

Endophytic Plant Growth-Promoting Rhizobacteria Promote *Dendrobium* Orchid Growth

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Dendrobium orchid is one of the major export products of Thailand. Endophytic bacteria inhabiting the roots have the ability to promote plant growth. They could be used to replace the chemical fertiliser. In this study, 42 bacterial isolates were isolated from the orchid roots and were screened for plant growth-promoting rhizobacteria (PGPR). We selected six isolates that exhibited plant growth promotion (PGP) abilities, including siderophores, IAA, and ACC deaminase production, phosphate-solubilisation, and nitrogen-fixation. The 16S rRNA gene sequencing identified these isolates as *Enterobacter* sp. and *Bacillus* sp. with a prominence of *Bacillus halotolerans*. All six isolates were confirmed to inhabit the orchid roots and were inoculated into the orchid hybrid (*D. anosmum* × *D. parishii*) seedlings. We found that *B. halotolerans* O-SWU-17 enhanced orchid seedling growth *in vivo*. Thus, *B. halotolerans* O-SWU-17 has great potential as a biofertiliser in enhancing orchid production.

Keywords: Plant Growth-Promoting Rhizobacteria; *Bacillus*; *Dendrobium*; Endophyte; Biofertiliser

I. INTRODUCTION

Orchids are one of the most widely used ornamental plants and their cut flowers are known for their colours and allure. The orchid-cut flower industry has seen an annual growth rate of 10–20% globally and has immensely contributed to the economy of several countries, including Thailand. Orchid cut flowers from *Dendrobium* spp. are not only in great demand in Thai markets, but are also among Thailand's top export items, with an export value of approximately 65–95 million USD per year. The cultivation of orchids relies on the application of expensive chemical fertilisers and pesticides, with an estimated 2,400 tons used per year at a cost of 5.4 million USD per year (Department of Agriculture, 2019). Typically, chemical fertilisers are applied to orchid plants via foliar and root spraying. The use of PGPR is an unexplored method for organically enhancing orchid growth and can reduce the cost of cultivation as an alternative to chemical fertilisers.

Endophytes are living organisms, usually bacteria or fungi, dwelling in plants. Some endophytes contain PGP traits and

colonise plant roots to effectively enhance plant growth (Santoyo *et al.*, 2016). The capability of nitrogen fixation and phosphate solubilisation by PGPR enhances the availability of macronutrients for plant uptake. PGPR-carrying indole-3-acetic acid (IAA) phytohormone production traits stimulate root growth. The 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase-producing bacteria can reduce the levels of ethylene phytohormones, which in turn reduces plant stress. Some PGPR produce siderophores, which are iron-chelating compounds. These rhizobacteria promote plant growth by increasing the amount of ferric micronutrients for plants around plant roots and reduce the proportion of phytopathogenic fungi via iron scavenging competition. Some rhizobacteria produce HCN, a toxic volatile compound, which can suppress plant pathogens. Some PGPR can trigger plant immunity known as induced systemic resistance (Olanrewaju *et al.*, 2017; Yang *et al.*, 2009).

Orchid mycorrhizal fungi are well-known PGP microorganisms. In nature, some terrestrial or epiphytic orchids need endophytic fungal symbionts to facilitate seed

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germination. For example, the seed germination of *Pecteilis susanna*, a Thai terrestrial orchid, could be promoted by co-culture with *Epulorhiza* sp. (Chutima *et al.*, 2011). Endophytic fungi of orchids, including *Spathoglottis affinis*, *Paphiopedilum bellatulum*, and *Phaius tankervilleae*, can produce IAA phytohormones that promote seed germination and root length (Chutima & Lumyong, 2012). In addition to enhancing seed germination, several endophytic fungi have PGP activities, and their elicitors promote orchid growth and development (Chand *et al.*, 2020). Alibrandi *et al.* (2020) found that bacteria in the genera *Pseudomonas*, *Pantoea*, *Rahnella*, *Staphylococcus*, *Sphingomonas*, *Microbacterium*, *Streptomyces*, *Fictibacillus*, and *Bacillus*, which express PGP characteristics, were endophytic bacteria of the terrestrial Mediterranean orchid. Although many PGPR capable of enhancing terrestrial orchid growth have been discovered, endophytic PGPR that promote epiphytic orchid growth have not been explored yet.

The objectives of this study were to screen for endophytic bacteria from orchid roots and to evaluate the characteristics of endophytic bacteria for plant growth promotion. We isolated endophytic bacteria from *Dendrobium* orchid roots. The endophytic bacteria were characterised for PGP traits, including the ability of nitrogen fixation and phosphate solubilisation, and the production of IAA phytohormone, siderophores, ACC deaminase, and hydrogen cyanide (HCN). The endophytic bacteria were also tested for *Dendrobium* orchid seedling growth enhancement.

II. MATERIALS AND METHOD

A. Isolation of Endophytic Bacteria

We isolated bacterial endophytes from the healthy *Dendrobium* spp. Including *Dendrobium* spp. 'Sonia Earsakul', *Dendrobium* spp. 'White Fairy', and *Dendrobium* spp. 'Burana Jade' which showed newly emerging shoots and roots. For each species, we used three plants whose new emerging roots were cut to around 3 cm long. The roots were surface-sterilised with 0.25% NaOCl and washed with sterile water three times. Then, one gram of the surface-sterilised roots was ground with a pestle and mortar and suspended in one millilitre of sterile water. The suspension was diluted before spreading on peptone agar, nutrient agar (NA), soil

extract agar (SEA), and sodium caseinate agar (SCA) plates. One millilitre of the last washed water (before grinding of roots) was spread on the NA plate to ensure the surface sterilisation process. The plates were incubated for two days, and the obtained bacterial colonies were further purified by cross-streaking twice. A single colony was collected for further analyses. The selected isolates were tested for the possibility of co-culturing microbial consortia using a dual culture assay.

B. Characterisation of PGP Traits of Endophytes

The endophytic bacteria were tested for nitrogen fixation and ACC deaminase production by culture on Jensen medium (a nitrogen-free medium) and Dworkin and Foster (DF) agar with 0.25 mM ACC as the sole nitrogen source, respectively. Nitrogen-fixing bacteria and ACC deaminase-producing bacteria can grow well in these selective media. The phosphate-solubilising bacteria were screened by spot inoculation on Pikovskaya's (PVK) medium. The positive isolates grew well and produced clear zones around their colonies in the PVK medium. The siderophore production of the isolates was tested by spot inoculation onto chrome azurol S (CAS) agar. The orange halo zone around the colony can be observed if siderophores are produced.

The IAA phytohormone production by isolates was tested by inoculation in nutrient broth (NB) containing 0.1 mg/mL tryptophan. After incubation for two days, the cell-free culture was tested using Salkowski's reagent. The presence of IAA was confirmed by the change of cell-free culture to pink colour. For HCN production, the isolates were cultured in NA containing 4.4 g/L glycine, where the inner lid of the plate was stuck to sterile filter paper soaked with picric acid solution. The change in colour of the filter paper from yellow to dark brown confirmed the presence of HCN.

C. Bacterial Colonisation Efficacy in *Dendrobium* Orchid Root

The selected isolates were cultured in peptone water for 24 h. Subsequently, the cell pellet was collected by centrifugation at 5000×g for 5 min. The pellets were resuspended in sterile water. A cell suspension of 1×10⁴ cells/mL was used for root inoculation of the hybrid *Dendrobium* cultivar (*D. anosmum* × *D. parishii*) from tissue culture conditions. After

inoculation, the inoculated plantlets were further cultured in tissue culture conditions with Vacin and Went (VW) medium, and the culture medium was changed every day. At one week post-inoculation, roots of the inoculated plantlets were surface sterilised with 0.25% NaOCl as described above. One millilitre of the last washed water was spread on the NA plate to ensure the surface sterilisation process.

D. Bacterial Identification

The bacterial isolates were analysed by colony PCR with the universal primers 27F and 1492R to amplify the 16S rRNA gene. The PCR product was purified with HiYield™ Gel/PCR DNA fragment extraction kit (RBC Bioscience, New Taipei City, Taiwan) and was sent to Macrogen Inc. (Seoul, Korea) for DNA sequencing with 27F, 518R, and 1492R primers. The sequencing results were analysed using Bioedit software, and the 16S rRNA gene of approximately 1,400 nucleotide long were run through BLASTN with the sequences in GenBank and EzTaxon databases. The phylogenetic tree was constructed using MEGA 10.2.6 software with a maximum likelihood program.

E. Efficacy of Isolates on *Dendrobium* Orchid Growth Promotion

The hybrid *Dendrobium* cultivar (*D. Anosmum* × *D. Parishii*) from tissue culture conditions was used for root inoculation with the selected isolates. There are two methods of inoculation as described below. The hardening process was performed by the method of Lakshanthi dan Seran (2019) with some modifications.

In Method 1, the seedlings were hardened at room temperature (around 28-32°C) under shade and high humidity conditions for two weeks. The seedlings were then weighed, and the number of roots and shoots were recorded. After that, the seedling was inoculated with a cell suspension of the selected isolate (10⁶ cells/mL) by root dipping. The seedlings were cultured in coconut flakes under shade for two months. Thus, the seedlings were hardened for two weeks before inoculation.

In Method 2, seedlings were recorded using the parameters described in Method 1. The seedlings were inoculated with the cell suspension by root dipping and then cultured in coconut flakes for two months under shade and high

humidity conditions. Thus, seedlings were inoculated before the hardening process.

There were four seedlings per treatment, and the experiment was performed independently three times. After inoculation for two months, the increasing weight, number of seedlings with one or more emerging shoots and roots, and surviving percentage were recorded. The data obtained from the three replications were pooled for statistical analysis. The increasing weight of the seedlings was analysed by analysis of variance (ANOVA) and comparison of the mean by Fisher's least significant difference (LSD) test.

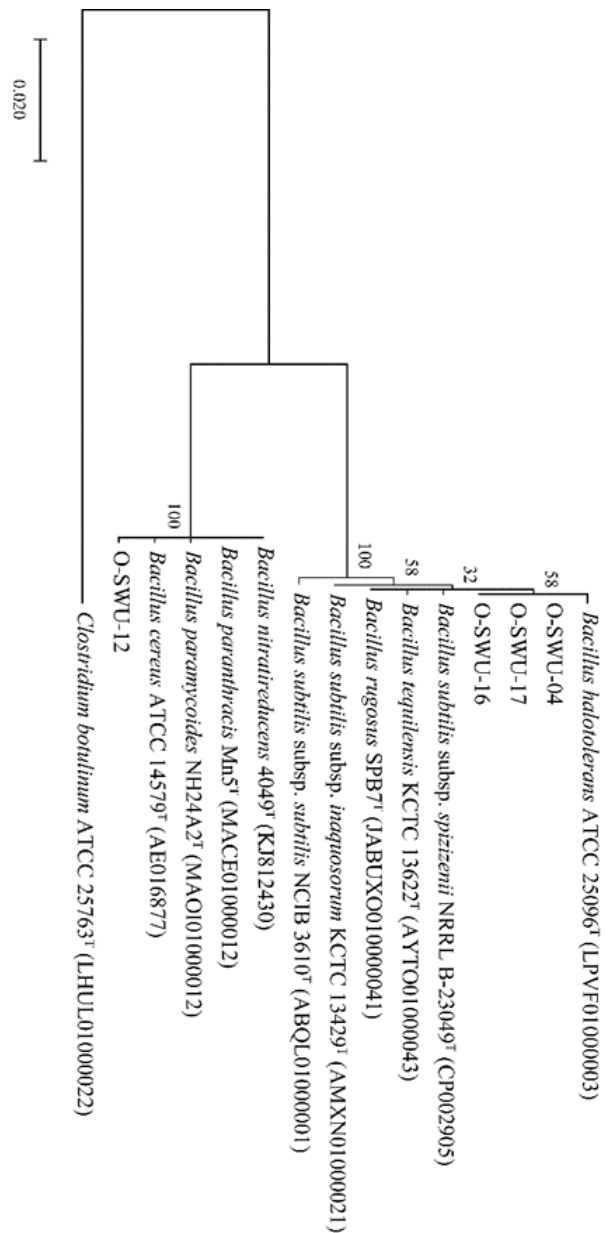


Figure 1. Phylogenetic tree of 16S rRNA gene of *Bacillus* isolates constructed by maximum likelihood method (1,000 bootstraps).

III. RESULTS AND DISCUSSION

The endophytic bacteria isolated from the roots of *Dendrobium* spp. were composed of 42 isolates obtained from NA (11), SEA (17), and SCA (14) medium agar plates. All the isolates were tested for PGP traits. Nitrogen fixation and phosphate solubilisation was detected in 23 and 15 endophyte isolates, respectively. Siderophores, IAA, ACC deaminase, and HCN were produced by 30, 25, 29, and 2 isolates, respectively. The isolates that exhibited all PGP characteristics, except for HCN production, were O-SWU-04, O-SWU-12, O-SWU-16, O-SWU-17, O-SWU-F1, and O-SWU-F2, which were chosen for further study. Additionally, dual culture tests showed that O-SWU-04 and O-SWU-17 inhibited the growth of O-SWU-12 cells.

Species level identification of the isolates was carried out by 16S rRNA gene analysis. We found that O-SWU-12 could be classified as *Bacillus* sp. (99.93% sequence similarity), whereas O-SWU-04, O-SWU-16, O-SWU-17 were similar to *Bacillus halotolerans* (100% similarity each). O-SWU-F1 and O-SWU-F2 could be classified as *Enterobacter* sp. (100 and 99.54% similarity, respectively). The phylogenetic tree is shown in Figure 1.

Table 1. Amount of the bacterial isolates found in the *Dendrobium* orchid roots.

Isolate	Endophytic bacteria (log CFU/g) ^{ns}
<i>B. halotolerans</i> O-SWU-04	8.550 ± 7.857
<i>Bacillus</i> sp. O-SWU-12	5.431 ± 4.880
<i>B. halotolerans</i> O-SWU-16	7.477 ± 6.864
<i>B. halotolerans</i> O-SWU-17	7.900 ± 6.795
<i>Enterobacter</i> sp. O-SWU-F1	8.837 ± 7.255
<i>Enterobacter</i> sp. O-SWU-F2	8.591 ± 7.969

Arithmetic means are shown with standard deviations. Ns indicates no significant difference ($P > 0.05$).

The isolates were investigated for their root colonisation potential. As shown in Table 1, all isolates could colonise the inner part of the orchid root; however, the potential of colonisation was not statistically significant between isolates ($P > 0.05$). It should be noted that the last wash water of each treatment was sterile, as no bacterial colonies were observed

upon culturing the wash water on the medium plate, which confirmed our hypothesis that the isolates were effective endophytic bacteria.

The potential of the isolates to promote orchid growth was evaluated in *Dendrobium* spp. hybrid seedlings. Among the endophytic isolates, *B. halotolerans* O-SWU-17 significantly enhanced seedling growth by increasing the fresh weight of the seedlings (Table 2). In addition, inoculation with *B. halotolerans* O-SWU-17 might promote shoot or root emergence. Notably, the inoculation method affects the survival rate of seedlings. A hardening process was required before the inoculation. As shown in Table 2, inoculation of the isolate before the hardening process (Method 2) decreased the survival rate. In Method 2, inoculation with *B. halotolerans* O-SWU-17 isolates did not reduce the survival rate, while other isolates did.

Table 2. PGP efficiency of endophytic isolates on *Dendrobium* spp. hybrid seedling growth.

Isolate	Increasing Weight (g) ¹	New shoot ^{1,2}	New root ^{1,2}	Surviving Percentage (Method 1)	Surviving Percentage (Method 2)
Control	0.313± 0.015 ^b	4	1	100	100
<i>B. halotolerans</i> O-SWU-04	0.313± 0.040 ^b	2	2	100	30
<i>Bacillus</i> sp. O-SWU-12	0.333± 0.004 ^{ab}	6	0	100	50
<i>B. halotolerans</i> O-SWU-16	0.279± 0.022 ^b	5	0	100	50
<i>B. halotolerans</i> O-SWU-17	0.384± 0.050 ^a	6	4	100	100
<i>Enterobacter</i> sp. O-SWU-F1	0.319± 0.043 ^b	6	1	100	50
<i>Enterobacter</i> sp. O-SWU-F2	0.316± 0.020 ^b	3	3	100	20

¹The results obtained from method 1. ²The total number of seedlings with one or more emerging shoots or roots. The increasing weight (g) of the seedlings are shown as mean ± standard deviation and were analysed by ANOVA with comparison of the mean by LSD test. Superscript letters indicate statistical significance ($P < 0.05$). Any two treatments sharing the same letter are not statistically different.

Molecular genetic studies of endophytic bacterial species found in *Dendrobium* spp. revealed that 699 genera of 22 bacterial phyla could be found in *Dendrobium catenatum*. Both the environment of the orchid cultivation and the *Dendrobium* species involved affected the variation in endophytic bacteria (Li *et al.*, 2017). For another commercial orchid, *Phalaenopsis*, Girija *et al.* (2018) investigated the endophytic bacterial diversity in the roots of orchids. They found that bacteria from the *Rubrobacter*, *Pseudomonas*, and *Acinetobacter* genera were abundant and bacteria from the *Bacillus* and *Enterobacter* genera were also found (0.14% and 1.78%, respectively). As endophytic bacteria with nitrogen fixation and plant hormone-producing abilities, *Sphingomonas paucimobilis* ZJSH1 promotes the growth of *Dendrobium officinale*, a medicinal orchid in China (Yang *et al.*, 2014). *Bacillus amyloliquefaciens* is reportedly an endophytic *Bacillus* with PGP traits in vanilla orchids (*Vanilla phaeantha*) and hybrid vanilla orchids (*V. planifolia* × *V. pompona*) (White *et al.*, 2014). A large number of endophytic *Bacillus* spp. have also been found in *Epipactis* spp. (terrestrial orchids) (Jakubská-Busse *et al.*, 2021). Additionally, all of the endophytic *Bacillus* species in this study showed plant growth promotion abilities *in vitro* and enhanced plant growth *in vivo*. This finding is consistent with a report by Jakubská-Busse *et al.* (2021), which stated that the spore-forming, gram-positive bacteria are mostly found in the orchid because of their ability to promote plant growth and tolerance to a nutrient-poor environment.

Bacillus spp. are well known as biofertilisers and have been applied in a variety of crop plants because of their strong PGP potential. *Bacillus velezensis* NJAU-Z9 can produce IAA, which enhances chilli pepper (*Capsicum annuum*) growth (Zhang *et al.*, 2018). *B. velezensis* SWUA08 exhibits PGP characteristics, including IAA and siderophore production, and phosphate solubilisation. Moreover, *B. velezensis* could protect the lime tree from citrus canker pathogens (Sudyoung *et al.*, 2020). *Bacillus amyloliquefaciens* WE15 and *Bacillus firmus* WD19 promote Chinese kale growth under polluted soil conditions (Sarawaneeyaruk *et al.*, 2019). Various *Bacillus* species, including *Bacillus cereus* PK6-15, *Bacillus subtilis* PK5-26, and *Bacillus circulans* PK3-109, isolated from desert plants promoted *Arabidopsis thaliana* growth under saline conditions (Bokhari *et al.*, 2019). Shah *et al.*

(2021) reported that *Bacillus* spp. PVL1 enhanced orchid root growth by producing IAA and protected the root from pathogens by producing ethyl iso-allocholate. The potential of *B. halotolerans* to promote plant growth has also been reported. *B. halotolerans* MSR-H4 exhibited nitrogen fixation, phosphate solubilisation, exopolysaccharide production, and IAA production abilities, and supported wheat growth under salt stress (El-Akhdar *et al.*, 2020). *B. halotolerans* SCCPVE07 promotes coriander (*Coriandrum sativum* L.) growth by successful colonisation of its plant roots, and enhances the accumulation of essential elements and bioactive compounds in plants. In addition, genes involved in PGP mechanisms were found in the *B. halotolerans* SCCPVE07 genome (Jiménez-Gómez *et al.*, 2020). In agreement with this study, *B. halotolerans* O-SWU-17 showed various PGP characteristics *in vitro*. The nutrient uptake support and IAA-producing ability of *B. halotolerans* O-SWU-17 might enhance *Dendrobium* seedling growth *in vivo* to increase both fresh weight and the number of new emerging shoots and roots.

The PGPR are usually used to enhance plant growth under harsh conditions such as drought, soil contamination, and soil salinisation. A high level of ethylene accumulation occurs in plants under stressful conditions, which in turn can stunt plant growth. ACC-deaminase-producing endophytic bacteria promote plant growth by reducing ethylene levels (Yang *et al.*, 2009). In this study, we used seedling growth under tissue culture conditions to study the potential of isolates without the adverse effects of natural bacteria. However, plant stress might occur, which causes damage to or the death of seedlings when the seedlings are transferred from tissue culture conditions to the natural environment. In this study, we expected that the isolates containing ACC deaminase-producing ability could improve seedling growth in both pre- and post-hardening conditions. The inoculation of *B. halotolerans* O-SWU-17 to the seedling did not reduce the survival rate in the pre- and post-hardening conditions and improved the growth only when the seedling passed the hardening process. This study provides early evidence that *B. halotolerans* O-SWU-17 could be used as a biofertiliser for *Dendrobium* spp., even in the early stages of growth.

IV. CONCLUSION

Endophytic bacteria from the genera *Bacillus* and *Enterobacter* were isolated from *Dendrobium* spp. roots. The selected isolates have plant growth promotion characteristics, including nitrogen fixation, phosphate solubilisation, and production of siderophore, IAA, and ACC deaminase. All the selected isolates could colonise the roots of *Dendrobium* spp. Among these isolates, *B. halotolerans* O-SWU-17 had the highest potential to promote *Dendrobium* hybrid seedling growth by increasing fresh weight and shoot

and root budding. The most suitable inoculation process was root dipping after hardening. Experimental results show that the use of *B. halotolerans* O-SWU-17 as an orchid biofertiliser is promising and should be explored further.

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VI. REFERENCES

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