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Compatible rhizosphere microbes mediated alleviation of biotic stress in chickpea through enhanced antioxidant and phenylpropanoid activities

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ABSTRACT

The study was conducted to examine efficacy of a rhizospheric microbial consortium comprising of a fluorescent *Pseudomonas* (PHU094), *Trichoderma* (THU0816) and *Rhizobium* (RL091) strain on activation of physiological defense responses in chickpea against biotic stress caused by the collar rot pathogen *Sclerotium rolfsii*. Results of individual microbes were compared with dual and triple strain mixture treatments with reduced microbial load (1/2 and 1/3rd, respectively, of individual microbial load compared to single microbe application) in the mixtures. Periodical studies revealed maximum activities of phenylalanine ammonia lyase [E.C. 4.1.3.5] and polyphenol oxidase [E.C. 1.14.18.1] and accumulation of total phenol content in chickpea in the triple microbe consortium treated plants challenged with the pathogen compared to the single microbe and dual microbial consortia. Similarly, the expression of the antioxidant enzymes superoxide dismutase [E.C.1.15.1.1] and peroxidase [E.C.1.11.1.7] was also highest in the triple microbial consortium which was correlated with lesser lipid peroxidation in chickpea under the biotic stress. Histochemical staining clearly showed maximum and uniform lignification in vascular bundles of chickpea stem sections treated with the triple microbes. The physiological responses were directly correlated with the mortality rate as least plant mortality was recorded in the triple microbe consortium treated plants. The results thus suggest an augmented elicitation of stress response in chickpea under *S. rolfsii* stress by the triple microbial consortium in a synergistic manner under reduced microbial load.

1. Introduction

Plants, in nature, are able to defeat attempts by microbes to infect them in most of the times. It is mainly because of the innate ability of plants to recognize potential microbial invaders and reprogram their defense systems appropriately. Plants provide a supportive environment for the beneficial soil microbes in the rhizosphere and in return the microbes provide several benefits to the plants by facilitating growth promotion and stress alleviation (Filippi et al., 2011; Mendes et al., 2011). In recent years considerable attention has been given to the rhizosphere microbes that can mediate induced systemic resistance (ISR), a state of enhanced defensive capacity whereby the plant's innate defenses are potentiated against subsequent biotic challenges (Conrath et al. 2002), in addition to their antagonistic activities. Bakker et al. (2007) also showed that plants under induced resistance state enhance their defensive capacity by mobilizing appropriate cellular defense responses before or upon pathogen attack. Punja (1985) long back suggested that host factors should be considered while trying to prevent establishment of infection of soil borne fungal pathogens by enhancing the levels of phenylpropanoid metabolites, a major defense pathway in the host tissues. Phenylalanine ammonia-lyase (PAL) is the first enzyme of the phenylpropanoid pathway that catalyses the transformation of L-phenylalanine into trans-cinnamic acid, by deamination (Dixon and Paiva 1995). Trans-cinnamic acid is the prime intermediary in the biosynthesis of phenolics and many of them are antimicrobial (Nicholson and Hammerschmidt 1992). It has been observed that a range of defense activities are elicited after attempted microbial infection such as activation of the phenylpropanoid pathway leading to deposition of lignin and phenolics beyond the infection sites (Shoresh et al. 2010) and induction of antioxidant enzymes including superoxide dismutase (SOD) (Singhai et al. 2011), peroxidase (POx) (Silva et al. 2004). Many of these defense activities are known to be augmented by plant growth promoting rhizobacteria (PGPR)-mediated ISR that protect the plants against diverse pathogens (Jetiyanon and Kloepper 2002; Silva et al. 2004; Jetiyanon, 2007). Antagonistic rhizosphere bacteria can elicit the phenylpropanoid pathway in a variety of crop plants that may result in reduced infection by their respective pathogens. Sarma and Singh (2003) showed

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that *Sclerotium rolfsii* is highly sensitive to some of the phenylpropanoid metabolites particularly to ferulic acid. Thus, it has been realized very strongly that the success of a plant in warding off an invading pathogen relies primarily on its ability to build a rapid line of defense. Previously we have demonstrated that non-pathogenic rhizobacteria such as *Pseudomonas* spp. (Singh et al., 2003; Sarma et al. 2002) as well as *Trichoderma* spp. (Singh et al. 2011) protect plants from diverse pathogens by triggering ISR in the host. Similarly, *Rhizobium* spp. have also been recently shown to trigger ISR like responses in their host plants to an extent against invading parasites (Mabrook et al. 2010; Mishra et al. 2006). Based on these findings, it is believed that combining such beneficial organisms can enhance the plant's innate resistance level against the invading pathogens more than their individual effort. To test this hypothesis, the present investigation was taken up to examine and document the host defense responses toward a consortium of microbes comprising fluorescent *Pseudomonas*, *Rhizobium* sp. and *Trichoderma* spp. in suppressing collar rot of chickpea (*Cicer arietinum*) caused by *S. rolfsii* in comparison to the individual effort by the same microbes.

2. Materials and methods

2.1. General procedures

The pathogen *S. rolfsii* was isolated by collecting sclerotia produced on infected collar regions of chickpea plants. Surface sterilization of sclerotia was done by dipping them in 1% (v/v) sodium hypochlorite (NaOCl) for five to ten seconds followed by three subsequent washings with sterilized distilled water (SDW). The sclerotia were then placed in Petri dishes containing potato dextrose agar (PDA) and incubated at $27 \pm 2\,^{\circ}C$ for 6 days. The cultures were purified by picking a single sclerotium and transferring into a new plate. The pathogen was maintained on PDA slants for further investigations. *Trichoderma* isolate THU0816 was obtained from National Botanical Research Institute (NBRI), Lucknow whereas the fluorescent *Pseudomonas* PHU094 and *Rhizobium* RL091 were isolated from rhizospheric region and root nodules of chickpea, respectively. The identities of the bacterial strains were confirmed by 16S rDNA sequence analyses. Compatibility between the strains of fluorescent *Pseudomonas* and *Rhizobium* was carried out on Nutrient Agar (NA) plates with one streaked in the center and the other sprayed using an atomizer after 24 h. Similarly, compatibility between *Trichoderma* and fluorescent *Pseudomonas*/*Rhizobium* was carried out on PDA. *Trichoderma* block (5 mm dia) cut with a cork borer was put in the center of the plate and both the fluorescent *Pseudomonas* and *Rhizobium* strains were streaked on either side of the block to check the compatibility. Absence of an inhibition zone in the interaction region of two organisms indicated compatibility.

For seed inoculation *Rhizobium* and *Pseudomonas* strains were grown overnight in yeast extract mannitol and King's B broth, respectively. One ml of each culture was pelleted by centrifugation and the supernatant was discarded. Cell pellets were washed with 1 ml phosphate buffer saline (PBS, 20 mM sodium phosphate, 150 mM NaCl, pH 7.4) and suspended in PBS to a cell density of 10^8 CFU ml^{-1} through optimization of optical density at 620 nm. Similarly, conidial suspension of *Trichoderma* was adjusted to 2×10^6 CFU ml^{-1}. Cell suspensions of the three strains were mixed in ratios 1:1 for dual consortia and 1:1:1 for triple consortium representing 1/2 and 1/3rd of each initial microbial load, respectively. The suspensions were vortexed to obtain homogenous suspensions of microbial mixtures. Chickpea (cv. Radhey) seeds were surface sterilized with 1% NaOCl solution for 5 min, followed by washing with SDW to remove traces of NaOCl. The moistened seeds were stored at room temperature ($25 \pm 2\,^{\circ}C$) for 24 h under aseptic condition to promote germination. Plastic pots (15 cm × 10 cm) were used for Sterile Soil Assay (SSA). Soil mixture containing sandy soil; vermicompost and farm yard manure (2:1:1) was autoclaved for 30 min for three consecutive days and filled with 1.5 kg pot^{-1}. For seed treatment, slightly germinated seeds of chickpea were coated with 15% (w/v) gum arabic (HiMedia, RM682) as an adhesive and uniformly coated with various combinations of cell suspensions and kept for air drying for 2 h under a stream of sterile air. The coated seeds were then planted in plastic pots with an average of six germinated seeds per pot at a depth of approximately 1.5 cm. Following seven suspensions prepared were used in the experiment: (i) *Rhizobium* (RL091), (ii) fluorescent *Pseudomonas* (PHU094), (iii) *Trichoderma* (THU0816), (iv) RL091 + PHU094, (v) RL091 + THU0816, (vi) PHU094 + THU0816, and (vii) RL091 + PHU094 + THU0816.

A potting mixture was inoculated with active mycelium and sclerotia of *S. rolfsii*, mass cultured in sterile cornmeal sand (240 g of clean quartz sand, 6.0 g of yellow cornmeal, and 75 ml of SDW) for 2 weeks at 25 °C (Abeygunawardhane and Wood 1975). The inoculum of *S. rolfsii* was added in the potting mixture at 50 g pot^{-1} after plants were of 4 weeks old. Untreated seeds sown in pots and inoculated with *S. rolfsii* served as positive control whereas untreated seeds sown in pots without inoculation of *S. rolfsii* served as true control. Five replications were maintained for each treatment and the experiment was repeated once. Data from the repeated experiments were pooled for analysis. Total number of germinated seedlings was recorded 10 days after sowing and a second observation taken after 5 weeks (2 weeks after pathogen inoculation) in order to determine per cent seedling mortality.

2.2. Determination of antioxidant activities

Leaves of the chickpea plants from all the treatments were harvested randomly at 24, 48, 72 and 96 h after pathogen inoculation. The collected leaves were then washed in running tap water and stored in a deep freezer (−80 °C) for biochemical analysis. Superoxide dismutase activity (SOD) [E.C.1.15.1.1] activity was measured by the method described by Fridovich (1974) using riboflavin/methionine system. For extraction and assay of SOD enzyme, 0.1 g leaf sample was homogenized with extraction buffer (2.0 ml; 0.1 M phosphate buffer containing 0.5 mM EDTA at pH 7.5) in a pre-chilled mortar and pestle. The homogenate was centrifuged at 15,000 × *g* for 20 min at 4 °C. The reaction was mixture consisted of 200 mM methionine, 2.25 mM nitroblue tetrazolium chloride (NBT), 3 mM EDTA, 100 mM phosphate buffer (pH 7.8), 1.5 M sodium carbonate, enzyme extract (200 μl) and the final volume was maintained to 3 ml. The reaction was started by adding 2 μM riboflavin (0.4 ml) and placing the tubes under two 18-W fluorescent lamps for 15 min. After 10 min, the reaction was terminated by switching off the light, and the tubes were then kept in dark. A complete reaction mixture without enzyme served as control. The absorbance was recorded at 560 nm on a UV-vis spectrophotometer (Thermo scientific UV-1). One unit of SOD activity represents the amount that inhibits nitroblue tetrazolium photoreduction by 50% under the assay conditions. Similarly, peroxidase (POx) [E.C.1.11.1.7] activity was measured spectrophotometrically with slight modification as described by Hammerschmidt et al. (1982) with pyrogallol as hydrogen donor. Briefly, leaf samples (0.1 g) were homogenized in ice cold 0.1 M phosphate buffer (5.0 ml; pH 7.0), at 4 °C. The homogenate was centrifuged at 16,000 × *g* at 4 °C for 15 min and the supernatant was used as the crude enzyme source. The reaction mixture was consisted of 0.05 M pyrogallol (1.5 ml), enzyme extract (50 μl) and 1% H_2O_2 (0.5 ml). The absorbance at 420 nm was recorded at 30 s intervals for 3 min. The enzyme activity was expressed as U min^{-1} g^{-1} fresh weight (FW). Further, lipid peroxidation (LPO) of the leaf samples was measured as the amount of malondialdehyde (MDA) determined by the thiobarbituric acid