



Anti-Biofilm Activity and Biocontrol Potential of *Streptomyces* Cultures Against *Ralstonia solanacearum* on Tomato Plants

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Abstract Biological control of phytopathogen is a promising approach when compared to the use of chemical agents. In the present study, seven *Streptomyces* cultures showing promising anti biofilm activity against *Ralstonia solanacearum* was mixed individually with farmyard manure. All the *Streptomyces* fortified farmyard manure (SFYM) were screened for plant growth promotion and control of bacterial wilt caused by *R. solanacearum* on tomato. Further, the ability of SFYM on stimulating the production of defense-related enzymes in *R. solanacearum*-inoculated tomato plants was investigated. When compared to the control tomato plants, the SFYM-treated plants had longer shoot and root length along with higher fresh and dry weight. The maximum level of chlorophyll was observed in the plants treated with strain UP1A-1 ($2.21 \pm 0.18 \text{ mg g}^{-1}$). Strain UP1A-1 also showed maximum of $96.8 \pm 1.4\%$ biocontrol efficacy in tomato plants challenged with *R. solanacearum*. In addition, the UP1A-1 treated tomato plants showed maximum accumulation of total phenolics ($3.02 \pm 0.09 \text{ mg g}^{-1}$) after 6 days of pathogen inoculation (DPI). Similarly, tomato plants treated with UP1A-1 showed highest level of peroxides, polyphenol oxidase and phenylalanine ammonia lyase during 1–9 DPI. Findings of present study revealed that the

Streptomyces culture UP1A-1 fortified farm yard manure could be applied as an eco-friendly alternative to synthetic agents for controlling bacterial wilt in tomato plants.

Keywords Tomato bacterial wilt · *Ralstonia solanacearum* · Biological control · *Streptomyces* · farm yard manure

Introduction

Tomato (*Solanum lycopersicon* L.) is one of the most important vegetable crops in the solanaceae family, with a global production of 188 million tons in 2018. India, after China, is the world's second-largest tomato producer [1]. The overall area for tomato cultivation and level of production are rising every year due to its potential health advantages and economic relevance. Tomato may readily be integrated into a balanced diet as a source of nutrients since they contain numerous health-promoting components such as carotenoids, vitamins A, C and E, antioxidants and phenolic compounds [2]. However, the wilt disease in tomato plants is a challenging problem. Among the various plant diseases, bacterial wilt caused by *Ralstonia solanacearum* has one of the most devastating symptoms resulting in massive production losses across the world. Tomato crop losses owing to bacterial wilt disease were estimated to be between 10 and 90% in India [3].

Ralstonia solanacearum is a debilitating plant vascular pathogen with a broad host variety [4]. Because of excessive extracellular polysaccharides (EPS) development within the vascular system, this pathogen causes typical wilting symptoms by colonizing xylem tissue and modifying water fluxes in the plant. N-octanoyl homoserine lactone (C8-HSL) is a major quorum sensing (QS)

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molecule generated by *R. solanacearum*, which promotes cell growth and biofilm formation [5, 6]. Potential bacteria such as *Bacillus*, *Pseudomonas* and *Streptomyces* inhibit QS through inactivation of C8-HSL signals by producing enzymes like AHL-lactonases and acylases [7–11]. Many microbicidal synthetic products, such as copper derivatives, have traditionally been used to combat bacterial wilt in tomato. However, conventional agrochemicals have not been very successful in controlling bacterial wilt, and antibiotic usage may result in the development of antibiotic-resistant strains. Alternative approaches such as applying biological control agents (BCAs) have proven to be successful and are now being used more often in the field. *Pseudomonas*, *Bacillus*, *Streptomyces* and *Trichoderma* are some of the potential BCAs widely used to control bacterial wilt disease [1, 12].

Streptomyces, the well investigated genus under the phylum Actinobacteria, is being explored in agriculture sector as plant growth promoters (PGP) and BCA. Due to their remarkable antagonistic behavior by producing different bioactive agents, certain *Streptomyces* have been used to manage soil-borne diseases [13]. Biocontrol products such as Mycostop, Actinovate, and Rhizovit by *S. griseoviridis* K61, *S. lidicus* WYEC 108, and *Streptomyces* sp. DSMZ 12,424, respectively are already developed to tackle fungal diseases in plants [14, 15]. Hence, members of the genus *Streptomyces* remain as promising microbial resources for the development of biocontrol agents and/or biofertilizers in agriculture. Nevertheless, the genus *Streptomyces* has not been investigated in detail as biocontrol agents for the management of bacterial wilt in tomato plants.

Induced systemic resistance (ISR) enzymes such as phenylalanine ammonia lyase (PAL), peroxidase (POX) and polyphenol oxidase (PPO) are involved in redirecting the flow of carbon from primary to secondary metabolism in plants. In addition, they act as key enzymes in the synthesis of phenolic compounds with antimicrobial activity [16]. When a pathogen is expressed, the ISR activates several defense mechanisms including the enhanced activity of POX, PPO, and PAL. POX is a broad-spectrum resistance enzyme that plays a role in plant-pathogen interactions. POX is believed to be one of the significant enzymes of the plant's biochemical protection against pathogens, and it contributes in self-regulation of plant metabolism following infection [17]. PPO is an oxidative enzyme that catalyzes the conversion of phenolic compounds into extremely poisonous quinones, which are essential for disease tolerance in plants [18]. The PAL enzyme, which converts l-phenylalanine to trans-cinnamic acid, a key product of phenylpropanoid metabolism and a critical step in the production of salicylic acid, protects against pathogen invasion [19]. Unfortunately, the kinetic

modifications in defense-related enzymes produced by *Streptomyces* cultures on tomato are very less understood. Hence, this work is undertaken to study the anti-biofilm and biocontrol potential of *Streptomyces* cultures against *Ralstonia solanacearum* on tomato plants.

Materials and Methods

Antagonists and Plant Pathogen

Antagonistic *Streptomyces* cultures were obtained from Division of Bioprospecting, Centre for Drug Discovery and Development, Sathyabama Institute of Science and Technology, Chennai, Tamil Nadu and India. The bacterial wilt causing pathogenic strain *Ralstonia solanacearum* (BRs_Gr) was used for *in-vitro* anti-biofilm study and pot culture study [20]. The antagonistic cultures were sub-cultured using YEME (Yeast Extract and Malt Extract) agar media at 28 °C. Similarly, the *R. solanacearum* was sub-cultured and maintained on *Pseudomonas solanacearum* medium amended with 1% TTC (2, 3, 5 triphenyl tetrazolium chloride) solution at 28 °C for further studies.

Anti-Biofilm Assay

Streptomyces cultures were grown in 20 ml of YEME broth in 100 ml conical flask at 28 °C in rotary shaker with 150 rpm for 7 days. After incubation, the supernatant was collected by centrifugation at 10,000 rpm for 15 min at 4 °C and used for biofilm inhibition study. Approximately 40 µl culture filtrate of biofilm-forming test pathogenic strain *R. solanacearum* (10⁸ CFU/ml) was inoculated into sterile 96-well polystyrene microtiter plate containing 60 µl LB broth supplemented with 100 µl of *Streptomyces* culture supernatant. In case of control treatment, 160 µl of LB broth with 40 µl culture filtrate of *R. solanacearum* was used and the plate was incubated at 37 °C for 16 h. After incubation, media was discarded from the 96 well plate and biofilm inhibition was observed by staining the wells with crystal violet, further dissolving with 95% ethanol. Quantification was done by UV–Vis spectrophotometer absorbed at 530 nm and the biofilm inhibition percentage was calculated using the formula:

$$\begin{aligned} & \% \text{ biofilm inhibition} \\ &= \left(\frac{A_{530} \text{ of biofilm in control} - A_{530} \text{ of biofilm in treatment}}{A_{530} \text{ of biofilm in control}} \right) \\ & \times 100 \end{aligned}$$

For microscopic imaging, anti-biofilm assay performed in 12-well polystyrene microtiter plate amended with 1 × 1 cm cover slip. The biofilm inhibition was observed

by staining the cover slip with crystal violet method described above and visualized by inverted bright-field microscope at magnifications of 40 X [21].

Pot Culture Study

Preparation of Streptomyces Enriched Farm Yard Manure (SFYM)

Streptomyces enriched farm yard manure (SFYM) was prepared with sterile talc powder, carboxy methyl cellulose (CMC) and well decomposed farm yard manure (FYM) in a standard ratio. A loopful of each *Streptomyces* culture (UP1A-1, UP1A-4, UP2A-9, UT2A-30, UT3A-39, UT4A-49 and UT6A-57) was inoculated into 200 ml of YEME broth and incubated in rotary shaker at 150 rpm for 7 days at 28 °C. To prepare SFYM, 200 ml of 7 days old *Streptomyces* suspension (10^8 CFU/ml or absorbance at 600 nm = 0.5–0.9) was mixed with one kg of sterilized talcum powder amended with 10 g of carboxy methyl cellulose (CMC) in sterile container. The freshly prepared talc formulation was then mixed with 50 kg of well decomposed FYM and the mixture was maintained under shaded conditions for 15 days with intermittent manual turn over once a day to enrich *Streptomyces* population in the FYM substrate.

Evaluation of SFYM for Plant Growth Promoting Properties

A fine red soil having the following characteristics: sand 14%; silt 39.5%; clay 24.2%; pH 7.03; EC (electrical conductivity) 1.39 dSm^{-1} ; OM (organic matter) 12.52%; total organic C 2.98%; total N 3.51%; P 0.94%; total K 1.64%, was taken. Twenty one days old tomato seedlings (cv. Meghdoot 2048, Syngenta, Pvt. Ltd) were transplanted into sterilized pots (15 × 15 cm) containing sterile mixture of red soil, coco peat powder and FYM (control treatment) or SFYM (*Streptomyces* treatment) at 2:1:1 ratio. Each pot contained approximately 300 g of soil mixture. Three tomato plants were maintained in each pot, and the experiment was repeated five times to examine the plant growth. Plants were irrigated 2 days once. After 30 days of treatment, plant growth parameters including shoot length, root length, fresh weight (FW), and dry weight (DW) were measured.

Measurement of Total Chlorophyll

The total chlorophyll from fresh tomato leaf samples was extracted using 80% acetone and quantified using spectrophotometric assay [22].

Biocontrol Study

Twenty one days old tomato seedlings (cv. Meghdoot 2048, Syngenta, Pvt. Ltd) were transplanted into sterilized pots (15 × 15 cm) containing mixture of sterile red soil, coco peat powder and FYM (control treatment) or SFYM (*Streptomyces* treatment) at 2:1:1 ratio. After 7 days, 20 ml of pathogenic suspension (1×10^8 CFU/ml or absorbance at 600 nm = 0.6) was inoculated into each pots by soil drench method [23] and incubated at 28–32 °C under controlled conditions. Three tomato plants were maintained in each pot, replicated five times in order to achieve completely randomized design. The wilt development on each tomato plant was calculated at regular intervals. The wilt percentage and biocontrol efficacy were calculated by the following formula,

Wilt percentage

$$= \left[\frac{\text{total no of plant wilted}}{\text{total no of plants inoculated}} \right] \times 100$$

Biocontrol efficacy =

$$\left[\frac{\text{total no of plant in treatment} - \text{total no of plant in control}}{\text{total no of plant in control}} \right] \times 100$$

Sample Collection for Enzyme Estimation

Tomato leaves were collected at various time intervals (1, 3, 6, and 9 days after pathogen inoculation) to estimate the plant defense-related enzymes. One gram of tomato leaves was homogenized using 1 ml of 0.1 M sodium phosphate buffer (pH 7.0) at 4 °C and the aqueous portion was collected by centrifugation at 12,000 rpm for 20 min at 4 °C. The supernatant was collected in a fresh tube to determine plant defense-related enzymes such as peroxidase (POX) [24], polyphenol oxidase (PPO) [25], and phenylalanine ammonia lyase (PAL) [26]. Total phenolic content was estimated according to the method of Zieslin and Ben-Zaken [27].

Data Analysis

The data were presented as the mean ± SE of several independent replicates. Analysis of variance (ANOVA) done by Duncan multiple post hoc comparison tests was performed in SPSS software version of 16.0. The values of $P \leq 0.05$ were considered as statistically significant.

Results and Discussion

Anti-Biofilm Activity of *Streptomyces* Cultures

In the present study, UP1A-1, UP1A-4, UP2A-9, UT2A-30, UT3A-39, UT4A-49 and UT6A-57 were selected based on their *in-vitro* plant growth promoting and antagonistic activity against *R. solanacearum* (data not shown).

The synthesis of extracellular polymeric substances (EPS) and various proteins by *R. solanacearum* was commonly believed to be responsible for biofilm formation. By causing blockage in the plant xylem, biofilm formation is one of the beneficial factors for pathogenic virulence [28]. In this present study, among seven *Streptomyces* cultures used for biofilm inhibition against *R. solanacearum*, UT2A-30 and UP1A-1 showed maximum anti-biofilm activity followed by UT6A-57, UP1A-4 and UT3A-39 (Fig. 1a). The bright field microscopic observation showed prominent anti-biofilm activity by all the seven *Streptomyces* against *R. solanacearum* (Fig. 1b).

Several studies reported anti-biofilm properties of bacteria and fungi against various pathogens [29–31]. Sabu et al. [32] reported that *Nocardioopsis* sp. can inhibit more than 90% biofilm formation by *Staphylococcus capitis* and *Staphylococcus haemolyticus*. Similarly, bioactive metabolites from *Streptomyces californicus* were found to inhibit more than 90% of *S. aureus* and MRSA biofilms [33]. In the current study, the culture supernatant of UT2A-30, UP1A-1, UT6A-57 and UP1A-4 showed more than 80% biofilm inhibition against *R. solanacearum* (Fig. 1a). This *in-vitro* study suggests that all seven *Streptomyces* cultures can reduce the virulence of *R. solanacearum* by suppressing biofilm formation.

Pot Culture Study

Promotion of Tomato Plant Growth

Plant growth promoting microbes are extensively researched because of their sustainable behavior in agriculture.

Members of the genus *Streptomyces* are well renowned among Actinobacteria in terms of their biocontrol and PGP properties. It exhibits such properties on various vegetable crops including tomato, either directly by the production of phytohormones, or indirectly by antagonizing plant pathogens [34]. In this study, the growth of tomato plants evaluated by shoot length, root length, fresh weight, and dry weight was substantially higher in the SFYM treated plants than in the non-inoculated control plants. The highest shoot length was recorded in the plant treated with UP1A-1 (15.44 ± 0.21 cm plant⁻¹) followed by UP1A-4 (12.72 ± 0.37 cm plant⁻¹) and UP2A-9 (12.5 ± 0.32 cm plant⁻¹) (Fig. 2a). Similarly, the application of UP1A-1 and UP2A-9 showed significant increase in the root length by 15.78 ± 0.58 and 11.72 ± 0.54 cm plant⁻¹, respectively relative to the non-inoculated control treatment (Fig. 2b). The maximum fresh and dry weight of tomato plants was recorded in the treatment of UP1A-1, which showed 10.89 ± 0.77 and 0.62 ± 0.04 g plant⁻¹, respectively. Next to that, UP2A-9 showed second highest fresh weight followed by UT4A-49, UT6A-57 and UP1A-4, when compared to control treatment (Fig. 2c and d). The highest level of chlorophyll was recorded on UP1A-1 (2.2 ± 0.18 mg g⁻¹) followed by UP2A-9 (1.61 ± 0.19 mg g⁻¹) (Fig. 2e). Our results are in agreement with the observations of Djebaili et al. [35] who reported that actinobacteria can be used for better plant growth promotion of tomato plants. Similarly, others findings also reported *Streptomyces* sp. used for pot culture study showing increased plant growth of tomato plants when compared to control plants [36]. These *Streptomyces* cultures will be the potential candidates for the development of soil nutrients and can be used as an alternate to chemical pesticides [37].

Biocontrol Study

Streptomyces are ubiquitous in nature and can protect host from disease causing phytopathogens by secreting various antagonistic molecules. In this study, *Streptomyces* cultures

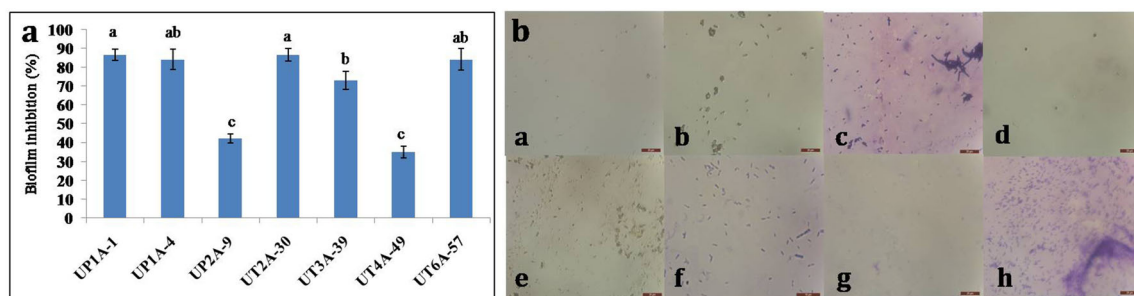


Fig. 1 Inhibition of biofilm formation by *Streptomyces* cultures against *Ralstonia solanacearum* (a). Percentage inhibition by spectrophotometer analysis (b). Microscopic visualization a UP1A-1, b UP1A-4, c UP2A-9, d UT2A-30, e UT3A-39, f UT4A-49, g UT6A-57 and h control

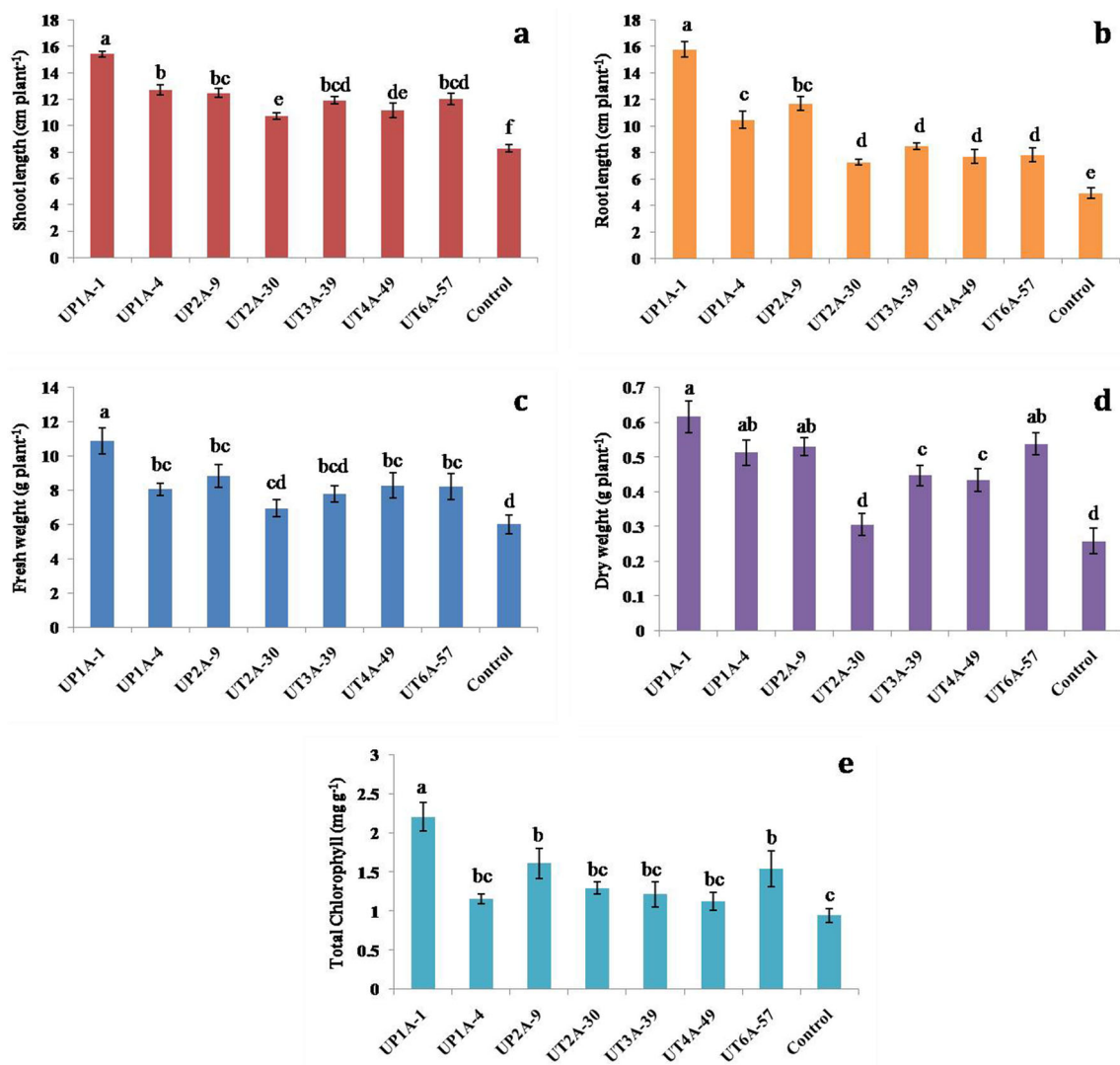


Fig. 2 Effect of *Streptomyces* fortified farmyard manure (SFYM) on the growth of tomato plants under pot culture study **a** shoot length, **b** root length, **c** fresh weight, **d** dry weight, **e** total chlorophyll

not only enhanced growth of tomato plants but also controlled the wilt caused by *R. solanacearum*. The seven *Streptomyces* cultures amended FYM exhibited varying degrees of protection ranging from 60 to 97%. The maximum biocontrol efficacy was exhibited by UP1A-1 (96.85%) and UP2A-9 (83.1%) cultures when compared to control (Table 1). Similar to this, *Streptomyces* culture NEAU-HV9 isolated from soil sample was reported that can control the tomato bacterial wilt up to 82% in pot culture study [38]. Also, *Streptomyces* culture LD120T showed 63.6% biocontrol efficacy in pot culture study on tomato plants against *R. solanacearum* [12]. Our present study showed that *Streptomyces* culture UP1A-1 was able to control more than 90% biocontrol efficacy on tomato against *R. solanacearum*. This observation revealed that *Streptomyces* strain UP1A-1 is a potential candidate for the

Table 1 Potential of *Streptomyces* cultures to induce resistance against *R. solanacearum* in tomato plants under pot study

Cultures	Disease incidence (%)	Biocontrol efficacy (%)
UP1A-1	2.96 ± 1.31 ^a	96.85 ± 1.40 ^a
UP1A-4	21.48 ± 2.54 ^b	77.75 ± 2.57 ^b
UP2A-9	16.29 ± 2.84 ^b	83.1 ± 2.99 ^b
UT2A-30	28.14 ± 3.04 ^c	62.84 ± 4.49 ^c
UT3A-39	17.03 ± 1.48 ^b	78.81 ± 2.22 ^b
UT4A-49	31.85 ± 2.13 ^c	59.71 ± 2.3 ^c
UT6A-57	19.25 ± 2.02 ^b	78.17 ± 2.85 ^b
Control	96.29 ± 1.77 ^d	–

Values are mean ± SE ($n = 5$). Values with the same letter within a column are not significant at $p \leq 0.05$ as per Duncan multiple post hoc comparison test

development of biocontrol agent against the wilt causing pathogen *R. solanacearum* on tomato crops.

Estimation of Total Phenolic and Defense Enzymes

Strains of *Streptomyces* were identified as intermediaries for decreasing plant disease incidence and increasing production in a variety of vegetable crops. The production of defense-related enzymes during the initial phases is important for plant disease control through cell-wall membrane and bioactive metabolites [39]. In this present study, *Streptomyces*-treated tomato plants showed impacted changes in the activities of defense related enzymes and total phenolic content. The maximal activity of these enzymes and total phenol occurred at different stages. Konappa et al. [40] have already demonstrated that treatment of tomato plants with antagonistic microbes develops systemic resistance through accumulation of total phenol and various plant defense enzymes against bacterial wilt. The estimation of phenol, POX, PPO, and PAL in tomato was also directly correlated with disease reduction study.

Higher phenol accumulation was observed in different *Streptomyces*-treated tomato plants when compared to control plant. Also tomato plants treated with UP1A-1 accumulated the maximum level of phenolic content when compared to the others at 6 DPI and 9 DPI. The accumulation of phenolic content increased after the pathogen inoculation and reached maximum level from 6 to 9 DPI in *Streptomyces* inoculated tomato plants while in case of control treatment, it started decreasing from 6 DPI onwards (Fig. 3a). This finding correlates with previous report

where the *Streptomyces* and fungal pathogen inoculated *Eucalyptus globulus* had increased phenolic content from 1 DPI-15 DPI whereas it showed decreasing strategy in control treatment [41].

Peroxidase induction in plants can enhance the disease resistance through oxidation of phenolic compounds by utilizing H₂O₂ and lignin biosynthesis. Fortification of the cell wall by lignin biosynthesis is crucial for mechanical defense against pathogens [42]. After *R. solanacearum* inoculation, the activity of POX increased in *Streptomyces* inoculated tomato plants, whereas the POX activity decreased in control plants. The tomato plants treated with UP1A-1 induced maximum amount of POX enzyme at 3 DPI. Next to that, UT6A-57 and UT3A-39 treated tomato plants showed maximum increasing POX activity at 6 DPI (Fig. 3b). Similar studies show that *Streptomyces* has induced POX in tomato [43], cucumber [44] and rice [45] as well. The susceptibility of tomato plants to *R. solanacearum* is positively correlated with low POX activity. POX, on the other hand, plays a significant role in plant resistance to *R. solanacearum* when activated early [40]. In present study, at 3 DPI, tomato plants treated with UP1A-1 showed this systemic response.

Polyphenol oxidase was used to oxidize different phenolic compounds without H₂O₂ and employed for plant disease control. During microbial invasion, they are essential in the oxidation of polyphenols into different antimicrobial metabolites such as quinones, as well as the lignification of plant cells [46]. The activity of PPO in *Streptomyces* treated tomato plants were found to be higher than the control plants and it reached maximum on early stage of pathogen invention at 1 DPI (Fig. 3c). Among the

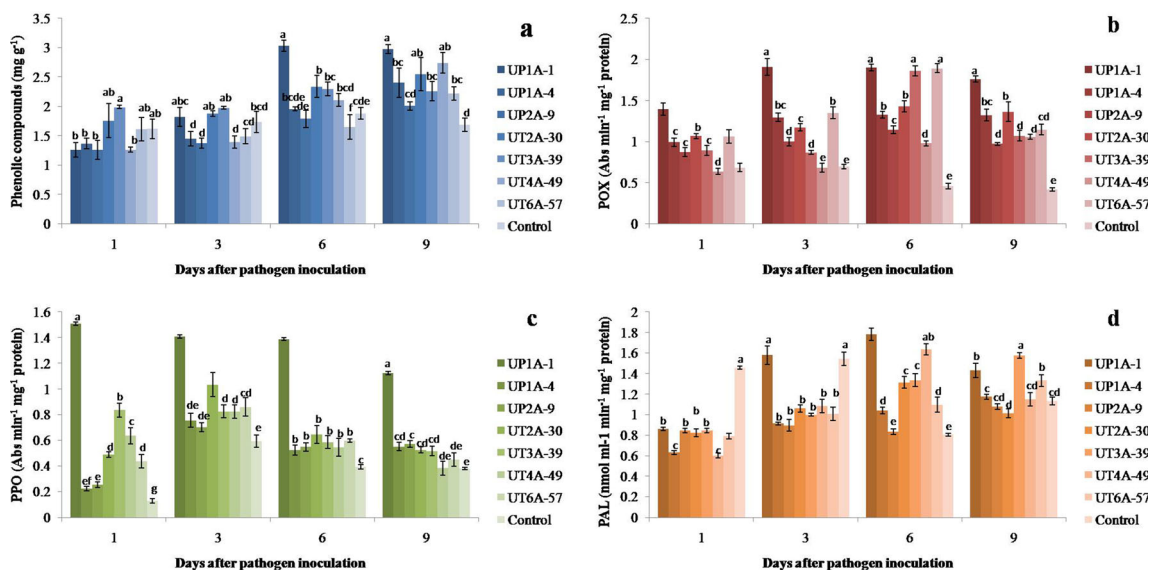


Fig. 3 Estimation of plant defense-related enzymes of tomato plants under pot culture study **a** total phenolic content, **b** peroxidase, **c** polyphenol oxidase, **d** phenylalanine lyase activity

Streptomyces treatments on tomato, the PPO activity was found to be higher in the plants treated with UPIA-3. The enzymes POX and PPO are involved in the activation of hypersensitive reactions in plant cells, as well as the modification of adjacent cell to prevent pathogen entry [43]. The findings of previous investigations also concur that tomato plants treated with beneficial microbes increased POX and PPO levels against the disease causing *R. solanacearum* pathogen [47].

The estimation of PAL enzyme was comparatively higher in control treatment than *Streptomyces* treated plants at 1 DPI. However, all *Streptomyces* treated tomato plants depicted increased PAL enzyme from 3 DPI onwards but in control treatment it showed a significantly decreasing strategy. It was observed that maximum PAL activity was induced in UPIA-1 treated tomato plants at 6 DPI followed by UT4A-49. The present study clearly observed that the PAL enzyme activity significantly increased in all *Streptomyces* inoculated tomato plants, whereas in control treatment it showed reduced PAL activity at the end of experiment (Fig. 3d). Induction of PAL activity by antagonistic microbes improved their biocontrol effect, according to several earlier findings [43, 47]. Such observations were also seen in this study in agreement with previous research findings showing that beneficial microbes can increase PAL enzyme in tomato infected with *R. solanacearum* [40, 47].

In conclusion, *Streptomyces* culture UPIA-1 fortified farmyard manure has the potential properties to control bacterial wilt and promote growth in tomato. Evaluation in field conditions is needed to be done to prove its further potentials.

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Authors' Contribution MK carried out sample collection, lab experiments, and writing of the manuscript. JJ gave design, supervision, and revision of the manuscript. RM and GV supervised the research work and revised the article. AS contributed in lab experiments and manuscript writing. BA contributed to work on particular pathogen *R. solanacearum* and to the experimentation. SK supervised the research work and revised the article. All authors have read and approved the final manuscript.

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Data Availability All datasets are presented in the main manuscript.

Declarations

Conflict of interest Authors do not have any competing interest.

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