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Endophytic *Streptomyces* spp. underscore induction of defense regulatory genes and confers resistance against *Sclerotium rolfsii* in chickpea

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HIGHLIGHTS

- Endophytic *Streptomyces* spp. triggered systemic resistance in chickpea under *S. rolfsii* stress.
- *Streptomyces* spp. primed plants also mitigate oxidative stress generated by *S. rolfsii*.
- PAL, PPO, and total phenolics accumulation was increased in *S. griseus* treatment.
- SOD, PO, APX and GPX was enhanced by priming with *S. griseus*.
- *S. griseus* induced gene expression of *SOD*, *PO*, *PAL*, *APX*, *CAT*, *CHI* and *GLU* in chickpea.

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ABSTRACT

This study evaluated the potential of endophytic *Streptomyces* spp. *viz*., *S. diastaticus*, *S. fradiae*, *S. olivochromogenes*, *S. collinus*, *S. ossamyceticus*, and *S. griseus* to trigger systemic resistance and mitigate oxidative stress in chickpea against *Sclerotium rolfsii*. The role of endophytes stimulating systemic resistance was evaluated by seed treatments in pathogen inoculated chickpea. Substantial increase in defense related enzymes such as phenylalanine ammonia lyase (PAL) and polyphenol oxidase (PPO), along with the accumulation of total phenolics and flavonoids in *S. griseus* was noticed in plants inoculated with *S. rolfsii*. Similarly, a significant increase of superoxide dismutase (SOD), peroxidase (PO), ascorbate peroxidase (APX) and guaiacol peroxidase (GPX) was observed in the same treatment which favors least lipid peroxidation in chickpea under pathogenic stress. In addition, under scanning electron microscope *S. griseus* primed plants showed undamaged tissue in the collar region. Furthermore, real-time polymerase chain reaction analysis of genes encoding *SOD*, *PO*, *PAL*, *APX*, catalase, chitinase and β-glucanase showed significant fold change which corroborated well with the above findings. These results indicate that the chickpea defense pathway is triggered after perception of endophytes to synthesize various enzymes, which led to an induced resistance against *S. rolfsii*.

1. Introduction

Reactive oxygen species (ROS) are a source of oxidative stress that are produced in plants under various biotic and abiotic stresses (Ahmad et al., 2008). Plants have been endowed with complex cellular mechanisms and varied defense machinery to protect themselves against several phytopathogens. These mechanisms become futile when pathogens infect the plants owing to dehydrogenases produced by pathogens that deactivate the plant immunity and inhibit or neutralize the effect of activated defenses (Kurabachew and Wydra, 2014). Therefore, priming plants with biotic elicitors prior to pathogen infection enhances the level of systemic resistance against a pathogen and results in reduce disease mortality (Beckers and Conrath, 2007). Research on induced resistance has highlighted the essential role of a few endophytic microorganisms in activating expression of defense-related genes (Wang et al., 2005; Gond et al., 2015).

Endophytic actinobacteria have been reported to produce a range of bioactive metabolites that play an imperative function in plant resistance (Qin et al., 2011). Several studies on beneficial microbes have shown that they have eliciting activities, leading

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to a variety of defense reactions in host plants in response to pathogen infection, including the secretion of defense related enzymes/genes such as peroxidase (PO), phenylalanine ammonia lyase (PAL), phenolic compounds and specific flavonoids along with induction of pathogenesis related (PR) proteins (Conn et al., 2008; Mercado-Blanco and Lugtenberg, 2014). Among many PR genes, the expression of chitinase and β-1,3 glucanase is known to be stimulated in various crops in response to fungal pathogens and their secretion is well correlated with induced resistance (El-Rahman et al., 2012). Antioxidants such as PO and polyphenoloxidase (PPO) are responsible for phenols oxidation which contributes to the reinforcement of cell barriers (Lavania et al., 2006). Another enzyme, PAL initiates the phenylpropanoid pathway, resulting in the biosynthesis of phytoalexins or phenolic compounds. Several studies have indicated that activation of phenylpropanoid pathway can offer protection against various phytopathogens (Ferrer et al., 2008; Zhang and Liu, 2015).

Host plants mitigate the level of ROS to some extent by antioxidant machinery through activities of various enzymes and maintain a homeostatic state of the free radicals. While sometimes this antioxidant machinery is overridden by the rapid and short-lived overproduction of ROS during biotic stress resulting in disease development. Hence, priming plants with beneficial actinobacteria is considered an effective strategy to induce resistance under biotic stress including excessive ROS generation (Kunoh, 2002). Actinomycetes are considered valuable for biocontrol of diverse plant pathogens *via* mechanisms of disease suppression and provide improved efficacy, consistency and reliability under field situations (Sharma, 2014). The regulation of antioxidant activities in the host by actinomycetes is scantily understood and some preliminary findings pointed out their function in the ROS-scavenging process (Singh et al., 2015b).

Chickpea (*Cicer arietinum*) is a leguminous crop that is attacked by various phytopathogens. Among them, *Sclerotium rolfsii* is one of the most devastating pathogen that transmits the disease to the collar region from root (Maurya et al., 2009). Initially the infection initiates from *S. rolfsii* hyphae which are closely appressed to the surface of epidermal region of susceptible host (Nene et al., 1987). Later infection of *S. rolfsii* increases as the numerous hyphae that invades the host tissues inter and intracellularly into the cell lumen, across the cells etc. The collar region gets covered by a profuse growth of *S. rolfsii* and affected host stem by showing shredding symptom. The pathogen is reported to cause maximum harm to the plants in the first two weeks of chickpea growth (Nene et al., 1987; Zarani and Christias, 1997).

The exact mechanism through which the *Streptomyces* spp. ameliorate the systemic resistance of primed plant is still unknown; hence efforts should be initiated to study the role of oxidative enzymes and phenolic compounds involved in interactions between chickpea plants treated with endophytic *Streptomyces* spp. and *S. rolfsii*. Precious reports highlighted the importance of actinomycetes in strengthening host plant defense to restrict pathogen invasion for sustainable agricultural systems (Goudjal et al., 2014; Zamoum et al., 2015). Therefore, the present investigation aims to gain a better understanding of the expression profiling of defense related enzymes/genes and antioxidants in a tripartite interaction of *Streptomyces*, *S. rolfsii* and chickpea.

2. Materials and methods

2.1. Microbial inoculums and seed priming with endophytic Streptomyces strains

Streptomyces diastaticus SP2, *Streptomyces fradiae* SP4, *Streptomyces olivochromogenes* SP5, *Streptomyces collinus* SP8, *Streptomyces ossamyceticus* SP10, and *Streptomyces griseus* SP12 were individually grown in glucose yeast malt (GYM) broth and incubated at 30 ± 2 °C for 7–10 days (Singh and Gaur, 2016). After incubation, the broth was centrifuged at 12,000×g for 5 min and the pellets were adjusted to 10^8 CFU mL^{-1} (in 0.85% saline). Pathogen *S. rolfsii* was grown in sterile cornmeal sand (80 g of clean quartz sand (SiO_2), 2 g of yellow cornmeal, and 25 mL of SDW; moisture level 50%) at 28 °C for 15 days (Rawn, 1991; Singh et al., 2015a).

Surfaced sterilized (2% NaOCl) chickpea seeds were coated with pure microbial suspensions with 10% (w/v) carboxyl methyl cellulose (HiMedia, India) and the seeds were kept at 23 ± 2 °C in a rotary shaker (70 rpm) for 3–4 h to facilitate the dispersion of isolates. Two trials were carried out in plastic pots (15 cm × 10 cm) containing 1.5 kg sterile soil (four germinated seeds per pot). The treatments were as followed (i) chickpea without pathogen (control), (ii) chickpea with pathogen (infected control), and (iii–viii) chickpea with *Streptomyces* isolates (SP2, SP4, SP5, SP8, SP10 and SP12 respectively) and pathogen. After 3 weeks, the pathogen was inoculated in the soil at the rate of 50 g per pot. The experiment was maintained in CSIR-National Botanical Research Institute, Lucknow and the pots were arranged in a complete randomized design in a set of twelve replicates under green house conditions. The experiment was terminated after 2 weeks of pathogen inoculation to record various physiological parameters such as plant weight, shoot height, root length, seeds, flowering, nodulation and plant mortality was measured according to Singh and Gaur (2016).

2.2. Determination of defense related enzymatic and antioxidant activities after seeds priming with endophytes

The chickpea seedlings were harvested for five consecutive (0, 1, 2, 3 and 4) days after pathogen inoculation (dapi). The protein content was estimated using bovine serum albumin (Sigma) as a standard (Bradford, 1976). For superoxide dismutase (SOD) activity, briefly the seedling (0.1 g) was homogenized in 1 mL of extraction buffer (0.1 M potassium phosphate buffer, 0.1 mM EDTA, 1% Triton X-100, 5% PVPP, and 2 mM β-mercaptoethanol) and centrifuged at 13,000×g for 15 min. The upper layer was mixed with the solution (0.1 M KPB containing Triton X-100, EDTA, L-methionine, nitroblue tetrazolium and riboflavin) and was incubated under 18-W fluorescent lamp for 15 min. The activity was observed at 560 nm according to Flohe (1984). PO assay was measured by homogenizing seedlings (0.1 g) in 0.1 M sodium phosphate buffer (pH 7.0; 1.5 mL) and centrifugation was done at 12,000×g for 10 min. The 25 μL supernatant was mixed with 0.05 M pyrogallol and 1% H_2O_2. The absorbance was recorded at 420 nm (Hammerschmidt et al., 1982). For lipid peroxidation (LPX) activity, the seedlings were homogenized using 20% trichloroacetic acid (TCA, w/v) with 1% TBA (w/v). The activity was measured at 532 nm and concentration of LPX was expressed as nmole g^{-1} FW (Ohkawa et al., 1979).

PAL activity was measured as μmol trans-cinnamic acid g^{-1} FW (Nagarathna et al., 1993). The seedlings were extracted in 25 mM sodium borate buffer (1 mL, pH 7.0) with β-mercaptoethanol (32 mM) and centrifuged at 10,000×g for 20 min. The reaction mixture containing enzyme extract (0.1 mL), borate buffer (0.5 mL; pH 8.7), distilled water (0.65 mL) and 0.1 mM L-phenylalanine (0.25 mL; pH 8.7) was kept at 32 °C for 30 min. The reaction was completed by addition of 1 M trichloroacetic acid and the absorbance was measured at 290 nm. Polyphenol oxidase (PPO) activity was calculated by homogenizing the seedlings in 0.1 M phosphate buffer (5.0 mL; pH 6.5) and centrifuged at 16,000×g. The supernatant (0.1 mL) was added to 0.01 M catechol and absorbance was recorded at 495 nm (Mohammadi and Kazemi, 2002). The total phenolic content (TPC) was calculated according to Zheng and Shetty (2000). A standard curve was prepared by differ-