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Analysis of Peroxidase and Polyphenol Oxidase Isozyme Profiles in Groundnut Plants Treated with *Streptomyces* sp.

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Isozyme analysis was carried out to determine whether Peroxidase (PO) and Polyphenol oxidase (PPO) activities were induced in groundnut (*Arachis hypogaea* L.) leaves by application of antagonistic *Streptomyces* sp. strains through seed and soil. PO isozyme analysis of protein extracts from control groundnut plants exhibited two isozymes (PO-1 and PO-2). Seed treatment or seed treatment plus soil application with *Streptomyces* sp. strain PDK strongly induced the activity of PO-2. In addition, two new PO isozymes (PO-3 and PO-4) were also appeared in the *Streptomyces* sp. strain PDK treated plants. No significant change in the PO isozyme profile of groundnut was observed in plants treated with other *Streptomyces* sp. strains *viz*., CBE and MDU through seed. However increase in the activity of PO-2 was observed in plants treated with MDU through seed plus soil. PPO isozyme analysis of protein extracts from control groundnut plants exhibited two isozymes (PPO-1 and PPO-2). An increase in the activities of both PPO-1 and PPO-2 compared to control was observed in plants treated with all three strains of *Streptomyces* sp. through seed or seed plus soil. The increased activities of both PO and PPO in groundnut plants upon treatment with *Streptomyces* sp. might have contributed to resistance of groundnut against stem rot caused by *Sclerotium rolfsii*.

Key words: *Arachis hypogaea*, Induced resistance, Peroxidase, Polyphenol oxidase, *Streptomyces sp*.

Biological control using antagonistic actinobacteria is one of the environmentally friendly methods of management of crop diseases (Doumbou *et al*., 2001). Phylum *Actinobacteria* constitute a morphologically diverse group, distinguished from other Gram-positive bacteria by their filamentous growth and GC-rich DNA (Lacey, 1997) . Among the actinobacteria, *Streptomyces* spp. are the most popular and found worldwide in soil and have been implicated in the antagonism of a wide variety of plant pathogenic bacteria, fungi and nematodes for their potential use as biological disease control agents (Doumbou *et al*., 2001). The actinobacteria are well known for their abilities to produce antibiotics and other secondary metabolites (Behal, 2000). The antagonistic actinomycete, *Streptomyces griseoviridis* is commercially available with the trade name Mycostop® to control *Alternaria* and *Fusarium* diseases in crucifers and *Fusarium* wilt in carnations (Tahvonen and Avikainen, 1987). Besides soil-borne diseases, foliar diseases can also be controlled by application of actinobacteria (Tahvonen and Avikainen, 1987). Activation of host biochemical defense responses is one of the mechanisms proposed for the biocontrol activity of actinobacteria against plant pathogens (Conn *et al*., 2008). Wei *et al*. (1991) demonstrated that non-pathogenic *Pseudomonas* spp. strains are capable of triggering plant-mediated resistance response in above-ground plant parts when applied on roots. This type of induced resistance is often referred to as rhizobacteria-mediated induced systemic resistance (ISR). Stem rot, also known as southern blight, southern stem rot, *Sclerotium* rot or white mold incited by *Sclerotium rolfsii* Sacc., is a serious soilborne disease in groundnut (*Arachis hypogaea* L.) (Mehan *et al*., 1994). We recently reported that seed treatment and soil application of *Streptomyces* sp. strains CBE, MDU and PDK significantly reduced the incidence of stem rot groundnut caused by *Sclerotium rolfsii* under greenhouse and field conditions (Adhilakshmi *et al*., 2013). ISR has been reported as one of the mechanisms by which actinobacteria reduce plant disease. Conn *et al*. (2008) demonstrated that inoculation of *Arabidopsis thaliana* with endophytic actinobacteria induced SAR and JA/ET gene expression. Hence the reduction in the stem rot incidence in groundnut upon treatment with *Streptomyces* sp. may be related to stimulation of host biochemical defense mechanisms. The objective of the present study was to understand the defense mechanism of groundnut plants treated with *Streptomyces* sp. against pathogens.

Materials and Methods

Fungal and bacterial cultures

Streptomyces sp. strains CBE, PDK, MDU, ANR and SA isolated and characterized from groundnut

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rhizosphere (Adhilakshmi *et al*., 2013) were used in this study.

Development of talc-based powder formulations of Streptomyces sp.

Talc-based powder formulations of *Streptomyces* sp. strains *viz*., CBE, PDK and MDU were developed as described by Vidhyasekaran *et al*. (1997). One kg of talc (hydrated magnesium silicate) powder was mixed with 10 g of carboxymethyl cellulose and the pH was adjusted to 7.0 by adding calcium carbonate. The mixture was then autoclaved for 30 min. *Streptomyces* sp. was grown in nutrient broth for 48 h at 28 ± 2 °C on a rotary shaker. Four hundred milliliters of *Streptomyces* suspension containing 9 x 10_8 CFU ml_{-1} was added to one kg of the sterilized talc mixture and mixed thoroughly. The formulation was packed with 35 % moisture content in polythene bags, sealed and stored at room temperature (28 ± 2 °C). The formulations, stored up to 30 days were used in greenhouse experiment.

Greenhouse experiment

Earthen pots (30 cm diameter) were filled up with 5 kg of soil and arranged on the greenhouse benches. The seeds of groundnut (cv. TMV7) were treated with the powder formulation of *Streptomyces* sp. at the rate of 1 g per 100 g seed and sown in the soil. In another set of pots, seed treatment was followed by soil application of talc-based powder formulation at 1 g per pot. Seeds mock-treated with the powder formulation without *Streptomyces* sp. were kept as control.

PO and PPO isozyme analysis

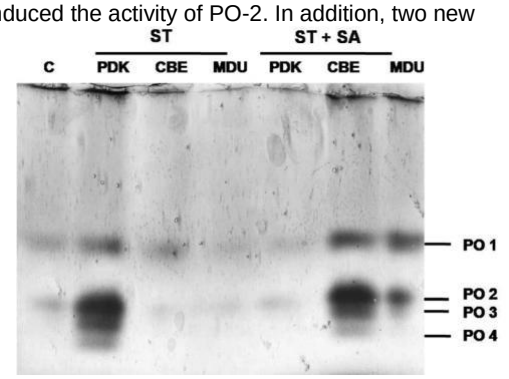
Leaves from the experimental plants were collected 20 days after treatment and frozen in liquid nitrogen and then stored at –80°C until further use. The proteins were extracted by homogenizing 1 g of tissue in 2 ml of 0.01 M potassium phosphate buffer (pH 7.0) at 4°C. The homogenate was then centrifuged at 10,000 g for 20 min at 4_oC in a refrigerated centrifuge and the supernatant was collected. Protein content in the enzyme extracts was measured as described by Bradford (1976) using bovine serum albumin as the standard.

PPO isoenzyme profiles were examined after running the samples on non-denaturing 12% polyacrylamide slab gels. After native PAGE, the gel was equilibrated for 30 min in 0.1 % p-phenylenediamine in 0.1 M potassium phosphate buffer (pH 7.0) followed by 10 mM catechol in the same buffer. The addition of catechol was followed by a gentle shaking which resulted in appearance of dark brown discrete protein bands (Jayaraman *et al*., 1987).

Results and Discussion

Induecd Systemic Resistance (ISR) has been reported as one of the mechanisms by which PGPR reduce plant disease and is functioning through the manipulation of the physical and biochemical properties of host plants (Pieterse *et al*., 2002). PGPR-elicited ISR has been demonstrated in many plant species, including *Arabidopsis* spp., bean, carnation, cucumber, radish, tobacco, and tomato (Van Loon *et al*., 1998). Several authors demonstrated that selected strains of non-pathogenic plant growth-promoting rhizobacteria, which colonize the rhizosphere of the plant, are capable of triggering a plant-mediated resistance response in above-ground plant parts (Pieterse *et al*., 2003). Shoresh *et al*. (2005) reported that *Trichoderma asperellum* (T203) modulates the expression of genes involved in the jasmonate/ethylene signaling pathways of ISR (Lox1, Pal1, ETR1 and CTR1) in cucumber plants. The authors further demonstrated that a subsequent challenge of *Trichoderma*-preinoculated plants with the leaf pathogen *Pseudomonas syringae* pv. *lachrymans* resulted in higher systemic expression of the pathogenesis-related genes encoding for chitinase, β-1,3-glucanase and peroxidase relative to non-inoculated, challenged plants and concluded that *Trichoderma* induced a potentiated state in the plant enabling it to be more resistant to subsequent pathogen infection.

PGPR are known to induce multiple biochemical defense mechanisms including induction of phenolics, phenylalanine ammonia-lyase, peroxidase, polyphenol oxidase, lignins, increased expression of pathogenesis-related (PR)-proteins and production of signal transduction molecules such as salicylic acid and ethylene (Ramamoorthy *et al*., 2002; Thangavelu *et al*., 2003). In the present study PO isozyme analysis of protein extracts from control groundnut plants exhibited two isozymes (PO-1 and PO-2) (Fig. 1). Seed treatment or seed treatment plus soil application with *Streptomyces* sp. strain PDK strongly induced the activity of PO-2. In addition, two new



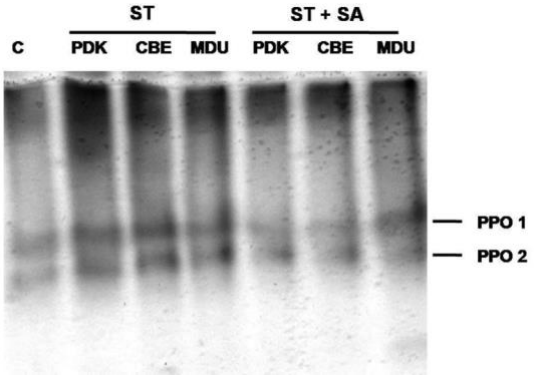
Fifty micrograms equivalent of protein was loaded on each lane. C- control; ST- seed treatment; SA- soil application. PDK, CBE and MDU denote the strains of *Streptomyces* sp.

Fig. 1. Peroxidase isoenzymes in protein extracts from groundnut plants treated with strains of *Streptomyces* spp.

PO isozymes (PO-3 and PO-4) were also appeared in the *Streptomyces* sp. strain PDK treated plants. No significant change in the PO isozyme profile of groundnut was observed in plants treated with other *Streptomyces* sp. strains *viz*., CBE and MDU through

seed. However increase in the activity of PO-2 was observed in plants treated with MDU through seed plus soil.

Peroxidases are involved in the lignification, suberification, polymerization of hydroxy proline-rich glycoproteins, regulation of cell wall elongation, wound healing and resistance against pathogens in plants (Yoshida *et al*., 2003). Increased activities of PO have been shown to be correlated with resistance reactions in many host-pathogen interactions (Hammerschmidt *et al*., 1982; Goncalves *et al*.,



Fifty micrograms equivalent of protein was loaded on each lane. C- control; ST- seed treatment; SA- soil application. PDK, CBE and MDU denote the strains of *Streptomyces* sp.

Fig. 2. Polyphenol oxidase isoenzymes in protein extracts from groundnut plants treated with *Streptomyces* sp.

2013). Furthermore, direct antifungal activity of plant peroxidases has been reported (Caruso *et al*., 2001). Zdor and Anderson (1992) observed an increase of peroxidase activity as well as an increase in the level of mRNAs encoding for phenylalanine ammonia-lyase and chalcone synthase in the early stages of interaction between bean roots and various bacterial endophytes. Khan *et al*. (2006) observed increased activation of class III peroxidase gene in wheat by *Pseudomonas* sp. strain MKB 158 in the presence of *Fusarium culmorum* compared with activation by the pathogen alone. Chen *et al*. (2000) also found that certain peroxidase isomers accumulated in cucumber roots in response to treatment with biocontrol *Pseudomonas* species. The increased PO activity in groundnut plants treated with *Streptomyces* sp. might be involved in lignin biosynthesis which in turn might have contributed to resistance against *S. rolfsii*.

Many phenols and their oxidation products such as quinones are highly toxic to the invading fungi (Sequeira, 1983). The enzymes PO and PPO catalyze the oxidation of phenolic compounds through a PPO-PO-H $_2O_2$ system (Srivastava, 1987). Polyphenol oxidases (PPO) are copper containing enzymes which are involved in catalyzing the oxygen-dependent oxidation of phenols to quinones (Vaughn and Duke, 2006). A number of studies have correlated the induction of PPO activity in plants with a resistance response (Srivastava, 1987; Velazhahan and Vidhyasekaran, 1994). The results of the present study indicate that control groundnut plants exhibited two PPO isozymes (PPO-1 and PPO-2) (Fig. 2). An increase in the activities of both PPO-1 and PPO-2 compared to control was observed in plants treated with all three strains of *Streptomyces* sp. through seed or seed plus soil. The induced PPO might have involved in the resistance of groundnut against *S. rolfsii* through oxidation of phenolic compounds to fungitoxic quinones. From the results of the present study, it can be concluded that the induction of systemic resistance in groundnut appeared to be an additional mechanism by which these *Streptomyces* sp. could protect the crop against stem rot disease.

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