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ARTICLE

Can metal-tolerant endophytic biocontrol agents promote plant-growth under metal stress?

Carrie Siew Fang Sim[1,2], Yuen Lin Cheow[1], Si Ling Ng[3], Adeline Su Yien Ting[1,2]*

[1] School of Science, Monash University Malaysia, Jalan Lagoon Selatan, 47500 Bandar Sunway, Selangor, Malaysia.
[2] Tropical Medicine and Biology Multidisciplinary Platform, Monash University Malaysia, Jalan Lagoon Selatan, 47500 Bandar Sunway, Selangor, Malaysia.
[3] School of Chemical Sciences, Universiti Sains Malaysia, 11800 USM, Penang, Malaysia.

ABSTRACT Five metal-tolerant endophytic isolates (*Bipolaris sp*. LF7, *Diaporthe miriciae* LF9, *Trichoderma asperellum* LF11, *Phomopsis asparagi* LF15, *Saccharicola bicolor* LF22), with known metal-tolerance attributes and biocontrol activities against *Ganoderma boninense*, were tested for growth-promoting activities independent of (*in vitro)* and associated with plants (height, weight, root mass and stem circumference) (*in vivo*). Results revealed that metal-tolerant endophytes did not significantly render benefit to host plants as plant growth was compromised by the presence of metals. Lower production of indole-acetic acid (0.74-21.77 μg mL^{-1}), siderophores (8.82-90.26%), and deaminase activities of 1-aminocyclopropane carboxylic acid (3.00-69.2 μmol mg $protein^{-1}$ hr^{-1}) were observed.
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***Corresponding author**
E-mail: adelsuyien@yahoo.com
adeline.ting@monash.edu

Introduction

Endophytes are a group of microorganisms known to colonize plant tissues asymptomatically. They render numerous benefits to the host plants, which include protecting host plants against pathogens, herbivores and insect pests (Rúa et al. 2013); enhancing tolerance towards unfavourable environmental conditions (Naveed et al. 2014; Zhang et al. 2011); and stimulating plant growth (Nascimento et al. 2016). Endophytes that are biocontrol agents (BCAs), typically have multiple mechanisms to suppress pathogen; via inhibitory compounds, induced host resistance, and by improving growth and vigour of host plants.

Several endophytic species have been reported to stimulate plant growth successfully. They include *Penicillium chrysogenum*, *P. crustosum*, *Piriformospora indica, Serratia quinivorans*, *Bacillus cereus*, *B. subtilis*, *Pseudomonas aeruginosa* and *Pantoea amanatis* (Sirrenberg et al. 2007; Nascimento et al. 2016; Hassan 2017; Wu et al. 2018). These endophytes stimulate plant growth by solubilising phosphate (*P. chrysogenum*, *P. crustosum, B. cereus*, *B. subtilis*), producing iron-chelating siderophores (*Ps. aeruginosa*), producing indole-acetic acid to stimulate root growth (*Pi. indica*), and by modulation of the hormone ethylene via the deaminase activity of 1-aminocyclopropane carboxylic acid (*Pa. amanatis*). Improved plant growth is important as it contributes to plant vigour and delays disease progression and the onset of disease symptoms (Ting 2014).

In this study, the growth-promoting activities of metal-tolerant endophytes were investigated under metal stress. Metal-tolerant endophytes are a new group of biocontrol agents that are hypothesized to render benefits to host plants in metal-laden soils. In earlier studies, these metal-tolerant endophytes were discovered to demonstrate biocontrol activities towards *Ganoderma boninense,* a causal agent of Basal Stem Rot disease of oil palm (Sim et al. 2019a, 2019b, 2019c). In those studies, the mechanisms of action were attributed to antifungal compounds produced. For this study, the ability of metal-tolerant endophytes in promoting plant-growth is determined. This observation is important as it suggests the potential role of metal-tolerant endophytic biocontrol agents in enhancing plant growth in the presence of metals and pathogen. Furthermore, various landscapes are now being used for oil palm planting; from desirable inland top-soils, to undesirable peat swamp and acid sulphate

soils. While inland terrain soils are suitable soils, peat and acid sulphate soils are acidic and has bioavailable forms of metal cations such as copper (Cu^{2+}), lead (Pb^{2+}), zinc (Zn^{2+}) and cadmium (Cd^{2+}), prompting the exploration of metal-tolerant endophytes. According to Zarcinas et al. (2004), Cu^{2+} levels in soils were between 10.1-47.2 mg kg^{-1}, for Zn^{2+} between 27.6-40.0 mg kg^{-1}, for Pb^{2+} between 23.9-29.0 mg kg^{-1}, and between 0.1-0.4 mg kg^{-1} for Cd^{2+}. With the presence of metals in soils, plant growth is generally affected due to metal toxicity. Therefore, to mitigate this, metal-tolerant endophytes with plant growth promoting properties (in addition to biocontrol potential) are investigated for potential as biocontrol agents. This is to enable the management of disease via plant growth promotion, albeit under metal stress conditions.

This study aims to establish the role of these endophytes in promoting plant growth, and this was evaluated under the influence of metal-stress, as the endophytes used are metal-tolerant endophytes. The endophytes were first tested via *in vitro* assays to determine their expression of plant growth-promoting attributes. The endophytes were exposed to conditions with and without metal stress, and the productions of the following compounds (or their activities) were quantified: indole-acetic acid (IAA), siderophore, phosphate solubilisation, and deaminase activity of 1-aminocyclopropane carboxylic acid (ACC). Two isolates with the most promising activities were subsequently selected for inoculation to oil palm ramets to validate their impact on growth (height, weight, root mass and stem circumference). This study is one of the few publications documenting the influence of metals on the growth-promoting activities of metal-tolerant biocontrol endophytes. This provides an overview of the possible use of these endophytes to improve plant growth and vigour, and in suppressing disease in metal-laden soils.

Materials and methods

Both *in vitro* and *in vivo* tests were performed to evaluate the growth promoting activities of the metal-tolerant biocontrol endophytes. The *in vitro* tests involved quantification of indole-acetic acid (IAA), siderophore production, phosphate solubilisation, and deaminase activity of 1-aminocyclopropane carboxylic acid (ACC) produced by the endophytes. In the *in vivo* assessment, growth parameters such as height, weight, root mass and stem circumference were measured to determine plant-growth promoting activities of two selected endophytes. Both *in vitro* and *in vivo* tests were carried out in conditions under metal-stress and in the absence of metal stress.

Culture establishment of endophytes and preparation of metal solutions

Five metal-tolerant endophytes previously identified by Sim et al. (2018) as *Bipolaris* sp. LF7 (GenBank accession no. KX510121), *Diaporthe miriciae* LF9 (GenBank accession no. KX398059), *Trichoderma asperellum* LF11 (GenBank accession no. KX510127), *Phomopsis asparagi* LF15 (GenBank accession no. KX510125) and *Saccharicola bicolor* LF22 (GenBank accession no. KX510132), were selected for this study. These endophytes were isolated from the phytoremediator plant *Phragmites* sp. and demonstrated strong antifungal activities towards the pathogen *Ganoderma boninense*: they were able to suppress disease development when applied to oil palm seedlings (Sim et al. 2019a, 2019b). The isolates were cultured, incubated and maintained on Potato Dextrose Agar (PDA, Merck) (7 days, 25 ± 2 °C) for subsequent tests.

Stock solutions of various metal solutions were prepared to 1000 mg L^{-1}. This includes for $Cd(NO_3)_2{\cdot}4H_2O$ (Aldrich Chemical), $Cu(NO_3)_2{\cdot}3H_2O$ (Riendemann Schmidt Chemical), $Pb(NO_3)_2$ (Emsure) and $Zn(NO_3)_2{\cdot}6H_2O$ (R&M Chemicals). Prepared stock solutions were diluted to concentrations of 10 and 25 mg L^{-1} (additional 50 mg L^{-1} for Cu^{2+}). The working solutions was adjusted to pH 5 using 0.1 M HCl and 0.1 M NaOH.

Production of indole-acetic acid (IAA)

Three mycelial plugs of each endophyte (5 mm diameter) were inoculated into 100 mL of Potato Dextrose Broth (PDB, Merck) supplemented with 100 mg L^{-1} tryptophan and incubated at 25 ± 2 °C for 28 days. After every 7-day interval, 5 mL of the culture was pipetted into a falcon tube and centrifuged (25 ± 2 °C, 12 000 rpm, 10 min). The supernatant (1 mL) was collected, mixed with 2 mL of Salkowski reagent, and incubated in the dark for 20 min prior to absorbance measurement at 535 nm (Babu et al. 2014). Standard curves were constructed from concentrations of 0-100 μg mL^{-1} and the IAA produced by the endophytes was determined. The influence of metals on the production of IAA was assayed by preparing a similar experimental set-up. The difference in this set-up is that that isolates were cultured in PDB supplemented with Cu^{2+}, Pb^{2+}, Zn^{2+}, Cd^{2+} (at concentrations of 10 and 25 mg L^{-1} for each metal, an additional 50 mg L^{-1} for Cu^{2+}). The supernatant obtained were subjected to similar assay conditions.

Production of siderophore

Production of siderophore by the endophytes was determined via qualitative and quantitative assays. For qualitative analysis, Chrome azurol S (CAS) was used as an indirect method to indicate the production of siderophore. This dye competes with siderophores for iron. The endophytes were first qualitatively assessed

for siderophore production using the modified half CAS blue agar method (Machuca and Milagres 2003). The CAS blue agar was prepared according to Louden et al. (2011). Half of the agar (PDA) was cut and replaced with the CAS blue agar. The mycelial plug of the endophyte (5 mm diameter) was subsequently inoculated onto the PDA and incubated (28 days, 25 ± 2 °C). At every 7-day interval for the next 28 days, colour changes of the CAS agar were observed and measured. Deep colourisation of the CAS dye indicated that more iron bonded to the dye due to lesser siderophore production/competition. In contrast, lighter dye intensity indicated higher siderophore production/competition as less iron is available to bind to the dye (lighter intensity). This reflected that more iron is uptake by siderophores. The influence of metals on the production of siderophore was determined by preparing a similar CAS blue agar set-up, with the CAS agar supplemented with Cu^{2+}, Pb^{2+}, Zn^{2+}, Cd^{2+} at 10 and 25 mg L^{-1} (and an additional 50 mg L^{-1} for Cu^{2+}).

The siderophore production by endophytes was also quantified. This quantitative assay is a less common approach used compared to the qualitative CAS blue agar technique. Nevertheless, it allows for the quantification of siderophores for comparative purpose. Three mycelial plugs of each endophyte were firstly inoculated into 100 mL PDB (28 days, 25 ± 2 °C). At every 7-day interval for the following 28 days, 1 mL of the supernatant was mixed with 1 mL of CAS solution and incubated for 60 min. Absorbance was read at 630 nm and siderophore production (%) was determined (Eq. 1) (Machuca and Milagres 2003). The influence of metals on the production of siderophore in broth was evaluated by preparing a similar set-up, using supernatant recovered from cultures established in PDB supplemented with Cu^{2+}, Pb^{2+}, Zn^{2+}, Cd^{2+} at 10 and 25 mg L^{-1} (and an additional 50 mg L^{-1} for Cu^{2+}).

$$\text{Production of siderophore (\%)} = \frac{\text{(Initial absorbance-Absorbance of sample)}}{\text{Initial absorbance} \times 100}$$

Production of siderophore (%) = (Initial absorbance-Absorbance of sample) / Initial absorbance × 100%

Phosphate solubilisation

The endophytes (mycelial plug, 5 mm diameter) were inoculated onto Pikovskya's agar (HiMedia Laboratories) and incubated (28 days, 25 ± 2 °C). Phosphate solubilisation is indicated by the formation of a clear halo zone on the agar. The phosphate solubilisation capacity of the endophytes is quantified as phosphate solubilizing efficiency (SE, %) (Eq. 2). The SE (%) were observed for day 7th, 14th, 21st and 28th (Srivastav et al., 2004). To determine phosphate solubilisation activities of endophytes under metal stress, the procedure was repeated using Pikovskya's agar supplemented with Cu^{2+}, Pb^{2+}, Zn^{2+}, Cd^{2+} (at 10 and 25 mg L^{-1}, and an additional 50 mg L^{-1} for Cu^{2+}.

$$\text{Solubilising efficiency (SE)(\%)} = \frac{\text{(Diameter of solubilization zone-Diameter of colony)}}{\text{Diameter of colony} \times 100}$$

Solubilising efficiency (SE)(%) = (Diameter of solubilization zone-Diameter of colony) / Diameter of colony × 100

Deaminase activity of 1-aminocyclopropane carboxylic acid (ACC) of endophytes

Three mycelial plugs (5 mm diameter) of each endophyte were inoculated into flasks containing 100 mL PDB (28 days, 25 ± 2 °C). The culture was allowed to grow and the mycelium was then filtered. The filtrate was collected and 200 µL was pipetted and reacted with 25 µL toluene and vortexed for 30 sec (Saleh and Glick 2001). Then, 20 µL of 0.45 M ACC of the enzyme ACC was added and incubated at 30 °C. After 10 min, the reaction was stopped with 1 mL of 0.56 N HCl. The lysates were centrifuged (10 000 *g*, 10 min), and 1 mL of the supernatant was pipetted and mixed with 800 µL of 0.56 N HCl and 300 µL of 2,4-dinitrophenylhydrazine. The mixture was incubated (30 min, 30 °C) and added with 2 mL of 2 N NaOH. The absorbance was measured at 540 nm and the ACC deaminase activity was calculated based on standard curves constructed. The ACC deaminase activities by endophytes under the influence of metals was assayed by preparing a similar set-up, using endophyte suspension recovered from cultures established in PDB supplemented with Cu^{2+}, Pb^{2+}, Zn^{2+}, Cd^{2+} at 10 and 25 mg L^{-1} (and an additional 50 mg L^{-1} for Cu^{2+}). The assay was carried out in a similar manner.

In vivo *plant growth promoting activities of selected endophytes*

Two endophytic isolates (*D. miriciae* LF9 and *T. asperellum* LF11) with the most promising results observed from the *in vitro* assays were selected. The isolates were first cultured in 250 mL PDB (14 days, 25 ± 2 °C). The mycelium was then homogenized using LabGen 125 homogenizer (Cole-Parmer, USA) and the homogenized mycelium was adjusted to a concentration of 10^6 of colony forming unit (cfu mL^{-1}) each. The adjusted inoculums were prepared in 100 mL and were used to inoculate the

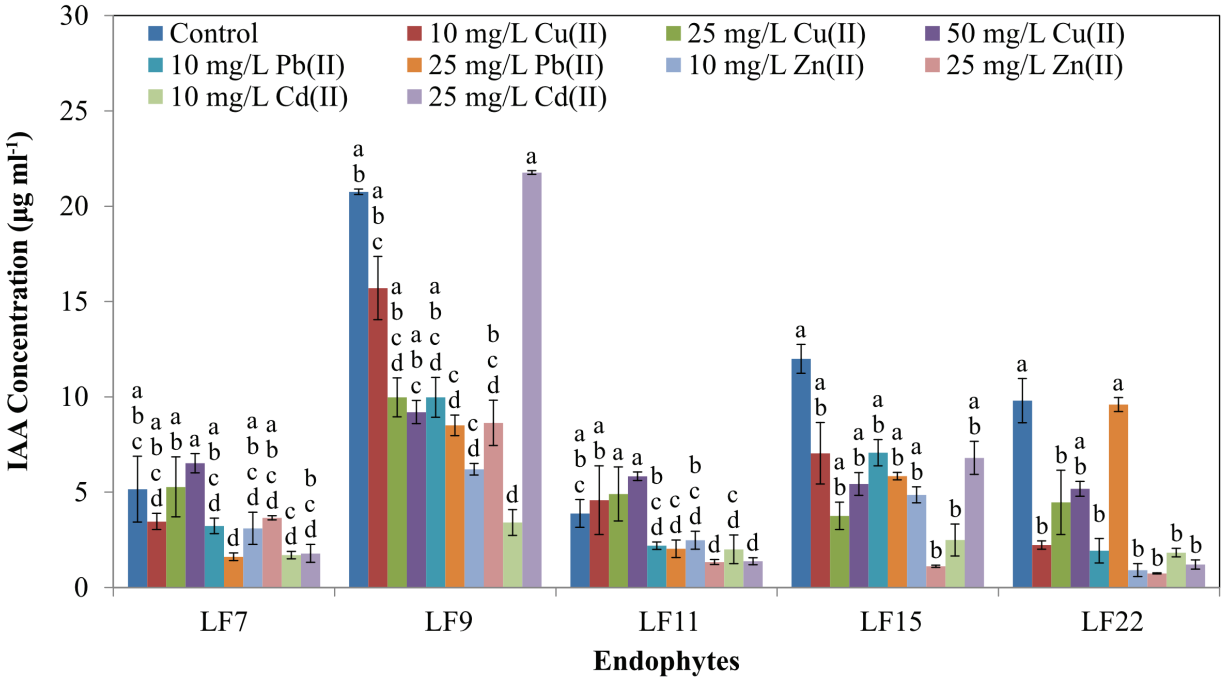

Figure 1. Indole-acetic acid (IAA) (µg mL^{-1}) produced by metal-tolerant endophytes (*Bipolaris sp*. LF7, *D. miriciae* LF9, *T. asperellum* LF11, *P. asparagi* LF15, *S. bicolor* LF22) after 28 days incubation with exposure to Cu^{2+}, Pb^{2+}, Zn^{2+} and Cd^{2+}. Controls were incubations without metal supplementation. Bars indicate standard deviations of means (±SD). Means with the same letters for each isolate are not significantly different ($HSD_{(0.05)}$).

soils via soil-drenching. The tissue-cultured ramets, at 3-4 leaf stage and 14-16 cm in height, were then planted into the drenched soils and allowed to grow in shade (28 ± 2 °C, 12 h photoperiod). For control, soil drenching was performed using sterile distilled water. Solutions for each metal (Cu^{2+}, Pb^{2+}, Zn^{2+}, Cd^{2+} at 10 and 25 mg L^{-1}; additional 50 mg L^{-1} for Cu^{2+}) were used to water the soil to create metal-laden soils. Controls were established with endophyte-free ramets watered using sterile distilled water. Growth parameters such as height, weight, root mass and stem circumference were measured at 7th, 14th, 21st and 28th day.

Statistical analysis

All the experiments/assays were carried out in triplicates, with data analysed using ANOVA (Analysis of Variance). The means and standard deviations were compared with Tukey comparisons ($HSD_{(0.05)}$) using the Statistical Packaging for the Social Science (SPSS) (software version 20.0).

Results and Discussion

Production of indole-acetic acid (IAA)

Lower levels of IAA were produced by endophytes subjected to metal stress (0.74-21.77 µg mL^{-1}) compared to non-metal conditions (control) (3.88-20.76 µg mL^{-1}). Among the isolates tested, *D. miriciae* LF9 was the least affected (3.41-21.77 µg mL^{-1} IAA production) whilst *T. asperellum* LF11 was the most susceptible to presence of metals as minimal IAA levels were detected (1.33-5.83 µg mL^{-1}) (Fig. 1). In the absence of metals, higher IAA production by *D. miriciae* LF9 (20.76 µg mL^{-1}), *P. asparagi* LF15 (11.99 µg mL^{-1}), *S. bicolor* LF22 (9.80 µg mL^{-1}), *Bipolaris* sp. LF7 (5.16 µg mL^{-1}) and *T. asperellum* LF11 (3.88 µg mL^{-1}) (Fig. 1) were detected. This suggested that the production of IAA and subsequently their growth promoting effect was influenced by the presence/absence of metals. Nevertheless, the production of IAA by all isolates under metal stress were somewhat maintained throughout the 28 days (Supplementary Fig. 1), although the IAA levels were lower than observations from endophytes in non-metal conditions.

It was evident that metals affected the production of IAA by the isolates. This was also observed by Acuna et al. (2011) in which the presence of metals such as Fe and Al reduced IAA production by *Bacillus* sp. and *Paenibacillus* sp.. The reduction in IAA production under metal stress was presumably attributed to the complexation of metal cations with IAA (Du et al. 2011). This suggested that metals lowered the production of IAA, hampering root elongation (Hilbert et al. 2012; Sukumar et al. 2012) and

Table 1 Chrome Azurol S (CAS) reaction rates of the isolates (mm/day) under the influence of metals after 28 days.

Endophytes	Metal concentrations (mg L^{-1})									
	Cu			Pb		Zn		Cd		Control
	10	25	50	10	25	10	25	10	25	0
Bipolaris sp. LF7	±	±	±	±	±	±	±	±	±	±
D. *miriciae* LF9	±	±	±	±	+	+	+	+	+	+
T. asperellum LF11	±	±	±	+	+	±	+	+	+	+
P. asparagi LF15	±	±	±	±	±	+	±	+	+	±
S. bicolor LF22	±	±	±	±	±	±	±	±	±	±

Note: ± for rates < 0.5 mm/day (mild) and + for rates between 0.5-1.0 mm/day (moderate)

consequently implicating water and nutrient uptake. It was also interesting to note that the metal tolerance attributes of the endophytes, did not render "immunity" to the isolates from the influence of metals on IAA production.

The production of IAA as a growth-promoting factor has been reported in various species of *Trichoderma* and *Phomopsis*. The low levels of IAA produced by *T. asperellum* LF11 (highest at 5.83 μg mL^{-1} IAA) was similar to observations by Gravel et al. (2007) that showed *T. atroviride* (another species of *Trichoderma*) producing only 6.20 μg mL^{-1} IAA. The production of IAA by *P. asparagi* LF15 in this study further strengthen the IAA-producing capacity of *Phomopsis* sp. This was also observed by Chen et al. (2011) and Chithra et al. (2017) in *Phomopsis* sp. isolated from *Piper nigrum,* and *P. liquidambari* from *Bischofa polycarp*, respectively. On the contrary, IAA production by *S. bicolor* LF22, *D. miriciae* LF9 as well as *Bipolaris* sp. LF7, were detected and reported for the first time. To summarize, results here highlighted that presence of metals generally reduced the production of IAA in endophytes, subsequently leading to absence or weak stimulation of root growth in the presence of metals. And the metal-tolerant endophytes were also not exempted from this metal-IAA production interaction.

Production of siderophore

The plate assay revealed that in the presence of metals, the reaction rates by *D. miriciae* LF9 and *T. asperellum* LF11 were generally moderate (0.5-1.0 mm/day) whilst *Bipolaris* sp. LF7, *P. asparagi* LF15 as well as *S. bicolor* LF22 showed mild CAS reaction rates (<0.5 mm/day) (Table 1). The reaction rates remained throughout the 28 days (Supplementary Table 1). The reaction rates for endophytes in CAS plates without metals remained similar as well (Table 1). Since the observation using CAS blue agar was deemed to be preliminary, presumptive and inconclusive, the quantitative test was performed.

The quantification of siderophore production revealed that under the influence of various metals, siderophore production was generally lower (8.82-90.26%) in most endophytes (except *T. asperellum* LF11) compared to non-metal solutions (66.75-84.44%). Isolates *S. bicolor* LF22, *P. asparagi* LF15, *D. miriciae* LF9 and *Bipolaris* sp. LF7, were susceptible to the presence of some of the metals, resulting in lower siderophore production; ranging from 8.82-80.51%, 0.08-78.20%, 32.81-76.05% and 21.94-78.24%, respectively (Fig. 2). In the absence of metals, siderophore production was generally higher for all isolates; *T. asperellum* LF11 (84.44%), *P. asparagi* LF15 (75.92%), *D. miriciae* LF9 (74.32%), *Bipolaris* sp. LF7 (73.70%) and *S. bicolor* LF22 (66.75%) (Fig. 2). Among the isolates, only isolate *T. asperellum* LF11 appeared to be less susceptible to metals with levels of siderophore production (19.21-90.26%) comparable to control. Nevertheless, siderophore production was maintained throughout the 28 days for the isolates, even in the presence of metal stress (Supplementary Fig. 2). The discovery of siderophore production by *P. asparagi* LF15, *D. miriciae* LF9, *Bipolaris* sp. LF7 and *S. bicolor* LF22 are relatively novel as limited reports are available on this. By contrast, the siderophore-producing attribute in *T. asperellum* LF11, is well known as it has been reported for other *Trichoderma* sp. such as *Trichoderma virens* (Babu et al. 2014). *Trichoderma* spp. are one of the most common filamentous fungi with siderophore-producing capacity, alongside *Aspergillus* spp. and *Penicillum menonorum* (Babu et al. 2015; Machuca and Milagres 2003).

The CAS assays also revealed that in some instances, the colour intensity does not reflect the CAS production of the endophytes. The lower concentrations of siderophore (from liquid medium) recorded, were attributed to the nature of this compound to form complexes with other metal cations other than the desired iron (Fe^{2+}) (Dimkpa et al. 2008; Aguado-Santacruz et al. 2012). Since metal cations are abundant in this study, the siderophores could have bind easily to any cations supplemented (Cu^{2+}, Pb^{2+}, Zn^{2+}, Cd^{2+}). The ability of siderophores to bind to metal cations not only aids in enhancing tolerance to metal-stress conditions, it is also a means of bioremediation. This mechanism has been exploited to bind toxic metals and consequently removing the toxic metals from the

environment. Thus, siderophore production is a highly valuable trait for biocontrol agents applied to metal-laden soils. With the production of siderophores, the biocontrol agents is expected to ameliorate the soil environment as toxic metals are removed, thus improving survivability of plants in metal-contaminated sites. Siderophores produced by biocontrol agents also deprives iron from pathogens, thus suppressing the growth of pathogens (Verma et al. 2011; Yu et al. 2011; Beneduzi et al. 2012).

Phosphate solubilisation

Four of the five endophytic isolates solubilised phosphate, in both the presence or absence of metals. Formation of clear halo zones was detected on Pikovskya's agar with and without metal supplementation. Results further suggested that phosphate solubilisation by the endophytes were higher in the presence of metals (3.94-79.82%) than in non-metal (17.64-20.83%) conditions. Higher phosphate solubilisation efficiency under metal stress was demonstrated by *P. asparagi* LF15 (18.81-79.82%) (Fig. 3). Solubilisation of phosphate was also regulated by *D. miriciae* LF9 (12.43-56.24%), *Bipolaris* sp. LF7 (3.94-34.00%) and *T. asperellum* LF11 (12.44-22.07%) (Fig. 3). The gradual increase in phosphate solubilisation by the isolates was observed throughout the 28 days (Supplementary Fig. 3). By contrast, the absence of metals resulted in inferior phosphate solubilisation activities by *D. miriciae* LF9 (20.83%), followed by *P. asparagi* LF15 (18.4%) and *Bipolaris* sp. LF7 (17.64%) (after 28 days). Interestingly, phosphate solubilisation was, however, not observed in *T. asperellum* LF11 (Fig. 3) in the absence of metal stress (control). This is presumably due to the rapid growth rate of LF11 (to the extent of overgrowing) on the metal-free agar. Phosphate solubilisation was also absent in *S. bicolor* LF22, when cultured in the absence and presence of metals, as no formation of halo zones were detected. This suggested that the isolate *S. bicolor* LF22 is not a phosphate solubilizer.

The findings in this study, which showed endophytes (except *S. bicolor* LF22) having generally higher phosphate solubilisation under metal stress, agreed with Zúñiga-Silva et al. (2015). Endophytes are proven to have similar capacity as other phosphate-solubilising fungi to solubilize

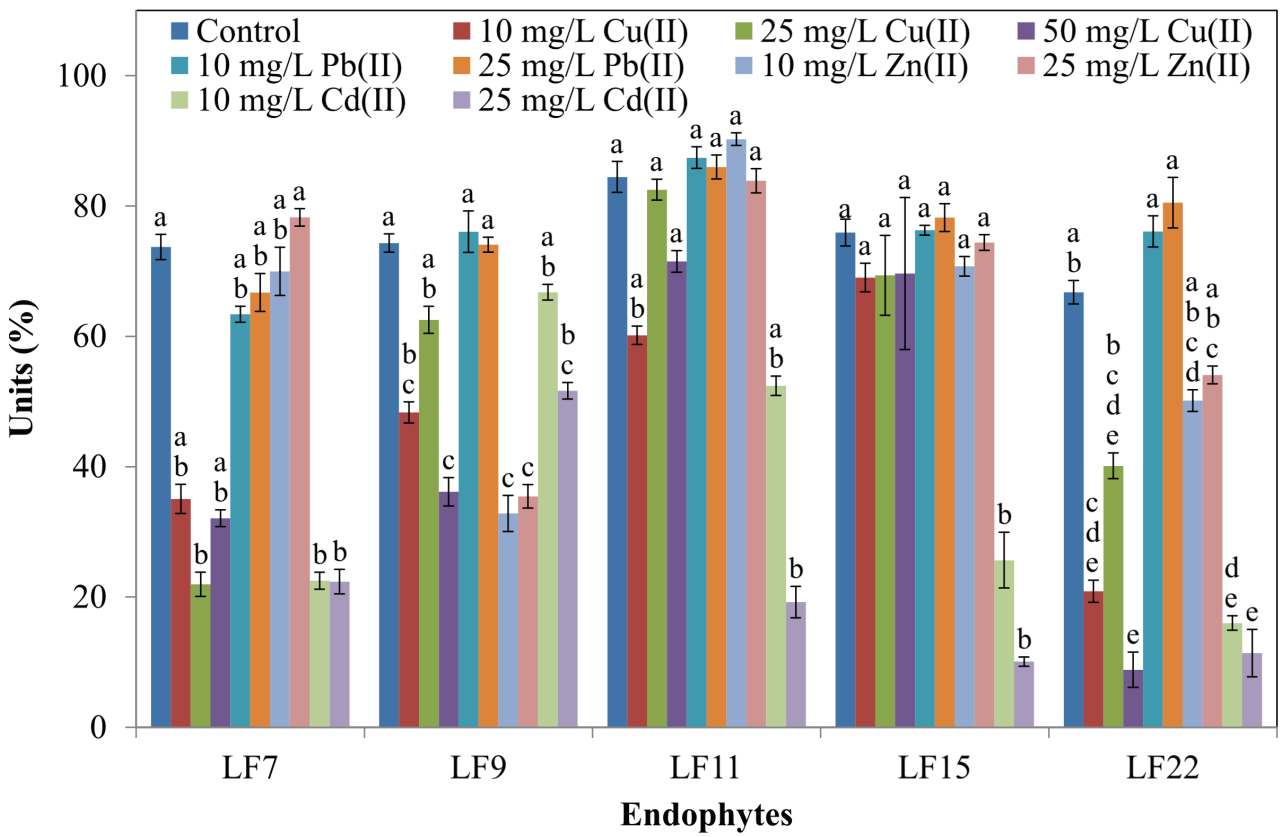

Figure 2. Siderophore production (%) by metal-tolerant endophytes (*Bipolaris sp*. LF7, *D. miriciae* LF9, *T. asperellum* LF11, *P. asparagi* LF15, *S. bicolor* LF22) after 28 days incubation with exposure to Cu^{2+}, Pb^{2+}, Zn^{2+} and Cd^{2+}. Controls were incubations without metal supplementation. Bars indicate standard deviations of means (±SD). Means with the same letters for each isolate are not significantly different ($HSD_{(0.05)}$).

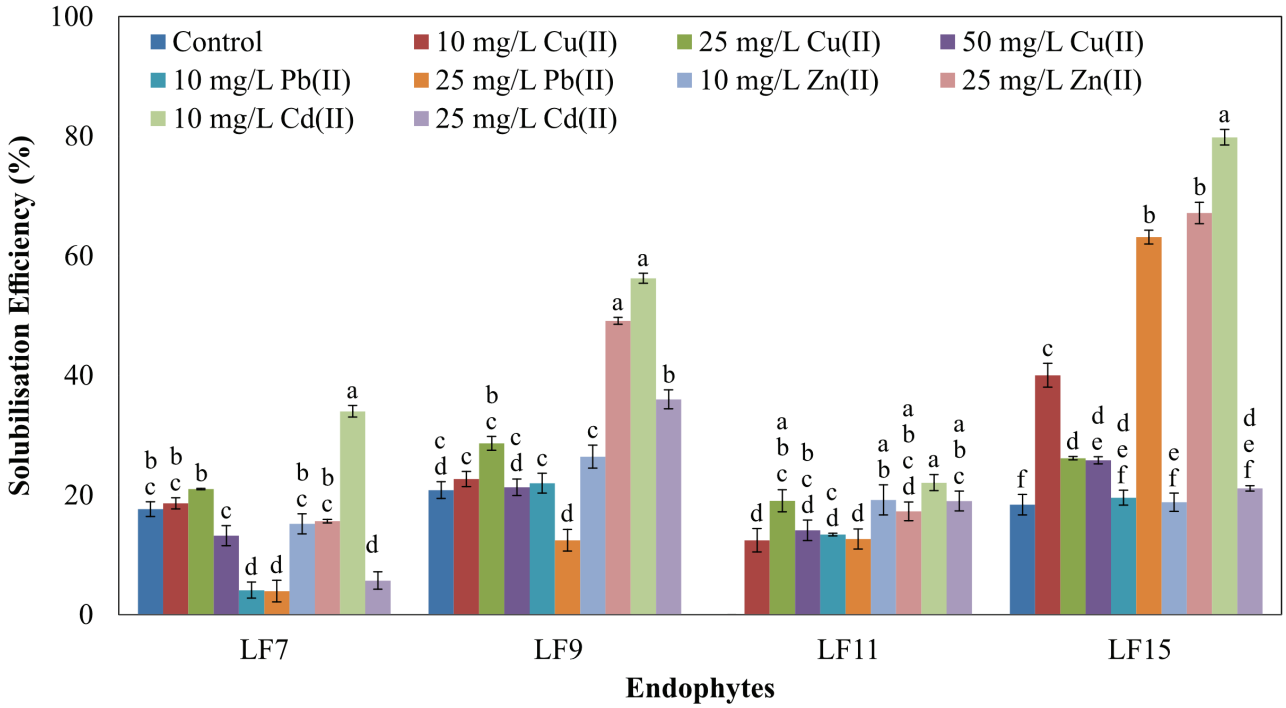

Figure 3. Phosphate solubilisation efficiency (%) by metal-tolerant endophytes (*Bipolaris sp*. LF7, *D. miriciae* LF9, *T. asperellum* LF11, *P. asparagi* LF15, *S. bicolor* LF22) after 28 days incubation with exposure to Cu^{2+}, Pb^{2+}, Zn^{2+} and Cd^{2+}. Controls were incubations without metal supplementation. Bars indicate standard deviations of means (±SD). Means with the same letters for each isolate are not significantly different ($HSD_{(0.05)}$).

phosphate for uptake and use. Phosphate-solubilising fungi acidify and secrete siderophores to solubilize the naturally-limited phosphate into available forms, particularly in response to metal stress, and this ultimately results in the dissolution of bound phosphate (Walpola and Yoon 2013). This study has revealed for the first time, the phosphate-solubilising potential in endophytic *Bipolaris* sp. LF7, *D. miriciae* LF9 and *P. asparagi* LF15, as well as the non-phosphate solubilising activity of *S. bicolor* LF22. Existing literature on this is limited, although studies on phosphate-solubilizing by *Trichoderma* sp. has been reported. This study, which revealed phosphate solubilisation by *T. asperellum* LF11 in both the presence and absence of metals agreed with Zúñiga-Silva et al. (2015) (using *T. atroviride*), suggesting that phosphate solubilisation could be a common trait for *Trichoderma* sp.. In short, the endophytes tested in this study were able to solubilise phosphate in both conditions (in the absence and presence of metal stress), except for *S. bicolor* LF22. The increased phosphate-solubilising activities under metal stress conditions suggested higher phosphate acquisition by endophytes when applied in metal-laden soils.

Deaminase activity of 1-aminocyclopropane carboxylic acid (ACC)

This assay measures the α-ketobutyrate, one of the products of the 1-aminocyclopropane carboxylic acid (ACC) deaminase activity. It was revealed that ACC deaminase activity in endophytes were generally lower when exposed to metals (3.00-69.2 µmol mg protein^{-1} hr^{-1}) as compared to the absence of metals (control) (19.17-54.30 µmol mg protein^{-1} hr^{-1}). Among the five isolates tested, *T. asperellum* LF11 was the least susceptible to metal stress, exhibiting the highest ACC deaminase activity (8.69-69.2 µmol mg protein^{-1} hr^{-1}) especially in the presence of Pb and Cd (Fig. 4). The ACC deaminase activity by *T. asperellum* LF11 is comparable to other *Trichoderma* species, such as *T. atroviride* and *T. virens* (Babu et al. 2014; Gravel et al. 2007). On the contrary, presence of metals severely impacted ACC deaminase activity in the other endophytic isolates, particularly *Bipolaris* sp. LF7. This isolate produced the lowest ACC deaminase activities in the presence of metals (3.00-17.54 µmol mg protein^{-1} hr^{-1}) (Fig. 4). Presence of metals was less detrimental on the ACC deaminase activities of the other three isolates. Isolates *P. asparagi* LF15 (6.23-38.26 µmol mg protein^{-1} hr^{-1}), *D. miriciae* LF9 (6.15-32.83 µmol mg protein^{-1} hr^{-1}) and *S. bicolor* LF22 (7.5-15.41 µmol mg protein^{-1} hr^{-1}) (Fig. 4) retained their ACC deaminase activities under metal stress, but the activities were significantly lower compared to ACC deaminase activities in the absence of metal. In the absence of metals, the highest activity was observed for *D.*

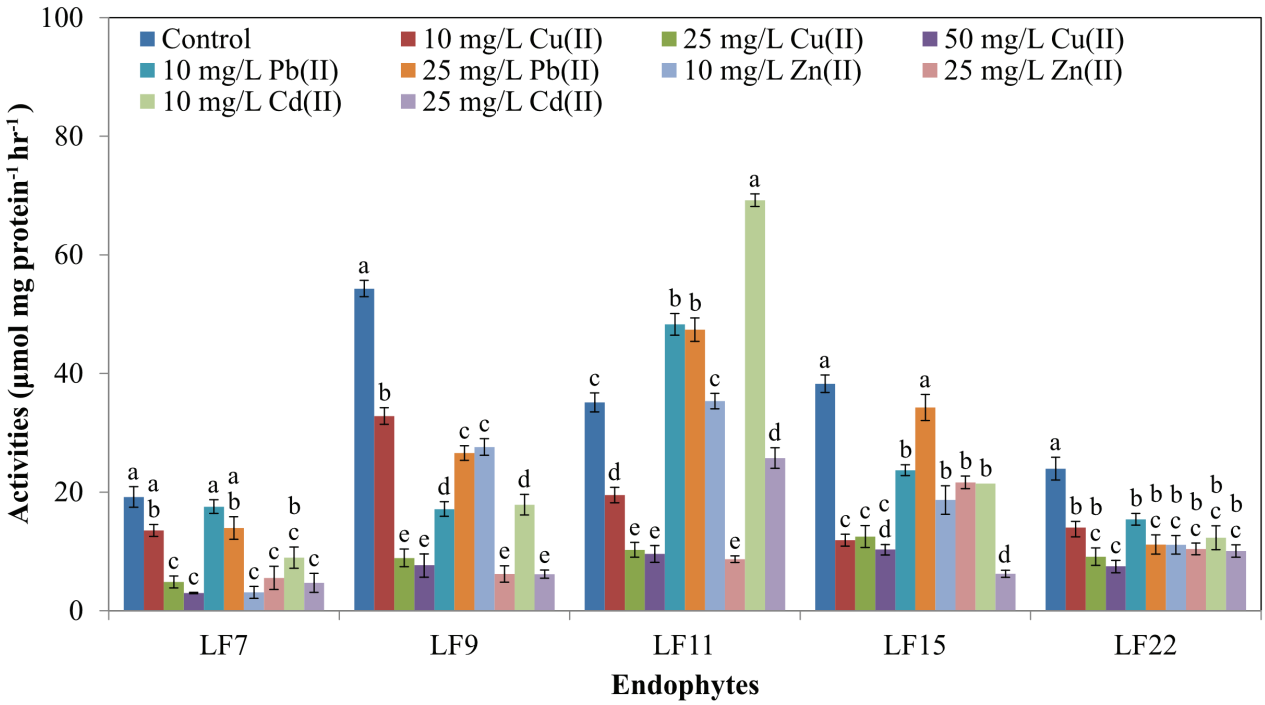

Figure 4. Activities of ACC deaminase (µmol protein mg^{-1} hr^{-1}) by metal-tolerant endophytes (*Bipolaris sp*. LF7, *D. miriciae* LF9, *T. asperellum* LF11, *P. asparagi* LF15, *S. bicolor* LF22) after 28 days incubation with exposure to Cu^{2+}, Pb^{2+}, Zn^{2+} and Cd^{2+}. Controls were incubations without metal supplementation. Bars indicate standard deviations of means (±SD). Means with the same letters for each isolate are not significantly different ($HSD_{(0.05)}$).

miriciae LF9 (54.30 µmol mg protein^{-1} hr^{-1}), followed by *P. asparagi* LF15 (38.26 µmol mg protein^{-1} hr^{-1}), *T. asperellum* LF11 (35.11 µmol mg protein^{-1} hr^{-1}), *S. bicolor* LF22 (23.95 µmol mg protein^{-1} hr^{-1}) and *Bipolaris* sp. LF7 (19.17 µmol mg protein^{-1} hr^{-1}) (Fig. 4). Observations based on time intervals indicated that the ACC deaminase activities were lower in the initial stages, gradually increasing in the next 28 days (Supplementary Fig. 4).

The present study showed that ACC deaminase activities by the endophytes decreased when metals were present as a stress factor. The inhibition of ACC deaminase activities by metals was not specific to just fungal endophytes, as similar observations have been reported on bacteria. Carlos et al. (2016) discovered *Serratia* sp. strain Mc107, having lesser ACC deaminase activities when cultured in the presence of Pb^{2+}, Cu^{2+} and Cd^{2+}. Only *Trichoderma* species appeared to have ACC deaminase activities that were not implicated by presence of metals. Additionally, the initial decreasing trend of ACC deaminase activities followed by a gradual increase in activities follows the model described by Glick et al. (2007). According to the model, the initial decreasing trend was possibly associated with the reaction of ACC deaminase upon ACC to prevent the precursor from converting to ethylene. The activities gradually diminished throughout 21 days in this study, as lesser ACC was available. Following that, environmental stress (metals, in this study) may have triggered follow-up production of ACC (Yang and Hoffman 1984), consequently resulting in a rise in the activities of ACC deaminase. The increase in ACC deaminase activities was to prevent the build-up of the precursor ACC and their further conversion to ethylene. As such, ACC deaminase activities aid in reducing ethylene levels in plants, which enhances plant growth. In general, the decreased ACC deaminase activities of the endophytes under metal stress suggested lower phytohormone modulation in metal-contaminated soils, which could implicate plant growth.

In vivo plant growth promoting activities of selected endophytes

In non-metal soils, improved growth was observed in ramets treated with both selected endophytes (*D. miriciae* LF9 and *T. asperellum* LF11). Of the two isolates, inoculation with *D. miriciae* LF9 showed better growth of ramets with increased height (4.68 cm), weight (1.14 g), root growth (0.39 g) as well as stem circumference (0.30 cm) (Fig. 5). Ramets inoculated with *T. asperellum* LF11 also exhibited similar positive influence; improved height (3.90 cm), weight (0.94 g), root mass (0.32 g) and stem circumference (0.18 cm) (Fig. 5). This further verified the

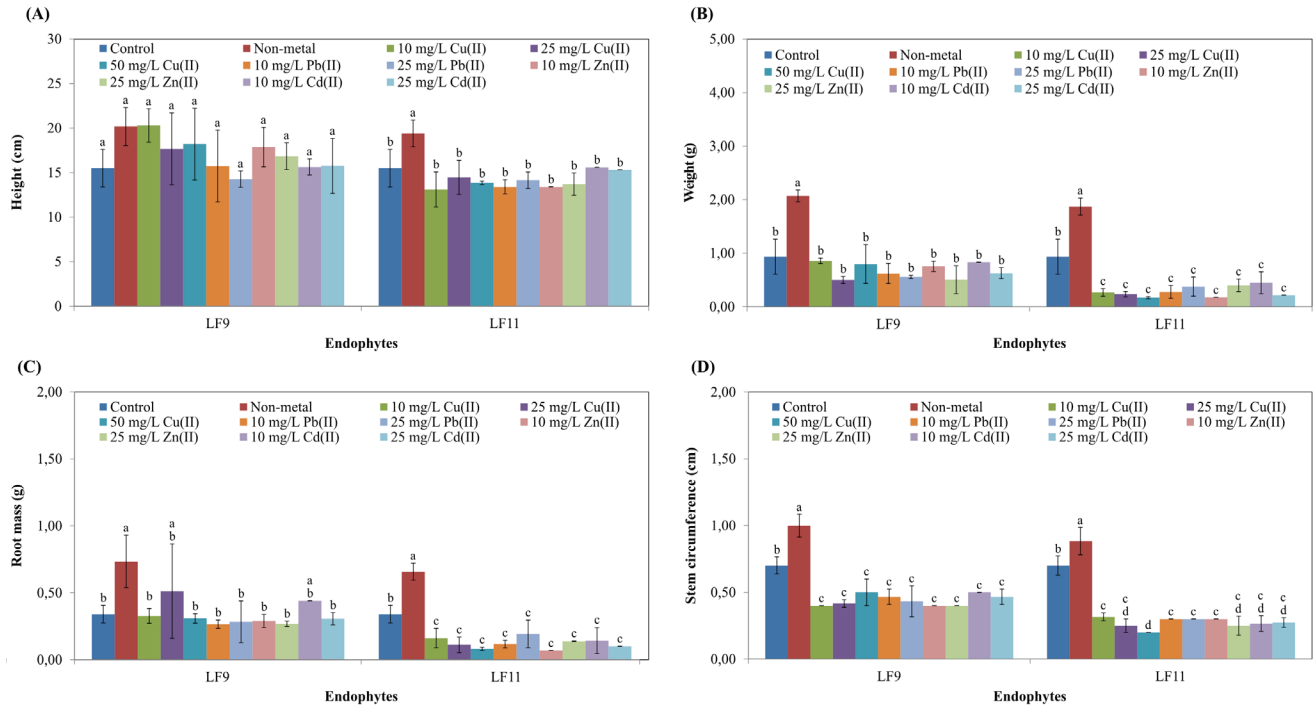

Figure 5. Growth parameters of (A) height (B) weight (C) root mass and (D) stem circumferences of ramets inoculated with metal-tolerant endophytes *D. miriciae* LF9 and *T. asperellum* LF11 in metal-laden soils consisting of Cu^{2+}, Pb^{2+}, Zn^{2+} and Cd^{2+}. Controls were endophyte-free ramets watered with sterile distilled water. Bars indicate standard deviations of means (±SD). Means with the same letters for each isolate are not significantly different ($HSD_{(0.05)}$).

growth-promoting ability of *Trichoderma* species, alongside *T. atroviride*, *T. harzianum* and *T. psedokoningii*, which were reported to effectively promote growth of tomato and maize (Babu et al. 2014; Gravel et al. 2007). However, in metal-laden soils, only *D. miriciae* LF9 appeared to have benefitted the ramets. Ramets inoculated with *D. miriciae* LF9 showed similar growth to ramets in non-metal soils with comparable height (0.13-4.80 cm), weight (0.08-0.43 g), and root mass (0.01-0.17 g) achieved. The stem circumference of the ramets was however affected (0.20-0.30 cm) (Figure 5). For ramets inoculated with *T. asperellum* LF11 and cultivated in soils with metals, poorer growth was observed compared to ramets in non-metal soils. In these ramets, inferior growth was evident with low growth measurements (height 0.10-2.4 cm, weight 0.49-0.76 g, root mass 0.15-0.27 g and stem circumference 0.38-0.50 cm) recorded (Fig. 5).

The *in vivo* findings were aligned to the *in vitro* results, which revealed lesser growth promoting activities when endophytes were exposed to metals compared to non-metal conditions. Ramets inoculated with *D. miriciae* LF9 were less susceptible to metal stress, but ramets treated with *T. asperellum* LF11 were not able to tolerate metal stress and this affected the growth of the ramets (low weight, root mass and stem circumference). This also revealed that the association of *Trichoderma* species with host plant may not necessarily be beneficial in metal-laden soils. The poor growths observed in ramets grown in soils supplemented with metals, is attributed to the effect of metals in inhibiting the production of IAA, siderophore, and ACC deaminase activities by the endophytic biocontrol agents (albeit at varying degrees). In the instance where the growth-promoting attribute is not inhibited, ramets continued to grow well. This explains the continued growth of ramets inoculated with *D. miriciae* LF9, as the phosphate solubilisation activity of LF9 in metal soils was detectable. This study also revealed that despite of the metal-tolerant attributes of the endophytes (Sim et al. 2018, 2019a, 2019b), the presence of metals did have an impact on the expression of plant growth-promoting activities. It also highlights that careful selection and investigation has to be performed, as not all metal-tolerant endophytes are ideal for application as biocontrol agents in metal-laden soil conditions.

Conclusions

The five endophytic isolates (*Bipolaris* sp. LF7, *D. miriciae* LF9, *T. asperellum* LF11, *P. asparagi* LF15, *S. bicolor* LF22)

demonstrated the potential to stimulate plant growth *in vitro* assays. This is a desirable attribute for biocontrol agents, as application of these bioagents would not only suppress disease development, but also to promote growth and strengthen the plant to delay disease progression. However, their beneficial role was generally affected by metal stress, with decreased IAA and siderophore production, and ACC deaminase activities. Hence, the metal-tolerant attributes of endophytes did not necessarily render significant benefits to the host-plants. Nevertheless, of the two isolates tested, *D. miriciae* LF9 showed better potential and prospect for further development as a biocontrol agent.

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