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RESEARCH

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A novel biocontrol strain *Pseudomonas canadensis* FRPC18 against box blight (causative agent *Calonectria pseudonaviculata*)

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Abstract

Background Recent box blight epidemics caused by *Calonectria pseudonaviculata* (*Cps*) have created the need for finding eco-friendly management strategies, especially in natural habitats. In the present research, the biocontrol activity of 26 bacterial isolates against *Cps* was investigated under laboratory and greenhouse conditions in a completely randomized design ($n = 3$; $P < 0.05$).

Results Strains FRPC18, FRPF4, and FRPF6 showed the most efficiency in the inhibiting *Cps* colony by 72.4, 46.8, and 34.2% through a dual culture test, respectively. The superior strain, FRPC18, identified belonged to *Pseudomonas canadensis*. FRPF6 volatile and FRPC18 non-volatile antifungal compounds resulted in the highest inhibition rate of 27.67 and 59.43%, respectively. Strain FRPC18 could also produce the maximum amount of chitinase, significantly, while the highest amount of siderophores was detected in FRPF4. There were non-significant differences among the bacterial strains in producing protease and pectinase. In the greenhouse, FRPC18 reduced box blight severity by 30% and had non-statistically significant differences with Serenade® as a biofungicide ($P < 0.05$).

Conclusion The results of this study suggest that *P. canadensis* may be a new biocontrol agent for controlling box blight in disease hotspots during epidemics in boxwood forests.

Keywords Boxwood, Disease severity, Greenhouse, *Pseudomonas fluorescens*, Siderophore

Background

Box blight caused by *Calonectria pseudonaviculata* (*Cps*) is a serious foliar disease infecting the *Buxus hyrcana* (boxwood) nurseries and forests in Iran (Yoldashkhan et al. 2018). The boxwood habitats in Iran's northern forests (Hyrcanian) have recently declined by over 40,000 ha due to the disease (Yoldashkhan et al. 2018). So far, several countries worldwide are affected, i.e., New Zealand, the UK, Italy, Belgium, Germany, Croatia, Spain, Austria, Norway, Denmark, Georgia, the Czech Republic, Ireland, France, Canada, the Netherlands, the USA, Turkey, and Iran (Gehesquiere et al. 2013).

Common strategies to control box blight include integrating chemical and cultural practices such as applying synthetic fungicides, using tolerant plant cultivars, and maintaining sanitation (LaMondia 2015). However, synthetic fungicides threaten the environment and human health and are forbidden to apply in forests. So, it is crucial to find environmentally friendly alternatives to suppress disease epidemics in large ecosystems like the Hyrcanian forests. Some effective alternatives include utilizing biorationals and humic compounds (Samavat et al. 2020) and implementing antagonistic bacteria and fungi (Yang and Hong 2018).

Several beneficial rhizobacteria belonging to *Arthrobacter*, *Bacillus*, *Serratia*, *Enterobacter*, *Pantoea*, and *Pseudomonas* have been known as antagonists of various

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fungal phytopathogens (Alizadeh et al. 2020). *Pseudomonas* species have become attractive biocontrol agents among antagonistic bacteria due to their ability to produce various secondary antifungal metabolites, broad environment and host plant adaptation (Sharifazizi et al. 2017). Among *Pseudomonas* species, *P. fluorescens*, *P. protegens*, *P. chlororaphis*, *P. syringae, P aureofaciens*, *P. putida*, *P. aeruginosa*, and *P. canadensis* are known as potent biological control agents (Yang and Hong 2018).

Antagonistic bacteria can suppress the plant pathogens' growth and activity through several mechanisms, such as inducing systemic resistance in host plants, competing for nutrients, colonization, and producing toxins, siderophores, cell-degrading enzymes (CDEs), and antimicrobial metabolites (Dimkic et al. 2022). The main bioactive compounds for the biocontrol of plant pathogens by *Pseudomonas* species are siderophores, biosurfactants, phloroglucinols, antibiotics (phenazines, 2,4-diacetylphloroglucinol, pyoluteorins, pyrrolnitrins, cyclic lipopeptides, hydrogen cyanide), CDEs (proteases, chitinases, and glucanases), and volatile organic compounds (Bonaterra et al. 2022).

So far, only the effectiveness of *P. protegens*, *Burkholderia cepacia*, *Bacillus subtilis*, and *Trichoderma koningiopsis* in controlling *Cps* has been investigated (Samavat 2022). Furthermore, Serenade® is a biological fungicide containing *B. subtilis* strain QST713, and it has been suggested to be used as a foliar application to prevent box blight disease in urban green areas (Abbasi and Weselowski 2014). Hence, the research objectives were to screen and identify promising antagonistic bacterial strains against *Cps* and evaluate the antifungal effects of their volatile and non-volatile metabolites, the possibility of siderophores and CDEs production, and their potential as biocontrol agents for box blight disease under *vivo* conditions.

Methods

Bacterial strains

The present study evaluated 24 bacterial isolates previously obtained from the rhizosphere of *Populus* spp. in the natural habitats of Tehran and Alborz provinces, Iran, between April 2020 and April 2021 for their antagonistic activities. *P. fluorescens* strains FRPF4 and FRPF6 were also obtained from the Beneficial Microorganisms Collection of the Research Institute of Forests and Rangelands, Tehran, Iran. According to the previous studies (Samavat and Rahimifard 2021), these two strains were isolated from *Populus nigra* rhizosphere and were superior for siderophore production and inorganic phosphate solubilization. For short-term preservation, all bacterial isolates were stored in Luria–Bertani medium (LB; Merck, Germany) containing 15% glycerol at − 20 °C.

Fungal isolate

The *Cps* isolate Cy-08 was donated by the Plant Pathology Department, College of Agriculture and Natural Resources, Science and Research Branch, Islamic Azad University, Tehran, Iran (Khazaeli et al. 2015). It was identified based on molecular investigations through ITS4 and ITS113F species-specific primers, pathogenicity tests, and morphological characteristics by Khazaeli et al. (2015). Isolate Cy-08 was cultured and maintained on standard Potato Dextrose Agar (PDA; Merck, Germany).

Fungicides

Serenade® (Bayer Company, UK) and tebuconazole (Fungus Fighter Concentrate®; Bayer Company., UK) were used as biological and chemical controls, respectively, according to the instructions provided for each product. These two fungicides were selected based on the Bayer Company recommendations for box blight control. Serenade with a suspension concentrate formulation is a foliar biofungicide that contains 1015.1 g/L *B. subtilis* strain QST 713 (a minimum of 1.05×10^{12} CFU/L). Fungus Fighter Concentrate with a suspo-emulsion formulation contains 25 g/L tebuconazole as an active ingredient. It is an enhanced systemic fungicide in a high concentrated form.

Dual culture test

All 26 bacterial isolates were evaluated for their potential to limit the mycelial growth of isolate Cy-08 through a dual culture test, according to Keel et al. (1992), with some modifications. A mycelial disk (5 mm diam.) from a week-old culture of isolate Cy-08 was placed at the center of the Petri plate (7 cm diam.). The PDA Plates were then incubated at 25 ± 2 °C in the dark. After 48 h, four spots per plate from a 48-h culture of each bacterial strain were spotted 0.5 cm from the edge of the plate and incubated at the same conditions until the control colony entirely encircled the plate. Based on the following formula, the inhibition percentage was calculated (Korsten et al. 1995):

$$I = \frac{R1 - R2}{R1} \times 100$$

Where I: percentage of inhibition (%), R1: colony radius of *C. pseudonaviculata* which grows in the opposite direction to the bacterial strain, and R2: colony radius of *C. pseudonaviculata* which grows toward the bacteria.

Bacterial isolates that could considerably reduce the colony growth of isolate Cy-08 were selected to

be identified at the species level and evaluated under greenhouse conditions.

Identification of the bacterial strain

Morphological properties of the superior strain (FRPC18), including colony color, cell morphology, and motility, were investigated on Nutrient Agar (NA) medium using an optical microscope (CX-33, Olympus, Japan). Conventional Gram staining was carried out as described by Claus (1992). Physiological and biochemical identification of the bacterial isolates was carried out according to Bergey's Manual of Systematic Bacteriology (De Vos et al. 2009).

A DNA extraction kit (SinaClon BioScience Co., Iran) was used to extract the chromosomal DNA for identifying the superior strains based on 16S rRNA gene amplification. The extracted DNA quantity and quality were tested using spectrophotometry and 1% agarose gel electrophoresis.

The PCR amplification was done with universal primers 1492R (5′-TACGGTTACCTTGTTACGACT-3′) and 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) (Lane 1991) to a total of 20 µL reaction volume, containing 10 µL of PCR Master Mix (Amplicon, Denmark) with 0.4 mM of each dNTP, 0.25 µL of each primer (10 uM), 1.5 mM $MgCl_2$, 3 µL (~100 ng) of template DNA, and 0.2 U/ul amplicon Taq DNA polymerase. The temperature cycling conditions on an Eppendorf® Mastercycler Gradient, Germany, were as follows: initial denaturation at 94 °C for five min, followed by 35 cycles of denaturation at 94 °C for one min, annealing at 56 °C for 45 s, and primer extension at 72 °C for one min, followed by a final extension at 72 °C for five min. The amplified PCR products were visualized by 1% agarose gel electrophoresis in TAE (Tris–acetate-EDTA) buffer stained with DNA-safe stain under UV light. Subsequently, the purified amplicons (using an agarose gel extraction kit (SinaClon BioScience Co., Iran)) were sequenced at SinaClon BioScience Co., Iran. The quality, arrangement, alignment, and diversity of the obtained sequences were checked using Geneious Prime Software (2019). To identify each isolate's species, the 16S rRNA gene sequences were compared to the NCBI (National Center for Biotechnology Information) GenBank database using nucleotide BLAST software (Altschul et al. 1990). All the sequences were uploaded into the mentioned database, and the accession numbers were issued.

Neighbor-joining trees were constructed using MEGA4 (Molecular Evolutionary Genetics Analysis) software (Tamura et al. 2007). The reliability of the phylogenetic trees was evaluated through bootstrap analysis with 1000 resampling repetitions.

Antifungal metabolites against *Cps*

Non-volatile metabolites

This experiment was carried out according to Win et al. (2022) with some modifications. Bacterial strains (FRPC18, FRPF4, and FRPF6) were cultured on Nutrient Broth (NB) at 27 ± 2 °C in a shaker incubator at 150 for 48 h. The cultural filtrates were obtained by centrifuging at 8000 rpm for 15 min and passing through a sterile 0.2 µm PES syringe filter (Roth, Germany). A filter paper (Whatman) was then soaked in 4 mL of each bacterial filtrate and put in a 9-cm petri plate. A 5-mm disk of *Cps* isolate Cy-08 was put at the center of the plates. The plates were incubated at 25 ± 2 °C in the dark. Cy-08 colony diameter was measured using a digital caliper after 1 week. The fungal isolate grown on the plate containing NB medium without bacterial culture served as the control.

Volatile metabolites

The suppression of Cy-08 colony growth through the production of volatile compounds by the bacterial strains was tested based on Kamal et al. (2015). The inhibition percentage was calculated as described in 2.2.1.

Siderophores production

The ability of the three superior strains (FRPC18, FRPF4, and FRPF6) to secret siderophores was investigated using Chrome Azurol S (CAS) agar media, according to Alexander and Zuberer (1991). The appearance of yellow to orange zones around the bacterial colonies confirms that the strain can produce siderophores. The ratio of halo diameter to colony diameter was calculated.

Hydrolytic exo-enzymes

Strains FRPC18, FRPF4, and FRPF6 were tested for the secretion of three different CDEs such as protease (Maurhofer et al. 1995), pectinase (Soares et al. 1999), and chitinase (Carrim et al. 2006). The appearance of clear halo zones around the bacterial colony indicated that the bacterial strain could produce certain hydrolytic enzymes. The ratio of halo diameter to colony diameter was recorded as an induction of the level of each CDE produced by the bacteria.

Sporulation induction in *Cps*

Sporulation induction in *Cps* was conducted using Glucose Asparagine Agar (GAA) media (Dhingra and Sinclair 1995) as described by Alfenas et al. (2013).

Preparation of the fungal inoculum

According to Henricot et al. (2008), conidia suspension of *Cps* isolate Cy-08 was prepared by adding 10 mL of

sterilized distilled water plus a few drops of Tween 20 to the culture surface. The suspension was then diluted to 4×10^5 conidia/mL.

Preparation of the bacterial suspension

One loop from the 48-h culture of the bacterial strains in Nutrient Agar (NA; Merck, Germany) medium was added to Luria–Bertani (LB; Merck, Germany) broth and put on a shaker incubator at 150 rpm for 48 h at 27 ± 2 °C. Afterward, the bacterial population was adjusted to 1×10^9 CFU/ml at 600 nm. Then, 0.1% carboxymethyl cellulose (CMC) as an adhesive was mixed with the suspension under sterile conditions. Finally, the obtained mixture was used for the greenhouse trials (Samavat et al. 2014).

Greenhouse trials

The Iranian Research Institute of Plant Protection, Tehran, Iran, donated 2-year-old boxwood seedlings. The leaves and stems of the seedlings were first surface-sterilized with 10% ethyl alcohol. They were planted in 3800 cm^3 pots with loamy clay soil. All pots were then randomly placed in a growth chamber set under controlled conditions (90% relative humidity, 16 h light/8 h dark, and 25/23 °C day/night). Plants were incubated in the chamber for 3 days before the experiments began. The bacterial suspensions and fungal inoculum were prepared as above. For each treatment, three seedlings were randomly selected from four pots containing three boxwood seedlings.

As described by Yang and Hong (2018), with some modifications, a suspension of each selected bacterial strain was sprayed on the foliage of each boxwood seedling 14 days and 6 h before Cy-08 inoculation. As a negative control, the 0.1% carboxymethyl cellulose suspension was sprayed only onto the untreated plants. Afterward, 20 mL/plant of the fungal conidial suspension was sprayed onto boxwood foliage. Seedlings were then irrigated every other day and monitored for box blight symptoms. After 10 days, the disease severity percentage (DS%) was calculated by evaluating three randomly selected branches of each boxwood seedling and recording the portion of infected leaves (including fallen ones) to the total number of leaves per branch.

Data analysis

Data achieved from the present research are shown as the mean ± SD. All experiments were performed in a completely randomized design (CRD) with three replications per treatment. All greenhouse experiments were repeated for two independent trials. Duncan's multiple range test (DMRT) ($P < 0.05$) and analysis of variance (ANOVA) were conducted using IBM SPSS Statistics 22, Chicago, USA.

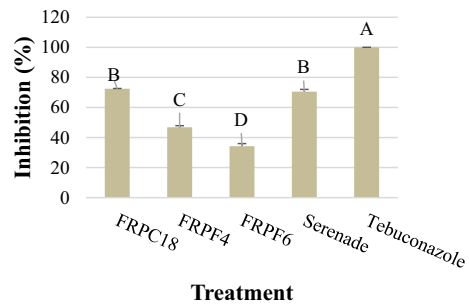

Fig. 1 Inhibition percentage of the *Cps* isolate Cy-08 mycelial growth by strains FRPC18, FRPF4, and FRPF6 compared to the biological and chemical controls ($P < 0.05$) ($n = 3$)

Table 1 Analysis of variance (ANOVA) for *Cps* isolate Cy-08 mycelial growth inhibition (%) by strains FRPC18, FRPF4, and FRPF6 ($P < 0.05$) ($n = 3$)

Source of variation	*DF*	Mean square	*F* value	*P* value
Treatment	4	1940.71	1944.27	0.000
Replication	2	2.48	2.49	0.145
Error	8	1.00		
Total	14			

Results

Dual culture test

Out of the 26 bacterial isolates tested, only the strains FRPC18, FRPF4, and FRPF6 showed an antibiosis mechanism in suppressing the hyphal growth of isolate Cy-08 (Fig. 1). FRPC18 was the most efficient against *Cps* with 72.4% inhibition. This strain had non-significant differences with Serenade® as a biological fungicide ($P < 0.05$). The chemical fungicide (tebuconazole) completely inhibited the fungal mycelial growth. The lowest inhibition rate of 34.2% was observed for strain FRPF6. Table 1 displays the results of the ANOVA analysis.

Identification of the bacterial strains

Results of the partial 16S rDNA sequence analysis demonstrated that the 16S rDNA sequences of strain FRPC18 are approximately 1464 bp in length and are closely related to *P. canadensis* (NR_156852.1) as shown in Fig. 2. The 16S rDNA sequences for FRPC18 were submitted to the NCBI, and the GenBank accession number of MW687114 was recorded. Hence, based on the morphological, physiological, biochemical, and molecular identification studies, strain FRPC18 was identified as *P. canadensis* (Table 2, Fig. 2).

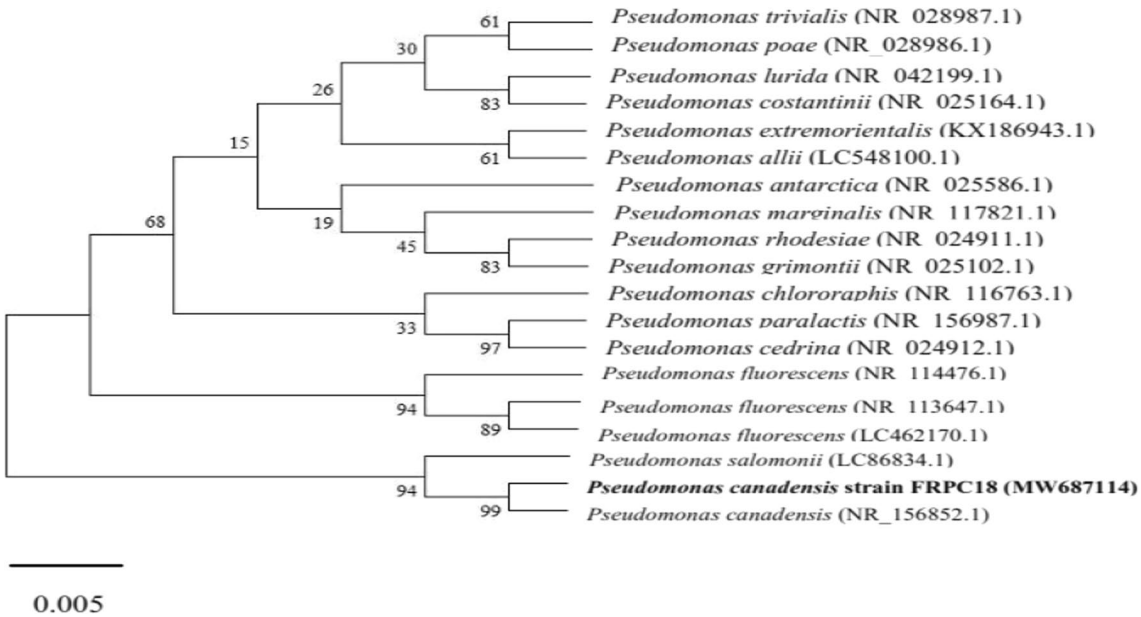

Fig. 2 Neighbor-joining phylogenetic tree based on 16S rRNA gene sequences of *Pseudomonas canadensis* strain FRPC18. The number of branches indicates a bootstrap value (> 50%) from 1000 replicates

Table 2 Morphological, physiological, and biochemical characteristics of strain FRPC18

Characteristic	FRPC18
Shape	Rod
Colony color	White
Motility (36 °C)	+
Gram staining	–
Nitrate reduction	–
Gelatin hydrolysis	+
Aesculin hydrolysis	–
Voges–Proskauer (V–P) test	–
Methyl Red (MR) reaction	–
Catalase	+
Oxidase	+
D-Glucose acid	+
Sucrose fermentation	+

"+" and "–" represent positive and negative reactions, respectively

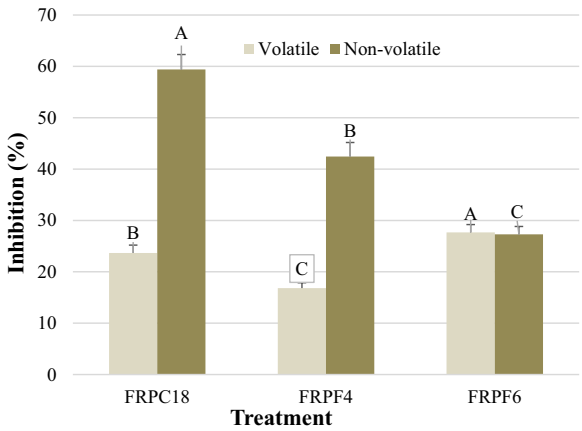

Fig. 3 Inhibition percentage of the *Cps* isolate Cy-08 mycelial growth by the volatile and non-volatile compounds produced by the strains FRPC18, FRPF4, and FRPF6 ($P < 0.05$) ($n = 3$)

Antifungal metabolites against *Cps*

Volatile and non-volatile compounds of the tested bacterial strains exhibited antifungal activities against *Cps*. Non-volatile and volatile metabolites of FRPC18 resulted in the inhibition rate of 59.43 ± 2.50% and 32.67 ± 1.94%, respectively (Fig. 3, Table 3).

Table 3 ANOVA for the volatile and non-volatile compounds produced by the superior bacterial strains ($P < 0.05$) ($n = 3$)

Source of variation	*DF*	Mean squares	
		Non-volatile	Volatile
Treatment	2	772.013**	90.028*
Replication	2	5.813	2.194
Error	4	6.207	1.778
Total	8		

* Significant at $P < 0.05$; **significant at $P < 0.01$

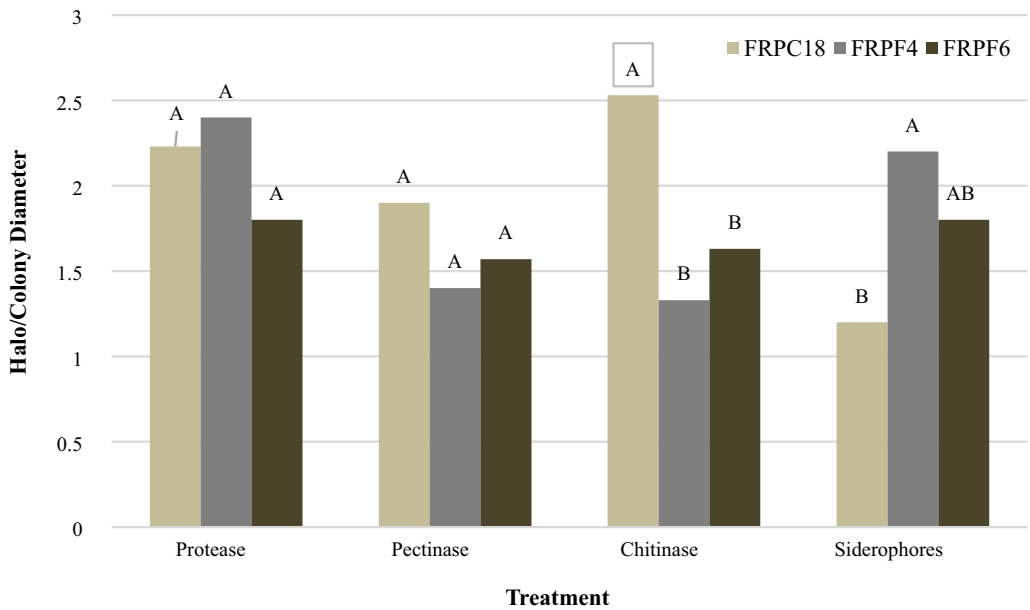

Fig. 4 The ratio of halo diameter to colony diameter as an induction of the level of CDEs and siderophores produced by the bacterial strains ($P<0.05$) ($n=3$)

Table 4 ANOVA for the CDEs and siderophores produced by the superior bacterial strains ($P<0.05$) ($n=3$)

Source of variation	*DF*	Mean squares			
		Protease	Pectinase	Chitinase	Siderophore
Treatment	2	0.28778^{ns}	0.19444^{ns}	1.17000**	0.76000*
Replication	2	0.05778	0.02111	0.06333	0.02333
Error	4	0.09278	0.05111	0.03833	0.06333
Total	8				

ns non-significant

* Significant at $P<0.05$; **significant at $P<0.01$

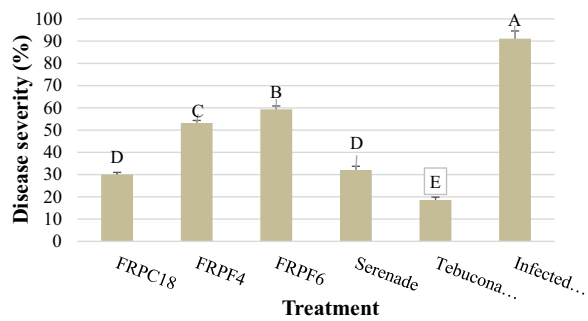

Fig. 5 Disease severity percentage of boxwood blight caused by *C. pseudonaviculata*, in seedlings treated with the suspensions of the superior bacterial isolates ($P<0.05$) ($n=3$)

Siderophores production

Based on the results, an orange halo was observed around the bacterial colonies on a CAS agar plate. So, it was found that the tested strains were capable of producing siderophores. The highest and lowest amounts of siderophores were produced by strain FRPF4 and FRPC18, respectively (Fig. 4, Table 4).

Hydrolytic exo-enzymes

As shown in Fig. 4, the three tested hydrolytic exo-enzymes (protease, pectinase, and chitinase) were produced by the superior strains. The highest amount of chitinase was detected in FRPC18. The bacterial strains did not show statistically significant differences in producing protease and pectinase with each other (Table 4).

Table 5 ANOVA for boxwood blight severity (%) caused by *C. pseudonaviculata*, in seedlings treated with the suspensions of the superior bacterial strains ($P<0.05$) ($n=3$)

Source of variation	*DF*	Mean square	*F* value	*P* value
Treatment	5	2071.92	515.43	0.000
Replication	2	0.88	0.22	0.807
Error	10	4.02		
Total	17			

Greenhouse trials

Based on the results, strain FRPC18 showed inhibitory effects as well as Serenade® ($P<0.05$) (Fig. 5). Tebuconazole as the chemical fungicide control, represented the

lowest box blight severity of 18.61% ± 1.23. As expected, the untreated control had the highest disease severity at 91.12% ± 2.23. The results of the ANOVA analysis are presented in Table 5.

Discussion

Although it seems that the best measure against box blight disease is to use chemical fungicides, it is an incompatible strategy with forest health. Therefore, the search for environmentally friendly alternatives to control the disease in natural habitats is required (LaMondia 2015). Among a variety of measures, biological control by beneficial microbes is an attractive alternative to the widespread use of synthetic chemicals.

Based on the results, it is feasible to control box blight by the application of some potent strains of *Pseudomonas* spp. Our in vitro findings revealed that three isolates (FRPC18, FRPF4, and FRPF6) out of 26 tested isolates could noticeably inhibit the mycelial growth of the pathogen at different rates in a dual culture assay. Accordingly, antibiosis may be considered as an effective mechanism against the pathogen. These results agree with those of Yang and Hong (2018), who reported that 153 strains out of 1547 tested bacterial strains inhibited *Cps* radial growth via possible mechanisms of antibiosis and or competition. In a dual culture test, Yang and Hong (2018) also displayed that *P. protegens* strain 34B6 decreased *Cps* mycelial growth by 66–77%. Samavat (2022) also revealed that four isolates (FRBP2, FRBS9, FRBS10, and FRBS15) out of 15 isolates belonging to *Bacillus* spp. have significantly suppressed *Cps* colony growth in a dual culture test. The highest (76.4%) and the lowest (26.5%) inhibition activities were related to *B. subtilis* FRBS9 and *B. pumilus* FRBP2, respectively. For the first time, Tambong et al. (2017) also isolated a novel species of *P. canadensis* (with type strain 2-92T). They reported its antagonistic activities against fungal pathogens like *Rhizoctonia solani* and *Gaeumannomyces tritici* under in vitro conditions.

Based on the results, volatile and non-volatile compounds produced by the three superior strains had antifungal effects on *Cps.* These results agreed with the data obtained by Wang et al. (2018). They reported that *B. subtilis* strain WXCDD105 filtrate significantly reduced mycelial growth of *Cladosporium fulvum* and *Botrytis cinerea*, the causal agents of tomato leaf mold and gray mold, respectively. Yang and Hong (2018) also found that superior antagonistic strains of *P. protegens* produced antifungal metabolites that decreased *Cps* infection and growth. Wang et al. (2021) also revealed that the volatile compounds produced by *P. fluorescens* ZX suppressed conidial germination and mycelial growth of *Penicillium italicum*. These bacterial volatiles and non-volatiles may contain various substances with antifungal properties produced in response to the target fungal pathogen. In this regard, Athukorala et al. (2010) reported that *P. chlororaphis* strain PA23 was able to produce both volatile (benzothiazole, nonanal, and 2-ethyl-1-hexanol) and non-volatile (phenazine and pyrrolnitrin) antibiotics against *Sclerotinia sclerotiorum.* Zhao et al. (2022) also stated that sulfur compounds and organic acids were the main active antifungal substances in *P. fluorescens* volatile compounds. Some other antifungal substances like benzoic acid ethyl ester and 3-methylbutanoic acid were also detected in *Pseudomonas* sp. volatiles.

It was also found that all the superior strains showed a positive CAS reaction with different rates. So, these strains were capable of producing siderophores. Samavat et al. (2014) also indicated that siderophore production was one of the antagonistic mechanisms of *P. fluorescens* and *P. aureofaciens* isolates in suppressing *R. solani*. Suresh et al.'s findings in (2022) also showed that *P. fluorescens* strain VSMKU3054, a potent biocontrol agent against *Ralstonia solanacearum*, formed a brown halo around its colony on CAS medium. Therefore, our results are in agreement with the other researchers'. Indeed, microbial siderophores control the plant pathogens by reducing ferric ions accessibility for the pathogen through ion chelation (Buyer and Leong 1986).

According to the results, protease, pectinase, and chitinase were produced by the superior strains at different rates. These mycolytic enzymes may play essential roles in the biocontrol ability of these bacteria against *Cps*. Protease destroys the fungal cell wall integrity by degrading the laminarin. Pectinase randomly hydrolyzes pectin α-1,4 glycosidic linkages in the fungal pathogens. Chitinase could also degrade C1 and C4 bonds in N-acetyl-glucosamine of chitin as a fungal structural polymer (Caiyun et al. 2021). Varatharaju et al. (2020) also introduced *Pseudomonas* sp. VSMKU4036 as an efficient biocontrol strain against several plant fungal pathogens like *R. solani*, *Macrophomina phaseolina*, *Scleotium rolfsii*, *Helminthusporium solani*, and *Fusarium oxysporum.* Based on their results, this strain could produce some CDEs such as protease, gelatinase, amylase, and pectinase under in vitro conditions. However, chitinase and cellulase were not produced by this strain. Kumar et al. (2022) also reported that *Pseudomonas stutzeri* produced chitinase and could inhibit the growth of *F. oxysporum* and *R. solani*. Accordingly, these findings supported obtained results.

Data from the greenhouse experiment indicated that foliar application of the superior strains significantly reduced box blight disease severity from 59.33 to 30%. Yang and Hong (2018) also demonstrated that *P. protegens* strain 14D5 reduced box blight disease severity by more than 50%, while strains 13D3 and 34B6 by

40 to 46% under greenhouse conditions. Additionally, Samavat (2022) reported that *B. subtilis* FRBS9 and *B. subtilis* FRBS10 caused the lowest disease severity and showed non-statistically significant difference with Serenade® ($P < 0.05$) under greenhouse conditions.

Conclusions

This research screened and identified *P. canadensis* strain FRPC18 as a promising biocontrol agent against *Cps*. It was found that this strain was capable of producing volatile and non-volatile antifungal compounds for biocontrol of box blight disease. As an antagonistic strain, it could produce several secondary metabolites, like antibiotics, siderophores, and CDEs (protease, pectinase, and chitinase). However, additional studies are warranted to elucidate its other mechanisms like inducing systemic resistance (ISR) and improving its biocontrol performance by appropriate formulations. Furthermore, as biocontrol properties are strain-dependent, more efficient strains belonging to *P. canadensis* and *P. fluorescens* should be studied. Finally, applying potent biocontrol agents would be an appropriate alternative to synthetic fungicides in the box blight hotspots in forest ecosystems during epidemics.

Abbreviations

CAS	Chrome azurol S
Cps	*Calonectria pseudonaviculata*
CDEs	Cell-degrading enzymes
ISR	Induced systemic resistance

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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