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DIAZOTROPHIC INOCULATION SUPPLEMENTED NITROGEN DEMAND OF FLOODED RICE UNDER FIELD CONDITIONS

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Supplementing N fertilizer demand of flooded rice (*Oryza sativa* L.) crop by integrated means is important area of research for economical and sustainable agriculture. For present field trial, three soil N rates (60, 90, 120 kg ha^{-1}) were applied to main plots. Before transplantation to randomized subplots, roots of rice seedlings were dipped for 1 h in water (control), *Azospirillum* and *Azotobacter* in 1:1 ratio (diazotrophs) or diazotrophs + 10^{-5} M L-tryptophan (L-TRP). Compared with control of seedling treatment, diazotrophs alone or in combination with L-TRP significantly ($P \leq 0.05$) increased straw and paddy yields at low (60 kg N ha^{-1}) and medium (90 kg N ha^{-1}) rates of N. However, paddy yield was only non-significantly different between 90 kg N ha^{-1} with diazotrophs alone and 120 kg N ha^{-1} with or without diazotrophs. At 90 and 120 kg N ha^{-1}, most of the studied plant growth and nutrient accumulation responses were statistically at par between seedling treatments with diazotrophs and diazotrophs + L-TRP. Nitrogen application was the main factor that influenced N concentration in plant tissues. However, seedling treatments also significantly increased N and P concentration in rice straw and grain. Maximum net value of the produce and value-to-cost ratio were achieved with diazotrophic inoculation at 90 kg N ha^{-1}. Conclusively, diazotrophic inoculation supplemented N demand of flooded rice by potentially fixing atmospheric N_2 and influencing nutrient dynamics in soil.
Keywords: Diazotrophs, L-tryptophan, Nitrogen, Rice

INTRODUCTION

Rice is one of the most important staple food for many developing countries (Rodrigues *et al.,* 2008). Due to price hike, the farmers of developing countries like Pakistan are finding it tough to produces economic yields from their agricultural farms (Meena *et al.*, 2003). Nitrogen is the most important required input for rice and other agronomic crops (Verma *et al.*, 2001). However, applied N is partly lost through different mechanisms; including ammonia volatilization, denitrification, and leaching (Choudhury and Kennedy, 2005). The efficiency of the applied urea-N in rice culture is very low; generally around 30–40% or even lower (Choudhury and Khanif, 2001 and 2004).

For environmental and crop sustainability concerns, the use of plant growth promoting rhizobacteria (PGPR) can play a crucial role (Shoebitz *et al.*, 2009). Plant growth promoting rhizobacteria facilitate plant growth by different means: adding N by biological fixation, releasing trapped nutrients from soil, increasing nutrient mobility to roots and promoting root growth (Marcel *et al.*, 2001) as well as through the solubilization of phosphate minerals (Freitas *et al.*, 1997). In rice growth, PGPR play an important role in many ways, such as by the synthesis of auxins, production of siderophores, solubilization of unavailable forms of various nutrients, synthesis of phytohormones, inhibiting ethylene synthesis, vitamins, enhancing stress resistance, mineralizing organic phosphate, solubilizing inorganic phosphate and improving nutrient uptake (Lucy *et al.*, 2004; Patten and Glick, 2002; Rodrigues and Fraga, 1999).

Effectiveness of symbiotic bacteria has been proved for leguminous crop (Zahir *et al.,* 2010). In non-leguminous crops, such as rice, free living diazotrophs are involved in N fixing asymbiotically and transfer it to plant roots and other plant parts (Saubidet *et al.*, 2002). These free fixers utilize rhizosphere carbon compounds and fix N for the plant. By using free-living diazotrophs, extending biological nitrogen fixation ability of non-legumes may be a useful technology for enhancing crop production (Rothballer *et al.*, 2006). A variety of non-symbiotic bacteria (*Azospirillum, Azotobacter, Bacillus* and *Klebsiella* sp.) are being evaluated worldwide for the same purpose (Naher *et al.*, 2009) and N fixers like *Azospirillum, Azotobacter, Burkholderia* and *Herbaspirillum* are being used in rice culture (Malik *et al.*, 2002).

Plant growth regulators (PGRs) are organic compounds, which have shown extensive impacts on growth of rice. Auxins, one of the five major divisions of PGRs, are naturally synthesized within plants and regulate plant growth at very low concentrations (Khalid *et al.,* 2006). In plants, indole acetic acid (IAA) is the most common auxin (Kende and Zeevaart, 1997). L-tryptophan (L-TRP), which is known as an auxin precursor, is involved in a variety of plant growth and development responses (Frankenberger and Arshad, 1995). Seed inoculation with 10^{-4} and10^{-5} M L-TRP improved 15 and 32% nodular mass plant^{-1}, respectively (Zahir *et al.*, 2010). Abbas *et al.* (2013) reported that seed inoculation with 10^{-4} M L-TRP improved chickpea plant weight and pod weight plant^{-1} up to 186 and 79%, respectively. The effectiveness of tryptophan-dependent auxin biosynthesis has been tested for improving yields of both legumes (Zahir *et al.*, 2010) and non-legumes (Zahir *et al.*, 2005; Naveed *et al.*, 2014).

Keeping in mind the above mentioned facts, a field trial was conducted to evaluate the response of rice to diazotrophic inoculation in presence or absence of L-TRP under varying N fertilizer applications to soil. Main aims of the study were to evaluate: i) potential of diazotrophs in supplementing N demand of rice crop; ii) efficiency of diazotrophs in presence of a plant growth promoter, L-TRP; iii) comparative economics of using diazotrophs and L-TRP treatments over sole application of N fertilization.

MATERIALS AND METHODS

Experimental setup: A field study was conducted at Ayub Agricultural Research Institute, Faisalabad (Pakistan). For pre-sowing soil characterization, randomized soil samples of 0–15 cm depth were composited, air dried, mixed thoroughly and passed through 2 mm sieve. The sandy clay loam had (in g kg^{-1}) 515, sand; 252, silt and 233, clay. Organic matter content, EC_e, pH_s, CEC, total N, Olsen P and extractable K were 8.5 g kg^{-1}, 1.27 dS m^{-1}, 8.1, 5.12 $cmol_c$ kg^{-1}, 0.7 mg g^{-1}, 7.33 mg kg^{-1} and 138 mg kg^{-1}, respectively.

For this experiment, rice (cv. Super Basmatti-2000) seeds were first grown in nursery. Then, five week old seedlings were transplanted to randomized experimental plots after root dipping for 1 h in following three solutions: (i) groundwater control, (ii) diazotrophic inoculum (10^8 cfu mL^{-1}) containing *Azotobacter* and *Azospirillum*in in 1:1 ratio, (iii) and a combination of the diazotrophic inoculum and 10^{-5} M L-TRP solution. The diazotrophic inoculum and L-TRP were kindly provided by Soil Bacteriology Section, Ayub Agricultural Research Institute, Faisalabad (Pakistan) and Soil Fertility and Plant Nutrition Laboratory, University of Agriculture, Faisalabad (Pakistan), respectively.

Soil N rates (60, 90 and 120 kg N ha^{-1}) were applied to main plots (13.0 × 23.5 m^2) whereas root dipping treatments were randomized in subplots (4.0 × 7.5 m^2). All the treatments were replicated thrice. In the experimental plots, rice seedling were sown with row-to-row and plant-to-plant distance of 22.5 cm.

Before transplantation, 80 kg P ha^{-1} as single super phosphate and 60 kg K ha^{-1} as sulfate of potash were applied uniformly to all plots. However, the mentioned N rates were applied as urea in two equal splits: before transplantation and 45 days after transplanting. During the cropping period, plots were flooded with canal water as and when needed. Weeds in all plots were controlled manually.

Agronomical measurements: At crop maturity, grains panicle^{-1} were counted in the field by observing ten homogenous panicles per replication. Harvested crop samples were manually threshed. Straw and paddy yields of each plot were recorded by using weighing balance. After removing rice paddy husk of each sample, 1000-grain weight was estimated.

Nutrient measurements: Straw and grain samples were washed with tap water followed by rinsing in distilled water. Washed samples were dried at 65°C in a forced-hot air driven oven (Eyela WFO-600ND, Tokyo Rikakikai, Tokyo, Japan) up to a constant weight. Plant and grain samples were finely ground with a Wiley mill fitted with stainless steel chamber and blades. Known weights of ground samples (about 0.2 g) were wet digested with sulphuric acid (H_2SO_4) and hydrogen peroxide (H_2O_2) (2:1ratio) by following the method described by Wolf (1982). Nitrogen concentration in the digests was determined by Kjeldhal method (Jackson, 1962). Phosphorus concentration in the samples was determined on UV–visible spectrophotometer (UV-1201, Shimadzu, Tokyo, Japan) at 410 nm after developing yellow color by vanadate-molybdate method (Chapman and Pratt, 1961).

Uptake of N and P in rice straw and grain was calculated by using following formula:

$$\text{Nutrient uptake} = [\text{Nutrient concentration (\%)} \times \text{yield (kg ha}^{-1})]/100$$

Total uptake (straw + grain) of N and P was calculated by adding contents of respective nutrient in straw and grain.

Economic and statistical analysis: For economic analysis of each applied treatments, value-to-cost ratio was calculated by dividing total value of produce (US$) with total expenditure on crop (US$).

All achieved data were subjected to analysis of variance. This was followed by least significant difference (LSD) test to analyze significance of treatment effects (Steel *et al.*, 1997).

RESULTS

Growth and yield: Soil N rates and seedling treatments significantly ($P \leq 0.05$) increased growth and yield attributes of rice crop (Table 1). In comparison with 60 kg N ha^{-1}, respective increase on an average basis at 90 and 120 kg N ha^{-1} was 8 and 12% in grains per panicle, and 14 and 15% in thousand-grain weight.

At 60 and 90 kg N ha^{-1}, diazotrophs and diazotrophs + L-TRP significantly increased straw and paddy yield over control of seedling treatment. At all applied rates of N, however, diazotrophs alone and diazotrophs + L-TRP were statistically at par for straw and grain yields.

Table 1. Grains panicle^{-1}, thousand-grain weight, straw yield and paddy yield of rice as influenced by application of N to soil and seedling treatments of root dipping in diazotrophic inoculum and L-tryptophan solution

Seedling treatments	Grain panicle^{-1}			Thousand-grain weight (g)			Straw yield (t ha^{-1})			Paddy yield (t ha^{-1})		
	Soil N rate (kg ha^{-1})											
	60	90	120	60	90	120	60	90	120	60	90	120
Control	104d	111b-d	121ab	15.5d	17.9a-c	18.7a	8.1d	9.6c	11.3a	2.46d	2.90bc	3.29a
Diazotrophs	110b-d	119a-c	117a-c	16.6b-d	19.0a	18.6a	9.7c	11.1ab	11.5a	2.76c	3.31a	3.15ab
Diazotrophs+L-TRP*	109cd	120a-c	124a	16.4cd	18.3ab	18.9a	10.1bc	11.6a	11.3a	2.84c	3.28a	3.23a

*L-TRP represents L-tryptophan

Table 2. Nitrogen and phosphorus concentrations in straw and grain of rice as influenced by application of N to soil and seedling treatments of root dipping in diazotrophic inoculum and L-tryptophan solution

Seedling treatments	Straw N concentration (mg g^{-1})			Grain N concentration (mg g^{-1})			Straw P concentration (mg g^{-1})			Grain P concentration (mg g^{-1})		
	N application (kg ha^{-1})											
	60	90	120	60	90	120	60	90	120	60	90	120
Control	3.70 d	5.87 b	6.43 ab	4.80 e	6.37 c	6.93 b	1.16 e	1.47 c	1.53 c	1.37 f	1.78 bc	1.74 cd
Diazotrophs	4.73 c	6.47 a	6.90 a	5.47 d	7.25 ab	7.52 a	1.34 d	1.65 ab	1.65 b	1.56 e	1.96 a	1.93 a
Diazotrophs + L-TRP*	4.83 c	6.40 ab	6.57 a	6.03 c	7.48 a	7.48 a	1.37 d	1.72 a	1.62 b	1.60 de	1.92 ab	1.93 a

*L-TRP represents L-tryptophan

Table 3. Nitrogen and phosphorus uptake in rice straw and grain as influenced by application of N to soil and seedling treatments of root dipping in diazotrophic inoculum and L-tryptophan solution

Seedling treatments	Straw N uptake (kg ha^{-1})			Grain N uptake (kg ha^{-1})			Straw P uptake (kg ha^{-1})			Grain P uptake (kg ha^{-1})		
	N application (kg ha^{-1})											
	60	90	120	60	90	120	60	90	120	60	90	120
Control	29.8 c	56.3 b	73.1 a	11.8 d	18.5 b	22.8 a	9.41 d	14.1 c	17.3 c	3.4 f	5.7 bc	5.2 cd
Diazotrophs	45.7 b	71.2 a	78.8 a	15.1 c	24.0 a	23.7 a	13.0 c	18.3 ab	18.9 ab	4.3 e	6.1 ab	6.5 a
Diazotrophs + L-TRP*	48.4 b	74.2 a	74.4 a	17.1 b	24.6 a	24.1 a	13.8 c	20.0 a	18.3 ab	4.6 d	6.2 ab	6.3 a

*L-TRP represents L-tryptophan

Table 4. Total (straw + grain) nitrogen and phosphorus uptake by rice plants as influenced by application of N alone and along with diazotrophs and L-tryptophan

Seedling treatments	Total N uptake (kg ha^{-1})			Total P uptake (kg ha^{-1})		
	N application (kg ha^{-1})					
	60	90	120	60	90	120
Control	42 d	75 b	96 a	13 c	20 b	23 a
Diazotrophs	61 c	95 a	103 a	17 b	24 a	25 a
Diazotrophs + L-TRP*	66 bc	99 a	99 a	18 b	26 a	25 a

*L-TRP represents L-tryptophan

Nitrogen and phosphorus in plant tissues: Soil N rates and seedling treatments significantly ($P \leq 0.05$) increased concentration of N and P in straw and grains of rice plants (Table 2). At 90 and 120 kg N ha^{-1}, concentrations of N and P in rice straw and grain were statistically at par; however, straw and grain nutrient concentrations were significantly greater at these N rates when compared with 60 kg N ha^{-1}. As compared with control treatment of seedlings, diazotrophs significantly ($P \leq 0.05$) increased N concentration in straw (13% increase) and grains (16% increase) of rice plants. Seedling inoculation with diazotrophs alone or in combination with L-TRP resulted statistically similar straw N concentrations. However, significantly greater grain N concentration was achieved with diazotrophs + L-TRP as compared to diazotrophs alone. On the other hand, diazotrophs alone or in combination with L-TRP significantly increased (about 12% increase) straw and grain P concentrations when compared with control of seedling treatments.

Table 5. Value-to-cost ratio on hectare basis

Treatments	Treatment Cost (US$)	Other Cost (US$)	Total Cost (US$)	Total Value (US$)	Value-to-cost ratio
60 kg N ha^{-1}	11	338	349	1109	3.18
60 kg N ha^{-1} + Diazotrophs	15	338	353	1241	3.51
60 kg N ha^{-1} + Diazotrophs + L-TRP*	18	338	356	1280	3.59
90 kg N ha^{-1}	22	338	360	1307	3.63
90 kg N ha^{-1} + Diazotrophs	26	338	364	1490	4.09
90 kg N ha^{-1} + Diazotrophs + L-TRP	29	338	367	1478	4.03
120 kg N ha^{-1}	34	338	372	1479	3.98
120 kg N ha^{-1} + Diazotrophs	37	338	375	1419	3.79
120 kg N ha^{-1} + Diazotrophs + L-TRP	40	338	378	1452	3.84

*L-TRP represents L-tryptophan

At 60 and 90 kg N ha^{-1}, diazotrophic inoculation significantly ($P \leq 0.05$) improved straw and grain N uptake (Table 3). Only at 60 N kg ha^{-1}, seedlings treatment with diazotrophs + L-TRP further improved grain N uptake. At all applied N rates, diazotrophic inoculation significantly improved straw P uptake, however additionally treatment of L-TRP had no further positive effect at any soil applied N rate. At 60 and 120 kg N ha^{-1}, grain P uptake was significantly improved by diazotrophic inoculation. As compared with diazotrophs alone, seedling treatment with diazotrophs + L-TRP significantly increased grain P uptake only at 60 kg N ha^{-1}.

Total (straw + grain) uptake of N and P was also significantly influenced by application of N to soil and seedling treatments (Table 4). Plant N and P uptake in aboveground biomass increased with incremental rates of soil applied N. As compared with control treatment of seedlings, diazotrophs alone or in combination with L-TRP significantly ($P \leq 0.05$) increased N and P uptake in aboveground parts of rice plants only at 60 and 90 kg N ha^{-1}.

Economic analysis of applied treatments: Net value of the produce ranged from US$ 1109 at 60 N ha^{-1} alone to US$ 1490 at 90 N ha^{-1} + diazotrophs (Table 5). Value-to-cost ratio was also maximum (4.09) at the later same treatment where net value of the produce was maximum. Value-to-cost ratio was minimum (3.18) at 60 N ha^{-1} alone where total incurred cost was also minimum. Maximum cost was incurred when 120 kg N ha^{-1} was combined with diazotrophs + L-TRP. At this treatment, achieved value-to-cost ratio was 3.98.

DISSCUSSION

A range of free-living diazotrophic PGPR participate in a synergistic interaction with a variety of crop plants. Biological N_2 fixation by diazotrophic bacteria is a spontaneous process where soil N is limited and the fixed N_2 promotes growth and yield of crop plants (Kennedy *et al.*, 2004). Moreover, the effectiveness of L-TRP dependent auxin biosynthesis has been tested for improving yields of non-legumes (Zahir *et al.*, 2005; Naveed *et al.*, 2014). In present study, however, additional treatment of L-TRP with diazotrophs was unable to further increase in plant growth.

Diazotrophic inoculation improved straw yield, thousand-grain weight, grains $panicle^{-1}$ and grain yield of rice (Table 1). This can be related to better N supply to plants at active tillering stage (Dixit and Patro, 1994) and more availability of N in a fixed form to rice plant by diazotrophic bacteria. Plant panicle length and thousand-grain weight were increased by seedling inoculation with diazotrophs (Anitha and Thangaraju, 2010; Biswas *et al.*, 2000a and b; Govindarajan *et al.*, 2008; Islam *et al.*, 2009; Zahir *et al.*, 2005 and 2007).Therefore, diazotrophs play a decisive role in rice grown in flooded conditions.

Increased in grain and straw N concentration (Table 2) can be related with increased availability of N either directly through N fertilizer application or indirectly through N_2 fixation by diazotrophs (Krishnakumar *et al.*, 2005). Apart from fixing N_2, PGPR may also mineralize organic phosphate or solubilize inorganic phosphates and ultimately may improve P uptake (Lucy *et al.*, 2004). Moreover, diazotrophs promoted root growth and therefore enhanced uptake of nutrients by plant (Panwar and Singh, 2000). In present study, diazotrophic inoculation and N application improved P concentration and contents plant tissues (Table 3). Such positive relation between N and P was previously reported by Krishnakumar *et al.* (2005). In roots, NH_4^+ absorption is coupled with P via symporters at plasma membrane (Surekha *et al.*, 1999). Moreover, H^+ may be released during absorption of NH_4^+ into root cells. The released H^+ may decrease soil pH of rhizosphere and increase mobility and solubility of soil P sorbed in various forms.

Economic analysis of data revealed that maximum outcome was achieved with diazotrophic inoculation at 90 kg N ha^{-1} (Table 5). Based on value-to-cost ratio, Wu *et al.* (2005) and Jilani *et al.* (2007) have also indicated that bio-fertilizers can be complementary to chemical fertilizers and their use can significantly decreased cost of production.

In short, the study strongly advocated diazotrophic inoculation of rice seedlings for economic production of flooded rice. At 90 and 120 kg N ha^{-1}, most of the studied plant growth and nutrient accumulation responses were statistically at par between diazotrophs alone and diazotrophs + L-TRP. Based on the achieved data, it can be concluded that diazotrophic inoculation can partly supplement N demand of rice and such seedling treatments can produce maximize economic yields at medium N rate (90 kg N ha^{-1}).

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