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Original Article

# Biocontrol Stem-end Rot Disease Agent, *Alternaria alternata* YZU, on Pitaya by Soil Phosphate Solubilizing Bacteria

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**Abstract:** Biocontrol of stem end disease agent, *Alternaria alternata* YZU, on pitaya is more interested in altering the application of chemical pesticides. This study was conducted to characterize antagonistic phosphate solubilizing bacteria (PSB) from rhizosphere soil for their biocontrol activities against *A. alternata* YZU under laboratory and greenhouse conditions. Six PSB isolated from 31 rhizosphere soil samples were tested for inhibiting the mycelial growth of *A. alternata* YZU in dual cultures. Six isolates were determined to inhibit the mycelial growth of *A. alternata* YZU. Among them, PSB31 presented the highest level of antagonistic activity against *A. alternata* YZU with a mean inhibition diameter of 0.64 ± 0.02 cm, while the other strains, including PSB11, PSB21, PSB41, PSB51, and PSB61 presented a weaker inhibition. The results also showed that the strain PSB31 was identified as *Bacillus* sp. strain PSB31 (Accession number: ON422095) and could control the mycelial growth of *A. alternata* YZU by secreting antifungal metabolites. Moreover, an *in vivo* antagonistic experiment of PSB31 on pitaya twigs showed a significant reduction of lesions on twigs than the control. The results suggested that the isolated PSB31 is a potential biological control agent. Further studies should be done to identify the biochemical basis of their activity against *A. alternata* YZU.

*Keywords: Alternaria alternata, Bacillus* sp, biocontrol, phosphate-solubilizing bacteria, rhizosphere soil.

## 1. Introduction

In Binh Thuan, Vietnam, there is a risk of fungal pathogens, particularly from stem-end rot on pitaya (*Hylocereus undatus*) caused by *Alternaria alternata* YZU, resulting in severe economic losses [1]. The control of fungal diseases attacking pitaya is mainly done by using chemical pesticides, which are hazardous to human health and contributes to environmental pollution, as well as in the induction of the resistance of phytopathogenic agents [2]. Hence, developing alternative methods to control this disease is strongly required. Biological control with soil bacteria has received significant attention as one of the

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non-hazardous pest management techniques against different plant pathogens [3]. For example, antagonistic bacteria such as *Bacillus subtilis* [4], *B. amyloliquefaciens* [5], and *Pseudomonas fluorescens* [6] or fungi such as *Trichoderma harzianum* [7] or yeast such as *Saccharomyces cerevisiae* [8] have been used to control the anthracnose disease on different plants under controlled research conditions. Among those, phosphate solubilizing bacteria (PSB) participate in biocontrol activity by producing plant growth hormones, stimulating induced systemic resistance (ISR), producing organic acids (mainly citric, oxalic, and gluconic acids) [9, 10], and producing hydrolytic enzymes such as phosphatase [11]. Additionally, PSB was also involved in promoting the growth of plants by acting in both roles of biofertilizers and biopesticides [11, 12]. Therefore, the identification of more PSB could be a promising alternative to control pathogens with high ecological versatility, such as *A. alternata*.

On the other side, the biological effectiveness of the antagonist against plant pathogens was strongly influenced by environmental factors such as climate change and water conditions, which are the crucial factors influencing microbial activity in natural systems [13]. These environmental factors interact with and directly affect the capability to grow and establish the biocontrol agent on the host. Some previous studies showed that the combined effects of these parameters play an important role in the genesis of microbial associations [7, 14, 15]. Therefore, it is reasonable to study the influence of temperature, pH, and water activity on the *in vitro* antagonism of PSB.

In biocontrol studies, there has been limited research on the biocontrol of *A. alternata* YZU, a causal of stem-end rot on pitaya, as well as there is a lack of comparative information on the effects of environmental factors on potential biocontrol of PSB against *A. alternata* YZU. Hence, the objectives of this study were to investigate the antagonistic ability of PSB from rhizosphere soil against *A. alternata* YZU; and to validate the effect of temperature, pH, and water activity during that antagonism.

## 2. Materials and Methods

### *2.1. Fungal Pathogen Strain*

Strain YZU of *Alternaria alternata* (accession number MN822486.1) was isolated from naturally infected pitaya presenting stem end rot symptoms. It was selected for its aggressiveness among several isolates found in different pitaya cultivars. *A. alternata* YZU originated from fields of pitaya plants of Binh Thuan (Vietnam), developed well in Potato Dextrose Agar, and was incubated ten days at 25 ˚C ± 2 ˚C before use. The identification was carried out by molecular method [1].

### *2.2. Isolation of Phosphate Solubilizing Bacterial Strains*

Six PSB were isolated using serial dilutions from rhizosphere soil taken from various agricultural zones of Nam Dinh province (Vietnam) using PVK media containing insoluble tricalcium phosphate as the sole source of phosphorus, allowing the selection of PSB. All isolates had been chosen for evaluating the antagonistic activity.

The Pikovskaya (PVK) medium with the following composition was used (g/L): glucose, 10; $(NH_4)_2SO_4$, 0.5; $MgSO_4.7H_2O$, 0.1; yeast extract, 0.5; KCl, 0.2; NaCl, 0.2; $FeSO_4.7H_2O$, 0.002; $MnSO_4.7H_2O$, 0.002; $Ca_3(PO_4)_2$, 5. The pH was adjusted at 6.5 (15 g of agar was added, for solid medium).

### *2.3. Molecular Identification of Selected PSB*

The total DNA of selected PSB was extracted using a Rapid Bacteria Genomic DNA Isolation Kit (Biobasic, Canada) as per the kit instructions. The PCR amplification of 16S rRNA was done with the extracted DNA by using the universal primers 27 F (5′-AGA GTT TGA TCC TGG CTC AG-3′), and 1492 R (5′-TAC GGT TAC CTT GTT ACG ACT T-3′). The amplification was done in a GeneAmp PCR System 2700 thermocycler

(Applied Biosystems, CA, USA) using the following program: 95 ˚C for 5 min; 30 cycles at 95 ˚C for 30 s, 55˚C for 30 s, and 72 ˚C for 90 s; and 72 ˚C for 7 min. The fragment of 16 S rRNA sequences (1.5 kb) was obtained and purified using the QIAquick PCR Purification Kit (Qiagen, USA). The purified 16S rRNA fragment was sequenced by First Base Company (Singapore). The obtained sequence was blasted on NCBI to identify the species. The sequences with high similarity (more than 99%) were used for multiple cluster alignment and phylogenetic analysis on MEGA software (v.7.2).

### *2.4. Evaluating the Antagonistic Activity of Isolated PSB*

The potential for biological control of six isolated PSB was assessed *in vitro*. The inhibited ability of PSB strains on *A. alternata* YZU was evaluated using the dual culture technique on potato dextrose agar (PDA, Potato infusion: the extract from 200 g of peeled potato; dextrose: 20 g/l; agar: 15 g/l; pH = 7.0-7.3). The antagonist and the phytopathogen were put on the opposite side of the Petri dish at the same distance from the periphery. Petri dishes with *A. alternata* YZU discs alone were considered as control. Plates were incubated at 28 °C for 7 days. There were three replicates for each treatment/control, and the experiment was repeated to confirm the results. The inhibition zone between *A. alternata* YZU and the bacterial strains was measured. The percentage inhibition of the radial growth (PIRG) of PSB was calculated as follows: $PIRG = [(R - r)/R] \times 100$. Where R: no inoculated bacterial colony region of fungal pathogen growth (control growth); r: growth of fungus in a dual culture [1].

### *2.5. Evaluating the Antagonistic Activity of Isolated PSB on the Pitaya Twigs under Laboratory Conditions*

The in vitro assays for biocontrol suggested the isolated PSB represented potential biocontrol agents against the *A. alternata* YZU. Hence, further experiments to study their antagonistic ability under laboratory conditions were carried out by the method described by Mohd et al., [16]. Briefly, the bacterial antagonist grown overnight (at OD = 1) was applied on pitaya twigs that were wounded by a sterile needle and put in a closed plastic box containing filter paper moistened with sterile water at the bottom.

The experiments were randomly designed in triplicates as follows: i) The wounded area of the pitaya twigs was sprayed with the bacterial suspension immediately before the placement of the PDA plug of *A. alternata* YZU on it; ii) The wounded area of the pitaya twigs was sprayed with the bacterial suspension for 24 hrs before placing the PDA plug of *A. alternata* YZU on it; iii) The wounded area of the pitaya twigs was placed the PDA plug of *A. alternata* YZU on it for 24 hrs before spraying the bacterial suspension; iv) The wounded area of the pitaya twigs was applied only with the bacterial suspension; and v) The wounded area of the pitaya twigs was applied only with the PDA plug of *A. alternata* YZU. After 15 days of inoculation, the observation for the symptom of stem-end rot disease was accessed by measuring the length of lesions produced.

### *2.6. Evaluating the Antagonistic Activity of Isolated PSB on the Pitaya Twigs in the Greenhouse*

The efficacy of antagonistic bacteria against A. alternata YZU was also assessed in a greenhouse [16]. A similar procedure described above was applied, except some modifications including 10 replications were used under the greenhouse conditions.

### *2.7. Statistical Analysis*

All experiments were conducted in triplicates. The PIRG of the phytopathogenic agent by the antagonists has been subjected to an analysis of variance (ANOVA) using the software STATISTICA for Windows v.6. The statistical significance of the results was determined by performing a test of Duncan's multiple ranges ($p < 0.05$). Results were expressed as mean ± standard deviation.

## 3. Results and Discussion

### *3.1. Antagonistic Activity of the Bacterial Strains*

In the *in vitro* dual culture antagonism test, among the six isolated PSB, only the strain PSB31 presented the highest level of antagonistic activity against *A. alternata* YZU with a mean inhibition diameter of 0.64 ± 0.02 cm. The other strains including PSB11, PSB21, PSB41, PSB51, and PSB61 presented weak inhibition effects on the mycelial growth of *A. alternata* YZU, with mean inhibition diameter of (0.25 ± 0.13, 0.22 ± 0.11, 0.26 ± 0.13, 0.26 ± 0.15, and 0.22 ± 0.13), respectively (Table 1).

Table 1. *In vitro* inhibition of growth of *A. alternata* YZU by single bacterial antagonists on PDA medium

| Strains | Mean diameter of inhibition zone (cm ± SD)[a] | Growth (cm ± SD) |
|---|---|---|
| PSB11 | 0.25 ± 0.13[b] | 7.2 ± 0.21[b] |
| PSB21 | 0.21 ± 0.11[b] | 7.4 ± 0.21[b] |
| PSB31 | 0.64 ± 0.02[a] | 3.9 ± 0.32[a] |
| PSB41 | 0.26 ± 0.13[b] | 7.3 ± 0.12[b] |
| PSB51 | 0.26 ±0.15[b] | 7.2 ± 0.31[b] |
| PSB61 | 0.22 ±0.31[b] | 7.1 ± 0.23[b] |
| *A. alternata* + sterile-distilled water | 0 | 9.0 ± 0.11[c] |

[a] Values in the same column with the same letter(s) are not significantly different as determined by the LSD test (p=0.01).

The results also showed that the PSB31 strain showed the highest PIRG of *A. alternata* YZU, which was reduced by 54.44% (Table 1). The antagonistic effects of the bacterial antagonists against *A. alternata* YZU are illustrated in Figure. 1.

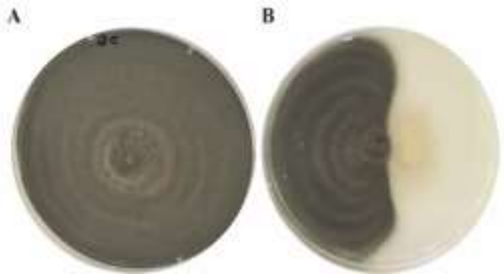


Figure 1. *In vitro* antagonistic activity of PSB against *A. alternata* YZU. Control (A), antagonistic activity of PSB31 strain (B).

Many studies have been done to exploit the application of PSB for biocontrol activities, and biofertilizers, to identify the different strains of PSB and their efficacy in P solubilization [17, 18]. However, the information on the application of PSB for biocontrol is limited. The antagonistic bacteria, PSB, which were tested in this study, effectively suppressed the mycelial growth of *A. alternata* in the *in vitro* assays, thus showing their biological control potential against the fungus *A. alternata*. Based on the *in vitro* results, among 7 isolated PSB, the PSB31 isolate effectively inhibited the mycelial growth of *A. alternata* in the *in vitro* assays. The inhibition of *A. alternata* mycelia might be due to the loss of cytoplasmic content resulting in the effects of the biocontrol agent on cell membrane permeability [19]. Based on the 16S rRNA sequence, the PSB31 strain resembles *Bacillus* sp. strain IMAU61039 (accession number: MF803700.1) with a nucleotide identity is 99.38%. The 16S rRNA sequence of PSB31 was deposited in Genbank with accession number ON422095.

Biological control plays a vital role in controlling the phytopathogen in sustainable agriculture [20]. *Bacillus* spp. such as *B. subtilis* and B. *amyloliquefaciens* have been used in commercial products for the biocontrol of plant disease due to their biocontrol potential and high stability in harsh environmental conditions caused by spore forms [21]. In this study, we found that *Bacillus* sp. strain PSB31 presented significant inhibition on the development of *A. alternata* YZU, the

phytopathogenic agent of the stem end rot of pitaya. This result is consistent with previous studies, which demonstrated biological control of *A. alternata* using *Bacillus* species [7, 14, 15]. The results of this study suggested that this PSB31 is a potential alternative method to chemical fungicide in inhibiting stem-end rot disease of pitaya.

### *3.2. Antifungal Activity of the Culture Filtrates of the Antagonistic Bacteria*

The culture filtrates of PSB31 significantly inhibited the mycelial growth of *A. alternata* YZU (Table 2). The results presented the reduction of *A. alternata* YZU growth by 60.67-62.92%. Especially, no significant differences were observed among the concentrations of culture filtrates (1.25, 2.5, and 5%) on the growth inhibition efficacy ($p > 0.05$).

Table 2. *A. alternata* YZU growth inhibition by the culture filtrate of the antagonistic bacteria

| Treatment | Culture filtrate concentration (%) | *A. alternata* YZU growth on PDA (cm ± SD) |
|---|---|---|
| PSB31 | 5 | $3.3 \pm 0.22^b$ |
| PSB31 | 2.5 | $3.5 \pm 0.25^b$ |
| PSB31 | 1.25 | $3.5 \pm 0.29^b$ |
| Control | 0 | $8.9 \pm 0.05^a$ |

[a] Values in the same column with the same letter(s) are not significantly different as determined by the LSD test ($P<0.05$).

The data showed the culture filtrate from the PSB31 strain significantly interfered with the growth of *A. alternata* up to 62.92%. A similar property was observed in *B. siamensis* strains LZ88 [15] and *B. megaterium* [22]. The culture filtrate of these strains was reported to inhibit mycelial growth, spore germination, and spore production in *A. alternata* [15, 22]. It indicated the secretion of antifungal metabolites by the bacterial antagonist, such as hydrolytic enzymes, bacteriocins, antibiotics, or other secondary metabolites [15, 23].

On the other hand, it is also well known that bacteria may also be good inducers of plant defense mechanisms besides striving for direct antagonistic effects on fungal and bacterial pathogens. Induced systemic resistance (ISR) can induce different genes to immunize the crop metabolically or mechanically by altering host physiology or metabolic responses, increasing cell wall strength, and enhancing the synthesis of plant defense chemicals. For example, *B. paralicheniformis* protected the plants from the attack of *A. alternata* through the induction of the systemic resistance of the plant [7]. Hence, our study provides evidence that the PSB31 strain influences the survival of *A. alternata*.

### *3.3. Disease Suppression Test on the Twigs under Laboratory and Greenhouse Conditions*

The results of laboratory and greenhouse experiments showed that spraying the PSB31 strain onto the wounded area of the pitaya twigs either 24 hrs before or immediately after fungal (*A. alternata*) inoculation was effective in inhibiting the stem-end rot disease severity in comparison to the control inoculated with the pathogenic fungus only (Figure 2). Moreover, the results also presented relatively better control efficiency when applying the EI-15 strain onto the wounded area of the twigs 24 hrs before fungal inoculation. However, control efficacy was slightly decreased under greenhouse conditions (Figure 2). These results suggested that applying the bacteria to the pitaya twigs before inoculating the phytopathogenic fungus should increase the biocontrol efficiency of stem-end rot disease development.

A central challenge to understanding antagonist-pathogen interactions is that we have little information on how environmental factors drive phenotypic variability in microbial function and physiology *in vitro* and how traits characterized in the lab may predict functional outcomes in nature [24]. Previous studies have demonstrated the important role of environmental factors during biocontrol processes. They influenced the biological life of the microbial species and the physiology/metabolism of pathogen antagonists and host plants [25, 26].

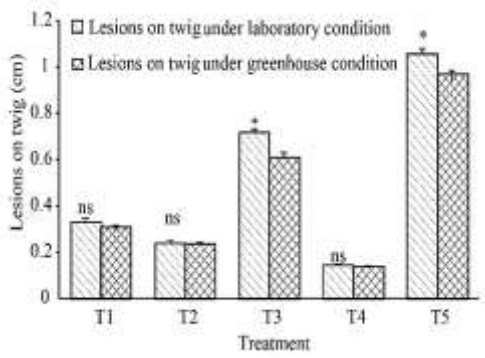


Figure 2. Effect of environmental conditions on the antagonistic ability of EI-15 against stem-end rot disease caused by *Alternaria alternata* on the twigs. Values are means ± SD (n = 10). "ns" indicates not significant ($p>0.05$), whereas the asterisk indicates a significant difference ($p<0.05$) in the size of the lesion present between treatments (paired t-test).

The laboratory and greenhouse experiments showed disease suppression when the PSB31 strain was applied 24 hrs before or followed immediately with fungal inoculation on the pitaya twigs. These results were consistent with previous studies that demonstrated the ability to suppress the phytopathogens by PSB [27-29]. Chowdhury et al., [28] showed that *B. amyloliquefaciens* could inhibit the development of head blight in spikelets or the growth of several phytopathogenic fungi and bacteria. The author also suggested a direct effect of the numerous antimicrobial secondary metabolites in suppressing pathogens and an indirect effect of triggered pathways of ISR by bacterial metabolites, such as surfactin and volatiles. Hence, the results suggested that PSB31 could be a potential biocontrol agent to control plant diseases.

## 4. Conclusions

*Six PSB isolated from* rhizosphere soil samples could differently inhibit the mycelial growth of the fungal phytopathogen *A. alternata*. PSB31 strain presented the highest level of antagonistic activity against *A. alternata* YZU, while a weak inhibition was observed by the PSB11, PSB21, PSB41, PSB51, and PSB61. The results also showed that PSB31 identified as a *Bacillus* strain was the most effective in suppressing stem end rot diseases' development on pitaya twigs under laboratory and greenhouse conditions. Hence, the PSB31 strain is a potential biological control agent and should be further explored in the future.

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