

Biological control of *Fusarium oxysporum* f. sp. *capsici* using allelopathic tolerant plant growth promoting rhizobacteria

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Abstract

Chili (*Capsicum annum* L.) is an important spice and one of the major cash crops of Pakistan. *Fusarium oxysporum* f. sp. *capsici* (FOC) is a soil-borne ascomycete fungus that causes wilt disease in chili. This study was carried out to assess the seven strains of plant growth-promoting rhizobacteria (PGPR) for their *in vitro* biocontrol potential against FOC. The PGPR were originally isolated from an allelopathic grass *Cenchrus pennisetiformis* Steud. These bacteria were used in an *in vitro* antagonistic assay against FOC. In general, all the seven bacterial species significantly inhibited the growth of fungal pathogen to variable extents. Among these, *Bacillus cereus* BP-CP3 showed the highest antagonistic potential and inhibited the growth of FOC by 55% followed by *B. cereus* BP-CP2 and *Bacillus subtilis* BP-CP5 causing 51% and 41% inhibition. The best *B. cereus* BP-CP3 strain was selected for GC-MS analysis of its secondary metabolites. The metabolites were prepared by incubating the bacterial strain in LB broth for five days. These metabolites were successively fractionated using *n*-hexane, chloroform and ethyl acetate. These fractions were subjected to GC-MS analysis. Five compounds were identified in *n*-hexane fraction. Among these, undecane was the most abundant compound followed by dodecane with peak areas of 47.81% and 29.14%, respectively. Likewise, four compounds were identified in chloroform fraction. The major compound in this fraction was hexadecanoic acid, methyl ester with 35.88% peak area followed by 9-octadecenoic acid, methyl ester, (E)- (29.53%). Three compounds were identified in ethyl acetate fraction with hexadecanoic acid, methyl ester (38.05%) as the predominant compound followed by 10-octadecenoic acid, methyl ester (31.98%). This study concludes that *B. cereus* BP-CP3 is a highly antagonistic strain against FOC.

Keywords: Antagonistic bacteria, Chili, *Fusarium* wilt, GC-MS analysis, Secondary metabolites.

Introduction

Chili (*Capsicum annum* L.) is among the most widely grown vegetable crops internationally. Worldwide production of chili was 4.3 metric tons in 2019. India accounted for 40.6% of production followed by Thailand (8.18%) and China (7.66%). Holding a share of 2.39% in the global market, Pakistan ranks 10th in chili production (Shaheen *et al.*, 2021). It not only gives a characteristic pungency to food but also plays a key role in agricultural economy globally owing to its high nutritional profile and diverse economic applications (Zou *et al.*, 2020). Its characteristics pungency is attributed to the presence of phenolic antioxidants and capsaicinoids (Olatunji and Afolayan, 2020). Other bioactive compounds of chili include different vitamins, flavonoids, carotenoids and polyphenols, which are known for their anticancer, antimicrobial, and anti-obesity activities (Duan *et al.*, 2025). Chili is susceptible to various fungal pathogens in the field, including *Fusarium oxysporum* f. sp. *capsica*, the cause of wilt. The affected plants show chlorosis of older leaves, downward epinasty of terminal shoots, subsequent wilting, and eventually death of the infected plant (Shaheen *et al.*, 2021).

Numerous strategies have been developed to control *Fusarium* wilt of chili. At present, generally fungicides and soil fumigation are applied to control soil-borne diseases of chili. Under laboratory conditions, carbendazim caused 92.4% reduction in mycelial growth of the wilt pathogen. The fungicide also markedly suppressed the population of the fungal pathogen under field condition (Madhavi *et al.*, 2006). However, the use of synthetic agrochemicals is generally discouraged due to their ill effect of human and animal health, and the environment (Gikas *et al.*, 2022). Thus, scientists are in search of alternative environment friendly strategies for the control soil-borne diseases. Biological control agents including the species of *Trichoderma* (Ali *et al.*, 2020; Khan and Javaid, 2020; Khan *et al.*, 2021), *Penicillium* (Javaid *et al.*, 2020), *Aspergillus* (Khan and Javaid, 2022), *Bacillus* (Sharf *et al.*, 2021), *Pseudomonas* (Zohaib *et al.*, 2024) and others, have been proved very effective in controlling soil-borne fungal pathogens namely *Fusarium oxysporum*, *Sclerotium rolfsii*, *Rhizoctonia solani* and *Macrophomina phaseolina*. The present study was conducted to evaluate the *in vitro* antagonistic behavior of seven bacterial species

previously isolated from the rhizosphere of an allelopathic grass *C. pennisetiformis*. The study was further extended to explore the chemical profile of metabolites of the most antagonistic bacterial species.

Materials and Methods

Procurement of PGPR

The seven PGPR strains utilized in this study were obtained from the Biopesticide and Biofertilizer Laboratory, Faculty of Agricultural Sciences, University of the Punjab, Lahore. The PGPR strains were originally isolated from the rhizospheric soil of *Cenchrus pennisetiformis*. These bacterial species included *Bacillus* sp. BP-CP1, *Bacillus cereus* BP-CP2, *B. cereus* BP-CP3, *Bacillus subtilis* BP-CP4, *B. subtilis* BP-CP5, *Alcaligenes faecalis* BP-CP6, and *Bacillus stercoris* BP-CP7 (Yousaf, 2024).

Procurement of fungal pathogen

The fungal pathogen, *F. oxysporum* f. sp. *capsici* was provided by Dr. Waheed Akram. The pathogen was originally isolated from the chili plants suffering from wilt disease and identified on morphological and molecular bases. The pathogen was subsequently refreshed on malt extract agar (MEA) plates at 28 °C for 7 days to be used in experimentation.

In vitro antagonistic interactions

The bacterial strains were grown in LB broth and incubated at 25 °C for 24 h to obtain fresh cultures. A 4 mm mycelial disc of FOC was placed in the middle of each Petri dish (9-cm) and the cultures of each of the seven bacterial strains were streaked around the plug separately. A control consisting of only the fungal pathogen plug in the middle was used for comparison. Five replicates of each treatment were made. The data regarding radial growth of the fungus was recorded when the pathogenic fungal isolate covered the plates of control treatment. The percent inhibition was calculated using the following formula:

$$\text{Inhibition (\%)} = (C - T) / C \times 100$$

C: Colony diameter of fungal pathogen in control plate.

T: Colony diameter of fungal pathogen in bacterial application.

Preparation of bacterial metabolites

To obtain the bacterial metabolites, the selected strain (*B. cereus* BP-CP3) was cultured in 100 mL of LB broth (0.5 g yeast, 1 g peptone, 1 g NaCl, 100 mL water) and incubated at 25 °C for 24 h. After incubation, the culture was passed through Whatman filter paper No. 1 to separate the bacterial cells from the supernatant. The resulting filtrate was then subjected to partial evaporation in a drying oven at 45 °C until the volume was reduced to

approximately 50 mL.

A liquid-liquid extraction was performed on the 50 mL bacterial filtrate using three different organic solvents. Initially, 20 mL of pure *n*-hexane was added to the filtrate, followed by vigorous shaking for 10 min to ensure thorough mixing. The mixture was then transferred to a separating funnel and allowed to stand undisturbed. Upon phase separation, the upper organic layer was collected while the aqueous phase was again partitioned with *n*-hexane twice. This procedure was subsequently repeated using 20 mL of chloroform, and 20 mL of ethyl acetate. The organic solvent layers were collected in separate vials (Javaid *et al.*, 2017). Each of the recovered organic fractions was passed through Whatman filter paper to remove any residual particulate matter. The clear extracts were stored in clean, tightly sealed glass tubes and labeled for GC-MS analysis. To prevent contamination from plastic-derived compounds such as phthalates, all extractions and transfers were performed exclusively using sterilized glassware.

GC-MS analysis

GC-MS analysis was carried out following the procedure described by Javaid *et al.* (2022). The samples prepared using three organic solvents were analyzed on a GC-MS-QP 2010 chromatograph. The ionization voltage was 70eV, m/z scan range 55-950 Da, fitted out with a capillary column DB-5 (30 m × 0.25 µm, film thickness 0.25 µm). The temperature of the oven was held at 45 °C for 1 min, thereafter automated from 45–100 °C at a rate of 5 °C min⁻¹, and held for 1 min, then increased up to 200 °C at a rate of 10 °C min⁻¹ and kept at the final temperature for 5 min. Helium served as the carrier gas. The injector temperature was 200 °C and that of the detector was 250 °C. The relative percentage of each volatile component was calculated using GC peak areas. Qualitative analysis was based on comparison of retention time, indices and mass spectra with reported values from the NIST Library 2010.

Statistical analysis

All the data were analyzed by one-way ANOVA. Means were compared by applying LSD test at P≤0.05 using computer software Statistix 8.1.

Results and Discussion

Antagonistic activity of PGPR

The seven PGPR strains showed highly variable antagonistic potentials against the fungal pathogen FOC. In general, all the bacterial strains variably and significantly inhibited the fungal growth over the control. Among these, the antagonistic activity of two PGPR strains namely BP-CP2 and BP-CP3 was highly pronounced with 51% and 55% inhibition in the fungal growth, respectively. Likewise, the antagonistic activity of BP-CP5 was

also remarkable with 41% inhibition in the growth of the pathogen. Two strains namely BP-CP6 and BP-CP7 proved less effective with 28% and 33% inhibition potential, respectively. BP-CP1 and BP-CP4 showed the least antagonistic potential and inhibited fungal growth by 18% and 20%, respectively (Fig. 1 and 2). These beneficial microbes manage phytopathogens through different direct or indirect mechanisms and play an important role in the growth and development of plants (Ahmad *et al.*, 2011). According to another research, it was found that the PGPR isolated from the rhizosphere of brinjal and chilies were not only efficient in controlling *F. oxysporum* and *Alternaria solani* but also boosted plant growth (Parasuraman *et al.*, 2022). Goswami and Deka (2020) conducted a research in which they concluded that the PGPR isolated from an allelopathic grass citronella, controlled the fungal pathogen *Sclerotinia sclerotiorum*. Zhao *et al.* (2010) reported that *Bacillus* spp. when used as biocontrol agents, produce various metabolites that help in their antifungal properties. PGPR application induced resistance in chili against attack of *Sclerotium rolfsii*, the causal agent of collar rot disease (Sharf *et al.*, 2021). Similarly, application of PGPR together with dry biomass of *Cirsium arvense* significantly reduced in vitro growth of *Rhizoctonia solani* and in vivo incidence of black scurf disease of potato (Zohaib *et al.*, 2024).

GC-MS analysis

Five compounds were identified in *n*-hexane fraction. Among these, undecane was the most abundant compound followed by dodecane with peak areas of 47.81% and 29.14%, respectively. The other three compounds were tridecane (9.04%), *o*-cymene (7.36%) and 7,9-di-tert-butyl-1-oxaspiro(4,5)deca-6,9-diene-2,8-dione (6.64%). Four compounds were identified in chloroform fraction. The major compound in this fraction was hexadecanoic acid, methyl ester with 35.88% peak area. Other compounds were 9-octadecenoic acid, methyl ester, (E)- (29.53%), and cyclononasiloxane, octadecamethyl- (22.93%). Methyl stearate was found as the less abundant compound with 7.97% peak area. Three compounds were identified in ethyl acetate fraction. Major compound in this fraction was hexadecanoic acid, methyl ester with 38.05% peak area. Other compounds were 10-octadecenoic acid, methyl ester (31.98%), and cyclononasiloxane, octadecamethyl- (29.96%) as shown in Table 1. Earlier, in *n*-hexane fraction of metabolites of *Bacillus cereus* HKS-1, the most abundant compound was undecane that induces systemic resistance in plants that make the plant to resist against a variety of pathogens (Jishma *et al.*, 2016). It is also extracted from plant essential oils and petroleum distillates and have its use in cosmetics,

lubricants and fuel. This compound can help reduce the need for synthetic pesticides and fungicides and promote more sustainable agricultural practices (Sidorova *et al.*, 2019). *Pseudomonas rhodidae*, another microbial species that produces undecane, also helped in controlling the disease while promoting plant growth in *Vigna radiata*. It has been proven through studies that undecane produced by different *Bacillus* species as a volatile organic compound possess the ability to inhibit fungal growth by disrupting the integrity of cellular membranes of fungi causing an intracellular leakage.

In the chloroform fraction, hexadecanoic acid, methyl ester was the most abundant compound. It possesses antifungal properties against a number of pathogens including *F. oxysporum*, *Aspergillus niger* and *Botrytis cinerea*. It protects cell membrane and reduces oxidative stress. It can boost induced systemic resistance (ISR) and increase stress tolerance of plants. The compound 9-octadecenoic acid, methyl ester, (E)- is found not only in *Bacillus* species but also in many strains of *Pseudomonas*. It is also produced by plant extracts and microbial and algal lipids. It interferes with the growth of fungi by interference with ergosterol biosynthesis Increased membrane permeability and ROS accumulation (Thankappan *et al.*, 2022).

Conclusion

B. cereus BP-CP2 and *B. cereus* BP-CP3 were found highly effective antagonistic PGPR species causing 51% and 55% inhibition in the fungal growth, respectively. Major compounds identified in metabolites of *B. cereus* were undecane; dodecane; hexadecanoic acid, methyl ester; 10-octadecenoic acid, methyl ester; and cyclononasiloxane, octadecamethyl-. Studies are needed to test the effect of individual possible antifungal compounds identified in this study, against FOC. Moreover, further plant-based and field validation is required before practical biocontrol efficacy of these bacterial strains can be concluded.

Acknowledgements

Dr. Waheed Akram provided the culture of *Fusarium oxysporum* f. sp. *capsici* that is highly acknowledged.

Contribution of authors

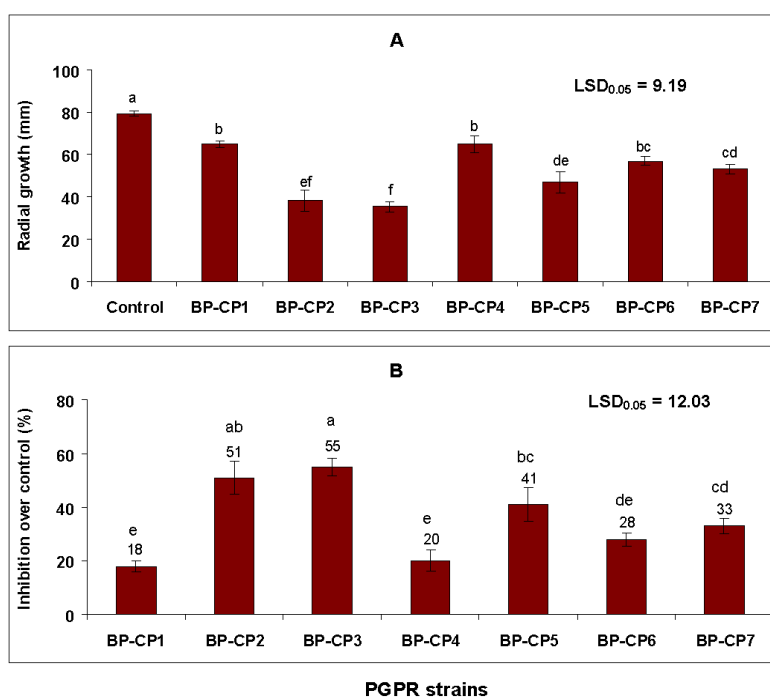
SA did experimental work. MA helped in helped in carrying out experiments. AS supervised the *in vitro* experiment. AJ supervised the whole work, did statistical analysis and wrote manuscript.

Conflict of interests

Authors declare that there is no conflict of interest.

Table 1: Compounds identified in *n*-hexane, chloroform and ethyl acetate fractions of metabolites of BP-CP3 through GC-MS analysis.

Names of compounds	Molecular formula	Molecular weight	Retention time (min)	Peak area (%)
<i>n</i>-Hexane fraction				
<i>o</i> -Cymene	C ₁₀ H ₁₄	134.22	5.658	7.36
Undecane	C ₁₁ H ₂₄	156.31	5.760	47.81
Dodecane	C ₁₂ H ₂₆	170.33	6.766	29.14
Tridecane	C ₁₃ H ₂₈	184.36	7.719	9.04
7,9-Di-tert-butyl-1-oxaspiro(4,5)deca-6,9-diene-2,8-dione	C ₁₇ H ₂₄ O ₃	276.4	12.662	6.64
Chloroform fraction				
Cyclononasiloxane, octadecamethyl-	C ₁₈ H ₅₄ O ₉ Si ₉	667.38	11.484	22.93
Hexadecanoic acid, methyl ester	C ₁₇ H ₃₄ O ₂	270.45	12.640	35.88
9-Octadecenoic acid, methyl ester, (E)-	C ₁₉ H ₃₆ O ₂	296.48	13.76	29.53
Methyl stearate	C ₁₉ H ₃₈ O ₂	298.50	13.902	7.97
Ethyl acetate fraction				
Cyclononasiloxane, octadecamethyl-	C ₁₈ H ₅₄ O ₉ Si ₉	667.38	11.484	29.96
Hexadecanoic acid, methyl ester	C ₁₇ H ₃₄ O ₂	270.45	12.645	38.05
10-Octadecenoic acid, methyl ester	C ₁₉ H ₃₆ O ₂	296.48	13.764	31.98

**Fig. 1:** Growth inhibition of *Fusarium oxysporum* f. sp. *capsici* due to interactions with different PGPR species isolated from the rhizosphere of *Cenchrus pennisetiformis*. Vertical bars show standard errors of means of five replicates. Values with different letters at their top show significant difference ($P \leq 0.05$) as determined by LSD Test.

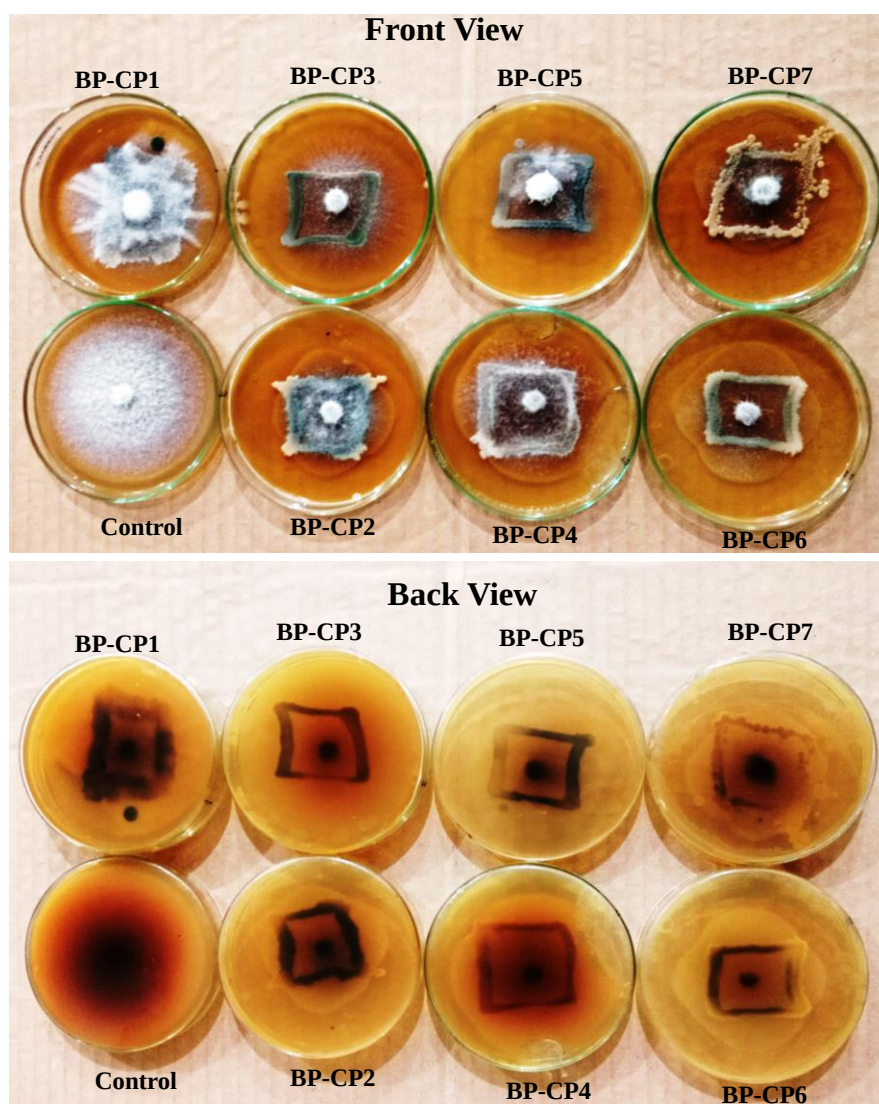


Fig. 2: Growth inhibition of *Fusarium oxysporum* f. sp. *capsici* due to *in vitro* interactions with different PGPR strains isolated from the rhizosphere of *Cenchrus pennisetiformis*.

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