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Screening of Endophytic Bacteria Isolated from Weed Plant to Biocontrol Stem Rot Disease on Pitaya (*Hylocereus undatus*)

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HIGHLIGHTS

- First report of endophytic bacteria isolated from weed (*Lactuca indica*)
- Endophytic bacteria, L115 strain, inhibit significantly the development of *Alternaria alternata*.
- L115 strain promotes the growth parameters of multiple plants compared to the control.

Abstract: Endophytic bacteria from weed are emerging as valuable alternatives for biochemical pesticides in sustainable agriculture. This study aims to discover the antagonistic effects of some endophytic bacteria isolated from weed (*Lactuca indica*) against *A. alternata*, a casual of stem end rot disease of pitaya. A total of 14 endophytic bacteria were isolated and four of them presented *in vitro* antagonistic activity against *A. alternata*. Of four, strain L115 significantly inhibited the pathogenic growth with a mean inhibition diameter of 12.67 ± 0.02 mm, while the other three (strain L110, L111, and L114) showed a weak inhibition. The results indicated that strain L115 could belong to the *Bacillaceae* family while the other three (strain L110, L111, and L114) had characteristics of the *Pseudomonadaceae* family. Interestingly, strain L115 showed positive results for phosphate solubilization, indole acetic acid (IAA), and biofilm production, whereas three other strains presented weak capabilities for phosphate solubilization, biofilm production and IAA production. In addition, the filtrate of strain L115 presented antifungal activity on biocontrol tests *in vitro*. Especially, strain L115

significantly increased seedling biomass of pitaya and tomato compared to the control. Hence, these results suggest strain L115 has the potential to be used as biocontrol agents against *A. alternata*. More studies should be done in the future to evaluate their efficiency in field conditions.

Keywords: Antagonistic activity; *Alternaria alternata*; Endophytic bacteria; *Lactuca indica*; pitaya.

INTRODUCTION

Pitaya is one of the main fruity cultivars in Vietnam. In recent years, the pest takes a part in decreasing productivity and the quality of products, especially the stem end rot diseases that caused by the fungus, *Alternaria* sp. Responsible for the significant economic losses on cultures before and after the harvest [1-3]. Famers have used chemical pesticides for decades to control plant diseases leading to serious environmental problems such as hazards to human health and animals, the target pests' resistance, and environmental pollution [4-7]. Biological control is a promising alternative using microorganisms such as fungus, bacterium, virus, protozoan, or algae to control the pathogenic agents [4,5]. It offers some eco-friendly properties to manage agriculture pests including less toxic, and specific on target organisms [6,7].

Among those, using endophytic bacteria is emerging alternative method to chemical pesticides. Endophytic bacteria are microorganisms that grow within plants without causing any obvious symptoms of infection or disease. They can accelerate the growth of their host plants and increase nutrient uptake [8] and/or protect their hosts by producing secondary metabolites that have the potential to kill or suppress the attack by pathogenic microorganisms or herbivores [9,10]. So, endophytic bacteria could be used as one of the biocontrol agents in sustainable crop production [11-13].

In agroecosystems, weeds exist parallelly with crops and are well adapted to the environment and grow or reproduce aggressively in association with crops [14]. *Lactuca indica* belongs to the *Compositae* family and is an edible medicinal plant widely distributed in Asian countries including Vietnam [15]. *L. indica* extract containing triterpene acetate, quinic acid derivatives, and flavonoids, which has been traditionally used as an anti-inflammatory, anti-bacterial, and anti-diabetic medicine [15]. The endophytic bacteria might play a role in the production of these metabolites. Hence, the aim of this study is to isolate bacteria endophytes from weed (*L. indica)* growing at Binh Thuan that have antagonistic activity on *A. alternata* causing stem-rot disease on pitaya at Binh Thuan, Vietnam.

MATERIAL AND METHODS

Isolation and purification of endophytic bacteria of *L. indica*

The weed (*L. indica)* was collected from field growing pitaya at Binh Thuan, Vietnam. Samples (leaves, roots, and stems) were disinfected as the following procedure: After washing under running tap water, plant sample were randomly cut from each part, and disinfected by immersing for 5 min in a solution containing 2% sodium hypochlorite and 1% Tween 20. Samples were then treated with a solution containing 70% ethanol for 2 min and consequently washed twice with sterile distilled water. Spread three aliquots of 100 µL of the second wash on LB plates to check the plant surfaces' sterility. The disinfected samples were placed between two sterile filter papers, then cut into 1x1 cm and plated on LB plates and incubated at 28^0C for one week. After incubation, the colonies with the most representative were selected and streaked twice on LB for purification. Different colony profiles based on morphological criteria (shape, color, elevation, diameter, and margin) were described.

The antagonist activity of bacterial isolates *in vitro*

Bacterial isolates were tested for antagonism against *A. alternata* on the PDA by the dual culture technique [16]. The bacterial strains are inoculated in place at opposite ends of the medium. A cylinder of 4 mm in diameter mycelium phytopathogenic is deposited in the center of the petri dish. The control contains only a phytopathogenic fungus. The petri dishes were incubated at 28^0C. The mycelial growth's inhibition was observed after five or seven days.

The antagonistic bacterial isolates were identified based on the characters of the cultural tests, morphological, and biochemical: The Gram reaction, respiratory-type with catalase and oxidase as described in the manual of Bergey's bacteriology [16].

Antifungal activity of extracellular filtrate

The overnight culture of the isolated bacterium with antifungal activity was used and filtrated to get extracellular filtrate. Then, biocontrol assays were carried out with the supernatant. After that, the plate incubation was done at 28°C for 120 h and verified every 12 h. The indicator for the antifungal activity of filtrate is the fungal growth inhibition.

Biofilm formation of isolated bacteria of *L. indica*

A total of 4 isolates were subjected to biofilm detection methods described by Christensen and coauthors [18], which is a qualitative method for biofilm detection. A loopful of test organisms was inoculated in 10 mL of LB in test tubes. The tubes were incubated at 370C for 24h. After incubation, tubes were decanted and washed with phosphate buffer saline (pH 7.3) and dried. Tubes were then stained with crystal violet (0.1%). The excess stain was washed with deionized water. Tubes were dried in an inverted position. The scoring for the tube method was done according to the results of the control strains. Biofilm formation was considered positive when a visible film lined the wall and the bottom of the tube. The experiment was performed in triplicate and repeated three times.

Phosphate solubilization assay

The phosphate solubilizing ability of antagonistic endophytes was determined by using Pikovskaya (PVK) agar medium [18]. Each individual antagonistic endophytic strain was placed on PVK plates using sterile inoculating sticks. The clear halo area around an endophyte colony was observed after incubating for 1 week at 300C. The phosphate solubilization index (PSI) was calculated in endophyte strains capable of producing a distinct clear halo as a zone of solubilization [19].

Indole-3-acetic acid (IAA) Quantification Assay

Antagonistic endophytes were grown in triplicate in 25 mL LB broth with 0.1% (w/v) L- tryptophan in 125-mL flasks for 4 days. After that, the bacteria cells were removed by centrifugation. One ml of the supernatant was incubated with 2 ml of Salkowski reagent (2 mL of 0.5 M $FeCl_3$, and 98 ml of 35% $HClO_4$) [20] for half an hour, and the optical density at a wavelength of 530 nm was observed [21, 22]. A standard curve was done with known amounts of IAA using Salkowski reagent and LB broth with tryptophan and without endophytes, and the IAA amount produced by each endophyte strain was calculated using the standard curve.

Greenhouse experiment

The L115 strain has been showed the antagonistic activity on the fungal disease of pitaya. To investigate the potential application of this train as a biocontrol agent, the different host plants have been used.

Pitaya and tomato seeds were surface separately sterilized with 2% sodium hypochlorite for 2 mins, rinsed thoroughly in sterile distilled water (SDW), and let dry on sterile discs of Whatman filter paper placed inside Petri dishes. Covering the pitaya and tomato seeds with the antagonistic bacterial suspensions (108 CFU mL-1) is carried out and then the bacterized seeds were incubated at room temperature at 28°C for 72 h before sowing. In parallel, a mixture that included equal parts of peat, sand, and field soil was prepared and autoclaved twice for 20 min at 120°C with 24 h between autoclavings. Plastic pots (6 × 6 × 5.5 cm) were filled with a sterile substrate. The bacterized seeds were added 1 mL of bacterial suspensions before covered with soil. For negative control, seeds were treated with SDW instead of the bacterial suspension. Pots containing the treated seeds were placed in the greenhouse according to a completely randomized block experimental design. For each treatment, 15 pots each containing three seeds were used. All treatments were designed in completely randomized experiments and 15 replicates for each treatment. Plant growth parameters were recorded 45 days after sowing, using parameters: percentage of seed germination; the fresh and dry weight of plants, roots and shoots. The experiment was repeated once.

Statistical analyses

Data analysis was done with significance ($P<0.05$) of treatment effects using one-way ANOVA followed by posthoc comparisons (Tukey's HSD).

Plant growth-promoting activity of endophytic bacteria was determined by calculating the percentage increases in seed germination and plant growth parameters. The significance of the results was determined by Duncan's tests.

RESULTS

Isolation of bacterial endophytes

A total of 14 endophytic bacteria were isolated from L. indica collected at different locations of Binh Thuan province during June 2019. The number of isolates recovered was 6 from roots (42.86%), 5 from stems (35.71%), 3 from leaves (21.43%). Of them, 4 isolates induced inhibition of growth of A. alternata (Table 1).

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

Strains	Mean diameter of inhibition zone (mm)[a]	Degree of antagonism[b]
L110	1.15 ± 0.11[b]	+
L111	1.21 ± 0.12[b]	+
L114	2.01 ± 0.11[b]	+
L115	12.67 ± 0.02[a]	+++
A. Alternata + sterile-distilled water		-

[a] Values in the same column with the same letter(s) are not significantly different as determined by the LSD test (P=0.01).
[b] Weak '+' (1-5.99 mm), moderate '++' (6-10.99 mm), strong '+++' (11-20 mm), inhibition effects on the growth of the pathogen.

As can be seen from Table 1, the L115 strain is remarkably effective compared to the control (illustrated as in Figure 1A and 1B), which produced the inhibition of mycelial growth of A. alternata with a mean inhibition diameter of 12.67± 0.02 (Table 1 and Figure 1B). Meanwhile, the other isolated strains including L110, L111, and L114 have generated a weak inhibition of A. alternata, with mean inhibition diameter of 1.15 ± 0.11, 1.21 ± 0.12, 2.21 ± 0.10, and 2.01 ± 0.11 mm, respectively. In addition, the result also showed the antagonistic activity of supernatant extracted from L115 culture in antagonizing A. alternata (Figure 1C).

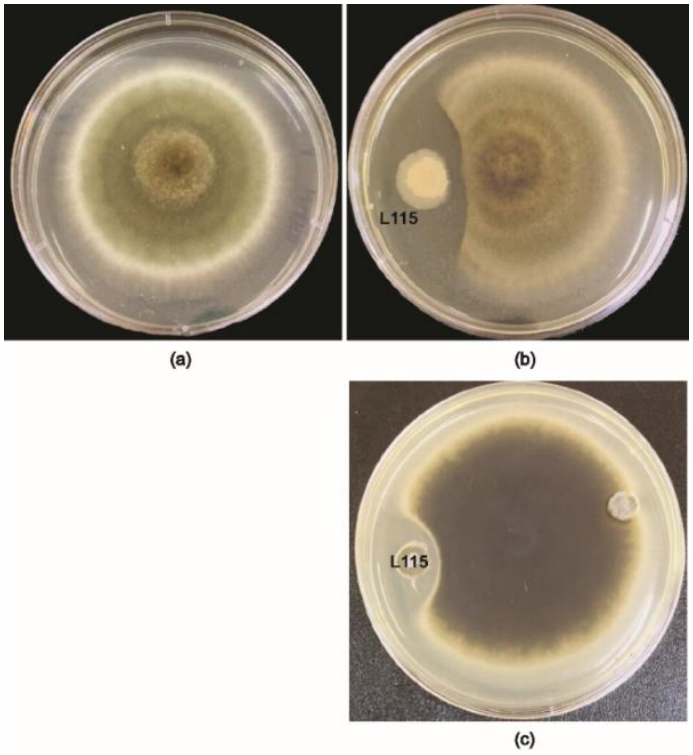

Figure 1. Antagonistic effects of L115 strain on mycelium growth of *A. alternata*. (a): control; (b): representative for antagonistic activity of L115 strain; (c): Effects of supernatant of L115 culture on mycelium growth of *A. alternata.*

Biochemical identification of endophytic bacteria isolated from L. indica

Based the Gram staining, 4 strains inhibited mycelial growth of phytopathogenic fungi A. alternata were divided into two groups: gram-positive and gram-negative (Tables 2). The are strictly aerobic bacilli of oxidase and catalase-positive, and they are all capable of reducing nitrate to nitrite or ammonia but unable to produce H2S.

Table 2. Biochemical and physiological characters of isolates isolated from *L. indica*

Strains Test	L110	L111	L114	L115
Citrate	+	+	+	-
Mobility	+	+	-	+
Mannitol	+	+	-	-
Glucose	-	+	-	-
Lactose	-	-	-	-
Methyl red	+	+	+	-
Voges Proskauer	-	-	-	+
Gelatinase	+	+	-	+
casein hydrolysate	-	+	-	+
starch hydrolysate	-	-	-	+

The data in Table 2 indicated the L115 is a gram-positive strain, is sporulation and belonged to the *Bacillaceae* family [17]. On the other side, the three bacteria including L110, L111 and, L114 are Gram-negative and belonged to the *Pseudomonadaceae* family [17].

IAA Production

It was reported that the bacteria can inhibit growth of pathogenic fungi by producing bioactive compounds such as IAA. Hence, screening IAA production might figure out the mechanism of antagonistic mechanism. The results indicated the variation of IAA amount produced by the different isolates when grew in LB medium supplemented with L-tryptophan (Figure 2). The results showed that the L115 strain produced the highest auxin levels (34.14 µg ml-1) and the next IAA producers were both L110 and L111 with IAA amount of 3.12 µg ml-1 and 4.31 µg ml-1, respectively. A negligible amount of IAA (<1.30 µg ml-1) was observed for the L114 strain.

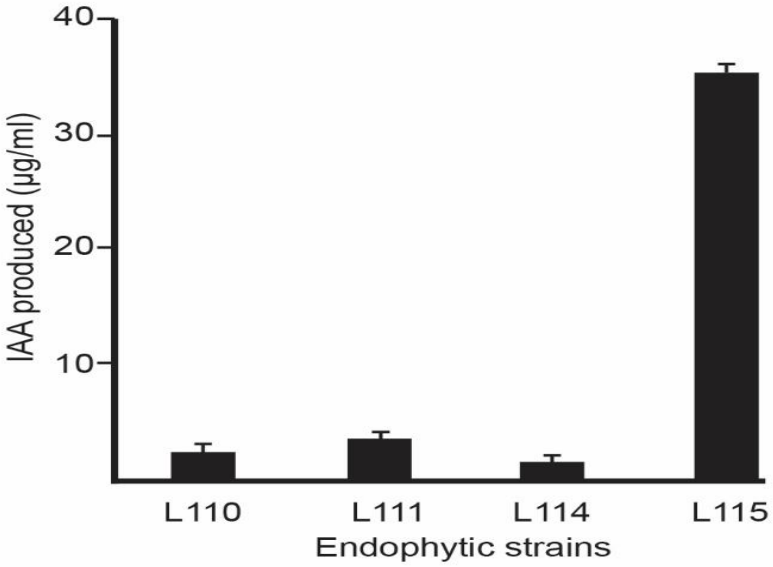

Figure 2. IAA production by the isolates. Strains were grown in triplicate for 4 days in TYC broth containing 0.1% L-tryptophan. The IAA amount produced by each endophyte strain was calculated using the standard curve

Phosphate solubilization of isolated bacteria of *L. indica*

It is reported that a halo area surrounded by endophyte colonies was a zone of phosphate solubilization, which was assessed by calculating PSI as described earlier [20]. As can be seen from Figure 3, several strains solubilized tricalcium phosphate more effectively than others. The best performing strains were L114 and L115 and scored more than 1.5 PSI. Strain L111 showed a relatively inconspicuous halo area. No solubilization was observed by strain L110.

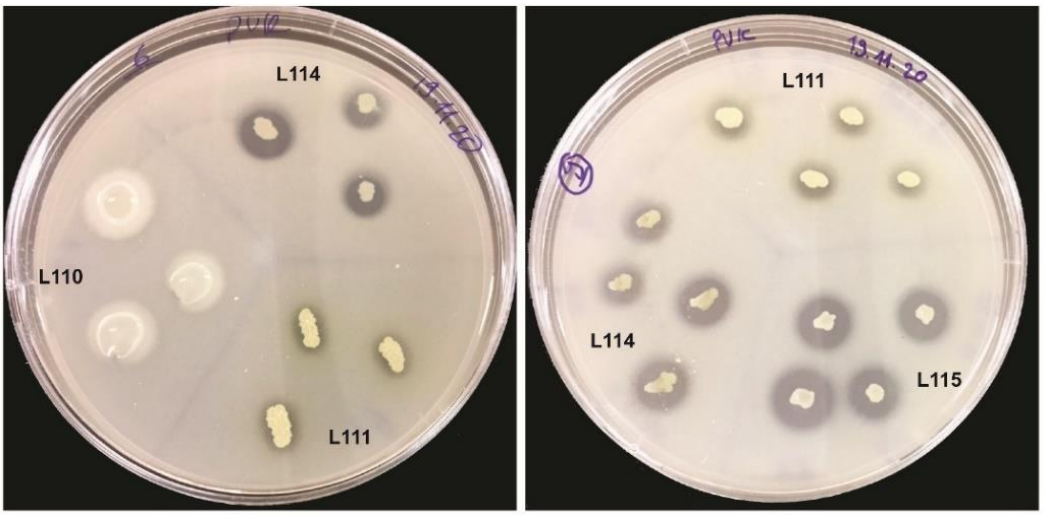

Figure 3. Tricalcium phosphate (PVK) plates showing the phosphate solubilization gradient: no solubilization (L110), moderate solubilization (L111), and high solubilization (L114 and L115).

Biofilm formation of isolated bacteria

Biofilm formation by plant-growth-promoting bacteria has been shown to play important roles not only in cycle nutrients but also in biocontrol of pests and diseases and consequently improves the productivity of crops [23-25]. For example, a Pleurotusostreatus-Pseudomonas fluorescens biofilm (FBB) increased the endophytic colonization of tomato (Lycopersiconlycopersicum) by P. fluorescens by over 1000%, compared to inoculation with P. fluorescens alone under in vitro conditions [24]. Hence, the biofilm formation of the isolates has been investigated. The results showed in Table 3 and Figure 4.

Table 3. Screening of antagonistic bacteria from *L. indica* for biofilm formation

STT	Strains	Ability of biofilm formation
1	Control	None
2	L110	Weak
3	L111	Weak
4	L114	Moderate
5	L115	High

As can be seen from Table 3, the number of strong biofilm producers was 1 (L115 strain), moderate and weak biofilm producers were 1 (L114) and 2 (L110 and L111) respectively.

It is well known that the biocontrol process mediated by plant growth-promoting rhizobacteria (PGPR) relies on multiple mechanisms. Among those, biofilm formation plays an important role in the ability of PGPR to control plant diseases (Figure 2). Our study showed that the L115 strain, belong to the Bacillaceae family, presented a strong biofilm formation (Figure 2E) while others (L110, L111, and L114) were members of Pseudomonadaceae family and showed a moderate and weak biofilm formation (Figure 2B, 2C and 2D) comparing to control (Figure 2A).

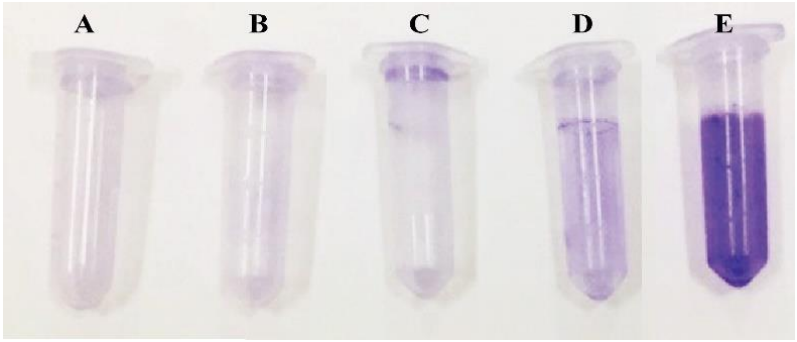

Figure 4. Biofilm formation of antagonistic bacteria from *L. indica.* A: Control; B: L110 strain; C: L111 strain; D: L114 strain; E: L115 strain.

Evaluation of plant growth promotion activity of endophytic bacteria

The results showed that all strains had positive effects on the germination of pitaya and tomato seeds. Bacterized seeds started to germinate early. The percentage of germinated seeds was greater than in the controls in vivo (Table 4). L115 was the most active one: at 10 d after bacterization more than 83% of the seeds were germinated as opposed to 51-53% of the controls. After 15 days, the germination rate of L115 treated seeds reached 100%, while this ratio of the seeds treated seperately with L110, L111 and L114 was from 77-94%. The seeds treated by SDW got the least effective in vivo with a germination rate was 75.5%.

Table 4. Mean percentage of germinated seeds at 5, 10 and 15 days after bacterization with endophytic strains *in vivo*.

Endophytic bacteria[a]	***In vivo* (bacterial suspension)**					
	Pitaya			**Tomato**		
	5 days	**10 days**	**15 days**	**5 days**	**10 days**	**15 days**
L110	3.5^{c}	52.1^{a}	80.2^{b}	3.5^{c}	53.2^{a}	84.3^{b}
L111	2.4^{b}	53.5^{a}	77.7^{a}	2.3^{b}	55.5^{a}	78.2^{ab}
L114	2.4^{b}	52.2^{a}	79.4^{b}	2.1^{b}	58.4^{b}	94.3^{c}
L115	5.6^{d}	83.3^{b}	100^{c}	5.2^{d}	83.5^{c}	100^{d}
Control	0^{a}	51.3^{a}	76.5^{a}	0^{a}	53.3^{a}	75.5^{a}

[a] Bacteria strains, Control = SDW=sterile distilled water.
[b] Data followed by the same letters are not statistically different according to Duncan test ($P< 0.01$).

The greenhouse results were illustrated as in Figure 5A and presented different results for the growth parameters in pitaya and tomato plants among the endophyte inoculated treatments (Figure 5B).

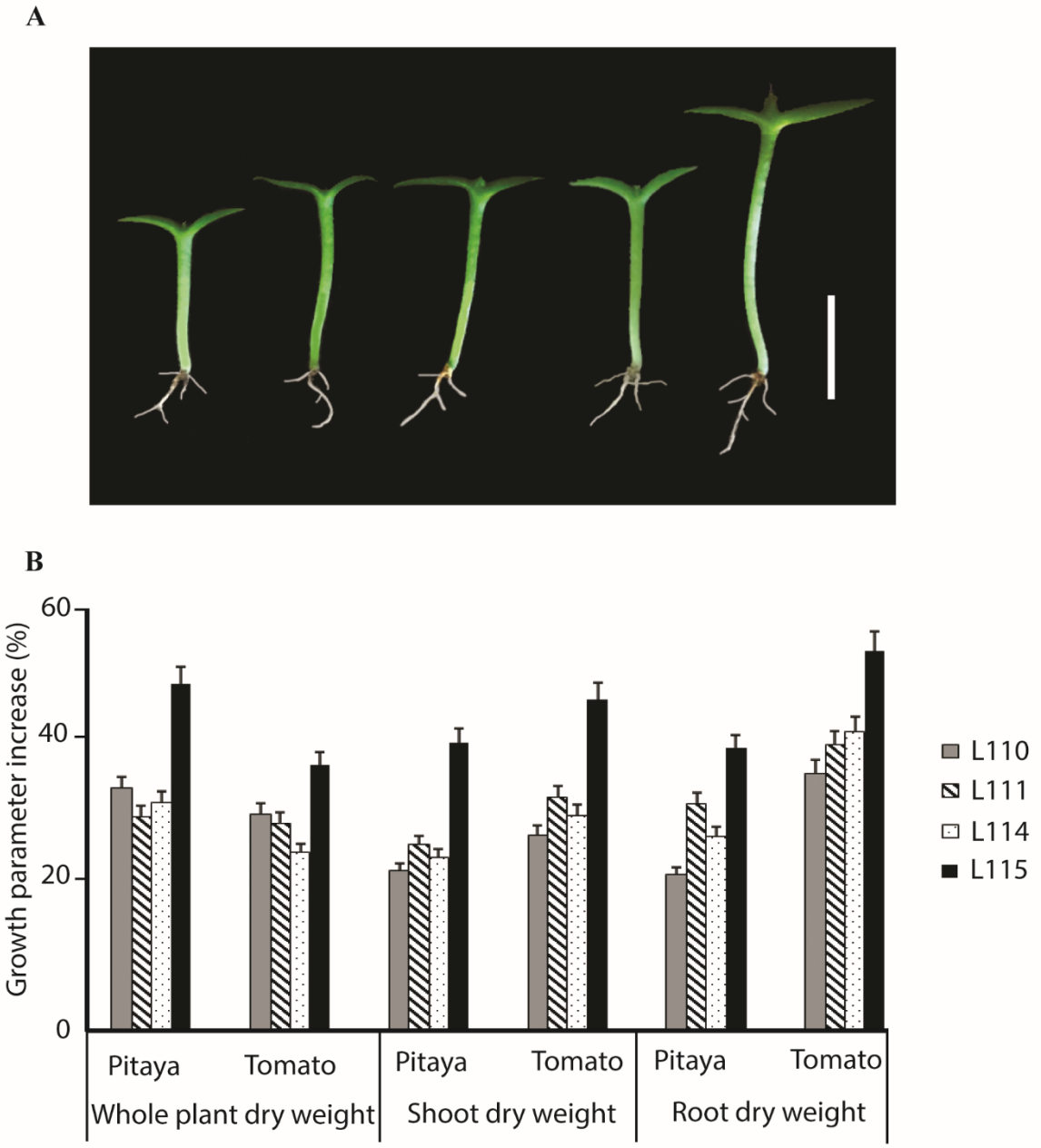

Figure 5. Endophytic bacteria improve the growth parameters of pitaya and tomato plants in greenhouse trials. A. Presentative for increasing the pitaya seedling length (From left to right is seeds immersed in SDW, L110, L111, L114, and L115). B. Illustration for improvement of plant biomass. Mean percentage increases of *in vivo* plant growth parameters for the most effective endophytic bacterial strains assessed in this study; scale bar: 1cm

The data presented in Figure 5 showed that the plants treated with the antagonistic endophytes had an improvement in plant growth parameters compared to the control, in which seeds were immersed in SDW. Among those, in both plants inoculated with strain L115 significantly improved for growth parameters. A weak enhancement of plant growth was also observed in both plants inoculated with strain L110, L111, and L114. The highest percentage of whole plant dry weight was also recorded in pitaya and tomato seedlings treated with L115 strain with an increase of 47.82 and 36.50%, respectively.

DISCUSSION

In this study, the biocontrol potential of weed endophytes that are associated with plant growth promotion was investigated. We used *in vitro* microbiological techniques to observe the antagonistic functionality of these endophytes on pitaya fungal pathogens. This study mainly aimed to investigate whether any of the weed endophytes have antagonistic activities over the growth of *A. alternata*, a casual of stem end rot disease on pitaya. The antagonistic strains were then further tested for additional growth-promoting activities

including IAA production, phosphate solubilization.

The application of endophytic bacteria for biocontrol of diseases is emerging as an eco-friendly approach in sustainable agricultural practices [26]. The endophytic bacteria and their metabolites were found to have promising potential in the control of pitaya pathogens and diseases [21,25,27]. In this study, 14 endophytic bacteria were isolated from weed (*L. indica*), but only 4 of them presented antifungal activity over the studied fungi through diffusible substances in agar. Further studies showed that strain L115 induced inhibition of mycelial growth of *A. alternata* greater than the other strains (L110, L111, and L114). Luu and coauthors [21] demonstrated that strain EC80, an endophytic bacterium isolated from the weed (*E. colonum*), exhibited the strongest inhibition on the mycelial growth of *A. alternata*, a casual of stem end rot disease on pitaya. The results are also consistent with some previous reports in which the *Bacillus* sp showed a strong potential of inhibition of mycelium growth of many fungal species [28]. Moreover, the result also indicated L110, L111, and L114 are *Pseudomonadaceae* that was also described as the biocontrol plant pathogenic fungi [29].

Previous studies have proved that the bacteria can inhibit fungal growth by producing bioactive compounds such as IAA, extracellular lytic enzymes, siderophores, salicylic acid, antibiotics, and volatile metabolites, such as hydrogen cyanide [30-33]. In this study, the single strain L115 had earlier shown an inhibited effect on biocontrol of *A. alternata*, and also presented the highest IAA-yield and enhanced plant growth parameters. These results suggested L115 strain may have a special function in promoting plant growth and enhancing resistance to disease. These results were consistent with previous reports [21, 34]. On the other hand, the results in this study also showed that the L115 strain produced lytic enzymes (phosphatase or phytase) that might play a role in inhibiting the growth of pathogenic fungi. It is consistent with the result, in which *A. faecalis* S18 and *B. cereus* produced lytic enzymes such as chitinases and/or proteases among other substances to inhibit the mycelial growth of pathogens and form an inhibition zone [34].

Moreover, the results from the antagonistic assay of filtrate might be explained due to the L115 strain producing the external metabolites (such as diffusible antifungal compounds) into inhibition zone. These compounds caused leakage of cellular electrolytes from the mycelium of *A. alternata* resulting in the shrinkage of hyphae. The leakage of electrolytes from fungal membranes has previously been reported as an indicator of cell membrane damage [35,36]. The results of the present study suggest the production of antifungal compounds as one of the possible mechanisms of action of these endophytic bacterial strains on the tested fungal pathogens.

It is well known that the biocontrol process mediated by plant growth-promoting rhizobacteria (PGPR) relies on multiple mechanisms. Among those, biofilm formation plays an important role in the ability of PGPR to control plant diseases [37]. Our results showed the biofilm formation of these bacteria that indicated the vital roles of bacteria cells in the inhibition of the disease. They may colonize in the seedlings forming biofilm and consistently exerting their antagonistic effect, which also requires further study to validate. It is said that successful colonization plays an important role in the function of antagonistic bacteria on the plant [38] and bacterial biofilms can ensure the long-term colonization of a host [39]. However, the plant was reported as the host of massive amounts of microbes [40], which might prevent the colonization and reduce the function of antagonistic bacteria. Therefore, an important requirement for maximizing the antagonistic efficacy of the bacteria is to optimize the inoculation conditions that promote biofilm formation and improves bacterial antagonists.

Using PGPR inoculants as biofertilizer provides a promising alternative/amendment to chemical fertilizers. The availability of soil microorganisms to convert insoluble forms of phosphorus to a soluble form is an important trait in plant growth-promoting bacteria for increasing yields. The current study shows that the inoculation of plants with endophytic bacteria with phosphate solubilization (PSB) and IAA production resulted in greater plant growth than un-inoculated plants. These results are consistent with some previous reports, in which the use of PSB enhanced growth, yield, and quality in many crops including walnut, apple, maize, rice, mustard, oil palm, aubergine and chili, soybean, wheat, sugar beet, sugarcane, chickpea, peanut and legumes, and potatoes [36,41]. It is proposed that the antagonistic bacteria in this study produced extracellular metabolites (such as GA) that resulted in the release of soluble phosphate and subsequently up-took by the plant [42]. This proposal is supported by some studies that reported that PSMs have shown to enhance P uptake, growth, and yield when applied to crop plants [43,44]. Moreover, the results also showed that these bacteria produced other plant growth promotion traits such as IAA production which may also have contributed to the enhanced growth of the inoculated plants and playing a role in the interaction between plant pathogens and host [44]. In addition, our data also presented the variation in promoting plant growth of pitaya and tomato by all tested strains. This might involve to the different interaction between endophytic bacteria and host plant such as the different colonization populations of antagonistic bacteria

around the roots of host plants. All of these results suggest the potential application of L115 in biocontrol of phytopathogens in future.

CONCLUSION

Endophytic bacteria from weed is a source of natural bioactive compounds for industry and agriculture. In this study, 14 endophytic bacteria were isolated from *L. indica* and four of them could differently inhibit the mycelial growth of *A. alternata.* Based on the morphological observation and biochemical results, the L115 strain could belong to the *Bacillaceae* family and the other threes (L110, L111, and L114) could be members of the *Pseudomondasea* family. Strain L115 strains inhibited strongly the mycelial growth with a mean inhibition diameter of 12.67 ± 0.02 mm while a weak inhibition was observed for the L110, L111, and L114. Furthermore, both L114 and L115 showed positive results in phosphate solubilization and biofilm tests and only L115 had a positive for the IAA test. In addition, the filtrate of L115 presented an antagonistic effect on A*. alternata* suggesting the vital role of extracellular metabolites in inhibiting the pathogenic growth. In greenhouse experiments, the L115 improved the seedling biomass. Hence, the L115 is potentially very useful for applications in the areas of agronomy and should be further explored in the near future.

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