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Phenols enhancement effect of microbial consortium in pea plants restrains *Sclerotinia sclerotiorum*

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HIGHLIGHTS

- Microbial consortium of *T. harzianum*, *B. subtilis* and *P. aeruginosa* was used.
- Their role in modulating phenols in presence of *S. sclerotiorum* was studied.
- Induction of phenolic compounds and their modulation in leaf and collar region was investigated.
- The phenylpropanoid metabolism was strongly involved in providing resistance.

GRAPHICAL ABSTRACT

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ABSTRACT

Microbial interactions occurring in rhizosphere may play important roles in providing protection against phytopathogens. Induction of phenolic compounds and their modulation in leaf and collar region was investigated upon challenge inoculation with *Sclerotinia sclerotiorum* in pea plants untreated and treated with beneficial microbes viz. *Bacillus subtilis* BHHU100, *Trichoderma harzianum* TNHU27 and *Pseudomonas aeruginosa* PJHU15 either singly or in the form of consortium. Changes in phenolic compounds in both leaves and collar region indicated that the induced response was systemic in nature altering the physiological status of the host plant. The phenylpropanoid metabolism was strongly involved in providing resistance against *S. sclerotiorum* challenge; especially in the plants treated with microbes in consortium, where the response was exaggerated in order to cope up with the biotic stress induced by the pathogen. Enhanced accumulation of phenolics viz., shikimic acid, gallic acid, chlorogenic acid, syringic acid, p-coumaric acid, cinnamic acid, salicylic acid, myricitin, quercetin and kaempferol occurred both in leaf and collar tissue.

1. Introduction

Sclerotinia sclerotiorum (Lib.) de Bary is an ascomycete, non-specific, cosmopolitan necrotrophic fungal pathogen, attacking more than 500 species of higher plants (Willets and Wong, 1980) and causes numerous soft rots of horticultural and agricultural crops. Extensive crop loss, wide host range and the general difficulty of controlling diseases caused by *S. sclerotiorum* have been consistently attracting the researchers for sustained research on this pathogen (Xiao et al., 2014; Kabbage et al., 2015).

Beneficial microbial interactions occurring in the rhizosphere can be involved in finding a suitable method for biological disease control and has thus received considerable attention (Jain et al., 2013a). Amongst the various mechanisms of successful disease control, induced resistance is a promising target regulated by a network of signal transduction pathways with phenols playing key role as

Abbreviations: BCAs, biocontrol agents; ISR, induce systemic resistance.

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signal molecules (Mandal et al., 2010). Phenolics are a class of plant secondary metabolites containing one or more hydroxyl derivatives of benzene rings. They are widely distributed in plants and are involved in host defense responses, growth, reproduction, pigmentation and for many other functions (Boudet, 2007). Phenolics and polyphenols refer to all secondary natural metabolites arising biogenetically from the shikimate-phenylpropanoids-flavonoids pathways in the plastid (Lattanzio et al., 2006), as by-products of the monolignol pathway and/or as breakdown products of lignin and cell wall polymers in vascular plants (Harborne, 1980; Carpita and McCann, 2000; Croteau et al., 2000). They induce resistance by providing metabolic plasticity and thus alleviate the stress caused by biotic and/or abiotic factors.

Phenolics are known in many host pathogen interactions (Nicholson and Hammerschmidt, 1992; Hammerschmidt, 2005). They are both antimicrobial (Gómez-Vásquez et al., 2004; Jung et al., 2004), and play crucial roles in host defense sensing and defense-triggering (Beckman, 2000). Amongst the various phenolic acids, the constitutive phenols are known to provide resistance either directly by exhibiting bactericidal and fungicidal activities (Cowan, 1999) or indirectly through activation of defense responses in the hosts (Harborne, 1998). Plant growth promoting microbes have been well studied for years for their plant growth-promoting effect via effective conquest of soil borne plant diseases through antibiosis, production of cell wall macerating enzymes, competition for space and nutrients and induced systemic resistance (ISR) (Mandal et al., 2010). Amongst various chemicals and compounds produced by plants, ISR is majorly mediated through production of several defense compounds belonging to phenolic acids and alkaloids (Lattanzio et al., 2006). There has been a large body of literature describing promising uses of plant associated microbes as effective agents for plant protection and health but what has largely remained unexplored is the exact mechanism of disease suppression especially with respect to production of phenolic acids. Greater accumulation of phenols may immunize plants against pathogenic stress (Matta et al., 1969). Increased accumulation of phenols was found to enhance host resistance in grapes, where phenolic stilbenes induced resistance to the fungal colonization (Jeandet et al., 2002). Similar increase in phenolic concentrations in plants treated with beneficial microorganisms was also observed in response to pathogen challenge (Sarma and Singh, 2003; Singh et al., 2011).

Previously, we have evaluated the potential of biocontrol agents (BCAs) viz. isolates of *Trichoderma harzianum* ARS culture collection number NRRL 30596, *Bacillus subtilis* JN099686 and *Pseudomonas aeruginosa* JN099685 in a microbial consortium for management of Sclerotinia rot of pea under greenhouse conditions and found decrease in plant mortality and increase in defense related, antioxidant enzymes and suppression of oxalic acid induced cell death (Jain et al., 2012; Jain et al., 2013b, 2014a). These microbes were also found to modulate nutritional and antioxidant potential of pea seeds and pericarp (Jain et al., 2014b). These results indicated that these microbes induce systemic resistance. The present study was aimed to compare the variation in phenolic profile of pea plants treated with above BCAs either singly or in consortium in both leaf and collar region upon challenge inoculation with *S. sclerotiorum*.

2. Materials and methods

2.1. In vitro screening of the isolated microbes for their growth promoting activities

P. aeruginosa PJHU15 (GenBank accession: JN099685), *B. subtilis* BHHU100 (GenBank accession: JN099686) and *T. harzianum* TNHU27 (ATCC No. PTA-3701) were isolated as mentioned in our previous study (Jain et al., 2012). These isolates were screened and selected on the basis of their antagonistic potential against *S. sclerotiorum* and used in consortium mode as they were compatible *in vitro* and *in vivo* as studied previously by giving seed treatment (Jain et al., 2012).

Pectolytic activity was detected on the medium containing pectin as described by Hankin et al. (1971). Amylolytic and proteolytic activities were detected in medium amended with soluble starch and gelatine, respectively (Hankin and Anagnostaksis, 1975). Lipolytic activity was assayed on the medium described by Sierra (1957) with sorbitan monolaurate (Tween 20) used as the lipid substrate. Chitinase activity was determined on solid agar plates with chitin prepared by method described by Campbell and Williams (1951).

Indole acetic acid (IAA) was quantitatively analyzed using the method of Loper and Scroth (1986) using different concentrations of tryptophan. Solubilization of tricalcium phosphate was quantified in liquid medium as described by King (1932).

2.2. Inoculum preparation

Flasks containing 100 ml of nutrient broth were inoculated with pure culture of *P. aeruginosa* PJHU15 and *B. subtilis* BHHU100 and were kept on a rotating shaker (150 rev min^{-1}) for 48 h at 27 ± 2 °C. Centrifugation at 6000*g* for 10 min at 4 °C was done to obtain bacterial pellets. Pellets were washed thrice with sterile distilled water and suspended in 4 ml of sterile distilled water. The final cell density was adjusted to 4×10^8 CFU ml^{-1} using a 'Thermo Scientific UV 1' spectrophotometer. Similarly, *T. harzianum* conidia were harvested from 6 days old potato dextrose agar (PDA) plates kept at 27 ± 2 °C. The final cell density of 2×10^7 CFU ml^{-1} was maintained for *T. harzianum* for giving seed treatment. The inoculums density was measured by plate counting method.

S. sclerotiorum used in the present study, was isolated from an infected pea plant as mentioned in our previous report (Jain et al., 2012). Multiplication of the pathogen was done on bajra seed meal-sand medium (bajra seed 250 g, washed white sand 750 g, distilled water 250 ml) at 25 ± 2 °C for 15 days (Sarma et al., 2007). The colonized culture was homogenized well in mortar and pestle prior to its use as inoculum.

2.3. Greenhouse experiment

For conducting the greenhouse experiment, 2:1:1 mixture of sandy soil, vermicompost and farmyard manure was sterilized for three consecutive days at 15 lbs pressure for 30 min followed by filling of plastic pots with 1.5 kg of soil mixture. Surface sterilization of pea seeds (*Pisum sativum* L. cv. Arkel) was done using 1% sodium hypochlorite for 30 s followed by rinsing twice with sterile distilled water and drying under a sterile air stream. Suspensions of the organisms were prepared in carboxymethyl cellulose (1%) followed by coating of the seeds with *B. subtilis*, *P. aeruginosa* and *T. harzianum* either singly or in dual or triple combinations. Seeds were soaked and left in the respective suspensions of organisms for 10 h [in case of consortia, equal amount of suspensions (v/v) were mixed], after which excess microbial suspension was drained off and the seeds were dried overnight in sterile Petri dishes. The following treatments were examined: (i) *B. subtilis* (BHHU100), (ii) *T. harzianum* (TNHU27), (iii) *P. aeruginosa* (PJHU15), (iv) BHHU100 + TNHU27, (v) BHHU100 + PJHU15, (vi) TNHU27 + PJHU15, and (vii) BHHU100 + TNHU27 + PJHU15. Apart from the above mentioned treatments, two sets of BCA untreated control plants were also maintained separately. Three pots for each treatment were maintained in green house for 4 weeks. After 4 weeks, all treatments except one set of control plants was challenged with