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Streptomyces mediated stimulation of defense related enzymes to increase the biocontrol resistance in *Capsicum annuum* L. against *Ralstonia solanacearum*

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Abstract The present study evaluated the selected rhizosphere soil *Streptomyces* cultures for the stimulation of defense enzymes in *Capsicum annuum* L. against *Ralstonia solanacearum.* Among the seven *Streptomyces* strains evaluated for the promotion of plant growth in pot experiments, strain UP1A-1 showed highest shoot length (16.8 cm), root length (12.1 cm), fresh weight (7.61 gm) and dry weight (0.81 gm). Similarly, the maximum amount of total chlorophyll was observed in the chili plant treated with the strain UP1A-1 (1.9 mg g^{-1}) and UT2A-30 (1.59 mg g^{-1}). The *Streptomyces* strains UP1A-1 (88.8%), UP2A-9 (87.0%) and UT6A-57 (83.2%) showed maximum biocontrol efficacy against *R. solanacearum*. The maximum phenolic content was observed in the chili pepper plants treated with the strain UP1A-1 at 9 days after pathogen inoculation (DPI). Also the plant defense related enzymes peroxides (POX) and polyphenol oxidase (PPO) significantly increased in UP1A-1 treated chili pepper at 3 DPI and 6 DPI. It is also noticed that initially at 1 DPI all treatments showed least amount of POX and PPO, later increased at 3 DPI and 6 DPI but suddenly started decreasing at 9 DPI in chili pepper. In conclusion, the current pot experiment indicated that the *Streptomyces* sp. UP1A-1 exhibited the maximum chili pepper growth, good stimulation of defense related enzymes and disease reduction against bacterial wilt and it could be a promising PGP and Biocontrol agent for chilli pepper plants against *R. solanacearum*.

Keywords: Chili pepper, Bacterial wilt, *Streptomyces*, Plant growth

Introduction

Chili pepper (*Capsicum annuum* L.) is the most extensively utilized and commercially significant spice crop in India (Tsopoe and Murry, 2020) which is the world's greatest producer of chili pepper, accounting for 50 to 60% of worldwide production (Geetha and Selvarani, 2017; Devi Priya, 2020). Chili

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pepper have a high nutritional value, as they are high in vitamins A and C in particular (Madala and Nutakki, 2020; Chakrabarty *et al.*, 2017). Chili pepper production has steadily declined over the last several decades due to certain bacterial and fungal diseases (Arora *et al.*, 2021; Islam *et al.*, 2020). *Ralstonia solanacearum* is a gram negative bacterial phytopathogen that causes wilt disease in a variety of plant species, including chili peppers (Singh *et al.*, 2021). Bacterial wilt is a devastating disease of solanaceae crops including chili pepper in India (Sakthivel *et al.*, 2016; Manda *et al.*, 2020; Kumar *et al.*, 2018). This pathogen will cause plants to wilt and finally die. Because they are infect plants from the soil where they may live for long periods of time. Hence the controlling of R. solanacearum infection is challenging. As a way of suppressing this disease in field, chemical control was ineffective (Guo *et al.*, 2021; Manickam *et al.*, 2021; Yuliar *et al.*, 2015). Biological control of phytopathogens using naturally antagonistic bacteria has considered as a promising phytopathogen control option to save the economically important crops. *Streptomyces* is a promising and proven biocontrol agent against various phytopathogens since it can generate a variety of secondary metabolites with diverse bioactive properties (Sharma *et al.*, 2021; Harir *et al.*, 2019; Olanrewaju and Babalola, 2019). Several *Streptomyces* species have been identified and used to suppress the bacterial wilt causing pathogen *R. solanacearum* (Ling *et al.*, 2020; Zhuang *et al.*, 2020; Zhao *et al.*, 2019). The synthesis of pathogenesis-related (PR) proteins, phytoalexins, changes in cell walls, and various defense related enzymes are all correlated to the development of inducible resistance in plants (Małolepsza, 2006). Plant defense related enzymes (chitinase, b-1,3-glucanase, peroxidase (POX), polyphenol oxidase (PPO), phenylalanine ammonia lyase (PAL), superoxide dismutase (SOD), catalase (CAT), lipoxygenase (LOX), ascorbate peroxidase (APX)) in the phenylpropanoid pathway are required for the synthesis of phytoalexins and phenolic compounds, which are related to ISR (Induce Systemic Resistance) (Alizadeh *et al.*, 2013). With this view the present work was attempted to study the Streptomyces mediated stimulation of defense related enzymes in *Capsicum annuum* L. against *Ralstonia solanacearum*.

Materials and methods

Description and formulation of biocontrol Streptomyces cultures

Based on our pervious study totally seven *Streptomyces* strains (UP1A-1, UP1A-4, UP2A-9, UT2A-30, UT3A-39, UT4A-49 and UT6A-57) were selected and used in this study. All the cultures were previously isolated from rhizosphere soil of solanaceae plants at Udhagamandalam, Tamil Nadu, India.

In vitro antiphyto pathogenic effect of all the seven cultures against *R. solanacearum* was reported earlier (data not shown).

The phytopathogen *R. solanacearum* (BRs_Gr) used in this study was kindly received from Division of Plant Protection, ICAR-Central Island Agricultural Research Institute, Port Blair, Andaman and Nicobar Islands, India. *Streptomyces* strains and BRs_Gr were cultured in ISP2 broth and casamino acid peptone glucose broth, respectively. *Streptomyces* suspension (density of 10^8 cfu/ml or absorbance at 600nm=0.5-0.9) was mixed with sterile talc powder at 200 ml per kg. Ten g of sterile carboxy methyl cellulose (CMC) was added into it to maintain the pH at 7. This formulated material was kept in sterile room for 15 days to enrich the maximum amount of *Streptomyces* in talc powder and used for pot culture study.

Chili pepper growth promotion

Twenty one days old chili pepper seedlings were transferred into sterilized pots with a mixture of sterile red soil, coco peat powder, and farm yard manure at 2:1:1 ratio. In the test pot, 300g of soil mixture enriched with 3g (1%) of *Streptomyces* enriched talc powder was taken, whereas only 300g of soil mixture was used for the control treatment. Three chili pepper plants were kept alive in each pot, and the experiment was repeated three times to assess plant growth. Various plant growth parameters including shoot length, root length, fresh weight (FW), and dry weight (DW) were measured after 30 days of treatment. The same procedure was followed for all the seven *Streptomyces* formulations.

Measurement of total chlorophyll

Hundred mg of fresh leaves from treated and control chili pepper plants were collected and smashed using 80% acetone. The obtained solution was centrifuged at 10,000 rpm for 5 min. Absorbance of the supernatants at 645 and 663 nm was measured using UV-Vis spectrophotometer (Thermo Scientific) using acetone as solvent control. Acetone solvent was used as control. Total chlorophyll is determined using the following formula described by Ni *et al.* (2009).

Total Chlorophyll = (Absorbance at 645) 20.2 + (Absorbance at 663) 8.02

Biocontrol study

Twenty one days old Chili pepper seedlings were transplanted into sterilized pots containing sterile red soil, coco peat powder, and FYM (alone for control treatment)/ FYM and *Streptomyces* enriched talc powder (for *Streptomyces* treatment) at 2:1:1 ratio. After seven days, each pot was inoculated with 20 ml of suspension ($1x10^8$ cfu/ml or absorbance at 600nm=0.6) *R. solanacearum* using the soil drench technique and incubated at 28-32 ℃ under controlled conditions. Three chili pepper plants were kept in each pot, which were then replicated three times for a completely randomized design. At regular time intervals, the wilt development was calculated (Guo *et al.*, 2004).

Wilt percentage = [total no of plant wilted/ total no of plants inoculated] x 100

Biocontrol efficacy = [total no of plant in treatment] − [total no of plant in control]/ total no of plant in control] × 100

Plant defense related enzyme estimation

Total phenolic content

The total phenolic content from 1 g chili pepper leaves was extracted with 10 ml of demineralized water and centrifuged at 10,000 rpm for 5 min. Then one ml of supernatant mixed with 5 ml of Folin- Ciocalteu reagent (0.2 N) and 4 ml of sodium carbonate solution (75 g/l). The suspensions kept in the dark for 120 min. Gallic acid used as a standard and the absorbance were taken at 760 nm (Zieslin and Ben-Zaken, 1993).

Peroxidase (POX)

Chili pepper leaves (1 g) homogenized using 1 ml of 10 mM phosphate buffer (pH 6.0) at 4 ℃ and centrifuged at 10,000 rpm for 5 min at 4 ℃. Addition of 100 ul supernatant to the fresh tube was further mixed with 0.25% guaiacol in 10 mM potassium phosphate buffer (pH 6.0) and 10 mM hydrogen peroxide. This suspension made up to 3 ml and the absorbance was measured spectrophotometrically at 470 nm for 1 min and after 30 s intervals the changes in absorbance measured at 420 nm for 3 min. The changes in the absorbance were expressed as min^{-1} mg^{-1} of leaves (Salla *et al.*, 2014).

Polyphenol oxidase (PPO)

Chili pepper leaves (1 g) homogenized with 10 mM phosphate buffer (pH 6.0) at 4 ℃ and centrifuged at 10,000 rpm for 5 min at 4 ℃. The reaction suspension prepared with the addition of 200 µl of leave extract, 1.5 ml sodium phosphate buffer (0.1 M and pH 6.5) and 200 µl of catechol (10 mM). Immediately, the spectrophotometer absorbance was taken at 420 nm for 1 min and the activity was expressed as change in absorbance $min^{-1}mg^{-1}$of leaves (Salla *et al.*, 2014).

Results

Chili pepper growth promotion

In this study, the growth of chili pepper plants in terms of shoot length, root length, fresh weight, and dry weight was substantially higher in the *Streptomyces* treated plants than in the non-treated control plants. The maximum shoot and root length was observed in plants treated with the *Streptomyces* strain UP1A-1 (16.8 and 12.1 cm plant^{-1}) followed by UT2A-30 (12.7 and 10.6 cm plant^{-1}) (Table 1). Also, the treatment UP1A-1 showed maximum fresh (7.61 g plant^{-1}) and dry weight (0.81 g plant^{-1}) than other treatments. Next to that, the treatments UT6A-57 and UT3A-39 showed the second and third maximum chili pepper fresh (6.94 and 5.50 g plant^{-1}) and dry weight (0.63 and 0.57 g plant^{-1}) than other treatments (Table 1).

The increased level of total chlorophyll content was noticed in all *Streptomyces* treated chili pepper plants than the control plant. Among them UP1A-1 (1.90 mg g^{-1}) treated plants estimated the highest level of total chlorophyll content followed by UT2A-30 (1.59 mg g^{-1}) and UP2A-9 (1.51 mg g^{-1}). The control treatment showed the least amount of total chlorophyll (0.99 mg g^{-1}) (Table 1).

Table 1. Effect of plant growth promoting parameters on *Streptomyces* inoculated chili pepper plants under pot culture study

Strains	Shoot length (cm plant^{-1})	Root length (cm plant^{-1})	Fresh weight (g plant^{-1})	Dry weight (g plant^{-1})	Total chlorophyll (mg g^{-1})
UP1A-1	16.8±0.84	12.1±0.78	7.6±0.69	0.8±0.09	1.9±1.97
UP1A-4	12.2±0.96	8.70.61	4.6±0.71	0.5±0.09	1.1±1.80
UP2A-9	11.6±0.73	9.8±0.51	5.4±0.93	0.4±0.08	1.5±1.44
UT2A-30	12.7±0.94	10.6±0.79	4.7±0.43	0.5±0.07	1.5±1.85
UT3A-39	11.8±0.88	9.4±0.70	5.5±0.68	0.5±0.09	1.1±1.81
UT4A-49	11.5±0.55	10.1±0.51	3.8±0.28	0.5±0.06	1.1±2.05
UT6A-57	11.3±0.35	9.1±0.75	6.9±0.74	0.6±0.08	1.4±1.30
Control	11.1±0.67	8.1±0.92	3.3±0.28	0.4±0.06	0.9±1.06

Biocontrol study on chili pepper

The results showed that the *Streptomyces* treated plants showed enhanced biocontrol efficacy than the control and reduced the wilt incidence caused by *R. solanacearum*. Among seven *Streptomyces* treatments, UP1A-1 (88.8%) showed the maximum biocontrol efficacy followed by UP2A-9 (87%)

and UT6A-57 (83.2%), respectively (Figure 1). Furthermore, a significant wilt incidence reduced by UP1A-1 and UP2A-9, when compared to other treatments.

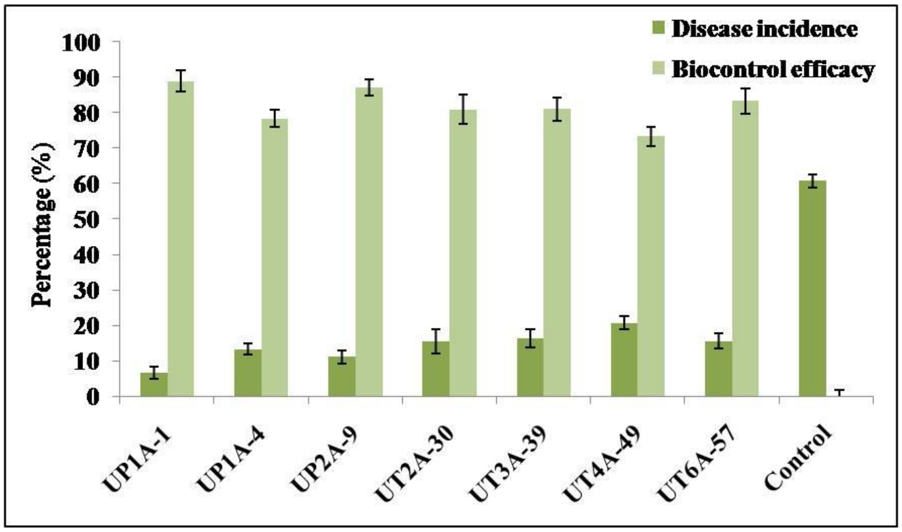

Figure 1. Bacterial wilt incidences and biocontrol efficacy of *Streptomyces* against *Ralstonia solanacearum* in chili pepper plants under pot study

Estimation of total phenolic and defense enzymes

There was a progressive and significant increase in total phenols activity in plants treated with *Streptomyces* and pathogen. Treatment of chili pepper plants with different *Streptomyces* treatments showed higher phenol accumulation when compared to control plant. The phenolic activity is more or less similar in all treatments including in control treatment at 1 DPI. Later it got raised until 9 DPI in all treatments whereas not much increased phenolic activity noticed in control treatment. The highest level of total phenolic activity observed in UP1A-1 treatments at 6 DPI and 9 DPI followed by UT4A-49 (Figure 2).

The POX activity increased in all *Streptomyces* inoculated chili pepper plants at 3 DPI and 6 DPI whereas in control treatment not much difference was observed. Later at 9 DPI it starts getting decreased in all treatments. Among seven different *Streptomyces* treatments, UP1A-1 treated chili pepper plants accumulated maximum POX activity at 3 DPI, 6 DPI and 9 DPI followed by UT2A-30, respectively (Figure 3).

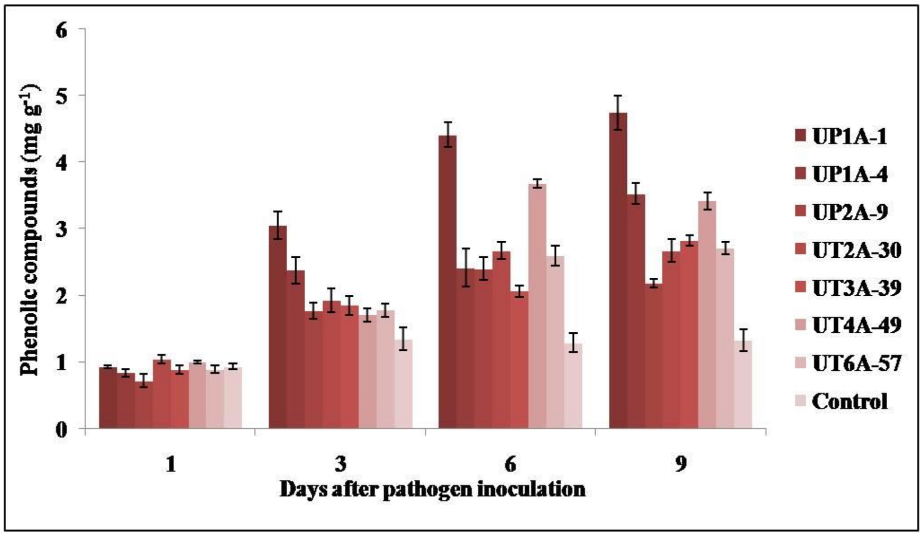

Figure 2. Estimation of total phenolic content chili pepper plants under pot culture study

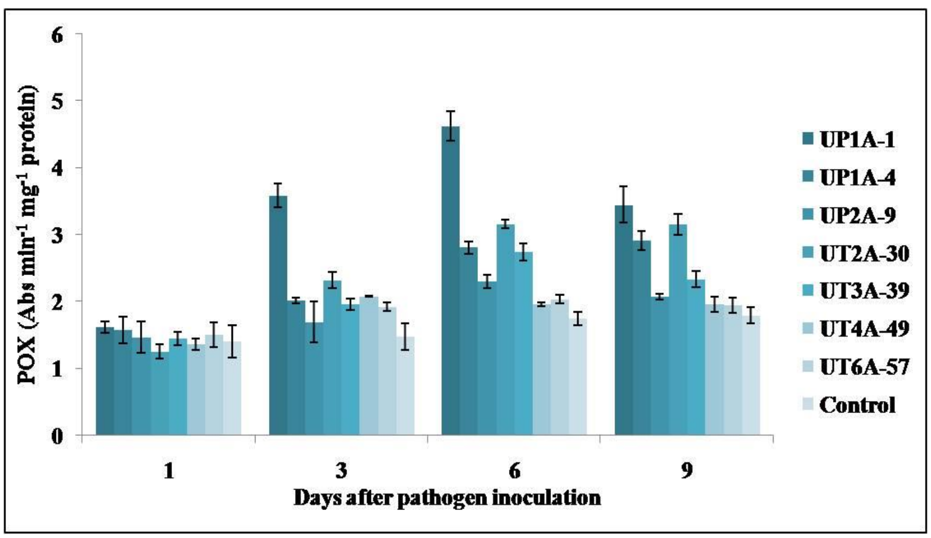

Figure 3. Estimation of peroxidase in chili pepper plants under pot culture study

Similarly, the maximum PPO activity also accumulated by *Streptomyces* inoculated chili pepper plants at 3 DPI, 6 DPI and 9 DPI than control treatment. The PPO accumulation among seven *Streptomyces* treated chili pepper plants, the maximum amount of PPO enzyme accumulated by

UP1A-1 at 3 DPI, 6 DPI and 9 DPI. Next to that, UP1A-4 treated chili pepper plants showed second maximum of PPO accumulation among others (Figure 4). The control treatment showed least phenolic, POX and PPO activity than *Streptomyces* treated chili pepper plants.

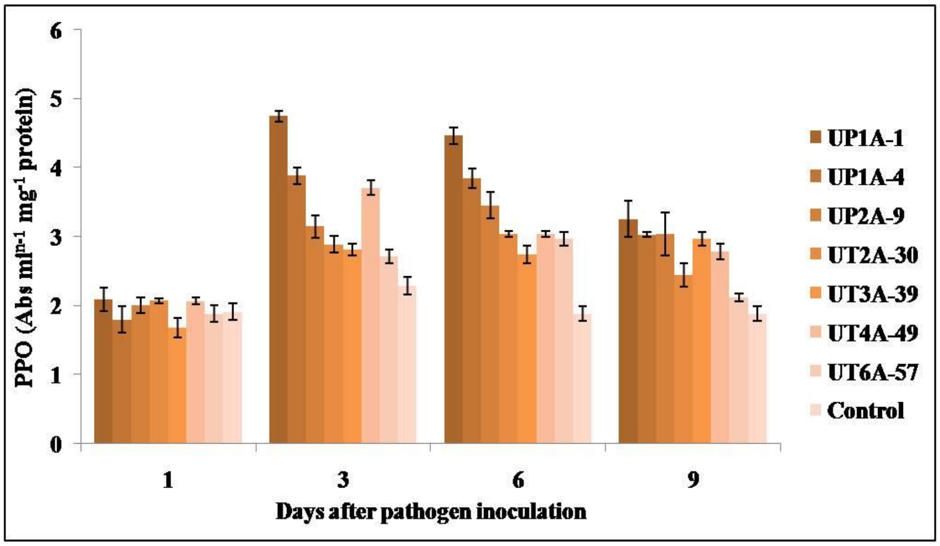

Figure 4. Estimation of poly phenol oxidase in chili pepper plants under pot culture study

Discussion

Many microorganisms such as including *Pseudomonas*, *Bacillus*, and *Trichoderma*, have been shown to promote plant growth and biocontrol efficacy against *R. solanacearum*. However, *Streptomyces* are less experimented for PGP and Biocontrol properties in chilli pepper plants (Amaresan *et al.,* 2011; Wuryandari *et al.,* 2016; Boukaew *et al.,* 2011). Thus, the present study aimed to utilize *Streptomyces* to promote chili pepper growth and biocontrol activity against *R. solanacearum*.

In chili pepper plants inoculated with *Streptomyces* were significantly enhanced plant growth parameters such as shoot, root length, fresh and dry weight than control treatment. In a pot study, the treatment UP1A-1 produced the highest plant growth parameters. This research also matched earlier findings that found that inoculating chili pepper plants with *Streptomyces* greatly boosted growth promotion in a pot trial (Srinivas *et al.,* 2020; Thilagam and Hemalatha, 2019; Boukaew *et al.,* 2011). In addition, the total chlorophyll content reported in this study was in agreement with others, indicating that *Streptomyces* inoculated chili pepper produced total chlorophyll content ranging from 0.98 to 1.15 mg g^{-1} (Thilagam and Hemalatha, 2019). Plant growth and resilience to different environmental variables may be monitored by the measurement of chlorophyll content (Pavlovic *et al.*, 2014). Chlorophyll is also used for the nitrogen accumulation and CO_2 absorption (Jinwen *et al.*, 2009). *Streptomyces*-treated chili plants in this study had a high chlorophyll content and improved plant development than control treatment. The visible symptom of plant stress is a decrease in chlorophyll concentration (Lichtenthaler and Babani, 2004). From the results of the current study, it is apparent that plant growth-promoting *Streptomyces* aids in the acquisition of required chlorophyll content for improved chili pepper plant vigor.

Aside from plant growth, Streptomyces inoculated chili pepper plants shown potential biocontrol effectiveness as well as increased phenolic and plant defense related enzymes including POX and PPO. The treatment UP1A-1 had the highest biocontrol efficacy and also accumulated maximum phenol, POX, and PPO in the chili pepper. Thus, the present findings proved that, *Streptomyces* inhibits bacterial wilt infection by boosting the systemic resistance mechanism with the assistance of their generated elicitors. This is also correlated to beneficial bacteria accumulating defense-related enzymes, which can boost plant resistance against infection (Enebe and Babalola, 2019; Andersen *et al.,* 2018). In comparison to control plants, *Streptomyces* treated plants become efficient in activating the cellular defense response rapidly and efficiently after pathogenic infection, according to a previous report (Abbasi *et al.,* 2019; Salla *et al.,* 2016).

Peroxidase is one of the important defense-related enzymes that play a role in biotic stressors in plants. It also plays an important role in plant defense by promoting the oxidation of phenolic compounds utilizing hydrogen peroxide (H_2O_2) as an electron donor (Zamocky *et al.,* 2001). Furthermore, POX is involved in the production of lignin, which plays a direct role in mechanical pathogen defense by fortifying the cell wall (Mandal and Mitra 2007). Increased POX activity has also been seen in solanaceae plants with bacterial wilt (Vanitha and Umesha, 2008) and in plants treated with *Streptomyces* as a biocontrol agent (Abbasi *et al.,* 2019). The susceptibility of chili pepper to *R. solanacearum* is likely the reason for the low POX activity. POX, plays an essential role in plant resistance to *R. solanacearum* when activated early (Senthilraja *et al.,* 2013). At 3 and 6 dpi, this significant result was observed in UP1A-1 treated plants in this study. Also POX activity was not increased in the control treatment when compared to the others. The synthesis of lignin in stimulated plants, which is a well-known defense reaction against *R. solanacearum*, may be linked to the oxidation of phenolic molecules by POX (Ramamoorthy *et al.*, 2001).

Polyphenol oxidases are used to oxidize a wide range of phenolic compounds without the need of H_2O_2. They are involved in the conversion of polyphenols to quinones which is also known as antimicrobial compounds and the lignification of plant cells after microbial invasion (Lattanzio *et al.*, 2006). This enzyme is believed to have a role in disease resistance because of this trait (Li and Steffens, 2002). PPO is essential in ISR mediated by PGPR in chili pepper, according to Yadav *et al.* (2021). PPO accumulation was greatest in chili pepper plants infected with UP1A-1 at 3 dpi, which coincided with total phenolic compound concentrations. Because UP1A-1 may metabolize phenolics to generate secondary molecules, this enzyme may have the capacity to modulate metabolism against *R. solanacearum.*

The activation of defense related enzymes produced by UP1A-1 may be the reason of the decreased wilt incidence of bacterial wilt in chili pepper. Therefore, *Streptomyces* strain UP1A-1 was able to elicite chili pepper by raising the two enzymes (PPO and POX) that are directly connected to disease incidence and stimulating the production of total phenolic compounds as part of defensive mechanisms. *Streptomyces* sp. UP1A-1 was shown to be a potential for use as a biological control agent against *R. solanacearum* in chili pepper cultivation in this study. Since *R. solanacearum* is such an aggressive bacterial wilt pathogen, more research will be done to evaluate the efficacy of *Streptomyces* sp. UP1A-1 in field conditions and to investigate different pathosystems.

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