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Antagonistic Potential of Endophytic Bacteria from Shallot against *Colletotrichum gloeosporioides* Causing Anthracnose Disease

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Abstract: Anthracnose, a highly destructive disease in fruit crops, is primarily caused by the widespread fungal pathogen *Colletotrichum gloeosporioides* (Penz.) Penz. & Sacc. Currently, chemical fungicides are commonly used to effectively prevent anthracnose infection and spread. However, the excessive and prolonged use of these chemicals has led to the development of resistance in pathogenic fungi. Moreover, chemical fungicides contribute to environmental pollution due to their slow degradation and non-specific toxicity to non-target organisms. As a result, researchers have increasingly focused on developing and implementing alternative methods that are less reliant on fungicides and more environmentally friendly. Endophytic bacteria have been acknowledged as potential biocontrol agents for effectively managing plant diseases. They provide safer alternatives for controlling fungal infections, offering a more environmentally friendly approach compared to chemical fungicides. The objective of this study was to examine the inhibitory effects of fourteen isolates derived from shallot plants on *C. gloeosporioides* through an in vitro dual culture assay. The isolates were purified and identified as various species belonging to the Bacillus genus. The inhibitory activity was assessed by measuring the percentage inhibition of radial growth. The results indicated that *B. velezensis* exhibited the highest antagonistic activity ($P<0.05$), reducing radial growth by 90% compared to the non-treated control. This was followed by *B. subtilis* at 80%, *B. cereus* at 39%, *B. megaterium* at 38%, and *B. licheniformis* with the lowest inhibition rate at 36%. Furthermore, the production of antifungal volatiles significantly contributed to the antagonistic activity ($P<0.05$), particularly with *B. licheniformis* volatiles inhibiting the mycelial growth of *C. gloeosporioides*. The results of this study indicate that Bacillus species exhibit significant antifungal characteristics and have the potential to be used as biocontrol agents for effectively managing Anthracnose in a variety of crops.

Keywords: Antagonistic, Endophytic bacteria, *Colletotrichum gloeosporioides*, Anthracnose

1. Introduction

Anthracnose disease, caused by *Colletotrichum* spp., is a significant factor that can result in substantial economic losses and have a profound impact on crop production worldwide. It has a detrimental effect on both yield and quality, leading to a reduction of marketable output ranging from 10% to 80%. This makes anthracnose the most economically consequential disease globally (Gomes et al., 2021). Among various tropical fruit crops, anthracnose is recognized as the most destructive disease, with *Colletotrichum gloeosporioides* being the primary causal agent. Fruits such as papaya, mango, banana, dragon fruits, strawberries, as well as certain fruiting vegetables like chili or pepper, are highly susceptible to this disease (Zakaria, 2021). Anthracnose affects all parts of the plant at all stages of growth. It manifests as small and irregular yellow, brown, dark-brown, or black spots on leaves, while fruits exhibit small, water-soaked, sunken, circular spots that darken over time. Inflorescence may develop dark lesion spots, while stems may experience dark brown sunken elongated lesions accompanied by pinkish spore masses in humid conditions. Additionally, canker

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and dieback can be observed on stems. However, symptoms are most prominent on ripening fruits, during preharvest, flowering and fruiting stages, as well as the postharvest period (Md. Nasir Uddin et al., 2018).

Endophytic bacteria are beneficial bacteria that live inside the living plant without harming their hosts. They usually localized in the roots and may spread to other parts such as leaves, flowers, stems and cotyledons after entering the plants and continue to reside in the tissue system. Endophytic bacteria inoculated from plants have shown strong antimicrobial activities against plant pathogens and have the ability to produce bioactive molecules, volatile compounds, secondary metabolites (Wu et al., 2021). They often reported as potent biological control agents as well as promoting plant growth (Ribeiro et al., 2021). Therefore, biological control using these endophytes is recognised as a safer alternative to chemical agents, long known as the primary strategy for fungal disease control despite its exposure risks, health and environmental hazards, persistence of residues, and development of tolerance. Endophytic bacteria of the genus Bacillus have been described to produce antifungal lipopeptides such as bacillomycin, gentamycin, iturin, fengycin and surfactin that are effective in suppressing the growth of one of the most important phytopathogens, *Botrytis cinerea* (de Moura et al., 2021).

Shallot (*Allium cepa* var. aggregatum) is the member of the allium family, an important vegetables crop just like onions and has been used worldwide as food. It is also known to have a lot of health and medicinal benefits, high source of antioxidant, anti-inflammatory and antimicrobial properties. Malaysia is currently focusing on agricultural initiatives such as shallot cultivation and adaptations in lowland areas and it is possible that attention is being given to exploring the biodiversity of the microbiome that exist in the agricultural environment, including the soil and plant tissues as well as discovering a better understanding of the beneficial microorganisms that contribute to plant growth, productivity and overall plant health. Shallot contains carbohydrates, vitamins, phenolic compounds with valuable nutritional and bioactive properties (Sun et al., 2019). Alliospiroside found in shallot showed antifungal activity against a wide range of fungi (Tatiana et al., 2020). Therefore, it is an opportunity to conduct more research related to shallot and endophytic bacteria for its biocontrol potential to combat plant diseases that is more environmentally friendly. Endophytic bacteria associated with shallot are still scarcely mentioned in scientific literature regarding their effect on plant pathogenic fungi. Hence, the present study aimed to evaluate the antagonistic potential of the isolated endophytic bacteria from shallot against *C. gloeosporioides* causing anthracnose disease.

2. Material and methods

2.1 Isolation and Identification of Endophytic Bacteria

The endophytic bacteria utilized in this study were isolated from different parts of shallot plants. The plant tissues were cut into small pieces and subjected to surface sterilization. This process involved immersing the tissues in ethanol (75% *v/v*) for 30 seconds, followed by a one-minute immersion in a sodium hypochlorite solution (1%). After sterilization, the tissues were rinsed with sterile distilled water and dried on a sterile filter paper. Subsequently, the dried tissues were placed onto nutrient agar (NA) medium in petri dishes and incubated under laboratory conditions for 24-48 hours. Pure bacterial cultures were obtained through successive subcultivation in NA medium. To identify the isolates, a molecular technique was employed, involving the extraction of bacterial genomic DNA using a Geneaid kit (GBB100) and PCR amplification of the 16S rDNA gene. The PCR products were separated using agarose gel electrophoresis and visualized under UV light. The amplified products were then sequenced, and the resulting sequences were compared to the NCBI database through a blast analysis to determine the identity of each isolate.

2.2 In vitro Screening for Antagonistic Activity

For the dual culture assay, potato dextrose agar (PDA) medium was prepared and poured into petri dishes with a diameter of 9 cm. The tested endophytic bacteria were streaked onto the PDA medium, maintaining a distance of 2 cm from the edge of the plate. In the center of the same petri dish, a fungal plug measuring 5 mm from a pure culture of *C. gloeosporioides* was placed. Petri dishes containing PDA and water served as negative control while benomyl served as positive control. This setup was replicated four times, and all plates were then incubated at room temperature. The growth and zone of inhibition between the fungus and bacteria were observed over a period of 7 days. To calculate the percentage inhibition of radial growth (PIRG) a standard formula was used as follows;

$$\mathrm{PIRG} = \frac{\mathrm{R1} - \mathrm{R2}}{R1} \times 100 \quad (1)$$

Where:
R1 = Radial growth of the fungus in the control plate (in centimeters)
R2 = Radial growth of the fungus in the dual culture plates (in centimeters)

2.3 Detection of antifungal volatiles production

Sealed plate method was used to conduct this assay. A petri dish containing NA medium were inoculated by spreading the tested bacterial isolate onto the agar. Petri dishes containing NA and water served as negative control while benomyl served as positive control. After 24 hours of incubation at room temperature, PDA medium was prepared in a separate petri dish and a fungal plug of *C. gloeosporioides* measuring 5 mm in diameter was placed at the center of the plate. The agar plate was then inverted and positioned over the bacterial culture. To ensure a sealed environment, the two plates were secured together using parafilm. The combined plates were subsequently incubated to allow for fungal growth. After 7 days of incubation, the diameter of the fungal mycelium was measured.

2.4 Experimental Design and Statistical Analysis

In both assays, the treatments were organized in a completely randomized design (CRD) with four replicates. All the collected data were subjected to analysis of variance (ANOVA). Significant differences between means were determined using the Least Significant Difference (LSD) test, with a significance level of $P<0.05$. The statistical analysis was performed using SAS software, specifically version 9.4.

3. Results and Discussion

Fourteen endophytic bacteria were isolated and identified (Table 1). Based on the rDNA sequencing analysis, the most frequently identified species among the isolates were *Bacillus subtilis* and *B. velezensis*, with 5 and 4 isolates, respectively. Following them were *B. cereus* with 2 isolates, *B. megaterium* with 2 isolates, and *B. licheniformis* with 1 isolate. Bacillus species have frequently considered as most abundant endophytic microorganism found in the rhizosphere of several plant species among other common species such as Pseudomonas and Burkholderia (Bolivar et al., 2021). *Bacillus subtilis*, *B. velezensis*, *B. licheniformis*, *B. cereus*, and *B. megaterium* have been widely acknowledged and reported as plant growth-promoting rhizobacteria (PGPR) with potential benefits in promoting plant growth and development. Additionally, these species have shown promise as biological control agents for managing plant diseases. Several studieshave recognized and highlighted the abilities of these Bacillus species in terms of their plant growth promotion and disease control capabilities (Pengfei et al., 2020; Hafiz et al., 2018).

Table 1: Identification of endophytic bacteria associated with shallot (*Allium cepa* var. aggregatum) according to 16S rDNA region of gene sequence.

Isolate No.	Bacillus Species Identity	Sequence Similarity (%)	GenBank Accession No.
1	*B. subtilis*	99	NR027752.1
2	*B. velezensis*	100	NR075005.2
3	*B. subtilis*	100	NR027752.1
4	*B. licheniformis*	100	NR118996.1
5	*B. subtilis*	100	NR027752.1
6	*B. cereus*	100	NR115714.1
7	*B. cereus*	100	NR115714.1
8	*B. megaterium*	100	NR117473.1
9	*B. velezensis*	100	NR075005.2
10	*B. velezensis*	100	NR075005.2
11	*B. subtilis*	100	NR027752.1
12	*B.megaterium*	99	NR117473.1
13	*B. velezensis*	99	NR075005.2
14	*B. subtilis*	100	NR027752.1

All the species tested in the dual culture assay exhibited antifungal activity and effectively inhibited the radial growth of *C. gloeosporioides* in vitro (P<0.05) as shown in Fig. 1. Among the tested species, *B. velezensis* displayed the highest percentage inhibition of radial growth (PIRG), with Isolate 9 at 90%, Isolate 13 at 89.25%, Isolate 10 at 86.25%, and Isolate 2 at 83.5%. *B. subtilis* also demonstrated significant inhibition, with Isolate 14 at 80%, Isolate 3 at 73%, Isolate 5 at 61.75%, and Isolate 11 at 59.75%. *B. cereus*, *B. licheniformis* (specifically Isolate 4), and *B. megaterium* (specifically Isolate 8 and Isolate 12) showed moderate levels of inhibition compared to the control. Previous studies have reported the potent antimicrobial activity of Bacillus species against various plant pathogens. *B. velezensis*, in particular, has been shown to have significant effects against pathogens such as from the genus Botrytis, Fusarium, Magnaporthe and Ralstonia (Tae et al., 2022). This antimicrobial activity is attributed to the presence of bioactive compounds or secondary metabolites produced by Bacillus species, such as surfactin, iturin, and fengycin (Munakata et al., 2022). These lipopeptides target cell membranes, leading to structural changes and cell destruction.

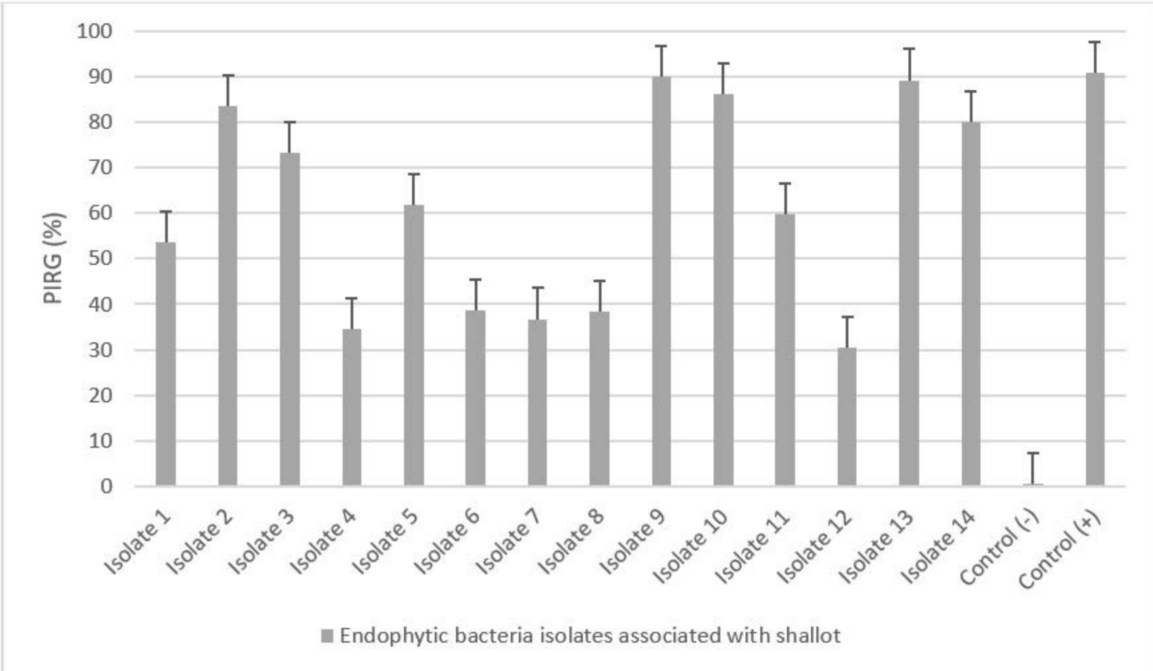

Fig. 1: Antagonistic activity of endophytic bacteria associated with shallot (*Allium cepa* var. aggregatum) on percentage inhibition of radial growth (PIRG) of *C. gloeosporioides* after 7 days of incubation. Vertical bars indicate standard error of means.

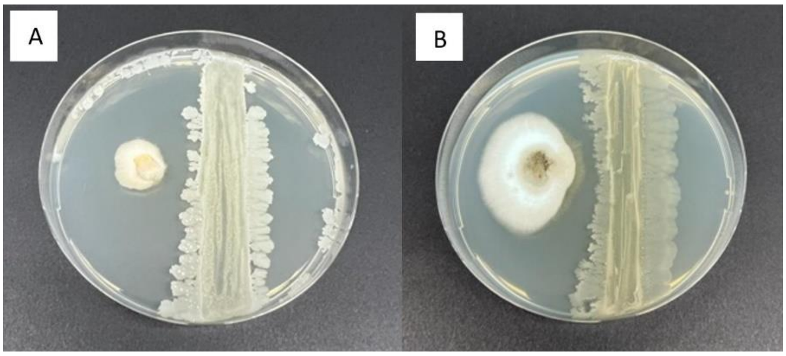

Fig. 2: Inhibition zone of *C. gloeosporioides* with (A) *B. velezensis* and (B) *B. subtilis* on dual culture assay plates after 7 days of incubation

The results shown in Fig. 2 indicate that all isolated endophytic bacteria were producing antifungal volatiles that significantly inhibit the mycelial growth of *C. gloeosporioides* at P <0.05. Antifungal activities of the *Bacillus* spp. volatiles varied for each species as determined by the sealed plate method. *B. licheniformis* (Isolate 4) volatiles showed the best reduction in mycelial growth of *C. gloeosporioides* followed by *B. velezensis* (Isolate 2 and Isolate 9) and *B. subtilis* (Isolate 14) while other isolates showed a moderate antifungal volatiles activities as compared to control. This is on the contrary of the antagonistic activity of *B. licheniformis* (Isolate 4) in the dual culture assay. The production of antifungal volatiles by tested endophytic *Bacillus* spp. suggested that those species have more than one mode of action in their antagonistic activities. According to Tiwari et al. (2021), Bacillus species often exhibit a range of beneficial interactions with their environment, including hyperparasitism, predation, stimulation of plant defense mechanisms, competition for resources, antibiosis, and the production of various secondary metabolites. These secondary metabolites include bacteriocins, lipopeptides, polyketides, and siderophores, which contribute to their antimicrobial properties. In a study conducted by Kai (2020), it was reported that endophytic bacteria from the genus Bacillus produce a wide array of volatile secondary metabolites. A total of 231 volatile compounds were identified, primarily consisting of hydrocarbons, ketones, alcohols, aldehydes, esters, acids, aromatics, as well as sulfur and nitrogen-containing compounds. These volatile metabolites contribute to the diverse biological activities exhibited by Bacillus species.

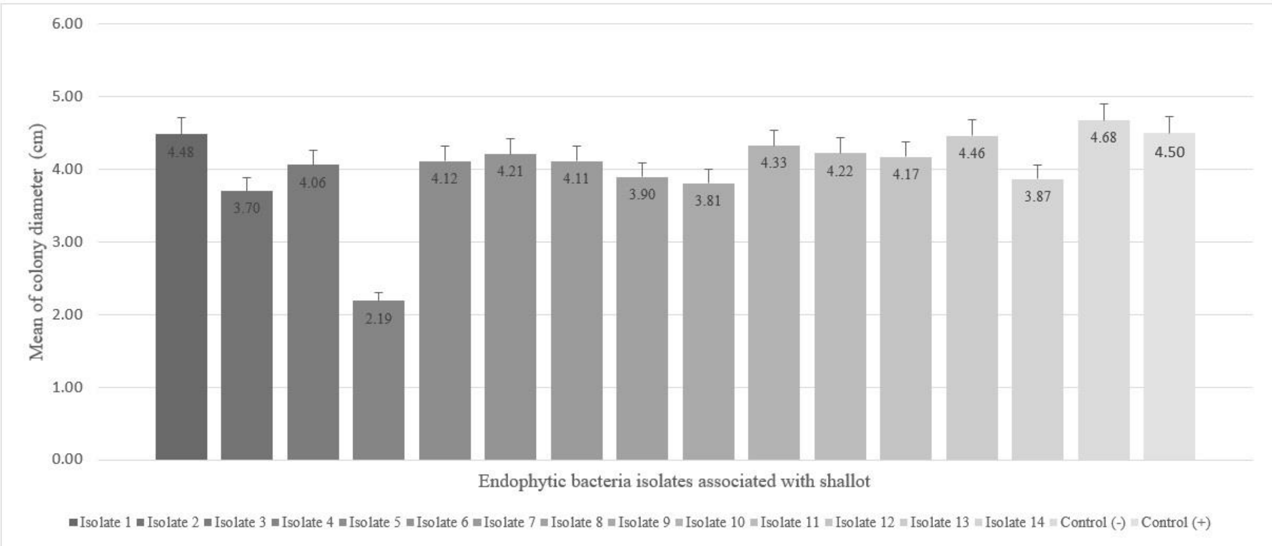

Fig. 3: Mycelial growth of *Colletotrichum gloeoporioides* in the presence and absence of adjacent endophytic bacteria associated with shallot (*Allium cepa* var. aggregatum). Vertical bars indicate standard error of means.

4. Conclusion

Based on our study, the endophytic bacteria isolated from shallot plants (*Allium cepa* var. aggregatum) mainly belong to the Bacillus genus, specifically identified as *Bacillus subtilis*, *B. velezensis*, *B. licheniformis*, *B. cereus*, and *B. megaterium*. All of these Bacillus species exhibited significant antagonistic activity against *Colletotrichum gloeosporioides*, as determined by their percentage inhibition of radial growth and the production of antifungal volatiles. Therefore, these isolated and identified endophytic bacteria hold potential as biocontrol agents for managing anthracnose caused by *C. gloeosporioides* in various crops. Further efficacy studies are currently being conducted to confirm their antifungal activity under field conditions.

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