In vitro and *in vivo* biological control of *Alternaria alternata* fungus by *Bacillus* spp. in Citrus fruit

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Abstract

Alternaria diseases pose a significant threat to the citrus industry worldwide. They are caused mainly by *Alternaria alternata* (Fr.) Keissl, and considered to be one of the prevalent diseases in Morocco, affecting both the quantity and quality of citrus fruit. The use of fungicides to manage this disease poses adverse effects on human health and the environment, as well as the potential development of new resistant strains. In response, biological control methods have gained attention as an alternative to chemical treatments. In this context, this study aims to investigate the antagonistic potential of three strains of *Bacillus* spp. (O1, O2, O3) against two strains of *Alternaria alternata* (Alt1 and Alt2), and compare their efficacy with two chemical fungicides: Imazalil (IMZ) and Azoxystrobin (AZT). *In vitro* experiments demonstrated significant inhibition of radial growth by all three bacterial strains against both *A. alternata* strains. The *in vitro* mycelial growth inhibition was particularly notable against the first strain, reaching approximately 60%, compared to 25% for the second. *In vivo* trials involving artificial inoculation of 'Valencia late' orange fruits with both Alternaria strains showed that *Bacillus* spp. effectively reduced disease development. *Bacillus* sp. O3 exhibited the highest inhibition percentage at 43%, with a highly significant difference observed compared to the control. As for the fungicides, Imazalil exhibited complete *in vitro* inhibition of *A. alternata* (100%) at 0.01 ppm, whereas the applied concentrations of azoxystrobin (250 ppm, 450 ppm, and 1000 ppm) notably reduced disease severity in inoculated citrus fruits by up to 55%. The assayed strains of *Bacillus* spp. displayed commendable efficacy in controlling the pathogen, yielding competitive outcomes similar to the effectiveness of the two approved fungicides.

Keywords: *Alternaria alternata,* citrus fruit*, Bacillus* spp.*,* fungicides

INTRODUCTION

Citrus fruit production has experienced significant growth worldwide, covering over 7.4 million hectares with an annual output surpassing 140 million metric tons. This industry is predominantly led by major producing countries such as Australia, Egypt, South Africa, Turkey, Portugal, USA and Morocco (Chen *et al*., 2019). In Morocco, the citrus fruit sector plays a vital socio-economic role, particularly in regions like Souss Massa, Gharb, Moulouya, Tadla and Haouz. Collectively, these areas contribute to over 93% of the national citrus cultivation, covering approximately 126,600 hectares and generating an average annual production of 2.6 million tons (MarocCitrus, 2023).

Despite playing a crucial role in Morocco's economy, the citrus sector faces various constraints, including the challenge posed by diseases, among which Alternaria rot. This disease is recognized as a latent infection that becomes active under suitable conditions, especially in the post-harvest period. In the orchard, elevated infection levels by *Alternaria* spp. can lead to premature color change and excessive fruit drop before harvest (Adaskaveg and Förster, 2014). As described by Akimitsu *et al.* (2003), Alternaria rot can affect all citrus species.

Two major diseases caused by Alternaria species on citrus fruits are Alternaria Black Rot (ABR) and Alternaria Brown Spot (ABS). In Morocco, these diseases has emerged as a significant fungal disease causing notable damage (Zelmat *et al*., 2021). ABS has also been reported in countries such as Peru (Marín *et al*., 2006), Iran (Kakvan *et al*., 2012), Italy (Aiello *et al*., 2020) and Greece (Elena, 2006), causing significant losses either pre- or post-harvest. Indeed, *A. alternata* and *A. arborescens* are the main species associated with citrus infections (Aiello *et al*., 2020; Zelmat *et al*., 2021). Aiello *et al*. (2020) identified *A. alternata* as the causal agent responsible for brown spot occurrences on various sweet orange and lemon cultivars in Italy. Therefore, these authors noted the occasional association of *A. arborescens* with brown spot on *Citrus sinensis*.

Globally, chemical products have been widely used to manage Alternaria rot. Fungicide treatments, including Quinone outside inhibitors (QoI) and demethylation inhibitors (DMI), have shown success in controlling ABS in Florida (Dewdney, 2020). However, several critical factors are limiting fungicide applications due to their hazardous effects. Additionally, the species of the Alternaria genus can develop resistance to these chemicals (Chitolina *et al*., 2021). In response to these challenges, alternative methods have been widely explored for combating *A. alternata*, including the use of biocontrol agents and resistant cultivars (Zelmat *et al*., 2022). *Bacillus* spp. gained recognition as highly effective biocontrol agents against *A. alternata* in citrus, as reported by Ferreira *et al*. (2020). Specifically, *Bacillus cereus* TV 85D has demonstrated remarkable potential in suppressing the growth of *A. alternata*. Furthermore,

the effective control of Alternaria brown rot (ABR) has been successfully achieved through the application of *B. amyloliquefaciens* on Valencia oranges (*Citrus sinensis* cv. Valencia), as demonstrated by Arrebola *et al.* (2010). This study investigates the efficacy of specific bacterial antagonists (O1, O2, and O3), isolated at IAV of Rabat, as biological control agents against Alternaria rot caused by *A. alternata*. Additionally, the study evaluates the effectiveness of two active substances, IMZ and AZX, against this fungus.

MATERIALS AND METHODS

Evaluation of the effectiveness of bacterial antagonists against *Alternaria alternata*

Origin of bacterial and fungal isolates

Three strains of bacteria belonging to the genus *Bacillus* spp., referenced as O1, O2, and O3, were used in this study to assess their antagonistic potential effect against two strains of *A. alternata* isolated from citrus fruits. These bacterial strains were isolated at the phytopathology laboratory of IAV Hassan II in Rabat, and they have previously demonstrated antagonistic activity against certain phytopathogenic fungi in prior research studies. *A. alternata* isolates were provided by the Phytopathology and postharvest quality laboratory at National Institute of Agricultural Research INRA of Kenitra.

Preparation of fungal suspensions

Conidial suspensions were prepared from pure cultures of *A. alternata* incubated at 25°C for 7 days on Potato Dextrose Agar (PDA) medium. Colonies were submerged with 10 ml of 0.05% sterilized Tween 20-distilled water. Then, suspensions were filtered using a sterile funnel through sterile rock wool to remove mycelial fragments. Subsequently, concentrations were adjusted to 10^5 conidia/ml using a hemocytometer technique.

Preparation of bacterial suspension

For bacterial inoculum, flasks containing 30 ml of LB liquid medium were sterilized, inoculated with each one of the bacterial strains, and incubated under continuous agitation at 25°C in darkness. After 48 hours, the culture was distributed into sterile tubes and centrifuged at 3500 rpm for 15 minutes. Then, the supernatant was discarded, and the pellet was resuspended in sterile physiological water. The concentration was adjusted to OD_{600nm} = 0.8 using a spectrophotometer (Islam *et al.*, 2019).

In vitro direct confrontation assays

To evaluate the antagonistic potential of bacterial isolates against *A. alternata*, a direct confrontation co-culture technique was employed, following the methodology outlined by Kurniawan *et al.* (2018). Mycelial discs, measuring 5 mm in diameter and obtained from the periphery of 7-day-old *A. alternata* colonies, were carefully placed onto PDA medium. Subsequently, antagonists were strategically positioned diametrically opposite to the mycelial discs, maintaining a separation of 2 cm. The application of antagonists involved depositing 20 µl of bacterial suspension. Controls were established using pure cultures of *A. alternata* without antagonists, following the protocol. Radial mycelial growth of the pathogen was measured after 3, 5, and 7 days of incubation at 25°C to quantify the inhibition rate exerted by the antagonists.

In vivo assays

All *in vivo* experiments were conducted as curative treatments using mature and healthy fruits of the 'Valencia Late' variety. Initially, the fruits underwent a comprehensive disinfection process with 10% sodium hypochlorite for 5 minutes. Subsequently, they were rinsed twice in sterile distilled water and allowed to air dry on sterile absorbent paper for an hour under aseptic conditions within a laminar flow hood.

Then, using a micropipette, 50 µl of a pre-prepared *A. alternata* spore suspension adjusted to 10^5 spores/ml was applied to the wound sites. The inoculated fruits were individually housed in plastic containers containing sterile filter papers moistened with 3 ml of sterile distilled water and were subsequently incubated at 25°C for a period of 10 days. After an initial 24-hour incubation period, a 20 µl volume of the previously prepared bacterial suspension was introduced to the inoculated wounds, constituting the curative treatment. Fruits inoculated solely with the pathogen were employed as control samples. The experiment was repeated three times for each antagonist. Measurements of the lesions were then taken using a graduated ruler, and the percentage inhibition of fungal growth was estimated following the previously described methodology.

Evaluation of the effectiveness of the active ingredients against *Alternaria alternata*

Fungicides tested

In this experiment, two fungicides were tested against *A. alternata* (Table 1).

Table 1: Active Ingredients Tested in this Study

Active Ingredient	Chemical Family	Active Ingredient Content
Azoxystrobine	Strobilurines	250 g/l
Imazalil	Imidazole	500 g/l

In vitro assays

Fungicide solution preparation

To evaluate the efficacy of fungicides under controlled conditions, concentrations ranging from 0.001 to 100 ppm were prepared from a stock solution. These precise volumes were then carefully dispensed into known quantities of sterile PDA medium to achieve the desired concentrations.

Culture medium preparation

The process of preparing Potato Dextrose Agar (PDA) culture medium supplemented with fungicides begins with the precise incorporation of the fungicides at desired concentrations. This involves adding fungicide solutions to sterilized culture medium while maintaining it in a state of supercooling at 40-45°C to prevent degradation of the fungicides due to heat. Continuous agitation ensures thorough mixing of the components. Subse-

quently, the culture media amended with fungicides or controls are aseptically dispensed into Petri dishes at a volume of 20 ml per dish and allowed to incubate for 24 hours at room temperature in the laboratory to minimize the risk of contamination.

For each concentration of fungicide, four Petri dishes were inoculated with a 5 mm diameter explant taken from the margin of a 7-day-old culture. Subsequently, these dishes were incubated at 25°C in darkness. Control samples were prepared under identical conditions but without the inclusion of fungicides. The concentrations tested for imazalil were [0, 0.01, 0.05, 0.1, 0.2, and 0.5 ppm], and for azoxystrobin, the concentrations tested were [0, 0.01, 0.1, 1, 10, and 100 ppm].

In vivo assays

The fruits underwent disinfection with 10% sodium hypochlorite for 5 minutes, followed by double rinsing in sterile distilled water. Subsequently, they were placed on sterile absorbent paper for 1 hour to facilitate drying. Once dried, the fruits were wounded at two equidistant points on the equatorial surface (3 mm deep). Using a micropipette, 50 µl of a spore suspension previously prepared and adjusted to 10^5 spores/ml was deposited onto the wounds. The fruits were then placed in plastic boxes containing sterile filter papers moistened with 3 ml of sterile distilled water and incubated at 25 °C. After 24 hours of incubation at 25°C, the wounds were treated with a volume of 100 µl of the fungicide solution.

Assessment method

The inhibition rate imposed by the antagonistic bacteria was calculated using the standard formula:

$$\% \text{ inhibition} = [(T0-T)/T0] \times 100$$

Where:

T0 (mm) is the diameter of fungal growth in the control.

T (mm) is the diameter of fungal growth in the presence of the antagonist.

Statistical Analysis

Statistical analyses were conducted using MINITAB software. The outcomes of this study were then subjected to one-factor analysis of variance (ANOVA). The comparison of mean values was executed through Tukey's test.

RESULTS

In vitro assessment of *Bacillus* spp. antagonists' effectiveness against *Alternaria alternata*

The *in vitro* findings of the assessment of *Bacillus* spp. antifungal activity against *Alternaria alternata* were illustrated in the Figure 1. The inhibition rates achieved by each bacteria strain against the two isolates of *A. alternata* (Alt1 and Alt2) were performed over varying durations. After seven days, the three bacteria strains O1, O2, and O3 reduced the *A. alternata* mycelium growth revealing an inhibitions rates ranged from 46% to 56% (*A. alternata* strain Alt1). Consequently, *Bacillus* spp. demonstrated a notably higher level of inhibition against *A. alternata* strain Alt1 (p=0.000).

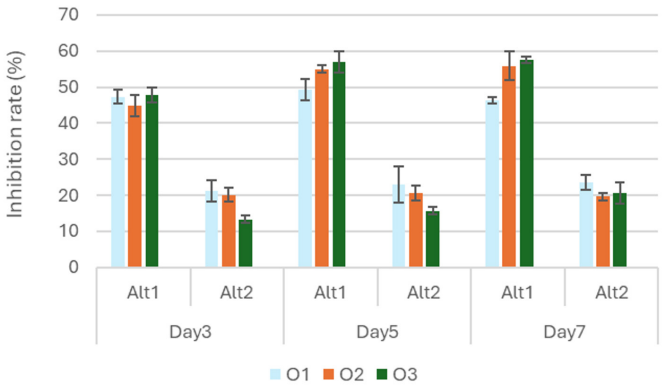

Figure 1: In vitro inhibition rates of Bacillus spp. against Alternaria alternata

In vivo assessment of *Bacillus* spp. antagonists' effectiveness against *Alternaria alternata*

Figure 2 illustrates the effect of *Bacillus* spp. on lesion diameter on 'Valencia late' oranges on the 10th day after artificial inoculation by both Alternaria strains Alt1 and Alt2, respectively. For the primary strain, the analysis of variance indicated a highly significant difference between the treatments (p=0.004), and Tukey's pairwise comparison highlighted that all bacterial treatments significantly deviated from the control. Regarding the second Alternaria strain, the analysis of variance also recognized a highly significant difference between the treatments (p=0.003). The *Bacillus* sp. O1 exhibited the highest inhibition percentage at 37%, followed by O2 (21%) and O3 (7%). Figure 3 provide a visual representation of the development inhibition of *A. alternata* on citrus fruits by the *Bacillus* spp.

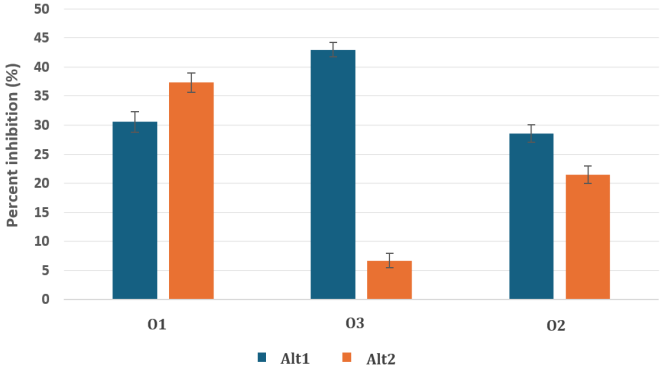

Figure 2: In vivo inhibition rates of Bacillus spp. against Alternaria alternata

Figure 3: In vivo inhibitory effect of bacillus spp. on the A. alternata development

In vitro assessment of fungicides effectiveness against *Alternaria alternata*

The findings of the *in vitro* assessment of azoxystrobin's impact on *A. alternata* were presented in Figure 4. Azoxystrobin was evaluated at five different concentrations: 0.01, 0.1, 1, 10, and 100 ppm against *A. alternata* strain Alt2. It was observed that azoxystrobin had a suppressive effect on the mycelial growth of the fungus, with inhibition ranging from 25% to 35%. In comparison to the control group, all four concentrations showed a significant effect (P=0.000). Moreover, there were no significant differences detected among the tested concentrations (p=0.37).

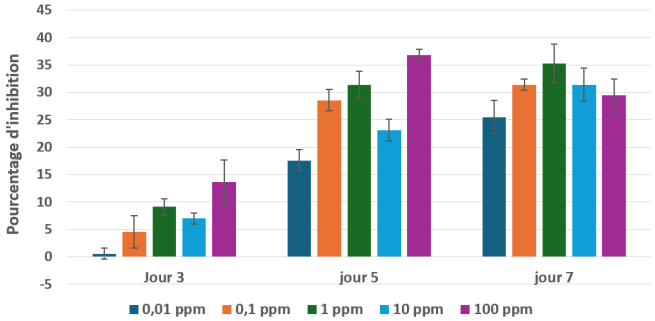

Figure 4: In vitro effect of the active substance 'Azoxystrobin' on the mycelial growth of Alternaria alternata

For the Imazalil, Figures 5 and 6 below illustrates the percentage of inhibition at different test rates. The analysis of variance revealing a notably significant difference, a pairwise Tukey analysis revealed three distinct groups with significant differences: (T and 0.01 ppm), (0.05; 0.1 ppm), and (0.1; 0.2 ppm). In fact, the 0.01 ppm dose did not show significant inhibition, whereas doses of 0.1 and 0.2 ppm demonstrated the highest potency, with inhibition percentages of 48.0% and 59.1%, respectively. The 0.5 ppm dose resulted in complete inhibition, reaching 100%.

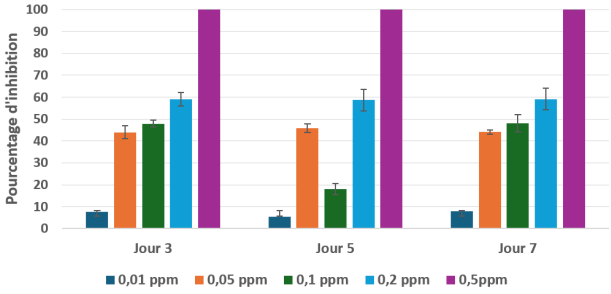

Figure 5: In vitro effect of the active substance 'Imazalil' on the mycelial growth of Alternaria alternata

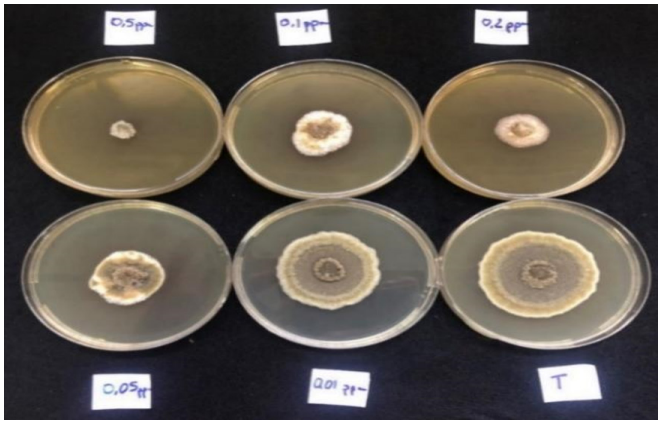

Figure 6: In vitro inhibitory effect of various doses of Imazalil on the mycelial growth of Alternaria alternata

In vivo assessment of fungicides effectiveness against *Alternaria alternata*

The *in vivo* trials were conducted to assess the effectiveness of Imazalil and Azoxystrobin in mitigating *Alternaria alternata* development on oranges using three applied concentrations. The results obtained are presented in Figures 7 and 8. The analysis of variance indicated no significant difference compared to the control for imazalil (p=0.890). In contrast, for azoxystrobin, the analysis of variance revealed a highly significant difference (p=0.000) when compared to the control. Remarkably, the inhibition of fungal growth (manifested as lesions on fruit) was substantial, ranging between 45% and 55%. A pairwise Tukey analysis further delineated two significantly distinct groups: the control on one hand, and on the other hand, the tested doses of azoxystrobin.

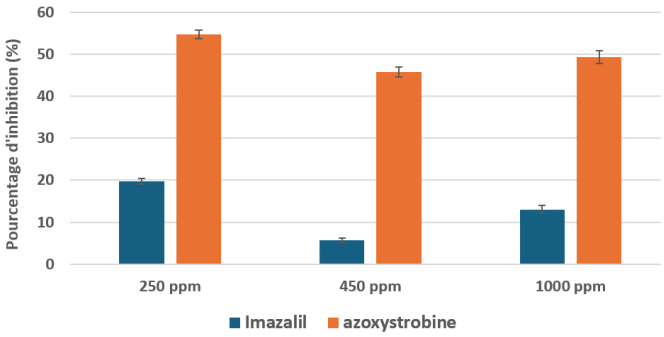

Figure 7: In vivo effect of the fungicides on the Alternaria alternata development

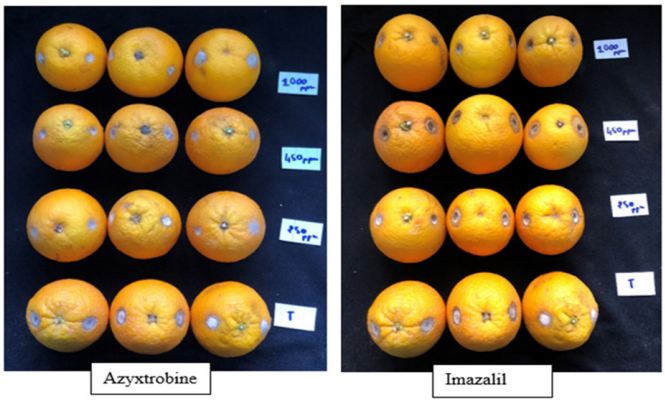

Figure 8: In vivo inhibitory effect of fungicides on Alternaria alternata development

DISCUSSION

Efforts are underway to minimize environmental impact and decrease chemical exposure for the population through the exploration of eco-friendly approaches. Biological control, utilizing microorganisms, emerges as a crucial option. It offers benefits such as absence of toxic residues, environmental compatibility, safe application, easy delivery, and cost-effectiveness. Various biocontrol methods have been devised for both pre- and post-harvest phases. Bacillus bacteria stand out for their effectiveness as biocontrol agents, producing diverse molecules capable of inhibiting several phytopathogenic fungi (Dunlap *et al.*, 2013). In this study, we aimed to evaluate the antagonistic effects of three Bacillus species against *Alternaria alternata*, both *in vitro* and *in vivo*. The results underscored the po-

tential of the three bacterial strains as effective antagonists, inhibiting the growth of *Alternaria alternata*. After a 7-day period, they demonstrated inhibition percentages of 60% and 23% against Alt1 (less virulent) and Alt2 strain (more virulent), respectively. This variance in inhibitory effects can be partially attributed to the differing levels of virulence between the two strains. Previous studies, such as Chen *et al*., (2020), have highlighted the remarkable antifungal activity of *Bacillus* spp., especially *Bacillus subtilis*, against citrus diseases caused by fungi, owing to their competitiveness for space and nutrients. In *in vivo* trials, for the first strain Alt1, all three bacteria exhibited substantial inhibition with O1 (42.9%), O3 (30.5%), and O2 (28.6%). For Alt2 strain, bacteria O1 (37.3%) demonstrated the most significant inhibition, followed by O2 (21.5%). *Bacillus velezensis* has been successfully utilized in postharvest preservation techniques for citrus fruits, effectively countering the citrus pathogens *Colletotrichum gloeosporioides* and *Penicillium digitatum* both in laboratory and in the field (Vu *et al*., 2023). Additionally, the incidence of *Botryosphaeria dothidea* on apples decreased by around 50% at 56 days after the application of *Bacillus amyloliquefaciens* PG12 (Chen *et al*., 2016).

In the intricate microenvironment of plants, Bacillus species exhibit rapid proliferation, effectively impeding pathogens and strengthening plant resistance (Herrmann *et al*., 2023). Research by Devi *et al*., (2019) indicates that *B. velezensis* produces various antifungal compounds, which are subsequently released into the surrounding environment. Moreover, previous studies have emphasized the vital role of Bacillus metabolites in its antifungal efficacy (Xue *et al*., 2023). Furthermore, recently, Zhou *et al*. (2024) highlight the significant inhibitory impact of antifungal proteins from the *B. velezensis* strain KL-2, particularly notable in their effectiveness against *A. alternata*.

In the literature, *Bacillus* spp. and fungi such as *Trichoderma* spp. have gained attention. However, some authors emphasize that citrus diseases associated with *A. alternata* still heavily rely on fungicide applications, with difenoconazole, Mancozeb and copper compounds reported as the most effective. Regarding active substances, IMZ and AZX were tested in the present study against *A. alternata* strain with more virulence. For Imazalil, *in vitro* results showed complete inhibition at the 0.5 ppm dose, 60% inhibition at 0.2 ppm, and 50% inhibition at 0.1 ppm. However, *in vivo* trials did not yield significant differences among the three doses, with an inhibition rate as low as 20%. Azoxystrobin was tested at five concentrations (0.01, 0.1, 1, 10, and 100 ppm) against *A. alternata* strain Alt2. *In vitro* tests demonstrated a 35% reduction in the fungus's mycelial growth. Furthermore, lesions developed on citrus fruits by *A. alternata* were diminished by 45% to 55%.

To summarize, the tested *Bacillus* spp. strains in our study demonstrated a commendable level of *A. alternata* pathogen control, albeit falling short of the efficacy demonstrated by the approved fungicide Imazalil. These findings suggest that strains of the pathogen (*A. alternata*) exhibit varying sensitivity to both antagonistic bacteria and conventional chemical fungicides, possibly influenced by the level of virulence inherent in each strain.

CONCLUSION

Alternaria alternata, responsible for citrus fruit diseases, poses a significant threat to the global citrus industry. Our study offers valuable insights into biocontrol methods to mitigate the severity of *A. alternata* in postharvest citrus fruits. The tested strains of *Bacillus* spp. demonstrated commendable efficacy in controlling the pathogen, revealing competitive outcomes comparable to the effectiveness of the two approved fungicides. This parity may stem from their ability to stably colonize citrus fruits, inhibiting hyphal growth and serving as protective agents. Strains of *Bacillus* spp. emerge as promising candidates for the development of biocontrol strategies integrated into pest management programs for managing *Alternaria alternata* in citrus. Such eco-friendly approaches not only aid in safeguarding food safety but also contribute to sustainable agricultural practices.

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