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Advantage of using PSIRB over PSRB and IRB to improve plant health of tomato

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ABSTRACT

Twenty-one isolates of phosphate solubilizing-indole acetic acid producing rhizobacteria (PSIRB), 20 isolates of phosphate solubilizing rhizobacteria (PSRB) and 42 isolates of indole acetic acid producing rhizobacteria (IRB) were isolated from 49 rhizospheric soil samples of tomato (*Lycopersicon esculentum* Mill.) collected from tomato growing regions of Karnataka. A method combining Pikovskaya's and Bric's technique was developed to isolate PSRIB, PSRB and IRB's. The selected isolates were further analyzed for their ability to solubilize calcium phytate. Based on the root colonization assays and the abilities of bacterial isolates to increase the seed germination and seedling vigor under laboratory conditions, five isolates were selected from each group for further studies. Under greenhouse conditions, all the selected rhizobacteria isolates significantly increased root length, shoot length, fresh weight, dry weight and total phosphorus content of 30-day-old-seedlings with respect to control. Isolate PSIRB1 and IRB36 significantly reduced the Fusarium wilt incidence over other isolates of same and other group, and the control. On the basis of results from laboratory and greenhouse studies, one bacterial isolate from each group was selected for plant growth and yield analysis studies. Isolate PSIRB2 showed increased plant height, fresh weight, number of fruits per plant and average weight of fruit over PSRB9, IRB36 and untreated controls. Studies on the nature of protection offered by these bacterial isolates following split-root technique revealed that the isolates PSIRB2 and PSRB9 had the ability to induce systemic resistance. One isolate, IRB36 appeared to protect the tomato seedlings through direct antagonism.

1. Introduction

The rhizosphere, representing the thin layer of soil surrounding plant roots and the soil occupied by the roots, support large and metabolically active groups of bacteria (Villacieros et al., 2003) known as plant growth promoting rhizobacteria (PGPR) (Kloepper et al., 1980). These free-living, root-colonizing bacteria have been studied from the past century as possible inoculants for increasing plant productivity (Kloepper et al., 1992). Several mechanisms have been postulated to explain how plant growth-promoting rhizobacteria (PGPR) stimulate plant growth. These mechanisms are broadly categorized as direct or indirect. Direct mechanisms elicit growth promotion by bacterial determinants, including production of plant hormones such as indole acetic acid (IAA) (Patten and Glick, 2002), gibberellic acid (GA) (Gutierrez-Manero et al., 2001) and cytokinin (Tien et al., 1979), phosphate solubilization (Idriss et al., 2002) and by symbiotic nitrogen fixation (Kennedy et al., 1997). Indirectly the plant growth promotion results from suppression of soil-borne and foliar pathogens through direct antagonism and/or by induced systemic resistance (ISR) (Schippers et al., 1987; Van Loon et al., 1998). These indirect mechanisms, such as suppression of harmful microorganisms and ISR, are normally recognized as having a role in biological control (Kloepper et al., 1992; Dobbelaere et al., 2003).

Promotion of root growth is one of the major markers by which the beneficial effect of PGPR is measured (Glick et al., 1995). Plant growth stimulating phytohormones produced by these rhizobacteria within the root zone increase the density and length of root hairs. Rapid establishment of roots, whether by elongation of primary roots or by proliferation of lateral and adventitious roots, is advantageous for young seedlings as it increases their ability to anchor themselves to the soil and to obtain water and nutrients from their environment, thus enhancing their chances for survival (Volkmar and Bremer, 1998).

Phosphorus is the second most abundant plant nutrient available in soil after nitrogen. Though soils usually contain high amount of total phosphorus, most of the phosphorus occurs in insoluble forms such as iron and aluminum phosphates in acidic soils or calcium phosphates in alkaline soils. On the other hand, phytate is an important reservoir of immobilized phosphate accounting for 20–80% of soil phosphorus (Richardson, 2001) and only a small portion (~0.1%) is available to plants. Conversion of the insoluble forms of phosphorus to a form accessible by plants, like orthophosphate, is an important trait of PSRB in improving the health of tomato (Hariprasad and Niranjana, 2009). Ability of phosphate solubilizing microorganisms to solubilize phosphorus

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complexes has been attributed to their ability to reduce the pH of the surroundings, either by releasing organic acids or protons. Phytase belongs to a special class of phosphomonoesterases and is produced by several soil microorganisms. It is involved in the stepwise degradation of phytate to lower phosphate esters which can be accessible by plants.

During the last couple of decades, the use of PGPR for sustainable agriculture has increased tremendously in various parts of the world. Significant increases in growth and yield of agronomically important crops in response to inoculation with plant growth promoting rhizobacteria (PGPR) have been reported (Chen et al., 1994; Amara and Dahdoh, 1997; Biswas et al., 2000; Khan and Khan, 2001; Asghar et al., 2002; Araujo et al., 2005). But attempts to use PGPR isolates that have multiple mechanisms in improving plant growth have been conducted by very few workers. Currently there is very limited knowledge regarding proper utilization of PGPR isolates with multiple mechanisms to improve plant health.

Vascular wilt caused by *Fusarium oxysporum* Schlechtend.: Fr. F. sp. *lycopersici* (Sacc.) W.C. Snyder and H.N. Hans, is an important disease of tomato and occurs throughout the world (Woltz and Jones, 1981). The fungus may cause great loss to tomato, depending upon the tomato cultivar and the environmental conditions (Khan and Akram, 1999). Currently many fungicides such as, benomyl, thiram, thiabendazole and carbendazim are used to manage the wilt fungus. But these fungicides adversely affect the useful soil microorganisms and environment. Biological control of Fusarium wilt can offer a potential alternative to chemical fungicide (Parker et al., 1985). Suppression of root disease by PGPR is attributed to its ability to colonize the host root, compete for space and nutrition and inhibit the growth of the fungus by producing antibiotics or siderophores. Apart from a direct antagonistic effect on soil-borne pathogens, some PGPR strains are also able to reduce disease in above-ground plant parts through ISR. PGPR-mediated ISR has been demonstrated in many plant species, e.g. Bean (*Phaseolus vulgaris* L.), carnation (*Dianthus caryophyllus* L.), cucumber (*Cucumis sativus* L.), radish (*Raphanus sativus* L.), tobacco (*Nicotiana tabacum* L.), tomato (*Lycopersicon esculentum* L.) and the model plant *Arabidopsis* (*Arabidopsis thaliana* (L.) Heynh.), against fungi, bacteria and viruses (Van Loon et al., 1998). The interactions between the pathogen and host plant induce changes in cell metabolism, primarily in the enzyme activities, including that of phenylalanine ammonia lyase (PAL), peroxidase (POX), polyphenol oxidase (PPO), lipoxygenase (LOX) and accumulation of phenolics (Ramamoorthy et al., 2002a; Silva et al., 2004; Girish and Umesha 2005). The increased level of defence related enzymes during ISR are known to play a crucial role in host resistance.

In the present study an attempt has been made to develop a simple methodology based upon the standard methods of Pikovskaya (1948) and Bric et al. (1991) to isolate PSIRB, PSRB and IRB in a single step and to determine the advantage of using PSIRB's in improving plant health and yield of tomato over PSRB and IRB. The second objective was to study the nature of protection offered by the selected rhizobacterial isolates against Fusarium wilt and involvement of defense related enzymes in ISR under greenhouse conditions.

2. Materials and methods

2.1. Plant materials

Seeds of tomato (*Lycopersicon esculentum* Mill.) cultivar 'Ruchi' were obtained from local seed agencies. Seeds were surface-sterilized with 1% sodium hypochlorite for 30 s and then rinsed in sterile distilled water, blot dried and used for the experiments.

2.2. Soil samples

Forty-nine rhizosphere soil samples were collected from tomato plants of various agro climatic regions of Karnataka, India between June and September, 2006. Rhizosphere soil samples were collected from healthy field-grown plants. For greenhouse studies, a sandy loam soil was collected from the experimental plot of Department of Applied Botany and Biotechnology, University of Mysore, India. The soil was dried and sieved to 2 mm before mixing it with cow dung manure (3:1 v/v).

2.3. Microorganisms and inoculum preparation

In order to isolate PSIRB, PSRB and IRB rhizosphere soil samples were serially diluted and suitable dilutions were spread plated onto modified Pikovskaya's agar medium [Glucose, 8.0; $Ca_3(PO_4)_2$, 5.0; Yeast extract, 5; $(NH_4)SO_4$, 0.5; KCl, 0.2; $MgSO_4$ $7H_2O$, 0.1; $MnSO_4$, 0.006; $FeSO_4$, 0.006; L-tryptophan, 0.5; Agar, 16 ($g\,l^{-1}$)]. Plates were overlaid with an 82-mm-diameter disk of nitrocellulose membrane after an initial incubation period of 24 h to enumerate total bacteria (Bric et al., 1991). All plates were incubated up to 4 days at 28 ± 2 °C, after which the membranes were removed from the plates and treated with Salkowski reagent (150 ml of concentrated H_2SO_4, 250 ml of distilled H_2O, 7.5 ml of 0.5 M $FeCl_3{\cdot}6H_2O$) (Gordon and Weber, 1951). The reaction was allowed to proceed until adequate color developed. Rhizobacterial isolates producing IAA were identified by the formation of a characteristic pink-red halo within the membrane immediately surrounding the colony. Further, Pikovskaya agar medium was incubated for 2–4 days at 28 ± 2 °C and the bacterial colonies were analyzed for a zone of clearance. The nylon membrane and Pikovskaya agar plates were analyzed side by side and the bacterial colonies showing both a zone of clearance on agar medium or pink-red halo in the nitrocellulose membrane or both, were pure cultured onto nutrient agar (NA) slants. All the selected bacterial isolates were further analyzed for their respective traits such as calcium phosphate (CP) solubilization in liquid medium (Alikhani et al., 2006), phytase activity on phytase screening medium (PSM) (Howson and Davis, 1983), phytase activity in culture filtrate (Shimizu, 1992; Idriss et al., 2002) and IAA production in Luria bertani (LB) broth supplemented with L-tryptophan (500 μg/ml) (Patten and Glick, 2002).

Finally the bacterial isolates were grouped into PSIRB; which showed IAA production, calcium phosphate solubilization and phytase activity, PSRB; which were positive for CP solubilization and phytase activity but negative for IAA production, and IRB; which produced IAA but were negative for CP solubilization and phytase activity. All selected bacterial colonies were cultured onto nutrient agar slants and maintained at 4 °C for further use.

Rhizobacterial inoculum for seed bacterization was prepared by growing the bacterial isolates in LB broth for 36 h on a rotary shaker at 150 rpm. Bacterial cell pellet from the log phase were obtained by centrifugation at 8000 rpm for 10 min and the bacterial concentration was adjusted spectrophotometrically to 1×10^8 cfu ml^{-1}.

Fusarium oxysporum Schlechtend.: Fr. F. sp. *lycopersici* (Sacc.) W.C. Snyder and H.N. Hans, was obtained from the culture collection of the Department of Studies in Biotechnology, University of Mysore, India and maintained on potato dextrose agar (PDA) slants at 4 °C till further use. Inoculum was prepared by culturing the fungus on PDA for 7 days in petri plates 28 ± 2 °C. A conidial suspension was prepared by pouring 20 ml of sterile distilled water in each Petri plate. The concentration of microconidia of *F. oxysporum* was adjusted to 1000 conidia ml^{-1} using an haemocytometer (Ramamoorthy et al., 2002a).