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Potentially biological control of *Bacillus* spp. against *Fusarium keratoplasticum* Geiser et al. 2013 causing wilt in pico chrysanthemum

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ABSTRACT

Fusarium wilt, caused by Fusarium spp., is considered one of the most serious diseases affecting pico chrysanthemum (Chrysanthemum spp.), leading to significant yield and quality losses. This study was conducted to identify the fungal pathogen causing wilt disease in pico chrysanthemum and to assess the antifungal potential of Bacillus spp. under in vitro conditions. The fungal isolate FC1 was recovered from infected plants and identified as Fusarium keratoplasticum through analysis of internal transcribed spacer (ITS) gene sequences together with morphological characteristics. Its pathogenicity was confirmed in accordance with Koch's postulates. All 41 Bacillus isolates exhibited antifungal activity against F. keratoplasticum in dual culture assays, with mycelial growth inhibition ranging from 20.0% to 52.2%. Volatile organic compounds (VOCs) and cell-free supernatants (CFS) produced by six promising isolates (CB3, CB10, CB31, CB32, CB35, and CB36) markedly suppressed both mycelial growth and spore germination of fungal isolate FC1. These findings suggest that the selected Bacillus isolates have strong potential as biological control agents for managing F. keratoplasticum-induced wilt in pico chrysanthemum.

1. INTRODUCTION

Chrysanthemum (*Chrysanthemum* spp.) is an ornamental plant of high economic value, widely cultivated in various forms, including cut flowers, potted plants, medicinal materials, and even as a food source (Hao et al., 2022; Mekapogu et al., 2022). However, chrysanthemum production is severely constrained by various diseases, among which *Fusarium* wilt is considered one of the most destructive (Liu et al., 2023a).

Fusarium wilt in chrysanthemum is caused by several species within the genus *Fusarium*, which have been reported across different regions (Thao et al., 2021; Liu et al., 2023a; Balamurugan et al., 2024). These pathogens can persist in soil or crop residues from previous seasons in the form of conidia or chlamydospores, posing substantial challenges for disease management (Bahadur, 2022).

Chemical fungicides are commonly used to play an important role in managing plant pathogens; however, their inappropriate or excessive use can lead to several risks, including environmental contamination, adverse effects on beneficial soil microorganisms (Zubrod et al., 2019), accumulation of chemical residues in agricultural products (Wang et al., 2023), and the development of fungicide-resistant pathogen populations (Naqvi et al., 2025). Therefore, there is an increasing demand for sustainable and environmentally safe approaches to disease management.

Under these circumstances, biological control based on beneficial microorganisms has been considered as a potential alternative strategy. *Bacillus* species are well known for their antagonistic activity against plant pathogens through various mechanisms, such as the production of antifungal compounds, siderophores, and hydrolytic enzymes, as well as the activation of systemic resistance in plants (Kim et al., 2019; Chen et al., 2024).

Pico chrysanthemum (*Chrysanthemum indicum*) is a popular ornamental variety characterized by its compact growth habit and diverse flower colors (Thao et al., 2023). Despite its commercial importance, studies on *Fusarium* wilt affecting piko chrysanthemum remain limited, particularly in identifying causal agents and applying biological control strategies.

The objectives of this study were to (i) identify the *Fusarium* species associated with wilt disease in pico chrysanthemum and (ii) evaluate the antifungal potential of *Bacillus* spp. isolates against these pathogens. The results of this study provide a scientific foundation for developing of environmentally friendly biocontrol agents for the management of *Fusarium* wilt in pico chrysanthemum.

2. MATERIALS AND METHOD

2.1. Fungal isolation and morphological characterization

Stem and root tissues of infected plants were excised and soaked in 1% sodium hypochloride solution for 45 seconds, then soaked in 70% ethanol for 30 seconds (Thao et al., 2021). Plant pieces were washed 3 times with sterile distilled water, blotted dry on sterile filter paper and inoculated on malt extract agar (MEA) supplemented with tetracycline (0.05 g/L) and streptomycin sulfate (0.1 g/L) (Sharma et al., 2018). The plates were incubated at 30°C in the dark for 4-7 days. Fungal colonies emerging from the infected plant tissues were subcultured on fresh MEA plates to obtain pure isolates. The morphological features of the fungal isolates were examined under a light microscope, focusing on hyphal structure and the morphology of microconidia and macroconidia. For colony characterization, fungal plugs (6 mm in diameter) were taken from the growing margin of the fungal isolates and transferred to fresh MEA plates. The plates were incubated at 30°C, and colony morphology and diameter were recorded daily for seven consecutive days.

2.2. Pathogenicity test (Koch's postulates)

The soil for growing chrysanthemum plants was autoclaved twice at 121°C for 40 minutes. Healthy chrysanthemum plants were individually transplanted into plastic pots (9×15 cm) containing sterile soil.

Fusarium isolates were cultured on MEA plates for 7 days. The fungal colonies were flooded with 10 mL of sterile distilled water and filtered through sterile cheesecloth to remove mycelial fragments. The concentration of conidia was adjusted to 10^6 conidia/mL using a modified Neubauer hemocytometer.

Sterile bamboo sticks were soaked in conidia suspension for 30 minutes. For the control treatment, bamboo sticks were soaked in sterile distilled water for 30 minutes. The inoculated sticks were pierced into chrysanthemum stems (Chen et al., 2020). Each treatment included three plants corresponding to 3 replications.

Plant symptoms were monitored at 24-hour intervals. The fungal isolates were considered pathogenic when the leaves wilted and the stems showed necrotic spots at the puncture sites.

The pathogens were re-isolated from symptomatic plant tissues using the same protocol described for initial isolation. The morphological characteristics of the re-isolated fungi were compared with those of the original isolates. The re-isolated fungi exhibited morphological features identical to the original isolates and were confirmed as *Fusarium* spp. causing wilt in pico chrysanthemum.

2.3. DNA extraction and molecular identification

Total genomic DNA of isolate FC1, which had been confirmed as a *Fusarium* causing wilt in pico chrysanthemum, was extracted following the protocol described by Al-Samarrai and Schmid (2000). The concentration and purity of the extracted DNA were determined using a NanoDrop spectrophotometer (Thermo Scientific, USA) by measuring absorbance at 260 and 280 nm. The ITS region of the fungal DNA was amplified using the universal primer pair ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') (Fujita et al., 2001). PCR amplification was carried out in a final volume of 25 µL containing PCR-grade water, 1× MyTaq DNA Polymerase buffer (Bioline, the UK), 0.4 µM of each primer, 1.25 U MyTaq DNA Polymerase (Bioline, the UK), and 2 µL of DNA

template. Amplifications were performed using an ABI 9800 Fast Thermal Cycler with the following cycling parameters: 94°C for 3 minutes; 35 amplification cycles consisting of 94°C for 30 seconds, 55°C for 30 seconds, and 72°C for 60 seconds; and a final extension at 72°C for 10 minutes. The PCR products were sequenced by a commercial sequencing service (DNA Sequencing Co., Ltd.). The resulting sequences were compared with existing references available in the NCBI database using BLASTn.

2.4. In vitro antifungal activity of *Bacillus* isolates against *F. keratoplasticum* FC1

In this study, 41 *Bacillus* isolates obtained from 12 cow manure samples (unpublished), were used in the experiment. *Bacillus* isolates were cultured on Luria–Bertani (LB) agar plates and incubated at 30°C for 24 hours. A loopful of bacterial biomass was suspended in 10 mL of sterile 0.85% NaCl solution. The bacterial concentration was adjusted to an optical density of 0.1 at 600 nm (OD_{600}) using a spectrophotometer.

Fungal plugs (6 mm in diameter) were taken from the actively growing margin of a *Fusarium* colony and inoculated into the center of MEA plates. The inoculated plates were incubated at 30°C for 24 h. After pre-incubation, four equidistant wells (6 mm in diameter) were made around the fungal colony using a sterile cork borer. A total of 20 µL of the prepared *Bacillus* suspension was pipetted into three wells, while the fourth well was filled with 20 µL of sterile 0.85% NaCl solution and served as a negative control. After incubation at 30°C for 5 days, the radial inhibition of fungal mycelial growth was determined according to the equation described by Han et al. (2015):

$$I\ (\%) = \frac{(R-r)}{R} \times 100$$

In which: I is the inhibition of the mycelial growth; R is the radial growth (mm) of the fungal colony in the control treatment; r is the radial growth (mm) of the fungal colony in the treatment with *Bacillus* spp.

2.5. Assessment of antifungal effects of volatile organic compounds and cell-free supernatants produced by *Bacillus* isolates against *F. keratoplasticum* FC1

Suspensions of strong antagonists (100 µL) were evenly spread on LB agar plates using sterile cotton swabs. LB agar plates without antagonistic bacteria were used as controls. Fungal plugs of isolate FC1 (6 mm in diameter) were inoculated in the center of MEA plates for the mycelial growth inhibition assay. For the spore germination inhibition assay, 100 µL of a *Fusarium* conidial suspension (10^5 conidia/mL) was spread uniformly over the surface of MEA plates. Each MEA plate inoculated with *Fusarium* (either plug or spore suspension) was inverted over a corresponding LB plate containing either *Bacillus* spp. or a sterile control (uninoculated with bacteria). The rims of the paired Petri dishes were sealed tightly with parafilm to prevent leakage of volatile organic compounds (VOCs). The sealed double-plate systems were incubated at 30°C for 2 days (spore germination test) or 5 days (mycelial growth test) (Lien et al., 2023).

After incubation, antifungal activity was evaluated by measuring the reduction in mycelial growth or spore germination. Inhibition rates were calculated using the following equations:

Rate of mycelial growth inhibition (%) = $(1 - \frac{D_t}{D_c}) \times 100$

Rate of spore germination inhibition (%) = $(1 - \frac{N_t}{N_c}) \times 100$

Where:

D_t and D_c represent the colony diameter in the treatment and control, respectively.

N_t and N_c represent the number of fungal colonies in the treatment and control, respectively.

Bacterial suspension (100 µL) was added to 20 mL LB broth, and incubated at 30°C on a shaker for 48 hours. Each experiment was repeated three times. The bacterial suspension was centrifuged at 10,000 rpm for 10 minutes at 4°C. The supernatant was filtered through a 0.22 µm cellulose filter. Cell-free supernatants (CFS) were mixed with MEA cooled to approximately 50°C to achieve a final concentration of 20% (v/v). The control plate was mixed with an equal amount of LB broth. Fungal plugs of isolate FC1 (6 mm in diameter) were inoculated in the center of the test medium plates. In the spore germination inhibition test, 50 µL of conidia suspension of isolate FC1 was spread evenly on the test medium plates. The plates were incubated at room temperature for 2 days for the spore inhibition test and 5 days for the mycelial inhibition test (Lien et al., 2023). The inhibition of radial mycelial growth and the inhibition of spore germination were calculated using the equation described above.

2.6. Data analysis

Data were entered and processed using Microsoft Excel 2016 software and statistically analyzed by ANOVA using Minitab 16 software with one-way ANOVA analysis of variance method with Tukey's HSD test at 5% significance level.

3. RESULTS AND DISCUSSION

3.1. Characterization of fungal isolate FC1 causing wilt of pico chrysanthemum

The fungal isolate FC1, obtained from the infected pico chrysanthemum plants, had round colonies; the upper surface exhibited spongy mycelium, light yellow in color when young, turning dark yellow with age. The lower surface displayed yellow pigmentation with a single concentric ring (Figure 1a). The average radial growth rate of the colony was 12.7±0.29 mm/day.

Microscopic examination revealed that the microconidia of FC1 were oval to kidney-shaped, measuring 7–11 × 3–4 µm (Figure 1b), while the macroconidia were characteristically sickle-shaped, possessing 2–3 septa and measuring 25–28 × 4–5 µm (Figure 1c). These morphological features are consistent with the typical characteristics of *Fusarium* spp., in which macroconidia are curved (sickle-shaped) and septate, and microconidia are generally unicellular with variable shapes ranging from oval to kidney-shaped (Thrane, 1999). Therefore, the isolate FC1 was preliminarily identified as belonging to the genus *Fusarium*.

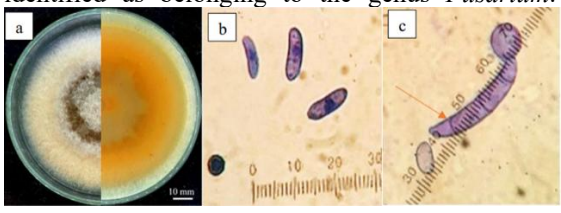

Figure 1. Macroscopic and microscopic characteristics of fungal isolate FC1

Upper and lower surface of colony of isolate FC1 (a); Microconidia of isolate FC1 (b); Macroconidia of isolate FC1 (red arrow) (c).

Pico chrysanthemum plants after artificial infection with isolate FC1 developed wilt symptoms. These included leaf and branch wilting, stem browning and collapse, and vascular tissue necrosis (Figure 2). Root necrosis and external mycelial growth were also observed. Symptoms appeared within 5 days post-inoculation. The fungus re-isolated from infected plant tissues showed colony and conidial morphology identical to those of isolate FC1, thereby fulfilling Koch's postulates and confirming FC1 as the causal agent of wilt disease in pico chrysanthemum.

Figure 2. Symptoms of *Fusarium* wilt in pico chrysanthemum plants artificially inoculated with isolate FC1

High-quality genomic DNA was successfully extracted from the fungal isolate FC1, with a concentration of 512.2 ng/µL and an A260/A280 ratio of 1.92. Subsequent PCR amplification targeting the ITS region using the ITS1–ITS4 primer pair produced distinct amplicons of approximately 500–600 bp, consistent with the expected fragment size (Figure 3). BLAST analysis of the ITS sequence of isolate FC1 revealed the highest similarity (99.6%) to *F. keratoplasticum* and *F. solani*. Conidia of *F. solani* are typically larger, with macroconidia ranging from 35.0–57.0 × 5.40–6.00 µm and microconidia from 9.00–26.0 × 4.80–5.90 µm (Chehri et al., 2015; James et al., 2022). In contrast, the smaller conidial size of FC1 more closely matches that of *F. keratoplasticum* (Chehri et al., 2015). The combination of the morphological observations and ITS region sequencing strongly supports the identification of isolate FC1 as *F. keratoplasticum* (Genbank accession number: PZ273786).

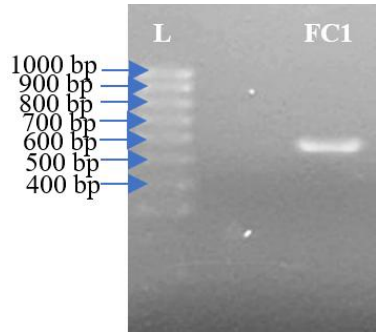

Figure 3. Agarose gel electrophoresis of ITS-PCR products from fungal isolate FC1 amplified with ITS1–ITS4 primers

Lane L: 100 bp Hyperladder Bioline; lane FC1: isolate FC1 showing a band of approximately 500–600 bp.

This study revealed that *Fusarium keratoplastocum* is a potential pathogen capable of causing severe damage to pico chrysanthemum. Consistent with our findings, previous studies have identified several *Fusarium* species, including *F. solani* (Liu et al., 2023a), *F. incarnatum* (Balamurugan et al., 2024), and *F. falciforme* (Thao et al., 2021), as major causal agents of wilt disease in chrysanthemum.

These findings suggest that the occurrence and prevalence of *Fusarium* spp. associated with wilt disease vary depending on host cultivars and geographical conditions. Therefore, understanding the diversity and distribution of these pathogens is essential to developing effective, targeted disease management strategies across different chrysanthemum varieties and growing regions.

3.2. Antifungal activity of *Bacillus* spp. against *F. keratoplasticum* FC1

All 41 bacterial isolates tested showed antifungal activity against *F. keratoplasticum* FC1 (Figure 4). The inhibition rate of mycelial growth ranged from 20% to 52.2%. Among these, isolate CB32 demonstrated the highest antagonistic ability with an inhibition rate of 52.2%, which was not statistically different from those of isolates CB3, CB10, CB31, CB35, and CB36.

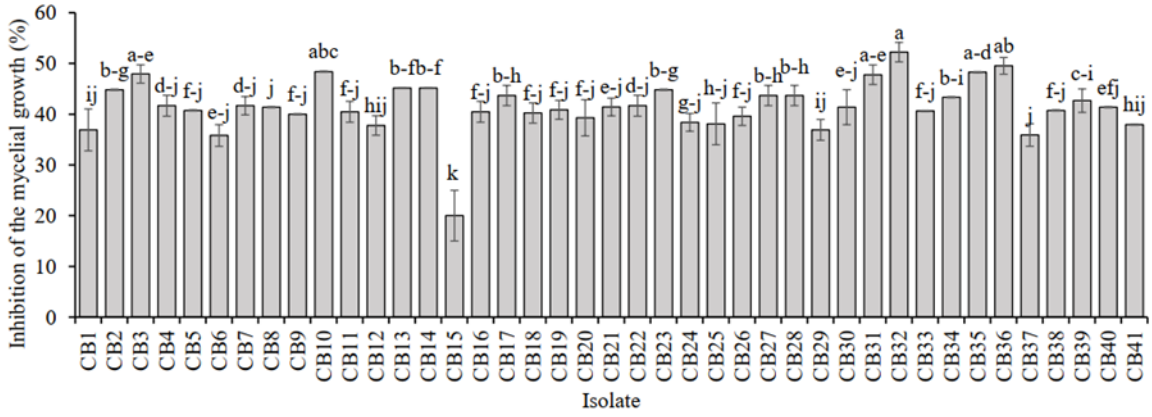

Figure 4. Antifungal activity of *Bacillus* isolates against *F. keratoplasticum* FC1 under in vitro conditions

Columns with the same letters are not significantly different according to Tukey's HSD test at the 5% significance level.

Previous studies have reported that members of *Bacillus* spp. are widely used as potential biocontrol agents with antifungal activity against *Fusarium*-induced wilt in various crops, including durum wheat (Zalila-Kolsi et al., 2016), potato (Liu et al., 2023b), chili (Iqbal et al., 2024), and cotton (Aslam et al., 2025). *Bacillus* spp. can effectively suppress pathogens through multiple mechanisms, including direct inhibition via competition for essential nutrients and the production of antifungal metabolites (Khan et al., 2017), as well as indirect mechanisms such as inducing systemic resistance in host plants (Khan et al., 2017; Chen et al., 2024).

In the present study, *Bacillus* isolates derived from cow dung exhibited strong antagonistic activity against *Fusarium keratoplasticum* causing wilt disease in pico chrysanthemum. These findings further highlight the biocontrol potential of *Bacillus* spp. in managing fungal diseases in both ornamental and agricultural crops. Notably, six isolates (CB3, CB10, CB31, CB32, CB35, and CB36) exhibited strong antagonistic activity and are promising candidates for developing biological control agents against Fusarium wilt in chrysanthemum.

3.3. Antifungal effects of volatile organic compounds and cell-free supernatants produced by strong antagonists against *F. keratoplasticum*

All six *Bacillus* isolates tested were capable of inhibiting both mycelial growth and spore germination of *F. keratoplasticum* isolate FC1 via volatile organic compounds. The inhibition rates of mycelial growth ranged from 40.7% to 60.3% (Figure 5a), while spore germination was inhibited at rates between 84.8% and 97.6% (Figure 5b). Among the isolates, CB32 and CB35 exhibited the strongest mycelial inhibition via VOCs, whereas CB3, CB10, CB35, and CB36 demonstrated the highest suppression of spore germination.

Similarly, the cell-free supernatants (CFS) derived from all six isolates showed inhibitory effects against isolate FC1. Inhibition of mycelial growth ranged from 24.4% to 63.2% (Figure 5c), and inhibition of spore germination ranged from 72.3% to 88.2% (Figure 5d). CFS from isolate CB10 exhibited the highest efficacy in suppressing mycelial growth, while the supernatants from isolates CB3, CB31, CB32, and CB36 were most effective in inhibiting spore germination. In addition, both CFS and VOCs from strong antagonists reduced the development of aerial mycelium and pigmentation in the fungal colonies.

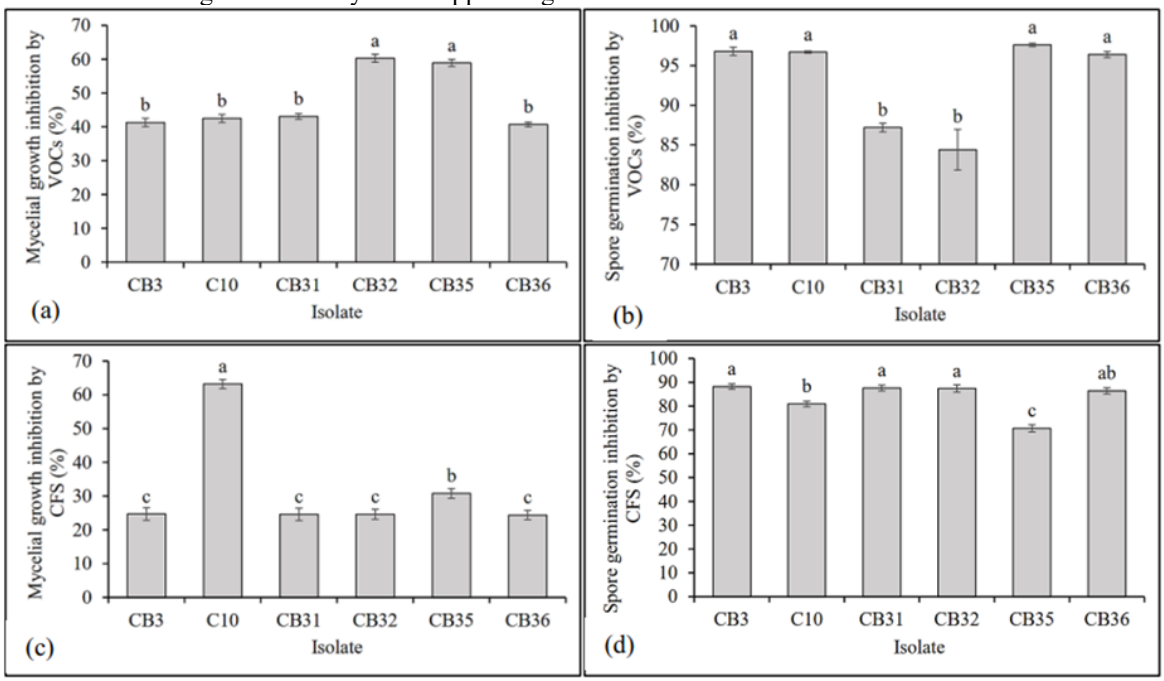

Figure 5. Mycelial growth and spore germination inhibition of *F. keratoplasticum* FC1 by VOCs and CFS derived from six antagonistic *Bacillus* isolates

Columns with the same letters are not significantly different according to Tukey's HSD test at the 5% significance level.

The antagonistic activity exhibited by the six selected isolates involves not only inhibition of mycelial growth but also interference with fungal development and morphogenesis. The observed multidimensional effects of both volatile organic compounds and cell-free supernatants suggest that their antifungal mechanisms are complex and potentially synergistic. The observations are in agreement with previous studies demonstrating the broad-spectrum antifungal activity of *Bacillus* spp. against a range of phytopathogens.

The antifungal capacity of *Bacillus* is largely attributed to the production of diverse bioactive metabolites, including hydrolytic enzymes and lipopeptides, which can degrade fungal cell walls and disrupt membrane integrity, thereby inhibiting pathogen growth and spread (Kim et al., 2019). In addition, siderophore production enables bacteria to sequester iron from the environment, creating conditions unfavorable for pathogen proliferation (Kim et al., 2019).

VOCs produced by *Bacillus* also play an important role in microbial interactions, functioning as signaling molecules in both intercellular and intracellular communication (Chandrasekaran et al., 2022). Moreover, these compounds have also been reported to inhibit numerous phytopathogenic fungi by inducing morphological abnormalities, including hyphal deformation, reduced conidial germination, and suppression of pigment biosynthesis (Chaves-López et al., 2015; Myo et al., 2019; Ghazala et al., 2022).

Taken together, these results indicate that the six *Bacillus* isolates are promising candidates for managing *Fusarium keratoplasticum*, the causal agent of wilt disease in pico chrysanthemum. The combined effects of live bacterial cells and their bioactive metabolites highlight their potential for

developing sustainable biocontrol strategies for ornamental crop protection. Future studies should evaluate the efficacy of these isolates under greenhouse and field conditions to comprehensively assess their performance under diverse environmental settings.

4. CONCLUSION

Fusarium keratoplasticum isolate FC1 was identified as the causal agent of wilt disease in pico chrysanthemum. Among the 41 *Bacillus* isolates evaluated, six isolates (CB3, CB10, CB31, CB32, CB35, and CB36) exhibited strong antifungal activity against FC1 through multiple mechanisms, including inhibition by live cells, volatile organic compounds, and cell-free supernatants. These findings suggest that the selected isolates are promising candidates for developing biological control strategies against *Fusarium keratoplasticum* in pico chrysanthemum.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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