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Biocontrol potential of *Pseudomonas syringae* against Emerging Phyto-Fungal Pathogens

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Fungal diseases caused by soil-borne pathogens like *Macrophomina phaseolina*, *Fusarium oxysporum* f. sp. *lycopersici* and *Sclerotium rolfsii* result in huge losses of crops including cereals, pulses, vegetables etc. hence threatening global food security. Bacterial biological control agents, such as strains of *Pseudomonas*, *Bacillus*, and *Streptomyces*, have been historically and currently utilized to manage soil-borne pathogens as eco-friendly alternatives in the quest for a sustainable solution.The antifungal potential of *Pseudomonas syringae* was assessed against the said phytopathogenic fungi through dual plate assay. The effect of bacterial metabolites was also assessed on the growth, biomass, and biochemical traits [total protein content (TPP), activity of catalase (CAT), peroxidase (POX), and polyphenol oxidase (PPO)] of the fungi. *P. syringae* significantly ($p \leq 0.05$) inhibited the growth of *M. phaseolina*, *S. rolfsii*, and *F. oxysporum* f. sp. *lycopersici* by 80, 75, and 48%, respectively, in the dual culture assays. The various concentrations of bacterial metabolites (2, 4, 6, 8, 10, ---- 20%) significantly ($p \leq 0.05$) suppressed the pathogens in a dose-dependent manner. Therefore, the biomass production of either *M. phaseolina* or *S. rolfsii* declined considerably by 65-100% with the increasing metabolite concentration from 2 to 10%, while for *F. oxysporum* f. sp. *lycopersici*, the biomass decreased by 50-100% with the concentration of 2 to16%. Inhibition in biomass production increased stress levels in the fungal cell, as indicated by enhancement in the biochemical enzymatic activity in the fungi. The study demonstrates that *P. syringae* effectively inhibits the growth of *M. phaseolina*, *S. rolfsii*, and *F. oxysporum* f. sp. *lycopersici*. These findings support the potential of *P. syringae* as an eco-friendly alternative to chemical fungicides, promoting sustainable agriculture by enhancing crop resilience and yield. Further research should focus on identifying the specific antifungal compounds in *P. syringae* metabolites responsible for this activity.

Keywords: Fungal physiology, Saprophytic gram-negative bacterium, Sclerotia, Wilt, FOC, Cereals, pulses.

INTRODUCTION

Fungal diseases, like blights and wilts, plague crops worldwide, reducing yields and causing billions in losses. These silent invaders rob plants of nutrients, weaken defenses, and ultimately threaten food security. Three particularly common culprits are Charcoal rot, Sclerotium wilt, and Fusarium wilt caused by *Macrophomina phaseolina*, *Sclerotium rolfsii*, and *Fusarium oxysporum* f. sp. *lycopersici*, respectively (Khurshid *et al.*, 2017; Siddique *et al.*, 2020; Shoaib *et al.*, 2022; Shoaib *et al.*, 2022; Shoaib *et al.*, 2019; Shoaib *et al.*, 2019). These soil-borne fungi exhibit extremely competitive saprophytic aptitude accounting for >50% of crop losses, particularly in the absence of appropriate disease management. Besides, threatening food security also contributes to rising food prices, impacting millions worldwide. *M. phaseolina* (MP) is known as a master of adaptation, targeting a wide range of crops, from essential food sources like soybeans and corn to towering sunflowers. The truly terrifying aspect of MP lies in its sclerotia. These hardened survival structures, akin to fungal time capsules, can lie dormant in the soil for a staggering decade or more. This extended dormancy allows MP to persist through harsh conditions and re-emerge to wreak havoc on unsuspecting crops (Siddique *et al.*, 2020; Shoaib *et al.*, 2022). *S. rolfsii* (SR), the culprit behind Sclerotium wilt, is another master of disguise. This soil-borne pathogen isn't picky about its victims, targeting a vast array of vegetables like potatoes, beans, and even ornamentals. Similar to MP, SR utilizes sclerotia as its weapon of long-term survival. These sclerotia,

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resembling tiny brown mushrooms, can remain viable in the soil for years, waiting for the opportune moment to germinate and unleash a web-like network of fungal threads that strangle and suffocate plant roots. SR's ability to infect a wide range of crops and persist in the soil makes it a formidable foe for farmers (Shoaib *et al.,* 2022; Shoaib *et al.,* 2019). *F. oxysporum f. sp. lycopersici* (FOL)**,** the cause of Fusarium wilt, is a tomato specialist with a particularly cruel strategy. This xylem-colonizing fungus acts like a microscopic burglar, infiltrating the plant's vascular system, essentially its water transport network. Once inside the xylem, FOL thrives, blocking the flow of water and nutrients throughout the plant (Khurshid *et al.,* 2017). Moreover, all three fungal pathogens can colonize and clog xylem vessels in addition to the secretion of several toxins and cell-wall degrading enzymes (Shoaib *et al.,* 2022). Managing these pathogens is challenging due to their persistence and adaptability (Khurshid *et al.,* 2017). Synthetic fungicides, while initially effective, often pose environmental risks by harming beneficial microbes in the soil and potentially contaminating water sources. Additionally, the overuse of these chemicals can lead to fungicide resistance, rendering them ineffective against the very pathogens they were designed to control (Awan *et al.,* 2022). This creates a critical need for exploring safer, eco-friendly solutions like biological control agents. Biological control bacterial agents (e.g., *Bacillus*, *Pseudomonas*, *Rhizobium*, *Azospirillum*, *Streptomyces*, etc.) through their multilayered approaches including antifungal action, root colonization, induction of systemic resistance in the plant, etc., guard plants and facilitate their growth (Basu *et al.,* 2021). Among the saprophytic Gram-negative bacterium, *Pseudomonas syringae*, stands out with a surprising dual role. While it's known as an agricultural pest causing diseases like bacterial blight, some strains may act as secret defenders (Bensaci *et al.,* 2011). It has the genes for building a weapon, the type III secretion system, and crafting harmful tools called effectors, it has a unique twist. Scientists suspect a special chemical code in its genes turns it into a "pacifist pathogen" capable of harm but hesitant to cause it. Despite uncertainties, studying its nature could unlock insights into bacterial evolution. This knowledge might revolutionize farming with sustainable practices and natural disease solutions, revealing hidden potential in familiar adversaries (Bensaci *et al.,* 2011; Passera *et al.,* 2019).

The distinctive characteristics observed in non-pathogenic *P. syringae* strains, including unique regulatory pathways, non-functional virulence, and the production of distinct antifungal metabolites, underpin their potential in disease management (Passera *et al.,* 2019). Many reports indicated the antifungal potential of *P. syringae* pv. *syringae* against many fungi, including *Penicillium expansum*, *P. italicum*, *P. digitatum*, *Botrytis cinerea*, and *Mucor* spp. causing mold on apples, citrus, and pears (Janisiewicz and Jeffers, 1997; Janisiewicz and March, 1992). Likewise, another antagonistic strain of *P. syringae* ESC-11 (commercialized as BioSave™ 110) has been shown to decrease banana crown rot caused by *Fusarium* spp. (*F. semitectum* and *F. moniliforme*) (Williamson *et al.,* 1999), potato dry rot caused by *F. sambucinum* (Kenwick and Jacobsen,1998). Moreover, *P. syringae* (ESC-11) has been recommended against post-harvest fungal decays on many fruits (Janisiewicz and March, 1992). Though the exact mode of disease control by *P. syringae* is still unidentified, these harmless bacteria are possibly out-competing pathogenic fungi for space and nutrients. However, strains of *P. syringae* pv. *syringae* were found to produce cyclic lipodepsinonapeptides with antifungal activity (Sorensen *et al.,* 1996). According to (Bensaci *et al.,* 2011), the antifungal action of the *P. syringae* pv. *syringae* occurs mainly through two classes of metabolites, the small cyclic lipodepsinonapeptides (syringomycins) and the larger cyclic lipodepsipeptide (syringopeptins SP22 or SP25). The antifungal properties of *P. syringae* strains, mediated by compounds like syringomycins, hold promise for sustainable disease management in agriculture. Further research is needed to understand their mechanisms and explore potential applications, highlighting the importance of leveraging these properties for effective agricultural practices. Therefore, the objective of the present study was to check the *in vitro* antifungal potential of *P. syringae* and its metabolites against three phytopathogenic fungi, including MP, SR, and FOL, through analyzing growth and biochemical changes in these fungi.

MATERIALS AND METHODS

A dual culture method was used to check the antifungal potential of *P. syringae* (FCBP-PB0405) against MP (FCBP 751), SR (FCBP 0011), and FOL (FCBP 0060) following the protocol described earlier (Awan *et al.,* 2023; Shoaib *et al.,* 2020). A mycelial plug (6 mm) of 7-days-old fungal culture was placed at the center of the Petri plate (9 cm) containing 25 mL of MEA (2 g Malt extract and 2 g agar/100 mL water), yielding a final depth of 4 mm. The *P. syringae* was seeded with a sterile needle at a distance of 2.5 cm from the fungal inoculum. A fungal disk, in the center of a plate without seeding the bacterium served as a control. After incubation for 7 days at 28 °C, the inhibition of mycelial growth was observed by measuring the diameter of the zone of inhibition (n = 3). The percentage inhibition of mycelial radial growth was calculated by the following formula:

$$\%\text{age inhibition} = \frac{\text{Control-Treatment}}{\text{Control}} \times 100$$

The antifungal potential of secondary metabolites of *P. syringae* was also checked, the bacterial metabolites were prepared in Luria-Bertani broth (tryptone 1.0%, yeast extract 0.5%, and NaCl 0.5%) following the protocol of (Shoaib *et al.,* 2020). The bacterial strain was inoculated in the broth and

grown at 37 °C with constant shaking for 3 days at 200 rpm. The broth was then centrifuged at 8,000 rpm for 30 min (4°C), followed by the collection of the supernatant after filtration through a 0.45 µm filter membrane. Ten concentrations (2, 4, 6, 8, 10, 12, 14, 16, 18, and 20%) of the extracted metabolites were prepared by mixing a measured amount of the extract in ME broth. The final volume of 5 mL of each concentration was taken in the test tube. The fungus was inoculated in each of the ten different concentrations of metabolites, which were incubated for 10 days at 28 °C. ME broth without bacterial metabolites served as a control. Each treatment was replicated thrice in a completely randomized design. Followed by 10 days of incubation, fresh biomass was filtered and dried at 50°C, and the dry weights of the fungal mat were measured in mg, while percentage inhibition was calculated using the same formula as mentioned above.

The fungal biomass was also analyzed for the total protein content (TPP), catalase (CAT), polyphenol oxidase (POX), and polyphenol oxidase (POX) activities after 4th, 5^{th,} and 6th day of inoculation following the protocols described previously (Shoaib *et al*., 2020). Data obtained for the fungal growth, biomass, and biochemical assays in the presence of biocontrol bacteria or metabolites were analyzed statistically by applying the LSD test (least significant difference) after applying one-way ANOVA. The changes in the biochemical markers were calculated by taking the average value for TPP, CAT, POX, and PPO against ten different concentrations of bacterial metabolites.

RESULTS AND DISCUSSION

In dual culture bioassays, *P. syringae* exhibited significant inhibition, with 80% radial growth suppression observed against *M. phaseolina* (MP), followed by 75% against *S. rolfsii* (SR), and 48% against *F. oxysporum* f. sp. *lycopersici* (FOL). These findings suggest the potential of *P. syringae* as a biocontrol agent against these fungal pathogens. Broth bioassays revealed that different concentrations (2, 4, 6, 8, ----- and 20%) of secondary metabolites of *P. syringae* significantly inhibited the growth of all three pathogenic fungi in a dose-dependent effect. Bacterial metabolites within the concentration range of 2-10% significantly ($p \leq 0.05$) decreased the biomass of MP and SR by 68-99% and 64-99%, respectively, while both fungi ceased to grow above 10% concentration. The biomass of FOL declined significantly by 50-99% with the increase in metabolite concentration from 2-16%, and no growth of the fungus was recorded with the further increase in metabolite concentrations. Regression analysis revealed the non-linear relationship between various concentrations of the bacterial metabolites and the fungal biomass with $R^2 > 0.87$ (Fig. 1 A-C).

The data of biochemical assays revealed that all investigated parameters were significantly increased with an increase in the incubation period (4, 5, and 6 days after inoculation) as well as metabolite concentration. Accordingly, the TPP and the activities of CAT, POX, and PPO were significantly increased by 10-50% and 30-70% in MP with an increase in the concentration of bacterial metabolite from 2 to 10% as compared to the control (without metabolite) (Fig. 2 A).

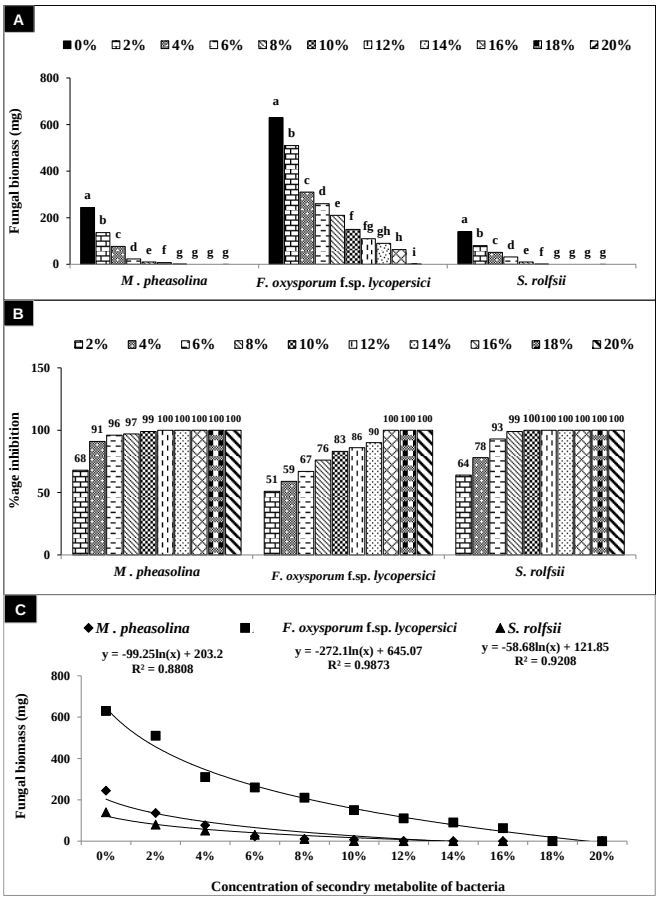

Figure 1. (A-C): Effect of different concentrations of secondary metabolites of *Pseudomonas syringae* on the biomass of different phytopathogenic fungi in malt extract broth medium. A: Effect on fungal biomass; B: Percentage of inhibition in biomass over control; C: Regression analysis for the relationship between different concentrations of *P. syringae* metabolites and biomass of different fungi. Values with different letters at their top show significant differences (P≤0.05) as determined by the LSD test.

The same trend was noticed in the biochemical attributes of SR, therefore, TPP and the activities enzymes improved from 50 to 100% with the increasing metabolite concentration range of 2-10% during different incubation periods over control (Fig. 2 B). In FOL, the total protein content was enhanced by up to 100% and the activities of the enzyme by up to 150% with an increase in the concentration of bacterial

secondary metabolites from 2-18% over the respective control (Fig. 2C).

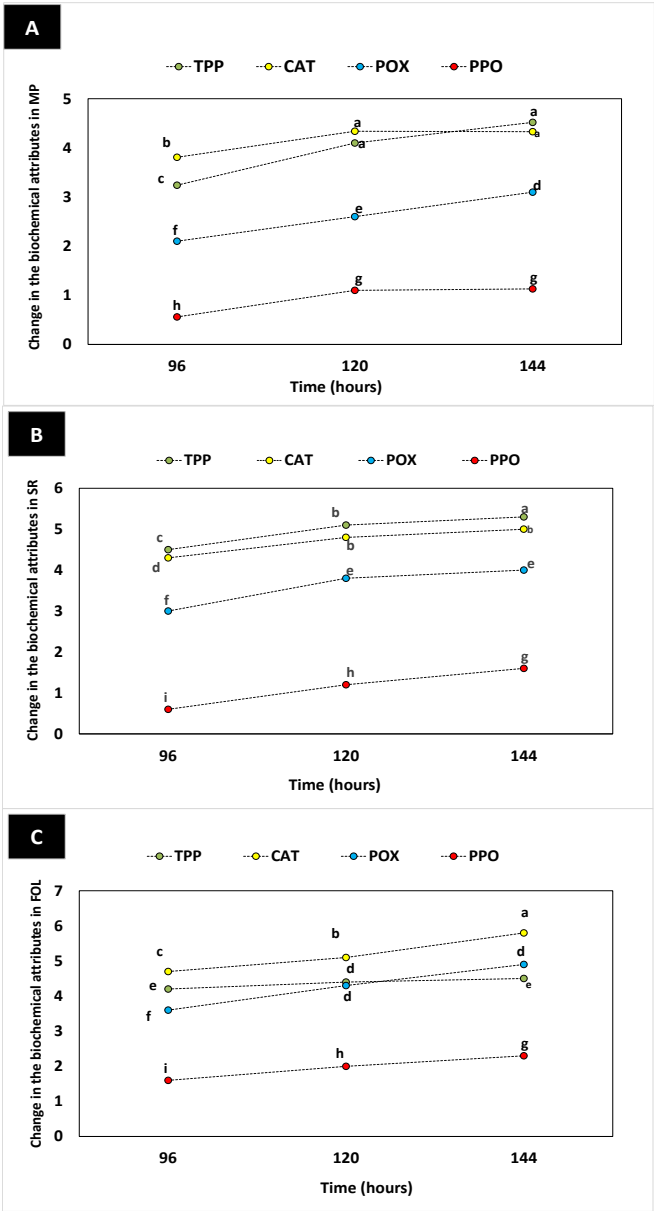

Figure 2. (A-C): Influence of secondary metabolites of *Pseudomonas syringae* on total protein content (mg/g) and antioxidant enzymes (unit/mg/g) in the biomass of *Macrophomina phaseolina* (MP), *Sclerotium rolfsii* (SR) and *Fusarium oxysporum* f.sp. *lycopersici* (FOL) at different time intervals. Values with different letters at their top show significant differences ($p \leq 0.05$) as determined by the LSD Test.

DISCUSSION

Increasing awareness of public concern regarding the deteriorating impact on the environment due to the continuing use of chemicals drives the quest for environmentally safe methods that will contribute to the goal of sustainability in agriculture (Shoaib *et al.,* 2019). The use of biological agents can be regarded as a suitable option for the management of diseases to fulfill the current demand for safe and healthy food along with concerns about environmental pollution (Awan *et al.,* 2022). The antifungal potential of many strains of *Pseudomonas* has been well-established, which besides functioning as bio-fungicidal agents promotes plant growth, and strengthens plant general health (Passera *et al.,* 2019).

In the current study, *P. syringae* significantly decreased the growth of MP, SR, and FOL in dual-culture assays. The growth inhibition was 80, 75, and 48% for MP, SR, and FOL, respectively. Many strains of *Pseudomonas* have been documented for their antifungal action against many phytopathogenic fungi due to their production of many phyto-regulatory and biocontrol substances (Lee *et al.,* 2003; Piechulla *et al.,* 2017). This result could imply that time lapse due to diffusion from the point of bacterial inoculum to the fungus might cause varying antifungal activity by the *P. syringae*.

Various concentrations (2-20%) of *P. syringae* metabolites significantly inhibited the biomass production of MP, SR, and FOL, hence altering the total protein content and activities of enzymes (CAT, POX, and PPO). The growth of both sclerotial-forming fungi was halted with 10% metabolite concentration, it was rather 14% concentration for FOL could be the result of the occurrence of different types of molecules in the bacterial metabolites, which might be more antifungal against sclerotial-forming fungi (Passera *et al.,* 2019). The inhibition in biomass production was accompanied by higher enzyme activities that could be the effect of the over-accumulation of reactive oxygen species to counter stress induced by volatile compounds in the bacterial metabolites. It was suggested that a higher amount of 1-4-octadiene in the antagonistic strain of *P. syringae* is responsible for antifungal activity (Stock *et al.,* 2000). Moreover, the antifungal action of *P. syringae* might be ascribed to their well-characterized small syringopeptin (Awan *et al.,* 2023; Sorensen *et al.,* 1996). The small lipodepsinonapeptide syringomycin E was reported to form ion-conducting membrane channels and distorted yeast membranes by increasing the efflux of K^+ and Ca^{++} (Stock *et al.,* 2000). Therefore, it could be speculated that toxins or other inhibitory compounds in the secondary metabolite of *P. syringae* might have disrupted the balance of fungal membrane ions due to oxidative stress as evidenced by the activities of elevated enzymes led to altered metabolism hence hindering the fungal growth.

***Conclusions*:** In vitro, P. syringae holds the promising potential to suppress the growth of M. phaseolina, S. rolfsii, and F. oxysporum f. sp. lycopersici in dual culture assays.

Various concentrations of bacterial metabolites (2, 4, 6, 8, 10, 12, ---20) also hold the potential to decrease the biomass of M. phaseolina, S. rolfsii, and Fusarium oxysporum f. sp. lycopersici by 68-99%, 64-99%, and 50-99%, respectively by altering fungal total protein content and activity of enzymes (CAT, POX, PPO). However, further research is needed to fully understand the mechanisms behind its "pacifist pathogen" nature and to ensure its safe and efficient application in agricultural systems.

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***Conflicts of Interest*:**

The authors declare that they have no conflicts of interest.

***Author's Contribution*:**

Muhammad Jarrar Ahmed: Experiment; Amna Shoaib: Supervision and drafted the manuscript; Qudsia Fatima: Helped in the writing manuscript; Barizah Malik: Analysis

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