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EVALUATION OF ANTIFUNGAL ACTIVITY OF *STREPTOMYCES PUNICEUS* RHPR9 AGAINST *MACROPHOMINA PHASEOLINA*, CAUSATIVE AGENT OF CHARCOAL ROT DISEASE IN CHILLI (*CAPSICUM ANNUUM* L.)

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ABSTRACT

A number of soil-borne fungi cause diseases in chilli crop that result in significant yield loss. Hence in the present study, *S. puniceus* RHPR9 was evaluated against soil borne pathogens such as *Macrophomina phaseolina, Fusarium oxysporum, Phytophthora capsici* and *Sclerotium rolfsii*. Strain RHPR9 was antagonistic to *M. phaseolina* (76±0.1%), *F. oxysporum* (62±0.1%), *P. capsici* (57±0.2%) and *S. rolfsii* (52±0.1%), with a severe inhibitory impact on fungal growth and development. Further evaluation against charcoal rot disease in chilli was done under greenhouse conditions and plant weight was recorded. In addition, enzymes (peroxidase (POX), polyphenol oxidase (PPO) and phenylalanine ammonia lyase (PAL)) responsible for induced resistance were characterized. It was observed that there was considerable increase of POX, PPO and PAL in chilli plants treated with *S. puniceus* when compared to control. This study elucidates that *S. puniceus* induced POX, PPO and PAL biosynthetic pathways and inhibited *M. phaseolina* and can be potential biocontrol agent.

Keywords **:** Antifungal activity, yield loss, *Capsicum annuum* L.

Introduction

Chilli (*Capsicum annuum* L.) is a major vegetable and spice crop, cultivated globally for its commercial value. It is known as the "miracle spice" since it is the most extensively used universal spice. The colour and pungent levels of Indian chilli are regarded to be world famous for two major commercial attributes. Because of the pigment, certain types are known for their red colour, while other quality factors in chilli include length, breadth and skin thickness. Production of chilli crop globally is estimated to be over 7 million tones and it is grown on 1.5 million hectares of land (Olatunji and Afolayan, 2018). India is the largest producer of chillies in the world (8.5 lakh tones) followed by China (4 lakh tones), Pakistan (3 lakh tones) and Mexico (3 lakh tones). Andhra Pradesh ranks first in India both in area and production with 2.04 lakh hectares and produce 323 thousand tones (Madhavi and Bhattiprolu, 2011). Agricultural researchers have studied various microorganisms with antagonistic properties against phytopathogens that can be used for biocontrol of many plant diseases (Köhl *et al*., 2019). Several plant diseases, including fungi that cause root and crown rot, damage the chilli plant. These pathogenic fungi have been found in practically every region of the globe where chillies are grown. In India, several plant pathogens, such as *Fusarium* spp., cause wilt (Hami *et al*., 2021) and root rot (Morid *et al*., 2012), while the fungus *M. phaseolina* causes root rot and wilting of chilli plants (Hussain *et al*., 2013), dry root rot of chilli by *Sclerotium rolfsii* (Madhavi and Bhattiprolu, 2011; Ganesan *et al*., 2007). Biological control is an ecologically acceptable strategy for plant disease management that may be combined with cultural and physical controls as well as the use of minimal chemicals to create a successful integrated pest management (IPM) system (Monte, 2001).

Actinomycetes are Gram-positive bacteria that generate mycelia and have a high guanine-cytosine ratio (51–78%). This group of microbes is the most important producers of antibiotics and novel metabolites (Hussain *et al*., 2018; Barka *et al*., 2016; Tobias Kieser *et al.,* 2000). Antibacterial agents such as actinomycins from *Streptomyces anulatus* (Waksman and Woodruff, 1993) are examples of commercial bioactive compounds developed from the genus *Streptomyces* that are employed in agriculture. Novobiocin from *Streptomyces niveus* (Kominek, 1972), antifungal compounds blasticidin from *Streptomyces griseochromogenes* (Cone *et al*., 2003) and kasugamycin from *Streptomyces kasugaensis* (Kasuga *et al*., 2017). *Streptomyces* spp. are notable for their effectiveness in biological control of plant disease pathogens (Gopalakrishnan *et al*., 2014; Passari *et al*., 2015). Objective of the present study was to evaluate RHPR9 against four different plant pathogens in plate culture conditions and its efficacy against charcoal rot disease of chilli crop.

Materials and Methods

Microorganism used

Streptomyces puniceus RHPR9 (MH512803) isolated from rhizosphere of medicinal plant (*Coscinium fenestratum*)

known for biosurfactant production and plant growth promotion (Ravinder *et al* published elsewhere) was used in this study.

Screening of S. *puniceus* RHPR9 against phytopathogenic fungi

Dual culture technique was used to determine the antagonistic effect of *S. puniceus* RHPR9 against phytopathogenic fungi (*Fusarium oxysporum, Macrophomina phaseolina, Phytophthora capsici* and *Sclerotium rolfsii*). Petri dishes (90 mm diameter) contained glucose casamino acids yeast extract (GCY) agar medium with the following composition (g/L): glucose (2.0), casamino acids (2.0), yeast extract (0.67), dextrose (2.0), L-tyrosine (0.9), agar-agar (20). Actively grown culture of strain RHPR9 was prepared by inoculating pure colony in GCY broth (3ml) and was streaked 3 cm away from the fungal plug (5mm) placed in the center of petri plate. The plates were then incubated for 5 days at 28±2 °C. The radial growth of phytopathogenic fungi in the direction towards strain RHPR9 was determined. Percentage inhibition of fungi was calculated using formula

$$\text{Percentage of Inhibition (I \%)} = \frac{\text{Control} - \text{Test}}{\text{Control}} \text{X100}$$

Screening of *S. puniceus* RHPR9 for different hydrolytic enzymes

S. puniceus RHPR9 was tested for production of amylase, cellulase and protease. Starch casein agar, carboxymethyl Congo red and 30% skimmed milk agar medium plates were used for screening of amylase, cellulase and protease respectively (Ribeiro dos Santos *et al*., 2012; Rathnan and Ambili, 2011; Boughachiche *et al*., 2016). Plates were incubated at 28±2°C for 4 days. The presence of halo around the colony indicated zone of hydrolysis.

Biocontrol studies

Chilli seeds were surface sterilized with 3% chlorax for 5-10 min. They were washed thoroughly with sterile distilled water. Pots (15×10cm) were filled with optimum soil and following treatments were done

T1: Uninoculated control

T2: *S. puniceus* RHPR9 culture

T3: Glycolipid biosurfactant

T4: Positive control (*M. phaseolina*)

T5: *S. puniceus* RHPR9 culture+ MP

T6: Glycolipid biosurfactant + MP.

Six seeds were sown in each pot and maintained in a greenhouse condition with four replications for each treatment. *M. phaseolina* inoculum was prepared by adding spores to sterilized sorghum seeds (250 gm taken in 1 litre conical flask) and incubated for two weeks under stationary conditions. Fungal inoculum obtained by this method was mixed with soil and then treated to 12 days old plants. All pots were watered at regular intervals. Leaves of these plants were used to estimate various enzymes responsible for Induced systemic resistance (ISR). For this, 2ml of 0.1M sodium phosphate buffer (pH 6.5) was homogenized with 0.5g leaf at 4 °C. The homogenate was centrifuged at 10,000 rpm for 2 minutes and the supernatant was utilized to calculate plant defense enzymes such as PO, PPO and PAL as mentioned below.

Peroxidase (POX)

The activity of peroxidase was evaluated by observing the oxidation of guaiacol in water containing of hydrogen peroxide, as reported by Hammer Schmidt, 1982. 1.8 ml of 0.1 M sodium phosphate buffer, 100 µl of 20 mM guaiacol, and 100 µl of enzyme extract were in the reaction mixer tube. To begin the reaction, 50 µl of 40 mM H_2O_2 was added to the reaction mixture. For 3 minutes, the maximum absorbance at 470 nm was recorded at 30 second intervals and enzymatic activity was reported as an increase in absorbance at 470 nm per min/mg protein.

Polyphenol oxidase (PPO)

Mayer *et al*., 1966 developed a technique for calculating polyphenol oxidase activity. 2.5 ml of 0.1M sodium phosphate buffer (pH 6.5) and 200 µl of enzyme extract were combined in the reaction mixer tube. The reaction was started by adding 200 µl of 0.01 M catechol (substrate) to the reaction mixer. At 495 nm for 3 minutes at 30 second intervals, the change in absorbance owing to catechol oxidation to benzoquinone (yellow colour) was recorded and enzyme activity was represented in OD at 495 nm /mg protein /min.

Phenylalanine ammonia lyase (PAL)

RW Whetten, 1992 technique to determine phenylalanine ammonia lyase activity was used. The synthesis of trans-cinnamic acid from L-phenyl alanine was used to determine enzyme activity. The assay mixture was made up of 0.1 ml enzyme extract, 0.5 ml 50 mM L-phenyl alanine (substrate), 3 ml 0.05 M borate buffer (pH 8.8), and incubated at 30 °C for 15 minutes before being stopped with 0.06 ml 6N HCl. At 290 nm, absorbance was taken against a blank (same volume of assay mixture without substrate). When compared to a standard graph of cinnamic acid, enzyme activity was represented as mol of trans-cinnamic acid (t-CA) mg protein/min.

Statistical Analysis

For each replication, a minimum of three plants were examined. The findings were computed with the control set to 100% to see if there was an increase or reduction in enzyme activity.

Results

Screening of *S. puniceus* RHPR9 against phytopathogenic fungi

S. puniceus RHPR9 inhibited all four phytopathogenic fungi used in this study. Maximum inhibition against *M. phaseolina* (Fig.1) was 76±0.3% followed by *F. oxysporum* (62±0.2%), *P. capsici* (57±0.1%) and *S. rolfsii* (52±0.1%) in a dual culture assay (Table 1).

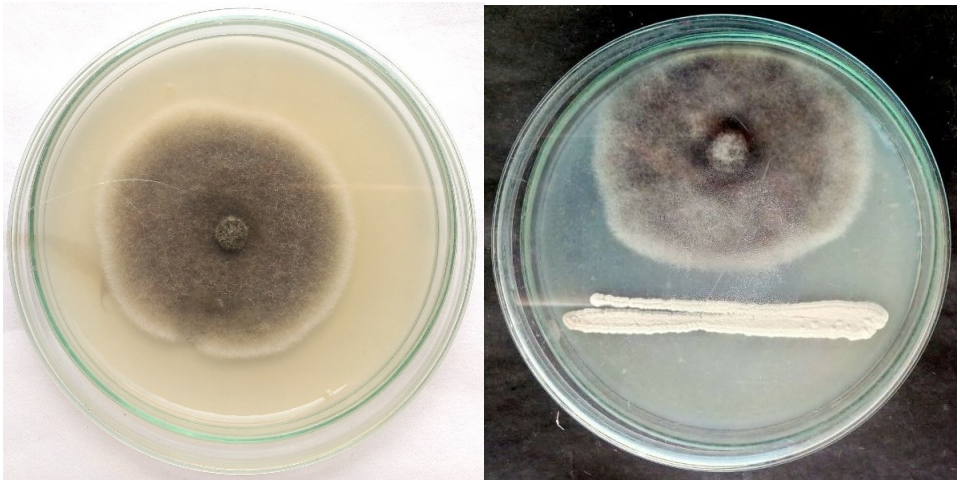

Fig. 1 : Antifungal activity of *S. puniceus* RHPR9 against *M. phaseolina*

Screening of *S. puniceus* RHPR9 for different hydrolytic enzymes

S. puniceus RHPR9 showed positive results for amylase, cellulase and protease, which was evident by the formation of halo zones on starch casein agar, carboxymethyl congo red and skimmed milk agar media (Fig. 2) & Table 1.

Table 1 : Percentage of inhibition of fungal pathogens by dual culture method and hydrolytic enzymes of *S. puniceus* RHPR9

Fungi	Zone of inhibition (%)	Amylase	Cellulase	Protease
F. oxysporum	62±0.2	+	+	++
M. phaseolina	76±0.3	++	+++	++++
P. capsici	57±0.1	++	+++	+++
S. rolfsii	52±0.1	+++	++	++

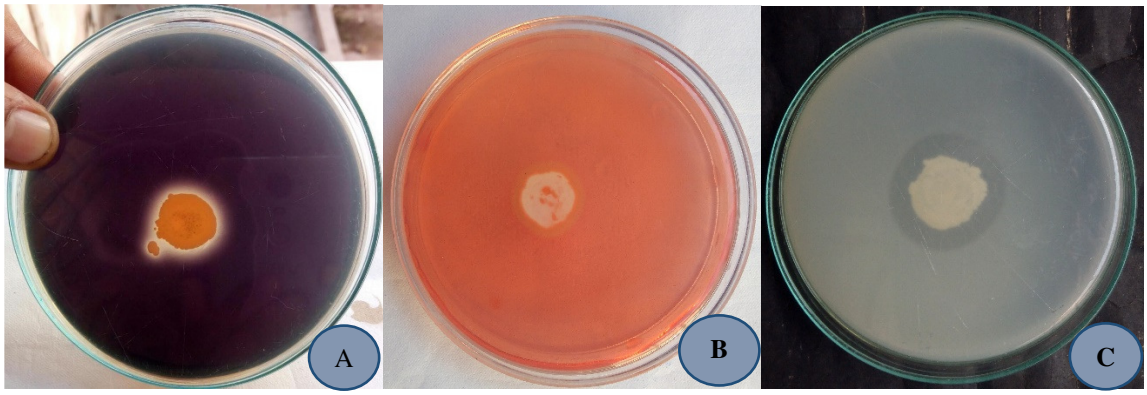

Fig. 2 : Qualitative screening for A. Amylase, B. cellulase and C. protease produced by RHPR9

Biocontrol studies

Peroxidase (POX) activity

Peroxide activity was assessed on the first and seventh day (before inoculation with fungus), the fourteenth and twenty-first day (after inoculation with fungus). The maximum PO activity was obtained on the 14th day, following which there was a little drop. When compared to plants treated with *M. phaseolina* alone, *S. puniceus* RHPR9 and MP treated plants elicited (2.9 folds) the highest peroxidase activity (pathogen control) (Fig. 4).

Polyphenol oxidase (PPO) activity

PPO activity was assessed on the first and seventh day (before inoculation with fungus), the fourteenth and twenty-first day (after inoculation with fungus). PPO activity rose from the 7th to the 14th day (after being challenged with *M. phaseolina*), then decreased somewhat at the 21st day. When compared to plants inoculated with *M. phaseolina* alone (pathogen control), *S. puniceus* RHPR9 and MP treated plants caused (1.8 folds) the highest polyphenol oxidase assay. Plants with no treatments showed the lowest enzyme activity (Fig. 5).

Phenylalanine ammonia lyase (PAL)

PAL activity was assessed on the first and seventh day (before inoculation with fungus), the fourteenth and twenty-first day (after inoculation with fungus). In comparison to plants treated with *M. phaseolina* alone, *S. puniceus* RHPR9 treated plants exhibited a substantial rise in PAL activity when challenged with *M. phaseolina*. In comparison to plants challenged with *M. phaseolina* alone (pathogen), *S. puniceus* RHPR9 (30 folds) produced the highest phenylalanine ammonia lyase (Fig. 6).

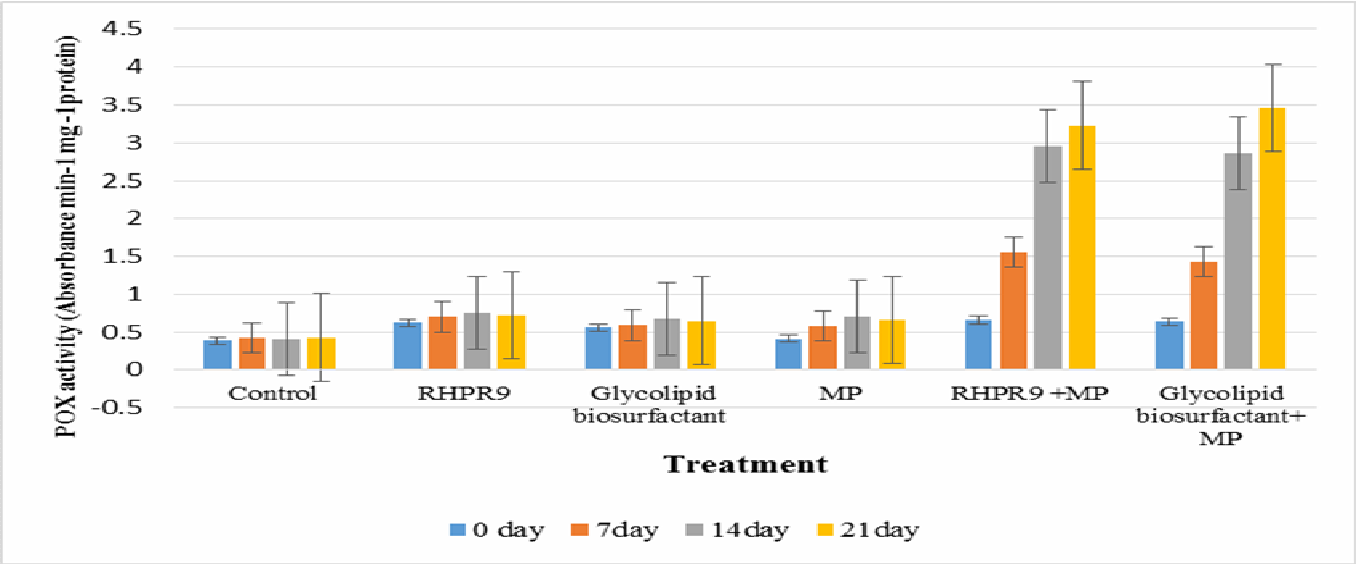

Fig. 4 : Peroxidase (POX) activity changes in chilli leaves; mean values were findings in three replicates. The bars reflect the standard errors.

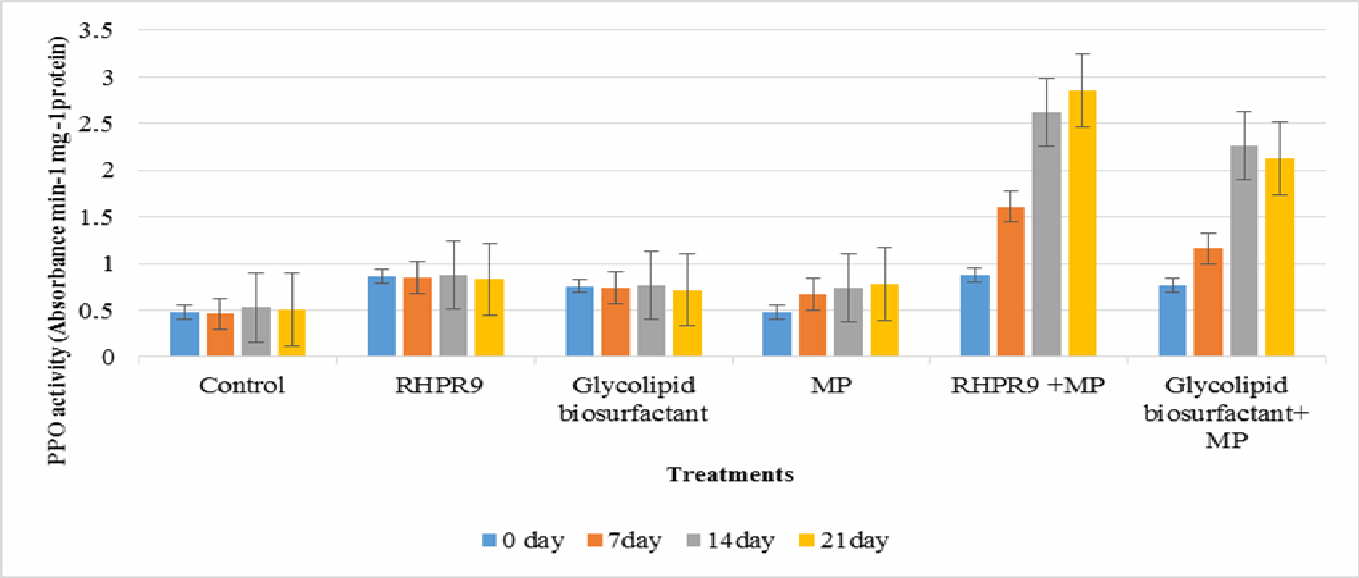

Fig. 5 : Polyphenol oxidase (PPO) activity changes in chilli leaves; mean values were findings in three replicates. The bars reflect the standard errors

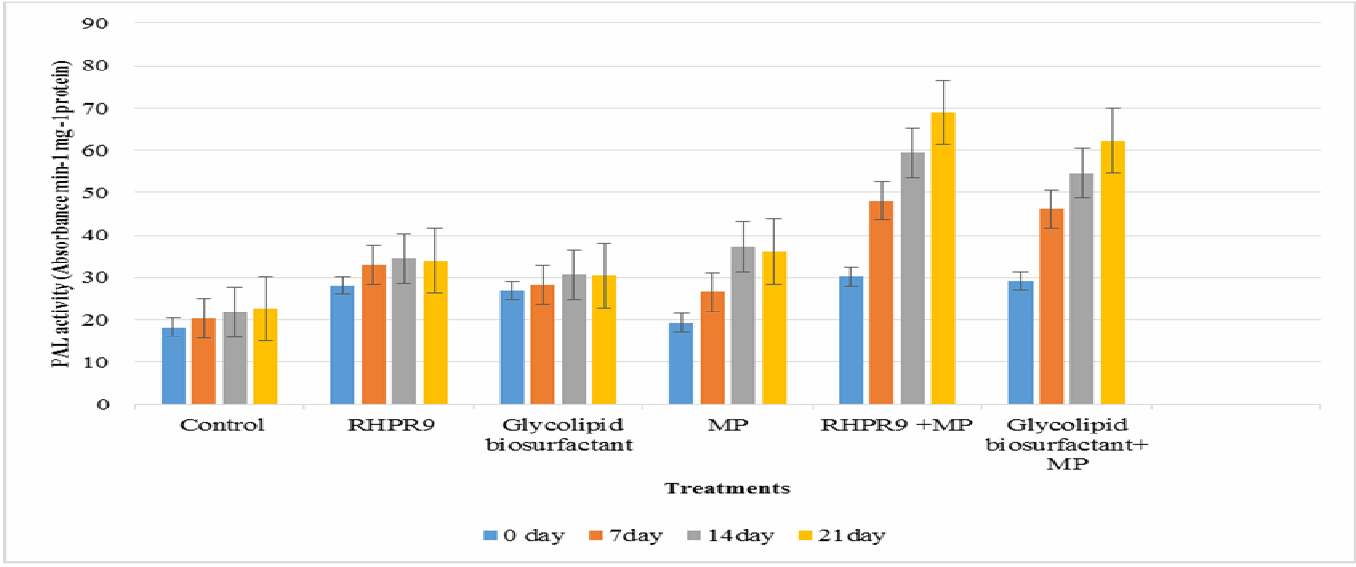

Fig. 6 : Phenylalanine ammonia lyase (PAL) activity changes in chilli leaves; mean values were findings in three replicates. The bars reflect the standard errors.

Discussion

Plants infected with a variety of fungal infections are a major stumbling block in agricultural output worldwide. Phytopathogens are often controlled with a variety of chemical insecticides and fungicides. These chemicals are not biodegradable and their long-term use causes them to stay in the soil. Several microorganisms with considerable antifungal capability can offer plants with "non-chemical" protection by suppressing pathogenic fungal development, which is an essential for long-term agricultural sustainability. In this study, actinobacterial strain isolated from rhizosphere of endangered medicinal plant (*C. fenestratum*) of Western Ghats in Karnataka known for PGP and biosurfactant activity was used. Previous study by Goveas *et al*., 2011 reported that actinobacterial endophytes from medicinal plants have plant growth promoting traits. Goveas *et al*., 2011 also revealed 41 endophytic fungi from *C. fenestratum.*

According to findings of this investigation, *S. puniceus* RHPR9 inhibited all the four pathogenic fungi used in this study. It was observed that strain RHPR9 showed significant inhibition of *M. phaseolina* (76.1%). Shrivastava *et al*., 2017 found that *Streptomyces aureofaciens* K20 inhibited growth of *M. phaseolina* up to 64.5%. Yadav *et al*., 2014 observed antifungal activity of *Streptomyces* sp. S160 against *M. phaseolina* (54.8%) in a similar study. It was also observed that strain RHPR9 produced different hydrolytic enzymes such as amylase, cellulase and protease. Production of hydrolytic enzymes by rhizobacteria enables inhibition of phytopathogens and aids in biological control (Goswami and Deka, 2020).

Plants have a well-organized and regulated defensive network of biochemical processes that may be induced in response to certain stimuli or signals. Novel plant protection tactics in agriculture include inducing innate biochemical defense systems in plants by exposing them with biocontrol agents (Henry *et al*., 2012). *Streptomyces* spp. are well-known for reducing disease prevalence in plants infected with fungal phytopathogens (Conrath *et al*., 2015). There are several mechanisms involved in antagonism exhibited by *Streptomyces* like production of antifungal metabolites, competes for nutrients, mycoparasitism and production of lytic enzymes such as amylase, cellulase and protease (Lee *et al*., 2010).

According to recent research, *Streptomyces* spp. may increase the formation of phenolic biochemical substances linked to host defense. Production of defense-related enzymes can prove a host's resistance to plant diseases. The plant genotyping, physiological circumstances and pathogen type all influence the rise in activity and aggregation of these enzymes. A sequence of morphological and biochemical changes induced by certain fungal strains promote synthesis of anti-pathogen defense molecules. In plants infected with *M. phaseolina*, treatment of chilli seeds with *S. puniceus* RHPR9 resulted in increased levels of PO, PPO and PAL.

S. puniceus RHPR9 induced plant defense related enzymes [Peroxidase (PO), Polyphenol oxidase (PPO), and Phenylalanine ammonia lyase (PAL)] on treatment with *M. phaseolina* in chilli leaves. When compared to pathogen control (*M. phaseolina* infected plants) at 14 days, the maximum activity of POX, PPO, and PAL elicited in chilli bacterized with *S. puniceus* RHPR9 was 2.9, 1.8 and 30 folds. The highest levels of defensive enzymes POX, PPO, and PAL were identified in *Trichoderma asperellum* + *Trichoderma harzianum* treated samples in chilli as compared with control samples, under challenged conditions, according to previous research (Yadav and Dubey, 2021). Inoculated seedlings demonstrated the strongest defensive enzyme activity with 1 to 1.5-fold rise in enzyme activity related to uninoculated seedlings (Abhayashree *et al*., 2017).

Conclusion

In this study, actinobacterial strain *S. puniceus* RHPR9 isolated from rhizosphere of *C. fenestratum* showed significant antifungal activity. *S. puniceus* RHPR9 with PGP and biosurfactant activity showed enhanced production of induced systemic resistance which was evident by production of PO, PPO, PAL. However, further studies at field level are needed for its recommendation as potential biocontrol agent.

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