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Research Article

Growth Promoting Effect of Endophytic Bacteria *Bacillus subtilis* From Leaves of *Vanda cristata* and Its Potential Impact on In vitro Growth of Orchid

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Abstract

The orchids are well known for their microbial association. *Bacillus subtilis* has great diversity in *Vanda cristata* which is regarded as a potential growth enhancer for the development of plants. The isolation of its strain was confirmed as *Bacillus subtilis* by morphological and molecular sequencing of DNA. The isolate was tested for indole acetic acid (IAA) estimation, ammonia production and phosphate solubilization in qualitative and quantitative manners. Its growth-promoting activities were tested on in vitro-raised plantlets of *Cymbidium aloifolium*. *B. subtilis* synthesized the IAA with and without a precursor (tryptophan). A more amount of auxin was found by using more amount of tryptophan. During the qualitative test for ammonia and phosphate solubilization, both compounds were synthesized by *B. subtilis*, which was further confirmed by the quantification method. Further, *B. subtilis* was used as a biotic elicitor in the in-vitro mass propagation of *C. aloifolium*. The development of root and shoot was found significant in the tryptophan-treated elicitor than the other compared medium combination. This study concludes that *B. subtilis* can be used as a biological elicitor to promote the in vitro growth of *C. aloifolium* and puts the spectrum on microbial relationships with orchid species.

Keywords: Ammonia synthesis, Auxin estimation, *Bacillus subtilis*, Biotic elicitor, Orchid, Phosphate solubilization

Introduction

All plants essentially interact and communicate with their surrounding organisms for their survival. In the case when all of the compatible partners benefit from their interactions, the interaction is called symbiosis (Pant et al., 2017). Over 90% of plants form a symbiotic relationship with a variety of environmental microorganisms and the association especially with bacteria and fungi, in different plant parts such as roots, stems and leaves (Pérez-Brocal et al., 2011; Shah et al., 2019a). Bacteria associated with orchids are recognized to have a great and often favourable impact on plant growth and development due to nitrogen fixation, production of plant growth regulators, improvement of water uptake and mineral nutrition, and biosynthesis of fungicidal and/or bactericidal substance and reduction the

number of phytopathogens (Ahmad et al., 2008; Deepa et al., 2010; Gulati et al., 2009). *Bacillus subtilis* is a spore-forming, motile, rod-shaped, Gram-positive and facultative bacteria which plays a significant role in plant growth by increased production of enzymes, phosphate solubilization, biocontrol activity, root nodulation, nitrogen fixation and ammonia production (Chauhan et al., 2015).

Orchids are considered the most desirable and valuable ornamental plants which have the highest commercial and horticultural value (Pant et al., 2016). However, the survival of orchids is nowadays severely threatened due to habitat loss, deforestation, diseases and pests, illegal trades, and commercial over-exploitation and cultivation (Pant, 2013; Swarts & Dixon, 2009). Thus, it is time to conserve and seek alternative means for the conservation and sustainable use of orchids by developing different in vitro techniques and uses of microorganisms for better growth and development. *Vanda cristata* is a monopodial and epiphytic orchid, widely distributed from Bangladesh, India, Nepal, and Bhutan to China at elevations of 600 – 2300 meters, which has high commercial and medicinal values (Pant, 2013; Timsina et al., 2016). However, there is a lack of information about the composition and functional activity of the orchid microbial communities. Only a few research groups are known for their studies on this subject (Tsavkelova et al., 2003; Tsavkelova et al., 2007a; Tsavkelova et al., 2007b; Wilkinson et al., 1989; Wilkinson et al., 1994) whereas the knowledge on interactions between the orchid and its "satellite" bacteria may add to an understanding of orchid biology and result in more effective cultivation, propagation, and conservation of these plants, particularly under artificial in vitro and greenhouse conditions. The current study focuses on the diversity, specificity, and functional activity of microbial communities associated with the different parts of the wild orchid *Vanda cristata* and their potential role to enhance the growth of in vitro-raised orchids.

Materials and Methods

Plant materials

Vanda cristata were collected from Khusma municipality, Parbat district, central hills of Nepal at an altitude of 1580 m on the host *Quercus semicarpifolia*. The bacterial endophyte was isolated from the leaves as per standard procedures (Bayman & Otero, 2006). In brief, leaves were washed thoroughly with tween 20 followed by running tap water to remove dust and debris and surface sterilized in a sequence of 70% ethanol for 1 minute, 1% $NaClO_2$ for 15 minutes and rinsed three times in sterile distilled water. The leaves were cut into small segments of 5 mm by using a sterile blade. The segments were placed in a petridish containing 15 ml of nutrient agar medium of pH 5.8. Before plating, imprints of the segments were made on the medium to check the efficiency of surface sterilization (Schulz et al., 2002). Samples were incubated at 25±2 °C with 12-hour alternate light and dark periods.

Identification and characterization

Pure cultures of bacterial isolates were confirmed by DNA barcoding sequences deposited in the gene bank database. A comparison of these sequences was done with the 16S RNA sequences of related bacteria using the online BLAST tool (https://blast.ncbi.nlm.nih.gov/Blast.cgi) (Shah et al., 2021).

Growth-promoting activities of *Bacillus subtilis* (PVL1)

Auxin estimation: The ability of bacterial endophytes to produce IAA was determined by Salkowski's reagent method (Bric et al., 1991). The endophytic bacterial strain was inoculated in Nutrient Dox broth at 27ºC. One disc (1 cm diameter) of each inoculum was added to 20 ml of nutrient broth medium containing 1-5 mg/l and without tryptophan and incubated for 10 days in a rotatory shaker. Five ml of each culture was collected from the incubating broth after 10 days and centrifuged at 6000 rpm for 30 min. One ml of the supernatant was mixed with one drop of Orthophosphoric acid and 2 ml of Salkowski's reagent (300 ml conc. H_2SO_4; 15 ml 0.5 M $FeCl_3$). The development of the pink colour indicated IAA production by the tested bacterial endophyte, which was further quantified by taking the optical density at 530 nm by using UV-spectrophotometer. The amount of IAA production was estimated by using a standard IAA graph. The IAA production was performed in a replicated manner.

Phosphate solubilization: For qualitative screening, 5 mm of actively growing colonies of endophytic bacteria (PVL1) isolates were inoculated in Pikovskaya's (PKV) agar plates. The halo zone of phosphate solubilization around bacterial colonies was measured in centimetres after 10 days of incubation. The colonies forming more than a 5 mm zone of solubilization were selected as efficient strains (Wahyudi et al., 2011). For a quantitative experiment, about a 5 mm diameter disc from the periphery of the actively growing bacterial colonies was inoculated in 100 ml PKV broth medium in conical flasks. The flasks were inoculated at 25±2ºC in a rotatory shaker at 130 rpm for 10 days. Five ml of cultured aliquot was centrifuged at 10,000 rpm for 10 minutes. Then 1 ml of supernatant was transferred to a 50 ml volumetric flask. This was followed by 5 ml sodium bicarbonate solution and added 10 ml distilled water. After that one drop of the p-nitrophenol indicator was added. The pH of the solution was adjusted to 5.0 by adding 2.5 M sulphuric acid. Then 8 ml of Murphy-Riley reagent was added and the volume was made up to 50 ml with deionized or distilled water. After incubation for 15 minutes, the intensity of the blue colour was measured on the UV-spectrophotometer at 730 nm (Murphy & Riley, 1962). The soluble phosphate 'P' was estimated from a standard curve of KH_2PO_4.

Qualitative analysis for ammonia production: Bacterial strain PVL1 were grown on Nutrient Dox medium for 10 days. After that, 10 ml of culture was collected from the incubating broth and centrifuged at 10,000 rpm for 10 minutes. Five ml of supernatant was transferred into the test tube and 1 ml of Nesselar's reagent was added to determine the ammonia production by endophytic bacteria. Where the change of colour from faint to yellow indicated the minimum ammonia production and the colour change from deep yellow to brownish, indicated the maximum ammonia production by *B. subtilis* (Singh et al., 2014).

Elicitor medium preparation

The broth containing *B. subtilis* (PVL1) was taken and centrifuged at 5000 rpm for 10 minutes and 2 % of the aliquot was mixed with the MS medium for making the elicitor's medium (Chand et al., 2020; Dong et al., 2008; Shah et al., 2019b). For this experiment, *Cymbidium aloifolium* was considered as a model plant.

Statistical analysis

The results presented are the means of the four independent replicates ± standard error of the mean (SEM). Data were analyzed by one-way ANOVA at a significant level of $p \leq 0.05$ with Posthoc and Tukey using IBM SPSS 20 Statistics.

Results and Discussion

In this study, the endophytic bacterial strain was identified as *Bacillus subtilis*, which was isolated from the leaves of *Vanda cristata*. This isolate showed characteristics of *Bacillus subtilis* physiologically or morphologically and including positive Gram reaction and catalase enzyme activities. *Bacillus subtilis* (Figure 1) was confirmed by the DNA sequencing method as a EU221672 with 97% query coverage (Shah et al., 2021).

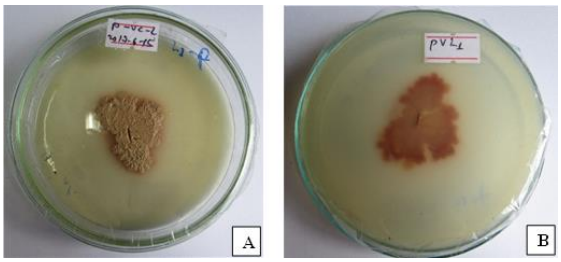

Figure 1: Bacterial colony (*B. subtilis*) A) overview colony and B) reverse colony.

Estimation of auxin synthesized by PVL1

B. subtilis isolated from *Vanda cristata* was used for the test of IAA synthesis. The recorded results revealed that the *B. subtilis* was able to synthesize IAA without precursor (tryptophan) or by using different concentrations of precursor in the growth medium.

Qualitative analysis of IAA

Selected *B. subtilis* used for the synthesis of IAA in Nutrient broth medium showed colour gradients according to their strength in different amounts of precursors (tryptophan). After the addition of Salkowski's reagent to the supernatant, the colour was changed to pink. The colour gradient was noticed differently according to the concentration of tryptophan used. The strongest intensity of pink colour was observed in *B. subtilis* at 5 mg/ml tryptophan, instead of that faint pink or no colour

was observed in strain without application of precursor (tryptophan) (Figure 2).

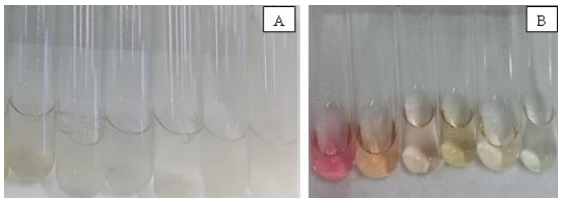

Figure 2: The qualitative test of auxin synthesized by bacterial strain (*B. subtilis*). A) without precursor and B) with precursor.

Quantitative analysis of auxin

B. subtilis was able to produce IAA without tryptophan or with tryptophan-supplemented conditions as an auxin precursor. However, the synthesis of IAA was directly proportional to the concentration of tryptophan used i.e., 5 mg, 2 mg, 1 mg and without tryptophan manners (Figure 2 and 3). *B. subtilis* produced significantly different amounts of IAA in the growth medium without and with different concentrations of tryptophan ($p \leq 0.05$). The highest amount of IAA was synthesized by *B. subtilis* strain in a growth medium containing 5 mg/ml tryptophan, in which 90.19 mg/l auxin was synthesized. Similarly, 17.42 mg/l and 9.87 mg/l auxin were synthesized in 2 mg/l and 1 mg/l tryptophan-containing growth medium respectively. Whereas the minimum concentration of auxin was synthesized by *B. subtilis* in without tryptophan conditions (Figure 3).

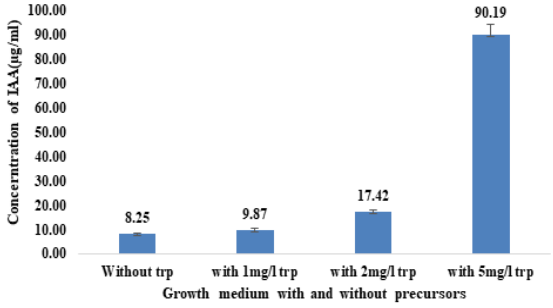

Figure 3: Auxin synthesized by PVL1 (*B. subtilis*) without precursor and with precursor (tryptophan) in different amounts 1, 2 and 5 mg/ml concentration. The bar represents mean ±SE (n=3) ($p<0.05$).

Qualitative analysis of ammonia synthesis and P-solubilization capacity

The qualitative test result (Table 1) showed that the intensity of ammonia synthesized by *B. subtilis* was isolated from *Vanda cristata*. The intensity was differentiated according to the color developed after the reaction with Nesselar's reagent. Similarly, *B. subtilis* was able to solubilize the complex form of phosphate into the simplest inorganic form. *B. subtilis* formed the halo zone around the colony according to their strength to solubilize supplemented phosphate compound, $Ca_3(PO_4)_2$. The strongest phosphate solubilizing capacity was shown by *B. subtilis* in 12 days of inoculation compared to the 7 days and 21 days of observations. The periodic observations for phosphate solubilization in 7 days found that no clear halo zone was formed around the *B. subtilis* colony. However, the clear and maximum halo zone was observed in 12 days observations. Whereas in 21 days of observations, the halo zone was found as equal to the 12 days of halo zone diameter.

Table 1: The strength of ammonia synthesis and phosphate solubilization by endophytic bacteria *B. subtilis.*

Endophytic strain	Phosphate solubilization		Ammonia synthesis
	Intensity	Diameter	
PVL1	+++	3 cm	+++

Quantification of Phosphate P-solubilizing capacity

From the qualitative test, it was assured that the maximum amount of complex form of phosphate was solubilized by endophytic bacterial strain in 12 days of inoculations. So, for quantification of solubilized inorganic phosphates, a maximum of 12 days of inoculated aliquots were used. The result (Figure 4) showed that *B. subtilis* was able to solubilize organic phosphates complex by 354 µg/ml. This result showed that *B. subtilis* helped the host plant by increasing inorganic phosphate availability in an epiphytic mode of habitat.

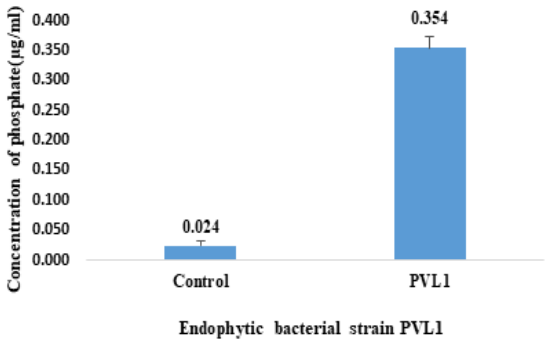

Figure 4: Soluble phosphate estimation for *B. subtilis* (PVL1) isolated from *Vanda cristata* (n=3) with ±SE.

Role of elicitors in in-vitro growth of *Cymbidium aloifolium*

The protocorms of *C. aloifolium* (60 days old) which were grown in in-vitro conditions on MS medium (Pradhan et al., 2014), were transferred in growth medium prepared by the combinations of MS and 2% biotic elicitor (*B. subtilis*). The growth rate was standardized by comparing results with control samples (only MS medium) and standard control media (MS with 1 mg/l IAA). The obtained results were compared in two parameters of the elicitor prepared with tryptophan and without tryptophan conditions. For this experiment, a total of 4 combinations of growth media were prepared with synthetic plant growth hormones and biological elicitors. The protocorms of *C. aloifolium* were transferred in this setup of medium for the shoot and root development for up to 16 weeks. Among all the treated conditions, PVL1 showed significant variation in the overall growth of *C. aloifolium*. For the development of shoot number, PVL1 treated with tryptophan-containing media was found effective. In this condition, the maximum number of the shoot was recorded. Similarly, PVL1 also showed a significant variation in shoot number production than the IAA and control medium. For the shoot length proliferation, PVL1 treated with tryptophan-containing medium showed significant variation ($p<0.05$). In these conditions maximum length of shoot of *C. aloifolium* was obtained. In comparison to this medium, the IAA-supplemented medium and control medium showed minimum shoot proliferation (Figure 5).

The effect of biological elicitors was also experimented with in the root proliferation of *C. aloifolium*. For the root number, PVL1 treated with tryptophan media showed significant variation ($p<0.05$). Similarly, PVL1 supplemented medium and IAA-supplemented medium showed a similar effect in root number proliferation. Whereas the control medium did not show significant variation from the other treated medium. For root length, PVL1 treated with tryptophan combinations showed significant variation in root length ($p<0.05$). In this condition, the maximum root length was recorded. However, PVL1 and IAA supplemented medium proliferate the shoot length in equal amounts whereas control media proliferate the shoot length in these two values. During observation periods, it was also noticed that the plant grown in different combinations had variations in pigmentation also. The plantlet grown in the PVL1 combination had dark green leaves compared to the plant grown in MS medium and IAA-treated medium (Figure 5).

Isolation of *Bacillus* species from the rhizosphere root was widely studied previously (Felici et al., 2008; Islam et al., 2012; Sivasakthi et al., 2014; Xie et al., 2014) but another zone has been neglected. In this study, it was of interest to obtain sufficient information including the diversity and plant growth-promoting activity of *B. subtilis* isolated from the wild orchid *Vanda cristata*. The root and rhizospheric portions of the host plant are of more reasonable areas for *B. subtilis* than the other morphological parts (Xie et al., 2014), however, this study also favours the maximum diversity of *B. subtilis* in the leaf portion. Little information is known about the composition and functional activity of the orchid-associated bacteria than the endophytic and mycorhizal fungi. Though rhizobacteria are recognized to have a great and often favourable impact on plant development (Martínez-Viveros et al., 2010) but knowledge of other parts of microbial association was also lacking. Plant growth-promoting rhizobacteria (PGPR) stimulates plant growth through the enhancement of phytohormones production, complex phosphate solubilization, ammonia synthesis and increasing the uptake of nitrogen (Wahyudi et al., 2011). *B. subtilis* have emerged as the largest species potentially the most promising group which has recently been used to enhance the ability of crop plants to promote growth and increase yields (Tsavkelova et al., 2007b). This study also supports the plant growth-promoting ability of endophytic bacteria *B. subtilis* in the case of orchids. It produces plant growth hormones with and without precursors but in the presence of tryptophan precursors, it synthesizes the maximum amount of auxin. Plant exudates supply the rhizosphere with tryptophan which is the main precursor in microbial IAA biosynthesis (Wachowska et al., 2006), IAA-producing bacteria transform IAA into auxin, increasing its exogenous level. Auxin is one of the crucial molecules, regulating most plant processes directly or indirectly, as was further proven when the auxin-producing *Bacillus* sp. inflicts a positive effect on *Solanun tuberosum* growth (Ahmed & Hasnain, 2010). The most active auxin in plants is indole-3-acetic acid (IAA). Previous researchers proved that *B. subtilis* produce IAA in the presence of precursor

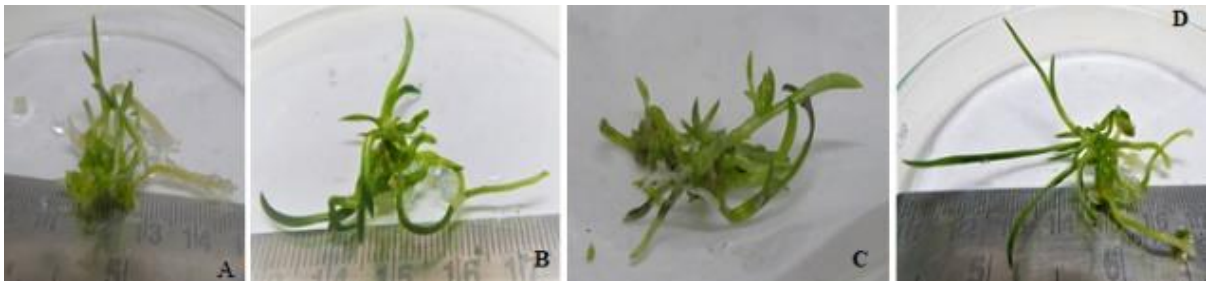

Figure 5: Morphological variation in experimented sample (protocorms of *C. aloifolium*) A) Control (MS), B) MS with IAA treated (1mg/l), C) MS with PVL1 and D) MS with Tryptophan treated PVL1.

tryptophan (Singh et al., 2014; Wahyudi et al., 2011). *B. subtilis* strain isolated from *Vanda cristata* is proven to be a versatile bacterium having manifold plant growth regulating beneficial activities such as the production of IAA (indole-3 acetic acid), P-solubilization, and ammonia production. This study also found that *B. subtilis* was also capable to solubilize Ca_3 (PO_4) supplemented in Pikovskaya's agar medium. The halo zone formed around the bacterial colony confirmed that *B. subtilis* was capable to solubilize complex formed of phosphates into inorganic forms. This result was verified by the quantitative analysis by spectrophotometry method. This study also showed that *B. subtilis* has the specific gene which initiates phosphate solubilization by producing acid phosphate and alkaline phosphatase enzymes. These enzymes are responsible for the solubilization of organic phosphates in the rhizosphere area of the host plant (Faires et al., 1999).

Endophytic bacteria associated with orchids can synthesize ammonia also, which enhances the growth of epiphytic orchids and helps to regulate all the metabolism necessary for their life cycle. Ammonia synthesized by bacteria was easily taken up by the host orchid. In the present study, it was found that *B. subtilis* isolated from *Vanda cristata* could produce ammonia in a broth medium at controlled conditions. Change in a colour gradient from transparent to brown after treatment with Nesselar's reagent indicated the production of ammonia in the solution by *B. subtilis* (Kole et al., 1988; Vejan et al., 2016). *B. subtilis* is one of the strongest-intensity plant growth-promoting rhizobacteria (PGPR) due to the abiotic stress tolerance in plants, nutrient fixation for easy uptake by the plant, plant growth regulators, the production of Siderophores, and the production of protective enzymes such as chitinase, glucanase, and ACC-deaminase for the prevention of plant diseases (Vejan et al., 2016). During this research, it is also found that the biotic extract of *B. subtilis* enhances the growth of in vitro-raised *Cymbidium aloifolium* by synthesizing phytohormones like auxin, phosphates solubilization and ammonia synthesis. When the bacterial elicitor treated with in vitro grown protocorms of *C. aloifolium* significantly enhance the overall shoot and root proliferation due to the presence of plant growth promoting compounds (auxin, inorganic phosphate and ammonia). The effect of bacterial elicitors grown in tryptophan provided medium showed significant effects on proliferation of root, shoot number and length of *C. aloifolium* than the control and synthetic auxin provided medium.

In the present research, elicitors medium prepared by *B. subtilis* with the precursors (tryptophan) was most proliferating medium for overall growth of root and shoot. It is found that bacterial elicitors increased the growth rate of plant and regulate the metabolism by secreting different chemical compounds which is not provided in growth medium (Chand et al., 2020; Shah et al., 2019a; Shah et al., 2019b).

Conclusion

The role of *Bacillus subtilis* in the growth of orchid during in vitro propagation can be a viable strategy for accelerating the mass propagation and acclimatization of plants by increasing biomass and compatibility. The biotechnological applications of bacterial inoculation technology in in-vitro and in-vivo orchid industries might contribute to the reduction in the use of synthetic hormones, leading to significant economic benefits on a long-term basis and being useful in conservation.

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