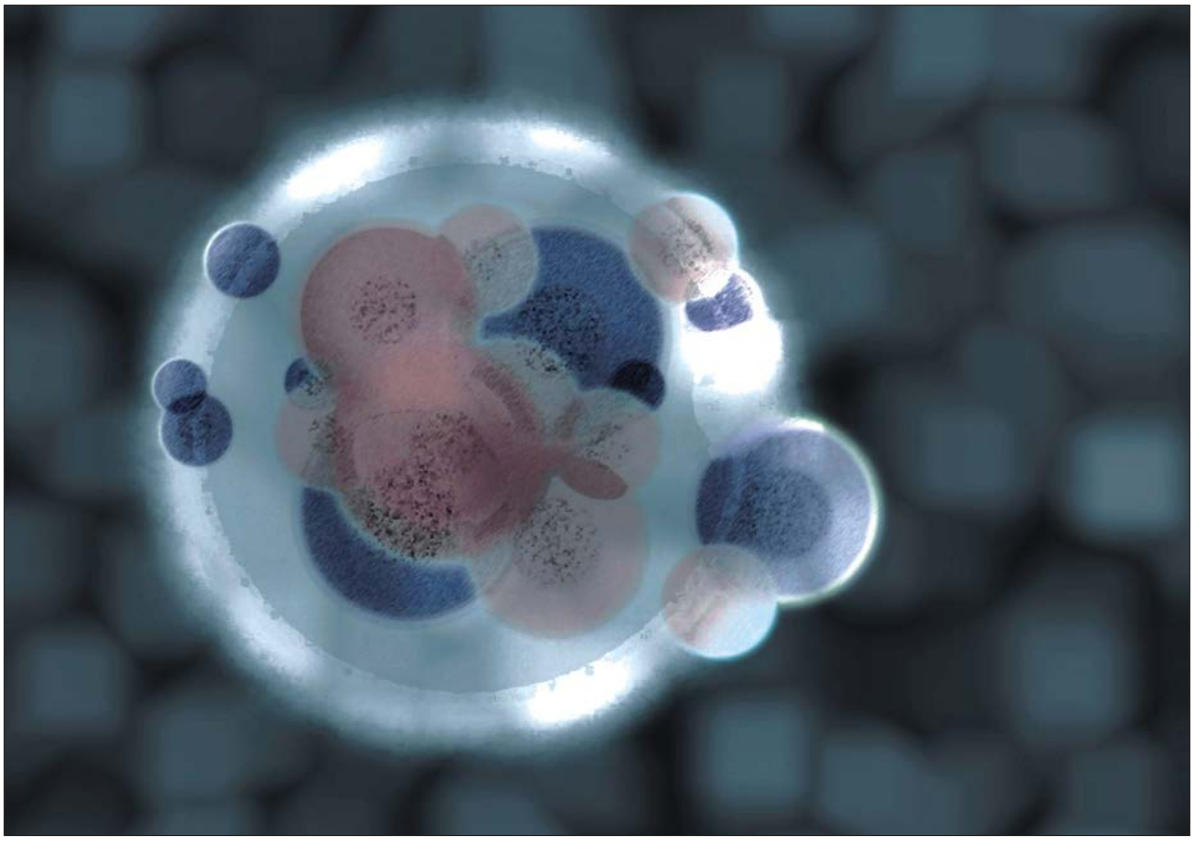

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Impact of Bio Inoculants Consortium on Rice Root Exudates, Biological Nitrogen Fixation and Plant Growth

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Abstract: The present study was conducted to investigate the effect of individual and microbial consortium viz., *Azospirillum lipoferum*-Az 204, *Bacillus megaterium* var. *phosphaticum* and *Pseudomonas fluorescens* Pf-1 on rice root exudates and plant growth under hydroponic culture conditions. Detailed investigations were made on the impact of bio-inoculants application on the influence of crop growth through production of total sugars, reducing sugars, amino nitrogen content, plant growth promoting substances in the root exudates and biological nitrogen fixation capacity. Through this study we have identified, the bioinoculants consortium improves the colonization potential, sustainability within the inoculants and enhances crop growth. We hypothesize that microbial consortium enhances plant growth positively by a multitude of synergistic mechanisms when compared to single inoculants application.

Key words: Root exudates, microbial consortium, plant growth promoting bacteria, hydroponics culture, $^{15}N_2$ studies, rice

INTRODUCTION

The chemicals secreted into the soil by roots are broadly referred to as root exudates. Through the exudation of a wide variety of compounds, roots regulate the soil microbial community in their immediate vicinity, encourage beneficial symbioses and change the chemical and physical properties of the soil (Nardi *et al*., 2000). Root exudates induce chemotactic responses which are essential in the early establishment, colonization and maintenance of rhizospheric populations. Under *in vitro* conditions, rice root exudates were found to exert a strong influence on motility of these bacteria towards plant roots (Bacilio-Jimenez, 2003).

A study conducted under gnotobiotic conditions has identified the plant root exudates as the source of nutrients and signaling molecules for efficient *Pseudomonas fluorescens* colonization in tomato crop (Simons *et al*., 1996). The bioinoculants influence growth and development of crop plants by influencing the physiological status such as phytohormone production (Chabot *et al*., 1996), N_2 fixation (Bashan and Hilguin, 1997), nutrient uptake and efficient use of nutrients (Chabot *et al*., 1996), antibiosis against phytopathogens (Handlesman and Staab, 1996), secretion of siderophores (Neilands and Leong, 1986), inducing systemic resistance (Tuzun and Kloepper, 1994) and morphological characteristics of inoculated roots (Biswas, 1998).

Free-living diazotrophs have been reported to improve nutrient uptake efficiency and to fix N_2 through associative and endophytic associations with graminaceous plants (Bashan and Hilguin, 1997). Nitrogen fixation and nitrogen use efficiency has a significant role because of its potential importance in sustainable agriculture, especially in cropping systems involving rotations of rice and legumes. PGPR benefits crop by their associative N_2 fixing activity and by their ability to change the phytohormone balance, thereby influencing plant physiology in ways that affect major nutrient uptake.

Soil acts as a habitat for diverse group of microorganisms. Plant root exudates enriched rhizosphere region of the soil attracts a variety of microorganisms. A strong interaction prevails between the group of microorganisms colonizing the rhizosphere region and plant roots. Bioinoculants, that can cater the different needs of growing plants act as a consortium along with other microorganisms in the rhizosphere. Understanding the interactions between consortium of microbial inoculants and plant systems will pave way to harness more benefits from microbial inoculants for improving plant growth and yield. Even though several studies were conducted about the interaction between bacterial

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inoculants and plant root exudates, studies on the interaction between microbial consortium and plant root exudates are very rare. In this context the study was undertaken as two experiments.

Both of the experiments were conducted without nitrogen source in the rooting media of plants with the objectives:

- To analyze and compare the ability of individual microbial inoculants and microbial consortium to effect uptake of N_2 nutrient, phytohormones production and growth as possible mechanisms of plant growth promotion in this beneficial PGPR - rice association.
- To test individual inoculants for their colonization potential and sustainability in the rhizoplane.
- To find out the changes in the composition of root exudates in response to bacterial inoculation.
- To assess the potentials and synergistic effect of microbial consortium on biological nitrogen fixation (BNF) through $^{15}N_2$ studies.

The effect of different microbial inoculants viz., *Azospirillum lipoferum, Bacillus megaterium* and *Pseudomonas fluorescens* and the consortium of the above three inoculants on rice root exudates and growth was studied under hydroponic conditions. Impact of bioinoculants on biological nitrogen content was studied through $^{15}N_2$ isotope tracer technique using acid washed sand as rooting media under aseptic conditions.

MATERIALS AND METHODS

Studies were conducted using the experimental facilities of Centre for Advanced Studies in Agricultural Microbiology, Tamil Nadu Agricultural University, Coimbatore, India in spring season of the year 2004.

Bacteria and seed material: Rice (*Oryza sativa* L. var. Co 43) seeds were obtained from the paddy breeding station, department of Agronomy, Tamil Nadu Agricultural University, India. The bacterial cultures *Azospirillum lipoferum*-Az 204, *Bacillus megaterium* var *phosphaticum* and *Pseudomonas fluorescens* Pf-1 were obtained from the culture collection centre of Department of Agricultural Microbiology, Tamil Nadu Agricultural University, India.

Germination of rice seeds: Uniform disease free rice (*Oryza sativa* L. var. Co 43.) seeds were dehulled gently, treated with ethanol (70%) for 3 min, rinsed three times with water, soaked for 3 min in a mixture of 0.1% HgCl and 3.0% NaCl, rinsed four times with water, soaked in water for an additional 4 h to completely remove sterilants and finally washed four more times with sterile water. Surface-sterilized seeds were germinated at 28°C on nutrient soft agar (0.8% agar) in an incubator for 3 days. Germinated seeds were used in the study as germinated seeds behave more reproducibly and similarly than using nongerminated seeds as such.

Specially designed apparatus for collection of root exudates-Hydroponic system: For the collection of root exudates, specially designed apparatus was used (Lee and Gaskins, 1982). All the operations for obtaining microbiologically controlled, hydroponic cultures of rice were performed using sterile materials and aseptic techniques at room temperature. Hydroponic cultures were prepared using autoclaved test tubes of diameter and length (40 and 500 mm, respectively) containing an strip of stainless steel mesh (size 20) positioned at the groove (5 cm from the top of the tube) on the upper fluid level. Hundred milliliter of Fahraeus N-free liquid plant growth medium (Fahraeus, 1957) was dispensed into each tube and capped with nonabsorbent cotton plugs for easy flow of filtered air and the entire set up was sterilized as such in an autoclave (Fig. 1).

Compatibility test: The bacterial cultures were investigated for their compatibility with each other by cross streak assay method. Nutrient agar medium was prepared, autoclaved and plated. Test organism was streaked at one end as a single streak and the plates incubated at 32°C for 48 h. After a robust growth of the test organism, the other cultures were streaked vertically to the test organism and plates were incubated at 32°C for one week.

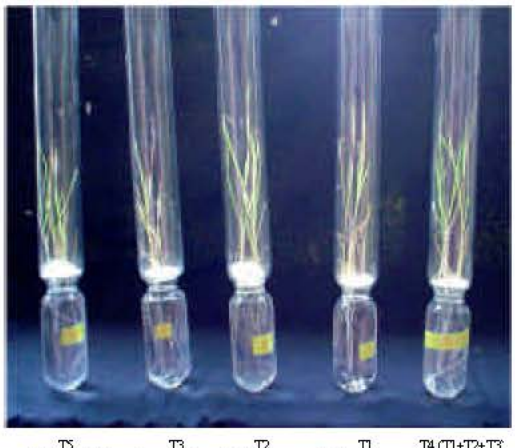

Fig. 1: Collection of root exudates under hydroponic culture conditions

Inoculation of bacterial cultures: Log phase cultures of *Azospirillum lipoferum*-Az 204 (N free malic acid broth) (Dobereiner, 1980), *Bacillus megaterium* var. *phosphaticum* PSB-1 (Pikovaskya's broth) (Pikovaskya, 1948) and *Pseudomonas fluorescens* Pf-1 (Liquid King's medium B (KB) (King *et al*., 1954) were grown in respective media and centrifuged at 10000 rpm for 10 min, the cell pellet washed in sterile phosphate buffered saline (0.14 M NaCl, 0.003 M KCl, 0.005 M Na_2HPO_4, 0.002 M KH_2PO_4; pH 7.0) and diluted to a final concentration of 10^6 CFU mL^{-1} in sterile phosphate buffered saline. In each tube three contamination free germinated seeds were placed on the steel mesh at the meniscus level so that the roots would have contact and grow into the medium. The roots of 3 day old plants were inoculated with 1 mL bacterial culture containing 10^6 CFU mL^{-1} as per the treatment schedule. For combined inoculation treatment, 1 mL of each of the inoculants was added to the base of the roots. Uninoculated plants were grown axenically as the control treatment. Ten tubes were used for each treatment. Enclosed tube cultures were grown in a growth chamber for 1 month, at 28°C, (14/10 h day/night cycles, 80% humidity).

Collection of root exudates: Root exudates were collected individually from each tube, clarified by centrifugation at 3000×G for 10 min, reduced to one tenth volume by freeze drying and used for further analysis.

Biochemical analysis of root exudates at 30th day after inoculation

Determination of total soluble sugars: To 1 mL of root exudate, 4 mL of freshly prepared anthrone reagent (200 mg anthrone in 100 mL of ice cold 95% H_2SO_4) was added. The tubes were placed in a boiling water bath for 10 min and then cooled. The absorbance of the resulting blue green solution was read at 630 nm in a DU-64 spectrophotometer against water blanks. Total soluble sugars present in the sample was calculated by referring to a standard curve prepared with glucose.

Estimation of reducing sugars and amino nitrogen content: Amount of reducing sugars in root exudates was estimated by Nelson- Somogyi method (Somogyi, 1952) and amino nitrogen content was estimated as described by Moore and Stein (1948).

Estimation of Indole Acetic Acid (IAA): The amount of IAA present in the root exudates was estimated by colorimetric method. Supernatants were obtained after centrifugation of root exudates at 10,000 *g* for 5 min. Two milliliter of Salkowski reagent (Gordon and Weber, 1951) (prepared with perchloric acid) was added to 1 mL of supernatant in a small glass test tube and incubated at room temperature for 30 min. The optical density of the solutions was quantified using a Beckman DU-64 spectrophotometer at 530 nm.

Estimation of gibberellic acid (GA): Gibberellic acid content in the root exudates was estimated spectrophotometrically by the method of Mahadevan and Sridhar (1982).

Determination of cytokinins: Cytokinin content of the root exudates was estimated by HPLC as described by Tien *et al.* (1979). HPLC system (Hitachi) equipped with an L6200 intelligent pump, D 2500 chromate integrator, C-18 ODS 2 stainless steel column was used in the study. Samples were injected into the HPLC with a solvent gradient of 30% methanol in water at a flow rate of 1.5 mL min^{-1} and the operating pressure was 1600 pounds sq $inch^{-1}$. Elutes were analyzed with UV-vis detector. Cytokinins were quantified by reference to the peak area authentic zeatin and kinetin standards (Sigma Chemical Co.).

Population dynamics at different days of observation: Microbial population from the root exudates was enumerated at 0, 15 and 30th days after inoculation. *Azospirillum lipoferum* Az 204 was enumerated through serial dilution and most probable number technique (Dobereiner, 1980). Population of Bacillus megaterium and *Pseudomonas fluorescens* was estimated through standard serial dilution and plate count method.

Biometric observations: After one-month time, the seedlings were removed and plant growth parameters of seedlings were recorded. The root length was measured from the base of the plantlet where the rooting starts to the tip of the longest root and expressed as cm $plant^{-1}$. The shoot length was measured from the base of the plantlet to the tip of the growing point and expressed as cm $plant^{-1}$. After 30 days the plant was carefully uprooted and washed free of root debris. Plant dry weight was obtained by keeping the plants in an oven at 60°C until reaching a constant weight.

Chlorophyll content: Chlorophyll content was determined as described by Wintermans and Demots (1965). One gram of leaf sample was homogenized and extracted with 95% ethanol and centrifuged. The absorbance of the extract was measured at 663 and 645 nm in a Beckman DU-64 spectrophotometer and results were expressed as mg g^{-1} of tissue.

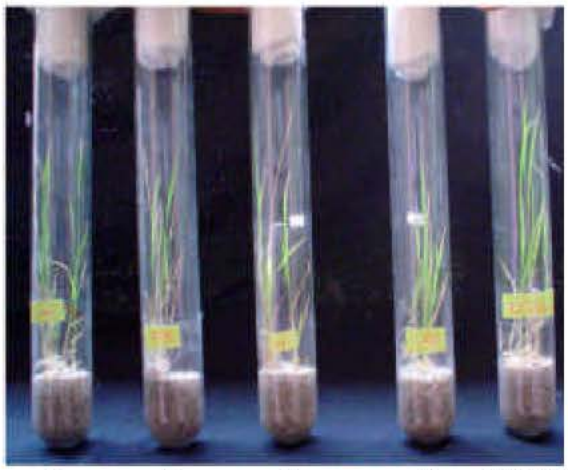

Fig. 2: $^{15}N_2$ studied to estimate the biological nitrogen fixation by inoculants

$$\text{Totalcholorophyll} = 20.0(A_{645}) + 8.02(A_{663}) \times \frac{V}{1000 \times W}$$

Where,

A = Absorbance at specific wavelengths
V = Final volume of chlorophyll extract in 95% ethanol
W = Fresh weight of tissue extracted

$^{15}N_2$ studies: For estimating total biological nitrogen fixation by the inoculants, $^{15}N_2$ urea was used (9.342 $^{15}N_2$ atom% excess) as a tracer. To Glass tubes (5×45 cm), 100 g of acid washed, sterile sand and 50 mL of plant nutrient solution (Fahreus, 1957) was added, plugged with cotton and autoclaved as such (Fig. 2). Seven surface sterilized pre germinated rice seeds were placed in each tube. Bacterial cultures were inoculated to each tube as per the treatment schedule as described earlier in the same paper. Filter sterilized stock solutions of $^{15}N_2$ urea was added to each tube to a final concentration of 10 ppm of $^{15}N_2$ in growth media. The experimental setup was incubated in a growth chamber for one-month period at 28°C (14/10 h day/night cycles, 80% humidity).

The whole plant was homogenized from which 1 g of plant samples was digested with appropriate reagent as per kjeldahl's method (Bremner, 1965) to convert organic nitrogen into ammoniacal form. On the completion of nitrogen distillation, ammonia was absorbed in 2% boric acid solution and titrated against 0.1 N H_2SO_4 to quantify and convert nitrogen to the form of ammonium sulphate. Later an excess of 1 mL of 0.1 N H_2SO_4 was added to acidify the contents. The acidified boric acid solution was evaporated at 60°C to cooled and stored in glass vials. Mass spectrometer (Micromass 622 V.G. isogas) was used for the $^{15}N_2$ assay and the ratio analysis was done as described elsewhere (Buresh *et al*., 1982).

Statistical analysis: The data were analyzed by Analysis of Variances (ANOVA) and the means were compared following Fisher's test of Least Significant Difference (LSD) to assess the effects of inoculation on plant biometric observations.

RESULTS

All the bioinoculants tested are compatible: All the inoculants used in the study were compatible with each other. In the cross streak assay, no inhibition zone around the colonies of each inoculant was found.

Root exudates content is altered by bioinoculants: Bioinoculants application has brought considerable change in the root exudates content (Table 1). In the absence of bioinoculants, fewer amounts of total sugars and negligible amount of amino nitrogen content was detected. Where as, when bioinoculants were applied, with increase in number of days, plants exert more amounts of organic substances in root exudates. Total sugars, reducing sugars and amino nitrogen content in the consortium applied treatments are higher than in

Table 1: Biochemical analysis of root exudates

Treatments	Total sugars (mg 10 mL^{-1})	Reducing sugars (mg 10 mL^{-1})	Amino nitrogen (mg 10 mL^{-1})
T_1 - *A. lipoferum*	4.2	1.5	0.50
T_2 - *P. fluorescens*	4.8	1.8	0.40
T_3 - *B. megaterium*	3.8	1.1	0.02
T_4 - Microbial consortium (T_1 + T_2 + T_3)	6.2	3.7	0.70
T_5 - Un inoculated control	2.3	0.7	0.01
SED	0.6542	0.1853	0.1247
CD (0.05)	1.50	0.4274	0.2877

Table 2: Estimation of plant growth promoting substances in root exudates

Treatments	IAA (μg mL^{-1})	GA (μg mL^{-1})	Cytokinin (ppm) Zeatin	Kinetin
T_1 - *A. lipoferum*	1.85	1.65	4.36	0.35
T_2 - *P. fluorescens*	2.40	1.95	6.55	0.60
T_3 - *B. megaterium*	0.45	0.35	2.53	0.15
T_4 - Microbial Contsortium (T_1 + T_2 + T_3)	2.65	2.52	7.15	0.65
T_5 - Un inoculated	0.25	0.08	0.24	0.05
SED	0.2288	0.1903	0.7469	0.1572
CD (0.05)	0.5277	0.4389	1.7223	0.3626

Table 3: Plant biometric observations

Treatments	Root length (cm)	Shoot length (cm)	Dry weight (mg plant^{-1})	Chlorophyll content (mg g^{-1})
T_1 - *A. lipoferum*	12.25	15.50	630	2.09
T_2 - *P. fluorescens*	8.20	15.30	580	2.03
T_3 - *B. megaterium*	4.30	6.10	227	0.63
T_4 - Microbial consortium (T_1+T_2+T_3)	20.30	16.30	720	2.30
T_5 - Un inoculated control	3.70	4.60	185	0.45
SED	2.28	2.96	127.66	0.2492
CD (0.05)	5.25	6.83	294.40	0.5748

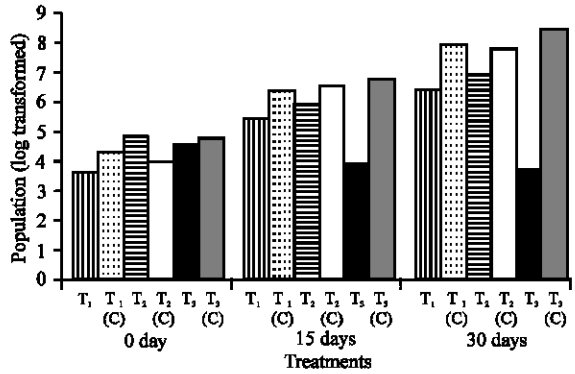

Fig. 3: Population of the inoculants in different treatments T_1 - *A. lipoferum*; T_2(C) - *A. lipoferum* (in consortium); T_2 - *P. fluorescens*; T_2 (C) - *P. fluorescens* (in consortium); T_3-*B. megaterium*; T_3(C) - *B. megaterium*

individual inoculant applied treatments. Diazotrophs applied treatments ((T_1, T_2 and T_4) recorded higher amounts of total sugars and amino nitrogen content. Non-diazotrophic inoculant (*B. megaterium*) has brought considerable increase in total sugars, but increase in amino nitrogen content was not detected (T_3).

Plant growth promoting substances found more in consortium treatment: Bioinoculants application has brought considerable increase in plant growth substances like IAA, GA and Zeatin and kinetin types of cytokinins. Even though, the same compounds were detected in uninoculated control treatment, bioinoculants application has brought several folds increase in growth promoting substances content. Among the individual bioinoculants treatments, *P. fluorescens* has brought higher amount of IAA (2.40 µg), GA (1.95 µg), zeatin (6.55 ppm) and kinetin (0.60 ppm). The results were followed by *A. lipoferum* and *B. megaterium* treatments (Table 2). Application of consortium of inoculants has brought more increase in plant growth promoting substances than any of the individual inoculants and the increase in IAA content over individual inoculant treatments was minimal.

Population dynamics changes over period: In the initial days after inoculation, the population increase was very minimal and it gradually increases up to 15 days (Fig. 3). After which there is a significant increase till it reached a maximum at 30 days after inoculation. At 30 days after inoculation, *B. megaterium* alone applied treatment recorded lowest population (4.66×10^3 CFU mL^{-1}) and in microbial consortium treatment (T_4), the same inoculant (*B. megaterium*) recorded the maximum population at all days of observation (6.29×10^4, 5.64×10^6, 0.35×10^8 CFU mL^{-1} at 0, 15 and 30th day, respectively). Like wise, the population of *Azospirillum* and *Pseudomonas* was less in the individual inoculant applied treatment when compared to the microbial consortium (0.43×10^6, 8.33×10^6 CFU mL^{-1}, respectively at 30 days after inoculation).

Consortium of inoculants brings in more plant growth: The results of the present experiment indicated positive response and significant increase in plant biometric parameters in bioinoculants applied treatments (Table 3).

Different treatments indicated variations in plant biometric observations. Maximum root length, shoot length, dry weight and chlorophyll content (20.30 cm, 16.30 cm, 720 mg plant^{-1} and 2.30 mg g^{-1}, respectively) were observed in plants inoculated with microbial consortium (T_4). Among the s ingle inoculants, *A. lipoferum* applied treatment has recorded maximum root length, shoot length, dry weight and chlorophyll content (12.25 cm, 15.50 cm, 630 mg plant^{-1} and 2.09 mg g^{-1}, respectively).

Plants get more nitrogen from consortium of inoculants: $^{15}N_2$ study conducted for the determination of biological nitrogen content revealed that, inoculation of biofertilizers increased the nitrogen content of the seedlings. Highest total plant nitrogen content (0.337%) and biological nitrogen fixation (19.340% Ndfa) was recorded in microbial consortium applied treatment. The lowest biological nitrogen fixation was recorded in plants inoculated with *P. fluorescens* (8.810% Ndfa) and biological nitrogen fixation was not detected in uninoculated control and in *B. megaterium* inoculated treatments (Table 4).

Table 4: $^{15}N_2$ isotope tracer studies to estimate biological nitrogen fixation

Treatments	Total nitrogen content (%)	Amount of N_2 fixed (mg Ndfa)	N derived from atmosphere (%Ndfa)
Azospirillum lipoferum	0.324	22.53	11.480
Pseudomonas fluorescens	0.320	13.25	8.810
Bacillus megaterium	0.255	0.00	0.00
Microbial consortium (T_1 +T_2+T_3)	0.337	34.27	19.340
Uninoculated control	0.120	0.00	0.00
SED	0.02	1.88	1.04
CD (0.05)	0.06	4.11	2.26

DISCUSSION

Through the biochemical analysis of root exudates, we have established that the total sugars, reducing sugars and amino nitrogen content in the consortium applied treatments are higher than the treatments in which individual inoculant was applied (Table 1). It is evident that the beneficial interactions between diazotrophic and non-diazotrophic inoculants have resulted in the increased total sugars, reducing sugars and amino nitrogen content. In *B. megaterium* inoculated and in uninoculated control treatment, amino nitrogen content was not detected. This is due to the inability of the phosphate solubilizing inoculants to fix nitrogen in the respective treatments.

Many plant growth-promoting bacteria, which stimulate the growth of roots, can produce at least small amounts of the auxin indole-3-acetic acid (IAA) (Dubeikovsky *et al*., 1993; Loper and Schroth, 1986; Patten and Glick, 1996). All the bioinoculants were positive for IAA production. Quantification by HPLC indicated a little increase in level of IAA in microbial consortium treatment than the individual inoculant treatment. Considered collectively, these results indicate that microbial inoculation can change the levels of IAA-like hormones in the rice root environment. Phytopathogens synthesize IAA constitutively predominantly via the indole-3-acetamide (IAM) pathway. But PGPR such as *Azospirillum* species synthesize IAA mainly via the indole-3-pyruvic acid (IPyA) pathway, which may be subject to more stringent regulation by plant metabolites (Costacurta and Vanderleyden, 1995; Patten and Glick, 1996). The PGPR used in the study is subjected to stringent regulation by plant metabolites hence in the consortium treatment the IAA production does not increase to higher levels even in microbial consortium. Hence plant growth was not negatively affected.

GAs produced by *Azospirilum* spp. play an important role in the early stages of plant growth in gramineae (Lucangeli and Bottini, 1997) by enhancing shoot growth through *in vivo* production of phytohormones (Lucangeli and Bottini, 1996) and root growth especially by increasing root hair density in physiologically active areas for nutrient uptake and water absorption (Fulchieri *et al*., 1993). Growth promotion in plants that is induced by *Azospirillum* infection may occur by a combination of both gibberellin production and gibberellin-glucoside/glucosyl ester deconjugation by the bacterium (Piccoli *et al*., 1997).

All these physiological effects of GA would explain the better growth (dry weight and plant length) in bioinoculants applied treatment than control. Bacteria capable of producing high amounts of IAA are probably often inhibitory to plant growth and can be reduced by the effects of gibberellins (Meador and Taylor, 1987; Walter, 1966), exemplifying the importance of gibberellins and auxins balance, which regulates cell development.

In the present study cytokinin amount is higher in consortium of inoculants applied treatments compared to the individual bioinoculants treatment. The beneficial effects of cytokinin can be correlated positively to plant growth, cytokinin influence on chlorophyll content (Rayle *et al*., 1982) and to the fact that low cytokinin decreases incorporation of N_2 into leaf proteins. Absence of nitrogen source in *Bacillus* alone applied and control treatments limits P uptake which inturn limits cytokinin production in plants (de Groot *et al*., 2001). Exogenously applied cytokinins increases yield, N, P and K content of rice grains in field trials (Zahir *et al*., 2001), which supports the hypothesis that bacterially supplied cytokinins can improve the growth of treated plants.

In the present study, throughout the days of observation, the population of all the bioinoculants gradually increases reaching a maximum population at 30 days. The gradual increase in the population of the inoculants can be correlated to the general increase in nutrient content of the root exudates as evidenced by increase in total sugars, reducing sugars and total nitrogen content. In the plant nutrient media, nitrogen source is not supplied hence level of root exudation is gradual till 15 days, a similar trend is also noticed in the multiplication of bioinoculants correlating with absence of nitrogen source.

When we compare the population of the individual inoculants, *B. megaterium* has recorded minimum population. The inability of *B. megaterium* to fix atmospheric nitrogen and the absence of nitrogen source in the hydroponic culture medium has counted for the lower population of *B. megaterium* in the individual inoculant applied treatment (T_3). But, in the microbial consortium applied treatment (T_4), *B. megaterium* recorded maximum population on all days of observation as it is a versatile carbon source utilizer and has shorter generation time compared to the other two inoculants. Inoculation of other diazotrophs along with *B. megaterium* has compensated the inability of *B. megaterium* to fix atmospheric nitrogen. The nitrogen fixed and evolved in the root hydroponic culture media is well utilized by *B. megaterium* as well as by the plant system. All these factors have played a role for the maximum population of *B. megaterium* in microbial consortium applied treatment. The capacity of quick multiplication has one main advantage, which is the capacity to compete for space with pathogens.

Once the population of diazotrophs starts to establish the symbiotic relationship between the plant and bioinoculants start to develop in a dual fashion i) due to synergistic activity among bioinoculants the production of phytohormones and biological nitrogen fixation increases ii) due to the availability of fixed nitrogen the plants start a normal metabolism and the production of root exudates increases and thereby increasing the population of all the bioinoculants in T_4 treatment. This indicates the beneficial relationship among the diazotrophic and non-diazotropic inoculants also creating a symbiotic relationship with the plants.

In individual inoculant treatment either the shoot growth or the root growth is pronounced, due to imbalance in the nutrient status. A balance in the nutrient status as well as plant growth promoting substances improves the overall plant growth and development.

Root elongation is inversely proportional to exogenous IAA concentrations above a threshold level of about 10^6 to 10^9 M (depending on the plant species) (Pilet and Saugy, 1985; Scott, 1972). In the microbial consortium nitrogen and phytohormones are available in the media, the plants can take up nitrogen and plant growth substances thereby increasing nitrogen content and chlorophyll content resulting in profuse plant growth cytokinin affect photosynthetic parameters directly (e.g., chlorophyll and photosynthetic protein synthesis and degradation, chloroplast composition) or indirectly (mediated by changes in growth, sinks for photosynthates) (Synková *et al*., 1997) and delay leaf senescence (Soejima *et al*., 1992), this fact accentuates the importance of cytokinin and plant growth. Low levels of cytokinins also affect shoot growth negatively (Rayle *et al*., 1982) as seen in *Bacillus* alone inoculated and control treatments. All these inoculants included in the study improve plant growth by various mechanisms; *Pseudomonas* secretes plant growth promoting substances, in addition to biological nitrogen fixation. Diazotrophs are known to enhance plant growth; the inoculant *B. megaterium* secretes different organic acids, which solubilizes inorganic phosphorus to available form. Biometric observations of the experiment indicate that consortium of inoculants can promote rice growth, most likely through mechanisms that involve changes in growth physiology of crops by transferring fixed N_2 and by improving nutrient uptake through modulation of hormone-linked phenomena, root morphology and mainly through BNF in microbial consortium.

The most useful methods for examining N_2 fixation in the field and in large greenhouse experiments are still the $^{15}N_2$ isotope dilution techniques (James, 2000). The amount of nitrogen fixed is highly variable and dependent on plant genotype and environmental conditions (Boddey *et al*., 1991). Twenty to twenty five% of the total nitrogen needs of rice can be derived from associative fixation (App *et al*., 1980; Roger and Ladha, 1992). The results of the present experiment are found in concurrence with the reports of Belimov *et al*. (1995), who conducted isotopic studies and observed that combined inoculation of nitrogen fixing and phosphate solubilizing organisms enhanced the absorption of P and N in barley plants.

The increase in biological nitrogen fixation due to inoculation of diazotroph with phosphate solubilizing microorganism can be correlated to the tripartite synergistic relationship between the three inoculated microorganisms. The amount of biological nitrogen fixed in microbial consortium is the cumulative amount of nitrogen fixed by *Azospirillum* and *Pseudomonas* but the amount of nitrogen incorporated *i.e*., the nitrogen use efficiency seems to be higher in microbial consortium than in single inoculant applied treatment. The higher nutrient uptake may be related to inoculation with Phosphate solubilizing bioinoculants which induce morphological changes in rice roots, especially increased root hair, number, thickness and length (Yanni *et al*., 1997; Biswas, 1998), thereby favoring higher nutrient uptake by exploration of a greater soil volume. These results indicate that the physiological status of rice may change due to inoculation with microbial consortium, enabling the plant to make more efficient use of nutrients.

CONCLUSIONS

The understanding of the biology of root exudation processes may contribute to devising novel strategies for improving plant fitness, it is increasingly clear that chemical composition of exudates can have dramatic influences on plant to microbe, microbe to microbe, by altering uptake of water, nutrients and determination of community structure in the rhizosphere. Root exudates analysis, plant biometric observations and population enumeration studies of this experiment provides new evidences about the interaction between plant roots and the bioinoculants.

The results of the present experiment indicate the complexity of interaction which is operative in the rhizosphere and proves that the synergistic effects of coinoculation. The combination of bioinoculants is a major cause for success of both the plant establishment and the sustainability of bioinoculants and confirms the beneficial effects of microbial consortium over conventional single inoculant application method. More studies are necessary to explore the possible mechanisms of enhanced plant nutrient uptake operative due to microbial consortium in the rhizosphere. Even though in hydroponic system microbial consortium performed well, the possibility of extending to field conditions, which is an intricate, more complex and wherein a multitude of synergistic, antagonistic interactions may take place with the endogenous population needs to be explored.

REFERENCES

App, A.A., I. Watanabe, M. Alexander, W. Ventura, C. Daez, T. Santiago and S. K. De Datta, 1980. Nonsymbiotic nitrogen fixation associated with the rice plant in flooded soils. Soil Sci., 130: 283-289.

Bacilio-Jimenez, M., 2003. Chemical characterization of root exudates from rice *(Oryza sativa)* and their effects on the chemotactic response of endophytic bacteria. Plant Soil, 249: 271-277.

Bashan, Y. and G. Holguin, 1997. *Azospirillu*m-plant relationships: Environmental and physiological advances (1990-1996). Can. J. Microbiol., 43: 103-121.

Belimov, A.A., A.P. Kojemiakor and C.V. Chuvarliyeva, 1995. Interactions between barley and mixed cultures of nitrogen fixing and phosphate solubilizing bacteria. Plant Soil, 173: 29-37.

Biswas, J.C. 1998. Effect of nitrogen fixing bacteria on growth promotion of lowland rice (*Oryza sativa* L.). Ph.D. Thesis, University of Philippines, Los Ban OS.

Boddey, R.M., S. Urquiaga, V. Reis and J. Dobereiner, 1991. Biological nitrogen fixation associated with sugar cane. Plant Soil, 137: 111-117.

Bremner, J.M. 1965. Inorganic Forms of Nitrogen. In: Methods of Soil Analysis, Part 2, C.A. Black, (Ed.) Madison, American Soc. Agronomy, USA, pp: 1979-1984.

Buresh, R.J., E.R. Austin and E.T. Craswell, 1982. Analytical methods in ^{15}N research. Fert. Res., 3: 37-62.

Chabot, R., H. Antoun and M.C. Cescas, 1996. Growth promotion of maize and lettuce by phosphate-solubilizing *Rhizobium leguminosarum* biovar *phaseoli*. Plant Soil, 184: 31-121.

Costacurta, A. and J. Vanderleyden, 1995. Synthesis of phytohormones by plant-associated bacteria. Crit. Rev. Microbiol., 21: 1-18.

de Groot C., L.F.M. Marcelis, R. Van den Boogaard and H. Lambers, 2001. A Growth and dry-mass partitioning in tomato as affected by phosphorus nutrition and light. Plant Cell Environ., 24: 1309-1317.

Dobereiner, J., 1980. Forage Grasses and Grain Crops. In: Methods for Evaluating Biological Nitrogen Fixation. Bergerson, F.J., (Ed.). John Wiley and Sons, New York. pp: 535-555.

Dubeikovsky, A. N., E.A. Mordukhova, V.V. Kochetkov, F.Y. Polikarpova and A.M. Boronin, 1993. Growth promotion of blackcurrant softwood cuttings by recombinant strain *Pseudomonas fluorescens* BSP53a synthesizing an increased amount of indole-3-acetic acid. Soil Biol. Biochem., 25: 1277-1281.

Fahreus, A., 1957. The infection of clove root hairs by nodule bacteria studied by a simple glass slide technique. J. Gen. Microbiol., 16: 374-381.

Fulchieri, M., C. Lucangeli and R. Bottini, 1993. Inoculation with *Azospirillum lipoferum* affects growth and gibberellin status of corn seedling roots. Plant Cell Physiol., 34: 1305-1309.

Gordon, S.A. and R.P. Weber, 1951. Colorimetric estimation of indoleacetic acid. Plant Physiol., 26: 192-195.

James, E.K., 2000. Nitrogen fixation in endophytic and associative symbiosis. Field Crops Res., 65: 197-209.

King, E. O., M.K. Ward and D.E. Raney, 1954. Two simple media for the demonstration of pyocyanin and fluorescein. J. Lab. Clin. Med., 44: 301-307.

Lee, K.J. and M.H. Gaskins, 1982. Increased root exudation of ^{14}C – compounds by *Sorghum* seedlings inoculated with nitrogen-fixing bacteria. Plant Soil, 69: 391-399.

Loper, J. E. and M.N. Schroth, 1986. Influence of bacterial sources of indole-3-acetic acid on root elongation of sugar beet. Phytopathology, 76: 386-389.

Lucangeli, C. and R. Bottini, 1996. Reversion of dwarfism in *dwarf-1* maize (*Zea mays* L.)and *dwarf-x* rice (*Oryzasativa* L.) mutants by endophytic *Azospirillum* spp. Biocell, 20: 223-228.

Lucangeli, C. and R. Bottini, 1997. Effects of *Azospirillum* spp. on endogenous gibberellin content and growth of maize (*Zea mays* L.) treated with uniconazole. Symbiosis, 23: 63-71.

Mahadevan, A. and R. Sridhar. 1982. Hydrolytic Enzymes. In: Methods in Physiological Plant Pathology, 2nd Edn., Sivakami Publishers, Madras, pp: 24-44.

Meador, D.B. and B.H. Taylor, 1987. Effect of early season foliar sprays of GA4+7 on russeting and return bloom of "Golden Delicious" apple. HortScience, 22: 412-415.

Moore, S. and W.H. Stein, 1948. Methods in Enzymology, Academic Press. New york. pp: 68.

Nardi, S., G. Concheri, D. Pizzeghello, A. Sturaro, R. Rella, G. Parvoli, 2000. Soil organic matter mobilization by root exudates. Chemosphere, 5: 653-658

Neilands, J.B. and S.A. Leong, 1986. Siderophores in relation to plant growth and disease. Ann. Rev. Plant Physiol., 37: 187-208.

Patten, C. L. and B.R. Glick, 1996. Bacterial biosynthesis of indole-3- acetic acid. Can. J. Microbiol., 42: 207-220.

Piccoli, P., C. Lucangeli, G. Schneider,and R. Bottini, 1997. Hydrolysis of [17, 17-2 H2] gibberellin A20-glucoside and [17, 17-2 H2] gibberellin A20-glucosyl ester by *Azospirillum lipoferum* cultured in a nitrogen-free biotin-based chemically defined medium. Plant Growth Regul., 23: 179-182.

Pikovskaya, R.I., 1948. Mobilization of phosphate in soil in connection with the activities of some microbial species. J. Microbiol., 17: 360-370.

Pilet, P. E. and M. Saugy, 1985. Effects of applied and endogenous IAA on maize root growth. Planta, 164: 254-258.

Rayle, L., C.W. Ross and N. Robinson, 1982. Estimation of osmotic parameters accompanying zeatin-induced growth of detached cucumber cotyledons. Plant Physiol., 70: 1634-1636.

Roger, P.A. and J.K. Ladha, 1992. Biological N_2 fixation in wetland rice fields: Estimation and contribution to nitrogen balance. Plant Soil, 141: 41-55.

Scott, T.K., 1972. Auxins and roots. Annu. Rev. Plant Physiol., 23: 235-258.

Simons, M., A.J. Van Der Bij, I. Brand, L.A. de Weger, C. Wijffelman and B.J.J. Lutenberg, 1996. Gnotobiotic system for studying rhizosphere colonization by plant growth promoting *Pseudomonas* bacteria. Mol. Plant - Microbe Interact., 9: 600-607.

Soejima, H., T. Sugiyama, K. Ishihara, 1992. Changes in cytokinin activities and mass spectrometric analysis of cytokinins in root exudates of rice plant *(Oryza sativa* L.). Comparison between cultivars Nipponbare and Akenohoshi. Plant Physiol,. 100: 1724-1729.

Somogyi, M., 1952. Determination of reducing sugars by Nelson-Somogyi method. J. Biol. Chem., 200: 245.

Synková, H., N. Wilhelmová, Z. Šesták, J. Pospíšilová, 1997. Photosynthesis in Transgenic Plants with Elevated Cytokinin Contents. - In: Handbook of Photosynthesis. Pessarakli, M. (Ed.). Marcel Dekker, New York - Basel - Hong Kong, pp: 541-552.

Tien, T., M.H. Gaskins and D.H. Hubbell, 1979. Plant growth substances produced by *Azospirillum brasilense* and their effect on the growth of pearl millet *(Pennisetum americanum* L.). Appl. Environ. Microbiol., 37: 1016-1024.

Tuzun, S. and J.W. Kloepper. 1994. Induced Systemic Resistance by Plant Growth Promoting Rhizobacteria. Ryder, M.H. *et al.* (Eds.). CSIRO, Adelaide, Australia, pp: 104-109.

Walter, T. E., 1966. Russetting and Cracking in Apples: A Review of World Literature. In: 1966 Annu. Rep. East Malling Station. East Malling Exp. Stn., Maidstone, England, pp: 83-95

Wintermans, J.F.G.M. and A. Demots, 1965. Spectrophotometer characteristic chlorophylls-a and b and their phenophytins in ethanol. Biochem. Biophys. Acta, 109: 448-453.

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

Zahir, Z.A., H.N. Asghar and M. Arshad, 2001. Cytokinin and its precursors for improving growth and yield of rice. Soil Biol. Biochem., 33: 405-408.