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The Positive Yield Response of Field-Grown Rice to Inoculation with a Multi-Strain Biofertiliser in the Hanoi Area, Vietnam

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Abstract

A multi-strain biofertiliser was found to provide statistically significant increases in rice yield in two out of three field trials in Vietnam. This biofertiliser contained three strains of bacteria selected from rice rhizospheres in paddies near Hanoi. The benefit possible for rice farmers from application of the inoculant biofertiliser was confirmed as a reliable effect by positive results in 65 farmer demonstrations over three seasons for both summer and winter rice crops, with the increases in grain yield compared to farm areas receiving urea alone usually much greater than 10 percent. Increases in the dose of biofertiliser organisms applied in the range 5.5–22.2 $\times 10^{12}$ cfu ha^{-1} had no significant effect suggesting that, with suitable quality control to ensure its effectiveness, costs of application could be reduced. The three biofertiliser strains were selected respectively for their ability to reduce acetylene (N_2 fixation), mobilise insoluble phosphates and to favour

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establishment of the other two under competition from other rhizosphere organisms. There is evidence of significant stimulation of early root and seedling growth and of panicle numbers and seeds per panicle as a result of applying biofertiliser but the precise mechanisms of increases in grain yield remains a topic for future research.

Keywords: Rice, biofertiliser, PGPR, *Klebsiella pneumoniae*, *Citrobacter freundii*, *Pseudomonas* spp.

1. Introduction

Biofertilisers have been used to increase legume crop performance for centuries with the direct transfer of soil from areas where particular crops were growing well to those where the crop was to be introduced. Not till 100 years ago was the rationale behind the practice described and from which inoculation with pure cultures of root nodule bacteria followed (Fred et al., 1932). Almost concurrently, inoculation with other biofertilisers developed with the discovery of improved crop growth following inoculation with *Azotobacter*. Unpredictable results were common with both systems (see Kennedy and Islam, 2001). The causes of those results associated with root nodule bacteria were later attributed to poor strains and too small an inoculum potential. The causes of variable responses with other organisms remain poorly understood. The situation is complicated by the wide range of plant growth promoting rhizobacteria (PGPR) organisms involved, the mixed nature of cultures, a poorly understood mechanism for their stimulation of plant growth, and claims for their benefits often based on unreplicated experiments. Recently, interest in biofertilisers has been heightened by studies of the positive effect of asymbiotic nitrogen-fixing organisms and in particular *Azospirillum* on plant growth (Okon and Labandera-Gonzalez, 1994; Kennedy and Islam, 2001).

With rice, *Azospirillum brasilense* (James et al., 2000), *Herbaspirillum seropedicae* (Baldani et al., 2000), *Burkholderia vietnamensis* (Van et al., 2000; Baldani et al., 2000) and *Rhizobium leguminosarum* bv. *trifolii* (Yanni and El-Fattah, 1999; Yanni et al., 2001) have all increased the yield of rice in field studies as a result of PGPR effects. Baldani et al. (2000) established by the ^{15}N tracer technique that *B. vietnamiensis* fixed 19% of the rice plant N from the atmosphere. Inoculation with *H. seropedicae* in field conditions also increased shoot and root length, individual grain weight of field-grown rice (Arangarasan et al., 1998). Mirza et al. (2000), working with super basmati, an aromatic rice, found that inoculation with *Herbaspirillum* increased grain yield by 44–90% in greenhouse conditions.

In Vietnam, experience is largely with biofertilisers based on studies by one of us (Nguyen Thanh Hien) from 1990. This work concentrated on making available to farmers a product which could both reduce their dependence on inorganic fertilisers and increase yields of rice. In the following years a considerable body of data was accumulated based largely on unreplicated trials established at many farm sites, which demonstrated a surprisingly consistent positive response to inoculation. The substitution of biofertiliser for more expensive chemical fertilisers, using local labour inputs, could become a significant element in the alleviation of poverty in these poor farming communities.

The experiments described in this paper, aimed to test the biofertiliser prepared at the Hanoi National University of Science, quantifying any responses obtained in replicated field trials conducted over three years. We took the opportunity to examine in a preliminary way the dose rates of biofertiliser required and their interaction with normal farmer inputs of urea and farmyard manure.

2. Materials and Methods

Sites and experimental design

A field trial was sown in the Hanoi area, Vietnam in July 1999, July 2000, and February 2001. All trials were established in alluvial soils of the Red River and those of 2000 and 2001 were in the same field at Dai Moi where the soil pH varied between 5.19 and 5.58 ($CaCl_2$). Applications of farmyard manure (FYM) had no effect on soil pH.

The trials were established in a split-plot design replicated four times with rates of biofertiliser applied to the subplots. The trials conducted in 1999 and 2000 had N levels as the main plots but in 2001, levels of FYM were allocated to the main plots. Plot sizes between replicates were not constant due to the shape of the fields but were at least 20 m^2 up to a maximum of 40 m^2. The amounts of fertiliser and biofertiliser applied to each plot were adjusted to allow for this variation. The plots were protected by well-prepared banks to minimise the movement of water between plots thereby reducing the possibility of uninoculated plots being contaminated with biofertiliser. A basal fertiliser of muriate of potash (MOP) at 55 kg ha^{-1} and triple superphosphate (TSP) at 417 kg ha^{-1} was applied in 1999 and 2000. In 2001 TSP was reduced to 208 kg h a^{-1}. In addition, in 1999 11.1 t ha^{-1} wet weight of FYM was included but in 2000 this amount was halved. In 2001 urea was applied to all plots at 55 kg h a^{-1} replacing FYM, which was a variable in this trial.

Planting geometry

In 1999, 3–4 plants were transplanted in rows 17 cm apart with hills at 14–17 cm spacing providing 42–45 hills of rice m^2. In 2000 the plantings were 14 cm apart in rows spaced at 21 cm. To simplify harvesting, in 2001 the hills were spaced at 45 hills per m^2 and statistical analysis of the number of hills per m^2 showed no significant difference between plots ($P<0.05$).

Biofertiliser

The biofertiliser contained three strains of bacteria (1N or 2N, 3C, 4P), selected from rice rhizospheres in the Hanoi area of Vietnam. In the trial of 1999, strain 1N was used but this was substituted for in the trials of 2000 and 2001 by strain 2N.

Suitable strains were initially selected for their ability to grow on nitrogen-free medium and to reduce acetylene to ethylene as an indication of potential N_2 fixation. Recently, more emphasis has been placed on PGPR effects stimulating root growth of seedlings. Strain 4P was selected as able to solubilise insoluble PO4 in an agar medium and strain 3C produced toxic extra-cellular compounds which inhibited 50% of a test group of 100 rhizosphere organisms. Growth of the other two biofertiliser strains was unaffected by strain 3C. This strain was included in the biofertiliser to aid the establishment of 1N or 2N and 4P in competition with other rhizosphere organisms.

Each of the three bacteria were grown in separate broth cultures and added to separate bags of carrier formulated by mixing clay soil 50%, rice husks 25%, sugar 1%, plus water and broth culture 24%. These separate cultures were mixed in the field immediately before use in the ratio of 10 parts 1N or 2N depending on the year of the trial: 10 parts of 4P: 1 part of 3C. Because strains 1N, 2N and 3C are difficult to count in the unsterile carrier, we were only able to make a direct count of 4P, which was 3×10^9 cfu g^{-1} carrier. We estimate that the numbers of 1N and 2N to be 1×10^8 and 3C to be 1×10^7 cfu g^{-1} carrier based on counts of their broth cultures.

Biofertiliser was applied to the seedlings at sowing at 25% 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 evenly by hand directly to the soil. To plots which were to remain uninoculated, uninoculated carrier was added at a rate equivalent to 222 kg ha^{-1}.

Microbial culture

Storage media for biofertiliser strains included (g/l): 4P, glucose, 10; $Ca_3(PO_4)_2$, 5.0; KCl, 0.2; $(NH_4)\ 2\ SO_4$, 0.5; $MnSO_4$, 0.01; $FeSO_4$, 0.01; $MgSO_4$.

$7H_2O$, 0.1; yeast extract, 6.5; Agar 20; H_2O, to 1000 ml; pH, 7.0. Strains N and 3C were grown on agar containing 15 g/l.

Media for plate counting contained per l, (NH_4) 2 SO_4, 0.5; NaCl, 0.2; glucose, 10.0; $Ca(PO_4)_2$, 5.0; trace element solution, 1 ml. Trace element solution contained H_3BO_3, 5.0; (NH_4) 2 MoO_4, 5.0; $AlCl_3$, 0.15; $ZnSO_4$, 0.2 per l. Fermentation media for 1N, 2N and 3C contained peptone, 5.0; rock phosphate, 5.0; fish sauce, 15 ml in 1000 ml of H_2O.

Tests for phosphate solubilisation were conducted in wells in agar plates by clearing of precipitated $Ca_3(PO_4)_2$. Multi-strain compatibility was assessed by scoring for the presence or absence of inhibition zones surrounding disks containing broth cultures of test strains when placed on agar media surface-inoculated with the indicator strain. Strains to be included in biofertiliser products were confirmed to be compatible whereas inhibition of other rhizosphere strains not included in a biofertiliser was regarded as positive.

Seedling production

Seedlings of rice variety 9830 for the trials in 1999 and 2000 were grown in plastic trays of tapered hexagonal thimble shaped units of 1.5 cm diam., filled with wet soil with seeds spread evenly over their surface. Seedlings were grown for 12 days before transplanting. In 2001 we used traditional field sown nursery bays to assist the farmers in planting a uniform three seedlings per hill. Separate seedling bays were used for each level of biofertiliser. Seedlings of rice variety Khang Dan, a shorter season variety than 9830 and better adapted to winter sowings, were transplanted 24 days after sowing rice sprouts.

Treatments 1999

Three levels of urea (0, 83 and 194 kg h a^{-1}) were applied to main plots, and four levels of biofertiliser (0, 111, 222 and 444 kg ha^{-1}) were applied to subplots.

Treatments 2000

Three levels of urea (0, 83 and 194 kg h a^{-1}) were applied to main plots, and four levels of biofertiliser (0, 55, 111 and 222 kg ha^{-1}) were applied to subplots.

Treatments 2001

Three levels of FYM (5,560; 11,120 and 22,240 kg h a^{-1}) were applied to main plots, and four levels of biofertiliser (0, 111, 222 and 444 kg h a^{-1}) were applied to subplots.

Sampling and harvest

Plants were sampled in each trial six weeks after transplanting. In 1999 and 2000 the number of tillers, root and tiller dry weight and plant height at 12 sampling points selected at random were recorded and in 2001 plant height and tiller dry weight were recorded at 10 sampling points.

At harvest, the number of panicles in three hills and the number of fertile and infertile seeds per panicle in five panicles were counted. Samples of 1000 seeds and grain yield from five, 1 m^2 quadrats in 1999 and 2000, and from 10 samples of 25 hills in 2001, were weighed and the results were expressed as kg h a^{-1}. In 2000 and 2001, the grain was analysed for total N content (%), and nitrogen uptake (kg ha^{-1}) by grain was calculated.

The data were analysed using the Genstat statistical package.

Farmers' tests of biofertiliser

We collected data from demonstration trials sown by farmers of the effect of adding biofertiliser to rice. In July 1999 three communes were included and 25 farmers participated. In July 2000 four communes and 20 farmers, and in January 2001 two villages and 20 farmers participated. Each farmer was asked to divide his field into two plots one to receive biofertiliser and half the normal fertiliser inputs, and the other to include all normal inputs and no biofertiliser. Costs of the two systems were recorded.

3. Results

Identification of biofertiliser strains

The strains 1N, 2N, 3C and 4P were identified as *Pseudomonas fluorescens*; *Pseudomonas fluorescens/putida*; *Citrobacter freundii* and *Klebsiella pneumoniae*, respectively using the API 20E strip tests (purchased from BioMerieux). The identification was further supported by observation of phenotypic characteristics such as gram reaction, shape and motility, biochemical characteristics such as production of fluorescent compounds in King B media, fermentation of carbohydrates and presence of the enzyme catalase.

Composition of farmyard manure

The composition of the farmyard manure used in the 2001 field trial is presented in Table 1. Farmyard manure is by its nature variable. The eight samples we analysed had a mean nitrogen value of 2.79% with a standard error of 0.27 or 10%.

Table 1. Composition of farmyard manure used in the field trial in 2001

Mean organic matter (%)	Total nitrogen (%)	Total phosphorus (%)	Total potassium (%)
33.66±2.389	2.79±0.271	0.72±0.106	1.04±0.105

Table 2. Effects of urea and biofertiliser on the grain yield of rice adjusted to 15% moisture in 1999

Urea (kg ha^{-1})	Biofertiliser (kg ha^{-1})	Mean* grain yield (kg ha^{-1})
0	0	6528
0	111	7254
0	222	7228
0	444	6702
83	0	5732
83	111	6730
83	222	6947
83	444	7081
194	0	6789
194	111	6466
194	222	6403
194	444	6603

*Means of 20 samples. LSD (P=0.05) = 542.

Field trial 1999

There was very little effect of treatment on the vegetative plant at 6 weeks. Biofertiliser had no significant effect on root dry weight, tiller dry weight but had a slight depressing effect (P=0.05) on plant height. The difference between the two extremes was only 3 cm or 4.4%. Urea increased plant height from 68 to 70.8 cm.

Application of urea reduced grain yield from 6933 kg h a^{-1} (mean yield in urea control plots) to 6623 and 6565 kg h a^{-1} when it was applied at 83 and 194 kg h a^{-1}, respectively. There was a significant interaction between urea and biofertiliser (P<0.05). At urea levels of 0 and 83 kg h a^{-1}, biofertiliser application at 111 kg h a^{-1} increased grain yield significantly although there was no response to increased doses of biofertiliser. When urea was applied at 194 kg ha^{-1} there was no effect of biofertiliser (Table 2).

Table 3. Effects of urea and biofertiliser on the grain yield of rice and N uptake by grain in 2000

Urea (kg ha^{-1})	Biofertiliser (kg ha^{-1})				Mean
	0	55	111	222	
Grain yield (kg ha^{-1})					
0	5087	4985	4645	4599	4829
83	6288	6252	5988	5884	6103
194	7076	6787	6690	6727	6820
Mean	6150	6008	5774	5737	
N uptake by grain (kg ha^{-1})					
0	46	44	39	38	42
83	52	59	49	51	53
194	61	68	59	62	63
Mean	53	57	49	50	

Grain yield: LSD (P=0.05) for urea means = 364.9, and for biofertiliser means = 272.0. N uptake by grain: LSD (P=0.05) for urea means = 4.5.

Field trial 2000

Urea stimulated both early tiller and root growth; at the 6 week sampling without added urea, mean tiller dry wt was 5.54 g but increased to 7.96 and 10.00 g with urea applied at 83 and 194 kg h a^{-1}, respectively. Root growth responded similarly with roots of 1.43 g without added urea and 2.09 and 2.71 g with added urea at 83 and 194 kg ha^{-1}, respectively.

At harvest neither the number of seeds per panicle or 1000 seed weight responded to treatment. Biofertiliser had a progressive negative effect on grain yield, which declined by 413 kg h a^{-1} or 6.7 % with the highest application (Table 3).

Grain yield increased significantly (P<0.05) due to application of each additional amount of urea. The increases were 1274 kg h a^{-1} (26.4%) and 717 kg h a^{-1} (11.8%). The response in grain yield was matched with a similar response in N uptake by grain, which also did not respond to the application of biofertiliser.

Field trial 2001

Applications of farmyard manure did not affect either vegetative growth or grain yield. Biofertiliser had an early effect on vegetative growth with both

Table 4. Effects of biofertiliser on tiller dry weight and plant height at 6 weeks, and number of panicles per hill, number of seeds per panicle and weight of 1000 seeds at harvest in 2001

Biofertiliser (kg ha^{-1})	Tiller dry wt. (g per hill)	Plant height (cm)	No. of panicles per hill	No. of seeds per panicle	Dry wt. of 1000 seeds (g)
0	10.83	62.5	4.4	194	18.411
111	12.07	66.7	4.6	197	18.624
222	13.52	65.8	5.1	200	18.726
44412.97	66.4	4.8	201	18.983	

LSD (P=0.05): tiller dry weight = 1.375, plant height = 1.98, number of panicles per hill = 0.42, number of seeds per panicle = 10.8, dry weight of 1000 seeds = 0.323.

Table 5. Effects of farmyard manure and biofertiliser on the grain yield of rice and N uptake by grain in 2001

FYM (kg ha^{-1})	Biofertiliser (kg ha^{-1})				Mean
	0	111	222	444	
Grain yield (kg ha^{-1})					
5,560	5476	6170	5890	5801	5834
11,120	5443	6360	6111	5979	5973
22,240	5764	5813	6116	5854	5888
Mean5561 b	6114 a	6039 a	5878 a		
N uptake by grain (kg ha^{-1})					
5,560	50.40	55.89	53.59	51.14	52.76 B
11,120	51.41	59.28	57.09	54.69	55.62 A
22,240	50.67	54.62	57.29	55.78	54.59 A
Mean50.83 b	56.60 a	55.99 a	53.87 a		

Grain yield: LSD (P=0.05) for biofertiliser means = 258.1. N uptake by grain: LSD (P=0.05) for FYM means = 1.669, and for biofertiliser means = 2.903. Means followed by a common small letter in a row and a common capital letter in a column for a parameter are not significantly different at 5% level by least significant difference (LSD).

plant height and tiller dry weight responding positively (Table 4). At harvest the number of seeds per panicle was unaffected by biofertiliser but the weight

of 1000 seeds responded directly to increasing the dose of biofertiliser. The difference between 0 and 444 kg ha^{-1} was significant ($P<0.05$).

Grain yield increased significantly due to biofertiliser application at 111 kg h a^{-1} (Table 5), but the response did not increase further by applying more biofertiliser. Yield increased by 553 kg ha^{-1} or 9.9 % with biofertiliser applied at 111 kg h a^{-1}. This increase was significant at the 0.1% probability level. Nitrogen uptake by grain (kg h a^{-1}) increased significantly due to biofertiliser application at 111 kg h a^{-1}, but beyond this rate there was no further increase. This increase was significant at a 1% level of probability. Farmyard manure (FYM) application at 11,120 kg h a^{-1} increased N uptake by grain significantly over the lowest rate of FYM, at the highest rate of FYM there was no further increase. The effect of FYM was significant at the 5% level of probability.

Farmers' tests of biofertiliser

All the results of the 65 farmers that were averaged by commune or village and presented in Table 6 showed an increase in yield resulting from biofertiliser. The overall mean increase was 15% (728 kg h a^{-1}) with extremes of 8.3–30.7%. The mean increase was valued at AUD$146. There was a supplementary saving in inputs in all but one commune and although its value was secondary to that from increased yield, averaged AUD$15 per ha. The mean total economic benefit was AUD$161 per ha which is a significant amount in this economy. The farmers with the highest rate of improvement increased their return by AUD$274 which included AUD$251 per ha from increased yield. None of the farmers appeared sufficiently confident to reduce fertiliser inputs to 50% of their normal practice. The mean reduction was only 7.6%.

4. Discussion

Biofertiliser increased grain yield significantly in two of the three field trials and in all 65 farmer demonstrations over three seasons. This consistency obtained in both winter and summer crops, and in 66 different sites is particularly significant. In the 1999 and 2001 field trials where we obtained a positive response, the dose of biofertiliser was not a factor suggesting that even the low dose rate may well provide more than the minimum inoculum potential required. The amount applied was large in terms of that used with legume inoculants although in their case the organisms are usually strategically applied in the vicinity of the seed. The highest rate of biofertiliser we used, 222 kg ha^{-1}, applied approximately 22.2×10^{12} cfu h a^{-1} and the lowest rate of 55 kg ha^{-1} applied 5.5×10^{12} cfu ha^{-1}. Legume inoculants for soybean, where the standards in Australia require 1×10^{9} cfu g^{-1} when used at the recommended

dose rate, add 5×10^{11} cfu ha^{-1}. Even at the lowest rate, biofertiliser adds some 22 times the number of useful organisms per unit area. These high rates of application affect the economics of its use so that further experimentation is warranted to determine a minimum effective dose for general use.

The interaction of other inputs with biofertiliser was not consistent. The best responses, those in 2001, were all obtained in conjunction with added urea. In 1999, the highest rate of urea (194 kg h a^{-1}), eliminated the biofertiliser response but at 83 kg ha^{-1} biofertiliser further increased yield.

Interestingly, added FYM did not affect yield in 2001 the only year in which it was a variable. FYM is nevertheless a key input in farmer sowings in north Vietnam, where it is applied in addition to 280 kg h a^{-1} urea. Using the N and P values form Table 1 as a guide, FYM contributed 30 kg h a^{-1} of N in 1999 trials and 15 kg ha^{-1} of N in 2000. In 2001 where FYM was a variable, approximately 15, 30 or 60 kg h a^{-1} of N was added depending on the amount applied to the main plots. The amount of P added in the FYM was approximately 25% of the amounts of N. These all represent high inputs against which to evaluate biofertiliser.

In the farmer sowings, although they are advised to halve inputs of urea to 140 kg h a^{-1}, the data on input costs indicated that they were reduced by only 7%, so that the consistent responses to biofertiliser the 65 farmers obtained were in the presence of relatively high levels of FYM, urea, TSP and MOP. It should be noted that in the 2000 season, when we failed to obtain a response to biofertiliser in our experiments, farmers using the same rice variety and biofertiliser in the same district all had a positive response.

5. Conclusions

Our trials and observed farmer demonstrations clearly indicated that biofertiliser, comprising strains 2N, 4P and 3C generally produced a significant increase in rice yield. The factors which caused the negative response in one trial in 2000 (Table 3) are not understood. In future trials, we intend to conduct more control tests to confirm the quality of the biofertiliser response, to obtain more dependable benefits and to learn from any failures.

However, this generally positive response in field studies, surprising in relation to the other trials and demonstrations, warrants further investigation, together with detailed studies of the mode of action of each bacterial genus in the inoculum, to establish the most economic rate at which to apply them and the factors which maximise their effect. The initial field trials conducted by the Biofertilizer Laboratory in the late 1980s using imported PGPR strains, such as azospirilla, were not regarded as successful in improving yields on rice farms near Hanoi. The bacterial strains used in this study were then selected

locally as likely to be better adapted to north Vietnamese conditions. In practice, the use of farmer trials has been regarded as the best measure of effectiveness of these biofertiliser products and the field experiments reported in this paper were conducted to verify their apparent effectiveness on farms.

In work to be reported elsewhere, the identification of the strains 1N, 3C and 4P as *P. fluorescens*, *Citrobacter freundii* and *Klebsiella pneumoniae* using API 20E tests has been confirmed by rDNA sequence analyses, although rDNA from strain 2N identified as possibly *P. putida* remains unsequenced. *Citrobacter freundii* is a human enteric organism and now that 3C has been identified as this species, caution is recommended. No problems were encountered in its use over several years in this work. A newer version of the biofertiliser product no longer includes strain 3C but future work will require that biofertiliser strains be shown to meet acceptable health and environmental standards that need to be established.

It is assumed that the positive yield responses may be associated with known properties of these inoculant strains of bacteria such as auxin (El-Khawas and Adachi, 1999; Xie et al., 1996) and cyotokinin (Zahir et al., 2001) production, leading to root growth stimulation as observed for rice inoculated with *Pseudomonas putida* (Hall et al., 1996), but this requires experimental confirmation. Whether nitrogen fixation is involved other than as a useful property enhancing the competitive ability of these strains (Ladha et al., 1987; Quispel, 1991; Malik et al., 1997) is not known. However, it is now essential to obtain a better understanding of the basis of these PGPR-biofertiliser effects, so that adequate quality control guaranteeing consistent yield responses on farms can be obtained (Kennedy and Roughley, 2002).

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