AUXIN-PRODUCING BACTERIA ISOLATED FROM THE ROOTS OF *Cattleya walkeriana*, AN ENDANGERED BRAZILIAN ORCHID, AND THEIR ROLE IN ACCLIMATIZATION[1]

Renato Fernandes Galdiano Júnior[2], Eliamar Aparecida Nascimbém Pedrinho[2], Tereza Cristina Luque Castellane[2] & Eliana Gertrudes de Macedo Lemos[3]

SUMMARY

Auxin-producing bacteria inhabit the roots of orchids and can bring benefits to the host plant. Plants of this family are multiplied by *in vitro* assimbiotic conditions and little is known about the role of these microorganisms for *ex vitro* acclimatization. Four auxin-producing rhizobacteria isolated from the specie *Cattleya walkeriana* were evaluated for their ability to promote survival and growth of *in vitro* germinated plantlets during *ex vitro* acclimatization. Partial sequencing of the 16S rRNA genes of bacteria cultures from root velamen of this epiphytic species identified them as *Bacillus*, *Burkholderia*, *Enterobacter* and *Curtobacterium*. The presence of indole compounds in the filtered supernatants of liquid cultures was quantified by colorimetric assay and confirmed by HPLC. Indole-3-lactic acid (ILA) and indole-3-acetaldehyde (IAAld) were present in high quantities, except in *Enterobacter* sp. cultures, where in indole-3-acetic acid (IAA) and indole-3-pyruvic acid (IPA) were more prevalent. These rhizobacteria were inoculated into asymbiotically-germinated plantlets of the host orchid, acclimatized in greenhouse for 90 days and assessed for their growth-promoting ability. The lowest ability to promote growth was observed for *Burkholderia* sp. and *Curtobacterium* sp., while *Bacillus* sp. and *Enterobacter* sp. improved growth in all evaluated characteristics and increased the percentage of plantlet survival. This

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[2] Post-graduatings of Programs in Agronomy (Genetic and Plant Breeding) and Microbiology. Laboratory of Biochemistry of Microrganisms and Plants, FCAV-UNESP. Via de Acesso Prof. Paulo Donato Castellane sn., CEP 14884-900, Jaboticabal (SP). E-mail: renatofgaldianojr@yahoo.com.br

[3] Professor of Biochemistry. Laboratory of Biochemistry of Microrganisms and Plants, Department of Technology, FCAV-UNESP. CNPq Scholarship. E-mail: egerle@fcav.unesp.br

study highlights the role of auxin-producing rhizobacteria and their benefits for growth promotion of a Brazilian orchid germinated in asymbiotic conditions, during acclimatization, when plant mortality is high, limiting orchid propagation.

Index terms: plant growth-promoting rhizobacteria; indolic compounds; Orchidaceae.

RESUMO: *BACTÉRIAS PRODUTORAS DE AUXINAS ISOLADAS DE RAÍZES DE* Cattleya walkeriana*, ORQUÍDEA BRASILEIRA AMEAÇADA DE EXTINÇÃO, E SUA FUNÇÃO NA ACLIMATIZAÇÃO*

Bactérias produtoras de auxinas habitam raízes de orquídeas e podem trazer benefícios para a planta hospedeira. Plantas dessa família são multiplicadas em condições assimbióticas in vitro *e pouco se conhece sobre a função desses microrganismos para a aclimatização* ex vitro. *Quatro rizobactérias isoladas da espécie* Cattleya walkeriana *foram avaliadas por sua capacidade de promoção do crescimento e sobrevivência de plântulas germinadas* in vitro *durante a aclimatização. Essas rizobactérias foram identificadas como* Bacillus, Burkholderia, Enterobacter *e* Curtobacterium, *com base no sequenciamento do gene 16S rRNA. A presença de compostos indólicos no sobrenadante filtrado de culturas líquidas foi quantificada por ensaios colorimétricos e cromatografia líquida de alta eficiência (CLAE). Ácidos 3-indol lático (AIL) e indol-3-acetaldeído (AIAld) foram encontrados em grande quantidade, exceto na cultura de* Enterobacter *sp., em que ácido 3-indol acético (AIA) e ácido 3-indol pirúvico (AIP) prevaleceram. As rizobactérias foram inoculadas em plântulas germinadas* in vitro*, aclimatizadas em casa de vegetação durante 90 dias e avaliadas quanto à sua capacidade de promover o crescimento.* Burkholderia *sp. e* Curtobacterium *sp. proporcionaram a menor eficiência para o crescimento, enquanto* Bacillus *sp. e* Enterobacter *sp. favoreceram incrementos em todas as características avaliadas e ampliaram a percentagem de sobrevivência. Este trabalho elucida a função de rizobactérias produtoras de auxinas e seus benefícios para a promoção de crescimento de uma orquídea brasileira germinada em condições assimbióticas durante a aclimatização – condição que confere alta letalidade e limitante para a propagação de orquídeas.*

Termos de indexação: rizobactérias promotoras do crescimento de plantas, compostos indólicos, Orchidaceae.

INTRODUCTION

Currently, there is a great interest in bacterial interactions with plants. The colonization of roots by microorganisms is, in many cases, beneficial to plant growth, development and productivity. The synthesis of plant hormones such as auxins, gibberellins, cytokinins (Tien et al., 1979; Ahmad et al., 2008) and polyamines (Cassán et al., 2009) by some microorganisms that inhabit the rhizosphere are considered to be involved in major microbial interactions with the host plants (Srinath et al., 2003; Yang et al., 2009). Bacteria that are beneficial to plants and free-living in soil have been called plant growth-promoting rhizobacteria (Kloepper at al., 1989; Solano et al., 2008).

Orchidaceae is one of the largest monocotyledonous families. It includes over 850 genera and 25,000 species and represents about 10 % of all phanerogamic plants, without counting the hybrids that have been created (around 100,000, more than of any other ornamental crop) (Roberts & Dixon, 2008). In Brazil and worldwide, the culture and trade of orchids has been based on predatory extraction, which, combined with ongoing urbanization and extended agricultural borders, has contributed to the imminent danger of extinction of different species.

The roots of Orchidaceae plants are composed of vascular cambium, cortex and velamen. The latter has lignified cells and can serve as a reserve source of water and nutrients and can function as protection (mechanical protection and solar radiation reflection), especially of roots of epiphytic orchids (Arditti, 2008). The velamen is permeable to gas and also serves as an important and specific niche inhabited by associative microorganisms (Tsavkelova et al., 2007a).

Interactions of orchids with their mycorrhizal fungi, essential for natural seed germination, have been subject of investigations with native species (Pereira et al., 2005, 2009). Although rhizobacteria are known to have a great and often favorable impact on plant development (Solano et al., 2008), little is known about composition and functional activity of the orchid-associated bacteria and only one paper

reported the occurrence of the N_2 fixing *Azospirillum amazonense* on roots of Brazilian orchids (Lange & Moreira, 2002).

The *in vitro* cultivation of plantlets, characterized by conditions of controlled light and high sugar and moisture contents, favors the reduction of photosynthetic activity, malfunction of stomata and impaired cuticle development (Hazarika, 2006). The process of acclimatization consists of the conversion of heterotrophic to autotrophic growth conditions and a gradual return to the natural growth characteristics of the plant. Consequently, the survival rate in the greenhouse environment is low and the loss of plantlets may be high enough to become a limiting factor in the process of micropropagation, especially for orchid plantlets that are mostly germinated under asymbiotic conditions *in vitro* (Arditti, 2008). The use of rhizobacteria to overcome the difficulties of this stage of propagation has improved the survival of plantlets cultured aseptically (Srinath et al., 2003; Thomas, 2004).

The aims of this study were to isolate and to identify auxin-producing rhizobacteria living in velamen of the *Cattleya walkeriana* orchid, to identify by HPLC the indole compounds produced by the bacteria and to evaluate their effectiveness in promoting growth and survival during *ex vitro* acclimatization of *in vitro* germinated plantlets.

MATERIALS AND METHODS

Isolation of rhizoplane and endophytic bacteria

Cattleya walkeriana root samples were collected from their natural habitat in the forest of Jaboticabal (21 ° 15 ' 17 " S and 48 ° 19 ' 20 " W, at an altitude of 605 m asl) in the State of São Paulo, Brazil. The root tissue was harvested during the summer. Roots were cut and disinfected according to the protocol described by Tsavkelova et al. (2007a).

For the isolation of rhizoplane bacteria, roots were cut into 2-5 mm segments in an aseptic chamber, placed in an autoclaved mortar, macerated in sterile saline solution (NaCl 0.8 %) and serially diluted (10^{-5}–10^{-9}). An aliquot (50 µL) of each dilution was transferred to Czapek agar medium (Tsavkelova et al., 2007a), supplemented with a filtered cyclohexamide antifungal solution (1.0 mg mL^{-1}).

Isolation of endophytic rhizobacteria was accomplished by surface desinfestation of the roots, according to Wilkinson et al. (1989). The roots were sliced with a scalpel blade into 1-2 mm pieces and transferred to plates containing the culture media. The sterility of the root surface was confirmed by plating them on Czapek agar medium.

The plates were then incubated at 30 ºC until the appearance of the colonies could be detected, and morphologically distinct colonies were collected, purified and then stored at – 80 ºC.

Colorimetric quantification of indolic compound production

The amount of indolic compounds secreted by bacteria into the culture medium was estimated by cultivating bacteria in Czapek liquid medium supplemented with L-tryptophan (Merck). Of the bacterial suspension, 5–10 % was transferred to 30 mL flasks containing 5 mL of the same medium supplemented with 200 µg mL^{-1} of tryptophan and maintained at 28 °C in the dark under constant agitation of 140 rpm. After growth for 48 h, the liquid cultures were centrifuged at 7,000 g at 4 °C for 10 min and the supernatants were collected.

The amount of indolic compounds per mL of culture was estimated adding 1 mL of culture supernatant to 2 mL of Salkowski's reagent (Gordon & Webber, 1951). The mixture was kept for 30 min in a dark environment until reddening, with darker red indicating a higher amount of indole compounds. The color intensity was measured at an absorbance of 530 nm (Asghar et al., 2002) in a Beckman DU 640 spectrophotometer (Caron, USA). The auxin concentration was estimated using a standard curve prepared with known amounts (1, 5, 10, 25, and 50 µg mL^{-1}) of filtered IAA (Sigma-Aldrich, USA) that generated the equation $\hat{y} = 0.016x + 0.3077$, with an $R^2 = 0.9845$.

Detection and quantification of indole compounds by HPLC

To confirm and quantify indole compounds in the culture supernatants, a high performance liquid chromatography (HPLC) assay was conducted. The four rhizobacterial isolates producing most indolic compounds were grown as described above, by the method modified by Tsavkelova et al. (2007b). HPLC was performed by injection of a 5 µL aliquot into a column of a reverse phase C18 HRC-ODS (Shimadzu, Kyoto, Japan) HPLC apparatus connected to a SCL 10A VP (Shimadzu) and a UV absorbance detector set to measure a visible signal at 254 nm. The mobile phase was composed of water: acetonitrile: acetic acid (40:60:1, v/v) at pH 2.8. The flow rate was 0.5 mL min^{-1} and the operation pressure 51 MPa. The presence of auxins was confirmed by comparison of peak retention times with those of authentic indolic standards added to the Czapek medium and extracted by the same procedures. Quantification was performed by comparing the weight of peaks and areas using the program CLASS-VT (Shimadzu). The standard curve was prepared in culture medium inoculated with known amounts of filtered indole-3-acetic acid, indole-3-pyruvic acid, indole-3-lactic acid, or indole-3-acetaldehyde (Sigma-Aldrich) at different concentrations

(0.25, 0.5, 1.0, 2.0, and 4.0 μg mL^{-1}), leading to the equation $\hat{y} = 9.085x + 0.147$, with $R^2 = 0.9995$.

PCR amplification of 16S rRNA genes and sequencing

Genomic DNA from bacteria was prepared using the QIAquick DNeasy® Blood & Tissue kit (Cat. Nº 69506) (Qiagen, UK) according to the manufacturer's recommendations. The 16S rRNA genes were amplified by PCR using the universal primers fD1 (5'-AGAGTTTGATCCTGGCTCAG-3') and rD1 (5'-AAGGAGGTGATCCAGCC-3') (Weisburg et al., 1991). The PCR mixture consisted of 1X PCR buffer [20 mmol L^{-1} Tris-HCl (pH 8.3), 50 mmol L^{-1} KCl], 200 μmol L^{-1} each of deoxyribonucleotides, 2.5 mmol L^{-1} $MgCl_2$, 2.5 pmol of each oligonucleotide primer; 20 ng of template DNA, 0.5 U of *Taq* DNA polymerase and Milli-Q sterile water to a final volume of 20 μL. Each reaction was performed in duplicate.

The amplification reaction was performed using the Gene Amp PCR thermocycler system (Applied Biosystems) with the following cycles: 3 min at 95 °C, 35 cycles (1 min at 95 °C, 1 min at 56 °C and 2 min at 72 °C), followed by a final extension at 72 °C for 5 min and stabilized at 4 °C. The PCR products were analyzed by running a 1.5 % agarose gel (Sigma) in 1x TEB buffer, stained with ethidium bromide, visualized under u.v. light and photographed with the GelDoc BioRad 101 photodocumentation system, which uses QuantityOne® software (BioRad™, USA). The PCR amplicon was purified using the enzyme SAP (Fermentas, Lithuania) according to the manufacturer's recommendations. Partial sequences of 16S rRNA genes were obtained using an automatic sequencer (ABI-PRISM 3700 DNA Analyzer, Applied Biosystems, USA) and primer fD1, along with DYEnamic ET Terminator cycle sequencing (Amersham Biosciences, USA) according to the manufacturer's recommendations.

To check the quality of sequences generated, the program "sequencing analysis 3.4" (Applied Biosystems) was used, which generated the electropherograms that were submitted to analysis by the packages Phred (Ewing et al., 1998) and Phrap (http://www.phrap.org). The selection of sequences was conducted using ContGen, with a minimum of 400 base pairs and with Phred quality > 20. For comparison with homologous sequences, the "nucleotide blast" function of the NCBI database (http://www.ncbi.nlm.nih.gov/blast) was used. The nucleotide sequences of the 16S rRNA genes determined in this study were deposited in the GenBank database (access numbers FJ664511 - FJ664514).

Assessment of the phylogenetic relationships of isolates including the related rate was conducted using Mega 3.0 (Kumar et al., 2004). The phylogenetic tree was constructed by the Neighbor-Joining method (Saitou & Nei, 1987), using the matrix obtained from the alignment of sequences acquired from the CLUSTAL algorithm X 1.81. Distances were calculated using Kimura (Kimura, 1980), and the 16S rRNA gene sequence of an Archaea isolate was used as outgroup.

Cattleya walkeriana in vitro seed germination, *ex vitro* inoculation and acclimatization

Closed capsules with mature seeds of *C. walkeriana* were desinfested using 1 % sodium hypochlorite and 70 % ethanol (v/v) (Arditti, 2008). Approximately 10 mg of seeds were placed in 300 mL vials containing 40 mL of MS nutrient medium (Murashige & Skoog, 1962). The vials were maintained in a growth chamber with controlled temperature and light (25 ± 2.0 °C, artificial light of 75 μmol^{-1} m^{-2} s^{-1} and a photoperiod of 16 h) for sowing and subsequent cultivation for 90 days.

After this period, when the protocorms had two leaflets, they were subcultured in MS medium with half the macronutrient concentration. Seedlings were grown for 60 days, subcultivated again on the same nutrient medium until 210 days after sowing and transferred to the *ex vitro* stage in a greenhouse.

Through serial dilutions and counting of colony-forming units, growth periods between 36–48 h were shown to result in bacterial concentrations of ~10^8 cells mL^{-1}, which were then used for inoculation. Homogeneous plantlets with 1.2 +/– 0.2 cm were selected and the root system was immersed for 30 min in the bacteria culture. The substrate was composed of sterilized coconut fiber and sphagnum (1:1, v/v) with the inoculum (10 mL for each 200 mL pot). A treatment without bacteria, but autoclaved deionized water only, was used as control.

The experiment consisted of five treatments with four isolates and one control. Each treatment consisted of four pots containing 10 plantlets in a randomized block design. The test was conducted in a greenhouse covered with a screen for the retention of 60 % of light and with a glass top. Watering occurred daily for 90 days, then the dry mass (roots and shoots) was measured, as was the number of roots. Root length were measured with a caliper. Leaf area was measured with a LI300 Area Meter (Lincoln, USA). Data were submitted for analysis of variance (ANOVA) using the software SAEG (www.ufv.br/saeg/), and averages were compared by the Tukey test at 5 % probability. To evaluate the leaf area and total dry mass, the formula IR = [(T - C)/C] 100 was used to calculate the increase rate, where IR = increase rate, T = treatment value, C = control value.

RESULTS AND DISCUSSION

Twenty-six bacteria were isolated and purified from the rhizoplane of the orchid *C. walkeriana,* of which 11 were epiphytic and 15 endophytic types (Table 1). Among IAA-positive bacteria, the four greatest auxin producers were selected for further testing (including two epiphytic and two endophytic).

After comparison with the 16S rRNA sequences available in the GenBank database, these four isolates were identified as *Burkholderia* sp., access number FJ664512 (AB191216, 99 % similarity); *Curtobacteria* sp. FJ664511 (EU741030, 99 % similarity); *Enterobacter* sp. FJ664513 (EU221358, 98 % similarity) and *Bacillus* sp. FJ664514 (FJ603032, 99 % similarity). A phylogenetic tree was generated with these bacterial sequences and homologous sequences indexed in the GenBank (Figure 1).

Tabe 1. Rhizobacterial affiliation of the isolates compared with 16S rRNA sequences in GenBank database and the microbial indolic compounds (IC) production in Czapek liquid medium supplemented with L-Tryptophan (200 μL^{-1}) measured by colorimetric assay

Root part	Isolate code	Homologous sequence in GenBank			IC production
		Species	Acc no.	e-value	
					$\mu g\ mL^{-1}$
	RzW31	*Bacillus* sp.	FJ174643	0	2.35 ± 0.7(1)
	RzW32	*Burkholderia* sp.	AB191216	0	22.2 ± 1.9
	RzW33	*Pantoea* sp.	AF130971	0	1.8 ± 0.4
	RzW34	*Bacillus* sp.	EU795037	0	9.6 ± 1.2
	RzW35	*Curtobacterium* sp.	EU741030	0	10.7 ± 0.4
Epiphytic	RzW36	*Enterobacter* sp.	EU554444	0	5.4 ± 0.8
	RzW51	*Pseudomonas* sp.	EF157292	0	-
	RzW52	*Bacillus* sp.	EU754025	0	8.6 ± 0.8
	RzW53	*Pantoea* sp.	DQ131851	0	8.8 ± 0.3
	RzW54	*Achromobacter* sp.	FJ810080	0	0.8 ± 0.3
	RzW55	*Bacillus* sp.	AB376080	$1e^{-6}$	-
	EndW37	*Enterobacter* sp.	EU221358	0	32.3 ± 2.7
	EndW38	*Pseudomonas* sp.	EF157292	0	6.4 ± 0.5
	EndW39	*Bacillus* sp.	EU836171	0	2.8 ± 0.6
	EndW40	*Bacillus* sp.	EU809481	0	2.6 ± 0.5
	EndW41	*Enterobacter* sp.	AM403125	0	1.6 ± 0.3
	EndW42	*Burkholderia* sp.	AB366336	0	10.4 ± 1.6
	EndW43	*Bacillus* sp.	FJ174643	0	2.35 ± 0.7
Endophytic	EndW44	*Pantoea* sp.	AF360974	0	7.4 ± 0.6
	EndW45	*Curtobacterium* sp.	EU740993	$1e^{-6}$	-
	EndW46	*Burkholderia* sp.	DQ150551	0	5.8 ± 0.6
	EndW47	*Enterobacter* sp.	AM403125	0	1.6 ± 0.3
	EndW48	*Bacillus* sp.	FJ603032	0	18 ± 0.2
	EndW49	*Burkholderia* sp.	AB366366	0	8.3 ± 0.2
	EndW50	*Pseudomonas* sp.	DQ854817	0	-
	EndW56	*Pseudomonas* sp.	EU073118	0	2.1 ± 0.1

(1) Averages of three independent experiments for each isolate in triplicate ± standard deviation.

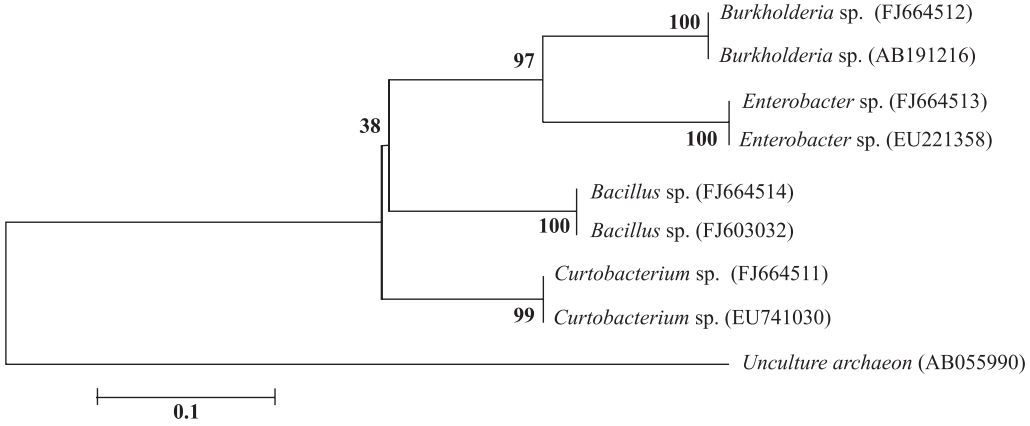

Figure 1. Phylogenetic tree based on 16S rRNA sequences using the Neighbor-Joining method to draw a relationship between rhizobacterial isolates and closely related bacteria listed in GenBank. The numbers on the nodes indicate bootstrap values. The 16S rRNA accession numbers are given within brackets. Bar = 0.1 substitution per site.

The number of bacterial isolates obtained was influenced by several factors, including simply the ability to be cultivated *in vitro*. Certainly, this approach provides only a partial picture of the bacterial community, which is larger and more diverse in the roots of epiphytic orchids in relation to the terrestrials due to the presence of velamen (Tsavkelova et al., 2007a). Environmental factors also play a role in the number of bacterial isolates obtained, including orchid habitat and cultivation conditions. In a pioneer study of bacteria isolated from orchid roots, Wilkinson et al. (1989) showed that bacterial abundance varied with season and age of the root tissues; additionally, they found that prokaryotic diversity was related to the taxon of the host plant.

Microbial root colonization can also be determined by assessing the composition of exudates, such as carbohydrates, organic acids, amino acids, and vitamins (Solano et al., 2008; Tsavkelova, 2007a), which promote both physical benefits (mucilages that protect the roots from friction with the growth substrate) and chemical benefits (attraction of beneficial microorganisms) for the plant (Nehl et al., 1996). The root exudates also enrich the rhizosphere with tryptophan, which is the main precursor in the microbial biosynthesis of IAA (Tien et al., 1979).

Family members of Enterobacteriaceae, Burkholderiaceae, Microbacteriaceae and Bacillaceae are known as plant-associated bacteria (Ahmad et al., 2008; Solano et al., 2008). *Bacillus* spp. and *Enterobacter* spp. were isolated as root endophytes of some species of terrestrial Western Australian orchids (Wilkinson et al., 1994), and the presence of *Bacillus* spp. symbionts was observed in the rhizosphere of tropical terrestrial and in the rhizoplane of epiphytic orchids (Tsavkelova et al., 2007a).

Differences among the amounts of IAA secreted in the bacterial cultures were identified using colorimetric and HPLC assays (Table 2). Considerable differences between these two analysis methods were observed (nearly eight-fold for the same bacteria). In agreement with our results, Crozier et al. (1988) compared the production of auxin in *Azospirillum* spp. cultures and also found great differences in the amount of IAA measured between the two techniques (more than 50-fold); HPLC detected IAA levels undetectable in the colorimetric assay. This discrepancy may be attributed to the fact that several indole compounds present in the liquid culture supernatants are more reactive to IAA alone when in Salkowski's solution (Tsavkelova et al., 2007b).

Thus, due to the simplicity of the colorimetric assay, this test may be recommended for selection of auxin-producing rhizobacteria, although a more accurate quantification of selected isolates must be performed by HPLC, a more cost and time-consuming technique. Future analyses may also include mass spectrometry, a very powerful method of detection, in order to confirm these indole compounds (Sachdev et al., 2009).

Indole-3-lactic acid (ILA), followed by indole-3-acetaldehyde (IAAld) were the most abundant indole compounds detected in the cultures of three strains tested in this study (Figure 2). During bacterial biosynthesis of IAA, some intermediaries can be converted to storage compounds that are important for transport and protection against enzymatic degradation of IAA; this can occur through the reduction of indole-3-pyruvic acid (IPA) and IAAld to ILA (Spaepen et al., 2007).

Since the discovery by Lewis Knudson in 1922 that orchid seeds can germinate in a culture medium containing mineral salts, agar, sucrose, amino acids, vitamins, growth regulators and other salts (Arditti 2008), asymbiotic germination has become an established and convenient method for obtaining plantlets of the Orchidaceae family for commercial or conservation purposes (Pereira et al., 2005; Stewart & Kane, 2006).

However, despite the rapid propagation and acquisition of a large number of uniform pathogen-free plantlets provided by asymbiotic methodologies, the acclimatization of *in vitro*-propagated orchids is characterized by high mortality of the plantlets during

Table 2. Rhizobacterial auxin production in Czapek liquid medium supplemented with L-Tryptophan (200 mg mL^{-1}) as measured by colorimetric (CA) and hight performance liquid chromatography (HPLC) assays. IAA, indole 3-acetic acid; IPA, indole 3-pyruvic acid; IAAld, indole-3-acetaldehyde; ILA, indole-3-lactic acid

Isolate code	Bacterial isolate	Production of indole compounds				
		CA	HPLC			
			Total	ILA	IAA + IPA	IAAld
				µg mL^{-1}		
RzW32	*Burkholderia* sp.	22.2 ± 1.9[1]	-[2]	1.75	-[2]	0.36
RzW35	*Curtobacterium* sp.	10.7 ± 0.4	1.7±0.5	1.24	0.11	0.35
EndW37	*Enterobacter* sp.	32.3 ± 2.7	4.15± 0.5	1.4	2.3	0.45
EndW48	*Bacilus* sp.	18 ± 0.2	2.33± 0.3	2.01	0.16	0.16

[1] Averages of three independent experiments for each isolate in triplicate ± standard deviation; Could not be quantified.

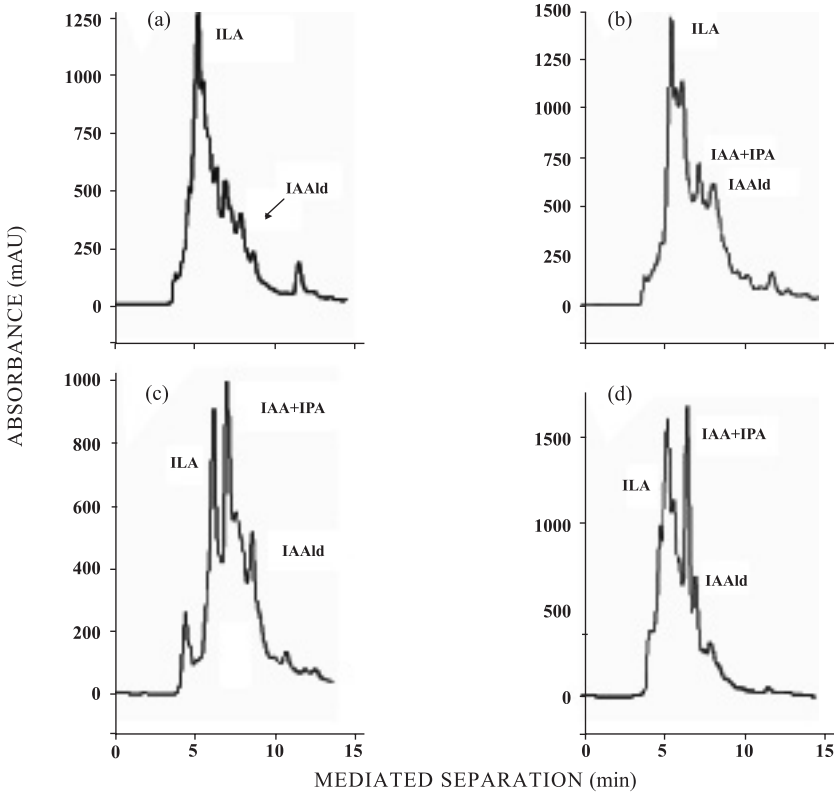

Figure 2. HPLC-mediated separation of indole compounds in rhizobacterial culture supernatants: (a) *Burkholderia* sp., (b) *Curtobacterium* sp., (c) *Enterobacter* sp. and (d) *Bacillus* sp. IAA, indole 3-acetic acid; IPA, indole 3-pyruvic acid; IAAld, indole-3-acetaldehyde; ILA, indole-3-lactic acid.

adaptation to *ex vitro* conditions (Hazarika 2006; Cha-Um et al., 2009). This increased mortality rate contributes to a reduction in the amount and a consequent increase in the costs of plantlets produced.

Bacillus sp. and *Enterobacter* sp. conferred orchids the highest survival percentages compared with the control treatment. *Burkholderia* sp. and *Curtobacteria* sp. however conveyed the highest plant mortality rates (Table 3). In the case of microorganisms that inhabit the rhizosphere, these results can be expected because bacteria that provide benefits or losses to host plants coexist in the same ecological niche. Accordingly, Nehl et al. (1996) have discussed the fact that individual rhizobacterial isolates can act to promote or inhibit plant growth according to environmental conditions, host genotype and mycorrhizal status.

Plantlets inoculated with *Burkholderia* sp. showed the lowest dry weight of shoots and foliar areas and a consequently increased leaf area and rate of plantlet survival (Table 2); thus, bacteria of this genus may

Table 3. Increased growth of *Cattleya walkeriana* inoculated with rhizobacterial isolates after acclimatization in a greenhouse for 90 days. Foliar area (FA), root number (RN), root length (RL), root part (RP), aerial part (AP), survival (S), foliar area (FA) and Total (T) increase rate

Treatment	FA	RN	RL	Dry mass		S	Increase rate	
				RP	AP		FA	T
	cm^2	no.	cm	mg		%		
Burkholderia sp.	3.7b	2.57c	6.35bc	24.52bc	17d	57.5	0	7.61
Curtobacterium sp.	4.7b	2.85b	5.33c	24.12bc	18.3c	55	12	5.13
Enterobacter sp.	8.5a	3.29 a	6.82 a	39.28a	32.25a	75	178.9	77.33
Bacillus sp.	4.9b	3.24a	6.67ab	26.5b	22.8b	80	27	22.2
Control	4.7b	2.47c	5.34c	23.36c	18.9c	60	0	0
CV (%)	5.12	8.45	6.62	4.46	1.7			

Means followed by the same letter within a column do not differ significantly ($p < 0.05$) by the Tukey test.

be pathogenic to orchids (Keith et al., 2005). The genus *Burkholderia* consists of a heterogeneous group of bacteria with extraordinary versatility that occupies different nutritional niches. In recent decades, *Burkholderia* sp. have been shown to be active participants in interactions of plants with other microorganisms, in that they benefit plant growth (PGPR), but promote the growth of certain plant pathogens as well (Poonguzhali et al., 2007). These responses can affect the density of isolated cells, possibly via complex and incompletely understood mechanisms.

Plantlets inoculated with an isolate of the *Enterobacter* sp. showed the greatest increase in leaf area, number and length of roots and dry mass. These characteristics were also reflected in higher rates of survival and growth increase (Table 3). Leaf area is a parameter that reflects significant growth of the plant, since leaves capture light energy from the sun and aid in the production of organic matter through the photosynthetic process. Plantlets inoculated with this isolate showed an increased shoot dry weight of the shoots, which consist of rhizomes and leaves, thus proving the effectiveness of this bacterium in inducing plant growth.

Particularly, the increase of the total biomass was higher in the treatments inoculated with *Enterobacter* sp. and *Bacillus* sp., which are endophytic bacteria. These kinds of microorganisms attract special interest, due to their biotechnological potential, once they live in the interior of plants and can produce numerous growth factors. As in the present research, Assumpção et al. (2009) isolated a endophytic *Enterobacter* sp. with high auxin production from soybean seeds, which efficiently promoted growth of the host species.

Although symbiotic germination determines the requirements of the asymbiotic growth of orchids (Stewart & Kane, 2006), aseptic *in vitro* culture is preferred because of ease, the simplicity of the materials involved and extensive knowledge about the technique (Arditti, 2008). As a result, the range of symbiotic microorganisms that associate with this plant family (mycorrhiza or rhizobacteria) remain poorly characterized (Pereira et al., 2005). The results of this study offer prospects for the use of rhizobacteria during the acclimatization of orchid plantlets, which is considered to be the bottleneck stage of their micropropagation.

This study highlights the role of auxin-producing rhizobacteria and their benefits to orchid plantlets during a normally lethal stage of propagation. Future research with these rhizobacteria, even associated with mycorrhiza will emphasize their importance in promoting growth and survival of Brazilian orchids germinated under asymbiotic conditions during *in vitro* germination or *ex vitro* acclimatization, when plant mortality is high, limiting the propagation of the Orchidaceae family.

CONCLUSION

Auxin-producing rhizobacteria *Enterobacterium* sp. and *Bacillus* sp. promoted the growth of *Cattleya walkeriana* plantlets during *ex vitro* acclimatization in greenhouse.

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