

Biocontrol of *Alternaria alternata* YZU, a causal of stem end rot disease on pitaya, with soil phosphate solubilizing bacteria

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Abstract. Stem end rot is the most destructive disease caused by *Alternaria alternata* YZU in pitaya-growing regions of Vietnam. This study was conducted to characterize antagonistic phosphate-solubilizing bacteria (PSB) from rhizosphere soil for their biocontrol activities against *A. alternata* YZU and evaluate the effect of temperature, pH, and water activity on that antagonism. Among seven PSB isolated from 45 rhizosphere soil samples, PSB31 (identified as *Bacillus* sp. strain IMAU61039, Accession number: MF803700.1) exhibited the highest antagonistic activity against *A. alternata* YZU with an average inhibition diameter of 0.65 ± 0.05 cm. The results also show that the strain PSB31 controlled the mycelial growth of *A. alternata* YZU by secreting antifungal metabolites. The most potent inhibitory activity was identified under *in vitro* conditions of 25 °C, pH 7, and a_w 1. The isolated PSB31 could be a potential biological control agent against *A. alternata* YZU.

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

1 Introduction

Pitaya (*Hylocereus Undatus*) is an important fruit crop grown well in Binh Thuan, Vietnam. In this region, however, there is a risk of fungal pathogens, particularly stem end rot caused by *Alternaria alternata* YZU, resulting in severe economic losses [1]. The control of fungal diseases attacking pitaya has been primarily performed by using chemical pesticides that are hazardous to human health, pollute the environment, and induce the resistance of phytopathogenic agents [2]. Hence, developing alternative methods to control this disease is crucial. Biological control with soil bacteria has received much attention as one of the 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 conditions. Among those, phosphate solubilizing bacteria (PSB) participate in biocontrol activity by producing plant growth hormones stimulating induced systemic resistance, forming organic acids (mainly citric, oxalic, and gluconic acid) [9, 10], and producing hydrolytic enzymes, such as phosphatase [11]. Additionally, PSB also contributed to promoting

plant growth by acting on both roles of biofertilizers and biopesticides [11, 12]. Therefore, identifying 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 crucial for microbial activity in natural systems [13]. These environmental factors interact with and directly influence the capability for growth and establishment of the biocontrol agent on the host. Several previous studies showed that the combined effects of these parameters play an essential 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 biological control studies, there has been limited research on biocontrol of *A. alternata* YZU, a causal of stem end rot on pitaya, and there is a lack of comparative information on the effects of environmental factors on potential biocontrol of PSB against *A. alternata* YZU. Hence, this study investigates the antagonistic ability of PSB from rhizosphere soil against *A. alternata* YZU and validates the effect of temperature, pH, and water activity during that antagonism.

2 Material and methods

2.1 Fungal pathogen strain

Strain YZU of *Alternaria alternata* (accession number MN822486.1) was isolated from naturally infected pitaya with stem end rot symptoms. It was selected for its aggressiveness among several isolates found in different pitaya cultivars. *A. alternata* YZU originated from pitaya plant fields in Binh Thuan (Vietnam) developed well in potato

dextrose agar and was incubated for ten days at 25 ± 2 °C before use. The identification was carried out with the molecular method [1].

2.2 Isolation of phosphate solubilizing bacterial strains

Seven PSB were isolated with the method of serial dilutions from rhizosphere soil taken from various agricultural zones by using the Pikovskaya media containing insoluble tricalcium phosphate as the sole source of phosphorus, allowing selection of PSB. All isolates were chosen for evaluating antagonistic activity.

The Pikovskaya medium with the following composition was used (g/L): glucose, 10; $(\text{NH}_4)_2\text{SO}_4$, 0.5; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.1; yeast extract, 0.5; KCl, 0.2; NaCl, 0.2; $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 0.002; $\text{MnSO}_4 \cdot 7\text{H}_2\text{O}$, 0.002, and $\text{Ca}_3(\text{PO}_4)_2$, 5. The pH was adjusted at 6.5 (15 g of agar was added to the solid medium).

2.3 Evaluating the antagonistic activity of isolated PSB

The potential for biological control of seven isolated PSB was assessed *in vitro*. The inhibited ability of PSB strains against *A. alternata* YZU was evaluated by using the dual culture technique on potato dextrose agar (PDA, potato infusion: 200 g/L, dextrose: 20 g/L, agar: 15 g/L, pH 7.0–7.3). The antagonist and the phytopathogen were put on the opposite sides of the Petri dish at an equal distance from the periphery. A Petri dish with *A. alternata* YZU discs alone was a control. The plates were incubated at 28 °C for seven days. Each treatment/control was performed in three replicates, and the experiments were repeated to confirm the results. The inhibition zone between *A. alternata* YZU and the bacterial strains was measured, and the percentage inhibition of the radial growth (PIRG) of PSB was calculated as follows:

$$\text{PIRG} = [(R - r)/R] \times 100$$

where R and r are the radial distance of the fungal pathogen growth for the control and the dual culture (mm) [1].

2.4 Evaluating effect of environmental factors on antagonistic activity of isolated PSB

Effect of temperature

The effect of temperature on the inhibition of mycelial growth of *A. alternata* YZU with antagonistic bacteria was conducted by incubating the inoculated dishes in the dark at temperatures 15, 20, 25, 27, and 30 °C for seven days [13].

Effect of water activity

The water activity (a_w) is defined as the ratio of the vapour pressure of water in the substance and the vapour pressure of pure water at the same temperature. It means water availability for biochemical reactions in the development of microorganisms. Different values of a_w have been tested (1, 0.95, 0.90, and 0.85) by adding glycerol to PDA [13]. Glycerol attaches to a part of water, making it unusable for microorganisms. The dual culture was carried out. The PIRG was calculated after seven days of incubation in the dark at 25 °C.

Effect of pH

The effect of pH was evaluated on PDA media [13]. The tested pH are 4, 4.5, 5, 5.5, 6, 6.5, 7, 7.5, 8.0, and 9.0.

The PDA medium was buffered to the desired pH with different buffers and adjusted by adding HCl or NaOH. The inoculated dishes were incubated at 25 °C. The PIRG was calculated after seven days of incubation.

2.5 Statistical analysis

All experiments were conducted in triplicates. The data were subjected to analysis of variance with the software STATISTICA for Windows v.6. The statistical significance of the results was determined by performing a test of Duncan's multiple ranges ($p < 0.05$). The results were expressed as mean \pm standard deviation.

3 Results

3.1 Antagonistic activity of bacterial strains

In the *in vitro* dual culture antagonism test, among the seven isolated PSB, only the strain PSB31 showed the highest level of antagonistic activity against *A. alternata* YZU with a mean inhibition diameter of 0.65 ± 0.05 cm. The remaining strains exhibited weak inhibition effects (Table 1).

The PSB31 strain showed the highest PIRG for *A. alternata* YZU, corresponding to a 54.44% growth reduction (Table 1). The antagonistic effect of the bacterial antagonist against *A. alternata* YZU is illustrated in Fig. 1.

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

Strains	Mean diameter of inhibition zone (cm \pm SD)*	Growth (cm \pm SD)
PSB11	0.26 ± 0.14^b	7.3 ± 0.52^b
PSB21	0.23 ± 0.15^b	7.5 ± 0.51^b
PSB31	0.65 ± 0.05^a	4.1 ± 0.41^a
PSB41	0.25 ± 0.12^b	7.2 ± 0.42^b
PSB51	0.27 ± 0.14^b	7.1 ± 0.34^b
PSB61	0.21 ± 0.11^b	7.3 ± 0.45^b
PSB71	0.24 ± 0.14^b	7.2 ± 0.55^b
<i>A. Alternata</i> + sterile-distilled water	0	9.0 ± 0.35^c

Note: * Values in the same column with the same letter(s) are not significantly different as determined by the LSD test ($p = 0.01$).

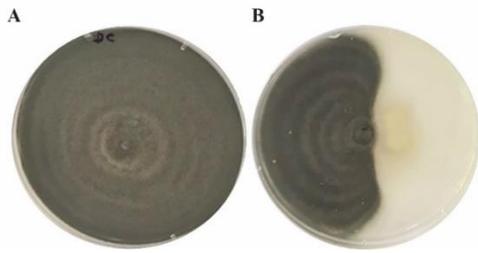


Fig. 1. *In vitro* antagonistic activity of PSB against *A. alternata* YZU: control (A), PSB31 strain (B)

Therefore, PSB31 was identified as *Bacillus* sp. strain IMAU61039 (Accession number: MF803700.1) and selected for further studies.

3.2 Antifungal activity of PSB31 culture filtrates

The strain PSB31 significantly inhibited the mycelial growth of *A. alternata* YZU (Table 2) with a 60.67–62.92% growth reduction. Especially, no significant differences were observed among the concentrations of culture filtrates (1.25, 2.5, and 5%) in terms of the growth inhibition efficacy ($p > 0.05$).

3.3 Effect of temperature on antagonistic activity of PSB31

All studied temperatures exhibited significant inhibition of PSB31, with the highest one (54.44%) at 25 °C (Fig. 2).

3.4 Effect of water activity on antagonistic activity of PSB31

The water exhibited significant inhibition against the mycelial growth of *A. alternata* YZU. The highest inhibition (71.23%) was found at a_w equal to 1. At lower water activity values, the inhibition was also lower, indicating that PSB31 plays an essential role in inhibiting the mycelial growth of *A. alternata* YZU, and radial growth inhibition of the phytopathogenic agent increases with water activities (Fig. 3).

3.5 Effect of pH on antagonistic activity of PSB31

The *in vitro* assay of pH revealed that the pH significantly affected the antagonistic potential against

A. alternata YZU (Fig. 4). pH 7 had the highest inhibitory effect on the growth of *A. alternata* YZU (51.14%); and the lowest effect (around 21%) was observed at pH 4, 4.5, and 9.

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

Treatment	Culture filtrate concentration (%)	<i>A. alternata</i> YZU growth on PDA (cm ± SD)*
PSB31	5	3.3 ± 0.22b
PSB31	2.5	3.5 ± 0.25b
PSB31	1.25	3.5 ± 0.29b
Control	0	8.9 ± 0.05a

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

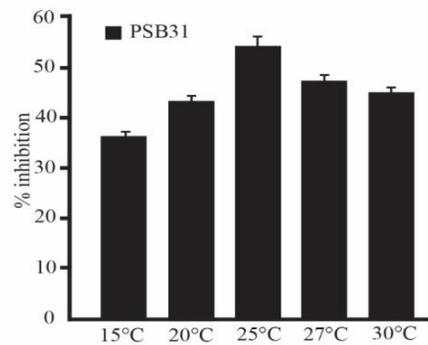


Fig. 2. Effect of temperature on growth inhibition against *A. alternata* YZU with PSB31 strain

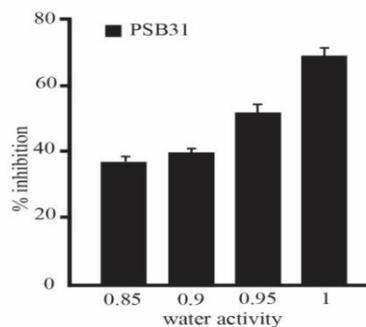


Fig. 3. Effect of water activity on growth inhibition against *A. alternata* YZU with PSB31 strain

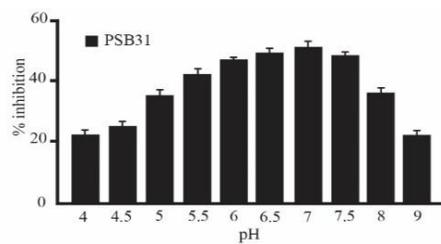


Fig. 4. Effect of pH on the inhibition of radial growth of PSB31 strain against *A. alternata* YZU

As can be seen from Fig. 4, the antagonistic effect of PSB on the phytopathogen increased with pH values up to neutral pH 7 and decreased in alkaline pH.

4 Discussion

Biological products for controlling plant diseases have been replacing fungicides. Stem end rot caused by *A. alternata* YZU is one of the most destructive diseases that threaten pitaya production in Vietnam and worldwide. In this study, isolated rhizosphere PSB were evaluated for biocontrol activities against *A. alternata*.

Numerous studies have been carried out to identify different PSB strains and their efficacy in phosphate solubilization and to exploit the advantages of PSB for biocontrol activities and biofertilizers [16, 17]. However, the information on the application of PSB for biocontrol is limited. The antagonistic bacteria tested in this study effectively suppressed the mycelial growth of *A. alternata* in *in vitro* assays, thus showing their biological control potential. Among seven isolated PSB, PSB31 (identified as *Bacillus* sp. strain IMAU61039, accession number: MF803700.1) 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 because of the effects of the biocontrol agent on cell membrane permeability [18].

In addition, our data also 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* [19]. The culture filtrate of these strains was reported to inhibit mycelial growth, spore germination, and spore production in *A. alternata* [15, 19]. This indicates the secretion of antifungal metabolites by the bacterial antagonist, such as

hydrolytic enzymes, bacteriocins, antibiotics, or some other secondary metabolites [15, 20].

On the other hand, it is well known that bacteria may also be good inducers of plant defence mechanisms besides striving for direct antagonistic effects on fungal and bacterial pathogens. Induced systemic resistance 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 defence chemicals. For example, *B. paralicheniformis* protected the plants from the attack of *A. alternata* by inducing the plant systemic resistance [7]. Hence, our study provides evidence that the PSB31 strain influences the survival of *A. alternata*.

Biological control is essential in controlling the phytopathogen in sustainable agriculture [21]. *Bacillus* sp., such as *B. subtilis* and *B. amyloliquefaciens*, have been used in commercial products for biocontrol of plant disease because of their potential for biocontrol and high stability under harsh environmental conditions caused by spore forms [22]. In this study, we found that *Bacillus* sp. strain IMAU61039 manifested significant inhibition on the development of *A. alternata* YZU, the phytopathogenic agent of the stem end rot of pitaya. This result is consistent with that of previous studies that demonstrated the biological control of *A. alternata* YZU by using *Bacillus* species [7, 14, 15]. The results of this study suggested that this PSB31 has the potential as an alternative to replace chemical fungicides in inhibiting the stem end rot disease of pitaya.

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 the traits found in laboratories may help predict functional outcomes in nature [23]. Our data showed how the PSB31 strain and environmental factors, such as temperature, water activity, and pH, affected the *in vitro* biocontrol against *A. alternata* YZU. Previous studies have demonstrated the vital role of the environmental factors during biocontrol processes, like how they influenced the biological life of the microbial species and the physiology/metabolism of pathogen antagonists and host plants [24, 25].

Temperature significantly affected the development of microorganisms and their biological activity. Previous studies were carried out to study the influence of environmental parameters on the development of *A. alternata* YZU and found that the optimal temperature was 25 ± 2 °C [7, 14]. In this study, the bacteria antagonist showed the inhibitory effect at all temperatures studied, and the most potent inhibition in the fungal radial growth was observed at 25 °C. Hence, the results agreed with those of previous studies [7, 14].

Regarding water availability, the results showed that water activity significantly affected mycelial growth, which was optimal between 0.95 and 1. It is also consistent with some previous studies on *Bacillus* sp. For example, the optimal growth of *B. amyloliquefaciens* was at a water activity of 0.960 and 37 °C [13], but it inhibited the growth of *A. flavus* and *F. verticillioides* at a water activity of 0.99, 0.97, 0.95, and 0.93 [26] and also inhibited the growth and aflatoxin B1 production by *Aspergillus section Flavi* at a water activity of 0.982 [27]. The results suggested that changes in water activity might be useful for improving the environmental competence of the microorganism in the environment.

As for the influence of pH on the biocontrol, the results revealed that the pH of the growth medium played a crucial role in the inhibition competence of organisms, which was low at acid pHs, increased by increasing pH values to 7 (neutral pHs), and then decreased in alkaline pHs. Previous studies demonstrated that pH 6 to 7 was optimum for the growth and inhibition ability of the majority of bacterial strains [28, 29]. We found that pH 6.5 was required for the optimum growth of PSB31. Therefore, the inhibitory effect of PSB31 against *A. alternata* YZU was investigated at pH 4.0–9.0. At pH 7.0, more than a 50% inhibition of the radial growth of *A. alternata* YZU was obtained, which decreased nearly to thirty per cent at pH 8.0 (Figure 4). Hence, the result indicated that the optimal pH for antagonistic activity was 7. A higher or lower pH considerably reduced the growth and the antagonistic activity of PSB31 against *A. alternata* YZU.

5 Conclusion

Seven PSB isolated from rhizosphere soil samples could inhibit the mycelial growth of *Alternaria alternata*. The PSB31 strain exhibited the highest antagonistic activity against *A. alternata* YZU. The results also show that the environmental factors significantly reduced inhibition of *A. alternata* mycelial growth under *in vitro* conditions. Hence, the PSB31 strain was a potential biological control agent and should be further explored in the near future.

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