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PSEUDOMONAS FLUORESCENS MEDIATED NUTRIENT FLUX IN THE BLACK PEPPER RHIZOSPHERE MICROCOSM AND ENHANCED PLANT GROWTH

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ABSTRACT

Pseudomonas fluorescens strains identified earlier were found effective in promoting growth in black pepper vines were evaluated for their nutrient mobilization capacity in the black pepper rhizosphere. P solubilization potency of the strains was proved *in vitro*. In *in planta* studies, the strains significantly increased the final dry matter production in the treated plants compared to the untreated control. The role of *P.fluorescens* mediated nutrient flux in the soil microcosm in plant growth promotion was confirmed with the higher uptake of nutrients by the bacterized plants after 90 days of treatment and planting. Significant uptake of nitrogen (N) and potassium (K) was noticed in IISR-13, IISR-11 and IISR-6 treated plants as it is related to the dry-matter yield. The uptake of K although was higher in bacterized plants, compared to the untreated check, only IISR-13 showed significantly higher levels. The bacterial population associated with the roots maintained a minimum population of log 6 even up to 90 days after treatment. The study revealed the enhanced nutrient mobilization in the rhizosphere of black pepper with PGPR treatment, which resulted in enhanced plant vigor.

INTRODUCTION

Black Pepper known as 'King of spices' is the most important and widely used spice in the world. Eco-friendly method of crop management is widely accepted. Microbial populations are key components of the soil-plant systems where they are immersed in a framework of interaction affecting plant developments. Plant Growth Promoting Rhizobacteria (PGPR) are soil and rhizosphere bacteria that can benefit plant growth by different mechanisms (Glick, 1995). Their use as natural bio-fertilizers is advantageous, not only from the economical, but also from the ecological point of view. The beneficial activities of PGPRs have been established in many crops including black pepper (Sarma *et al*, 2000). A large proportion of phosphorus in soil is present in an insoluble form and therefore not available for plant nutrition. The ability to convert insoluble P to an accessible form like orthophosphate is an important trait for a PGPR for increasing plant yields (Rossolini *et al*, 1998). Reports on non-symbiotic nitrogen fixation by *Acetobacter* spp. in sugarcane *Herbaspirillum* spp. in rice and *Azospirillum* spp. in cereals (Dobereiner, 1992, App *et al*, 1980, Gillis *et al*, 1989, Boddey *et al*, 1991) have stimulated appreciation for the importance of rhizobacteria in plant production and crop protection. Apart from P solubilization and biological nitrogen fixation, improvement of other plant nutrients uptake and

phytohormone production like IAA is some examples of mechanisms that directly influence plant growth (Glick et al, 1995)

In the present study the efficacy of five strains of *Pseudomonas fluorescens* were evaluated for mobilizing the essential nutrient uptake in black pepper and thereby enhancing the total plant vigor.

MATERIAL AND METHODS

The crop and the microorganisms used

Two to three leaf stage cuttings of black pepper *cv. Karimunda* rooted in sterile coir pith was used for the study. The PGPR strains, *P. fluorescence*, IISR-6, IISR-8, IISR-11, IISR-13, IISR-51 were obtained from the repository of biocontrol agents maintained at the Indian Institute of Spices Research, Calicut. Unless otherwise stated, the culture medium used was King's B agar.

Treatment and Planting

The bacterial strains were mass multiplied in nutrient broth by incubation at 28^{0}C for 48 h. The cells were pelleted at 7000 rpm for 10 min. Cells were resuspended in 10 mM $MgSO_4$ and diluted in such a way to get log 8 cfu/ml of the final suspension. Root bacterization was carried out by dipping the root system of the black pepper cuttings in the bacterial suspension for 30 min.

The cuttings were replanted in sterile potting mixture (Soil: Sand: FYM @ 2:1:1) and the bags were drenched with 25 ml of the bacterial culture. The six treatments included five strains of *P. fluorescens* and an untreated control. Three replicates were maintained for each treatment and each replicate had two plants.

Sampling

The planting medium was collected before treatment & planting in order to quantify the total and plant-available NPK. The soil sampling was also done on 30, 60 and 90 days after treatment by destructive sampling by collecting the soil adhering to the close proximity of the black pepper roots, sieved and dried before analysis. The total dry weight of the plants in each treatment was taken on the final day of sampling that is on the 90th day for estimation of total and available quantity of NPK.

Population dynamic of the introduced bacteria in the black pepper root /rhizosphere

Two grams of roots of the plants from each treatment were collected immediately after bacterization on the 0th day and the viable population of the introduced *P. fluorescens* was estimated by serial dilution method. The population was determined also after 30, 60 and 90 days after planting. The plating of the dilutions were performed in King's B agar after amending the media with appropriate antibiotics since the strains used were specifically resistant to certain antibiotics. The plates were incubated at 28^{0}C for 48h. The bacterial colonies appeared were counted.

In vitro studies on P solubilization

The phosphate-solubilizing test was done in both solid as well as in liquid medium. Medium used was Pikovskaya's medium (Pikovskaya, 1948). Agar plates were prepared and the bacterial strains were spot inoculated at the centre of the plates and incubated for 5-6 days. The plates were observed for clearing zone around the colony and the diameter of the clearing zone was measured.

100 ml of Pikovskaya's broth taken in conical flask was sterilized by autoclaving. The bacterial strains were inoculated and incubated for 48 h. at 28°±2°C at 200 rpm in rotary shaker. Medium without inoculation served as control. The culture was centrifuged at 7000 rpm for 20min at 4°C. The supernatant was collected and the pellet was discarded. 1ml of the supernatant was taken in a test tube and diluted by adding 6ml of distilled water. Then 2ml of chloromolybdic acid was added and 1ml of chlorostanous acid (Prepared by 10g of Stannous chloride was dissolved in 25ml of conc. HCl. 1ml from this was diluted with 132ml of distilled water.) was added to the mixture. The absorbance of this reaction mixture was read at 660 nm. From the standard graph, the quantity of phosphates released from tricalcium phosphate by the bacteria was obtained.

Estimation on Total and available NPK in the soil and plant.

The total N in soil was estimated by diacid digestion and Kjeldahl distillation. The total P and K were analyzed by triacid digestion and estimation by standard procedures (Jackson, 1967). The plant-available quantity of N in soil was estimated by alkaline $KMnO_4$ distillation (Subbaiah and Asija, 1956) and P & K by Bray's 1 and NH_4OAc extractions and estimated by following standard procedures using flame photometer (Jackson, 1967).

The plant samples were digested in sulphuric acid and N was estimated by Kjeldahl's distillation. The P & K were estimated by following standard procedures outlined by Jackson (1967) in samples digested by wet oxidation method.

RESULTS

P solubilization *in vitro*

In the Pikovskaya's agar plates, *P.fluorescens* strains produced 1.8 to 3.3 cm of clearing zones indication the P solubilization. IISR-8 showed lowest ability in plate (1.8 cm) while a maximum clearing zone was observed with IISR-6 (3.3 cm) the broth assay implicated the micrograms of phosphate released to the medium by the bacterial strains from tricalcium phosphate. The intensity of colour developed was directly proportional to the amount of phosphate released. 0.5 – 0.6 ppm of phosphate was released by the strains of *P. fluorescence* used.

Population dynamics of the introduced bacteria.

On the 0th day of treatment the population size of the strains per gram of root tissues was in the order of log 8-9 while it reduced to log 6-7 upon 30 days of treatment. This population was maintained till the end of study period without considerable fluctuation.

Total dry matter production

There was significantly higher dry matter yields in plants treated with each strain of *P. fluorescence* compared to the untreated. There was 23.64 – 40.83 % increase in dry matter production in PGPR treated plants compared to the control, highest by IISR-51 followed by IISR13 and IISR 6.

Uptake of NPK by the plants

The total uptake of NPK was obtained by multiplying the percent quantity of the mineral with the total dry weight of the plant. All the strains were found to ease the uptake of NPK by the plant roots. The total quantity of NPK in the *P. fluorescence*

treated plants was higher compared to that in the untreated control. Except IISR-8, all the strains significantly increased the total quantity of N in plants (11.10 – 13.39 mg), highest being by IISR-13 followed by IISR-11 and IISR-6. Highest P accumulation was observed in plants treated with *P. fluorescence* IISR-6 (1.95 mg) and the lowest by IISR-8, which was on par with the untreated plants (0.88 mg). Significant increase with respect to the control plants was noticed with IISR-6, IISR-13 and IISR-51. Even though higher, the PGPR strains except IISR-13 did not have significant levels of K in treated plants.

Quantity of total and available NPK in soil over time

The total quantity of NPK at the same time the available quantity did not show any significant change over time.

DISCUSSION

All the strains used in the study were found to solubilize complex forms of P to the plant available form in the *in vitro* studies conducted. The strains also mobilized higher uptake of N & K in the treated plants. The strains used in the study are proven biocontrol and growth promoting agent in different spice crops viz black pepper, ginger and cardamom. The ability to mobilize the essential nutrients required for the plant in the rhizosphere becomes an added quality of these bioinoculants.

The population of the introduced bacteria did not come down below log 6 cfu/g of root, over the period of study. The cause of this population maintenance may be because of the constant availability of nutrients in the rhizosphere especially from the plant roots as root exudates. There are several reports of the root exudates of the plants supporting the enhanced proliferation of rhizosphere microflora. The exudates contain mainly low molecular organic compounds such as sugars, amino acids and organic acids (Buchnen and Kinsel, 1992). Additionally organic substances are made available to soil microbes through constant root and root hair turn over (Leinweker *et al*, 1995). This rhizodeposition enhances microbial activity and therefore increases the rate of nitrogen mineralization in the soil (Vinton an Burke, 1995, Bradley and Fyles, 1996). Even then, due to the high complexity of the soil ecosystem, many aspects of the interactions between plants and soil microorganisms still are poorly understood (Kilham, 1994, Kapulnik, 1996)

Improvement of nutrient availability and other mechanisms (Kapulnik, 1996) by rhizosphere bacteria result in significant increase in plant growth (Suslow *et al*, 1979). Microorganisms are critical for the transfer of P from poorly available forms and are important for maintaining P in readily available pools. The present study proved the *P. fluorescens* mediated P solubilization and thereby enhanced uptake by the plants, which resulted in increased plant biomass. The microbe mediated P mobilization in plants may be through i) an increase in the surface area of roots ii) by displacement of sorption equilibria that results in increased net transfer of phosphate ion in to soil solution or an increase in the soil mobility of organic forms of P and iii) through stimulation of metabolic processes that are effective in directly solubilizing and mineralizing P from poorly available forms of inorganic and organic P (Jones, 1998). The microorganisms used in the study are found producing siderophores (Diby Paul, *et al* 2001) which may in turn release P from complex forms of P through ligand exchange reactions by chelating metal ions associated with the bound P. Processes

such as rhizosphere acidification, exudation of organic acids and secretion of phosphatase from plant roots occur in response to P deficiency and are established mechanisms by which plants require P (Randal, *et al* 2001). The soil P is also utilized by the microbes to meet their own requirements (Oberson, *et al* 2001). Incubation studies using labeled phosphate have highlighted that microbial P in turn get in to the soil solution and is available to the plant (Oehl, 2001, Oberson *et al*, 2001).

The increased biomass in these PGPR treated plants may also be attributed to the production of antibiotics, lytic enzymes, HCN and siderophores (Diby Paul *et al*, 2001, 2002) produced by these strains thus inhibiting the growth of deleterious microorganisms in the rhizosphere. The phytohormone, IAA production by these *P. fluorescens* strains (data unpublished) also may be contributing to enhanced biomass production in black pepper.

There also found an increase uptake of N by the treated plants. A biological alternative for the extensive use of nitrogen fertilizers is the interaction between PGPR and the plant roots. The main forms of nitrogen taken up by plants are as either ammonium (NH_4^+) or nitrate (NO_3^-). Nitrogen, which is available to plants, may come from decomposition of organic matter, biological fixation of nitrogen and from additions of nitrogen in organic and inorganic fertilizers. The inorganic nitrogen taken up by the plants is converted to organic compounds of amino acids. The ability of *Pseudomonas* species to fix nitrogen is still debated. Hans Vermeiren (1999) proved that a diazotrophic rice endophyte, *Pseudomonas stutzeri* fix nitrogen and provide, thus improving plant growth. Sipirin (2000) found that the *Vetiver* could grow and survive without nitrogen and phosphorus application especially in the infertile soil with the help of diazotrophs including the genera of *Pseudomonas*.

Even though not significant, higher levels of K uptake was noticed in PGPR treated plants. K is one of the essential nutrient for plant growth. Good K nutrition favors the rapid turn over of inorganic nitrogen in to proteins (Koch, *et al* 1974). Even though there is no direct influence of rhizosphere microbes in K mobilization, the enhanced uptake of the same in the bacterized plants would have effected from the higher root / root hair growth, increased root surface area and better root health supported by the PGPRs.

CONCLUSION

The application of *P. fluorescens* strains black pepper rhizosphere resulted in easy mobilization of the essential nutrients in the rhizosphere microcosm and resulted in enhanced uptake of the same, which reflected in increased plant biomass. These biocontrol bacteria can be explored for the effective management of black pepper for enhanced plant growth and disease suppression.

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