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Evaluation of biocontrol potential of fluorescent *Pseudomonas* associated with root nodules of mungbean

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ABSTRACT

Thirty two isolates of fluorescent *Pseudomonas* and sixteen rhizobia were isolated from the root nodules of mungbean (*Vigna radiata*) collected from the experimental fields of Karachi University. *Pseudomonas* and rhizobia were characterized for their plant growth promoting and biocontrol potential through the determination of *in vitro* activity against root rotting fungi viz., *Macrophomina phaseolina, Fusarium solani, Fusarium oxysporum* and *Rhizoctonia solani* and nematicidal activity against the second stage juveniles of *Meloidogyne javanica. Pseudomonas* isolates NAFP-19, NAFP-21, NAFP-22, NAFP-27, NAFP-31 and NAFP-32, and rhizobial isolates NFB-102, NFB-103, NFB-107 and NFB-109 inhibited the growth of all the test fungi and showed maximum nematicidal activity against second stage juveniles of *M. javanica.* Strains of *Pseudomonas* (NAFP-19, NAFP-31 and NAFP-32) and rhizobia (NFB-103, NFB-107 and NFB-109) used as a soil drench significantly reduced root rot disease under screen house condition and thereby enhanced plant growth and yield in mungbean. In field plot experiments, mixed or separate application of NAFP and rhizobia showed effective control of *M. phaseolina* and *F. oxysporum* with some protection from *F. solani* and *R. solani*.

1. Introduction

Rhizobia are of considerable scientific and economic interest because of their ability to fix atmospheric nitrogen in leguminous plants (Hynes and O'Connel, 1990; Sprent, 2001). These bacteria have the unique capacity to induce the formation of root nodules in the host plant by the production of specific signal molecules called Nod factors (Lerouge et al., 1990; Spaink et al., 1991; Truchet et al., 1991). The rhizobia, inside the nodules then convert nitrogen into ammonia for uptake by host plants while legumes provide nutrients to rhizobia (Spaink, 2000). Besides nitrogen fixation some isolates of rhizobia can reduce attack by soilborne root infecting fungi both in leguminous and non-leguminous plants (Ehteshamul-Haque and Ghaffar, 1993; Siddiqui et al., 1998a, 1998b).

Plant growth promoting rhizobacteria (PGPR) have a beneficial effect on plants. Besides direct biological control of soilborne pathogens, they induce systemic resistance in plants against pathogens and improve plant growth by producing growth regulators and increasing nutrition and water uptake in plants (Bhattacharyya and Jha, 2012). Of the various rhizosphere bacteria, those belonging to fluorescent *Pseudomonas* are aggressive colonizers of the rhizosphere of various crop plants and have broad spectrum antagonistic activity against soilborne plant pathogens (Siddiqui and Ehteshamul-Haque, 2001; Siddiqui et al., 2000; Weller et al., 2002; Whipps, 2001). Raajmakers and Weller (1998) reported the role of 2, 4-diacetyl-phloroglucinol, an antifungal metabolite from species of fluorescent *Pseudomonas* in root disease suppression. The production of antibiotics and the competition for iron by the release of siderophores have been shown to be active mechanisms for the control of root infecting fungi (Weller, 1988). Biocontrol potential of fluorescent *Pseudomonas* associated with the rhizosphere, rhizoplane (Siddiqui et al., 2000, 2001) and endoroot (Tariq et al., 2009; Afzal et al., 2013) is well documented.

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However, the biocontrol role of fluorescent *Pseudomonas* associated with root nodules received less attention (Batool et al., 2013; Issar et al., 2012). Moreover, in the past it was thought that the root nodules of leguminous plants contain only rhizobia. But recent research studies have indicated that besides rhizobia, root nodules also contain other bacteria (Pandya et al., 2013). The present report describes the isolation and characterization of fluorescent *Pseudomonas* associated with root nodules of mungbean (*Vigna radiata*) and their biocontrol potential against root rotting fungi of mungbean.

2. Materials and methods

2.1. Collection of plant material for the isolation of fluorescent Pseudomonas and rhizobia

Root nodules from mungbean (*Vigna radiata*) plants grown in different experimental fields of Karachi University were collected and brought to the laboratory and kept at 4 °C until isolation of bacteria was done within 24 h.

2.2. Isolation of fluorescent Pseudomonas and rhizobia from root nodules

Roots of the test leguminous plants were washed under running tap water. A well formed, healthy pinkish nodule surface was sterilized with 1% mercuric chloride for 5 min and then repeatedly washed with sterile water. The nodule was then washed in 70% ethyl alcohol for 3 min followed by washing with sterile water. The nodule was crushed in a small aliquot of sterile water with the help of a sterile glass rod. The nodule suspension was streaked onto Petri dishes containing S-1 medium supplemented with trimethoprim (Gould et al., 1985; Bashan et al., 1993) for the isolation of fluorescent *Pseudomonas.* Dishes were incubated for 3 days at 28 °C. Bacterial colonies that fluoresced under UV light at 366 nm were purified on King's B agar medium (King et al., 1954). For the isolation of rhizobia, the suspension was streaked onto YEMA medium supplemented with 2.5 ml of 1% congo red/liter. After incubation for one week, distinct colonies (gummy white-creamy) of rhizobia were picked up and transferred to agar slants (Subba Rao, 1977). Bacteria were identified after reference to Bergey's manual (Holt et al., 1994; Brenner et al., 2005). Identification of selected fluorescent *Pseudomonas* was also made on the basis of "16S rDNA" and internal transcribed spacer "ITS1" sequences (Spilker et al., 2004; Locatelli et al., 2002; Anzai et al., 2000).

2.3. In vitro test against root infecting fungi

Dual culture plate method was used to determine antifungal activity of bacterial strains (Drapeau et al., 1973). The bacterial strains were streaked on one side of the Petri dishes containing Czapek's Dox agar at pH 7.2. On the other side of the Petri dishes, a 5 mm diameter disc of mungbean root rotting fungi *Fusarium solani, Fusarium oxysporum*, *Rhizoctonia solani, Macrophomina phaseolina* which were maintained on Potato dextrose agar (PDA) were inoculated. The dishes were incubated at 28 °C and zone of inhibition were recorded from 3 to 7 days (depends upon the growth of test fungus).

2.4. In vitro juvenile mortality test

Pseudomonas isolates were grown on King's B broth, while rhizobia were grown on YEM broth at 30 °C for 72 h in the dark and centrifuged at 3000 rpm for 20 min. The pellets were discarded and the culture filtrates were collected in the beaker for use. One milliliter of freshly hatched second stage juvenile suspension (20 juveniles) and 1 ml of a cell free culture filtrate of bacterial strains were transferred in glass cavity blocks making three replicates of each strain of *Pseudomonas* and rhizobia and kept at 26 ± 5 °C. Mortality of second stage juveniles was recorded after 48 h. The nematodes were considered dead if they did not move when probed with needle (Cayrol et al., 1989).

2.5. Screen house experiment

Non-sterilized sandy loam; pH 8.0, with a moisture holding capacity of 40% was obtained from the field of Department of Botany, University of Karachi and transferred into 12 cm diameter earthen pots at 1 kg of soil per pot. The soil had natural infestation of 3–6 sclerotia of *Macrophomina phaseolina* g^{-1} of soil, as determined by wet sieving and dilution technique (Sheikh and Ghaffar, 1975), 5–10% colonization of sorghum seeds was used as bait for *R. solani* (Wilhelm, 1995) and 3000 cfu g^{-1} of soil of a mixed population of *Fusarium solani* and *F. oxysporum* as determined by a soil dilution technique (Nash and Snyder, 1962). Six mungbean {*Vigna radiata* (L.) Wilczek} seeds were sown each per pots after applying 25 ml (10^8 cfu/ml) bacterial suspension of NAFP-19 (3.3×10^8 cfu/ml), NAFP-32 (2.5×10^8 cfu/ml), NAFP-31 (1.4×10^8 cfu/ml), NFB-103 (3.6×10^8 cfu/ml), NFB-107 (3.4×10^8 cfu/ml) and NFB-109 (2.2×10^8 cfu/ml) into each pot. Plant not received bacterial suspension served as control. While carbendazim (200 ppm) at 25 ml per pot served as positive control against root rotting fungi. The experiment was conducted in complete randomized block design with four replicates. After germination, four seedlings were kept in each pot and excess were removed.

The experiment was terminated after 45 days and the plant height and fresh weight of the roots and shoots was recorded. To determine the root-infecting fungi, the roots were washed in running tap water, surface sterilized in 1% Ca $(OCl)_2$ and five 1 cm long root pieces were inoculated onto PDA plates containing penicillin (100,000 units/L) and streptomycin sulfate (0.2 g/L). The plates were incubated at room temperature and the incidence of root infecting fungi was recorded as follows;

$$\text{Infection (\%)} = (\text{No. of plant infected by a fungus} / \text{Total number of plants}) \times 100$$

2.6. Field plot experiment

The experiments were conducted at Crop Diseases Research Institute, Pakistan Agricultural Research Council, Karachi University Campus, Karachi in plots of 2 × 2 meters in complete randomized block design with four replicates. The field soil had a natural infestation of 5–16 sclerotia/g of soil of *Macrophomina phaseolina*, 5–12% colonization of *R. solani* on sorghum seeds used as baits and 2500 cfu/gm of soil of mixed population of *F. oxysporum* and *F. solani* as determined by soil dilution. Cell suspension of fluorescent *Pseudomonas* NAFP-19 (2.3×10^8 cfu/ml), NAFP-32 (3.5×10^8 cfu/ml) and rhizobia NFB-103 (4.7×10^8 cfu/ml) and NFB-109 (3.8×10^8 cfu/ml) were drench separately in planting rows at 100 ml/m. In another set, mixed suspension of rhizobia and fluorescent *Pseudomonas* were also applied in rows. Thirty seeds of mungbean were sown in each row. Carbendazim (200 ppm) at 200 ml/m row served as positive control against root rotting fungi. The plants were watered 2–3 days intervals depending upon requirement of plants. To determine the efficacy of fluorescent *Pseudomonas* and rhizobia on the root pathogens and plant growth, plants were uprooted (4 plants from each replicate) after 45 days of