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Bioprospecting and selection of growth-promoting bacteria for *Cymbidium* sp. orchids

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ABSTRACT: Inoculants containing bacteria which promote growth in plants can increase productivity and both the economic and the environmental cost in plant crop systems. Similarly, in the flower and ornamental plant sector, the use of diazotrophic bacteria is a promising approach for improving orchid propagation from tissue culture to the ex vitro environment. We isolated diazotrophic bacteria from the roots and leaves of *Cymbidium* sp. The isolates were used to inoculate *Cymbidium* sp. plantlets during acclimatization in the nursery. After 150 days, plants were collected and their morphological and nutritional characteristics assessed. Eight bacterial strains were isolated containing traits that promote plant growth: *Bacillus thuringiensis*, *Burkholderia cepacia*, *Burkholderia gladioli*, *Herbaspirillum frisingense*, *Pseudomonas stutzeri*, *Rhizobium cellulosilyticum*, *Rhizobium radiobacter*, and *Stenotrophomonas maltophilia*. The isolated *Herbaspirillum frisingense* and *Stenotrophomonas maltophilia* increased 26 % and 29 % in dry matter in *Cymbidium* sp. plants, respectively, compared to the control. In addition, *H. frisingense* led to higher contents of N and P, by 68 % and 28 %, respectively, than those found in the control plants. These isolates, therefore, have potential for application as biostimulants and biofertilizers to promote growth and development of *Cymbidium* sp. during acclimatization.

Keywords: orchidaceae, diazotrophic bacteria, phosphate-solubilizing bacteria, endophytic bacteria, acclimatization

Introduction

Sustainable agriculture requires strategies that increase productivity and minimize environmental damage. Among the new sustainable technologies being developed, the formulation and application of inoculants and/or biofertilizers containing bacteria that promote growth in plants have shown promising results in a variety of crops (Hallmann et al., 1997). The promotion of plant growth through bacterial inoculation is mediated in part by biological nitrogen fixation as well as by factors such as the solubilization of rock phosphate, synthesis of phytohormones and siderophores, and the biological control and systemic resistance of host plants (Hallmann et al., 1997; Hardoim et al., 2008; Ryan et al., 2008; Singh et al., 2011).

The expansion of the flower and ornamental plant industry, in association with the use of diazotrophic bacteria as growth and plant protection agents, has encouraged research into not only bacteria-orchid interactions but also isolating, characterizing, and re-introducing growth-promoting bacteria into the cultivation environment. In order to cultivate *in vitro* propagated plants, for example, inoculants containing growth-promoting bacteria might be of value for accelerating the development of plantlets and decreasing the long acclimatization period (Baldotto et al., 2010; Faria et al., 2013).

Among cultivated orchids, *Cymbidium* sp. hybrids form a large group of epiphytic, terrestrial, and rhizomatous plants that originate in Asia (Choi et al., 2006). Since they are popular ornamental plants, they require propagation methods that ensure quick formation of a large number of vigorous and healthy plantlets. In this respect, *in vitro* propagation is an appropriate method, as it allows for the production of a large number of plantlets free of pests and diseases, with requirement for little space and the possibility of establishing production and marketing schedules (Chugh et al., 2009). Strategies that accelerate the growth of *Cymbidium* sp. plantlets during acclimatization are attractive, and offer the possibility of reestablishing the association between orchids and beneficial microbiota.

This study was undertaken in order to (1) isolate diazotrophic bacteria found in the roots and the leaves of orchids; (2) assess diazotrophic bacterial strains for their ability to solubilize phosphate and zinc oxide, and synthesize indole compounds; (3) identify the bacterial isolates; and (4) evaluate the growth and development of *Cymbidium* sp. plantlets in response to bacterial inoculation during acclimatization.

Materials and Methods

Plant material for bacterial isolation

The study was carried out in Florestal, in the state of Minas Gerais, in Brazil (19°53′22″ S, 44°25′57″ W, 776 m above sea level). Samples of roots and leaves of *Cymbidium* sp. orchids were collected from the orchid collection at Florestal. The one mother plant is kept in a greenhouse, and is grown in a ceramic pot containing coconut fiber as a substrate.

Isolation of diazotrophic bacteria

Endophytic diazotrophic bacteria were isolated as previously described by Döbereiner et al. (1995) with a

number of alterations. One gram of root sample and 1.0 g of leaf sample were macerated in 9.0 mL of saline solution (NaCl, 8.5 g L^{-1}). Serial dilutions of this suspension were obtained by adding 1.0 mL of the suspension to 9 mL of the saline solution; this series was concluded at 10^{-6} dilution. Aliquots (100 µL) from the different dilutions were transferred in triplicate to glass bottles containing 5 mL of the semi-solid, semi-selective culture media JMV (mannitol 5.0 g L^{-1}, K_2HPO_4 0.6 g L^{-1}, KH_2PO_4 1.8 g L^{-1}, $MgSO_4.7H_2O$ 0.2 g L^{-1}, NaCl 0.1 g L^{-1}, $CaCl_2.2H_2O$ 0.02 g L^{-1}, $Na_2MoO_4.2H_2O$ 0.002 g L^{-1}, $MnSO_4.H_2O$ 0.00235 g L^{-1}, H_3BO_3 0.0028 g L^{-1}, $CuSO_4.5H_2O$ 8.0 × 10^{-5} g L^{-1}, $ZnSO_4.7H_2O$ 0.002 g L^{-1}, bromothymol blue (0.5 % solution in 0.2 M KOH) 2 mL, iron ethylenediamine tetraacetic acid (FeEDTA) (solution 1.64 %) 4 mL, biotin 1.0 × 10^{-4}, HCl- pyridoxine 2.0 × 10^{-4} and agar 2.1 g L^{-1}, pH between 4.2 and 4.5), JMVL (mannitol 5.0 g L^{-1}, K_2HPO_4 0.6 g L^{-1}, KH_2PO_4 1.8 g L^{-1}, $MgSO_4.7H_2O$ 0.2 g L^{-1}, NaCl 0.1 g L^{-1}, $CaCl_2.2H_2O$ 0.02 g L^{-1}, $Na_2MoO_4.2H_2O$ 0.002 g L^{-1}, $MnSO_4.H_2O$ 0.00235 g L^{-1}, H_3BO_3 0.0028 g L^{-1}, $CuSO_4.5H_2O$ 8.0 × 10^{-5} g L^{-1}, $ZnSO_4.7H_2O$ 0.002 g L^{-1}, bromothymol blue (0.5 % solution in 0.2 M NaOH) 2 mL, FeEDTA (solution 1.64 %) 4 mL, biotin 1.0 × 10^{-4}, HCl- pyridoxine 2.0 × 10^{-4}, yeast extract 0.02 g L^{-1} and agar 1.6 g L^{-1}, pH between 5.0 and 5.4), NFb (malic acid 5.0 g L^{-1}, K_2HPO_4 0.5 g L^{-1}, $MgSO_4.7H_2O$ 0.2 g L^{-1}, NaCl 0.1 g L^{-1}, $CaCl_2.2H_2O$ 0.01 g L^{-1}, $Na_2MoO_4.2H_2O$ 0.002 g L^{-1}, $MnSO_4.H_2O$ 0.00235 g L^{-1}, H_3BO_3 0.0028 g L^{-1}, $CuSO_4.5H_2O$ 8.0 × 10^{-5} g L^{-1}, bromothymol blue (0.5 % solution in 0.2 M KOH) 2 mL, FeEDTA (solution 1.64 %) 4 mL, agar 1.6 g L^{-1}, at pH 6.8), JNFb (malic acid 5.0 g L^{-1}, K_2HPO_4 0.6 g L^{-1}, KH_2PO_4 1.8 g L^{-1}, $MgSO_4.7H_2O$ 0.2 g L^{-1}, NaCl 0.1 g L^{-1}, KOH 4.5 g L^{-1}, $CaCl_2.2H_2O$ 0.02 g L^{-1}, $Na_2MoO_4.2H_2O$ 0.002 g L^{-1}, $MnSO_4.H_2O$ 0.00235 g L^{-1}, H_3BO_3 0.0028 g L^{-1}, $CuSO_4.5H_2O$ 8.0 × 10^{-5} g L^{-1}, $ZnSO_4.7H_2O$ 0.002 g L^{-1}, bromothymol blue (0.5 % solution in 0.2 M KOH) 2 mL, FeEDTA (solution 1.64 %) 4 mL, biotin 1.0 × 10^{-4} g L^{-1} and HCl- pyridoxine 2.0 × 10^{-4} g L^{-1}, agar 1.7 g L^{-1}, at pH 5.8), and LGI (sacarose 5.0 g L^{-1}, K_2HPO_4 0.2 g L^{-1}, KH_2PO_4 0.6 g L^{-1}, $MgSO_4.7H_2O$ 0.2 g L^{-1}, $CaCl_2.2H_2O$ 0.02 g L^{-1}, $Na_2MoO_4.2H_2O$ 0.002 g L^{-1}, KOH 4.5 g L^{-1}, bromothymol blue (0.5 % solution in 0.2 M KOH) 2 mL, FeEDTA (solution 1.64 %) 4 mL, biotin 1.0 × 10^{-4} g L^{-1} and HCl-pyridoxine 2.0 × 10^{-4} g L^{-1}, agar 1.4 g L^{-1}, pH between 6.0 and 6.2) (Baldani et al., 1986; Baldani et al., 1996; Döbereiner et al., 1995), all without added nitrogen. The formation of a typical aerotaxic film on the surface of the medium was observed 7 days after incubation in a growth chamber at 30 °C and was considered positive growth. Subsequently, bacteria grown at greater dilution factors were transferred to new semisolid media, where they were grown for 7 days, followed by plating on the appropriate solid medium. Individual colonies with different morphological characteristics were transferred to fresh semi-solid media and then to solid DYGS medium to check the purity of the isolates (Döbereiner et al., 1995). Once purified, the colonies were kept in sterile distilled water.

Evaluation of the capacity to synthesize indole compounds

Bacterial isolates were grown in liquid DYGS medium for 24 h at 30 °C and 120 rpm. To evaluate the synthesis of indole compounds, 10 µL of bacterial cultures were transferred to plates containing 1/10 TSA (Trypticase Soy Agar) medium (Brick et al., 1991). After the transfer, the medium was covered with a nitrocellulose membrane and incubated at 28 °C for 24 h. Subsequently, the membrane was transferred to another plate, saturated with Salkowski solution (Gordon and Weber, 1951), and incubated at room temperature for 30 min. Formation of a reddish halo on the membrane indicated the presence of indole synthesized by the bacteria. Three replicates for each bacterial strain were conducted.

Evaluation of the calcium phosphate and zinc oxide solubilization capability

Bacteria were grown in DYGS liquid medium for 24 h at 30 °C and 120 rpm. Bacterial solution samples of 20 µL were placed on Petri plates with solid culture media containing insoluble phosphate (10.0 g L^{-1} glucose, 5.0 g L^{-1} of NH_4Cl, 1.0 g L^{-1} $MgSO_4.7H_2O$, 4.0 g L^{-1} of $CaHPO_4$, 15.0 g L^{-1} of agar, pH 7.2) or zinc oxide (10.0 g L^{-1} glucose, 1.0 g L^{-1} $(NH_4)_2SO_4$, 0.2 g L^{-1} of KCl, 0.1 g L^{-1} K_2HPO_4, 0.2 g L^{-1} of $MgSO_4.7H_2O$, 1.0 g L^{-1} ZnO, 15.0 g L^{-1} agar, pH 7.0), and incubated at 30 °C for 72 h (Baldotto et al., 2010). The solubilization activities for calcium phosphate and zinc oxide were evaluated by examining the plates for the presence of a translucent halo around the solubilizing colonies on each medium. Three replicate experiments were carried out for each bacterial strain.

Identification of bacterial isolates

A number of bacterial isolates were identified by analyzing fatty acid methyl esters (GC-FAME) (Sasser, 2006) at Viçosa, in the state of Minas Gerais. Bacterial isolates were grown on TSA medium for 24 h at 30 °C, and then again in fresh medium for a second incubation. A cell sample of approximately 3 mg was collected to extract fatty acids. Fatty acids were extracted and obtained using the Instant Fame kit (Midi, Newark, DE), following the manufacturer's recommendations. The Sherlock® Microbial Identification (Midi, Newark, DE) system was used to determine the composition of the bacterial fatty acids. Following extraction and quantification, the fatty acid profile was compared with a library to identify the sample.

Further bacterial isolates were identified by sequencing the 16S rRNA gene at Piracicaba, in the state of São Paulo. Genomic DNA was extracted from bacterial isolates using the protocol described by Stirling (2003). The 16S rRNA gene was amplified with the following oligonucleotide primers for Eubacteria (5'-AGA GTT TGA TCC TGG CTC AG-3') and rD1 (5'-AAG GAG GTG ATC CAG CC-3') (Weisburg et al., 1991). The 16S rRNA gene amplifications were conducted in a final volume of

25 µL that contained 5 pmol of oligonucleotide primers, 200 µM of each dNTP, 1 × Taq buffer, 1.5 mM $MgCl_2$, 2 U Platinum Taq polymerase DNA and 10 ng DNA. The reaction was initiated with 3 min of denaturation at 94 °C, followed by 30 cycles of denaturation at 94 °C for 1 min, annealing at 55 °C for 30 s, extension at 72 °C for 30 s, and final extension at 72 °C for 32 min. The sequencing polymerase chain reaction (PCR) of the 16S rRNA fragments was conducted in a final volume of 10 µL with 5 pmol of initiator oligonucleotides, 1.0 µL of 2.5 × buffer; 3.0 µL of Big Dye Terminator 30 Cycle Sequencing v.3 (Applied Biosystems, USA). The oligonucleotides used were the same as in the previous amplification (Weisburg et al., 1991). Amplification conditions were 2 min of denaturation at 96 °C, followed by 30 cycles of denaturation at 95 °C for 20 s, annealing at 55 °C for 15 s, and extension at 60 °C for 1 min. After amplification of the fragments, unincorporated dNTPs were eliminated by precipitation. The reading of the labeled bases was performed on an Automatic Sequencer ABI Prism 3130 Genetic Analyzer, which uses capillary electrophoresis to separate and detect the amplified fragments. The sequences were compared in the BLAST database (NCBI).

Plant material for greenhouse experiments

Cymbidium sp. plantlets of the Angelica variety were grown in glass bottles containing MS medium (Murashigue and Skoog, 1962) with no added growth regulators or vitamins. To carry out the subsequent experimental steps, plantlets with approximately 1.0 g of fresh matter were selected.

Bacterial growth and inoculation

The bacteria were grown in DYGS liquid medium in a shaker for 24 h, at 30 °C and 120 rpm, and inoculation was performed by immersing the orchid plantlets in 50 mL of bacterial medium for 2 h, with further application of the same bacteria on the substrate (Baldotto et al., 2010). The control was immersed in autoclaved liquid DYGS medium. Later, the propagative materials were transferred to 1.0 dm^3 plastic pots (two plantlets per pot) containing Bioplant® commercial substrate for acclimatization in a greenhouse for 150 days. Each pot was fertilized every 15 days with 5 mL liquid mineral fertilizer BeG Orquídeas®.

Growth analysis

After acclimatization, the plants were collected for measurement of the following variables: number of leaves; plant height, which was measured by the distance between the lower internode to the leaf apex using a tape-measure: base diameter, measured with a Starrett 727 digital caliper; fresh matter from the root and aerial parts; and dry matter of the root and aerial parts, which were obtained after material had been kiln dried under forced air ventilation at 65 °C for 48 h. Total fresh matter, total dry matter, and the relationship between root and aerial parts were calculated.

Nutritional analysis

After drying, the leaves of the orchids were ground in a Wiley mill coupled to six 50 cm^2 mesh sieves. Next, the powder obtained was subjected to sulfuric acid digestion combined with hydrogen peroxide and the total contents of nitrogen (N), phosphorus (P), potassium (K), calcium (Ca) and magnesium (Mg) were determined. For the measurement of N, Nessler's method was used; P content was obtained by molecular absorption spectrophotometry (colorimetry, at a wavelength of 725 nm) after a reaction with vitamin C and ammonium molybdate; K was measured by using flame photometry, and the Ca and Mg contents were obtained by atomic absorption spectrophotometry. The contents of N, P, K, Ca, and Mg were estimated by multiplying the dry matter of the aerial part by the nutrient content.

Statistical analysis

The experiment was conducted in a random block design with six replicates and each experimental unit consisted of a pot containing two plants. The data were analyzed by analysis of variance (ANOVA) and LSD *t*-tests using the SISVAR software program v. 5.4 at Lavras, Brazil, with significance set at the 5 % probability level.

Results and Discussion

We found that at least eight strains of diazotrophic bacteria were naturally inhabiting leaves and roots of *Cymbidium* sp. and differed in their ability to promote plant growth (Table 1). Analysis of fatty acid methyl esters (FAME-GC) and sequencing of the 16S rRNA gene identified the isolated bacterial strains as *Bacillus thuringiensis*, *Burkholderia cepacia*, *Burkholderia gladioli*, *Herbaspirillum frisingense*, *Pseudomonas stutzeri*, *Rhizobium cellulosilyticum*, *Rhizobium radiobacter*, and *Stenotrophomonas maltophilia*. In addition to the biological fixation of atmospheric nitrogen, most of the isolates showed potential for solubilizing calcium phosphate and zinc oxide and for synthesizing indolic compounds, which are predecessors of the auxin class of phytohormones and reflect the ability of these bacteria to promote plant growth (Table 1).

These results are consistent with the work of Wilkinson et al. (1989; 1994) who presented the first descriptions of an association between orchids and plant growth-promoting bacteria; these authors isolated *Pseudomonas*, *Bacillus*, *Xanthomonas*, *Arthrobacter*, and *Kurthia* bacteria with the potential to synthesize an auxin class phytohormone. Subsequently, Tsavkelova et al. (2001; 2003; 2004) isolated *Acinetobacter*, *Bacillus*, *Cellulomonas*, *Flavobacterium*, *Gluconobacter*, *Micrococcus*, *Mycobacterium*, *Pseudomonas*, *Rhodococcus*, and *Streptomyces* bacterial genera from *Acampe papillosa* and *Dendrobium moschatum* orchids. Recently, Faria et al. (2013), isolated strains belonging to the *Paenibacillus* genus from *Cymbidium eburneum*. Notably, bacteria with biotechnological potential inhabit plants belonging to the Orchidaceae family.

Growth data (Table 2) of acclimatized *Cymbidium* sp. indicated that a number of bacterial strains helped the growth and adaptation of plantlets under *ex vitro* conditions. The bacteria *Stenotrophomonas maltophilia* and *Herbaspirillum frisingense* promoted the greatest increases in growth of *Cymbidium* sp., indicated by an increase in total dry matter of 29 % and 26 %, respectively, compared to control plants. The acclimatization of *Cymbidium* plants propagated *in vitro* is a slow process (Chugh et al., 2009) which might benefit from applications of both biostimulants (Baldotto et al., 2014) and, as verified in this work, and plant growth-promoting bacteria.

The bacterial genus *Stenotrophomonas* comprises gram-negative, rod-like species. *Stenotrophomonas maltophilia* is found in a variety of environments and geographical regions, and it promotes growth or is a symbiotic agent in several species of plants, such as sugarcane (Beneduzi et al., 2013), fruit from the Brazilian Cerrado (Dias et al., 2015) and agricultural crops in Korea (Park et al., 2005). According to Jeong et al. (2010), this bacterial species shows a high capacity for producing indole acetic acid (IAA), an observation also verified in several other species of plant growth- promoting bacteria (Spaepen et al., 2007). As IAA has a beneficial effect on plant growth, our observation here that inoculation of *Cymbidium* sp. with *Stenotrophomonas maltophilia* gave the highest resultant relative increase in dry matter may be due to hormone activity.

The bacterial isolate *Herbaspirillum frisingense* caused the highest increases in the nutritional contents of N and P, of 68 % and 28 %, respectively, compared to control samples (Table 3). Representatives of the genus *Herbaspirillum* are considered mandatory endophytic bacteria and have a low survival rate in the soil. *Herbaspirillum frisingense* has biological nitrogen fixation capability, and is a potential candidate for calcium phosphate and zinc oxide solubilization and indolic compound synthesis (Kirchhof et al., 2001; Montañez et al., 2012; Straub et al., 2013). Potential for the promotion of growth was reflected in the relative increases in dry matter and nutritional contents of N, P and K obtained in our greenhouse experiment.

Bacteria belonging to the genus *Bacillus* can be easily isolated from the soil and rhizosphere of various plants (Seldin et al., 1998). Many *Bacillus* species contribute to the health of plants in many ways, such as

Table 1 – Identification and characteristics of growth-promoting bacteria isolated from *Cymbidium* sp.

Identification of bacterial isolates	TI[1]	PTI[2]	CMI[3]	CPS[4]	ZOS[5]	SIC[6]	RIDM[7]
							%
(UFV11362) *Bacillus thuringiensis*	GC-FAME	Root	NFB	Yes	ND	Yes	2
(UFV12251) *Burkholderia cepacia*	GC-FAME	Leaf	JMVL	Yes	ND	ND	12
(UFV12141) *Burkholderia gladioli*	16S rRNA	Leaf	JMV	Yes	Yes	ND	-26
(UFV11541) *Herbaspirillum frisingense*	16S rRNA	Root	LGI	Yes	Yes	Yes	26
(UFV11521) *Pseudomonas stutzeri*	GC-FAME	Root	LGI	Yes	Yes	Yes	5
(UFV11442) *Rhizobium cellulosilyticum*	16S rRNA	Root	JNFb	Yes	Yes	ND	5
(UFV12321) *Rhizobium radiobacter*	GC-FAME	Leaf	NFB	Yes	Yes	ND	10
(UFV11261) *Stenotrophomonas maltophilia*	16S rRNA	Root	JMVL	ND	ND	Yes	29

[1]Technique to identify bacterial isolates: GC-FAME analysis of fatty acids methyl esters; 16S rRNA sequencing of gene 16S rRNA with percentage of similarity in GenBank of 99 %; [2]PTI = plant tissue used in isolation; [3]CMI = culture medium used for isolation; [4]CPS = calcium phosphate solubilization; [5]ZOS = zinc oxide solubilization; [6]SIC = synthesis of indole compounds; [7]RIDM = relative increase in dry matter of *Cymbidium* sp. plants inoculated with respect to the control treatment (RIDM: 100 (-x + y) / y, where x and y are the average of the treatments compared); ND = not detected.

Table 2 – *Cymbidium* sp. growth characteristics in response to inoculation of plant growth-promoting bacteria.

Treatment	Growth Characteristics[1]									
	NL	DB	PH	FMAP	FRM	DMAP	DRM	TFM	TDM	R/AP
		cm		g						
Control	9 ab	0.638 ab	14.3 abc	2.251 ab	4.127 bc	0.371 ab	0.248 bc	6.378 bc	0.619 bc	0.699 b
Bacillus thuringiensis	10 a	0.576 ab	14.9 ab	2.456 ab	4.299 bc	0.040 a	0.229 bc	6.755 bc	0.632 abc	0.578 b
Burkholderia cepacia	9 ab	0.700 a	15.1 a	2.456 a	5.154 ab	0.403 a	0.297 bc	7.813 ab	0.695 ab	0.725 b
Burkholderia gladioli	8 b	0.550 b	12.8 c	1.761 b	3.079 c	0.269 b	0.190 c	4.840 c	0.460 c	0.740 b
Herbaspirillum frisingense	10 a	0.688 a	14.3 abc	2.906 a	5.984 a	0.448 a	0.329 ab	8.890 a	0.778 ab	0.755 b
Pseudomonas stutzeri	9 ab	0.625 ab	15.9 a	2.523 a	4.279 bc	0.397 a	0.254 bc	6.801 bc	0.651 ab	0.665 b
Rhizobium cellulosilyticum	9 ab	0.613 ab	14.4 abc	2.446 ab	5.121 ab	0.377 ab	0.275 bc	7.567 ab	0.652 ab	0.737 b
Rhizobium radiobacter	9 ab	0.675 ab	15.4 a	2.748 a	3.938 bc	0.425 a	0.256 bc	6.686 bc	0.681 ab	0.603 b
Stenotrophomonas maltophilia	9 ab	0.663 ab	12.8 bc	2.491 a	4.908 ab	0.391 a	0.409 a	7.399 ab	0.800 a	1.233 a
CV (%)	10	14	10	20	22	19	28	19	18	43

[1]Growth Characteristics: NL = number of leaves; DB = diameter of the base; PH = plant height; FMAP = fresh matter of the aerial part; FRM = fresh root matter; DMAP = dry matter of the aerial part; DRM = dry root matter of; TFM = total fresh matter of the plant; TDM = total dry plant matter; R/AP = root and aerial part ratio. Averages followed by the same letter in the columns are not statistically different as per the LSD *t*-test with a 5 % probability.

Table 3 – Nutritional composition of *Cymbidium* sp. in response to inoculation of plant growth-promoting bacteria.

Treatment	Nutritional Characteristics[1]				
	N	P	K	Ca	Mg
	mg per plant				
Control	15.681 bc	6.273 bc	6.671 ab	10.877 b	1.762 bc
Bacillus thuringiensis	15.315 c	5.876 bcd	6.189 bc	10.742 b	1.954 ab
Burkholderia cepacia	16.149 bc	4.974 cd	6.453 abc	13.214 b	2.400 a
Burkholderia gladioli	13.849 c	4.812 d	7.090 a	17.242 a	1.954 ab
Herbaspirillum frisingense	26.314 a	8.041 a	7.502 a	11.047 b	1.512 c
Pseudomonas stutzeri	17.472 bc	6.410 b	6.407 abc	10.519 b	1.760 bc
Rhizobium cellulosilyticum	16.047 bc	5.770 bcd	6.705 ab	12.581 b	1.808 bc
Rhizobium radiobacter	20.104 b	5.833 bcd	6.304 abc	11.184 b	1.779 bc
Stenotrophomonas maltophilia	17.408 bc	4.664 d	5.285 c	10.771 b	1.770 bc
CV (%)	15	13	10	13	10

[1]Nutritional composition: Contents of N, nitrogen; P = phosphorus; K = potassium; Ca = calcium; Mg = magnesium. Averages followed by the same letter in the columns do not differ between themselves as per the LSD *t*-test with 5 % probability.

biological nitrogen fixation and biological phytopathogen control agents (Lacey et al., 2001). *Bacillus thuringiensis* is known for its use in biological control programs (Sadfi et al., 2001); however, there are only a few reports of use of this bacterial species as an endophytic growth promoting microorganism, for example, in the banana tree "prata-anã" (Andrade et al., 2014) and when isolated from soybean root nodules (Bai et al., 2002). Andrade et al. (2014) also identified the calcium phosphate solubilization and IAA production capability of diazotrophic bacteria belonging to the genus *Bacillus* which was also identified in this study (Table 1).

Two bacterial strains belonging to the genus *Burkholderia* were isolated from *Cymbidium* sp. leaves. Bacteria from the genus *Burkholderia* are gram-negative in the form of motile rods, with three or more flagella. Currently, this genus includes 62 species with great functional diversity. Species within this genus may be plant growth-promoters or human, animal, and vegetable pathogens (Eberl and Vandamme, 2016), as in the case of *Burkholderia gladioli*, which was isolated in this study and characterized as pathogenic in orchids by Keith et al. (2005), a fact that may be related to the negative effect on the relative increase of dry matter after inoculation. In addition, the *Burkholderia* bacteria identified here had the ability to solubilize calcium phosphate, consistent with the results from Rodríguez and Fraga (1999).

The genus *Pseudomonas* is characterized as being composed of gram-negative, aerobic, and mobile bacilli and stands out because of its great nutritional versatility in agricultural production systems and its ability to grow in a wide variety of environmental conditions (Peix et al., 2009). *Pseudomonas stutzeri* was isolated from cucumber (Islam et al., 2016), rice (Pham et al., 2017), sunflower (Pandey et al., 2013), and has significant potential for biological nitrogen fixation. Diazotrophic bacteria from the genus *Pseudomonas* can solubilize calcium phosphate (Rodríguez and Fraga, 1999) and zinc oxide, and can synthesize IAA (Islam et al., 2016), which was also observed in this study.

The *Rhizobium* genus is composed of microorganisms generally identified as rhizobia and gram-negative bacteria, and is usually related to nitrogen-fixing bacteria that form nodules on leguminous plants (Stroschein, 2010). Singh et al. (2015) isolated *Rhizobium radiobacter* from corn and identified its growth-promoting activity. Recently, Diez-Mendez et al. (2015) identified the most efficient approach to biological nitrogen fixation from co-inoculation of *Rhizobium cellulosilyticum* with beans. Isolated bacterial strains from *Cymbidium* sp. were identified as belonging to the genus *Rhizobium* and have the ability to solubilize calcium phosphate, consistent with the findings of Rodríguez and Fraga (1999).

The above findings indicate that the inoculation of orchid plantlets with diazotrophic bacteria during *in vitro* propagation can be a viable strategy for accelerating the acclimatization of plants; this approach can, thus, reduce production costs by improving growth and nutritional efficiency. The promotion of plant growth as a result of bacterial metabolic processes, the focus of this study, has the potential to contribute to achieving sustainability in the agribusiness sector. Therefore, inoculating orchid plantlets with selected bacterial strains (*Herbaspirillum frisingense* and *Stenotrophomonas maltophilia*) could provide the floriculture and ornamental plant sector with a greater competitive edge in the production and marketing of their agricultural products. The biotechnological applications of bacterial inoculation technology in agriculture might contribute to the reduction in use and consequent impact of fertilizers, leading to significant economic benefits.

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