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Research Article

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Assessment of Various Microbiological and Plant-Based Antagonists against Fusarium Wilt Pathogen *Fusarium oxysporum*

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ABSTRACT

The wilt disease of sesame (*Sesamum indicum* L.) caused by *Fusarium oxysporum is* one of the most significant fungal diseases that affect yield production. In this study, two different approaches, namely *in vivo* and *in vitro*, were focused on, in which different microbiological and plant-based antagonists were investigated after 3, 5, and 7 days of incubation to inhibit the development of *F. oxysporum* in sesame. The results of this study revealed great varietal differences in the growth inhibition of *F. oxysporum* at all growth stages. In *in vitro* studies, *Bacillus subtilis* was more effective in growth inhibition (98.67%) of *F. oxysporum* among the bacterial antagonists. In fungal antagonists, *Aspergillus niger* showed a great potential of maximum inhibition in mycelium growth (92.43%) and in plant-based antagonists, *Cannabis sativa* exhibited maximum antifungal activity (85.5%) as compared to control. However, the highest bacterial antagonistic activity was evaluated comparatively to fungal and plant-based antagonists. Whereas, *in vivo*, the study showed the highest antifungal activity (38% - 69%) of selected fungal antagonists, followed by plant-based antagonists (34.17% - 65%) and bacterial antagonists (20% - 48%). This study would help the agrochemical companies to develop microbial and plant-based fungicides that are non-phytotoxic and eco-friendly for the management of fungal diseases, as compared to chemical fungicides that are harmful to consumers and pollute the environment.

Keywords: *Fusarium oxysporum*, microbiological antagonists, plant-based antagonists, sesame.

INTRODUCTION

Sesame is an ancient crop of oilseed, which is cultivated primarily for oil and protein contents. It is commonly known as “Til”. Sesame is grown in 70 countries worldwide, including 26 in Africa and 24 in Asia, with an annual global production of around 3 million tons. The top five producers contribute to almost 70% of total global production. Sesame is cultivated in 65 districts of Pakistan as an irrigated or rain-fed crop, especially in the Punjab (Nayyar et al., 2018).

Antioxidants that are present in sesame are sesamolin, sesamin, and sesamol. This crop is being used for nutritional, therapeutic, and industrial purposes. Sesame has antioxidant and anti-cancerous activity (Ara et al., 2017). The wilt disease of sesame is initiated by a soil-associated fungus *Fusarium oxysporum*, which is one of the most significant soil-borne fungal diseases that affects root, stem, and foliar components, causing economic yield loss (Belay, 2018). Sesame is affected by a variety of pests and diseases that lower its production and badly affect the quality and quantity of the seed. The crop is being affected by 72 fungi, 7 bacteria, 1 mycoplasma, 38 pest species, and 29 insect species that cause diseases in sesame (Egnoyu et al., 2005).

Sesame is a commercial crop worldwide due to its numerous uses. However, contamination brought on by fungus-based agricultural pests is a serious issue (Ojiambo et al., 2003). For plant disease management, using traditional methods such as chemical herbicides, pesticides, and fertilizers is not an eco-friendly approach, as they leave harmful residues that cause pollution and the development of resistant organisms (Naher et al., 2014). To overcome these issues, biological approaches are playing the most significant role, which mostly include the use of microorganisms to control hazardous pathogens that cause plant

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disease without upsetting the ecological balance (Mohiddin et al., 2010). Moreover, the use of these biological approaches removes the economic burden of purchasing and applying fungicides for the farmers. In light of the environmental and economic challenges posed by chemical fungicides, there is a growing urgency to explore sustainable and environmentally acceptable substitutes for managing fungal attacks in sesame crops (Mohiddin et al., 2010).

Numerous studies have reported the activity of bacteria as bio-control agents against pathogens. Bacterial antagonists have different advantages, such as faster multiplication and rhizosphere competence. One of the *in vitro* investigations has shown that *Trichoderma* spp. can dramatically limit the growth of *F. oxysporum. T. viride* and *T. harzianum* were the most effective isolates for inhibiting *F. oxysporum* growth *in vitro*. *T. viride* provided the greatest reduction in the severity of *Fusarium* wilt disease, followed by *T. harzianum* (Mahmoud and Abdalla, 2018). Different studies revealed that *T. harzianum*'s capacity to parasitize other fungi led to its acceptance as a proven fungal antagonist (Lubaina and Murugan, 2015). Mahmoud et al., (2016) investigated that secondary metabolites like pyocyanin are produced by *Pseudomonas aeruginosa* can halt the fungal electron transport chain and show antifungal activity. For this purpose he employed the pure pigment as a bioactive compound to investigate *F. oxysporum's in vitro* antagonistic efficacy against root-rot diseases of agronomic crops. The clinical strain of *P. aeruginosa* shows promise for usage in agricultural applications as a biopesticide or fungicide to protect crops (Mahmoud et al., 2016). One of the studies proves that *Pseudomonas fluorescens* can produce the antibiotic chemical phenazine, which can stop the growth of some plant diseases (Has and Defago, 2005). Previously, *Chenopodium album* extracts were reported to be highly effective against *Sclerotium rolfsii, Ascochyta rabiei, Macrophomina phaseolina, Rhizoctonia solani,* and *F. oxysporum* (Alkooranee et al., 2020). *C. sativa* shows antimicrobial activity against *Staphylococcus, Bacillus subtilis, Pseudomonas aeruginosa,* and *Escherichia coli. C. album* showed antifungal activity against the basal rot disease caused by *F. oxysporum* in onions (Rauf and Javaid, 2013). A study showed that *Malva neglecta* has potent activity against bacterial and fungal species (Alqurashy, 2017). Therefore, the main purpose of this research was to evaluate the microbiological and plant-based antagonists against the *Fusarium* wilt pathogen as an eco-friendly, inexpensive, and sustainable disease management practice.

MATERIALS AND METHODS

Collection of Pathogenic Fungus and Bioagents

The identified strain of test pathogenic fungus (*F. oxysporum*) and selected bioagents (*Bacillus subtilis, P. fluorescens,* and *P. aeruginosa*, *T. harzianum, T. viride,* and *Aspergillus niger*) were collected from the First Fungal Culture Bank of Pakistan (FCBP), University of the Punjab, Lahore. These cultures were preserved at 4°C until used. Fresh leaves of selected weeds viz., *C. sativa, C. album,* and *M. neglecta* were collected from different localities of Tehsil Pasrur, district Sialkot, Pakistan. Plant material was brought to the laboratory for validation in a zipper bag to the Department of the Biological Sciences, University of Sialkot. Fresh leaves of selected weeds were thoroughly washed with tap water, and shade dried for 15-20 days. Dried leaves of selected weeds were crushed into fine powder by using a kitchen grinder and stored at 4°C until used.

Preparation of Plant Extracts and Potato Dextrose Agar (PDA): For plant extracts, the Maceration technique of Azwanida (2015) was followed. Ten grams of each plant powder was soaked in 100 ml of water in 250 ml Erlenmeyer flasks, and these flasks were left at room temperature for three days. This process was used to break plant cells for the release of soluble phytochemicals in the solvent. After three days, the mixture was filtered using Whatman filter paper number 3. PDA powder (39g) was mixed with distilled water (1 L) and autoclaved for 15 minutes at 121ºC and 15psi. After the autoclave, the PDA was cooled down and poured into Petri dishes. PDA was supplemented with antibiotics (streptomycin) 100mg/1000ml. These Petri dishes were inoculated with *F. oxysporum,* wrapped properly with parafilm tape, and incubated at 28±1 ºC (Azwanida, 2015).

In-vitro* Evaluation of Bioagents Against *F. oxysporum

The *in vitro* inhibitory efficacy of plant extracts against the pathogenic fungus *F. oxysporum* was assessed using the poisoned food technique. For each treatment, in sterilized Petri dishes, 10 ml of each plant extract was mixed into 10 ml of sterilized PDA and these dishes were agitated in a circular motion to mix the extracts in PDA homogenously. These Petri dishes were inoculated with a 5mm disc of fungal mycelium and incubated at 28±1 ºC for 7 days (Adhikari et al., 2018).

The antifungal activity of the leaf extract was measured by using a formula:

Percentage inhibition = (dc-dt)/dc × 100

dc represents the average increase in fungal growth in the control; dt represents the average increase in fungal growth in the treated.

Selected bacterial antagonists (*B. subtillis, P. fluorescens,* and *P. aeruginosa*) and fungal antagonists (*A. niger, T. harzianum,* and *T. viride*) were used to evaluate their sensitivity against test fungus *F. oxysporum* using a disc of mycelial having

5 mm diameter of a developing culture in the center of the Petri dish containing PDA. A disc (5 mm in diameter) of freshly grown cultures of *A. niger, T. harzianum, T. viride, B. subtillis, P. fluorescens,* and *P. aeruginosa* was placed at a constant distance opposite to the other edge of the same Petri dishes and incubated at 28C° for 7 days. Petri dishes were inoculated with the test pathogen as those without bioagents, and used as a control. Once the surface of the control dishes had been completely covered by fungi, the inhibition zones of bacterial and fungal growth in the treatment were assessed. The following formula was used to determine an antagonist's capacity to prevent *F. oxysporum* growth (Hassan et al., 2021):

Percentage inhibition of mycelial development =[H-N/H] 100

Where H denotes the diameter of non-treated mycelium development, and N denotes the diameter of the treated mycelium development. All experiments were performed in triplicate with a suitable control. The fungal mycelial growth was recorded after 3, 5, 7, and 10 days of incubation.

In-vivo* Evaluation of Bioagents Against *F. oxysporum

To infest purified pots (9 cm in diameter) with refined soil, 2.5 ml of *F. oxysporum* suspension (10^6 spores/ml) was added. *F. oxysporum* and fungal antagonists were grown separately on PDA. Bacteria were grown on nutrient agar media. Conidia were collected using a sterile brush and sterilized water. The mycelium was removed by filtering the mixture through four layers of sterile cheesecloth. When seven days passed, equal volumes of bacterial and fungal opponent inoculum were distinctly added to all pots, and both were then carefully watered. The control pots contained solely *F. oxysporum* inoculum. Each treatment included three replicates, and ten sesame seeds were sown in each pot. Plants were watered and checked daily. The percentage of seedlings that were dampened off before or after emergence and the severity of the wilt disease were analyzed.

Disease severity = [(severity of disease on all plants/ total no. of plants assessed)]/ highest severity scale× 100 (Nayyar et al., 2018). All experiments were performed in triplicate for each treatment.

Statistical Analysis

A two-way analysis of variance (ANOVA) was used, followed by a post hoc multiple comparisons (Fisher's least significant difference test) to compare the treatment means across all of the trial data. Data are also presented as mean ± standard deviation (SD). Values were considered statistically significant at $P<0.05$.

RESULTS

In-vitro* Evaluation of Bioagents Against *F. oxysporum

For the study containing microbial antagonists, the result of bacterial antagonistic activity was found not significant ($P>0.05$), while fungal and plant-based antagonists indicated significant differences ($P<0.05$) for the mycelium growth inhibition of *F. oxysporum* after 3, 5, and 7 days of incubation. The least mycelium growth was measured at 3 days, followed by 5 and 7 days, respectively (Figure 1, 2).

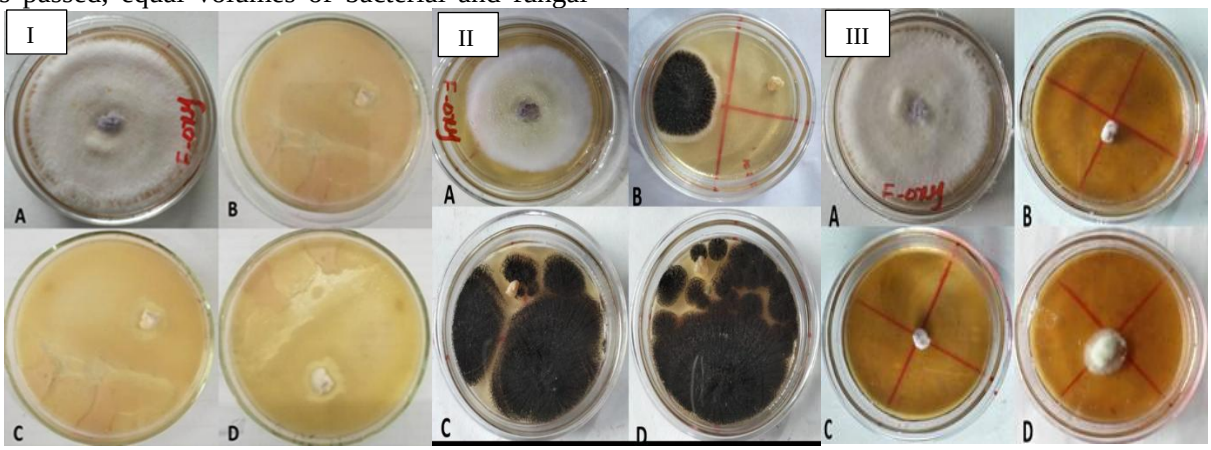

Figure 1. Antagonistic activity of bioagents (I) *B. subtilis,* (II) *A. niger* and (IIII) *C. sativa* on mycelium growth of *F. oxysporum* (A) Control (B-D) antifungal activity after 3,5 and 7 days of incubation respectively.

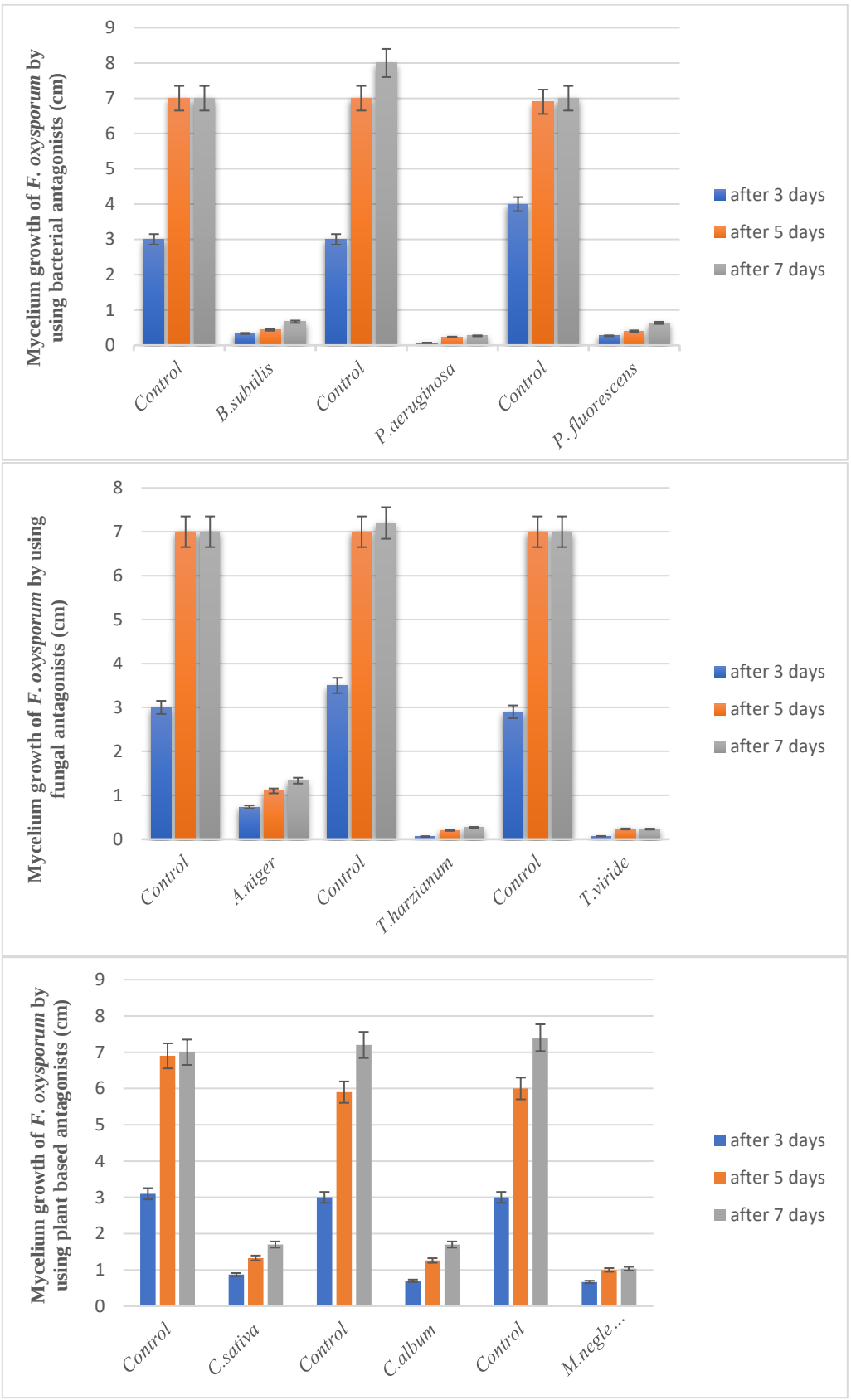

Figure 2. Mycelium growth of test fungus *F. oxysporum* by using bioagents.

Based on the results obtained, *B. subtilis* highly inhibited (98.67%) the growth of *F. oxysporum* as compared to *P. aeruginosa* and *P. fluorescens*. While *P. fluorescens* showed (96.49%) lesser inhibition. In fungal antagonists, maximum inhibition (92.43%) of *F. oxysporum* was observed after 7 days of incubation by *A. niger* while the other two showed lesser inhibition. In which *T. harzianum* revealed the least inhibition (70.78%). Whereas, among extracts of all weeds, *C. sativa* exhibited maximum antifungal activity (85.5%) as compared to other weeds. While minimum inhibition (79%) was observed in *M. neglecta* (Table 1).

Table 1. Average Inhibition percentage of *F. oxysporum* through antagonists

Antagonists	Species	Mean%	S.D	Probability value	Significant
Bacterial	*B. subtilis*	98.67	2.139	0.9	P>0.05
	P. aeruginosa	97.56	1.624	0.11	
	P. fluorescens	96.49	1.391	0.5	
Fungal	*A. niger*	92.43	3.972	0.7	P<0.05
	T. viride	73.70	9.000	0.01	
	T. harzianum	70.78	9.023	0.01	
Weeds	*M. neglecta*	79	6.858	0.0030	P<0.05
	C. album	83	6.665	0.020	
	C. sativa	85.5	33.086	0.043	

Results are represented as the average mean and standard deviation of 3 replicates. The mean difference is significant at the 0.05 level.

In-Vivo* Evaluation of Bioagents against *F. oxysporum

In *an in vivo* study, results of fungal, bacterial, and plant-based antagonistic activity exhibit significant (P<0.05) antifungal potential against the *Fusarium* wilt pathogen of sesame as compared to their control**.** Relative antifungal activity from selected antagonists followed the order of Fungal antagonists > Plant-based antagonists > Bacterial antagonists. Whereas, among extracts of weeds, *C. album* exhibited maximum (65%) antifungal activity. In the case of bacterial antagonists, maximum (48%) inhibition of *F. oxysporum* was observed by *P. aeruginosa*, while *P. fluorescence* and *B. subtilis* showed lesser inhibition as compared to the control. Similarly, In the case of fungal antagonists, maximum (69.33%) inhibition of *F. oxysporum* was observed by *A. niger* while *T. viride* showed (38.33%) least inhibition (Figure 3).

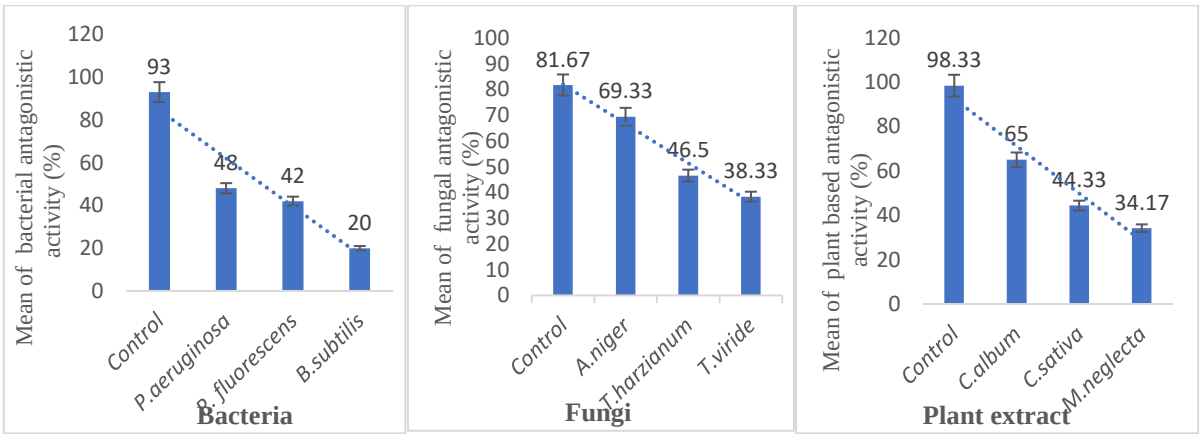

Figure 3. Antagonistic activity of bioagents against *Fusarium oxysporum* in an *in vivo* growth

DISCUSSION

Sesame is a commercial crop worldwide due to its numerous uses at the industrial level. However, infestation of pathogenic *F. oxysporum* is a serious issue in sesame crop production. The use of microbiological, i.e., bacterial, fungal, and plant-based antagonists against *F. oxysporum* is the best biological approach to inhibit the growth of the *Fusarium* wilt pathogen in sesame.

In this *in vitro* study, the order of effectiveness of bacterial antagonists was observed as *B. subtilis* > *P. aeruginosa* > *P. fluorescens*. One of the studies also showed that isolates of *B. subtilis* demonstrated competitive action against *F. oxysporum* and showed more than 50% mycelial inhibition (Gajbhiye *et al*. 2010). One of the researchers also reported that *B. subtilis*, *P. aeruginosa*, and *T. viride* reduced the occurrence of wilt disease in sesame seeds (Hassan et al., 2021), which supports the results of the current study. Whereas, fungal antagonists showed their effectiveness as *A. niger* > *T. harzianum* > T. *viride*. These results are consistent with Zulqarnain *et al*. (2020), who reported that *A. niger* showed the highest (51.5 ±1.1 %) growth inhibition against *F. oxysporum*. However, in weeds inhibition rate was observed as *C. sativa* > *C. album* > *M. neglecta*. One of the studies revealed that *C. sativa* contains a high concentration of bioactive compounds such as terpenes, cannabinoids, flavonoids, and phenols, which have strong antifungal activities and may be beneficial in halting the growth of *Aspergillus flavipes* (Singh et al., 2018). Swain *et al*. (2016), also used leaf extracts of *C. sativa* to create gold nanoparticles that were also efficient against species of *Fusarium*. Sharma et al., (2011) also reported the use of *A. niger* (74%), *Trichoderma* spp. (62%) *Pseudomonas* spp. (27%) and *Bacillus* spp. (31%) as a biocontrol agent for the management of *F. oxysporum lycopersici*, which caused wilt disease in tomatoes in their *in vitro* study. All these studies support the results of our *in vitro* study during this research.

Results of the *in vivo* study revealed that all selected weeds significantly showed antifungal potential against the *Fusarium* wilt pathogen. Among extracts of all weeds, *C. album* exhibited maximum antifungal activity as compared to other weeds. Various studies also revealed that *C. album* has alkaloids, flavonoids, saponins, terpenoids, and steroids in its aerial part, which possess significant antifungal potential (Pandey and Gupta, 2014). Rauf and Javaid (2013) also reported the antifungal efficacy (24 - 80%) *of C. album* against *F. oxysporum*. Javaid *et al*. (2020) also investigated strong antifungal activity in leaf, root, and fruit extracts of *C. album*, which led to suppression of *A. alternata* biomass by 23-95%, 29-96%, and 9-94%, respectively. These studies also confirm that *C. album* has the potential for antifungal properties. In the case of bacterial antagonists, maximum inhibition of *F. oxysporum* was observed by *P. aeruginosa*, while the other two showed lesser inhibition. *P. aeruginosa* is known for producing soluble pyocyanin pigment, which has the ability to halt the electron transport chain of fungi and hence, show antifungal activity (Mahmoud *et al*., 2016). Among fungal antagonists, maximum inhibition of *Fusarium oxysporum* was observed by *A. niger* while the other two showed lesser inhibition. Concluding this relative antifungal activity from selected antagonists, followed the order of Fungal antagonists > Plant-based antagonists > Bacterial antagonists in our *in vivo* study. According to Patel et al. (2021), *T. viride* also inhibits *M. phaseolina* by causing systemic resistance in sesame plants and the synthesis of enzymes and secondary compounds that break down the plant's cell walls. Therefore, *T. viride* is also a promising biocontrol agent for controlling the disease known as charcoal rot in sesame (Patel et al., 2021), which confirms the results of *T. viride* as a biocontrol agent used in this study.

CONCLUSION

The current research revealed that a successful inhibition of the growth of pathogenic *F. oxysporum* is possible by using the antagonistic potential of selected microbial and plant-based antagonists. In the case of *in vitro* studies, maximum inhibition was observed by the *B. subtilis*, whereas *in vivo* study showed the highest anti-fungal activity of *A. niger*. These biocontrol agents also offer sustainable and eco-friendly alternatives to manage the *Fusarium* wilt pathogen in sesame crops by reducing the reliance on harmful synthetic chemicals. Further biological approaches are needed to find the possible microbial and plant-based antagonists that possess more antifungal properties against the growth of *F. oxysporum*, as indicated in the present research.

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