uptake of nutrients such as P, N, zinc, copper and iron. These inocula stimulate plant growth through a range of mechanisms that improve nutrient acquisition and inhibition of fungal plant pathogens (Biró et al. 2000; Artursson et al. 2006; Toljander et al. 2006). When applied directly to the soil, FRBs increased N and P availabilities by ca. 2-fold and 15-fold, respectively, and showed a high nitrogenase activity, even under a very high soil NO_3^- concentration, compared to the monocultures (Seneviratne and Jayasinghearachchi 2005).

Figure 2. A fungal–rhizobial biofilm (FRB) on a wheat root. The FRB may act as a 'pseudonodule', fixing N_2 biologically on the roots of this non-legume.

The biofilmed inocula can be effectively used in biosolubilisation of rock phosphate. This was demonstrated by developing biofilms from *Penicillium* spp., *Pleurotus ostreatus* and *Xanthoparmelia mexicana*, a lichen fungus which increased the P solubilisation up to ca. 230% compared to the fungus-only cultures (Jayasinghearachchi and Seneviratne 2006a; Seneviratne and Indrasena 2006). Furthermore, the biofilmed inocula can also be used for successful establishment of introduced beneficial micro-organisms in plants for biocontrol of diseases etc. This was confirmed in vitro by a *Pleurotus ostreatus – Pseudomonas fluorescens* biofilm that increased endophytic colonisation of tomato by *P. fluorescens*, a biocontrolling agent, by over 1,000% compared to inoculation with *P. fluorescens* alone (Jayasinghearachchi and Seneviratne 2006b). As such, diverse forms of the biofilmed inocula may satisfy the future demand of augmented crop productivity with increased N_2 fixation, nutrient uptake and biocontrol of diseases.

FBBs of beneficial endophytes were observed to produce higher acidity and PGP hormones than their mono- or mixed cultures with no biofilm formation (Bandara et al. 2006). The higher acidity is generally important for pathogen suppression. Further, there was a significant negative relationship between pH and the production of indoleacetic-acid-like substances (IAAS) in liquid culture media of FBBs, but not in mixed cultures with no biofilm formation of a large collection of microbes (Figure 3). Thus, when biofilms are formed, high acidity reflects high IAAS production. As such, the highest microbial effect may not be achieved by the conventional practice of plant inoculation with monocultures or mixed cultures of effective microbes, but rather by biofilmed inocula.

In conventional inoculant technology of microbial monocultures, a major problem yet to be addressed is the poor survival of introduced micro-organisms in the soil due to various environmental stress factors. Biofilmed inocula were observed to help their rhizobia survive at high salinity (400 mM NaCl) and tannin concentrations (0.4 mM tannic acid) by 10^5-fold and 12-fold, respectively, compared to rhizobial monocultures (Seneviratne et al. 2007). Their higher tolerance than the monocultures for low pH, chromium and predation by earthworms was also noted. It has been reported that the formation of microcolonies and the production of toxins are effective mechanisms that may allow bacterial biofilms (e.g. *Pseudomonas aeruginosa*) to resist protozoan grazing and so persist in the environment (Matz et al. 2004). Similar observations of Burmolle et al. (2006) revealed that, in multispecies biofilms, the synergistic interactions cause an enhancement of biofilm formation and increased resistance to antimicrobial agents. Bacterial cells are protected from antimicrobial agents in biofilms through the formation of persister cells—a highly protected state adopted by a small fraction of the outermost cells of a biofilm (Roberts and Stewart 2005).

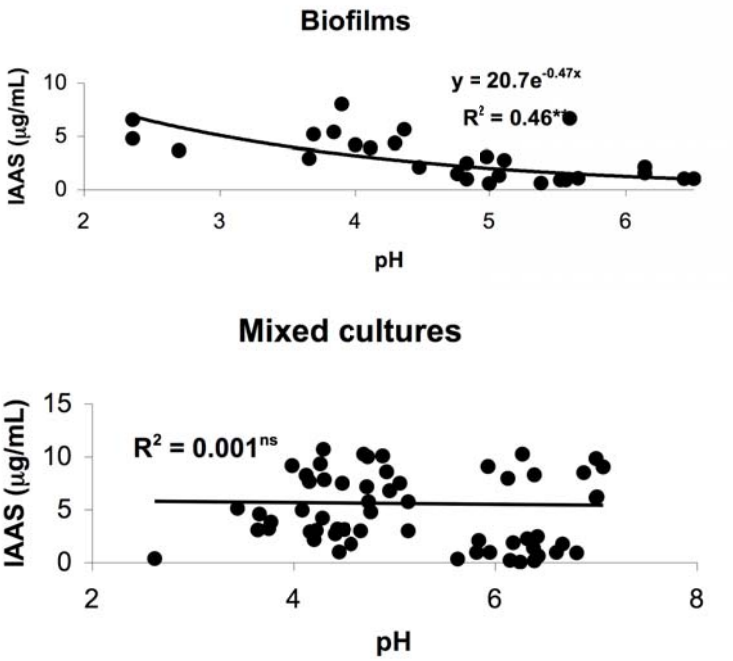

Figure 3. Relationships between pH and indoleacetic-acid-like substances (IAAS) production in liquid culture media by fungal–bacterial biofilms or mixed cultures with no biofilm formation of a large collection of microbes.

Conclusions and future research needs

Studies reported here have shown that FBBs/FRBs are more effective in their biological performance than mono- or mixed microbial cultures, and perhaps also multispecies bacterial or fungal biofilms on abiotic surfaces. The soil application of FRBs as biofilmed inocula appears to be important if soil fertility is to be sustained in nutrient-depleted lands, and survival of rhizobia is to be improved in the soil in the absence of their hosts. However, applications of this biotechnology are scarce because it is still understudied. Selection of combinations of microbes for highest efficiency, simultaneous biofertilising and biocontrolling activities is a key in future research in this technology. Thus, more research should be done under laboratory and field conditions in order to optimise biofilmed inocula for various crops.

References

Artursson V., Finlay R.D. and Jansson J.K. 2006. Interactions between arbuscular mycorrhizal fungi and bacteria and their potential for stimulating plant growth. Environmental Microbiology 8, 1–10.

Artursson V. and Jansson J.K. 2003. Use of bromodeoxyuridine immunocapture to identify active bacteria associated with arbuscular mycorrhizal hyphae. Applied and Environmental Microbiology 69, 6208–6215.

Bandara W.M.M.S., Seneviratne G. and Kulasooriya S.A. 2006. Interactions among endophytic bacteria and fungi: Effects and potentials. Journal of Biosciences 31, 645–650.

Bashan Y. 1998. Inoculants of plant growth-promoting bacteria for use in agriculture. Biotechnology Advances 16, 729–770.

Biró B., Köves-Péchy K., Vörös I., Takács T., Eggenberger P. and Strasser R.J. 2000. Interrelations between *Azospirillum* and *Rhizobium* nitrogen-fixers and arbuscular mycorrhizal fungi in the rhizosphere of alfalfa in sterile, AMF-free or normal soil conditions. Applied Soil Ecology 15, 159–168.

Burmolle M., Webb J.S., Rao D. Hansen L.H., Sørensen S.J. and Kjelleberg S. 2006. Enhanced biofilm formation and increased resistance to antimicrobial agents and bacterial invasion are caused by synergistic interactions in multispecies biofilms. Applied and Environmental Microbiology 72, 3916–3923.

Frey-Klett P., Garbaye J. and Tarkka M. 2007. The mycorrhiza helper bacteria revisited. New Phytologist 176, 22–36.

Jayasinghearachchi H.S. and Seneviratne G. 2004a. Can mushrooms fix atmospheric nitrogen? Journal of Biosciences 23, 293–296.

Jayasinghearachchi H.S. and Seneviratne G. 2004b. A bradyrhizobial-*Penicillium* spp. biofilm with nitrogenase activity improves N_2 fixing symbiosis of soybean. Biology and Fertility of Soils 40, 432–434.

Jayasinghearachchi H.S. and Seneviratne G. 2006a. Fungal solubilization of rock phosphate is enhanced by forming fungal-rhizobia biofilms. Soil Biology and Biochemistry 38, 405–408.

Jayasinghearachchi H.S. and Seneviratne G. 2006b. A mushroom-fungus helps improve endophytic colonization of tomato by *Pseudomonas fluorescens* through biofilm formation. Research Journal of Microbiology 1, 83–89.

Matz C., Bergfeld T., Rice S.A. and Kjelleberg S. 2004. Microcolonies, quorum sensing and cytotoxicity determine the survival of *Pseudomonas aeruginosa* biofilms exposed to protozoan grazing. Environmental Microbiology 6, 218–226.

O'Toole G., Kaplan H.B. and Kolter R. 2000. Biofilm formation as microbial development. Annual Review in Microbiology 54, 49–79.

Roberts M.E. and Stewart P.S. 2005. Modelling protection from antimicrobial agents in biofilms through the formation of persister cells. Microbiology 51, 75–80.

Roesti D., Gaur R., Johri B.N., Imfeld G., Sharma S., Kawaljeet K. and Aragno M. 2006. Plant growth stage, fertilizer management and bio-inoculation of arbuscular mycorrhizal fungi and plant growth promoting rhizobacteria affect the rhizobacterial community structure in rain-fed wheat fields. Soil Biology and Biochemistry 38, 1111–1120.

Seneviratne G. 2003. Development of eco-friendly, beneficial microbial biofilms. Current Science 85, 1395–1396.

Seneviratne G. and Indrasena I.K. 2006. Nitrogen fixation in lichens is important for improved rock weathering. Journal of Biosciences 31, 639–643.

Seneviratne G. and Jayasinghearachchi H.S. 2003. Mycelial colonization by bradyrhizobia and azorhizobia. Journal of Biosciences 28, 243–247.

Seneviratne G. and Jayasinghearachchi H.S. 2005. A rhizobial biofilm with nitrogenase activity alters nutrient availability in a soil. Soil Biology and Biochemistry 37, 1975–1978.

Seneviratne G., Zavahir J.S., Bandara W.M.M.S. and Weerasekara M.L.M.A.W. 2007. Fungal-bacterial biofilms: their development for novel biotechnological applications. World Journal of Microbiology and Biotechnology 24(6), 739–743.

Toljander J.F., Artursson V., Paul L.R., Jansson J.K. and Finlay R.D. 2006. Attachment of different soil bacteria to arbuscular mycorrhizal fungal extraradical hyphae is determined by hyphal vitality and fungal species. FEMS Microbiology Letters 254, 34–40.

Vilain S. and Brözel V.S. 2006. Multivariate approach to comparing whole-cell proteomes of *Bacillus cereus* indicates a biofilm specific proteome. Journal of Proteome Research 5, 1924–1930.

Future perspectives for biofertilisers: An emerging industry needing a scientific approach

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Abstract

The occurrence of peak oil supply and the resulting increase in urea prices provide ideal socioeconomic conditions for the adoption of new technology able to improve the efficiency of nitrogen utilisation by crops like rice and wheat. The application of biofertilisers containing catalytic amounts of inoculant micro-organisms, such as the product BioGro studied in this project, represents one possible means of meeting this demand. There are a number of caveats that must be attached to any proposal to promote the application of inoculant biofertilisers on a large scale. Several major economic and technological hazards have been identified during this ACIAR project that threaten success unless specific actions are taken to minimise their risk. The commercial profitability of biofertiliser production has been limited more by the rate of adoption of the technology by farmers than by the technological capacity to provide good quality mother cultures. Reasons for the slow rate of adoption include lack of financial credit and doubts concerning the efficacy of the biofertiliser.

By contrast, chemical fertilisers in Vietnam are promoted by sales companies in liaison with government extension agents, often using company loans repayable at harvest. Successful introduction of biofertilisers will require similar credit arrangements during a concerted and sustained period of demonstration and extension, innovative commercial production and technological support to ensure quality control for the overall process. One model showing promise would be introduction of the technology with consistent expansion of its use based on the franchising of local commercial producers, who would be supplied with effective cultures and protocols for production and sales. This process would also be subjected to rigorous quality control to ensure efficacy of the products and protection of human and environmental health.

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Introduction

To grow rice productively, Vietnamese farmers have typically needed to apply at least 100 kilograms of fertiliser nitrogen (N) per hectare to harvest each crop. Until recently, such fertilisers have been inexpensive and their cost to farmers was often subsidised. But it has been clear for some time that such practices using excessive inputs of urea were not sustainable for both economic and environmental reasons, and programs such as the 'Three Reductions' for seed, fertilisers and pesticides were implemented with incomplete success in the Mekong Delta (Tran 2008).

In the past few years the price of N fertilisers such as urea has trebled as oil prices continue to reach record highs. Yet no more than 40 kg N/ha is recovered in the rice crop and the remainder is directly lost to the environment as ammonia or as nitrate in groundwater, or is evolved as the greenhouse gas, nitrous oxide. More than 30 million Vietnamese people involved in rice production are now being affected economically by this problem of increasing input costs (Figure 1).

Better management with split applications of fertilisers can have some beneficial effects on improving the efficiency of use of applied N, but there is a trade-off in increased labour needs as farm workers are being attracted away from the land to other manufacturing industries. Any new technology that could improve the efficiency of use of fertilisers, reducing the total input costs and improving environmental health, would significantly increase the wellbeing of the rural poor in these areas.

Innovation

The research output of this project has shown that this problem of high cost and waste of N can be lessened if steps are taken to improve the efficiency of N use by rice plants. More efficient uptake of N can be achieved by ensuring that specific micro-organisms are present in the root zone of rice plants (Kennedy 2008). The strains of microbes present in the biofertiliser known as BioGro were isolated from rice paddies near Hanoi by Professor Nguyen Thanh Hien of Hanoi College of Science, Vietnam National University (Nguyen 2008). These microbes were selected for properties such as plant growth promotion by hormone production, the capacity to fix atmospheric nitrogen (N_2) and an ability to mobilise phosphorus from insoluble forms. These properties together allow rice growing with lower inputs of fer-

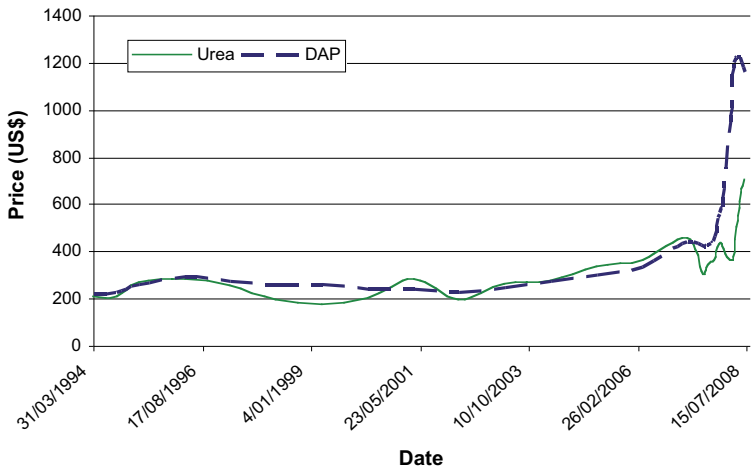

Figure 1. Price of urea and diammonium phosphate (DAP) fertilisers (US$), using annual data from the United States Department of Agriculture (04/1994 – 04/2007); <http://www.ers.usda.gov/Data/FertilizerUse/> and monthly data from The Market (04/2007 – 06/2008); <http://www.fertilizerworks.com/fertreport/>

tilisers such as urea and superphosphate. All the microbial strains except the yeast have been identified to species level by our group of colleagues at the University of Sydney, and their biological safety and effectiveness in BioGro have been established by a large number of laboratory tests. These micro-organisms were introduced to rice plants by inoculation of seedlings or of rice paddies at a rate of about 1 g (a million million (10^{12}) cells) per ha. This pioneering technology has been verified as effective by the research results of this ACIAR project in Vietnam.

More than 20 field experiments were performed in northern and southern Vietnam during the project, proving the high rate of effectiveness of BioGro (Pham et al. 2008a; Tran et al. 2008). For example, Phan and Tran (2008) clearly show that rice can be grown in Tay Ninh province with only half as much urea-N supply, some 50 kg/ha less, as that needed to give maximum yields. Under some conditions inoculation with BioGro can even lead to extra rice yields of the order of 10–15% as well as the reduction of input costs for urea-N. These results support earlier field trials in northern Vietnam that demonstrated similar fertiliser reductions and yield increases (Nguyen et al. 2003). Understanding the agronomic conditions that support such general increases in yield requires further research. It should be noted that the inocula contain negligible quantities of plant nutrients, even when the carrier materials used to deliver them are taken into account. Instead, by biologically multiplying their numbers in the rice plant rhizosphere, the catalytic effect of the microbes on nutrient mobilisation and uptake by rice plants becomes enormously amplified.

Although the technology itself is innovative, this project was also innovative in several other ways. While the use of inoculant biofertilisers has been proposed before, the active micro-organisms in most available products are not disclosed and little or no quality control of the product is assured, leading to unreliable results. BioGro now involves a unique production system characterised by a high degree of quality control with defined operating protocols and ongoing validation of effectiveness. Information on the microbial strains in the product is freely available and scientifically proven. Any microbial strains posing a likelihood of risk to human, animal, plant or environmental health have been eliminated. Furthermore, a number of different production–supply systems were investigated in this project in order to overcome the supply and demand variability that often impedes new technology uptake (Marsh and Nguyen 2008; Tran et al. 2008). Currently, no integrated production systems that include built-in quality control are known to exist for any biofertiliser product to be used on non-legume crops. A production model capable of being commercially franchised is now proposed to speed up the rate of adoption of this inoculant biofertiliser technology.

Reducing risks

Economic risks

The economic risks in this technology are related to profitable production of BioGro by the biofertiliser manufacturer, the feasibility of profitable sales to farmers and the profit gain by farmers that dictates their capacity to purchase the product on a sustainable basis (Tran et al. 2008). In general, these economic risks are not unique to this application of technology, but there are some special features that need consideration. In order to minimise economic risks, the following strategy is envisaged: the supply chain arrangements associated with BioGro production and the economic risks will be considered as a single commercial package, requiring defined equipment items and special protocols to be developed together with a set of standard quality controls for monitoring the success of the process.

These proceedings contain recommendations regarding commercial production models. The concept that this biofertiliser production package could be franchised is advanced here as a step towards sustainable commercialisation, based on the field results in Vietnam and elsewhere during the past 10 years (Figure 2). If supported by sufficient training of extension personnel and commercial producers, with a parent company providing advice and quality control, many of the risks to the introduction of the technology could be reduced to acceptable levels.

The capacity of farmers to afford even the reduced costs of using biofertiliser is also of concern, and it is possible that systems providing farmers with access to micro-credit for short-term loans during the crop production cycle may need to be promoted. Non-government organisations (NGOs) such as World Vision have previously provided cash loans to farmers involved in collaborative projects with the Institute of Agricultural Sciences (IAS) in the central coast region of Vietnam.

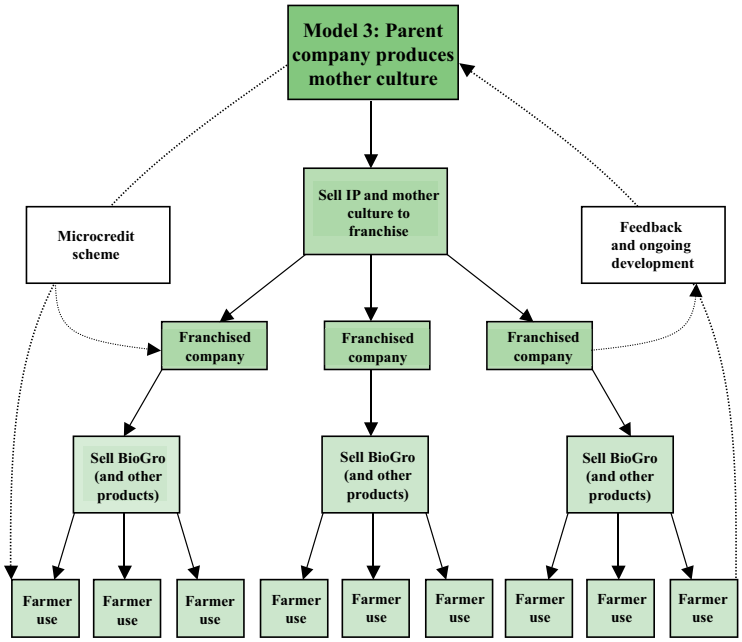

Figure 2. Proposed production–supply chain for sustainable uptake of inoculant biofertiliser technology

Technological risks

The technological risks identified relate to the safety of the inoculants, the manufacture of effective inoculants and their successful agronomic application. The definition of a biofertiliser, with which BioGro complies, is given in Vietnamese legislation as: 'addressing all safety matters related to human, animal, plant and environmental health' (Pham et al. 2008b). Risks in manufacture should be strongly mitigated by the quality control manual (QCM) developed as an output of the ACIAR project terminating in 2008. The QCM will contain details of methods developed during the project (Kecskés et al. 2008) that allow identification and quantification of the beneficial microbial strains in BioGro. The manual will also contain methods for guaranteeing and demonstrating the effectiveness of the agronomic application of the inoculant biofertiliser products on farms. A detailed description of methods for conducting and analysing farmer field trials as part of the process of extension will also be included. These clearly demonstrate the benefits for farmers from BioGro, expressed as increases in rice yields with lower fertiliser inputs.

Human safety

Several strains that raised questions regarding human health (Nguyen et al. 2003) were excluded because of such doubts. These strains, such as *Citrobacter freundii* and *Klebsiella pneumoniae,* were originally employed in BioGro because of their strong plant growth promoting (PGP) effects. They were used successfully for several years without reported adverse effects, and the degree of actual risk was probably extremely low given that these strains were isolated from rice soils in the first place. Similar reasons prevented the application of *Burkholderia vietnamiensis,* also shown to be an effective PGP organism for rice (Van et al. 2000), as a commercial inoculant in North America. However, it is essential for the good name of these biofertiliser products that all strains used be properly identified as done here (Kecskés et al. 2008; Deaker et al. 2008) and excluded if of even slightly doubtful human safety. All strains now employed in BioGro meet this criterion.

Sustainable growth potential

The major challenge regarding sustainability and growth will be the effectiveness of commercialisation of biofertilizer production. The current best model of production based on the ACIAR project is one with mother or starter cultures purchased by biofertiliser producers from the Biofertilizer Action Research Centre (BARC), who can then sell the product multiplied at least 100-fold in quantity to farmers using readily available carrier materials such as peat, high-organic matter soils or other composted materials. Biofertiliser effectively replaces N fertiliser, which can then be applied at a reduced rate without sacrificing yield.

Prior experience in the north of Vietnam indicates that enterprising producers will come forward, but sustaining production will depend on generating a sustainable market demand. Given the rapidly increasing price of N fertiliser, farmers will now be more willing than ever to consider this product instead of urea. Globally, given the urgent need to reduce the production of greenhouse gases such as nitrous oxide (400 times as significant as carbon dioxide in warming effect per molecule), the environmental benefits of less factory production and transport of urea fertiliser justifies the application of locally produced biofertiliser. Indeed, the technology may ultimately attract greenhouse reduction credits.

Dr Cong and Professor Hien at the final workshop in Hanoi in October 2007, discussing a critical point about BioGro's action

It is predicted that a large extension project will allow operation on a scale adequate to enable the technology to reach a 'take-off' stage, where production of biofertiliser will be considered profitable in a sustainable fashion. The key to financing the operation will be to expand production at such a rate that adequate new cash flow will be generated as profits are made. There is a clear role for microcredit to be made available to farmers to cover input costs, with repayment possible at harvest. One of the production models for an earlier version of BioGro in the north of Vietnam involved an NGO providing cash to establish a factory and maintain the supply of biofertiliser (Marsh and Nguyen 2008). But this model had no scope for expansion and could only continue with significant production while the NGO provided cash inputs.

Currently, the IAS team led by Phan Thi Cong works in close collaboration with World Vision Vietnam, who have assisted with training and decision support for farmers to improve soil fertility in the central coast region. Throughout this collaboration, World Vision has provided loans and cash to poor farmers. A more dynamic model that includes franchising, favoured by the prospect of rapidly rising prices of chemical fertilisers continuing into the future, can take advantage of a greater sense of entrepreneurship in southern Vietnam.

For training programs to promote the BioGro technology, both the IAS and Mekong Delta Developing Research Institute (MDI) have a mandate to extend technology of all kinds to farmers in southern Vietnam. They are well placed to carry out such promotion. The IAS and MDI also have significant microbiological expertise in their institutions and will be able to provide support to BioGro manufacturers for both production methods and quality control of their products. Vu Thuy Nga of the National Institute of Soils and Fertilizers (Hanoi), associated with BARC, and Tran Thi Kim Cuc of IAS can fulfil specific roles related to quality control procedures. Two months' special training at the University of Sydney in early 2009 has already been funded under the AusAID Leadership Fellowship Award scheme.

While an extension project can only realistically target a cohort of several thousands of rice farmers, the scope for further expansion of the application of BioGro or of competitor products of similar effectiveness is virtually unlimited. Given that approximately 30 million Vietnamese people are involved

in growing rice, there is extraordinary scope for the technology in Vietnam. Although it can be expected that many rice growers will take up other occupations as Vietnam's wealth increases, this need not diminish the use of these biological products as the technology could easily be adapted to more automated systems of rice production. Moreover, if the production–supply model is demonstrated to be successful, it could logically be applied to a range of scientifically reliable and transparent microbial products for other agricultural industries, both nationally and internationally.

Financial viability

To ensure the financial viability of biofertiliser application, it is essential that a supply chain for profitable production and application of inoculant biofertilisers on farms be established. The current global conditions of increasing fertiliser prices linked closely to the increasing price of oil provide a very favourable environment for success of this technology. Indeed, for poor farmers there is now an imperative need for alternatives to more expensive fertilisers such as urea and superphosphate, and more efficient use of urea-N will be most valuable.

A significant uptake of the technology in the Mekong Delta and the central southern coastal region, together with proof that it is likely to be effective over a period of 2–5 years, will provide a compelling demonstration of this approach. If rice production can be maintained or even increased, a flow-on effect would be anticipated to other areas of Vietnam and to other rice-producing countries. In these circumstances the potential scale for growth is very large.

Taken province by province with a 50% uptake rate, the number of beneficiaries is potentially in the millions, even if remaining within Vietnamese borders. In these conditions the franchising concept for biofertiliser production may be particularly effective as a means of more rapidly spreading the technology. If capital to manufacture the inoculants can be obtained locally for moderate levels of production, it is anticipated that economic viability might be enhanced by the sense of community involvement and the local cash flow generated. The increasing availability of micro-credit would also be important in allowing farmers to make outlays for biofertiliser in a similar way as they have done when financed by chemical fertiliser companies in the past.

Possible income generated by the production and sale of biofertilisers can be estimated from known production costs of BioGro and sell-on costs to farmers at US$150/t (estimated at 25% of the cost of equivalent chemical-N fertiliser, i.e. currently US$600 for urea). Considering an anticipated service area containing 10,000 ha of rice production using 100 kg/ha of BioGro, production and sale of 100 t of BioGro per season per franchise is estimated. This is equivalent to a gross income of US$15,000 per season, with a nominal net profit of US$1,500 per season per franchise after transport costs, worker salaries and production and sale costs. Such a minimum income may be sufficient to ensure franchise uptake and sustainable production to supply farmers' needs.

Current estimates of the financial benefit to individual farmers project an average annual profit increase of more than 10% with uptake of this technology (Marsh 2008). These figures are based on average reduction in use of N-fertiliser afforded by the technology, and are likely to increase with oil price increase.

Agronomic viability

Financial viability will also depend on constant improvements to the agronomic performance of the technology. Currently, BioGro contains four microbial strains (three bacteria and a yeast), and has normally been applied in significant quantities in carrier (ca. 200 kg/ha) at similar rates to fertilisers. This is partly as insurance to maintain cell numbers against the need for a shelf life of at least 1 month for the

Project team and review panel at Ba Vi factory in Ha Tay province near Hanoi, October 2007

product before application. Application of a large quantity of carrier also ensures more even distribution of the microbes and may be significant for adequate pricing. However, since only 1 gram of microbial cells is actually needed per hectare as inoculant, there is now an opportunity to substantially reduce the cost of BioGro and the quantity of carrier if delivery of sufficient numbers of microbes can be verified, that is if cell numbers can be increased. This is most likely if the use of biofertilisers increases and more experience is gained in obtaining optimal results.

Conclusion

The prospects that inoculant biofertilisers can be applied on a significant scale are greater than ever before. The main drivers for success will be the increasing price of chemical fertilisers and the need to minimise the production of greenhouse gases such as carbon dioxide and nitrous oxide. Achieving such an 'evergreen revolution' will still be extremely challenging but there are many indicators that it can now succeed.

References

Deaker R., Mijajlovic G. and Casteriano A.V. 2008. Estimating the most probable number of bacteria in multistrain biofertiliser inoculants using a multiple-tube fermentation test. These proceedings.

Kecskés M.L., Rose M.T., Tran Thi Kim Cuc, Nguyen Kim Oanh, Michel E., Lauby B., Rakotondrainibe M., Casteriano A.V., Palágyi A., Krishnen G., and Kennedy I.R. 2008. Identification and quality control of BioGro inoculant biofertiliser strains. These proceedings.

Kennedy I.R. 2008. Overview of research on efficient nutrient use in rice production using inoculant biofertilisers. These proceedings.

Marsh S.P. 2008. BioGro and farm-level benefits. These proceedings.

Marsh S.P. and Nguyen Thanh Hien 2008. Models of commercial biofertiliser production: Experiences with BioGro. These proceedings.

Nguyen Thanh Hien 2008. The product BioGro and improvements in its performance. These proceedings.

Nguyen Thanh Hien, Deaker R., Kennedy I.R. and Roughley R.J. 2003. The positive yield response of field-grown rice to inoculation with a multi-strain biofertiliser in the Hanoi area, Vietnam. Symbiosis 35, 231–245.

Pham Van Toan, Vu Thuy Nga and Luong Huu Thanh 2008b. Adoption and regulation of biofertiliser technology for rice production. These proceedings.

Pham Van Toan, Vu Thuy Nga and Nguyen Van Thanh 2008a. Evaluation of varietal difference and N–P–K combinations on the effectiveness of BioGro in rice cultivation and on rice quality. These proceedings.

Phan Thi Cong and Tran Dang Dung 2008. Interaction effects of BioGro with nitrogen and phosphorus on grain yield and nutrient uptake of rice in light-textured soils of southern Vietnam. These proceedings.

Tran Thanh Be 2008. Challenge of the 'Three Reductions' program in Vietnam: The potential role of inoculant biofertiliser technology. These proceedings.

Tran Thanh Be, Pham Thi Phan and Marsh S.P. 2008. Farmer extension trials using BioGro for rice production in the Mekong Delta. These proceedings.

Van Van Tran, Berge O., Ngo Ke S., Balandreau, J. and Heulin, T. 2000. Repeated beneficial effects of rice inoculation with a strain of *Burkholderia vietnamiensis* on early and late yield components in low fertility acid sulphate soils in Vietnam. Plant and Soil 218, 273–284.

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