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FIELD APPLICATION STRATEGIES FOR THE INOCULANT BIOFERTILIZER BIOGRO SUPPLEMENTING FERTILIZER NITROGEN APPLICATION IN RICE PRODUCTION

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□ *Biofertilizer research for rice in Vietnam has focused on the isolation and selection of strains that can fix nitrogen, solubilize inorganic phosphates, stimulate plant growth, and breakdown soil organic matter. This paper assesses the consistent positive effect of BioGro on grain yield and agronomic parameters, including the rates and times for its application, the need for continued inoculation of crops grown in the same site, varietal differences, and nitrogen (N), phosphorus (P), and potassium (K) combinations on the effectiveness of BioGro. The commercial biofertilizer, BioGro, consists of four strains, one formerly considered as nitrogen fixing,* Pseudomonas fluorescens, *a soil yeast strain,* Candida tropicalis *is P-solubilizing, and two other bacilli,* Bacillus amyloliquefaciens *and* Bacillus subtilis, *potentially breaking down cellulose, protein, and starch. All four strains contribute to plant growth promoting rhizobacteria (PGPR) effect as shown by enhanced root growth. BioGro can be produced in local factories providing there is technical backup in the supply of starter culture and quality control of the final product.*

Keywords: rice, biofertilizer, plant growth promotion

INTRODUCTION

Rice is the staple food for more than half of the human population. It is estimated that the rice cropped area was 158 M ha in 2010, and providing

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about 19.3% of the total worldwide calorie intake (IRRI, 2011). For rice cultivation, indeed agriculture in general, to remain productive and sustainable, a balance between the crop's nutritional needs and maintenance of soil fertility must be met. Nitrogen (N), phosphorus (P), and potassium (K), in particular, comprise the macronutrients of critical importance to plant development and function. Over the last 50 years, agriculture has relied on artificial inputs of these elements to soil to increase plant growth and grain yields. Total global inputs of N, P, and K to rice systems have increased from 32 MT in 1961 to 156 MT in 2008 (IFA, 2011).

Although successful in achieving higher food output, this greater reliance on chemical fertilizers has inadvertently affected non-target ecosystems through increased incidence of eutrophication and accelerated emissions of greenhouse gases causing environmental pollution problems (Choudhury and Kennedy, 2005; Choudhury et al., 2007). Moreover, because the price of chemical fertilizers is directly linked to those of fossil fuels, international economic instabilities place pressure on the livelihood of farmers who rely solely on chemical fertilizers for plant nutrition. More efficient nutrient use promoted by such biofertilizer products can provide a means to address these problems.

Soil microbes play an important role in regulating plant nutrient acquisition, through biological nitrogen fixation, mineral weathering, nutrient mobilization, organic matter recycling, competition with pathogenic species, and plant growth promotion (PGP) via biochemical exchange of nutrients and other plant growth substances. Microorganisms that exhibit favorable combinations of these traits can be encouraged to increase crop nutrient use efficiency and minimize disease infection, thereby increasing overall vigor and potential grain yield whilst minimizing environmental effects of excess nutrients. Significant progress has been made over the past two decades in the design and application of inoculant biofertilizers that contain one or more (non-rhizobial) PGP microorganisms (Kennedy et al., 2004; Govindarajan et al., 2008). Numerous field experiments have demonstrated the potential for single strain inoculant biofertilizers to reduce fertilizer requirements and enhance yields of rice, including applications of azospirilla, pseudomonads, enterobacteria and rhizobia (Table 1).

More recently mixed inoculants containing several different bacterial strains have been investigated for their PGP effects. Govindarajan et al (2008) found that the strain *B. vietnamiensis* (MGK3) gave higher rice yield increases when combined with each of diazotrophs *Gluconacetobacter diazotrophicus* LMG7603, *Herbaspirillum seropedicae* LMG6513, *Azospirillum lipoferum* 4B LMG4348 (yield increase of 9.5–23.6%) than when inoculated onto rice seeds alone (yield increase of 5.6–12.2%). Nguyen et al. (2002) recorded significant yield increases in northern Vietnam using a mixed inoculant biofertilizer called BioGro containing the strains shown to be *Klebsiella pneumoniae* (4P), *Pseudomonas fluorescens* (1N) and *Citrobacter freundii* (3C) as

TABLE 1 Effect of various single inoculants on rice yield compared with non-inoculated controls

PGPR	Application method	Hypothesized major PGP effect	Yield increase over non-inoculated crop (%)	Geographical location	Reference
Azospirillum brasilense (Wb3)	Seedling root dipping	Nitrogen fixation	56	Pakistan	Malik et al., 2002
Azospirillum lipoferum (N4)	Seedling root dipping	Nitrogen fixation	32	Pakistan	Malik et al., 2002
Pseudomonas fluorescens (Pf1)	Seed inoculation with talc powder formulation	Biocontrol of rice blast pathogen (*Pyricularia oryzae*)	25–84[a] 3–33[b]	Tamil Nadu, India	Vidhyaskaran et al. (1997)
Burkholderia vietnamiensis (TVV75)	First inoculation: seed dipping, second inoculation: seedling dipping at transplanting	Nitrogen fixation, pathogen inhibition and siderophore production	13–22[c]	Mekong delta, Vietnam	Trân Van et al. (2000)
Rhizobium leguminosarum bv trifolii (E11)	Seedling inoculation	Modulation of root architecture for increased NUE	15–41[d]	Nile delta, Egypt	Yanni et al. (2001)

[a]mean of control (no fungicide application) vs treatment, range given of four independent experiments conducted in four different years.
[b]mean of control (carbendazim fungicide application) vs treatment, range given of four independent experiments conducted in four different years.
[c]Percentage increase in yield varied among locations.
[d]mean of control vs treatment differences at 4 different N-fertilizer levels (0, 48, 96 and 144 kg N ha^{-1}), range given of three independent experiments conducted in three different years.

identified at the University of Sydney (Kecskés et al., 2008). A newer version of the same product, containing *Pseudomonas fluorescens* (1N), *Bacillus subtilis* (B9), *Bacillus amyloliquefaciens* (E19), and *Candida tropicalis* (HY) increased the efficiency of fertilizer N use and grain yield significantly during the less rainy season in southern Vietnam, although this effect was less significant at higher fertilizer N when solar insulation was more limited (Phan et al., 2009).

Despite these promising reports, the potential yield increases afforded by biofertilizers can be inconsistent, with variable efficacy under different environmental and agronomic conditions (Bashan and Dubrovsky, 1996). For example, Diaz-Zorita and Fernandez-Canigia (2009) recently analyzed results from a comprehensive collection of nearly 300 independent field trials over 6 years, which examined the effect of *Azospirillum brasilense* inoculation on dry land wheat production. They found that the proportion of trials exhibiting a significant grain yield response to inoculation ($P < 0.10$) varied between 49 and 75%, with a mean yield response of 7.5% greater than that of the untreated controls. No significant response to inoculation was found under strongly limiting growth conditions like soils with shallow profiles in semiarid regions or when crops were low yielding. A greater positive contribution from the inoculation treatment was observed with moderate water shortage. Because positive and consistent grain yield benefits to inoculation were observed under a wide range of production conditions, the authors conclude that the contribution of *Azospirillum* complements adequate resource availability rather than substituting for those resources.

More information of this type is required if use of inoculant biofertilizers are to become reliable agronomic tools in rice production. In particular, we identified a lack of field-based information regarding the effect of biofertilizer application timing, biofertilizer application rate and the persistence of PGP effects from one season to the next. Furthermore, agronomic responses need to be linked to economic analyses in order to ensure uptake on the basis of increased gross margins, particularly if fertilizer reductions rather than grain yield increases are the goal of inoculation. This paper therefore addresses the hypothesis that the timing, rate and re-application of the inoculant biofertilizer BioGro can be optimized to maximize yield increases whilst minimizing economic and labor costs associated with inefficient bio- and chemical fertilizer applications. Previous investigations showed varietal difference in N fertilizer response in rice crop (Choudhury and Bhuiyan, 1991; Choudhury et. al., 1997). It was assumed that the effect of BioGro on rice crop might differ depending upon varieties and fertilizer application rates. Based on this assumption, this study also included field experiments to evaluate the varietal difference and NPK fertilizer combinations on yield and some other agronomic parameters of the rice crop.

MATERIALS AND METHODS

Biofertilizer

Before 2005, BioGro contained three strains of bacteria (1N, 4P, and 3C) selected from rice rhizospheres in Hanoi area of Vietnam. From 2005 a combination of four strains has been used – 1N, HY, B9, and E19. *Pseudomonas fluorescens* (1N) was selected for its ability to grow on nitrogen-free medium and to reduce acetylene to ethylene as an indication of potential N_2- fixation. A soil yeast, *Candida tropicalis* (HY) and was selected as for its ability to solubilize insoluble phosphate (PO_4) in an agar medium and its ability to produce more significant biomass in peat. Two other strains *Bacillus subtilis* (B9) and *Bacillus amyloliquefaciens* (E19) were selected for their ability to breakdown protein, cellulose and starch. These strains were identified at the University of Sydney by a combination of cultural and molecular techniques and their identify confirmed at the German Collection of Micro-organisms and Cell Cultures (DSMZ), Braunschweig, Germany (Kecskés et al., 2008).

Each of the four organisms were grown in separate broth cultures, and separately added to the carrier formulated by mixing peat (clay soil greater than 25% organic matter) 75%, plus water and broth culture 24% and sugar 1% and incubated for 48 h at 30°C. These four carriers were mixed before use in equal proportions and then constituted as the biofertilizer of these experiments. Biofertilizer strains are required to exceed 10^6 viable cells g^{-1} and BioGro is prepared to exceed 10^7 cells g^{-1}, equivalent to an application rate of 10^{12} cells of each strain ha^{-1}.

Biofertilizer was applied in the nursery by mixing with seeds at sowing at 15% of the rate to be used in the field plots to which they were to be transplanted. Biofertilizer was applied to the field plots by spreading the carrier evenly by hand directly to the soil. Carrier peat without microbial strains was added at an equivalent rate of 270 kg ha^{-1} to plots that were to remain uninoculated.

Microbial Culture

Storage media for biofertilizer strains included (g L^{-1}): (1) HY, glucose, 10; calcium phosphate [$Ca_3(PO_4)_2$], 5.0; potassium chloride (KCl), 0.2; ammonium sulfate [$(NH_4)_2SO_4$], 0.5; manganese sulfate ($MnSO_4$), 0.01; iron sulfate ($FeSO_4$), 0.01; magnesium sulfate heptahydrate ($MgSO_4.7H_2O$), 0.1; yeast extract, 6.5; Agar, 20; water (H_2O), to 1000 mL; pH,7.0. (2) 1N was grown on agar containing Tryptic Soy Broth, 30; Agar, 15; H_2O, to 1000 mL; pH, 7.0. (3) B9**,** E19 were grown on agar containing peptone, 10; tryptone, 3; sodium chloride (NaCl), 3; Agar, 15 in 1000 mL of H_2O; pH7.

Fermentation media (g l^{-1}): (1) HY as for storage, (2) 1N peptone, 5.0; fish sauce, 15 ml in 1000 ml of H_2O. (3) B9, E19 were grown in the media containing peptone, 10; fish sauce, 20 ml in1000 ml of H_2O; pH 7.0.

Media for plate counting (1) HY contained (g l^{-1}): $(NH_4)_2SO_4$, 0.5; NaCl, 0.2; glucose, 10.0; Ca_3 $(PO_4)_2$, 5.0; trace element solution, 1 ml. in 1000 ml of H_2O. Trace element solution contained : H_3BO_3,5.0; $(NH_4)_2MoO_4$,5.0; $AlCl_3$, 0.15; $ZnSO_4$,0.2 per l. (2) 1N was grown on agar containing glucose,10; peptone,3.0; yeast extract, 2.0; trace element solution, 10 ml; agar,16 in 1000 ml of H_2O. Trace element solution: $(NH_4)_2SO_4$, 5.0; $MgSO_4$ 1.0; NaCl, 1.0; KH_2PO_4, 2.0; calcium chloride ($CaCl_2$), 1.0 in 1000 ml of H_2O. (3) B9, E19 were grown in the media containing KH_2PO_4, 0.5; $MgSO_4.7H_2O$, 0.2; $FeSO_4.7H_2O$, 0.01; $CaCl_2$, 0.01; casein, 10; agar, 15 in 1000 mL of H_2O.

Tests for phosphate solubilization were conducted on solid media containing precipitated calcium phosphate [$Ca_3(PO_4)_2$] agar plates. Phosphorus solubilization was determined by the production of zones of clearing around isolated colonies. Similarly, assessing breakdown of organic substance was conducted in on solid media containing casein by the production of zones of clearing around isolated colonies.

FIGURE 1 Harvesting seedlings for transplanting.

Seedling Production

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

Sites and Experimental Design

Field trials were conducted in the Hanoi area, Vietnam in 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 ($CaCl_2$). Three types of experiments (continuous application of BioGro, rates of BioGro application and timing of BioGro application) were carried out by Hanoi University of Science. Another experiment on varietal difference on BioGro response was conducted at Thanh Tri, near Hanoi in two consecutive seasons of 2006 by the Vietnam Academy of Agricultural Science, Hanoi. The same organization conducted another experiment on the effects of NPK combinations on BioGro response of quality rice during four consecutive seasons of 2005–2006.

FIGURE 2 Experimental plots as in Trials 1 and 2.

Continuous Application of BioGro

This experiment was initiated in spring 2005, and was continued in the following two seasons (summer 2005 and spring 2006). The experiment was conducted in randomized complete block design (RCBD) replicated four times (Figure 2). Plot sizes were 40 m^2 and were protected from cross contamination from adjoining plots by asbestos roof tiles that were sunken 20–30 cm into the soil. Blanket fertilizer doses of potassium chloride (KCl) (muriate of potash, MOP) at 56 kg ha^{-1} before flowering, thermo phosphate (TP) at 200 kg ha^{-1} and urea at 55 kg ha^{-1} were broadcasted and spread evenly using rakes at transplanting. Rice seedlings were planted at a spacing of 21 cm × 14 cm.

The experiment was started in the spring of 2005 to investigate the effect of inoculation with BioGro on number of panicles per hill, number of fertile seeds per panicle, weight of 1000 seeds and grain yield. There were two treatments (with and without BioGro). BioGro was applied in two splits (40 kg ha^{-1} during seed sowing + 200 kg ha^{-1} during transplanting). In the following season (summer 2005), the effect of BioGro inoculation in two successive rice crops grown in the same plots compared with a single inoculation was evaluated. In the third season (spring 2006) investigations were done to evaluate the effect of inoculation of three successive rice crops grown in the same plots compared with a single inoculation.

Optimum Rates of BioGro Application

This was studied in experiments conducted in six farmers' fields. Individual fields were divided into two plots: one to receive biofertilizer at one of four rates and half the normal fertilizer input and the other to include the normal fertilizer input but with no added biofertilizer. Plot sizes varied between 91 and 210 m^2. Rice seedlings were transplanted at a spacing 21 cm × 14 cm. Trial was conducted in spring 2006 on plots on 6 farms in 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^{-1}) were applied at transplanting. Treatment plots of farmers 1 and 2 received 200 kg ha^{-1} of BioGro, treatment plots of farmers 3 and 4 received 100 kg ha^{-1} of BioGro and treatment plots for farmers 5 and 6 received 50 kg ha^{-1} of BioGro. Control (0 kg ha^{-1} of BioGro) was included at all the farmers' plots. Considering the farmers as replications, the control had six replications while each level of BioGro has two replications. Data were analyzed accordingly using an un-balanced treatment structure.

Timing of BioGro Application

This experiment was conducted in spring 2007 to evaluate the effects of higher amounts of BioGro and the timing of the application on yield of rice. The experiment was conducted on three farms in Dai Mo village near Hanoi. An uninoculated control was also established on each farm. In farm one, a 150 m^2 plot was used for the uninoculated control and another 150 m^2 plot used for the rice seedlings treated with BioGro at 38 kg ha^{-1} in the nursery area. In farm two, a 120 m^2 plot was used for the uninoculated control treatment and another 120 m^2 plot was used for rice treated at the seedling stage with 38 kg ha^{-1} and at transplanting with BioGro at 278 kg ha^{-1}. In farm three, a 203 m^2 plot was used for the uninoculated control treatment and another 203 m^2 plot was used for rice treated at the seedling stage and at transplanting as in Farm 2, plus 139 kg ha^{-1} BioGro at 28 days after transplanting.

Varietal Difference in BioGro Response

Treatments

The experiment was 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, VD8; and three quality varieties: LT2, HT1, BT7). Three treatments used in the experiment were T1: 100% NPK + farmyard manure (FYM) as control, T2 : BioGro + 50% NP +100% K+ FYM, and T3 : BioGro + 30% NP + 100% K + FYM.

Nitrogen fertilizer rates for spring and summer were 260 kg and 220 kg urea ha^{-1}, respectively. In both seasons, 450 kg triple super phosphate (TSP), 180 kg muriate of potash (KCl), 10 tones farmyard manure (FYM), and 283 kg BioGro ha^{-1} were used.

Experimental Design

The experiment was laid out in a split plot design with treatments in the main plots and varieties in the sub plots. The unit sub-plot was 10 m2. Three replications were used. The spacing for transplanting was 15 cm × 15 cm for both seasons. Seed sowing, transplanting and harvesting dates were 20 January, 22 February, and 22 May 2006, respectively for spring season while the respective dates for summer season were 20 June, 12 July, and 3 October 2006.

Fertilizer Applications

Full amounts of FYM and P was applied at transplanting time in both the seasons. N and K were applied in two splits (50% at transplanting + 50% at active tillering stage) in both the seasons.

BioGro Application

BioGro was applied in two splits (83 kg ha^{-1} during seed sowing by mixing with seeds + 200 kg ha^{-1} applied on experimental plots during transplanting). The formulation of BioGro was 1N + HY + B9 + E19 at the ratio of 1:1:1:1. 1N stands for *Pseudomonas fluorescens*. HY (*Candida tropicalis*) is a soil yeast. B9 stands for *Bacillus subtilis* and E19 stands for *Bacillus amyloliquefaciens*. Both B9 and E19 can breakdown cellulose, protein and starch.

Intercultural Operations and Harvesting

Necessary intercultural operations including weeding and pest controlling were done 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.

Effects of NPK Combinations in BioGro Response

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 fertilizers with BioGro2 on a 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% NP + 100% K + FYM
T3: 50% NP + 100% K + FYM
T4: 75% NP + 100% K + FYM
T5: 100% NPK + FYM (Control)
T6: BioGro + 25% NP + 100% K + FYM
T7: BioGro + 50% NP + 100% K + FYM
T8- BioGro + 75% NP + 100% K + FYM
T9- BioGro + FYM

The N, P and K rates were 120 kg N ha^{-1}, 75 kg phosphorus pentoxide (P_2O_5) ha^{-1} and 60 kg potassium oxide (K_2O) ha^{-1}, respectively. The FYM rate was 8 t ha^{-1} and the BioGro rates were 417 and 278 kg ha^{-1} in summer and spring seasons, respectively.

Experimental Design

The experiment was laid out in a randomized complete block design with three replications. The unit plot size was 20 m^2. The spacing for transplanting was 15 cm × 15 cm. The seed sowing dates were 19 January 2005, 12 June 2005, 3 January 2006, and 13 June 2006 for spring 2005, summer 2005, spring 2006, and summer 2006, respectively while the respective transplanting dates were 20 February 2005, 21 July 2005, 3 February 2006, and 22 July 2006. The harvesting dates were 26 June 2005, 21 November 2005, 18 June 2006, and 19 November 2006 for spring 2005, summer 2005, spring 2006, and summer 2006, respectively.

Fertilizer Applications

Full amounts of FYM and P was applied at transplanting time in all the four seasons. Both N and K were applied in two splits (50% at transplanting + 50% at active tillering stage).

BioGro Application

The formulation of BioGro2 has been described above. BioGro was applied in two splits: 78 kg ha^{-1} during seed sowing by mixing with seeds for both summer and spring + 200 kg ha^{-1} applied on experimental plots during transplanting in spring while the second installment was 339 kg ha^{-1} for summer.

TABLE 2 Effect of BioGro on grain yield of rice and some agronomic parameters in the spring crop 2005

Treatment	Grain yield (t ha^{-1})	Number of panicles $hill^{-1}$	Number of fertile seeds $panicle^{-1}$	1000 seeds weight (g)
Control (without BioGro)	7.39	5.90	188.90	19.00
BioGro	7.96**	7.20**	222.00*	19.03
F probability	0.018	0.023	0.074	0.649
LSD (0.05)	0.38	0.96	—	—
LSD (0.10)	—		28.83	—
CV (%)	5.1	16.8	15.5	0.7

**and *indicate significantly higher over control at 5% and 10% probability levels, respectively.

Intercultural Operations and Harvesting

Necessary intercultural operations including weeding and pest controlling were done 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.

Harvesting

For all the experiments mentioned above, the rice crop was harvested at maturity to record grain yield and yield components. The grain yields of all trials were adjusted to 14% moisture content.

Statistical Analyses

All the data were analyzed at University of Sydney using GenStat (VSN International, Oxford, UK) version 7 (Payne et al., 2003).

RESULTS

Continuous Application of BioGro

In the first season, inoculation with BioGro increased grain yield significantly over the control at 5% level of probability (Table 2) 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 1000 seeds was unaffected by BioGro.

In the second season, fresh application of BioGro increased grain yield and number fertile seeds per panicle significantly at 5% probability level over un-inoculated control while there was no significant residual effect of BioGro (Table 3). The weight of 1000 seeds was unaffected by the treatments.

In the third season, fresh application of BioGro increased grain yield significantly at 10% probability level over un-inoculated control (Table 4).

TABLE 3 Effects of BioGro application on grain yield, 1000 seeds weight and number of seeds per panicle, summer crop 2005, Hanoi University of Science

Treatment*	Grain Yield (t ha^{-1})	1000 Seeds Weight (g)	Number of Fertile Seeds $Panicle^{-1}$
Control 1	6.12	19.12	138.0
Control 2	5.69	19.21	154.7
Treatment	6.63	19.25	207.2
F Probability	0.011	0.145	0.007
LSD (0.05)	0.50	—	35.46
CV (%)	7.5	1.1	19.3

*Control 1: No Biofertilizer at all, Control 2: Biofertilizer was applied in the first crop, not in the current crop, Treatment: Biofertilizer was applied continually for 2 crops.

Residual of BioGro application for two continuous seasons was noticeable. It (control 3) increased grain yield by 0.76 t ha^{-1} over un-inoculated control although the difference was not statistically significant. Number of panicles/hill increased significantly by fresh application of BioGro over un-inoculated control at 5% probability level.

Rates of BioGro Application

BioGro applied at 50, 100, or 200 kg ha^{-1} increased grain yield significantly compared with uninoculated control at 5% probability level (Table 5). Increasing the amount BioGro from 50 to 100 or 200 kg ha^{-1} did not further increase yield. There was no consistent effect of BioGro on the number of fertile seeds per panicle although the highest amount (200 kg ha^{-1}) did increase the number of fertile seeds per panicle over all other treatments significantly.

TABLE 4 Effects of BioGro application on grain yield and yield components of rice, spring crop 2006, Hanoi University of Science

Treatment*	Grain yield (t ha^{-1})	Number of panicles $hill^{-1}$	Number of fertile seeds $Panicle^{-1}$	1000 seeds weight (g)
Control 1	5.99	6.25	202.8	19.71
Control 2	5.45	6.40	176.4	19.55
Control 3	6.75	6.70	171.1	19.91
BioGro	7.32	7.90	186.7	19.55
F Probability	0.054	0.013	0.247	0.145
LSD (0.05)	—	0.95	—	—
LSD (0.10)	1.10	0.77	—	—
CV (%)	9.5	24.8	24.7	1.7

*Control 1: No Biofertilizer at all, Control 2: Biofertilizer was applied in the first crop, not in the second and third crops, Control 3: Biofertilizer was applied in the first and second crops, not in current crop (third crop), BioGro: 1N + HY + B9 + E19 was applied at the ratio of 1:1:1 in current crop at 240 kg/ha. 1N stands for *Pseudomonas fluorescens/Pseudomonas putida*. HY is a yeast. B9 and E19 can breakdown cellulose, protein and starch.

TABLE 5 The effect of the amount of BioGro applied on grain yield, the number of panicles per hill and the number of fertile seeds per panicle, spring 2006

Amount of BioGro applied (kg ha^{-1})	Grain yield (t ha^{-1})	Number of panicles $hill^{-1}$	Number of fertile seeds $panicle^{-1}$
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

Timing of BioGro Application

On the first farm where a single application of BioGro at 38 kg ha^{-1} was used in the nursery only, grain yield was increased by 9% (Table 6). On a second farm where a total of 316 kg ha^{-1} of BioGro was applied in a split application the increase in yield was by 80%. When three applications of BioGro were made on a third farm, the increase was 39%. Grain yield obtained by two or three applications were similar, suggesting more than two applications of BioGro were not needed. However, much of the variation in percent increase over the control was due to the variation in the yields in control plots among the farms. This illustrates the frequent effect of basal soil fertility on the effectiveness of biofertilizer application suggesting this needs to be measured.

TABLE 6 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^{-1})	Grain yield (t ha^{-1})		Increase in yield with inoculation (t ha^{-1})	Percentage increase
		Uninoculated	Inoculated		
1/Inoculated once in nursery	38	4.35	4.75	+0.4	9
2/Inoculated twice –in nursery and at transplanting	316	3.46	6.24	+2.78	80
3/Inoculated three times - in nursery, in field at transplanting and 28 days later	455	4.44	6.17	+1.73	39

BioGro: 1N + HY + B9 + E19 was applied at the ratio of 1:1:1:1. 1N stands for *Pseudomonas fluorescens/Pseudomonas putida*. HY is a yeast. B9 and E19 are bacteria capable to breakdown cellulose, protein and starch.

FIGURE 3 Seedlings at 12 days age with one application of BioGro.

BioGro's PGPR Effect

Where rice seedlings are prepared within nursery areas, as in the spring crop of 2007 PGPR effects can be monitored at transplanting. 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 6).

Varietal Difference in BioGro Response

In spring 2006, interaction effect of variety and treatment was not significant however, individual effects of variety and treatment were significant (Table 7). BioGro with 50% NP gave statistically similar yield as obtained with 100% NP without BioGro indicating that BioGro has the potential to save 50% NP fertilizers in spring season. A further reduction in NP (30% NP with BioGro) reduced grain yield significantly. There were significant differences among the varieties in producing grain. The highest grain yield (6.48 t ha^{-1}) was obtained with VD8 followed by KD18 (6.05 t ha^{-1}), and the lowest yield (4.89 t ha^{-1}) was noticed in LT2.

In the summer of 2006, interaction effect of treatment and variety was significant (Table 8). While treatment effects were not significant in LT2

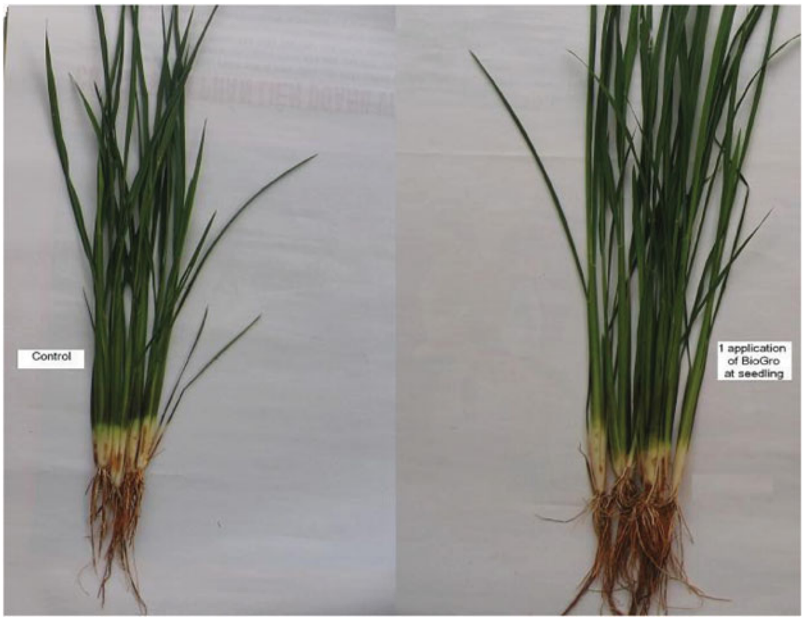

FIGURE 4 Rice at tillering stage inoculated once at seedling stage.

FIGURE 5 Rice at harvest inoculated once at seedling stage.

TABLE 7 Effect of BioGro (in combination with chemical fertilizers) and variety on grain yield of rice at Thanhtri, Hanoi, spring crop 2006

Variety	Treatment*			Mean
	T1	T2	T3	
	Grain yield (t ha^{-1})			
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	5.79 A	5.84 A	4.91 B	

Means followed by a common capital letter in a row or a common small letter in a column are not significantly different at 5% level by least significant difference (LSD) test.

The interaction effect of 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. *Treatments have been described in Materials and Methods section.

and AYT01, there were significant effects of treatments in the other four varieties. In HT1, BioGro with 30% NP gave statistically similar yield as obtained in 100% NP without BioGro while in BT7 reduced rates of NP (either 50 or 30%) with BioGro produced significantly lower amount of grain compared with 100% NP. In KD18, BioGro with 50% NP gave statistically similar grain yield as obtained with 100% NP, a further reduction in NP (30% NP) decreased grain yield significantly. The most interesting finding is that in VD8, BioGro with 50% NP gave significantly higher grain yield over 100% NP. In the same variety, BioGro with 30% NP produced significantly lower amount of grain compared to 100% NP. Varietal differences in grain

TABLE 8 Effect of BioGro (in combination with chemical fertilizers) and variety on grain yield of rice at Thanhtri, Hanoi, summer crop 2006

Variety	Treatment		
	T1	T2	T3
	Grain yield (t ha^{-1})		
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

Values followed by a common capital letter in a row or a common small letter in a column are not significantly different at 5% level by LSD test.

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 9 Effects of BioGro and chemical fertilizers on grain yield of rice, Haihau, Namdinh 2005–2006

Treatment	Grain yield (t ha^{-1})			
	LT3-Spring 2005	Tam Xoan-Summer 2005	LT3-Spring 2006	Tam Xoan-Summer 2006
FYM	3.16*	2.20*	2.51*	2.27*
25% NP + 100% K + FYM	3.44*	2.33*	2.74*	2.35*
50% NP + 100% K + FYM	3.75*	2.47*	3.12*	2.42*
75% NP + 100% K + FYM	4.15*	2.90^{ns}	3.79^{ns}	2.67^{ns}
100% NPK + FYM (Control)	4.63	2.78	4.13	2.73
BioGro + 25% NP + 100% K + FYM	3.65*	2.50*	2.88*	2.30*
BioGro + 50% NP + 100% K + FYM	3.73*	2.78^{ns}	3.25*	2.51*
BioGro + 75% NP + 100% K + FYM	4.03*	2.93^{ns}	3.97^{ns}	2.74^{ns}
BioGro + FYM	3.04*	2.18*	2.16*	2.16*
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

*Significantly different from control, ^{ns}not significantly different from control.

production varied among the treatments. For example, KD18 and VD8 were statistically similar in T1 and T3 while VD8 produced significantly higher amount of grain over KD18 in T2.

The results showed that the interaction effect of treatments and variety was not significant in spring 2006 while it was significant in the following season (summer 2006) indicating the seasonal impacts on the effectiveness of BioGro in different varieties. In general grain yields were higher in spring 2006 compared to summer 2006. The intensity of solar energy, which is a seasonal attribute, might influence the activity of micro-organisms as well as N response of different rice varieties. This should be verified by correlating solar radiation data with the experimental findings for both the seasons. This might also contributed in higher grain yields in the spring season.

Effects of NPK Combinations in BioGro Response

In the first season (spring 2005), BioGro with any combination of NP failed to produce statistically similar yield as obtained with 100% NP without BioGro while in the second season (Summer 2005) BioGro with 50% NP gave statistically similar yield as obtained with 75% and 100% NP without BioGro (Table 9). In the following two seasons of 2006, this beneficial effect was not noticed. Full fertilized treatment and 75% NP without BioGro produced statistically similar yields in three seasons (summer 2005, spring 2006, and summer 2006). So, it is recommended to reduce the N fertilizer rate to maximum 90 kg ha^{-1} in order to evaluate the effectiveness of BioGro on the quality rice varieties (LT3 and Tam Xoan).

DISCUSSION

The data from previous field trials and farmers' tests (Nguyen et al., 2003) and from others (Phan and Tran, 2008; Phan et al., 2009; Pham et al., 2008) showed that BioGro consistently increased grain yield, reduced the need for fertilizer N and P and farmyard manure (FYM), and increased farmers profit from cost savings and yield increase. The trials have been conducted in different soils from northern to the southern Vietnam with similar results. The increases in yield could be the result of Biological Nitrogen Fixation (BNF), P-solubilization, and PGPR.

Criteria proposed by Nguyen Thanh Hien for strain selection are:

They should be the most abundant of their kind in the soil

They should have high activity for a particular desirable character e.g. nitrogen fixation, phosphate solubilizing activity etc. All strains should have PGPR effect

They should be fast-growing allowing growth on non-sterile media

They must be non-pathogenic to plants and animals

They should be reselected periodically to ensure their characteristics are maintained

The effectiveness of BioGro depends on the quality of biofertilizer product (Kennedy and Roughley, 2002). The one negative response in a trial in 2000 emphasized the importance of quality control of the biofertilizer to ensure reliable results. The quality of biofertilizer depends on the number of the cells g^{-1} of carrier and the characteristics of each strain. Details of appropriate checks and standards have been published elsewhere as a quality control manual.

All four strains in BioGro have a PGPR effect –stimulating root development, producing thicker and longer roots with greater dry matter in the seedling stage. Better root development and enhanced nutrient uptake as a result are key factors in expressing the beneficial effects of PGPR (Mia et al., 2002), contributing to the yield increase (Table 5) and allowing a 50% reduction in applied fertilizer.

All rates of BioGro (50, 100, and 200 kg ha^{-1}) applied at transplanting increased grain yield significantly over control at 5% probability level. This suggests that application rates of 50 kg ha^{-1}, supplying similar numbers of root nodule bacteria as when inoculating soybeans (5×10^{11} cfu ha^{-1}) in Australian legume inoculants, could be sufficient. This would further reduce the input costs for farmers however confirmation that commercial biofertilizer products contain sufficient numbers of PGP organisms would be essential.

Because of limitations in replication and variation between the sites in this study, it is difficult to draw definite conclusions from the trial comparing timing of BioGro applications and the amount applied. However it is clear from these results that inoculation had a beneficial effect at all sites, indicated by 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 the timing of BioGro applications are warranted. An interim recommendation would be for a maximum of two applications (nursery plus paddy field) to be made in early stages, a total application of 100 kg ha^{-1}.

Despite evidence for some residual effect of BioGro inoculation on later crop, a new inoculation of the same field in every rice-growing season was shown to be necessary if the maximum benefit from inoculation by BioGro was to be achieved. Because this data is obtained from one site only near Hanoi, more detailed studies will be required to confirm the general applicability of this finding. Because these strains have been isolated from soils where they were a significant component of the soil micro flora and because there is a relatively short interval between successive rice crops in many areas it would be reasonable to expect that some cells would survive in sufficient numbers to have some biological effect on the next crop. However, the principle of such inoccupation (Balandreau, 2002) is the provision of high numbers of specific strains at the same time as the rice roots are developing, providing them with a competitive advantage regarding access to carbon exudates from rice roots. The persistence of BioGro in after rain fed crops would be worthy of further investigation as would the effect of different break crops or fallow on numbers with time but it is anticipated that repeated inoculation is necessary.

Data of Phan and Tran (2008) showed that application of BioGro significantly increased total N and P uptakes by rice. Increase in N uptake might be due to increase in fertilizer N uptake, BNF or improved access and uptake of soil N due a more extensive root system. The fertilizer N uptake can be quantified by using ^{15}N-enriched urea (Choudhury and Khanif, 2001, 2009). The%BNF contribution can be measured by ^{15}N isotope dilution technique (Fried and Middelboe, 1977; Kurdali et al. 2007). Malik et al. (2002) reported that two species of the genus *Pseudomonas* (*Pseudomonas stutzeri* K1 and *Pseudomonas* 96–51) fixed atmospheric N (%Ndfa were 19.5 and 3.0, respectively) while rice seedlings were inoculated with these organisms resulting in significantly increased rice grain yields over control. As BioGro contained 1N (*Pseudomonas fluorescens*), there might be some BNF contribution giving an increase in total N uptake by rice if its ability to fix N_2 can be confirmed. This can be quantified by using the ^{15}N tracer technique. The effect of BioGro on fertilizer N uptake by rice plants could also be quantified by the ^{15}N tracer technique using labeled urea.

In Thanthri, the interaction effect of treatments and variety was not significant in spring 2006 while it was significant in the following season (summer 2006) indicating the seasonal impacts on the effectiveness of

BioGro in different varieties. In general, BioGro has the potential to save 50% NP fertilizers in the spring season. In summer season, the beneficial effect of BioGro was noticed in four varieties (HT1, BT7, KD18, and VD8) while it was not observed in the other two varieties (LT2 and AYT01). The most interesting finding is that in VD8, BioGro with 50% NP gave significantly higher grain yield over 100% NP. 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 amount of grain over KD18 in T2. In general grain yields were higher in the spring compared to summer indicating seasonal impacts on grain production. The intensity of solar energy, which is a seasonal attribute might cause variations in grain yield between the seasons.

In Haihau, The beneficial effect of BioGro was noticed in only one season out of four seasons. The failure of getting the beneficial effect of BioGro in the three seasons needs to be investigated. Full fertilized treatment and 75% NP without BioGro produced statistically similar yields in three seasons (summer 2005, spring 2006, and summer 2006). So, it is recommended to reduce the N fertilizer rate to maximum 90 kg ha^{-1} to evaluate the effects of BioGro on quality rice.

CONCLUSION

These results confirm our earlier data on the effectiveness of applying BioGro to rice (Nguyen et al., 2002, 2003). The effectiveness of BioGro could be a result of PGPR, N fixation, and P-solubilization. Studies based on ^{15}N are recommended for the future to quantify the fertilizer N uptake as well as the possible 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 high numbers of the BioGro strains are present in the biofertilizer product. There are seasonal impacts on the effectiveness of BioGro on different varieties.

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