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Effect of some root associative bacteria on germination of seeds, nitrogenase activity and dry matter production by rice plants

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ABSTRACT

An experiment was conducted under laboratory conditions with five efficient strains of root associative bacteria of which two N_2-fixing bacteria *viz.* *Azotobacter* sp. (strain AS_2) and *Azospirillum* sp. (strain AM_3), one phosphate solubilizing *Bacillus* sp. (strain BP_3), one sulphur oxidizing *Thiobacillus* sp. (strain BT_1) and one sulphur mineralizing *Bacillus* sp. (BC_5) isolated from the root surface of rice (*Oryza sativa* L. cv. IR-36) to investigate the effect of combined inoculation of the non-diazotrophic root associative bacteria (BP_3, BT_1 and BC_5) in presence of N_2-fixing bacteria on the rate of germination of rice seeds, nitrogenase activity (C_2H_2 reduction) of the roots and dry matter accumulation of the rice seedlings grown in a synthetic medium for 30 days. Inoculation of non-diazotrophic root associative bacteria in combination with N_2-fixing bacteria significantly stimulated the rate of germination of seeds as well as the nitrogenase activity of the microbes present in the roots and dry matter accumulation of rice seedlings. After 10 days of inoculation, the maximum seed germination (96.3%) was recorded with the combined inoculation of AS_2, AM_3, BP_3 and BT_1. This was followed by the combined inoculation of AS_2, BP_3, BT_1 (92.7%); AS_2, BP_3, BT_1, BC_5 (92.3%); AS_2, BT_1 (90.7%); AM_3, BP_3, BT_1 and BC_5 (90.3%), respectively. Nitrogenase activity of roots and dry matter production of the 30 day old seedlings were increased concurrently and the effects were more pronounced with the combined inoculations of AS_2, AM_3, BP_3, BT_1 followed by AS_2, BP_3, BT_1; AS_2, BP_3, BT_1, BC_5 and AM_3, BP_3, BT_1, BC_5.

Key words: Diazotrophs, Dry matter production, Germination of seeds, Inoculation effect, Nitrogenase activity, Phosphate solubilizer, Rice, Root associative bacteria, Sulphur bacteria, Synthetic medium.

The root system of any higher plant is closely associated with a vast community of active microorganisms which interact with each other for their metabolites. Consequently the fertility of soil and production of the crops are significantly influenced by the proliferation and metabolism of these active microorganisms present in the rhizosphere (Alexander, 1978). In rice rhizosphere, the availability of major plant nutrients *viz.* nitrogen, phosphorus and sulphur, is greatly monitored by the proliferation and activities of N_2-fixing bacteria (Das and Saha, 2005), phosphate solubilizing microorganisms (Das *et al.*, 2003) and sulphur oxidizing/mineralizing microorganisms (Saha *et al.*, 1995) closely associated with the rice roots. Besides transformation of plant nutrients, the rhizosphere microorganisms also elaborate different growth promoting substances (Arshad and Frankenberger, 1998) which are preferably utilized by the associated microbes as well as plant roots for their growth and metabolism. In the present study, an experiment has been conducted to investigate the inoculation effect of two non-symbiotic N_2-fixing bacteria (*Azotobacter* and *Azospirillum*) either alone or in combination with phosphate solubilizing, sulphur oxidizing and sulphur mineralizing bacteria on germination of seeds, nitrogenase activity (C_2H_2 reduction) of the microbes present in the roots and dry matter accumulation by the rice plants grown on a synthetic medium under laboratory conditions.

MATERIALS AND METHODS

Five efficient strains of root associative bacteria *viz.* *Azotobacter* sp. (strain AS_2), *Azospirillum* sp. (strain AM_3) as non-symbiotic N_2-fixing bacteria, *Bacillus* sp. (strain BP_3) as a phosphate solubilizing bacterium, *Thiobacillus* sp. (strain BT_1) and *Bacillus* sp. (BC_5) as sulphur oxidizing and mineralizing bacteria respectively, were isolated from the root surface of rice (*Oryza sativa* L. cv. IR-36) following the methods as outlined by Das and Saha (2003). The bacterial strains were purified in their respective media through repeated sub-culturing and microscopic examination and were identified up to their generic level following the guide of Skerman (1967) as modified by Bowie *et al.* (1969).

Rice seeds (*Oryza sativa* L. cv. IR-36) previously surface sterilized with 0.1% mercuric chloride followed by alternate washing with sterile distilled water and 70% ethyl alcohol, were inoculated with the diazotrophs (strains AS_2 and AM_3) either alone or in combination with the other non-diazotrophic root associative bacteria (BP_3, BT_1 and BC_5) by dipping the seeds in heavy suspensions (containing more than 10^9 cells/ml) of the bacterial cultures for 1 hour followed by drying in shade. The inoculated seeds were then placed on sterile wet filter paper in sterile petriplates and were incubated at 25 ± 1ºC temperature and 95 ± 1% humidity in presence of sufficient light in a growth chamber (Bittencourt *et al.*, 1995) for 10 days. There were all together 27 treatments including the uninoculated control. Each treatment was replicated thrice. The number of germinated seeds was counted after 3, 5, 7 and 10 days of incubation.

An agar tube culture experiment with the germinated rice seeds was conducted with the inoculation of efficient bacterial strains, having the same treatment as stated above. After germination, one seedling from each treatment was inoculated with the respective inoculants combinations by dipping rice roots in bacterial suspensions as stated above for 1 hour followed by air drying in shade for 30 minutes. Seedlings inoculated with bacterial cultures were then transplanted separately in 50 ml sterile semisolid plant nutrient agar medium (Murasighe and Skoog, 1962) in culture tubes under laboratory conditions. The composition of the medium was (per liter): NH_4NO_3, 1.65 g; KNO_3, 1.9 g; $CaCl_2,2H_2O$, 440 mg; $MgSO_4,7H_2O$, 370 mg; KH_2PO_4, 170 mg; KI, 0.83 mg; H_3Bo_4, 6.2 mg; $MnSO_4,4H_2O$, 22.3 mg; $ZnSO_4,7H_2O$, 8.6 mg; $Na_2MoO_4,2H_2O$, 0.25 mg; $CaCl_2,6H_2O$, 0.025 mg; $FeSO_4,7H_2O$, 27.8 mg; $CuSO_4,5H_2O$, 0.025 mg; Na_2-EDTA, 37.3 mg; inositol, 100 mg; nicotinic acid, 0.5 mg; pyridoxine-HCl, 0.5 mg; thiamin, 0.1 mg; IAA, 1-30 mg; kinetin, 0.4 -10 mg; glycine, 2 mg; sucrose, 30 g and agar 8 g. The pH of the medium was adjusted to 5.7. After 30 days of transplanting, the seedlings were carefully uprooted from the medium and the roots were washed with sterile distilled water. Thereafter, nitrogenase activity of the roots was determined through acetylene reduction assay (ARA) (Ghosh and Saha, 1993), with the help of a gas chromatograph (HP model 5730A) fitted with a glass column packed with porapak R (80-100 mesh) and equipped with a flame ionizing detector. The operating temperature of the oven and the flow rate of carrier gas (N_2) were adjusted to 80ºC and 60 ml/min, respectively. The dry matter content of the 30-day old rice plants was also determined following standard methods.

RESULTS AND DISCUSSION

Effect on seed germination

Inoculation of *Azotobacter* (strain AS_2) and *Azospirillum* (strain AM_3) singly or in combination with the phosphate solubilizing *Bacillus* (strain BP_3), sulphur oxidizing *Thiobacillus* (strain BT_1) and sulphur mineralizing *Bacillus* (strain BC_5), significantly stimulated the germination of rice seeds as compared to uninoculated control (Table 1). The bacterial combinations AS_2, AM_3, BP_3, BT_1 and BC_5 significantly increased the germination of seeds from 3rd day onwards. In general, the maximum germination of seeds was recorded with the inoculation of the bacterial strains after 5 and 7 days of inoculation and the combined inoculation of phosphate solubilizing *Bacillus* (strain BP_3), sulphur oxidizing *Thiobacillus* (strain BT_1) and sulphur mineralizing *Bacillus* (strain BC_5) with *Azotobacter* (strain AS_2) and/or *Azospirillum* (strain AM_3) showed better effect as compared to their single inoculation. This indicated that non-symbiotic N_2-fixing bacteria along with other root associative non-diazotrophic bacteria (strains BP_3, BT_1 and BC_5) increased the availability of nitrogen, phosphorus and sulphur as well as some growth promoting substances released by the microorganisms (Park *et al.*, 2005) in the vicinity of the seeds which subsequently stimulated the germination of rice seeds to a greater extent. It was also revealed that the effect of the diazotrophs (strains AS_2 and AM_3) on seed germination was more pronounced when they were in association with the other root associative non-diazotrophic bacteria responsible for releasing mineral phosphorus, sulphur and other growth promoting substances for the growth and metabolism of the rhizosphere microorganisms as well as the young seedlings germinating from the inoculated seeds. Similar observations were also reported by earlier workers (Thakuria *et al.*, 2004). After 10 days of inoculation, the maximum seed germination (96.3%) was recorded with the combined inoculation of $AS_2 + AM_3 + BP_3 + BT_1$. This was followed by the combined inoculation of $AS_2 + BP_3 + BT_1$ (92.7%), $AS_2 + BP_3 + BT_1 + BC_5$ (92.3%), $AS_2 + BT_1$ (90.7%) and $AM_3 + BP_3 + BT_1 + BC_5$ (90.3%), respectively.

Effect on nitrogenase activity (C_2H_2 reduction)

Inoculation of *Azotobacter* (strain AS_2) and *Azospirillum* (strain AM_3) either alone or in combination with phosphate solubilizing *Bacillus* (strain BP_3), sulphur oxidizing *Thiobacillus* (strain BT_1) and sulphur mineralizing *Bacillus* (strain BC_5) increased the nitrogenase activity (C_2H_2 reduction) of the microbes present in the roots of the rice seedlings as compared to the uninoculated control (Table 2) and the stimulation was more pronounced when the diazotrophs were inoculated in combination with the other non-diazotrophic root associative bacteria (BP_3, BT_1 and BC_5) rather than their single inoculation. This indicated that phosphate solubilizing and sulphur oxidizing/mineralizing bacteria had a proto-cooperative association with the non-symbiotic N_2-fixing bacteria (*Azotobacter* and *Azospirillum*) in the root rhizosphere of rice plants resulting in an increase in their activities which augmented the nitrogenase activity of the diazotrophs (strain AS_2 and AM_3) in the root zone to a great extent (Das and Saha, 2005). This was in agreement with the earlier workers (Park *et al.*, 2005) who reported that the versatile groups of microorganisms isolated from the rice rhizosphere had a significant effect on the nitrogenase activity of the rice roots when the organisms were inoculated in combination. As compared to uninoculated control, the highest increase in nitrogenase activity was recorded (185.9%) in the rice roots when the seedlings were inoculated with AS_2, AM_3, BP_3 and BT_1 in combination. This was followed by the combined inoculation of $AS_2 + BP_3 + BT_1$ (165.9%), $AS_2 + BP_3 + BT_1 + BC_5$ (127.4%) and $AM_3 + BP_3 + BT_1 + BC_5$ (87.4%), respectively. It was also revealed that the effect of *Azotobacter* (strain AS_2) in association with other non-diazotrophic bacteria was more pronounced than that of *Azospirillum* (strain AM_3). This indicated that the activity of *Azotobacter* was highly stimulated due to the presence of other root associative non-diazotrophic bacteria that could have released more amounts of phosphorus, suluphur and other growth promoting substances to the diazotrophs as well as to the plants for their growth and metabolism (Park *et al.*, 2005). This supported the existence of more compatible association of the non-diazotrophic root associative bacteria with *Azotobacter* than that with *Azospirillum*.

Effect on dry matter production

Sustaining the earlier reports (Thakuria *et al.*, 2004), the dry matter production of the rice plants were concomitantly increased with the nirogenase activity of the rice roots. This indicated that greater N_2-fixation together with greater solubilization of insoluble phosphates, oxidation of inorganic sulphur and mineralization of organic sulphur in the root rhizosphere of the rice plants by the active microbes significantly increased the availability of nitrogen, phosphorus and sulphur in the root zone (Table 2). This accelerated the metabolic activities of the plants resulting greater accumulation of dry matter content of the plant as compared to the uninoculated control during the incubation period. Among the treatments, the maximum increase in the accumulation of dry matter was recorded with the inoculation combination AS_2, AM_3, BP_3 and BT_1 (77.5%) followed by AS_2, BP_3 and BT_1 (76.1%), AS_2, BP_3, BT_1 and BC_5 (60.6%), and AM_3, BP_3, BT_1 and BC_5 (36.6%), respectively as compared to the uninoculated control.

The results of the present investigation thus clearly indicated that the inoculation of

Table 1 Effect of inoculation of diazotrophs and some root associative bacteria on the germination (in per cent) of rice seeds

Treatments	Incubation period in days			
	3	5	7	10
Control (uninoculated)	28.7	30.7	56.0	75.3
Azotobacter (strain AS_2)	25.0	57.3	76.0	82.0
Azospirillum (strain AM_3)	23.3	65.3	79.0	79.3
P-solubilizing *Bacillus* (strain BP_3)	26.3	52.3	78.3	85.0
S-oxidizing *Thiobacillus* (strain BT_1)	23.3	69.7	76.7	81.7
S-mineralizing *Bacillus* (strain BC_5)	24.3	61.3	77.7	84.3
$AS_2 + AM_3$	29.3	73.0	79.0	82.3
$AS_2 + BP_3$	27.7	75.3	82.3	85.3
$AS_2 + BT_1$	24.0	75.7	86.7	90.7
$AS_2 + BC_5$	26.0	65.0	75.0	83.0
$AM_3 + BP_3$	22.3	67.7	73.7	84.0
$AM_3 + BT_1$	27.7	69.0	82.0	87.7
$AM_3 + BC_5$	23.3	71.0	83.0	85.7
$AS_2 + AM_3 + BP_3$	25.0	73.7	81.7	86.7
$AS_2 + AM_3 + BT_1$	23.3	56.7	75.3	85.0
$AS_2 + AM_3 + BC_5$	22.7	64.0	74.6	85.7
$AS_2 + BP_3 + BT_1$	38.0	84.0	88.7	92.7
$AS_2 + BP_3 + BC_5$	25.7	70.7	84.3	87.7
$AS_2 + BT_1 + BC_5$	27.0	68.0	78.0	83.0
$AM_3 + BP_3 + BT_1$	25.0	70.0	81.0	84.7
$AM_3 + BP_3 + BC_5$	23.0	69.0	75.0	78.3
$AM_3 + BT_1 + BC_5$	22.0	59.3	72.7	80.0
$AS_2 + AM_3 + BP_3 + BT_1$	39.7	85.0	87.7	96.3
$AS_2 + AM_3 + BP_3 + BC_5$	25.0	63.7	73.0	79.7
$AS_2 + BP_3 + BT_1 + BC_5$	29.3	77.3	85.7	92.3
$AM_3 + BP_3 + BT_1 + BC_5$	26.0	64.3	81.0	90.3
$AS_2 + AM_3 + BP_3 + BT_1 + BC_5$	22.7	65.0	78.0	85.7
Mean	26.1	66.8	78.6	84.9
CD at 5%	Treatments 3.3; Incubation days 1.9; Interaction 10.1			

Table 2 Effect of inoculation of diazotrophs and some root associative bacteria on nitrogenase activity (C_2H_2 reduction) of roots and dry matter production of rice seedlings grown in a synthetic medium

Treatments	C_2H_2 reduced by roots (nmol/100 mg/hr)	Dry matter produced (30 DAS) (mg/plant)
Control (uninoculated)	135	71
Azotobacter (strain AS_2)	190	99
Azospirillum (strain AM_3)	173	94
P-solubilizing *Bacillus* (strain BP_3)	162	83
S-oxidizing *Thiobacillus* (strain BT_1)	169	87
S-mineralizing *Bacillus* (strain BC_5)	165	88
$AS_2 + AM_3$	211	101
$AS_2 + BP_3$	144	93
$AS_2 + BT_1$	182	92
$AS_2 + BC_5$	148	77
$AM_3 + BP_3$	135	102
$AM_3 + BT_1$	183	79
$AM_3 + BC_5$	171	82
$AS_2 + AM_3 + BP_3$	200	76
$AS_2 + AM_3 + BT_1$	175	86
$AS_2 + AM_3 + BC_5$	204	85
$AS_2 + BP_3 + BT_1$	359	125
$AS_2 + BP_3 + BC_5$	233	90
$AS_2 + BT_1 + BC_5$	194	98
$AM_3 + BP_3 + BT_1$	147	99
$AM_3 + BP_3 + BC_5$	181	99
$AM_3 + BT_1 + BC_5$	159	103
$AS_2 + AM_3 + BP_3 + BT_1$	386	126
$AS_2 + AM_3 + BP_3 + BC_5$	179	97
$AS_2 + BP_3 + BT_1 + BC_5$	307	114
$AM_3 + BP_3 + BT_1 + BC_5$	253	111
$AS_2 + AM_3 + BP_3 + BT_1 + BC_5$	192	78
CD at 5%	39.3	11.2

root associative bacteria significantly stimulated the germination of seeds, nitrogenase activity (C_2H_2 reduction) of the microbes present in the roots and the dry matter accumulation by rice seedlings grown in a synthetic medium. It was also revealed that the combined inoculation of the organisms responded better than their single inoculation and the response was more pronounced when the diazotrophic bacteria (*Azotobacter* than *Azospirillum*) were inoculated in combination with the non-diazotrophic bacteria (phosphate solubilizing *Bacillus*, *Thiobacillus* and sulphur mineralizing *Bacillus*). Between the two non-symbiotic N_2-fixing bacteria, the compatibility of the non-diazotrophic bacteria was better with *Azotobacter* than with *Azospirillum*.

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