EVERALDO DA SILVA CRUZ

TAXONOMY AND PHYLOGENY OF MYCORRHIZAL FUNGI ASSOCIATED WITH Gomesa recurva (ORCHIDACEAE) AND CHARACTERIZATION OF ENDOPHYTIC BACTERIA OF ORCHIDS

Dissertação apresentada à Universidade Federal de Viçosa, como parte das exigências do Programa de Pós-Graduação em Microbiologia Agrícola, para obtenção do título de Magister Scientiae.

Orientadora: Maria Catarina M. Kasuya

Coorientadores: Meiriele da Silva
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Assentimento:

Everaldo da Silva Cruz

Everaldo da Silva Cruz
Autor

Maria Catarina Megumi Kasuya

Maria Catarina Megumi Kasuya
Orientadora

A Deus,

Aos meus pais,

Aos meus irmãos,

Aos meus amigos,

Dedico.

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BIOGRAFIA

Everaldo é filho de Maria Aparecida da Silva Cruz e Antônio Raimundo da Cruz. Nasceu em Inhapim, Minas Gerais. Formou-se no ensino médio, em 2010, na Escola Estadual Manoel Joaquim Teodoro, em Imbé de Minas, Minas Gerais.

Em 2012, ingressou no curso de Agronomia pela Universidade Federal de Viçosa. De outubro de 2014 a julho de 2018 foi estudante iniciação científica no Laboratório de Associações Micorrízicas. No período de 2015 a 2018 desenvolveu pesquisas pelo projeto Protax, desenvolvendo projetos relacionados a associações micorrízicas de orquídeas.

Em julho de 2018 concluiu o curso de Agronomia e em agosto de 2018 ingressou no Programa de Pós-Graduação em Microbiologia Agrícola. Retomou as pesquisas do projeto Protax sob orientação da professora Maria Catarina Megumi Kasuya no Laboratório de Associações Micorrízicas.

RESUMO

CRUZ, Everaldo da Silva, M.Sc., Universidade Federal de Viçosa, setembro de 2020. **Taxonomia e filogenia de fungos micorrízicos associados à Gomesa recurva (Orchidaceae) e caracterização de bactérias endofíticas de orquídeas.** Orientadora: Maria Catarina Megumi Kasuya. Coorientadores: Meriele da Silva e Olinto Liparini Pereira.

As orquídeas são dependentes de uma fonte externa de carbono, devido à ausência de tecido de reserva em suas sementes. Dessa forma, para obter carbono e outros nutrientes, associam-se com fungos mutualistas em seu sistema radicular, os quais são conhecidos como fungos micorrízicos orquidoides. Estes fungos são caracterizados por formar pelotons, um conjunto de enovelados de hifas no interior das raízes. Além disso, são capazes de colonizar as células do embrião das sementes de orquídeas e podem permanecer até a fase adulta da planta. As orquídeas podem associar-se com outros microrganismos como bactérias endofíticas, que podem trazer diversos benefícios como promoção de crescimento, devido a produção de fitohormônios, fixação biológica de nitrogênio, solubilização de fosfato, produção de sideróforos, entre outros. Este trabalho teve como objetivos (1) estudar a diversidade taxonômica de fungos micorrízicos associados a Gomesa recurva (2) identificar e caracterizar bactérias endofíticas associadas à Gomesa recurva R. Br. e Zygopetalum maxillare Lodd. Pelas análises filogenéticas e morfológicas, todos os isolados de G. recurva pertencem à uma nova espécie de Ceratobasidium. Foram obtidos cinco isolados de bactérias para cada espécie de orquídea. Pelo sequenciamento da região 16S rRNA, verificou-se que pertenciam aos gêneros Pseudomonas e Paraburkholderia, os quais, pelos testes in vitro, mostraram que possuem capacidade de realizar a fixação biológica de nitrogênio, de produzir fitohormônio ácido indol-3-acético (AIA) e solubilizar de fosfato de cálcio. Os isolados de fungos e bactérias obtidos apresentam potencial para serem utilizados para a produção de mudas de orquídeas.

Palavras-chave: Filogenia. Fungos. Bactérias. Fitohormônios.

ABSTRACT

CRUZ, Everaldo da Silva, M.Sc., Universidade Federal de Viçosa, September, 2020. **Taxonomy and phylogeny of mycorrhizal fungi associated with Gomesa recurva (Orchidaceae) and characterization of endophytic bacteria of orchids.** Advisor: Maria Catarina Megumi Kasuya. Co-advisors: Meriele da Silva and Olinto Liparini Pereira.

Orchids are dependent on an external source of carbon, due to the absence of reserve tissue in their seeds. Thus, in order to obtain carbon and other nutrients, they associate with mutualistic fungi in their root system, which are known as orchidoid mycorrhizal fungi. These fungi are characterized by forming pellets, a set of entwined hyphae inside the roots. In addition, they are able to colonize the embryo cells of the orchid seeds and can remain until the adult stage of the plant. Orchids can associate with other microorganisms such as endophytic bacteria, which can bring several benefits such as growth promotion, due to the production of phytohormones, biological nitrogen fixation, phosphate solubilization, production of siderophores, among others. The objective of this work was (1) to study the taxonomic diversity of mycorrhizal fungi associated with Gomesa recurva (2) to identify and characterize endophytic bacteria associated with Gomesa recurva R. Br. And Zygopetalum maxillare Lodd. By phylogenetic and morphological analyzes, all isolates of G. recurva belong to a new species of Ceratobasidium. Five bacterial isolates were obtained for each species of orchid. By sequencing the 16S rRNA region, it was found that they belonged to the genera Pseudomonas and Paraburkholderia, which, by in vitro tests, showed that they have the capacity to perform biological nitrogen fixation, to produce indole-3-acetic acid phytohormone (AIA) and solubilizing calcium phosphate. The isolates of fungi and bacteria obtained have the potential to be used for the production of orchid seedlings.

Keywords: Phylogeny. Fungi. Bacteria. Phytohormones.

SUMÁRIO

1. INTRODUÇÃO GERAL

As orquídeas pertencem à família Orchidaceae, sendo uma das maiores do reino vegetal. Distribui-se em um amplo número de espécies e gêneros, sendo 27.000 espécies e aproximadamente 900 gêneros (Govaerts et al., 2016). Possuem uma larga distribuição global, estando presente em 5 continentes, à exceção na Antártida (Cribb et al., 2003). No Brasil, as orquídeas estão distribuídas em 2805 espécies e 252 gêneros, sendo a Mata Atlântica e Amazônia os biomas com maior diversidade de espécies (http://floradobrasil.jbrj.gov.br/reflora/floradobrasil/FB179).

As orquídeas são cultivadas principalmente para fins ornamentais e algumas espécies nativas alcançam elevados valores comerciais (Medina et al., 2009), o que pode levar a coletas para a venda ilegal de espécies nativas. Este fator, juntamente com a degradação de seus habitats naturais, resulta em um número elevado de espécies listadas como em risco de extinção (Martinelli & Moraes, 2013). No Brasil, 176 espécies de orquídeas estão ameaçadas de extinção (MMA, 2014), o que corresponde a 6,27% das espécies catalogadas no país. O Quadrilátero Ferrífero, por exemplo, localizado na região de Mata Atlântica em Minas Gerais, sofre com a degradação antrópica, mas abriga grande diversidade de orquídeas nativas (Marent et al., 2011; Barros et al., 2016).

Em condições naturais, as orquídeas possuem dificuldade de germinação de suas sementes, devido à ausência de tecido de reserva, sendo presente apenas o embrião e o tegumento no corpo da semente, o que as tornam dependentes de uma fonte externa de carbono (Rasmussen, 1995). Portanto, as orquídeas associam-se com fungos mutualistas em seu sistema radicular, conhecidos como fungos micorrízicos, que possuem importância para germinação e estimulação do crescimento vegetal (Rasmussen & Rasmussen, 2009).

Essa interação entre fungos especializados e raízes de orquídeas, denominada associações micorrízicas orquidoides (Peterson et al. 2004), caracteriza-se por um conjunto de enovelados de hifas fúngicas no interior das raízes, denominando péloton (Rasmussen & Rasmussen, 2014). Os fungos micorrízicos orquidóides também são capazes de colonizar as células do embrião das sementes de orquídeas e, consequentemente, formar os pélotons, que seguem nas raízes até a forma adulta da planta (Dearnaley, 2007, Rasmussen & Rasmussen, 2009). Os pélotons são degradados pelas células do embrião, resultando na liberação de nutrientes, principalmente carbono, que são exigidos em grande quantidade para a germinação e desenvolvimento nos primeiros estágios (Dearnaley et al. 2012).

Fungos que formam esse tipo de associação com orquídeas, são pertencem principalmente ao grupo dos rizoctonióides (Garc et al., 2006). Esse grupo polifilético é composto por espécies cosmopolitas, que são relatadas como patógenos de plantas, saprófitas e, ou endofíticos (Oberwinkler et al. 2013). Entre as principais famílias de fungos micorrízicos que colonizam as orquídeas estão Serendipitaceae, Sebacinaceae, Ceratobasidiaceae e Tulasnellaceae (Weiß et al., 2016). Em relação aos gêneros comumente formando associação micorrízica com orquídeas encontram-se os gêneros Tulasnella, Ceratobasidium e Sebacina (García, 2006).

Nos estudos taxonômicos de fungos do tipo rizoctonióides, existem problemas relacionados ao uso de apenas a morfologia para delimitar novas espécies dentro desse gênero (Andersen, 1990). Dessa forma, para implementar os estudos desse grupo, ferramentas de filogenia molecular vêm sendo utilizadas para o estudo taxonômico, que, associadas com o estudo morfológico, são capazes de discriminar espécies de fungos micorrízicos orquidóides (Linde et al., 2017).

As orquídeas podem, também, associar-se com outros microrganismos, a exemplo de bactérias endofíticas (Vendramin et al., 2010, Chutima et al., 2011). Essas bactérias têm a capacidade de induzir a germinação in vitro de sementes de orquídeas (Tsavkelova et al., 2007). A interação com bactérias endofíticas podem trazer outras vantagens para as plantas, incluindo a fixação biológica de nitrogênio, solubilização de fosfato, antibiose, melhoria da condição hídrica e nutricional das plantas, além da produção de fitohormônios (Mehnaz & Lazarovits 2006, Tsavkelova et al., 2007).

Apesar de estudos evidenciarem a importância das bactérias endofíticas para a germinação e o desenvolvimento de orquídeas, pouco se sabe sobre a composição e atividade funcional dessas bactérias relacionadas às orquídeas (Li et al., 2017).

Diante disso, este trabalho consistiu em investigar a diversidade taxonômica de fungos micorrízicos associados à Gomesa recurva R. Br., orquídea nativa da Mata Atlântica Mineira, bem como isolar, identificar e caracterizar bactérias endofíticas de raízes de duas espécies de orquídeas: Zygopetallum maxillare e G. recurva, em relação a capacidade de fixação de nitrogênio, solubilização de fosfato e produção de ácido indol acético (AIA).

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2. Capítulo 1: Written according to Phytotaxa

A new mycorrhizal species of Ceratobasidium (Ceratobasidiaceae) associated with roots of the epiphytic orchid Gomesa recurva from the Brazilian Atlantic Florest

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Abstract

A new species of Ceratobasidium was isolated from the roots of Gomesa recurva, an epiphyte orchid from the Brazilian Atlantic Forest. Morphological and phylogenetic analyzes based on the ITS DNA sequence, show that this new isolated species is different from the reported species of Ceratobasidium. All descriptions are provided for the new species.

Keywords: Basidiomycota, Orchidaceae, Phylogeny

Introduction

Orchidaceae is the largest family among angiosperms, with around recognised 27,000 species (Govaerts et al. 2016). Brazil stands out in biodiversity of orchids with approximately 2400 species, which them 60% are endemic and most occur on Atlantic Forest (Neto et al. 2013). Despite the great diversity, orchids are frequently threatened of extinction. The IUCN Global Red List have been assessed 1641 orchids (ca. 6% of orchids), which them five are extinct and 747 are threatened, and also include 72 species occurring in Brazil: three critically endangered, six endangered and six vulnerable (IUCN, 2020). These numbers may be higher, since only 6% of orchids were evaluated.

The dependence of orchids on mycorrhizal fungi in the earliest stages of development is well known (Smith and Read 2008), and there are studies on symbionts diversity in adult phase, as well as the specificity of interaction (Zettler et al. 2005, Meng et al. 2019, Thixton et al. 2020, Gao et al. 2020). However, most studies have focused on temperate regions and terrestrial orchids, while works on tropical and epiphytic orchids are scarce.

In Brazil, researches on mycorrhizal fungi in orchids have reported mainly Tulasnella species associated with adult plants (Pereira et al. 2003, 2005, Nogueira et al. 2005, Pereira et

al. 2009, 2014, Freitas et al. 2020) and a few studies have showed Ceratobasidium species in epiphytic orchids (Pereira et al. 2005, Valadares et al. 2011).

Although less investigated, the genus Ceratobasidium is an important symbiont of orchids (Gónzalez et al. 2016). Some studies on tropical orchids have described Ceratobasidium species as the single mycobiont, suggesting interactions highly specific (Otero et al. 2002, Graham and Dearnaley, 2012, Valadares et al. 2012). This high specificity of fungus-orchid interaction emphasizes the importance that Ceratobasidium can play in symbiotic germination programs and further conservation of species.

In a survey of cultivable mycorrhizal fungi associated with Gomesa recurva roots, nine isolates of Ceratobasidium were obtained. Herein, based on morphological and molecular analysis we introduce a new species: Ceratobasidium sp. VIC 47413.

Material and Methods

Sample collection and isolation

Root samples of Gomesa recurva were collected from the Parque Estadual Serra do Brigadeiro (PESB), state of Minas Gerais, Brazil (Fig. 1.). The samples were taken to the Laboratório de Associações Micorrízicas (LAMIC), Departamento de Microbiologia/Universidade Federal de Viçosa, subsequently washed in running water to remove impurities. The fragments were surgace-sterilized in ethanol 70 % for 1 min, sodium hypochlorite 2 % for 6 min and three successive washes in sterile distilled water. The roots were cut transversely into fragments of approximately 0.5 mm and examined under a stereomicroscope, for the removal of the peloton. The pelotons were isolated in plates containing Potato Dextrose Agar (PDA) culture medium with antibiotics (chloramphenicol 1 $mg.mL^{-1}$) and the plates were incubated at 25 ºC in the dark. Axenic cultures were preserved on silica gel and deposited in the culture collection of the Universidade Federal de Viçosa, Coleção Oswaldo Almeida Drummond (COAD). The specimens were deposited at the Herbarium of the Universidade Federal de Viçosa (VIC).

Figure 1. A, Atlantic Forest fragment, natural habitat of Gomesa recurva at Parque Estadual da Serra do Brigadeiro, Minas Gerais, Brazil (PESB); **B**, Gomesa recurva.

Morphology

To describe the characteristics of the fungus and colony, the isolates were grown in PDA at 25 ºC in the dark for 3 days. Measurement of the colony diameters were performed with a digital caliper. The nuclear condition was observed in young hyphae after staining with SYBR Green I (Meinhardt et al. 2001). The formation of monilioid cells was observed in the culture grown on Corn Meal Agar (CMA) at 25 º in the dark, for 1 week (Pereira et al. 2005). Observations, measurements, and photographic images of these microscopic fungal structures were recorded using an Olympus BX53 light microscope, with Olympus Q-Color5TM high-resolution color digital camera and differential interference contrast (DIC) lighting.

DNA extraction, PCR amplification and sequencing

Genomic DNA was extracted from the mycelium of fungi grown in PDA at 25 ºC, using the Nucleospin®Soil extraction kit (MACHEREY-NAGEL GmbH & Co. KG) following the manufacturer's guidelines. The nuclear ribosomal Internal Transcribed Spacer (ITS) region was amplified using the primer pair ITS1 (5’-TCC GTA GGT GAA CCT GCG G-3’) e ITS4 (5’-TCC TCC GCT TAT TGATAT GC-3′) (White et al. 1990). The polymerase chain reaction (PCR) conditions were for a final volume of 50 µL. Were used: 20-30 ng of genomic DNA sample, 1X of buffer, 2 mM $MgCl_2$, 0.2 µM of primer, 0.4 mM of dNTP, 1 U of Taq Dna polymerase. The PCR reaction was performed in a thermocycler. The PCR conditions consisted of initial denaturation at 95 ºC for 2 min; followed by 39 cycles of denaturation at 95 ºC for 1 min; annealing at 50 ºC for 1 min; extension at 72 ºC for 1 min and final extension at 72 ºC for 10 min.

The PCR products were visualized for fragment size and quality on 1.5 % agarose gel stained with ethidium bromide. Subsequently, the PCR product was purified and sequenced. The DNA sequences were analyzed for quality in the Geneious Prime® 2020.0.5 software (https://www.geneious.com).The sequences were deposited in GenBank (http://www.ncbi.nlm.nih.gov/Genbank/) (Table 1).

Phylogenetic Analysis

Consensus sequences were compared in the GenBank nucleotide database using the BLASTn algorithm. Similar sequences were downloaded in FASTA format and aligned with the sequences of the isolates using the online portal MAFFT v.7 (http://mafft.cbrc.jp/alignment/server/index.html) (Katoh & Standley 2013). The sequences resulting from the alignment were manually adjusted in the MEGA software v.7.0.26 (Kumar et at. 2016). The phylogenetic tree was built from the ITS-5.8S region of rDNA sequences.

The Markov Chain Monte Carlo method was used in all sequences for Bayesian Inference (BI) analyzes. Using the MrModeltest 2.3 software (Nylander 2004), nucleotide replacement models were determined. According to the calculations of the probability scores, the models were selected according to the Akaike Information Criterion (AIC). Thus, the results recommended a HKY + G model for ITS, Dirichlet's state frequency distribution was (1,1,1,1) and the rate variation distributed in gamma. Phylogenetic analysis was performed using the CIPRES portal (Miller et al. 2010) and using MrBayes v.3.1.1 software (Ronquist et al. 2003). Two sets of four MCMC chains were run simultaneously, starting in random trees for 1,000,000 generations and sampling every 1,000th generation. The first 25 % of the trees were discarded as a burning phase for each analysis. The subsequent probabilities (Rannala & Yang 1996) were determined from the remaining trees and are shown to the left of each node. Maximum likelihood (ML) analysis was implemented using RAxML-HPC v.8 on XSEDE (8.2.12) available on the CIPRES portal. Parameters for maximum probability were defined for rapid booststrapping and analysis was performed with 1000 repetitions. The trees were visualized in FigTree V1.4.4 (Rambaut 2009).

Results

Phylogeny

The alignment of the ITS region consisted of 62 strains (including the outgroup sequence) (Table 1), with a total length of 729 characters (including alignment gaps). Among these, 229 were informative characters about parsimony, 394 variables and 239 conserved. From the phylogenetic analyzes and morphological characteristics (see below), it is possible to identify the new species of Ceratobasidium sp. VIC 47413. The tree generated with the Bayesian analyzes is shown below (Fig. 2). The tree generated from the RAxML analysis was similar to that of the Bayesian analysis (Fig. 2), therefore, it is not shown.

TABLE 1. GenBank accession numbers of Ceratobasidium isolates included in this study.

Ags	Species	Isolates	Host/Substrate	Origin	GenBank accession no. (ITS)
AGA	–	C-662	–	Japan	AF354092
AGBo	–	SIR-2	Ipomoea batatas	Japan	AF354091
AGT	–	4Oit-800	Reseda odorata	Japan	AB196661
AGA	–	–	–	–	DQ279052
AG-K	–	–	–	Netherlands	DQ279056
AG-L	–	FK02-1	Soil	Japan	AF354093
AG-C	–	–	–	Netherlands	DQ279046
AGO	–	FK06-2	Soil	Japan	AF354094
AGO	–	–	–	Netherlands	DQ279045
AG-G	–	–	–	Netherlands	DQ279049
AGBo	–	–	–	Netherlands	DQ279057
CAG6	–	BN74	Erigeron	USA	AF354083
AG-I	–	IMI 375130	–	United Kingdom	DQ279064
AGH	–	STC-9 s	Soil	Japan	AF354089
AGH	–	–	–	Netherlands	DQ279065
AG-S	–	–	Pittosporum sp.	USA	AJ427400
AGQ	–	C-620	Soil	Japan	AF354095
AGBa	–	C-460	Oryza sativa	Japan	AF354088
AGD	–	C-610	–	Japan	AF354090
CAG1	–	BN1	Grass	USA	AF354086
AGD	–	–	–	Netherlands	DQ279060
AG-Bb	–	–	–	Netherlands	DQ279058
AG-Ba	–	–	–	Netherlands	DQ279059

–	Ceratobasidium sp. VIC 47416	CGR1C	Root of G. recurva	MG, Brazil	MT796438
–	Ceratobasidium sp. VIC 47417	CGR1F	Root of G. recurva	MG, Brazil	MT796439
–	Ceratobasidium sp. VIC 47415	CGR1A	Root of G. recurva	MG, Brazil	MT796444
–	Ceratobasidium sp. VIC 47418	CGR1G	Root of G. recurva	MG, Brazil	MT796445
–	Ceratobasidium sp. VIC 47414	CGR3B	Root of G. recurva	MG, Brazil	MT796446
–	Ceratobasidium sp. VIC 47419	CGR1I	Root of G. recurva	MG, Brazil	MT796440
–	Ceratobasidium sp. VIC 47420	CGR1J	Root of G. recurva	MG, Brazil	MT796441
–	Ceratobasidium sp. VIC 47421	CGR1O	Root of G. recurva	MG, Brazil	MT796442
–	Ceratobasidium sp. VIC 47413	CGR3H[a]	Root of G. recurva	MG, Brazil	MT796443
–	C. albasitensis	Eab - T2	Crocus sativus	Spain	AJ427398
–	C. anceps	–	Pteridium aquilium	United Kingdom	AJ427402
–	C. angustisporum	–	Pterostylis mutica	South Australia	AJ427403
–	C. bicorne	1231	Polystrichastrum formosum	Filand	AF200514
–	C. bulbillifaciens	–	Bark of Fraxinus	Germany	KC336072
–	C. cereale	–	Poa annua	USA	AF063019
–	C. cereale	Sequence 17 from the patente WO01151653	–	Switzerland	AX195391
–	C. cereale	Sequence 17 from the patente WO01151653	–	Switzerland	AX195385
–	C. cereale	Sequence 17 from the patente WO01151653	–	Switzerland	AX195387
–	C. cereale	Sequence 17 from the patente WO01151653	–	Switzerland	AX195390
–	C. cereale	Sequence 17 from the patente WO01151653	–	Switzerland	AX195392
–	C. cereale	–	Secale cereale	Germany	AJ302008
–	C. cereale	–	Triticum aestivum	Germany	AJ302009
–	C. cereale	99,125	Agrostis palustres	Canada	AF222793
–	C. cereale	645	Wheat	Turkey	KC590547
–	C. chavesanum	DK2c2a	Diospyros kaki	SP, Brazil	EU810049

–	C. chavesanum	DK11c2a	Diospyros kaki	SP, Brazil	EU810047
–	C. cornigerum	–	Erigeron canadenses	USA	AJ301902
–	C. cornigerum	–	Juniperus sp.	USA	AJ301900
–	C. cornigerum	–	Pittosporum sp.	USA	AJ302006
–	C. cornigerum	–	Taxus sp.	USA	AJ301901
–	C. cornigerum	–	Festuca sp.	USA	AJ301903
–	C. cornigerum	–	Pittosporum sp.	USA	AJ301899
–	C. niltonsouzanum	CS1032	Camellia sinensis	SP, Brazil	EU810029
–	C. niltonsouzanum	MPM 201	Azadirachta indica	PI, Brazil	KX870111
–	C. noxium	–	Coffea arabica	India	EU810056
–	C. papillatum	–	Sarcochilus dilatatus	Australia	AJ427401
–	C. ramicola	–	Pittosporum sp.	USA	AJ427404
–	C. sphaerosporum	–	–	–	DQ278943

Ags: Anastomosis groups

[a]Ex-type strain

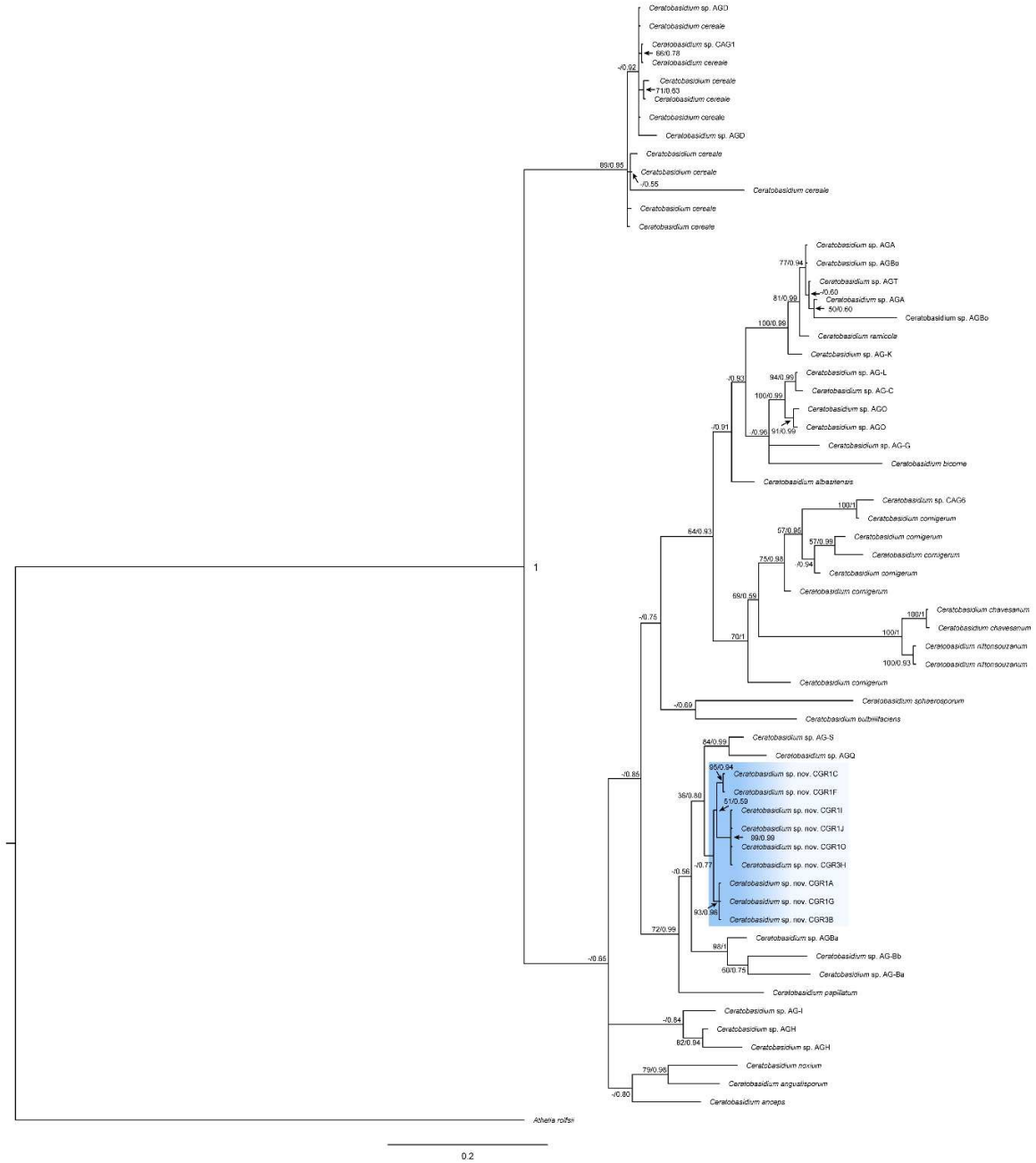

Figure 2. Bayesian phylogenetic tree inferred with alignment of the nucleotide sequences of the ITS region. RAxML boostrap support (MLBS,> 50) and bayesian posterior probabilities (BPP > 0.50) are indicated at the nodes (MLBS / BPP). Sequences of ex-type are indicated. The tree is rooted in Athelia rolfssi.

Taxonomy

Ceratobasidium sp. VIC 47413 (to be proposed as new species) (Fig. 3)

Mycobank: -

Systematic position: Basidiomycota, Agaricomycotina, Agaricomycetes, Cantharellales, Ceratobasidiaceae.

Type: — BRAZIL. Minas Gerais: Parque Estadual Serra do Brigadeiro, from roots of Gomesa recurva R.Br. (Orchidaceae), September 2019, Cruz, E.S. (**holotype** VIC 47413, ex-type living culture COAD 3147).

Etymology: — Name derived from the plant host species Gomesa recurva.

Colonies growing 51 mm in 3 d, flat with entire margin, dense aerial mycelium, white; reverse of the colony white to cream. Hyphae are regularly septated branching out at right angles, 3.2 – 6.1 µm in diameter ($\bar{X}$ ± SD = 4.7 ± 0.59 µm), hyaline, with binucleated cells and thick walls. Hyaline monilioid cells, elongated in the shape of a barrel, 10,1 – 12,7 µm de diam. ($\bar{X}$ ± SD = 11.1 ± 0.76 µm), branched chain with more than four cells. Sexual morphotype not observed.

Additional specimens examined — BRAZIL. Minas Gerais: Parque Estadual Serra do Brigadeiro, from roots of Gomesa recurva R.Br. (Orchidaceae), COAD 3148, 3149, 3150, 3151, 3152, 3153, 3154, 3155, September 2019, Cruz, E.S.

Notes: — Ceratobasidium sp. VIC. 47413 is a sister taxon to C. papillatum Warcup & P.H.B. Talbot (1980). The sister taxon is morphologically differente from C. gomesae por diferenciação na espessura da hifa. Ceratobasidium papillatum has hyphae smooth, 4-7 µm in diameter, hyaline, long cells, with binucleated cells, and there is a marked tendency to form hyphal strands. C. gomesae has hyphae regularly septated, 3.2 – 6.1 µm in diameter, hyaline, with binucleated cells and does not form hyphal strands.

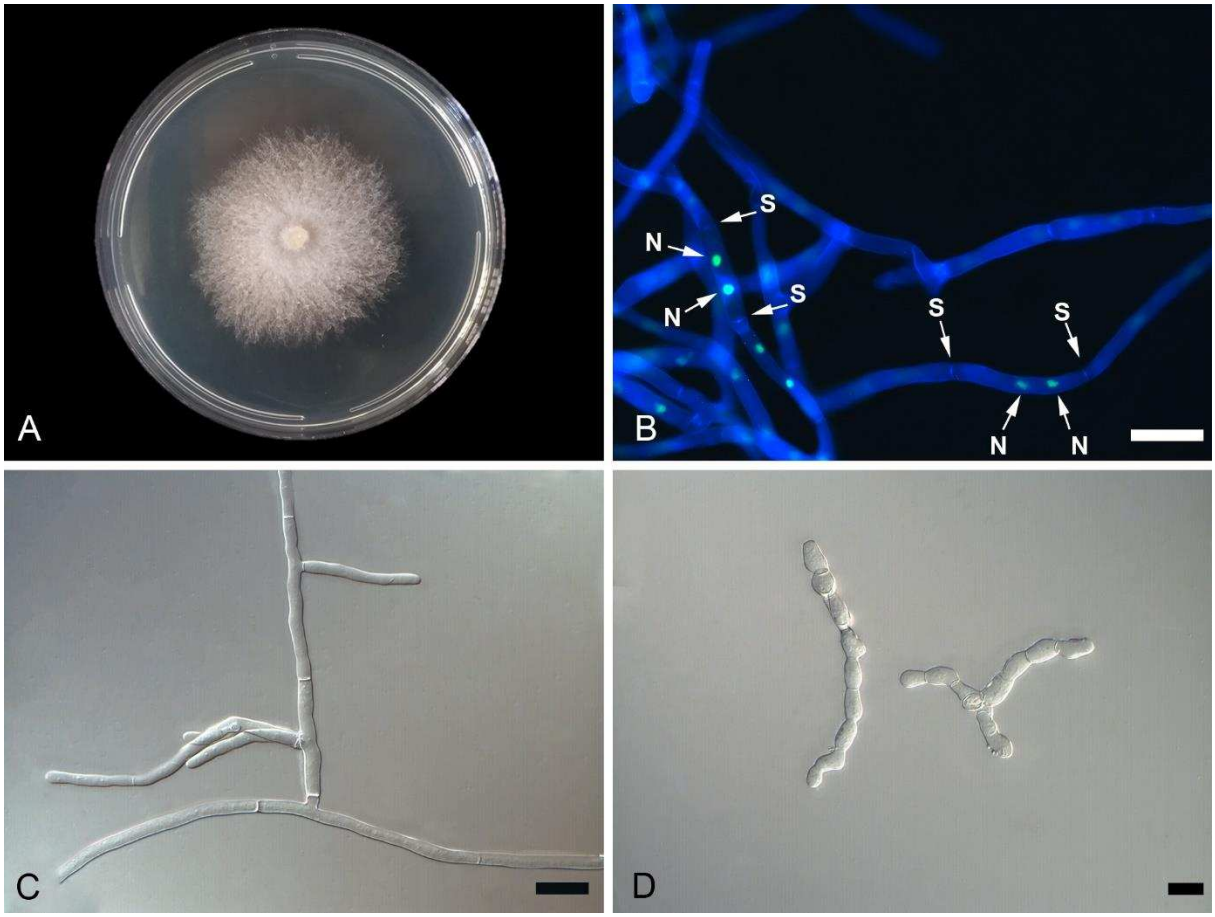

Figure 3. Ceratobasidium sp. VIC 47413. **A**, Three-day-old PDA culture in 9 cm of diameter Petri Dish; **B**, Hyphae stained with SYBR Green I showing binucleate cells (N= nuclei; S= septa); **C**, Hyphae with branching at right angles; **D**, Monilioid cell chains in CMA. Scale bars C and D = 20 µm; B = 25 µm.

Discussion

Morphological and phylogenetic data about the study describes a new species of Ceratobasidium: Ceratobasidium sp. VIC 47413, colonizing the root tissue of Gomesa recurva, an epiphytic orchid from the Brazilian Atlantic Forest. The new species is distinguished from others using sequences from the ITS rDNA region as well as morphological data.

Orchids generally form mycorrhizal associations with basidiomycetous fungi (Rasmussen 2002; Dearnaley 2007), most of which include the genus Rhizoctonia (Bogoure et al. 2005, Waterman & Bidartondo 2008), where they generally include the genus Ceratobasidium (Smith & Read 2008). Certatobasidium species can infect other plant families causing diseases, such as white-thread blight (Wolf & Back 1927, Benchimol et al. 2001, Benchimol & Bastos 2004, Costa et al. 2013, Melo et al. 2018).

The new species Ceratobasidium sp. VIC 47413 formed a monophyletic group supported by 80 % bootstrap with AG-S and AG-Q. Sharon et al. (2008) compared ITS rDNA sequences of AGs, suggesting that if the proposed isolates are in the 95 to 100 % range in similarity and within a clade of a given AG or subgroup, it may indicate that a given isolate belongs to that AG. The similarity of the proposed new species with AG-S and AG-Q was <95 % (80 %). In addition, it has a group supported by 56 % bootstrap with AGBa, AG-Ba, AG-Bb and distant group supported by 99 % bootstrap with C. papillatum.

Eight Ceratobasidium species are known to occur in Brazil, Ceratobasidium anceps on Citrus and Hevea, Ceratobasidium lantanae-camarae on Lantana camara, Ceratobasidium noxium on Piper nigrum, Ceratobasidium ochroleucum on Cinnamomum zeylanicum, Eugenia stipitata, Heliconia psittacorum and Tabebuia serratifolia, Ceratobasidium tradescantiae on Tradescantia fluminensis, Ceratobasidium stevensii on Theobroma grandiflorum and Ceratobasidium chaveanum and Ceratobasidium niltonsouzanum on coffee plants (Ceresini et al. 2012, Melo et al. 2018, Mendes et al. 2020). All of these species are reported as plant pathogenic, most of them causing severe symptoms as leaf blight but also growing on leaves leading to any noticeable damage to colonized organs. Nevertheless, for some species no molecular information are available, due to old descriptions based only on herborized material (e.g. C. lantanae-camara) or due to the lack of culture for highly specialized biotroph (e.g. C. tradescantiae). Ceratobasidium sp. VIC 47413 is phylogenetically different from all Ceratobasidium spp. available (Fig. 2). Isolates of the new Ceratobasidium species formed a well distinguished clade, phylogenetically closed related to Ceratobasidium papillatum. However, the new species do not have a tendency to form hyphal strands as marked well observed for C. papillatum (Warcup & Talbot, 1980).

Ceratobasidium spp. have already been reported in associations with orchids worldwide, mainly with temperate species, with a lack of information regarding to tropical species. Otero et al. (2002, 2004) isolated Ceratobasidium species associated with tropical epiphytic orchids from Puerto Rico. In Brazil, Pereira et al. (2005) and Valadares et al. (2011) identified and demonstrated a high specificity of both Oncidium flexuosum and Coppensia doniana in relation to fungi of the genus Ceratobasidium. Graham & Dearnaley (2012) showed the strict specificity of a rare species of Australian epiphytic orchid (Sarcochilus weinthalii) with only a single species of Ceratobasidium. All these works on tropical epiphytic orchids suggests a possible moderate to highly specificity of Ceratobasidium species to its hosts,

corroborating for the studies of preservation of this species in natural environments for tropical orchid conservation.

Conclusion

Phylogenetic analyzes, together with morphological characteristics, showed the composition of fungal associates related to G. recurva. Only one genus of fungi was found, including the description of a new species of Ceratobasidium. This is the first work describing a new species of Ceratobasidium in G. recurva, an orchid from the Brazilian Atlantic Forest. The maintenance of these species can help in the conservation strategies of Brazilian orchids.

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3. Capítulo 2: Written according to Rhizosphere

Characterization of endophytic bacteria in roots of Gomesa recurva R. Br. and Zygopetalum maxillare Lodd. (Orchidaceae)

Abstract

The Orchidaceae family has great economic and ecological importance, but there is little information about its association with rhizobacteria in Brazilian Atlantic Forest. This study aimed to isolate, identify and characterize endophytic bacteria growing in roots of two orchid species, Gomesa recurva and Zygopetalum maxillare, native to the Atlantic Forest. Ten bacterial isolates were obtained in NFb selective medium, five from each orchid species, which were identified by sequencing the 16S rRNA gene. Furthermore, they were characterized in terms of the ability to nitrogen fixation, phosphate solubilization and indole-3-acetic acid (IAA) production. These isolates belong to genera Pseudomonas and Paraburkholderia. All isolates confirm their ability to fix atmospheric nitrogen by qualitative analysis. Moreover, five isolated from G. recurva and one from Z. maxillare presented high phosphate solubilization index (S.I > 4) and standing out as early in this process. All isolates are promising to produce IAA, with emphasis on two isolates, with production of 14 and 14.3 $\mu g\, mL^{-1}$. Our results showed that in roots of G. recurva and Z. maxillare there are endophytic bacteria that should be helping these orchids in the nitrogen and phosphate nutrition as well as root growth. The ten isolates are promising bacteria to be used during seedlings production of orchids.

1. Introduction

Orchids is the largest family among angiosperms, Orchidaceae, distributed in about 27,000 species (Govaerts et al., 2016). Species of this family are associated with mutualistic fungi in their root system, known as mycorrhizal fungi, which are important for germination and stimulation of plant growth (Rasmussen & Rasmussen, 2009).On the other hand, other symbiotic microorganisms in orchids, especially endophytic ones, are focused mainly in fungi (Novotná et al., 2018), while studies based on endophytic bacteria are still scarse (Glick, B., 2012; Liu et al., 2017; Glazebrook et al., 2018). Some promising studies have already been done with growth-promoting bacteria isolated from orchids, but there are no studies on orchids from the Atlantic Forest (Tsavkelova et al., 2005, 2007).

Plant growth-promoting rhizobacteria (PGPR) are soil microorganisms that live close to plant roots, which are influenced by root exudates (Compant et al., 2010). These bacteria were first studied in 1978 by Kloepper and Schroth and described as PGPB (Kloepper & Schroth, 1978). Under the influence of the plant in the rhizospheric soil, the bacteria penetrate and colonize the root cells and form a symbiotic relationship with their host (Rashid et al., 2012). PGPR is commonly able to promote plant growth by various mechanisms and factors, direct or indirectly, such as by nitrogen fixation capacity, inorganic phosphate solubilization and phytohormone production, aiding plants in nutrient acquiring (Rashid et al., 2012). However, few studies have been investigating the potential of orchids endophytic bacteria in promoting the growth of these species (Faria et al., 2012). Thus, studies on the potential of bacterial isolates in orchids should be carried out, to assess the ability to promote growth, based on the production of phytohormones, nitrogen fixation and phosphate solubilization for future application in seedling growth.

In this study, we isolated, identified and characterized endophytic bacterial from the roots of two epiphytic species of orchids, Gomesa recurva R. Br. and Zygopetalum maxillare Lodd. from the Atlantic Forest. The characterization was to evaluate the ability of these isolates for nitrogen fixation, inorganic phosphate solubilization, and indol acetic acid (IAA) production. These characteristics may be of fundamental importance for production of orchids seedlings.

2. Material and Methods

2.1. Study area and plant material

Root samples of Gomesa recurva and Zygopetalum maxillare (Fig.1) were collected from the Parque Estadual Serra do Brigadeiro, located in the city of Araponga, state of Minas Gerais, Brazil. It is located in the Atlantic Forest and has an altitude that varies between 1300 m to 1900 m altitude.

Fig. 1. Representation of the two species of epiphytic orchids. (**A)**, Gomesa recurva; and (**B)** Zygopetallum maxillare.

2.2. Isolation

Root samples were taken to the Laboratório de Associações Micorrízicas (LAMIC), Microbiology Department, Federal University of Viçosa (UFV), washed in running water to remove impurities. The fragments underwent superficial disinfestation with alcohol 70 % for 1 min, sodium hypochlorite 2 % for 6 min and three successive washes in sterile distilled water. Twenty root fragments from each orchid formed a composite sample, separately. These samples were macerated in microtubes, added with 1 mL of 0.85 % saline solution. Serial dilutions were made and 100 µL of samples was transferred from the 10^{-5} dilution to a solid selective medium for diazotrophic bacteria (NFb) (Döbereiner et at., 1995).

2.3. Molecular identification

The pure culture of bacteria was used for DNA extraction using the thermal method according Baratto & Megiolaro (2012). The bacterial cells were suspended in 1 mL of ultrapure water (Milli-Q®) in 2 mL microtubes, these tubes were vortexed for 5 s and then they were taken to the boiling bath at 95ºC for 5 min. After this step, the microtubes were placed immediately in the ice bath for 5 min. After the thermal shock, the microtubes were centrifuged at 10.000 rpm for 5 min. At the end of this step, 600 µL of the supernatant was removed and the precipitate was discarded (Rowlands et al., 2006). For amplification of 16S ribosomal RNA genes, primer pairs 27f were used (ccgaattcgtcgacaacAGAGTTTGATCMTGG) and 1492r (cccgggatccagcttTACCTTGTTACGACTT) (Lane, 1991). The polymerase chain reaction (PCR) conditions were for a final volume of 50 µL. Were used: 20-30 ng of genomic DNA sample, 1X of buffer, 2 mM $MgCl_2$, 0.2 µM of primer, 0.4 mM of dNTP, 1 U of TaqDNA polymerase. The PCR reaction was performed in a thermocycler. The PCR conditions consisted of initial denaturation at 94 ºC for 5 min; followed by 35 cycles of denaturation at 94 ºC for 30 s; annealing at 53ºC for 1 min; extension at 72 ºC for 1 min and final extension at 72 ºC for 5 min.

2.4. Nitrogen fixation

For the evaluation of the nitrogen fixation capacity, bacteria previously isolated in solid NFb medium, were inoculated into test tubes containing 15 mL of semi-solid NFb medium (gL^{-1}: malic acid, 5: K_2HPO_4, 0.5; $MgSO_4.7H_2O$. 0.2; NaCl, 0.1; $CaCl_2.2H_2O$, 0.02; KOH, 4.5; and in mL: micronutrient solution, 2; bromothymol blue solution (0.5 % in 0.2 KOH), 2; FeEDTA solution (1.64 % solution), 4; and vitamin solution, 1; pH 6.5 (Döbereiner et al., 1995).

2.5. Phosphate solubilization

For evaluation of inorganic phosphate solubilization capacity, the culture medium was GL medium proposed by Sylvester-Bradley et al. (1982), containing (gL^{-1}): glucose, 10; yeast extract, 2; agar-agar, 15 was added to this medium: NH_4Cl, 5g; NaCl, 1; $MgSO_4.7H_2O$, 1; $CaHPO_4$ (tribasic calcium phosphate), 0.8; pH 7.2, in order to form precipitated calcium phosphate. For the dissolution of tribasic calcium phosphate, 10 mL of 2 % HCl was used. In each Petri dish (9 cm diameter, 20 mL culture media), two 5 mm spot of bacteria was inoculated about 2 cm from the bord of Petri dish. Each isolated was tested in three plates and incubated at 28 ºC was evaluated for 15 days.

Based on the solubilization indexes, the bacteria were classified as low (S.I. < 2), medium (2 < S.I <4) and high solubilization (S.I > 4) (Hara & Oliveira, 2004). The bacteria were classified as early, whose solubilization started until the third day; late, after the third day and not solubilizing, to those that did not show solubilization until the fifteenth day of evaluation (Hara & Oliveira, 2004).

2.6. Production of IAA

For laboratory tests in relation to the production capacity of Indole-3-acetic acid (IAA), the culture medium Trypticase Soy Agar 10 % (TSA), containing in gL^{-1}, was used: Soy peptone, 0.5; tryptone, 1.5; NaCl, 2.5; and plus, L-Tryptophan, 1; pH 7.3. The bacterial isolates were inoculated in liquid TSA in Erlenmeyer, incubated at 28 ºC under constant agitation at 125 rpm. After 72 h, 2 mL aliquots of bacterial suspension were transferred to a microtube and centrifuged at 12,000 rpm for 3 min, and 1 mL of the supernatant was transferred to new microtubed and 1 mL of Salkowsky's reagent was added, the samples were kept for 30 min in the dark. Absorbance were taken on a 530 nm spectrophotometer (Pharmacia Biotech) and adjusted with a standard curve using commercial IAA (Merch).

2.7. Statistical analysis

The inorganic phosphate solubilization and prodution of IAA observed in each bacterial isolate were subjected to analysis of variance (ANOVA). Subsequently, the Scott-Knott multiple comparison test (post-hoc) using a significance level of 0.05. All analyzes and graphics were performed in the R 3.6.3 program (R Core Team, 2020) with the ExpDes and igraph libraries (Csardi, 2006).

3. Results

From the root material of G. recurva and Z. maxillare, 10 isolates (Figs. 3) of endophytic bacteria were obtained. From G. recurva, all bacteria were belonging to the genus Pseudomonas (GR05, GR06, GR08, GR09, GR10) were isolated. From Z. maxillare, two species of the genus Pseudomonas (ZM04, ZM05) and 3 species of the genus Paraburkholderia (ZM01, ZM07, ZM08) were isolated. Both genera are gram-negative, presented rapid growth, with the incubation period varying from 1 to 3 days, at 28 ºC in PDA medium.

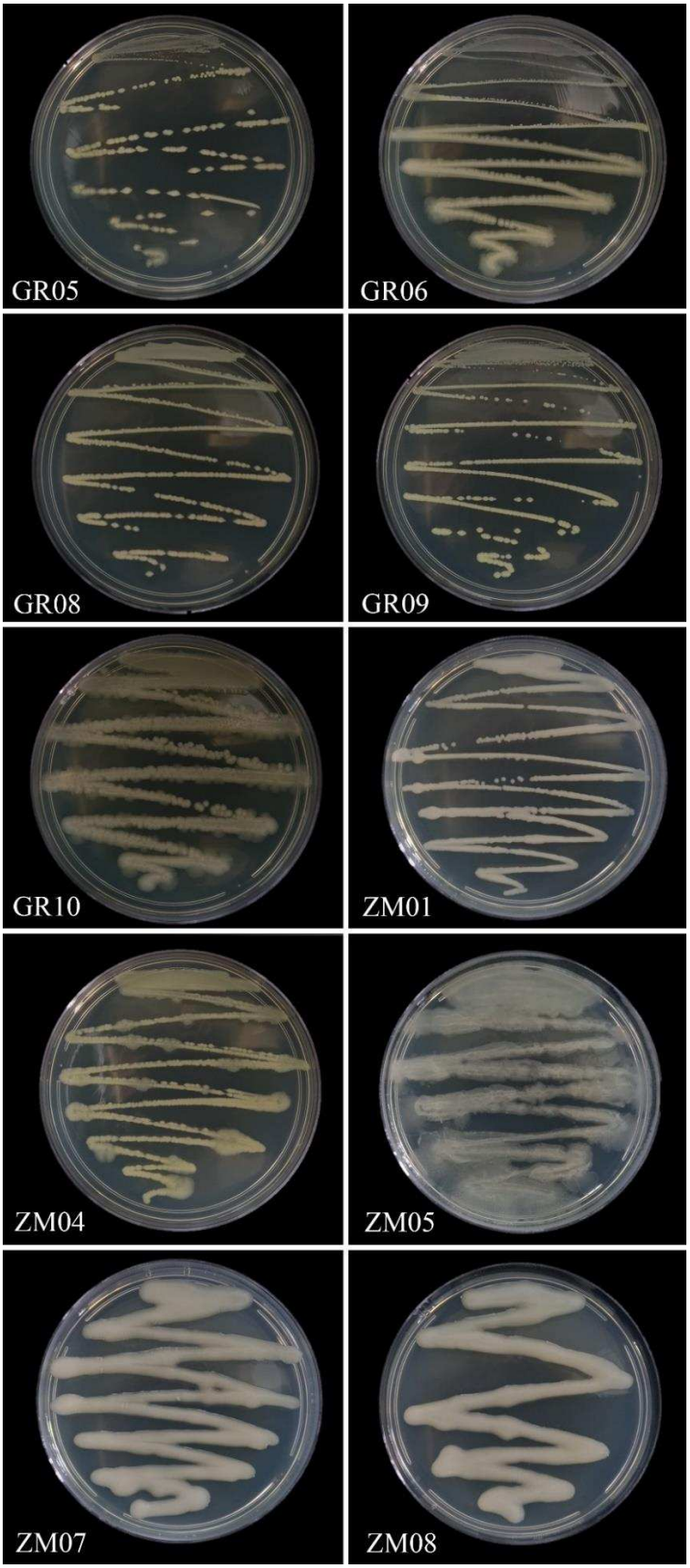

Fig. 3. Plant growth promoter rhizobacteria isolated from roots of Gomesa recurva (GR05-GR10) and Zygopetallum maxillare (ZM01-ZM08).

3.1. Molecular identification

The isolates of G. recurva (GR05, GR06, GR08, GR09, GR10) were identified as species of the genus Pseudomonas, while isolates of Z. maxillare, two species were identified, Pseudomonas (ZM04 and ZM05) and Paraburkholderia (ZM01, ZM07 and ZM08) (Table 1).

Table 1. Molecular identification of endophytic rhizobacteria isolated from Gomesa recurva and Zygopetalum maxillare and their code and genBank acession number.

Code	Host	Genus	GenBank Accession nº.
GR05	G. recurva	Pseudomonas	MT680629
GR06	G. recurva	Pseudomonas	MT680630
GR08	G. recurva	Pseudomonas	MT680631
GR09	G. recurva	Pseudomonas	MT680632
GR10	G. recurva	Pseudomonas	MT680633
ZM01	Z. maxillare	Paraburkholderia	MT680634
ZM04	Z. maxillare	Pseudomonas	MT680635
ZM05	Z. maxillare	Pseudomonas	MT680636
ZM07	Z. maxillare	Paraburkholderia	MT680637
ZM08	Z. maxillare	Paraburkholderia	MT680638

3.2. Nitrogen fixation

Bacterial isolates capable of assimilating nitrogen grew in NFb medium forming a light-colored growth halo, showing a positive result for the test (Fig. 4). Moreover, there is a change in the color of the culture medium to blue, indicating that there was a change in pH to basic, since the medium has the blue pH indicator of Bromothymol, which has this characteristic at high pH.

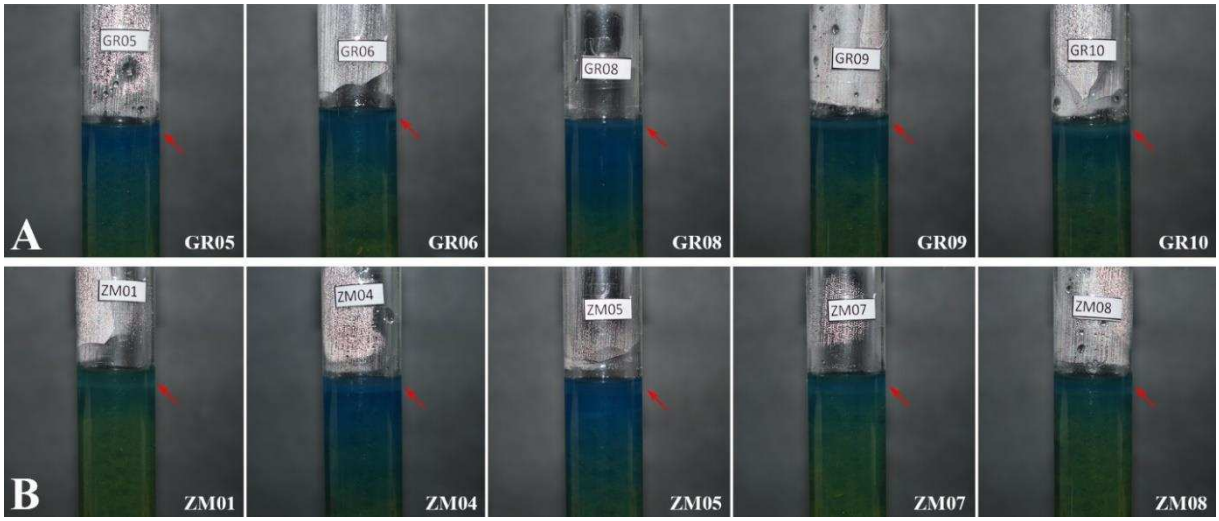

Fig. 4. Nitrogen assimilation test in NFb medium, showing the ability of endophytic rhizobacteria isolated from Gomesa recurva (GR05-GR06) and Zygopetalum maxillare (ZM01-ZM08), demonstrated by the change of green to blue color and the formation of the light halo indicated by the arrows.

3.3. Phosphate solubilization

All isolates were able to solubilize calcium phosphate in vitro in solid culture medium. The isolates formed translucent halos around the colony, indicating that the inorganic phosphate had solubilized. Of these, 60 % of the isolates had a high index (GR10, GR09, GR08, GR06, GR05 and ZM07); 30 % had an average index (ZM05, ZM08 and ZM04) and 10 % a low index (ZM01) (Table 2). Although forming three distinct groups within the solubilization index, there was a statistical difference between the isolates according to the solubilization index of each one. Based on the Scott-Knott test, six groups were then separated (Table 2), with only isolate GR10 as the greatest solubilizer among the other isolates. Only one isolate was characterized as late in relation to those according to the beginning of the solubilization of the tricalcium phosphate (Table 2).

3.4. IAA production

All of ten isolates showed capacity to produce the hormone indole-3-acetic acid in 10 % TSA medium, plus L-tryptophan. The production ranged from 3.5 to 14.3 $\mu g\, mL^{-1}$ (Table 2). The isolates that performed the best according to the Scott-Knott statistical test were ZM05 and ZM04, which differed statistically from the others, but not from each other. These isolates were obtained from the orchid species Z. maxillare. All G. recurva isolates did not differ statistically from each other, maintaining a uniform pattern in the production of the hormone (Table 2).

Table 2: Phosphate solubilization (P-Ca) capacity, showing days for stat P solubilization, Solubilization index (S.I.) and indole-3-acetic acid (IAA) production by10 endophytic rhizobcteria isolated from Gomesa recurva and Zygopetalum maxillare.

P-Ca				IAA	
Isolates	Start Solubilization (1) (day)	S.I. medium*	S.I. level**	Isolates	Concentration * (µg mL^{-1})
GR10	1	6,16 a	High	ZM05	14,3 a
GR09	1	5,26 b	High	ZM04	14,0 a
GR08	1	5,21 b	High	GR10	7,6 b
GR06	1	5,07 b	High	GR05	7,6 b
GR05	2	4,22 c	High	GR06	7,5 b
ZM07	6	4,07 c	High	GR08	6,5 b
ZM05	1	3,51 d	Medium	GR09	5,8 b
ZM08	1	2,79 e	Medium	ZM08	4,0 c
ZM04	1	2,11 f	Medium	ZM07	3,6 c
ZM01	3	1,73 f	Low	ZM01	3,5 c

*values follow by the same letter in the column had no statistical variation in the 0.05% Scott-Knott test.

** values above 4 demonstrate high solubilization capacity, values between 2 and 4 demonstrate medium capacity and values below 2 demonstrate low solubilization capacity.

4. Discussion

The present study characterized endophytic bacteria from the root tissue of epiphytic orchids G. recurva and Z. maxillare, native to the Brazilian Atlantic Forest. Studies of endophytic bacteria in both epiphytic and terrestrial orchids are present worldwide (Tsavkelova et al., 2007, 2011; Galdiano et al., 2011; Gontijo et al., 2018; Herrera et al., 2020). However, this type of study is scarce for orchids from the Atlantic Forest, which houses many endemic and endangered species.

The nitrogen fixing bacteria in G. recurva and Z. maxillare was restricted to the genera Pseudomonas and Parabukholderia. This pattern can also be observed in terrestrial orchids, where orders of Pseudomonadales and Burkholderiales have been observed (Gontijo et al., 2018; Herrera et al., 2020). These bacteria were also found in Pholidota articulata Lindl. (epiphyte orchid) (Tsavkelova el al., 2007). It is important to highlight that the genus Burkholderia has recently been associated with clinically important bacteria, and environmental nitrogen fixers species have been transferred to a new genus Paraburkholderia (Swana et a., 2014). So, Burkholderia cited in the literatures before 2014, may be Paraburkholderia.

The genus Burkholderia was identified occurring in epiphyte orchid Cattleya walkeriana Gardner (Galdiano et al., 2011). The nitrogen fixing capacity in Burkholderia has been also observed associated to rice, maize roots (Gillis et al., 1995; Estrada et al., 2002), sugar cane and corn (Reis et al., 2004). Environmental isolates of Burkholderia found in orchid species have shown potential for promoting plant growth (Tsavkelova et al., 2007; Gontijo et al., 2018). In addition, other endophytic bacteria associated with Australian orchids capable of promoting plant growth, such as Bacillus, Xanthomonas, Arthrobacter and Kurthia have been described (Wilkinson et al., 1989 and 1994). Paenibacillus isolated orchid Cymbidium eburneum Lindl. could promote the growth of orchid plants tested in vitro (Faria et al., 2013). Pseudomonas stutzeri isolated from Cymbidium sp. (Gontijo et al., 2018) and rice (Desnoues et al., 2003) has also demonstrate the ability to fix atmospheric nitrogen.

All isolates showed the ability to solubilize phosphate in culture medium. Both of bacteria were also isolated from species of orchid Cymbidium sp., but no solubilization ability were measured (Gontijo et al. 2018). Burkholderia species isolated from rice root have shown to have a solubilization index (SI) ranging from 2.34 to 2.95 (Estrada et al., 2012), while our isolates showed SI varying from1.73 to 4.07, being Pseudomonas more efficient presenting S.I. above 4 (high solubilization), with a diameter of the halos ranging from 16.6 to 21.8 mm. This value is similar to presented by two strains of Pseudomonas putida, which presented the halo diameter of 18.21 and 21 mm (Rosas et al., 2006).

All isolates were able to produce indole-3-acetic acid in a medium supplemented with L-tryptophan. Production of IAA by strains of Pseudomonas isolated from orchids Calanthe vestita Wall. Ex Lindl., Dendrobium moschatum (Buch. -Ham.) Sw., Acampe papillosa (Lindl.) has also been observed (Tsavkelova et al., 2005). These authors have also showed that the most active strains in the production of IAA isolated from orchids were Bacillus, Pseudomonas and Agrobacterium, and that the maximum IAA is accumulated in the stationary phase. However, they observed that Burkholderia sp. isolated from the rhizoplane has lower efficiency than Pseudomonas. PGPR associated with C. walkeriana demonstrate the ability to produce indole compounds, ranging from 0.8 to 32.3 μg mL^{-1} (Galdiano et al., 2011), which show a great variation among isolates.

PGPR is very important for the microbial ecology of orchids contributing with production of phytohormones that promote plant growth (Tsavkelova et al., 2003, 2005, 2007). In addition, in vitro test is important for epiphytic orchids from the Atlantic Forest since the

inoculation of these bacteria can promote the growth and development of orchid seedlings (Faria et al. 2012).

In the future, a study of the genomic material extracted from the root system of these orchids associated with metagenomic techniques, and compared with the isolations obtained, may corroborate with the understanding of the profile of the microbial community associated with the two species of orchids from the Atlantic Forest. The study of the microbial community in the forophytes, where these orchids grow, may show the relationship between the endophytic bacterial microbiota of G. recurva and Z. maxillare with substrate.

5. Conclusion

According to the present study, this is the first work involving endophytic Pseudomonas and Paraburkholderia colonizing root tissue of G. recurva and Z. maxillare, epiphytic orchids from the Brazilian Atlantic Forest. The bacterial isolates demonstrated a potential ability to promote plant growth, due to nitrogen fixation, phosphate solubilization and auxin production (IAA). These bacteria could be tested for germination and growth of orchids in vitro, being of fundamental importance for maintenance and reintroduction of native species of orchids. As they have shown biotechnological potential, in a future they can be used as commercial inoculants for agricultural and ornamental crops.

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