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BIOTECNOLOGIAS APROPRIADAS AO USO E CONSERVAÇÃO DE *EPIDENDRUM FULGENS* BRONGN (ORCHIDACEAE): CONDIÇÕES IDEAIS DE CULTIVO, FIXAÇÃO DE GANHO GENÉTICO E IDENTIFICAÇÃO DE MICRORGANISMOS ASSOCIADOS

Florianópolis
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“Faço do meu canto a neura existencial / O conteúdo do cotidiano, o dia a dia da vida / A eletrônica está substituindo o coração / A inspiração passou a depender do transistor / O poeta de aço, de poesia programada, é demais para os meus sentimentos, tá sabendo?”

(Arnaud Rodrigues & Chico Anysio)

RESUMO

Considerando a grande diversidade de orquídeas existentes no Brasil, o valor ornamental de muitas delas, e a necessidade do emprego de biotecnologias dependentes de uma estrutura laboratorial para a sua adequada propagação massal, o objetivo deste trabalho foi: desenvolver e aprimorar protocolos adequados à micropropagação e à fixação de ganho genético, bem como o estudo de microrganismos associados à *Epidendrum fulgens* Brongn (Orchidaceae), a fim de possibilitar seu uso e dar subsídios à sua conservação. Foram desenvolvidos quatro capítulos que abordaram: (i) a influência dos comprimentos de onda da luz e a troca da atmosfera *in vitro* e biorreatores de imersão temporária em todas as etapas da micropropagação de *E. fulgens;* (ii) a relação entre o padrão de ploidia dos tecidos ou órgãos usados como explantes para a indução de estruturas semelhantes a protocormos (PLB) com a ploidia das plantas regeneradas destes PLB; (iii) o isolamento, identificação e caracterização de fungos associados à protocormos e raízes de *E. fulgens*. Os principais resultados obtidos foram: (i) a ventilação natural da atmosfera *in vitro* é detrimental nas etapas iniciais da micropropagação e benéfica nas etapas de crescimento de plântulas, enquanto as fontes de luz LED nos comprimentos de onda azul e vermelho têm efeitos positivos em todas as etapas. O uso de biorreatores de imersão temporária resulta num incremento adicional na produtividade e na qualidade final das mudas; (ii) todos os tecidos/órgãos analisados são endopoliplóides. Foram desenvolvidos protocolos de indução de PLB a partir de folhas, raízes e bases de protocormos de *E. fulgens.* As plantas regeneradas a partir dos PLB de explantes foliares mantiveram a mesma ploidia das plantas obtidas de sementes, indicando que o protocolo é adequado para a fixação de ganho genético da espécie; (iii) um total de quatorze isolados fúngicos foram obtidos, pertencentes à três espécies de basidiomicetos e quatro de ascomicetos. Os três basidiomicetos, duas possíveis novas espécies de *Tulasnella* e uma da ordem Sebacinales, foram capazes de promover a germinação simbiótica de sementes de *E. fulgens in vitro. Serendipita restingae* sp. nov. (Sebacinales) é descrita como uma nova espécie de fungo micorrízico de orquídea, a qual foi demonstrada ser também capaz de colonizar a raiz de plantas de diferentes famílias botânicas.

Palavras-chave: LED; micorrizas; micropropagação; citometria de fluxo; orquídea.

ABSTRACT

Considering the great diversity of orchids existing in Brazil, the ornamental value of many of them, and the need to use biotechnologies dependent on a laboratory structure for their adequate mass propagation, the main objective of this work was: to develop and improve protocols suitable for the micropropagation and the fixation of genetic gain, as well as the study of microorganisms associated with *Epidendrum fulgens* Brongn (Orchidaceae), in order to enable its utilization and provide subsidies for its conservation. Four chapters were developed that addressed: (i) the influence of the wavelengths of light and the exchange of the *in vitro* atmosphere and temporary immersion bioreactors in all stages of *E. fulgens* micropropagation; (ii) the relationship between the ploidy pattern of the tissues or organs used as explants for the induction of protocorm-like bodies (PLB) with the ploidy of the plants regenerated from these PLB; (iii) the isolation, identification and characterization of fungi associated with *E. fulgens* protocorms and roots. The main results obtained were: (i) the natural ventilation of the *in vitro* atmosphere is detrimental in the initial stages of micropropagation and beneficial in the stages of plantlet growth, while the LED light sources in the blue and red wavelengths have positive effects in all steps. The use of temporary immersion bioreactors results in an additional increase in productivity and in the final quality of the seedlings; (ii) all tissues / organs analyzed are endopolyploid. PLB induction protocols were developed from *E. fulgens* leaves, roots and protocorm bases. Plants regenerated from PLB of leaf explants maintained the same ploidy as plants obtained from seeds, indicating that the protocol is suitable for fixing genetic gains in the species; (iii) a total of fourteen fungal isolates were obtained, belonging to three species of basidiomycetes and four ascomycetes. The three basidiomycetes, two possible new species of *Tulasnella* and one of the Sebacinales order, were able to promote the symbiotic germination of *E. fulgens* seeds *in vitro. Serendipita restingae* sp. nov. (Sebacinales) is described as a new species of orchid mycorrhizal fungus, which has also been shown to be able to colonize the roots of plants from different botanical families.

Key-words: LED; mycorrhizae; micropropagation; flow cytometry; orchid.

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LISTA DE ABREVIAÇÕES

®	Registered trademark
2,4-D	2,4-Dichlorophenoxyacetic acid
2iP	2-isopentenyladenine
C	C-value
Car	Carotenoids
CFL	Sysvania® Cool White Fluorescent
Chl_a	Chlorophyll *a*
Chl_b	Chlorophyll *b*
CV	Cycle value
DR	Philips GreenPower TLED Deep red/White/Medium Blue
ESP	Estrutura semelhante à protocormo
FC	Flow cytometry
FL	Sysvania® Cool White Fluorescent
FR	Philips GreenPower TLED Deep red/White/Far red/Medium Blue
FW	Fresh weight
FWN2	Fresh weight of protocorms in stage N2
FWN3	Fresh weight of protocorms in stage N3
FWN4	Fresh weight of protocorms in stage N4
FWN5	Fresh weight of protocorms in stage N5
G1	Refers to the G1 stage of the cell cycle
GC	Germplasm collection
GI	Growth index
GLM	Generalized linear models
ITS	Internal transcribed spacer
LED	Light-emitting diodes
LFDGV	Laboratório de fisiologia do desenvolvimento e genética vegetal
LSU	Large subunit
MOTU	Molecular operational taxonomic units
Mbp	Mega base pairs
N0	No germinated seed
N1	Germinated/embryo swelling
N2	Protocorm with apical meristem
N3	Protocorm with first leaf
N4	Plantlet stage; formation of first root
N5	Plantlet with more than one leaf and root
NL	Number of leaves
NR	Number of roots
NS	Number of shoots
NV	Natural ventilation
PAR	Photosynthetic active radiation

PCA	Principal component analysis
PDA	Potato dextrose agar
pg	Picograms
PGR	Plant growth regulator
PGS	Plantlet growth stage
PI	Propidium iodide
PLB	Protocorm-like body
propabnormal	Proportion of abnormal (hyperhydricity) protocorms
propN1	Proportion of germinated/embryo swelling
propN2	Proportion of protocorms with apical meristem
propN3	Proportion of protocorms with first leaf
propN4	Proportion of plantlets with formation of first root
propN5	Proportion of plantlets with more than one leaf and root
PTFE	Polytetrafluoroethylene
s.d.	Standard deviation
SGS	Seed germination and initial protocorm development stage
STOM	Number of stomata
STOMO	Number of opened stomata
TCL	Thin cell layers
TDZ	Thidiazuron
TM	Trademark
UV	Ultraviolet
VEL	Number of roots with velamen
W	Philips GreenPower TLED White/High Blue

SUMÁRIO

INTRODUÇÃO E JUSTIFICATIVA

O desenvolvimento da presente tese é basicamente resultado de duas características marcantes existentes na família Orchidaceae: (i) a grande variação morfológica de suas flores; (ii) o tamanho reduzido de suas sementes. Estas duas características, resultantes de um longo processo coevolutivo com fungos e insetos, são a espinha dorsal da pesquisa realizada na presente tese de doutorado.

As orquídeas, ao contrário de outras monocotiledôneas petalóides, possuem flores incrivelmente variáveis (MONDRAGÓN-PALOMINO; THEISSEN, 2009). Com poucas exceções, o labelo é sempre diferente de outros órgãos do perianto, sendo adornado com calos, glândulas e padrões distintos de coloração a fim de atrair insetos polinizadores (MONDRAGÓN-PALOMINO; THEISSEN, 2009). Esta variação morfológica nas flores das orquídeas não atrai tão somente os insetos. Esta grande variabilidade é refletida também em um apelo ornamental, motivo pelo qual muitas orquídeas são conhecidas e cultivadas.

No entanto, a produção de mudas de orquídeas é um processo que exige o emprego de biotecnologia. E isto se deve ao tamanho reduzido de suas sementes. As sementes de orquídea têm um peso unitário de 0,31 a 24 µg e não possuem endosperma (ARDITTI; GHANI, 2000) e são conhecidas como "*dust seeds*". As *dust seeds* não ocorrem exclusivamente em orquídeas (ERIKSSON; KAINULAINEN, 2011), mas em todas as espécies desta família botânica é obrigatória a ocorrência de uma fase micoheterotrófica (RASMUSSEN; RASMUSSEN, 2014). Portanto, todas as sementes de orquídeas precisam de uma associação micorrízica para a germinação.

Até a metade do século XIX se sabia muito pouco sobre a reprodução sexual de orquídeas, sendo praticamente impossível a germinação artificial das sementes, o melhoramento genético e o desenvolvimento de híbridos (ARDITTI, 1967). Apenas em 1922, Lewis Knudson publicou o primeiro trabalho descrevendo a germinação assimbiótica de sementes de orquídeas (KNUDSON, 1922). O trabalho de Knudson foi revolucionário, pois foi a base de tudo o que temos atualmente em termos de orquídeas ornamentais. Após a germinação assimbiótica foi possível a produção em larga escala de mudas de orquídeas, o melhoramento genético e o desenvolvimento de variedades híbridas (YAM; ARDITTI, 2009). Desta maneira, para toda e qualquer

orquídea que se deseja propagar eficientemente, existe a necessidade de uma estrutura laboratorial.

Na presente tese propusemos uma pesquisa aprofundada sobre uma espécie negligenciada de orquídea. *Epidendrum fulgens* Brongn é uma espécie de orquídea nativa da Mata Atlântica, amplamente distribuída na vegetação de Restinga (PINHEIRO *et al.*, 2010). A alta variabilidade na morfologia e cor das flores dessa espécie fez com que seja considerada uma “planta para o futuro” pelo Ministério do Meio Ambiente do Brasil (MÜLLER, 2011).

Como propomos o uso de um recurso genético negligenciado, devemos propor conjuntamente todas as medidas necessárias para que isto aconteça, inclusive para que não ocorra sua exploração indiscriminada e erosão genética da espécie. Desta maneira, a pesquisa foi estruturada a fim de abordar tanto a elaboração de protocolos eficientes para a propagação e o melhoramento genético da espécie, como para gerar informações básicas que auxiliem na sua conservação. Também estudamos as relações da espécie com fungos endofíticos e micorrízicos tanto para incorporá-los no co-cultivo com a espécie como para gerar informações sinecológicas que auxiliem futuras medidas de conservação.

Desta forma, objetivou-se: (1) gerar um protocolo eficiente de micropropagação da espécie, com foco no uso de biotecnologias a fim de reduzir os custos de produção e melhorar a qualidade das mudas; (2) gerar um protocolo eficiente de clonagem e produção de plantas poliplóides, a fim de aumentar o valor ornamental da espécie; (3) conduzir um estudo sobre os fungos endofíticos e micorrízicos associados à espécie de modo a utilizá-los no co-cultivo com a espécie e também dar subsídios a sua conservação. Com isso, a tese foi dividida em quatro capítulos distintos.

No capítulo 1, estudou-se a influência de diferentes espectros de luz e da ventilação natural em todas as etapas do cultivo *in vitro* de *E. fulgens,* assim como o emprego de um biorreator de imersão temporária (BIT) para a fase final de cultivo. Mostrou-se que as quatro fontes de luz utilizadas e a ventilação natural da atmosfera *in vitro* afetam diferentemente as fases iniciais, de germinação e desenvolvimento de protocormos, e finais, de crescimento de plântulas. O emprego do BIT adicionou ganhos extras tanto na taxa regenerativa quanto na qualidade das mudas. O protocolo desenvolvido possibilitou melhorar a qualidade das plântulas, tanto em termos morfológicos como bioquímicos. Também mostrou-se que é possível diminuir o tempo

de cultivo e os custos de produção finais, o que faz do protocolo desenvolvido excelente do ponto de vista de aplicação prática para a realidade atual.

No capítulo 2, realizou-se um mapeamento aprofundado da ploidia de diferentes tecidos e órgãos de *E. fulgens.* Utilizaram-se estes tecidos e órgãos mapeados para desenvolver protocolos de micropropagação para a espécie a fim de tanto gerar ganhos genéticos com a obtenção de regenerantes poliplóides, como possibilitar a fixação de ganhos genéticos obtidos pelo melhoramento convencional. Os resultados mostraram que todos os tecidos e órgãos analisados de *E. fulgens* são endopoliplóides e que o padrão de endopoliploidia possui ampla variação em diferentes partes da planta. Um protocolo eficiente de indução de estruturas semelhantes à protocormos (ESP) foi desenvolvido para a micropropagação da espécie usando explantes com padrões contrastantes de ploidia. Analisou-se a estabilidade citogenética das plântulas regeneradas com o protocolo proposto e mostrou-se que ele é eficiente na manutenção da ploidia.

Nos capítulos 3 e 4 foram procedidos o isolamento e a caracterização morfológica e molecular de fungos associados às raízes e protocormos de *E. fulgens.* Verificou-se que a espécie é altamente prolífica em termos de associações simbióticas e foram encontradas diferentes espécies de fungos associadas com *E. fulgens* em diferentes etapas de seu ciclo de vida, a maioria deles ainda desconhecida para a ciência. Foi descrita uma nova espécie de fungo micorrízico, a qual foi denominada de *Serendipita restingae*, em homenagem à vegetação de Restinga. Mostrou-se que esta nova espécie possui propriedades de um promotor de crescimento, inclusive em plantas de outras famílias botânicas.

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Capítulo 1 - Light quality and natural ventilation have different effects on protocorm development and plantlet growth stages of the *in vitro* propagation of *Epidendrum fulgens* (Orchidaceae)

ABSTRACT

Micropropagation laboratories still face productivity problems associated to high costs and low efficiency production. Improvements of *in vitro* culture protocols are essential to and increase productivity. However, they should also take in account the reduction of costs in order to ensure their practical application in commercial laboratories. The aim of the present work was to evaluate the influence of the physical environment on the *in vitro* seed germination, protocorm development and plantlet growth of the orchid *Epidendrum fulgens.* Specifically, we used light sources of different wavelengths in combination with natural ventilation of the *in vitro* atmosphere (NV) and accessed their influence in all steps of the micropropagation protocol, from the germination to plantlet growth, as well as for *ex vitro* acclimatization, with the aim to improve productivity levels and the quality of resulting plantlets. Additionally, we compared the use of a temporary immersion bioreactor (Plantform^{TM}) and containers with semi-solid medium and NV for the final steps micropropagation. We evaluated the physical parameter effects on the velocity of development of protocorms, and the productivity and quality of plantlets, accessing both morphological and biochemical features, as number of organs, velamen formation, stomatal density and functionality, as well as photosynthetic pigments and microscopic features of leaves. For seed germination and initial development of protocorms, NV was found to be detrimental while blue and red LED lights accelerated the development of protocorms and fluorescent lights increased their fresh weight. For plantlet growth NV was responsible not only to enhance productivity, as higher fresh weight and root number, but also the quality of plantlets, stimulating the development of velamen epidermis in roots and increasing photosynthetic pigments. NV also induced the formation of sclerenchyma tissue in leaves and leaves produced higher number of opened stomata. The use of Plantform^{TM} further increased the fresh weight and the leaf, root and shoot number, as well as produced thicker cuticle on leaves, although it did not modify leaf pigments content or stomata number in comparison with NV on semi-solid agar medium. The results allowed the determination of suitable modifications for each step the micropropagation protocol of

E. fulgens to accelerate the process, improve the quality of the resulting plantlets and reduce the production costs.

Key-words: LED lights; chlorophyll content; stomata; velamen; temporary immersion bioreactor.

INTRODUCTION

Current micropropagation methods are usually laborious and expensive, demanding constant transfer of plant material to fresh medium to ensure adequate development (HOSAKATTE NIRANJANA MURTHY, KEE-YOEUP PAEK, 2018). Cost reduction is the most important demand for micropropagation in this century, which can be achieved either by increasing multiplication index or by decreasing costs directly linked to the technique (CARDOSO; SHENG GERALD; TEIXEIRA DA SILVA, 2018). However, the complete picture is not that simple. Increasing micropropagation efficiency usually demands specific culture media formulations for each cultivar, which further demands skilled labor to avoid errors. In *Phalaenopsis*, for example, skilled labor represents more than 60% of micropropagation costs (CHEN, 2016).

In this context, the manipulation of the physical factors related to micropropagation can be efficient for both reducing direct costs and increasing productivity. Several internal and external physical elements regulate the growth and development of *in vitro* plants, and light is one of the most important (DUTTA GUPTA; JATOTHU, 2013).

Cool White Fluorescent Lamps (CFL) are still widely used as light sources in plant micropropagation, despite its many negative aspects, such as high energy consumption and heat production, as well as light emission in not photosynthetically active wavelengths (BATISTA *et al.*, 2018; DUTTA GUPTA; JATOTHU, 2013). Furthermore, these lamps have a short lifespan and do not produce stable light spectra and intensity throughout their life time (DARKO *et al.*, 2014). Nevertheless, CFL are yet the most employed sources of light for orchid micropropagation (HANUS-FAJERSKA; WOJCIECHOWSKA, 2017).

There is a tendency to replace CFL by Light-emitting Diode Lamps (LED), as, progressively, studies show their advantages for micropropagation (BATISTA *et al.*, 2018). Compared to traditional light sources, they consume less energy, have greater

durability, produce less heat and allow the emission of light in narrow and specific wavelengths, which makes them suitable for horticultural illumination (DARKO *et al.*, 2014). Modern LED systems developed exclusively for plants allow the dynamic control of the intensity and the quality of light (HANUS-FAJERSKA, E WOJCIECHOWSKA, 2017). They can be useful to improve morphological and biochemical characteristics of *in vitro* seedlings, such as better rooting, larger leaf area, higher frequency of stomata and increased contents of chlorophyll, sugars and starch, although these responses are dependent on the species (DUTTA GUPTA; JATOTHU, 2013).

Many studies with the use of LED for plant growing were carried out with food crops, but they can also be useful for ornamental plant species (MASSA *et al.*, 2008). For orchid micropropagation, LEDs were found to be superior to CFL, despite many studies show that the responses to the light spectra are highly dependent on the species, variety and even on the developmental stage (HANUS-FAJERSKA, E WOJCIECHOWSKA, 2017), which highlights the need to define optimal conditions for each case.

Another physical factor that can affect *in vitro* plant growth and development is the atmosphere composition (ZOBAYED, 2005). The *in vitro* and *ex vitro* environments are quite different. Conventional micropropagation avoids gas exchanges with the external environment, resulting in higher humidity levels, accentuated fluctuations on CO_2 concentration and accumulation of ethylene that further suppress photosynthesis and water and nutrient uptake (SALDANHA *et al.*, 2012; ZOBAYED, 2005).

The natural ventilation of the internal atmosphere may be achieved with the use of membranes that allow gas exchange between *in vitro* and *ex vitro* environments, providing better conditions to photosynthesis and resulting in better growth rates (KOZAI, 2010). For *Cattleya walkeriana*, seedlings cultivated under NV were more able to control water loss when exposed to the external environment (DA SILVA *et al.*, 2016). *Doritaenopsis* orchids cultivated with NV presented higher photosynthesis rates and increased starch and sugar concentration (SHIN; PARK; PAEK, 2014).

Another way of achieving *in vitro* atmosphere ventilation is by the regular forced exchange of internal atmosphere through air pumping, using temporary immersion bioreactors (ETIENNE; BERTHOULY, 2002). As they are based on the use of liquid media, the control of physical parameters, as pH, temperature and atmosphere is

facilitated, allowing the automation of procedures and decreasing the cost of orchid production (HOSAKATTE NIRANJANA MURTHY, KEE-YOEUP PAEK, 2018). Orchid micropropagation in bioreactors is also advantageous over other methods in regard to explant manipulation, labor costs and production per area (EKMEKÇIGIL *et al.*, 2019). In general, plant costs can be reduced by more than 40% with their use (CARDOSO; SHENG GERALD; TEIXEIRA DA SILVA, 2018). In orchids, they were successfully used to scale up the steps of propagules production and plantlet growth for attending the commercial demand (GAO *et al.*, 2014). They can also be used to proliferate protocorm-like bodies and to collect secondary metabolites from the liquid media (YANG, F. *et al.*, 2015).

The aim of the present work was to evaluate the influence of different light sources in combination with NV in all steps of the micropropagation protocol, from the germination to plantlet growth, with the aim to increase *in vitro* productivity and the quality of resulting plantlets. We also compared the use of a temporary immersion bioreactor (Plantform^{TM}) with NV for the final steps of *in vitro* plant growth. Both morphological and biochemical features were accessed. *Epidendrum fulgens* was used as a model plant due to its ornamental appeal and the little knowledge about their *in vitro* cultivation. The following questions were addressed: (1) Do the light source and NV really play important roles in improving quality of plantlets and increasing productivity? (2) Are the light source and NV equally important for the different steps of *in vitro* orchid production? (3) The use of Plantform^{TM} can further increase productivity and quality of final plantlets in comparison with NV?

We discuss the obtained data considering the ecological features of the natural habitat of *E. fulgens* to explain the results. Considerations about the feasibility and practical application for commercial propagation were taken in account to propose a practical protocol for mass propagation of this species with reduced costs, high yields and improved quality of resulting plantlets.

MATERIAL AND METHODS

In order to evaluate the influence of light sources and NV in all steps of *in vitro* growing stages, from germination to plantlet growth, we performed three different experiments that are described separately below.

EXPERIMENT 1 - LIGHT SOURCES AND NATURAL VENTILATION FOR SEED GERMINATION AND INITIAL PROTOCORM DEVELOPMENT STAGE (SGS)

Seeds were collected from mature fruits of plants grown in greenhouse and surface-sterilized according to Voges *et al.* (2014). In a laminar flow chamber, ≈ 0.5 g of seeds were suspended in 1L of liquid MS medium (MURASHIGE; SKOOG, 1962), supplemented with vitamins (MOREL; WETMORE, 1951), 3% sucrose and maintained under constant agitation with a magnetic stirrer. This step was previously established in order to inoculate standard seed number per culture flask by pouring a volume of the liquid media with the seed suspension. With an automatic media dispenser (DMC-100, Tecnopon®), 27 mL of the medium seed suspension were poured in 300 mL capacity polypropylene (PP) culture flasks. They were closed either with sealed PP lids, to avoid gas exchange, or with a commercial lid equipped with a polytetrafluoroethylene (PTFE) filter that allow the *in vitro* NV in a rate of 4 dm^3 day^{-1}, according to manufacturer information (Combiness™). The flasks were maintained at 25 ± 2°C and 16/8h photoperiod. Four different light sourced were used to provide the cultures with 70 µmol $m^{-2}s^{-1}$ of photosynthetic active radiation (PAR) levels.

1- Sysvania® Cool White Fluorescent Tube (CFL);

2- Philips GreenPower TLED White/High Blue (W);

3- Philips GreenPower TLED Deep red/White/Medium Blue (DR);

4- Philips GreenPower TLED Deep red/White/Far red/Medium Blue (FR);

The wavelengths emitted for each light source were measured with a spectrofluorometer and are available in Sup Fig 1. The experiment was installed in a 4x2 factorial scheme, with 8 treatments (NV x light sources) and 6 repetitions, each consisting of a flask with 81±18 seeds. Twelve weeks after sowing, the data of seed germination and proportion of normal and abnormal (hiperhydric) protocorms was accessed. The normal protocorms were classified according to their developmental stage:

N1= germinated/embryo swelling;

N2= protocorm with apical meristem;

N3= protocorm with first leaf;

N4= plantlet stage; formation of first root;

N5= plantlet with more than one leaf and root;

The number and the fresh weight of protocorms in each category were accessed. The last fully expanded leaf from plantlets in stage N5 from each treatment, was excised and used for the determination of stomatal density on abaxial surface, as well as the number of opened stomata. For that, leaves were detached and used for performing epidermis printing on microscope slides using one drop of ethyl cyanoacrylate (WILSON; PUSEY; OTTO, 1981). Leaf stomatal density was performed in 3 different random leaf regions (1 mm^2), on three leaves from each treatment.

EXPERIMENT 2 - LIGHT SOURCES AND NATURAL VENTILATION FOR PLANTLET GROWTH STAGE (PGS)

Plantlets at initial stage of development, with the first leaf and root (stage N4), were selected from each treatment of the SGS experiment and transplanted to 870 mL capacity polypropylene flasks containing 100 mL of MS medium supplemented with vitamins (MOREL; WETMORE, 1951), 3% sucrose and 2 g L^{-1} Phytagel®. Ten protocorms were planted in each flask. The flasks were closed using the same methods as for SGS, either with sealed PP lids, to avoid gas exchange, or with a commercial lid equipped with a PTFE filter that allow the *in vitro* NV in a rate of 11.5 dm^3 day^{-1}, according to manufacturer information (Combiness™). Plantlets were grown under the same four different light treatments and conditions described before, i.e., they were maintained under the same treatments they were obtained since the seed germination. Experimental design consisted of a completely randomized design in a 4x2 factorial scheme, with 8 treatments (NV x light sources) and 4 repetitions, each consisting of a flask with 16 protocorms.

After 12 weeks of growth, morphological and biochemical aspects of each plant were accessed. The number of leaves (NL), shoots (NS), roots (NR) and roots with velamen (VEL) were determined, as well as the fresh weight (FW). The last fully expanded leaf from 6 plantlets from each treatment was used for stomatal density and functionality analysis using the same methodology previously described for SGS. The leaves were also used for photosynthetic pigments quantification, as described below.

EXPERIMENT 3 - PLANTFORM™ AND NATURAL VENTILATION FOR PLANTLET GROWTH

The plantlet growth stage (PGS) was also compared using two different *in vitro* cultivation systems. For that, sixteen N4 stage plantlets where transferred to either 4 L capacity polypropylene culture containers or to 4 L capacity temporary immersion bioreactor (Plantform™) (WELANDER *et al.*, 2014). The polypropylene containers were poured with 400 mL of gelled MS media and sealed with lids containing PTFE filters that allow NV in a rate of 54 dm^3 day^{-1}, according to manufacturer information (Combiness™). The Plantform™ bioreactor contained the same amount of liquid MS liquid medium and were programmed for forced atmosphere renovation for 3 min every 3 hours, by sterile air pumping. The cultures were maintained 25 ± 2°C and a 16/8h photoperiod provided by CFL tubular lamps (70 µmol $m^{-2}s^{-1}$). After 12 weeks of growth, the same data for the PGS experiment were collected.

Chlorophyll and carotenoids contents

Plantlets from the two PSG experiments were used for this analysis. A sample of 20 mg of leaf tissue from the central leaf region was incubated for 2 hours at 65°C in microtubes with 1.4 mL de dimethylsulfoxide, as the methodology adapted from Barnes *et al.* (1992). After incubation, 200 µl aliquots of each sample were transferred in triplicates to 96 cells microplates and analyzed in spectrometer, where it was collected absorbance data from 665, 649 and 480 nm wavelengths. The chlorophyll *a* (Chl_a), chlorophyll *b* (Chl_b), and carotenoids (Car) concentrations (µg mL^{-1}) were determined with the equations proposed by Wellburn (1994):

$$Chl\_a = 12.47A_{665.1} - 3.62A_{649.1}$$

$$Chl\_b = 25.06A_{649.1} - 6.5A_{665.1}$$

$$Car = (1000A_{480} - 1.29\ Chl\_a - 53.78\ Chl\_b)/220$$

Histoanatomical features

Plantlets from the two PSG experiments were used for this analysis. Leaf sections (≈ 0.25 cm^2) from de central region of leaves, including the central grove, were fixed in glutaraldehyde (2.5% v/v) in sodium phosphate buffer (0.1 M, pH 7.2) for 24 h. The samples were dehydrated in ethanol series (20%, 40%, 60%, 70%, 80%, 90% and

100%) for 30 min each, embedded with historesin (Leica Historesin, Heidelberg, Germany) and polymerized. Transversal slices (5 µm) were performed in a microtome, stained with toluidine blue and analyzed in an Olympus® BX-40 microscope. Observed microfeatures were the leaf central grove thickness, and sclerenchyma and cuticle formation. Measurements were performed with ImageJ software (SCHNEIDER; RASBAND; ELICEIRI, 2012).

ACCLIMATIZATION ASSAY

Plantlets from the two PSG experiments were used for this experiment. For the acclimatization, 30 plantlets from each treatment were placed in 72 cell cappacity styrofoam trays containing one pebble expanded clay per cell, which was used only for plant fixation. No substrate was used. Acclimatization took place in a greenhouse with 50 shading and sprinkler irrigation 3 times per day. Plants were fertirrigated once a week with half-strenght Hoagland´s solution. After 8 weeks, the survival rate was accessed and plants were considered acclimatized when new roots and leaves were produced.

STATISTICAL ANALYSIS

An exploratory analysis was conducted for the data from the SGS experiment using a multivariate approach. In order to determine the relationships among the measured variables and the light and NV treatments, a principal component analysis (PCA) was performed using a correlation matrix using FactoMineR package (LÊ; JOSSE; HUSSON, 2008) in the R environment (R core team, 2019), which resulted in final analyses with two principal components that explained 84.9% of total variation. The total fresh weight of protocorms was compared with ANOVA ant Tukey post-hoc test (p=0.05).

For the two experiments of PGS, data was analyzed with one or two-way ANOVA and Tukey post-hoc test (p= 0.05). Variables for which residues were not homoscedastic, according to Bartlett test at 5% of significance, were compared with Kruskal-Wallis and Dunn post-hoc test (p=0.05). All analyses were performed on the R environment (R core team, 2019).

RESULTS

LIGHT SOURCES AND NATURAL VENTILATION FOR SEED GERMINATION AND PROTOCORM DEVELOPMENT STAGE (SGS)

The x axis from the PCA analysis contains the largest eigenvalue and corresponds to the first component or dimension. It accounts for the greatest amount of variance in the samples. The fluorescent light treatments were in opposite sides of this axis in relation to the LED treatments, which means they showed different influences on the variables (Figure 1a). On the second dimension, the y axis, LED treatments without NV (Conventional cultivation system) were grouped on the inferior quadrant, while LED treatments with NV were far distant on the superior left quadrant. The variables are represented by the vectors, and right angles less than 90° between two vectors mean they were positively correlated, while right angles higher than 90° means they were negatively correlated. For example, the proportion of protocorms on initial stages of development (propN1 and propN2) were positively correlated with each other and negatively correlated with the proportion of plantlets (propN4 and propN5). Furthermore, the propN1 and propN2 were grouped with LED treatments with NV, meaning these treatments have a positive influence to raise the proportion of protocorms on initial stages of development. In this case, LED lights with NV were responsible to slower rates of development. On the other hand, LED treatments without NV were linked with faster developmental rates and higher proportion of fully developed plantlets (propN5). Exceptions were the CFL treatments, both with and without NV, which were grouped with vectors of variables that indicate higher fresh weight and intermediary velocity of development. CFL were linked with highest fresh weights for all developmental stages as well as for total fresh weight. However, they were also linked with higher proportion of hiperhydric protocorms.

The PCA analysis results were confirmed by the stacked bar plot of the proportion of protocorms on each developmental stage in relation to light and NV treatments (Figure 1b). The proportion of fully developed plantlets (stages N4 and N5) was more than 50% for all treatments under LED lights without NV. That means that more than 50% of the seeds that germinated reached the plantlet stage within three months or less after sowing. CFL treatments showed intermediate results, but with a minor difference between the presence and absence of NV.

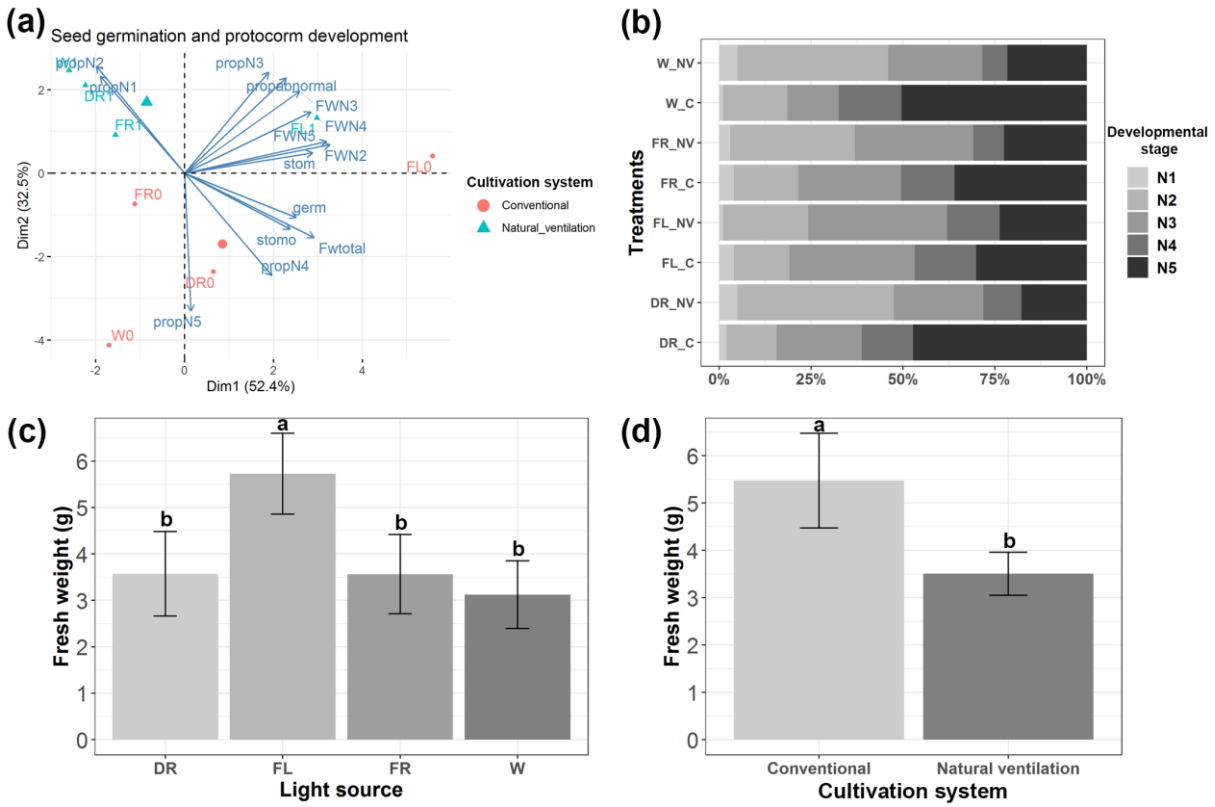


Figure 1: *Epidendrum fulgens* seed germination and protocorm development (SGS) under different sources of light and natural ventilation. a) Principal component analysis (PCA) showing the ordination of the measured variables for (SGS). The x and y axis represents the first and second dimensions of the analysis, that together explain 84.9% of the total variation. FL = fluorescent light; W= blue LED; DR= deep red LED; FR= far red LED. Numbers 0 and 1 associated with light treatments represent the conventional and NV systems, respectively. FW= fresh weight; prop=proportion; stom and stomo= number of total stomata and opened stomata, respectively; propabnormal= proportion of abnormal/hiperhydric protocorms; N1:N5= the stage of development (see material and methods). b) Proportion of protocorms under each developmental stage, as indicated. NV= natural ventilation; C= conventional, i.e. without natural ventilation. c) Total fresh weight of protocorms, from N1 to N5, for each light treatment. d) Total fresh weight of protocorms, from N1 to N5, for each cultivation system. Bars are confidence intervals (p= 0.05). Different letters on top of error bars indicate significant differences according to post-hoc tests (p≤0.05).

In relation to the fresh weight, the PCA results were also confirmed by the ANOVA and Tukey tests (Figure 1c). No interaction between light and cultivation system was observed. In that case, it is confirmed that the CFL treatments resulted in higher FW of protocorms. That was also the case of the cultivation system, where protocorms without NV had higher FW (Figure 1d).

LIGHT SOURCES AND NATURAL VENTILATION FOR PLANTLET GROWTH STAGE (PGS)

The effects of light and NV treatments were different for the PGS. The number of shoots was significantly affected, with interaction between light and NV treatments

(Figure 2a). No differences on the number of shoots were observed among the treatments of light or NV (Figure 2b). The number of roots per plant (NR) and fresh weight (FW), as well as significant interaction between light and NV treatments were observed. All plantlets grown with NV had higher NR (Figure 2c). The highest NR was obtained for plantlets grown under deep red LED lights with NV. The NR on CFL treatments, even without NV was also high, although it should be noted that were more variable, which can be noted by the wider confidence intervals. That means the root production by plantlets grown under CFL light was not as stable as for the other light treatments. The number of roots with velamen (VEL) also had a significant interaction between factors, but that occurred only because no velamen formation was observed for LED treatments without NV (Figure 2d). However, all the LED treatments with NV produced higher VEL. All plantlets grown with NV had higher FW (Figure 2e). There was no significant interaction between light and NV factors for the number of stomata and opened stomata.

The biochemical and microscopic differences were also significantly affected by light and NV treatments, but no interaction between factors was recorded. The number of stomata and opened stomata was not altered by the different light treatments. However, plantlets grown with NV leaves produced a lower number of stomata and a higher number of opened stomata (Figure 2f). The chlorophyll a (Chl_a) and carotenoids (Car) concentration was higher on plantlets grown with NV and lower for plantlets grown under far-red LED (Figure 2g-h). No significant differences were observed for the chlorophyll b content.

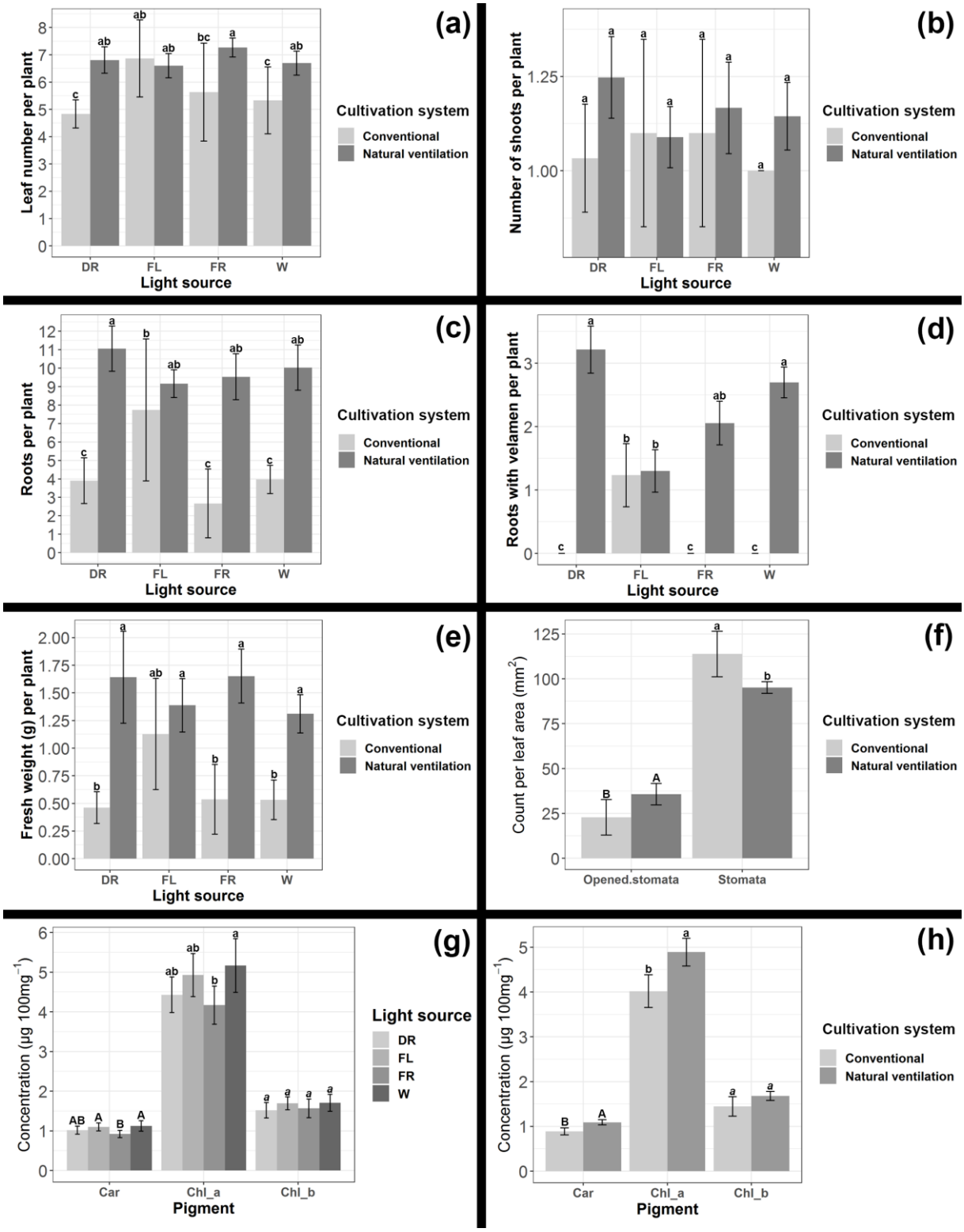


Figure 2: Effects of light sources and NV on plantlet growth stage (PGS) of *E. fulgens*. a) Number of leaves per plant. b) Number of shoots per plant. c) Number of roots per plant. d) Number of roots with velamen per plant. e) Fresh weight per plant (g). f) Number of stomata and opened stomata (1 mm^2 leaf area). g-h) Carotenoids and chlorophyll *a* and *b* concentration (µg 100 mg^{-1}) in relation to light and cultivation system treatments, respectively. Error bars represent confidence intervals (p=0.05). Different letters on top of error bars indicate significant differences according to post-hoc tests (p≤0.05).

Micromorphological changes were observed in leaves from the plantlets grown with or without NV and under the different lights. Sclereids were only observed in leaves of plants grown with NV, except for the CFL light treatment (Figure 3). The central vascular bundle of plants grown with NV had also higher content of sclerenchyma.

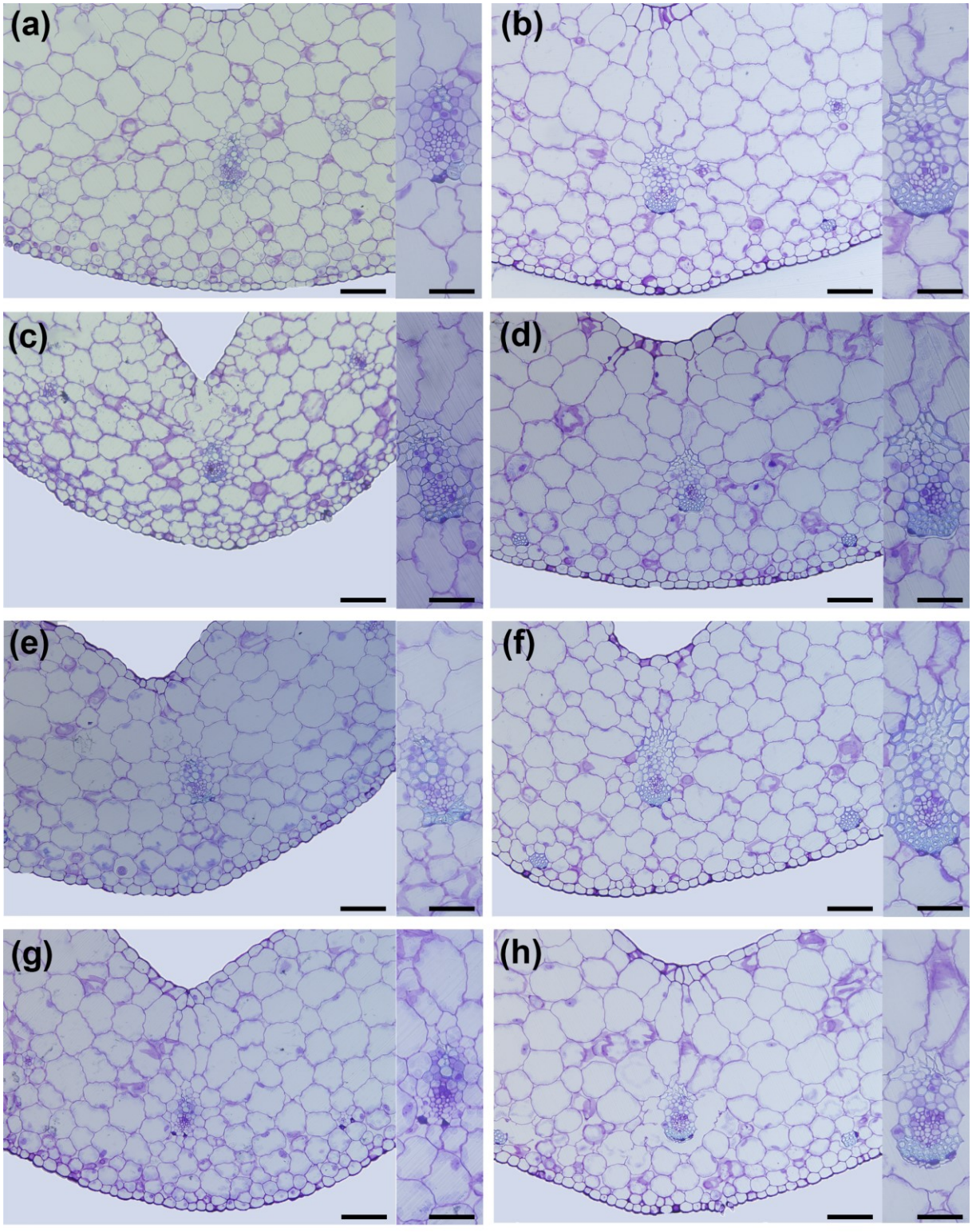


Figure 3: Light microscopy micrographs of leaves of *E. fulgens* from the PGS. Plantlets were grown with or without natural ventilation (NV) under different light sources. a-b) Fluorescent light with and without NV, respectively. c-d) Deep red LED with and without NV, respectively; e-f) Far-red LED with and without NV, respectively. g-h) White LED with and without NV, respectively. Bars from the main images = 100 µm. On the right of each image is presented a closer view of the leaf central vascular bundle (bars=50 µm). Note the absence of screleids close to the abaxial epidermis for the treatments without NV, and the relative abundance of sclerenchyma in the vascular bundles of the leaves from plantlets grown with NV.

PLANTFORM™ AND NATURAL VENTILATION FOR PLANTLET GROWTH STAGE (PGS)

The comparison between Plantform™ and containers with NV showed that the cultivation system significantly affected the growth and the quality of the plantlets (Figure 4a-b). Variables related to plant growth, as FW, NS, NR and NL were higher for plantlets grown in Plantform™ (Figure 4 c-e). The NR was almost 2-fold higher in plants grown in Plantform™. The cultivation system had no impact on other variables, such as the number of stomata and opened stomata or on photosynthetic pigments contents (Figure 4f-g). The cuticle was thicker in plantlets grown in Plantform™ (Figure 4h).

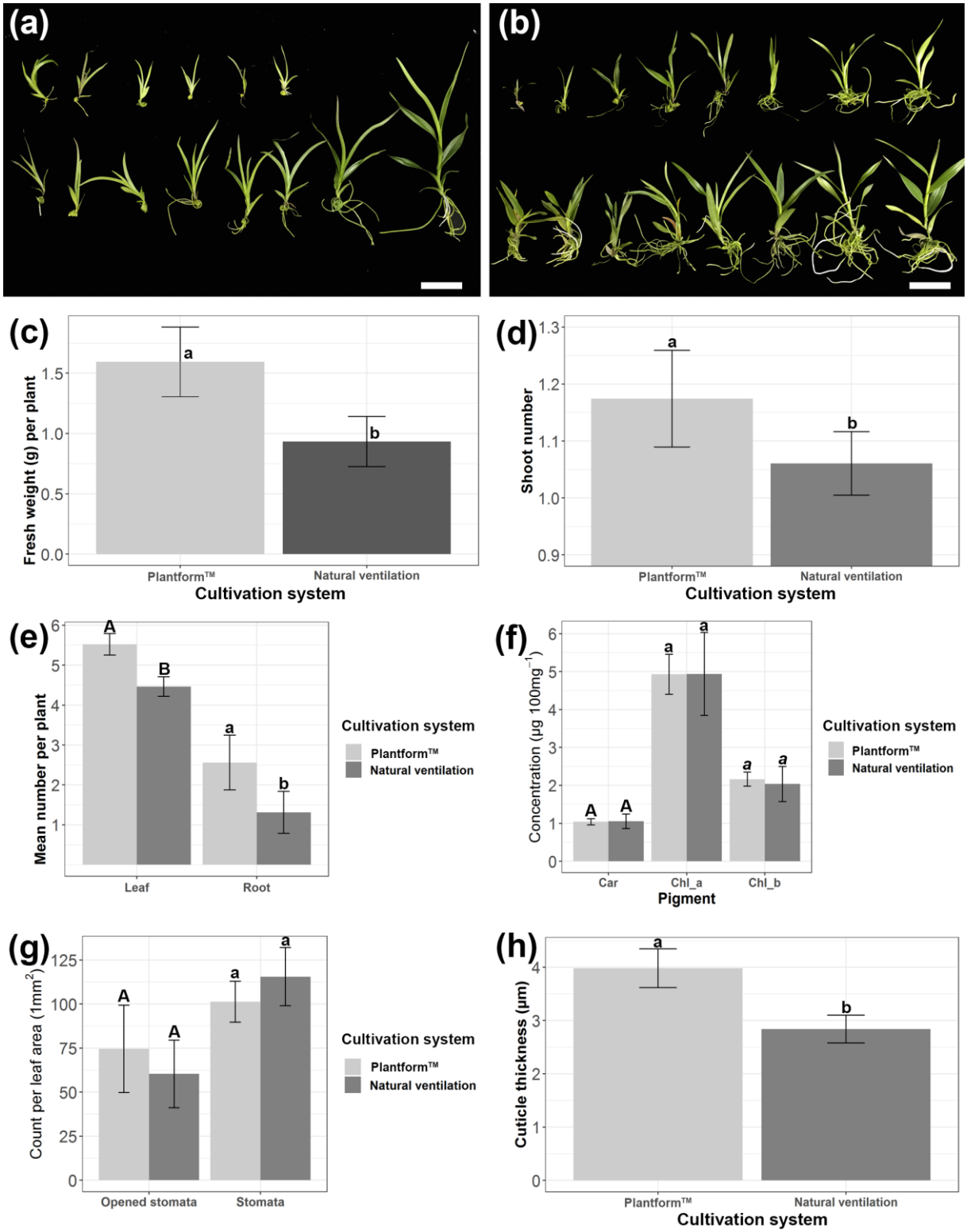


Figure 4: Comparison of *E. fulgens* plantlets grown in temporary immersion bioreactor (Plantform™) and in containers with natural ventilation. a) Plantlets grown in container with natural ventilation. b) Plantlets grown in Plantform™. c) Mean fresh weight per plant (g). d) Mean shoot number per plant. e) Mean number of leaves and roots per plant. f) Carotenoids and chlorophyll *a* and *b* concentration (µg 100 mg^{-1}). g) Number of stomata and opened stomata (1 mm^2 leaf area). h) Cuticle thickness (µm). Error bars represent confidence intervals (p=0.05). Different letters on top of error bars indicate significant differences according to post-hoc tests (p≤0.05).

ACCLIMATIZATION

Acclimatization is normally a critical step in plant micropropagation protocols, as high mortality rates are experienced when transferring *in vitro* plants to *ex vitro* conditions (CHANDRA *et al.*, 2010). However, in the present work, the acclimatization plantlets from both PGS experiments resulted in 100% survival rates, indicating that this step is not an issue for this orchid species, and that the proposed *in vitro* physical modifications evaluated play no role on the *ex vitro* survival rates of the plantlets.

DISCUSSION

THE EFFECTS OF THE LIGHT SOURCES ARE POSSIBLY RELATED WITH THE CHARACTERISTICS OF *E. FULGENS* NATURAL HABITAT

Epidendrum fulgens is a terrestrial orchid that occurs in sandy plains along the coast of Brazil, from the states of Rio Grande do Sul (24°50.886´S) to Rio de Janeiro 28°12.538´S) (PINHEIRO *et al.*, 2010). It inhabits shrubby sand dunes and rock outcrops (SUJII; COZZOLINO; PINHEIRO, 2019), almost always exposed to direct sunlight, which suggests it has greater affinity for high luminosity.

Considering the characteristics of its natural habitat, we expected to find differences between the light treatments tested in this study during all stages of plant development. The sunlight contains almost the same amount of photons from the blue, red and green spectra (SMITH; MCAUSLAND; MURCHIE, 2017). The first leaves of the canopy absorb most of the blue and red wavelengths, and the resulting transmitted light is enriched in green and far-red spectra (FIORUCCI; FANKHAUSER, 2017).

Since *E. fulgens* has high affinity to full sunlight, it was expected that the light treatments containing higher proportions of blue and red wavelengths would be most suited for all stages of micropropagation. And that was really the case summarizing the results of the present study. With the exception of the CFL light, which emit high proportion of light in the green wavelength (Sup. Figure 1), more than 50% of the seeds germinated under the W and DR treatments developed into fully plantlets within 12 weeks or less (Figure 1b).

It is important to note that CFL treatments were directly linked to an increase in FW. One plausible explanation is the high green light produced by the fluorescent lights

(Sup. Figure1). Green photons are capable of reaching chloroplasts much deeper into the mesophyll, and their scattering inside the leaf accomplishes the ‘detour effect’, or the lengthening of photons through the vertical leaf profile enhancing their chance to be absorbed (SMITH; MCAUSLAND; MURCHIE, 2017). In summary, it means that green light is able to drive photosynthesis with quantum efficiency similar to that of red light (SMITH; MCAUSLAND; MURCHIE, 2017). That effect can be easily pictured in round photosynthetic structures such as protocorms.

Furthermore, the results from PGS also, in summary, point to betters morphological and biochemical profiles for plantlets grown under higher proportions of blue and red wavelengths. Bottom leaves receives higher proportions of green light than top leaves (KAMI *et al.*, 2010), which means the ‘detour effect’ also occurs within the canopy (SMITH; MCAUSLAND; MURCHIE, 2017). That explains why the plantlets grown under CFL lights without NV had higher FW and NR, and were the only to produce roots with velamen in this condition. In this case, probably the bottom leaves contributed more to this increase. In this study, we used only top leaves for the chlorophyll analysis, and the increase in the Chl_a and carotenoids observed were significantly linked to the different light treatments. The absorbance range of carotenoids is greater for green wavelengths, covering a spectrum region where the chlorophyll is not so efficient (SMITH; MCAUSLAND; MURCHIE, 2017).

In future studies, we recommend the sampling of leaves distributed in the canopy, since the light effect on pigment concentration can be further increased in bottom leaves. In tomato, for example, green light supplementation increased plant biomass and yield, and the chlorophyll a/b ratio and carotenoids concentrations were only recorded for leaves located in the middle leaf layer (KAISER *et al.*, 2019). There are not many studies in regard to the effect of green light in orchids, but in *Cymbidium insigne*, it was found to increase protocorm-like bodies proliferation as well as shoot and root production (NAHAR; HAQUE; KAZUHIKO, 2015). The effects of green light in plant production are nowadays been reconsidered, and regarding this aspect, fluorescent lights still have their value.

It is also important to note that other important features from plantlets should be considered, as their resistance to biotic stresses. That feature was not the focus of the present study, as no diseases were observed during acclimatization and, therefore, the 100% percentage survival of the plantlets can only be interpreted in terms of abiotic

stress resistance. Nevertheless, light effects on plant resistance to the biotic stresses is an important factor, and we recommend that future studies should extend the *ex vitro* period analysis to include this factor. Plants have a 'light memory' that can trigger systemic resistance induction when they are subjected to excess light levels, especially in the red spectrum (KARPIŃSKI *et al.*, 2013). Therefore, the *in vitro* light quality received by the plants can have effects that go beyond the survival rate during acclimatization. In that aspect, the UVB radiation is also known affect plant defense against pathogens and herbivory (BALLARÉ, 2014).

NATURAL VENTILATION IS POSITIVE FOR PLANTLET GROWTH, BUT IT RETARDS SEED GERMINATION AND PROTOCORM DEVELOPMENT

As far as we are aware, there are no studies regarding the influence of light or atmosphere composition on *E. fulgens* sexual reproduction. Under natural conditions, its asexual reproduction through vegetative off-shoots produced on floral stalks is frequently observed, but no studies about its sexual reproduction have been conducted under natural conditions. However, gene flow via pollen is high among different *E. fulgens* populations (PINHEIRO *et al.*, 2011), suggesting sexual reproduction is frequent in nature. It was expected that our results on asymbiotic seed germination and initial protocorm development would show different results than the obtained. In the natural habitat of *E. fulgens*, the *´Restinga',* plants are exposed to constant winds, floods, drought, high salinity and low nutrient levels (SCARANO, 2002), which means that NV was expected to be beneficial for all stages of plant development, including SGS.

However, the results showed that NV was detrimental for the initial stages of protocorm development, especially when associated LED light sources, irrespective of the wavelength. It should be considered, though, that under natural conditions orchid seeds germinate only symbiotically, when associated to mycorrhizal fungi (RASMUSSEN; RASMUSSEN, 2014), which makes a comparison with artificial asymbiotic seed germination *in vitro* quite shallow.

Orchid symbiotic protocorms were found to accumulate both the enzyme ACC synthase, involved in ethylene biosynthesis, and an ethylene receptor (VALADARES *et al.*, 2014), suggesting this hormone plays an important role in protocorm development. The addition of exogenous ethylene increased the germination of orchid

seeds (MIYOSHI; MII, 1995), and this hormone is produced by many orchid mycorrhizal fungi (RASMUSSEN; RASMUSSEN, 2009). That might be one of the reasons why protocorms developed faster in the absence of NV in this study. One of the changes expected when using membranes that allow the NV of the *in vitro* atmosphere is the low accumulation of ethylene (KOZAI, 2010). One explanation for the results of the present study is that ethylene is favorable to protocorm development in *E. fulgens.* As oxygen is required to convert 1-aminocyclopropane-1-carboxylic acid to ethylene (MOSHKOV *et al.*, 2008), another explanation is that the lower oxygen levels promoted by the liquid medium was responsible for lowering ethylene production.

For PGS, however, NV and played a favorable role on the better development of plants. That is in accordance with the harsh characteristics of the natural environment of *E. fulgens,* and similar results were also found for the epiphytic orchid *Cattleya walkeriana* (DA SILVA *et al.*, 2014). In conventional micropropagation systems, the high humidity level reduces the plants transpiration and, consequently, the nutrient uptake and transport, as observed in *Pfaffia glomerata* plants grown in flasks with NV (SALDANHA *et al.*, 2012). Therefore, the firs explanation is that the low humidity levels in NV stimulate transpiration, and plants absorb more nutrients from the media.

It is known that the NV of *in vitro* affects photosynthesis rate, stomata formation and functionality, and can serve as a hardening and preparation of the plants for acclimatization (ZOBAYED, 2005). In the present study, the chlorophyll a and carotenoids content were also significantly higher in plantlets from the NV treatments. Under drought stress, it is generally known that the chlorophyll a/b ratio is increased (ASHRAF; HARRIS, 2013), but that was not observed in our data (data not shown).

As the internal atmosphere is renewed in NV treatments, not only CO_2 and oxygen levels are balanced with the external atmosphere, but also internal volatiles are expelled, as ethylene (KOZAI; KUBOTA, 2001). In *Catasetum fimbriatum*, the addition of exogenous ethylene stimulated root hair production and inhibited velamen formation (RODRIGUES *et al.*, 2014), which could also be the case for *E. fulgens*, as velamen was found for all treatments of light under NV.

THE EFFECTS OF LIGHT QUALITY AND NATURAL VENTILATION ARE ALSO SIGNIFICANT AT CELL AND TISSUE LEVEL

Plantlets responded microscopically to the light and to changes on the *in vitro* atmosphere. NV increased the size of leaf central vascular bundle and lead to the formation of fibers in subepidermal position (Figure 3). Sclerenchyma cells are responsible for providing protection and support to plants (CRANG; LYONS-SOBASKI; WISE, 2019), which means plants from the NV treatments have a better developed structure to support mechanical damage, for example. Additionally, sclerenchyma cells in Rice were shown to be the target site for adjustments in the defense against pathogens invasions (LI *et al.*, 2020).

Another treatment-derived microfeature observed in plantlets in the present study was the decreased total stomatal density and the increased number of opened stomata in plants grown with NV. As the conventional *in vitro* atmosphere contains high humidity, the plants are not subjected to dehydration. The higher number of opened stomata suggests their functionality is increased under NV conditions. High stomata number can be detrimental when the plants are exposed to the *ex vitro* conditions (CHANDRA *et al.*, 2010). The higher humidity also retards the development of cuticle, epicuticular wax deposition and functional stomata, which can influence in plant acclimatization success (CHANDRA *et al.*, 2010).

PLANTLET GROWTH AND QUALITY CAN BE FURTHER INCREASED BY THE USE OF PLANTFORM™ BIOREACTOR

The efficiency of temporary immersion bioreactors for plant micropropagation is undeniable, and new models are been developed to overcome the problems with the their high initial cost (GEORGIEV *et al.*, 2014). The Plantform™ bioreactor was designed to save shelf space, have a greater interior size to growing cultures and to provide easy handling and avoid contamination (WELANDER *et al.*, 2014). Its advantage over conventional micropropagation has been shown to many different plant species as *Vaccinium* (WELANDER *et al.*, 2017), *Salix viminalis* (REGUEIRA *et al.*, 2018), *Capparis spinosa* (GIANGUZZI; BARONE; SOTTILE, 2020), Olive (BENELLI; DE CARLO, 2018), as well as to increase secondary compounds produced by plants (SKRZYPCZAK-PIETRASZEK *et al.*, 2019; SZOPA *et al.*, 2017).

As far as we are aware, this is the first report of the use of Plantform™ for orchids. The plantlets grown in Plantform™ presented higher number of leaves, roots, shoots and fresh weight when compared to plantlets grown in a same size container (4 L) with natural ventilation. Microscopic analysis of leaves also showed they presented significantly thicker cuticles. Orchids avoid water loss either by increasing cuticle thickness or by storing water in pseudobulbs (YANG, S.-J. *et al.*, 2016). As *E. fulgens* doesn't have pseudobulbs, cuticle thickness must be of main importance for this species to avoid drought stress. The cuticle also serves as a physical barrier to protect plants against irradiation and pathogens (SERRANO *et al.*, 2014). These overall improved characteristics indicate the plantlets produced in Plantform™ are not only bigger, but of better quality.

WHILE THE PRODUCTIVITY AND THE QUALITY OF PLANTLETS ARE INCREASED, THE COST OF PRODUCTION IS REDUCED

LEDs have evolved from rare and expensive sources of light to one of the most widely used electronic equipment (DUTTA GUPTA; JATOTHU, 2013). In ornamentals micropropagation, the energy reduction costs by substituting fluorescents by LEDs can reach 75%, without considering further electric savings in the cooling system (MILER *et al.*, 2019). According to Cardoso *et al.* (2018), to decrease the costs of micropropagated plantlets, one should mandatorily not only consider the reduction of costs directly associated with the technique, but also the increase of the production efficiency. In the present study both factors were considered, and we were able to propose an applicable protocol with reduced costs and increased yields, with both quantitative and qualitative aspects of plantlets taken into consideration. We also contemplated the particularity of each *in vitro* cycle to propose simple changes that do not add any laborious technique, but result in significant advantages.

Bioreactors were shown to be experimentally feasible for the micropropagation of many orchid species (LEYVA-OVALLE *et al.*, 2020; RAMÍREZ-MOSQUEDA; IGLESIAS-ANDREU, 2016). However, their commercial application is still an issue. Despite the need to reduce labor costs, bioreactors are not extensively used in commercial micropropagation in Germany, which alternatively transfers part of the production to countries with lower salaries, building strategic partnerships and founding sister companies (HUTTER; SCHNEIDER, 2019).

One of the main reasons to avoid using bioreactors in commercial micropropagation is the contamination rates. In sugar cane, for example, this problems are been overcome by supplementing the culture media with already known antimicrobial compounds, as Vitrofural® (MARTÍNEZ RIVERO *et al.*, 2020). The sugar withdrawal in combination with antimicrobials is also an alternative (LU *et al.*, 2020). These alternatives deserve future attention to assure the commercial use of bioreactor for orchid micropropagation.

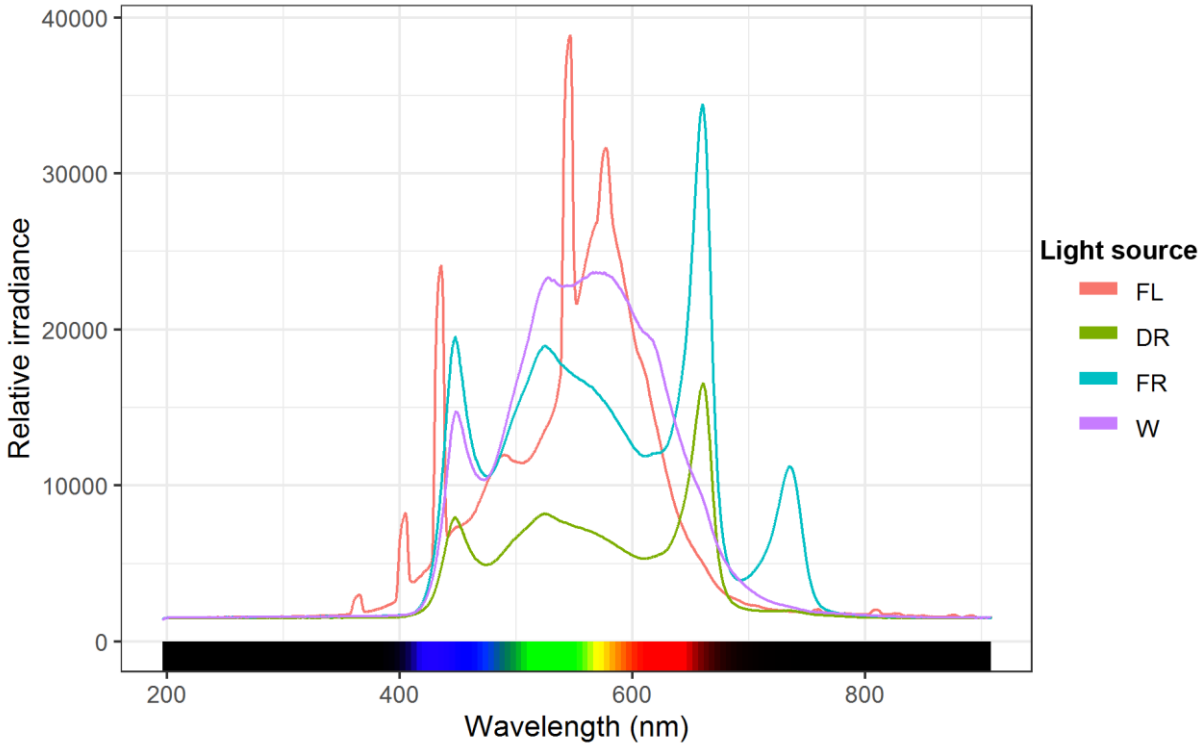
CONCLUSIONS

The exchange of *in vitro* physical conditions, with the use of NV membranes and different light sources affects differently the protocorms development and the plantlet growth stages of the *Epidendrum fulgens* micropropagation.

For early *in vitro* stages, as seed germination and initial protocorm development, NV has negative effects, as protocorms developed faster without NV. LED lights with higher proportion of blue and red wavelengths are the most suitable for SGS.

For PGS, the use of membranes that allow the NV improves plantlet growth and quality, including the content of photosynthetic pigments and microscopic features on the leaves that indicate higher resistance to biotic and abiotic stresses. LED lights are also suitable for PGS, as they can improve plantlet quality. CFL lights are also suited for PGS, although their energy consumption should be taken in account.

The use of Plantform™ is better for PGS, as they increase all variables related to plantlet development, such as number of roots, shoots and leaves, as well as total fresh weight. Plantlets have also a thicker cuticle, which can improve their resistance to biotic and abiotic stresses.



Supplementary figure 1: Relative irradiance of each wavelength (arbitrary units) for the different light sources used. FL= Fluorescent, DR= deep red LED, FR= far-red LED, W= white LED.

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Capítulo 2 - Protocorm-like body induction and plantlet regeneration from endopolyploid explants of *Epidendrum fulgens*: does pre-existing ploidy variation in explants affect the cytogenetic stability of regenerated plantlets?

ABSTRACT

The induction and regeneration of protocorm-like bodies (PLB) is an *in vitro* morphogenetic pathway used in orchid micropropagation with many benefits over conventional techniques. However, the somaclonal variation, which can result both from the tissue culture process itself and also from pre-existent variations in the explants, is a problem that may arise. As orchids commonly have somatic tissues with endopolyploidy, i.e., the coexistence of cells with different ploidy levels within the same individual, the pre-existent variation is a primary concern when choosing explant sources for micropropagation. Knowing the accurate endopolyploidy pattern existing in different explant sources can be helpful to prevent somaclonal variation and also to generate polyploid regenerants. Polyploid orchids are valuable, once this condition improves flower size, color, number and durability. The objective of the present work was to perform a screening of the ploidy levels of different orchid organs and tissues, to develop PLB induction protocols using explants with contrasting ploidy patterns, as well as to elucidate the influence of the pre-existing explants' ploidy variation on the cytogenetic stability of regenerants. *Epidendrum fulgens*, an orchid species with high ornamental value with occurrence in the sand plains of the coastal region from south and southwest regions in Brazil was used as a model plant, as it is an example of a neglected genetic resource. Flow cytometry (FC) was used to analyze the ploidy patterns in pollinia, petals, labella, leaf bases, leaf tips, root tips, protocorms bases and protocorms apexes of *E. fulgens*. The proportions of different cytotypes were measured and a cycle value (CV) was calculated for each organ/tissue. All FC analyzed organs/tissues were used as explants for the *in vitro* induction PBL. Leaves were used to define the best plant growth regulator (PGR) concentration for PLB induction, which was subsequently used for PLB induction from protocorm bases and root tips. Plants were regenerated from PLB induced from different explants and their ploidy level was compared with seedlings obtained from asymbiotic seed germination.

The ploidy screening showed the existence of contrasting ploidy patterns in the different tissues/organs analyzed. Endopolyploidy was detected in all samples, with C-values ranging from 1C to 16C. 1C values were only detected in pollinia. Protocorm bases presented the highest CV, followed by root tips. Labella and leaves showed intermediary CV, while petals and protocorm apexes contained the lowest CV. Flower parts showed high levels of oxidation when used as explants for PLB induction. Pollinia germinated in all PGR concentrations tested, but failed to produce PLB or callus. Only one PLB was recorded from a labellum explant, but it oxidized, not allowing its proliferation or regeneration. Leaf explants were suitable for defining the best conditions for PLB induction, with the highest induction rate estimated at 10 µM thidiazuron (TDZ) concentration. When the best TDZ concentration was used with protocorm bases, leaves and root tips, PLB induction rates obtained where 92%, 22% and 0.92%, respectively. Plantlets were more easily regenerated from PLB induced from protocorm bases than from leaves and were hardly obtained from root tips. Cytogenetic stability of PLB-regenerated plantlets obtained from leaf explants showed they have the same ploidy level of control seedlings

Key-words: Protocorm-like bodies; endopolyploidy; flow cytometry; pollinia; partial endoreplication.

INTRODUCTION

Brazil holds a privileged position in relation to orchid species diversity, with essentially 10% of the world´s species (*World Checklist of the Monocotyledons,* 2019), of which 60% are endemic (Brazilian Flora Checklist, 2018). Many of these species are of high ornamental and horticultural value (VAN DEN BERG *et al.*, 2009). Nevertheless, the ornamental plant market is composed basically by Asiatic species and hybrids (CHEN, W-H.; CHEN, 2007).

The Brazilian´s great biodiversity and appropriate climate creates appropriate conditions for the development of the ornamental plants chain market (POMPELLI *et al.*, 2007). However, it is necessary an extensive work on selection and prospection of native plants with high ornamental potential, allied with the use and development of modern biotechnologies in order to develop plants with high value for the ornamental

market (POMPELLI *et al.*, 2007). The genetic improvement of flower attributes, and the development of novel floral combinations are targets for researchers all over the world (HOSSAIN, KANT, *et al.*, 2013), whom together with flower breeders and producers, warn for a possible collapse of a such restricted flower market (WINKELMANN, GEIER e PREIL, 2006). In this context, the development of appropriate technologies for the mass production of orchid plants is important for meeting the ornamental market demand and for biodiversity conservation (HOSSAIN, KANT, *et al.*, 2013).

The induction of protocorm-like bodies (PLB) is a morphogenetic pathway used for orchid micropropagation. The terminology was first used by Morel in 1960 (ARDITTI, 2008) to describe their structural similarity to orchid protocorms. It is a promising technique that could replace conventional micropropagation as it allows the clonal conservation, breeding and propagation of elite plants with high phytosanitary quality (CARDOSO; ZANELLO; CHEN, 2020). PLB are also commonly used as targets for genetic transformation (HSIEH *et al.*, 2020; LIU *et al.*, 2017).

The use of such morphogenetic pathway, however, has some disadvantages. Main limitations are the low repeatability of protocols due to genotype-dependent responses and the occurrence of somaclonal variations (CARDOSO; ZANELLO; CHEN, 2020). Therefore, it is crucial to define protocols for the induction and regeneration of PLB for each specific species or variety considering not only its multiplication efficiency, but also the genetic stability of the regenerated plantlets.

Somaclonal variation may be caused by genetic variation, as point mutations, insertions and deletions and also ploidy alterations (WECKX; INZÉ; MAENE, 2019). They can also arise as a result of the tissue culture technique or due to pre-existing variation in the explants (BAIRU; AREMU; VAN STADEN, 2011). In regard to the first type, the number of micropropagation cycles using PLB is often reduced to fewer than three generations to avoid somaclonal variations (HSU *et al.*, 2019). The pre-existing variations in orchid tissue culture, on the other hand, are less frequently considered in most protocols. Orchid micropropagation protocols adopt many different types of explants, as roots (KERBAUY; ESTELITA, 1996; PICOLOTTO *et al.*, 2017), floral stalks (CHEN, L.-R.; CHEN; CHANG, 2002), leaves (CHEN, J. T.; CHANG, 2006), and shoot tips (ROY *et al.*, 2007), but whether the use of such variable explant sources may lead to variations on regenerated plantlets is still an issue.

The pre-existing variation should receive even more attention in orchid tissue culture, as many species are endopolyploid (FUKAI; HASEGAWA; GOI, 2002; HO *et al.*, 2016; LIM; LOH, 2003; TEIXEIRA DA SILVA, J. A.; TANAKA, 2006; YANG; LOH, 2004). Endopolyploidy is characterized by the coexistence of cells with different ploidy levels within the same tissue or organism (BAROW; MEISTER, 2003). Additionally, the problem with the ploidy levels of explants is even more complicated, as orchid somatic tissues can undergo partial endoreplication, a feature unique of the Orchidaceae (TRÁVNÍČEK *et al.*, 2019), which occurs in all orchid subfamilies with the exception of Apostasioideae (BROWN *et al.*, 2017; TRÁVNÍČEK *et al.*, 2015).

On the other hand, breeders have already used endopolyploidy of explants as a tool for obtaining polyploid plants. Murashige and Nakano (1966) isolated polyploid cells from the pith of tobacco plants and regenerated polyploids. Polyploid azaleas were also successfully regenerated from endopolyploid petal margins (SCHEPPER *et al.*, 2004). More recently, polyploid *Phalaenopsis* were obtained using the endopolyploid base of protocorms (CHEN, W. H.; TANG; KAO, 2009).

Polyploid induction is common used in ornamental plant breeding to obtain novelties and to overcome crossing barriers (EECKHAUT *et al.*, 2018). Polyploidy is particularly important for orchids as they result in bigger flowers, more intense color and scent (RODRIGUES; BRUZI, 2015), better agronomic traits (CHUNG, H. H. *et al.*, 2017) and stress resistance (EECKHAUT *et al.*, 2018). Polyploid plants are been extensively employed on the genetic improvement of *Phalaenopsis,* either directly as commercial products due to its shorter flower stalks and high quality flowers (CHEN, W. H.; TANG; KAO, 2009), or to restore the fertility of triploid hybrids (CHEN *et al.,* 2011). Likewise, Hawaiian *Dendrobium* production was improved with the use of polyploid cultivars (KUEHNLE, 2007).

Hence, the accurate knowledge of the genetic uniformity of the entire plant in order to select explants for tissue culture is necessary not only to avoid somaclonal variation (BAIRU; AREMU; VAN STADEN, 2011), but it can also be used as a tool for the genetic improvement.

The objective of the present work was to perform a screening of the ploidy levels of different orchid organs and tissues, to develop PLB induction protocols using explants with contrasting ploidy patterns, and to elucidate the influence of the pre-existing explants' ploidy variation on the cytogenetic stability of regenerants.

MATERIAL AND METHODS

DNA PLOIDY LEVEL OF ORGANS AND TISSUES

Flow cytometry was used to analyze the endopolyploidy level of the following organs/tissues:

1- Pollinia;
2- Petals;
3- Labellum;
4- Root tips;
5- Leaf base;
6- Leaf tip;
7- Protocorm base
8- Protocorm apex;

Nuclei extraction and isolation

Pisum sativum cv. Ctirad (Fabaceae) (2C = 9.09 pg DNA) was used as an internal reference standard for the flow cytometry analysis. Seeds of *P. sativum* were kindly provided by Dr. Jaroslav Doležel from the Institute of Experimental Botany of the Czech Academy of Sciences.

Nuclei from the samples and leaves of the reference standard (~50 mg) were simultaneously extracted by chopping with a razor blade (GALBRAITH *et al.*, 1983) on 2 mL ice-cold Otto-I buffer (OTTO, 1990). The nuclei suspension was filtered through a 40 µm nylon mesh (BD Falcon) and centrifuged at 150g for 5 min. The supernatant was removed with a pipette and the pellet was resuspended after the addition of 100 µL of fresh ice-cold Otto-I buffer. The nuclei suspension was stained in the dark for 30 min in 500 µL of Otto-II buffer (OTTO, 1990) supplemented with 50 µg mL^{-1} of propidium iodide (PI; Sigma-Aldrich) and RNase (Sigma-Aldrich).

Propidium iodide fluorescence intensity was measured with a BD FACSCanto™ II flow cytometer, equipped with an Argon Laser (488 nm), at the Laboratório Multiusuário de Estudos em Biologia at the Universidade Federal de Santa Catarina (LAMEB/UFSC). The position of the peaks from the samples and the reference standard was settled by analyzing a first run with each sample separately. The G1

peaks were assigned to a specific channel and the equipment voltage and gain were kept constant throughout the analyses.

Flowing software 2.5.1 was used to process the data. First, we analyzed dot-plots of fluorescence intensity on a linear scale vs. forward scatter light in a logarithmic scale. A polygonal region including all PI stained nuclei was created on the dot-plots from which gated histograms of fluorescence intensity in linear scale were created. Linear regions were created on histograms to gate and obtain descriptive statistics of only intact nuclei.

The numbers of nuclei in each endopolyploid level from the samples were used to calculate the cycle value (BAROW; MEISTER, 2003), according to the following formula:

Cycle value = (0 · n2C + 1 · n4C + 2 · n8C + 3 · n16C) / (n2C + n4C + n8C + n16C)

Where, n2C, n4C, n8C and n16C are the numbers of nuclei with C-values 2C, 4C, 8C and 16C, respectively.

The genome size was calculated based on the ratio between the 2C fluorescence intensity peaks from the samples and the internal reference standard. The value was multiplied by the DNA C-value of the reference standard (DOLEŽEL; BARTOŠ, 2005). To convert DNA content in picograms (pg) to base pairs (bp), we considered that 1 pg = 0.978 x 10^9 bp (DOLEŽEL *et al.*, 2003).

PROTOCOL FOR PLB INDUCTION AND PLANTLET REGENERATION

Experiments for PLB induction were performed using all the organs/tissues used on the endopolyploidy analysis. They are described separately below.

PLB induction using flower parts as explants

Flower buds were collected before anthesis and superficially sterilized with ethanol 70% for 1 min and sodium hypochlorite 0.5% for 5 min, followed by 3 rinses in sterile distilled water. Buds were opened under sterile conditions and the petals, labella and pollinia were excised. Explants were inoculated in Petri dishes containing 25 mL of half-strength MS medium (MURASHIGE; SKOOG, 1962) supplemented with 20g L^{-1} sucrose, 250 mg L^{-1} polyvinylpyrrolidone (PVP) and different concentrations of plant

growth regulators (PGR). The PGR compositions were the four different treatments of the experiment:

1- 20 µM Thidiazuron (TDZ);
2- 30 µM TDZ;
3- 9.3 µM 2-isopentenyladenine (2iP) + 36 µM 2,4-Dichlorophenoxyacetic acid (2,4-D);
4- Control medium without PGR.

Petals and pollinia were inoculated intact. Labella were cut in half, and ovaries were sliced in 1 mm thin cell layers (TCL). The numbers of explants inoculated per treatment were 32 for pollinia and labella and 64 for petals. Each explant was considered as a repetition. After 60 days after inoculation data of PLB induction and oxidation rates were collected. For pollinia, the medium length of pollen tube was also measured.

Defining the best TDZ concentration for PLB induction from leaf explants

Leaves from *in vitro* plantlets were used to define the optimal concentration of PGR for PLB induction. Then, roots and protocorm bases were inoculated on this optimal concentration to compare the induction rate between the three different explants, as described below.

For obtaining *in vitro* plantlets, *E. fulgens* seeds were harvested from mature pods, sterilized and sown as described by Voges *et al.* (2014). After four months of sown, plantlets with 4-5 leaves and roots were selected for the extraction of leaf explants. Leaves (≈ 1 cm) were inoculated with the abaxial face down in petri dishes containing half-strength MS medium (MURASHIGE; SKOOG, 1962), supplemented with 20 g L^{-1} sucrose and different concentrations of TDZ (0, 3, 6, 9, 12 and 15 µM). Eight leaves were inoculated per Petri dish, and a minimum of 40 leaves per treatment. The Petri dishes were sealed and kept in the dark at (25 ± 2°C).

PLB induction using different explants

The best TDZ concentration for leaves was used to induce PLB from protocorm base and root tips. The explants were obtained from the asymbiotic seed germination as previously described. After one month of sown, homogenous protocorms, with shoot

apex and before the formation of the first leaf, were selected and used as explants. A 1 mm width TCL from the protocorm base was excised from each protocorm under sterile conditions and inoculated in test tubes containing 5 mL of MS medium supplemented with 20 g L^{-1} sucrose and 10 µM TDZ. The apexes were discarded. Root tips and leaves (≈ 1 cm) were obtained from four-month-old *in vitro* plantlets, as previously described, and inoculated in test tubes with the same medium used for protocorm bases. A total of 109 root tips, 49 protocorm bases and 32 leaves were inoculated.

Data analysis

Eight weeks after inoculation data from oxidation, PLB induction rates, and number of PLB per explant were collected. Statistical analysis was performed with generalized linear models (GLM) using binomial distribution and logit link function, considering explants as categorical, and growth regulator concentrations as explanatory variables. All GLM analyses and figures were produced on the R environment (R Core Team, 2019) using the ‘‘car’’ (FOX; WEISBERG, 2011), ‘‘MASS’’ (VENABLES; RILEY, 2002) and “ggplot2” (WICKHAM, 2016) packages.

Microscopic features of primary and secondary PLB

Leaves with PLB and PLB clusters were fixed in glutaraldehyde (2.5%) in sodium phosphate buffer (0.1 M, pH 7.2) for 24 h and then dehydrated in ethanol series (20%, 40%, 60%, 70%, 80%, 90% and 100%) for 30 min each, followed by critical point method drying in liquid carbon dioxide (EM CPD 030/Leica, Germany). Samples were fixed in aluminum stubs with double-sided sticky carbon tape, coated with gold (EM SCD 500/Leica, Germany) and examined at an accelerating voltage of 10 kV in a scanning electron microscope (JEOL, JSM-6390LV) at different resolutions and magnifications. The analysis was performed at LCME/UFSC, Florianópolis, Brazil.

Histoanatomical features of PLB

Leaves with PLBs were collected four months after inoculation and fixed in glutaraldehyde (2.5%) in sodium phosphate buffer (0.1 M, pH 7.2) for 24h and then dehydrated in ethanol series (20%, 40%, 60%, 70%, 80%, 90% and 100%) for 30 min

each. The dehydrated samples were embedded with histo-resin (Leica Historesin, Heidelberg, Germany) and polymerized. Transversal slices (5 µm) were performed in a microtome, stained with toluidine blue and analyzed in an Olympus® BX-40 microscope.

CYTOGENETIC STABILITY OF PLANTS REGENERATED FROM PLBS

For plant regeneration, induced PLB were transferred to test tubes containing 10 mL of PGR-free MS media, supplemented with 1.5 g L^{-1} activated charcoal and maintained in a 16/8h photoperiod at 25°C. Subcultures were performed every 8 weeks, and plants were obtained after ≈24 weeks of growth, in average.

The ploidy status from plantlets regenerated from PLBs obtained from leaves, protocorm bases and root tips was analyzed with flow cytometry using the same methodology previously described. Their ploidy level was compared with the *P. sativum* as an internal reference standard, and asymbiotically seed-derived plantlets were used as a control for ploidy level. Thirty PLB-regenerated plantlets obtained from leaves and from protocorm bases and one plantlet obtained from root tip were analyzed. Data from the flow cytometer were analyzed using Flowing software 2.5.1.

RESULTS

DNA PLOIDY LEVEL OF ORGANS AND TISSUES

The flow cytometry analysis of the different tissues and organs showed that all organs and tissues analyzed are endopolyploid. Five different cytotypes were detected, with C-values ranging from 1C to 16C (Figure 1). Cells with 1C DNA C-value were observed only in pollinia, which correspond to the haploid vegetative nuclei. In all other organs/tissues, cells with C-values ranging from 2C to 16C were observed, implying the occurrence of at least 3 endocycles.

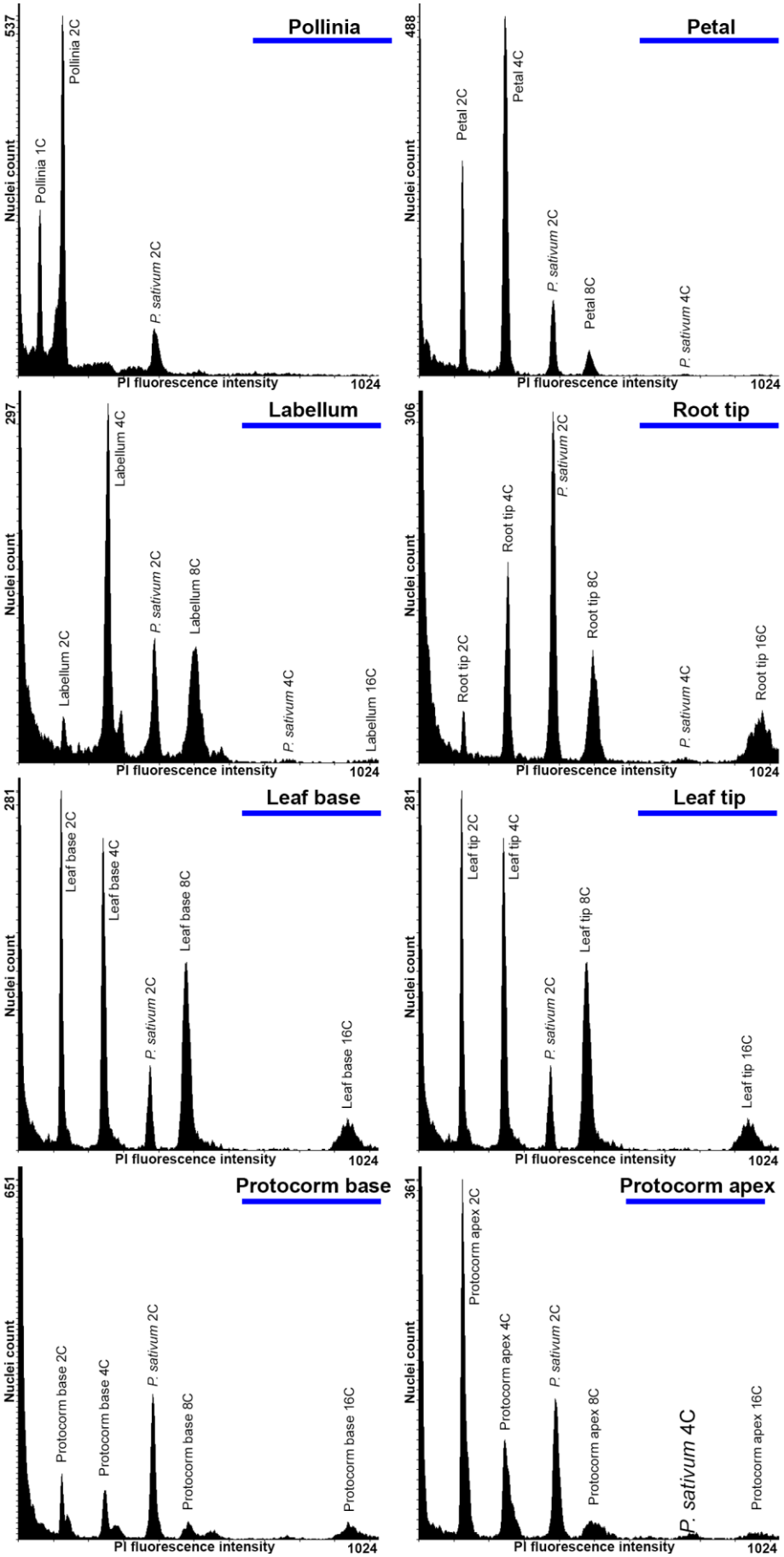
Pollinia
537
Nuclei count
Pollinia 1C
Pollinia 2C
P. sativum 2C
PI fluorescence intensity
1024
Petal
488
Petal 2C
Petal 4C
P. sativum 2C
Petal 8C
P. sativum 4C
Labellum
297
Labellum 2C
Labellum 4C
P. sativum 2C
Labellum 8C
P. sativum 4C
Labellum 16C
Root tip
306
Root tip 2C
Root tip 4C
P. sativum 2C
Root tip 8C
P. sativum 4C
Root tip 16C
Leaf base
281
Leaf base 2C
Leaf base 4C
P. sativum 2C
Leaf base 8C
Leaf base 16C
Leaf tip
281
Leaf tip 2C
Leaf tip 4C
P. sativum 2C
Leaf tip 8C
Leaf tip 16C
Protocorm base
651
Protocorm base 2C
Protocorm base 4C
P. sativum 2C
Protocorm base 8C
Protocorm base 16C
Protocorm apex
361
Protocorm apex 2C
Protocorm apex 4C
P. sativum 2C
Protocorm apex 8C
P. sativum 4C
Protocorm apex 16C

Figure 1: Linear scale histograms of relative fluorescence intensity obtained after flow cytometry analysis of propidium iodide-stained nuclei isolated from different organs and tissues of *Epidendrum fulgens*.

The ratios between sample peaks were always close to 2.0, which indicated complete endoreduplication (Table 1).

Table 1: Flow cytometric profiles of different organs and tissues of *Epidendrum fulgens*

		Ratios between 2C peaks	Ratios between individual peaks			
	Cycle value	from sample and *P. sativum*	2C/1C	4C/2C	8C/4C	16C/8C
Organ / tissue		Mean ± s.d.	Mean ± s.d.	Mean ± s.d.	Mean ± s.d.	Mean ± s.d.
Pollinia	0	0.317 ± 0.007	1.965 ± 0.012	—	—	—
Petal	0,840	0.330 ± 0.015	—	1.987 ± 0.016	1.989 ± 0.013	2.051 ± 0.035
Labellum	1,341	0.334	—	1.984	1.979	2.050
Leaf tip	1,452	0.326 ± 0.002	—	2.000 ± 0.004	1.974 ± 0.000	1.970 ± 0.006
Leaf base	1,348	0.330	—	1.978	1.962	1.994
Protocorm base	1,953	0.340	—	1.979	1.983	1.857
Protocorm apex	0,863	0.328	—	1.977	1.984	1.970
Root tip	1,696	0.321 ± 0.003	—	2.021 ± 0.030	1.968 ± 0.012	1.960 ± 0.005

The proportion of each cytotype was variable among the different organs/tissues (Figure 2). Cells with 16C DNA content were less frequent in petals and labellum (1.12 and 2.62%, respectively). On protocorm base, however, they represented 49.08% of the total cells. Root tips also contained higher numbers of 16C (28.26%). On the other hand, 2C cells where quite scarce in labellum (7.93%), while on protocorm apex they correspond to more than half of the cytotypes. Noteworthy, endopolyploidy pattern between protocorm apex and base regions was contrasting.

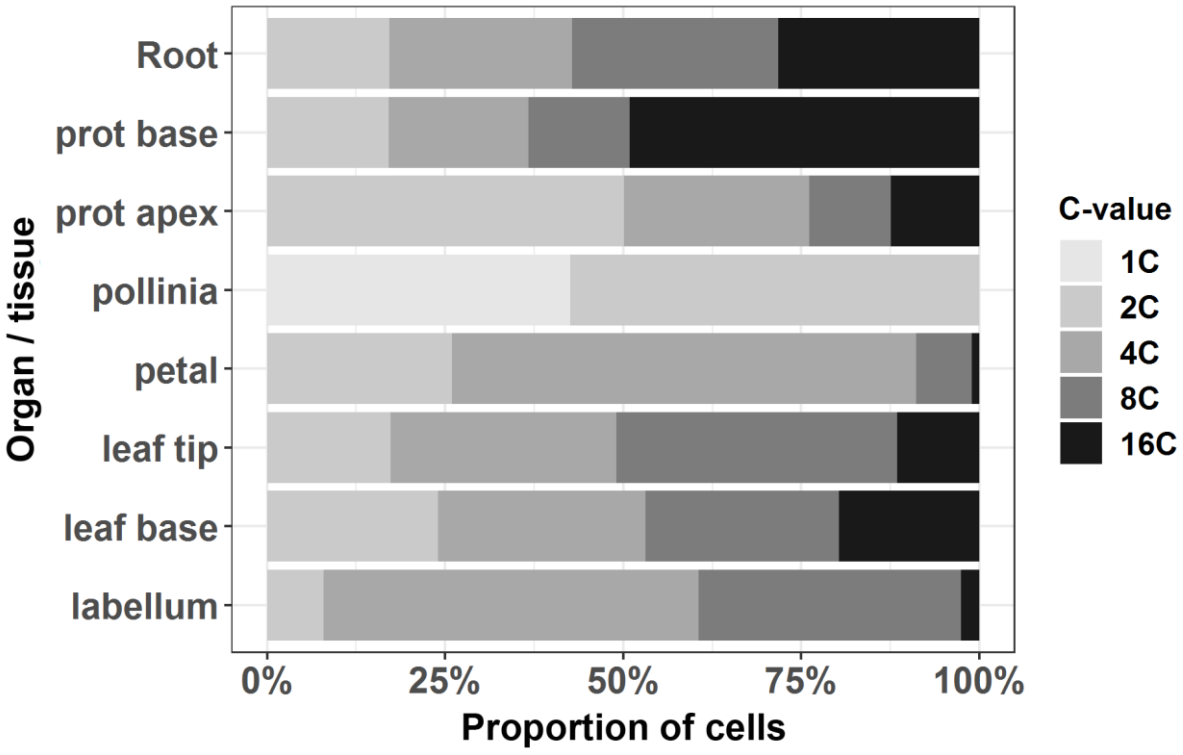

Figure 2: Proportion of cells with different DNA C content on different organs and tissues of *Epidendrum fulgens.*

These differences in the proportion of cytotypes resulted in significantly different cycle values for the different organs and tissues analyzed (Table1). Protocorm bases presented the higher cycle value, followed by root tips. Leaf tip and base presented intermediate values, while protocorm apex and petals exhibited values lower than 1.0.

GENOME SIZE ESTIMATION

The nuclear genome size of *Epidendrum fulgens* calculated from the 2C peaks of all organs and tissues gave the same results, with a variation of only 2.04%. The estimated DNA C-value was 1.492 ± 0.031 pg = 1459 ± 30 Mbp.

PLB induction using flower parts as explants

The attempt to use flower parts as explants for PLB induction showed that the method of using *ex vitro* flower buds was efficient in terms of asepsis. No contamination was observed on the introduced material (Figure 3a). However, only one PLB was obtained from flower parts, more specifically from labellum (Figure 3b), although we were not able to regenerate it, as it oxidized after a few days. Petals and labellum showed to be highly susceptible to early oxidation. Two weeks after inoculation, more than 60% of petals and labella oxidized, which reached 100% of them after another four weeks (Figure 3c). Therefore, petals end labella were not suitable to regenerate PLB or callus tissue under the tested conditions.

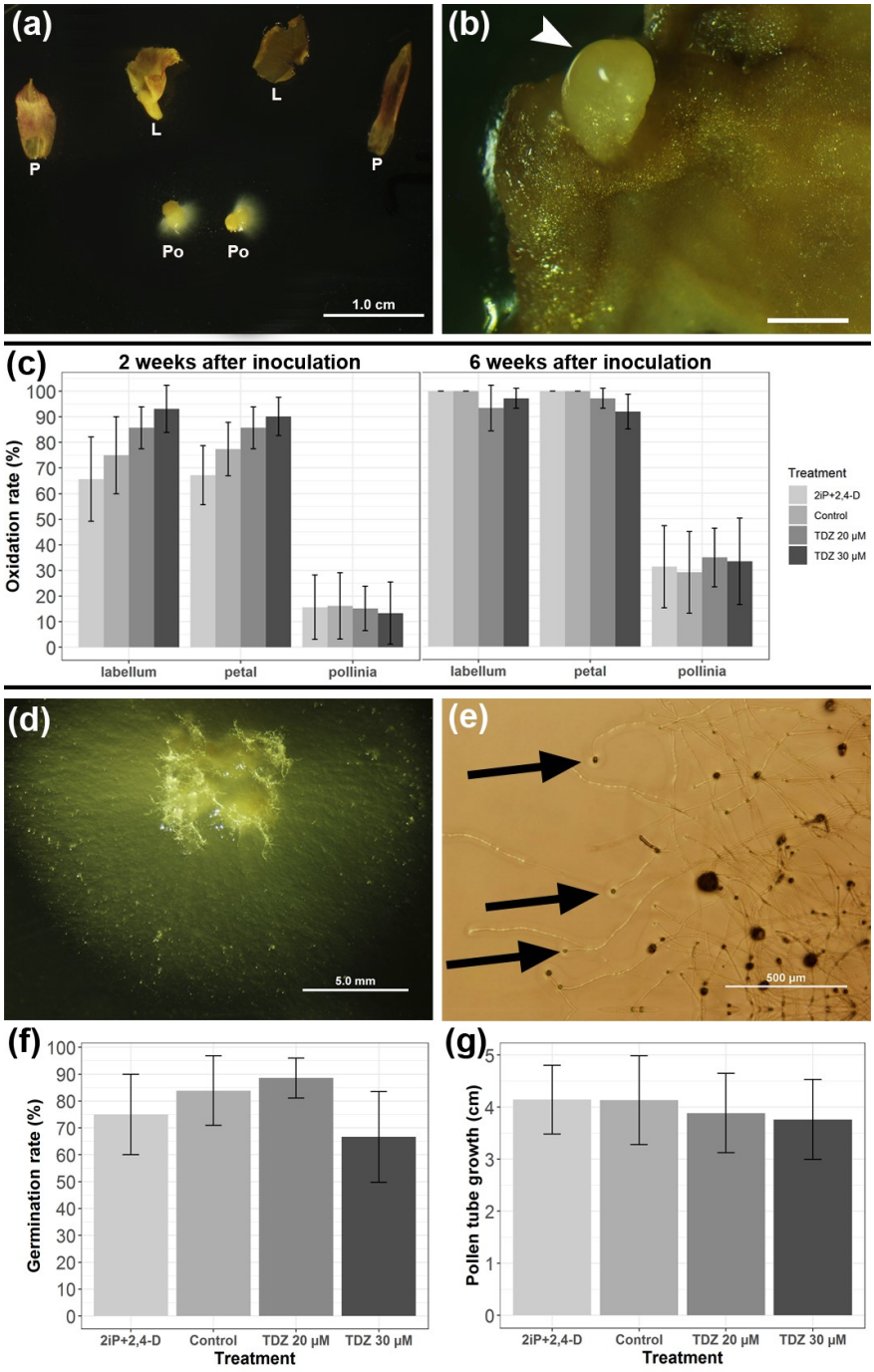

Figure 3: PLB induction using *Epidendrum fulgens* flower parts as explants. a) Petals (P), labella (L) and pollinia (Po) 1 week after inoculation on MS/2 medium supplemented with 20 µM TDZ. b) A primary PLB induced from the labellum. c) Explant oxidation rates two (left) and six (right) weeks after inoculation. d) A germinated pollinium 4 weeks after inoculation in MS/2 medium without PGR. e) Details of germinating pollen grains seen under the microscope. f) Pollinia germination rate in media supplemented with different PGR. g) Pollen tube growth in media supplemented with different PGR. Error bars in (c), (f) and (g) are confidence intervals (p = 0.05).

On the other hand, pollinia were more recalcitrant to oxidation and were able to germinate in all treatments tested (Figure 3c-e). The pollen tubes reached more than 2 cm radius after six weeks of inoculation (Figure 3g). Neither the germination rate nor

the pollen tube growth varied significantly between treatments. Nevertheless, no PLB or callus were observed from the pollinia or from germinated pollen grains.

PLB induction from leaves

Leaves from young *in vitro* plantlets were responsive for PLB induction. Initial PLB were observed after 4 weeks of culture (Figure 4a-b). The PLB were globular shaped, with smooth surface aspect, and were formed directly from the leaf tissue, without an intermediating callus (Figure 4c). Eight weeks after inoculation, primary PLB elongated (Figure 4d). Secondary PLB were formed on the top and at the base of primary PLB (Figure 4e). Histo-anatomical analysis showed that primary PLB had a main meristematic region as well as secondary meristematic regions localized at the base and close to the peridermis (Figure 4f).

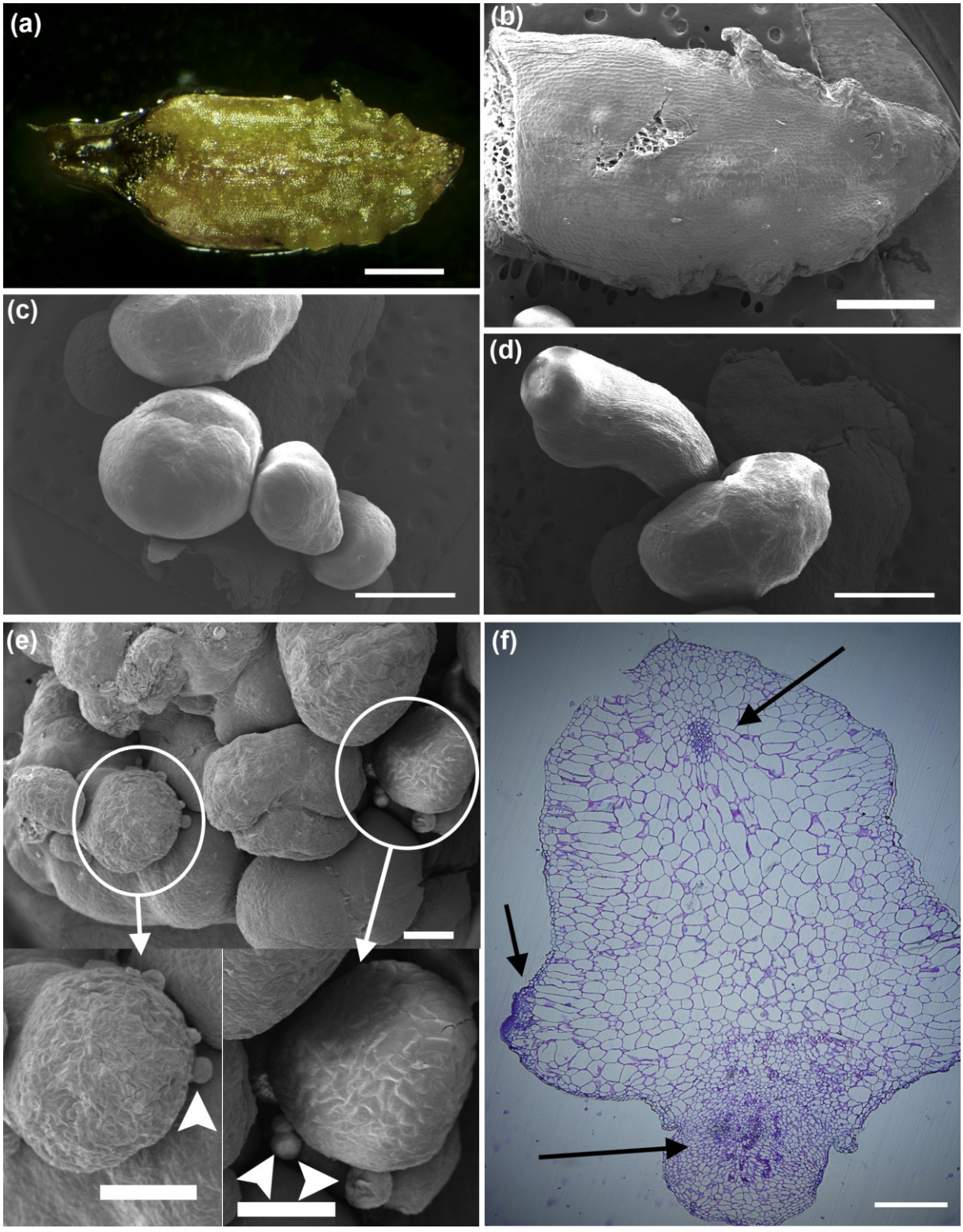

Figure 4: Morphological and histoanatomical features of *Epidendrum fulgens* protocorm-like bodies (PLB). a) A leaf explant after 4 weeks of inoculation in MS medium supplemented with 10 µM Thidiazuron showing initial PLB induction. bar = 1 mm. b) Scanning electron micrograph of the same explant (a) showing details of the initial PLB induction from the epidermis leaf tissue. bar = 1 mm. c) Globular shaped primary PLB 4 weeks after inoculation. bar = 1 mm. d) Primary PLB elongating without leaf primordia formation. bar = 1 mm. e) Secondary PLB formation (arrowheads) from primary PLB. Bottom images are magnified details from the main micrograph. Bars = 500 µm. f) Histoanatomical feature of a primary PLB stained with toluidine blue. The PLB is surrounded by a well-defined cell layer

(peridermis). The central meristem (upper most arrow) and secondary meristematic regions (arrows) are characterized by small cells with little cytoplasm that are intensively stained by toluidine blue.

They were induced on adaxial and abaxial surface, mainly at the leaf base (Figure 5d) and less frequently on leaf tips (Figure 5c) and at the top of the leaf blade (Figure 5a-b). When in the dark, PLB were pale white with a smooth surface (Figure 5e). When transferred to PGR-free medium and exposed to light they rapidly became dark green in color and initiate the production of leaf primordia (Figure 5f). A complete representation of the PLB-to-plantlet regeneration process can be observed in Picture 5g.

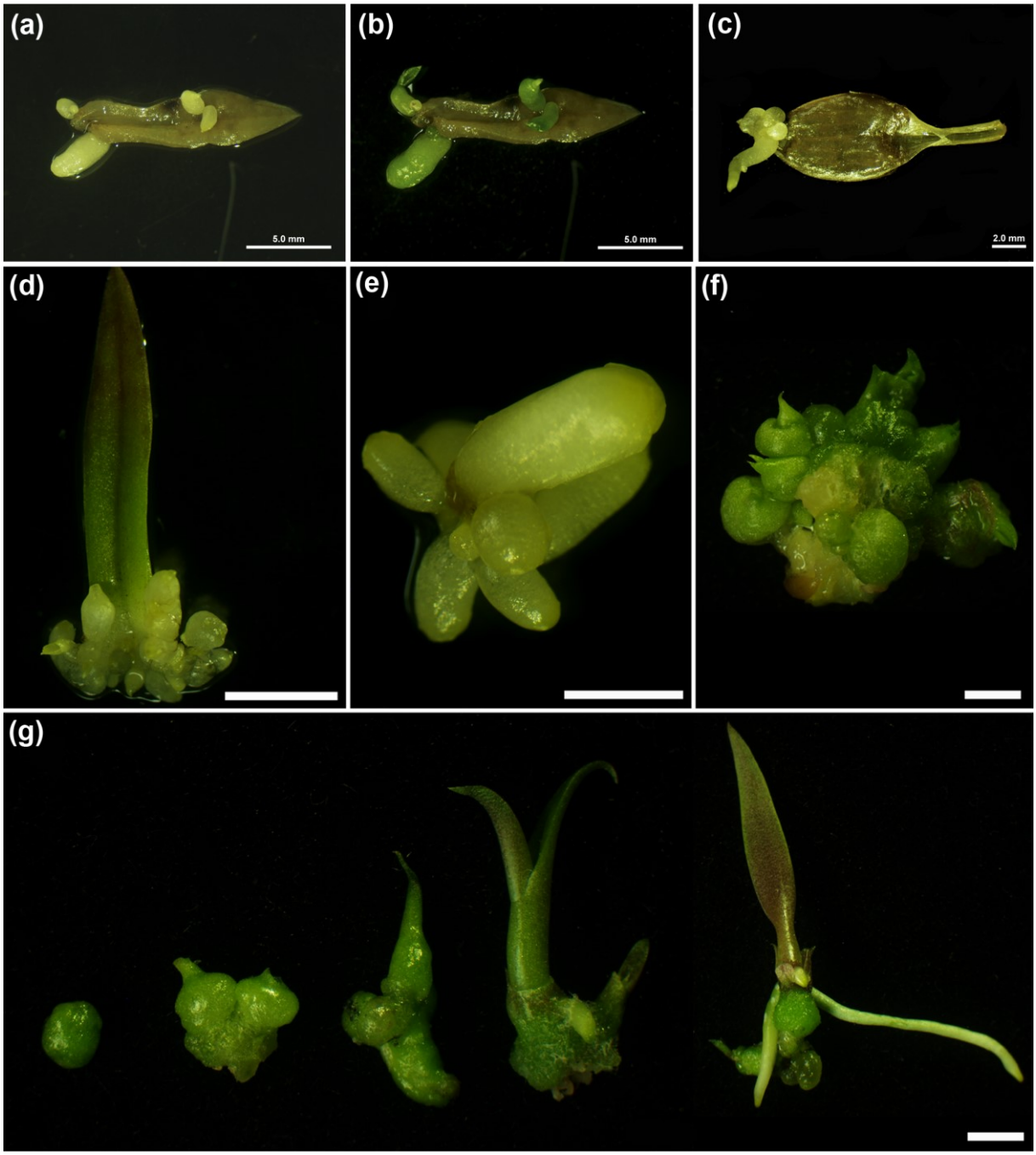

Figure 5: PLB induction and plantlet regeneration from leaf explants. a-b) PLB induced at the leaf base and in the top of the leaf blade, before (a) and after (b) light exposition. c) PLB induced at the leaf tip. d) A responsive leaf explant with many PLB at the base region. Bar = 5 mm. e) An individualized PLB cluster before light exposition. Bar = 2 mm. f) A compact cluster of PLB 2 weeks after transference to PGR-free medium and light exposition. Note the formation of leaf primordia. Bar = 2 mm. g) The detailed process of plant regeneration from PLB, from the globular stage (left) to plantlet with fully expanded leaf and adventitious roots (right). Bar = 0.5 cm.

The best TDZ concentration for PLB induction

The GLM analysis showed that PLB induction rate and average number of PLB per leaf explant varied according to TDZ concentration in culture medium (Figure 6). PLB can be obtained from leaves even in medium without TDZ, at a low frequency and with small number of PLB per explant, though. The PLB induction frequency showed to be a quadratic trend across the TDZ concentrations tested, with the higher induction rate estimated at 10 μM (Figure 6a).

On the other hand, the average number of PLB per leaf explant grows linearly with increasing TDZ concentrations. The best-fitted model suggests that the number of PLB can be further increased when concentrations higher than 15 μM are used, yet the frequency of induction decreases at concentrations higher than 10 μM (Figure 6b). Oxidation was not a concern for PLB induction from leaf explants, as the oxidation rates were the same across the TDZ concentrations tested (Figure 6c).

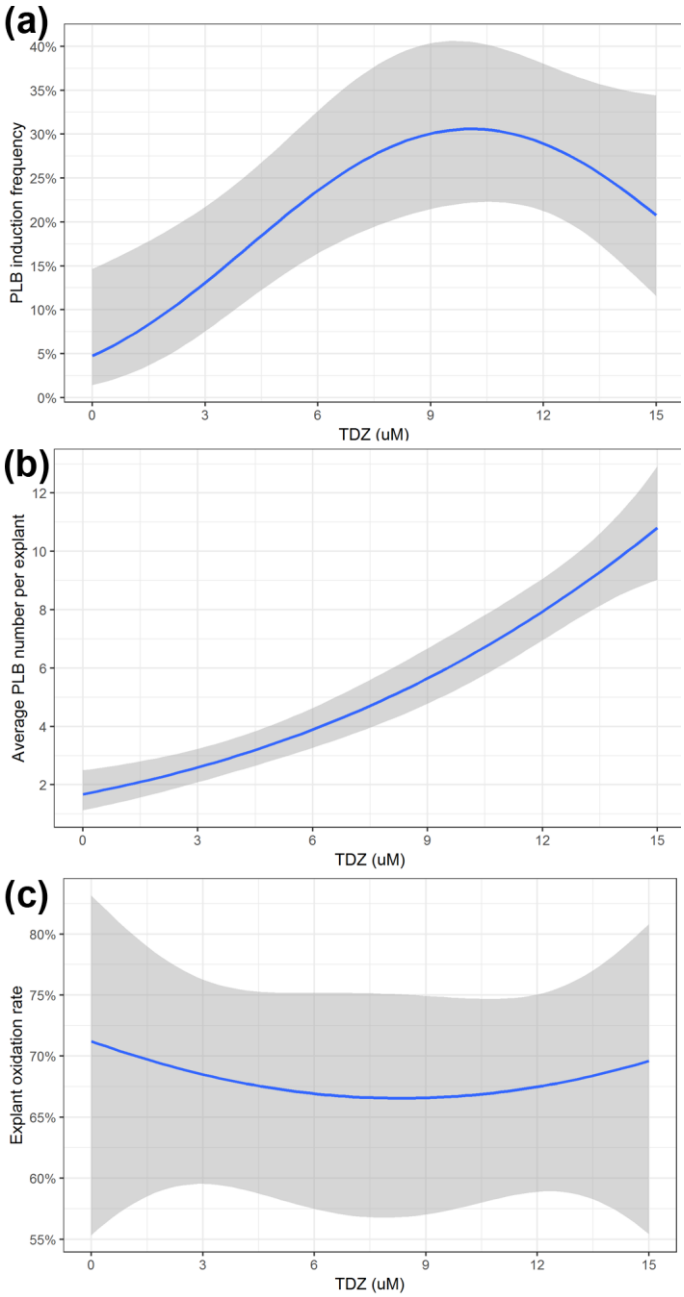

Figure 6: Estimated trends for protocorm-like body (PLB) induction frequency (a), average number of PLB (b), and explant oxidation rates (c) from *Epidendrum fulgens* leaf explants under increasing concentrations of TDZ (0, 1, 3, 9, 12 and 15 µM). Data collected after 8 weeks of culture. Shaded bands indicate 95% confidence envelopes.

The obtained results suggest that in medium supplemented with 10 µM TDZ, about 30% of PLB induction rate is obtained (22 to 40%), with an average number of 5 PLB per explant.

Comparison between leaves, root tips and protocorm bases

The comparison between root tips, protocorm bases and leaves using the best TDZ concentration experimentally defined showed a high response variation. Protocorm bases were the most responsive explants for PLB induction, with more than 90% induction rate (Figure 7g). PLB were white and globular shaped when in the absence of light, and were induced mainly from the protocorm base epidermal tissue (Figure 7a). When exposed to light they became green and continued to proliferate (Figure 7b). Plants were obtained after only one subcultivation step to PGR-free medium (Figure 7c), which was faster in comparison to leaf-derived PLB (data not shown).

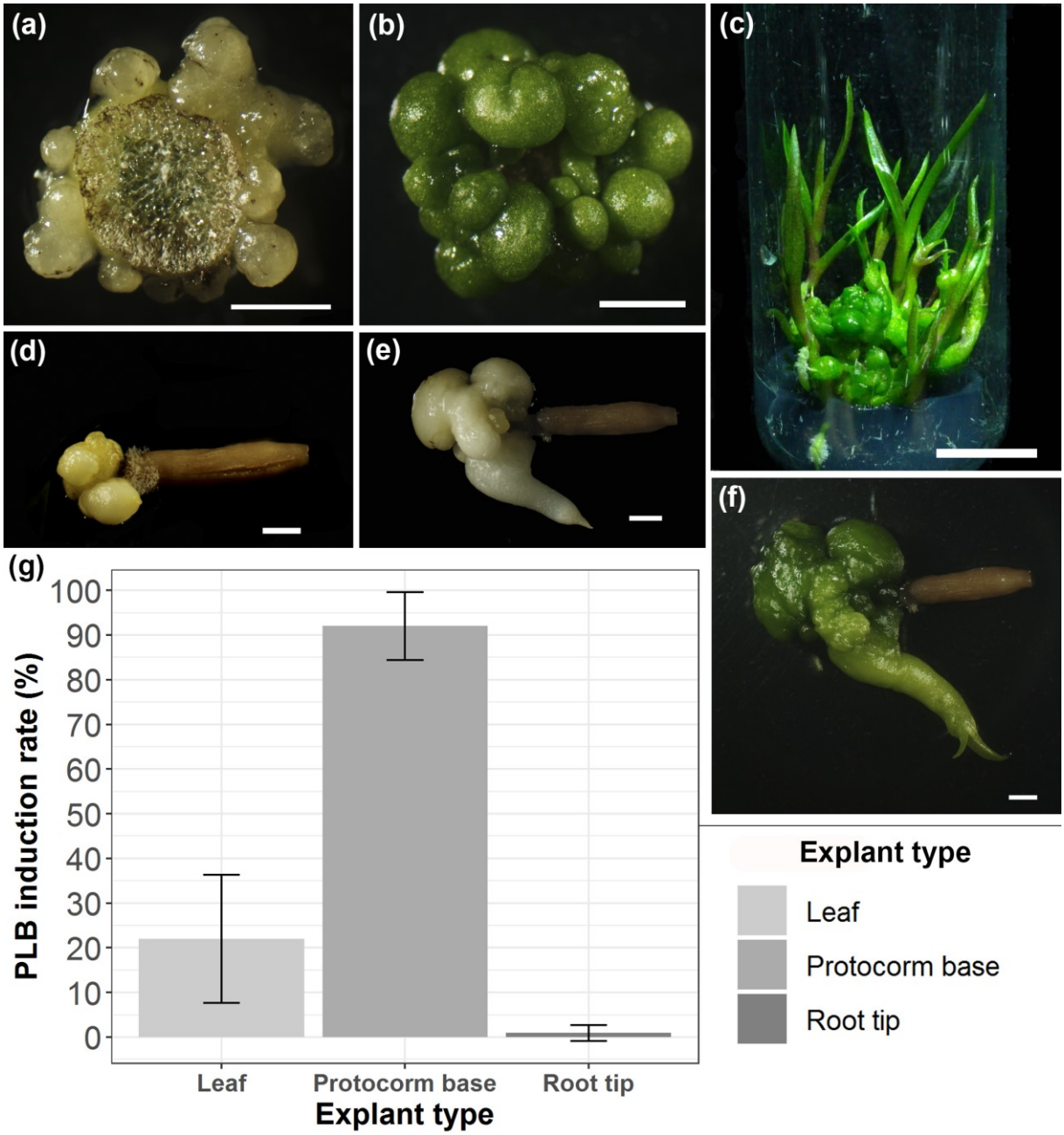

Figure 7: PLB induction and plant regeneration from protocorm bases (a-c) and root tips (d-f). a) A 1mm width TCL from the protocorm base 4 weeks after inoculation on MS/2 medium supplemented with 10 µM TDZ. Note that the globular white-color PLB are induced directly from the epidermal tissue. b) PLBs acquire a dark green color 2 week after transfer to PGR-free medium and light exposition. c) Plantlets obtained from a TCL from the base of a protocorm. d) A root tip explant with PLB forming at the apical meristem region. Note the velamen and root hairs close to the PLB. e) PLB started to elongate before transfer to light. f) A shoot with leaf primordia was formed 9 months after transfer to PGR-free medium and light exposition. g) PLB induction rate comparison between leaves, root tips and protocorm bases in MS/2 medium supplemented with 10 µM TDZ. Bars are confidence intervals (p = 0.05).

Only one PLB was obtained from root tip explants (Figure 7d-e) out of 109 inoculated root tips, which is less than 1% induction rate (Figure 7g). It was induced at the calyptra, and was extremely slow growing. We were able to regenerate a shoot from the root-derived PLB only after eight months of culture in PGR-free medium

(Figure 7f). No adventitious root was produced from the shoot even after one year of culture (data not shown). PLB induction rate on leaves was 22% (Figure 7g), which is within the GLM analysis confidence envelope and shows that the model is precise.

PLANT REGENERATION FROM PLB AND CYTOGENETIC STABILITY

Plantlets formation was considered after the observation of adventitious roots. Regeneration took place after transferring PLB to PGR-free media under 16h photoperiod. It took ≈12 and ≈24 weeks for protocorm bases and leaf-derived PLB, respectively, to regenerate plantlets. Root-derived PLB is extremely slow and no plantlets were yet observed.

The PLB-to-plantlet regeneration process was very similar to the observed for plantlets obtained from seeds, with the difference that plantlets were not individualized, but clustered, once that PLB clusters are not friable and easily separated from each other (Figure 5).

CYTOGENETIC STABILITY OF PLB-REGENERATED PLANTLETS FROM LEAF EXPLANTS

To date, six leaf-derived PLB were cytogenetically analyzed. The flow cytometry analysis of PLB-regenerated plantlets in comparison with seed-obtained control plants showed that they maintained the same ploidy levels (Figure 8).

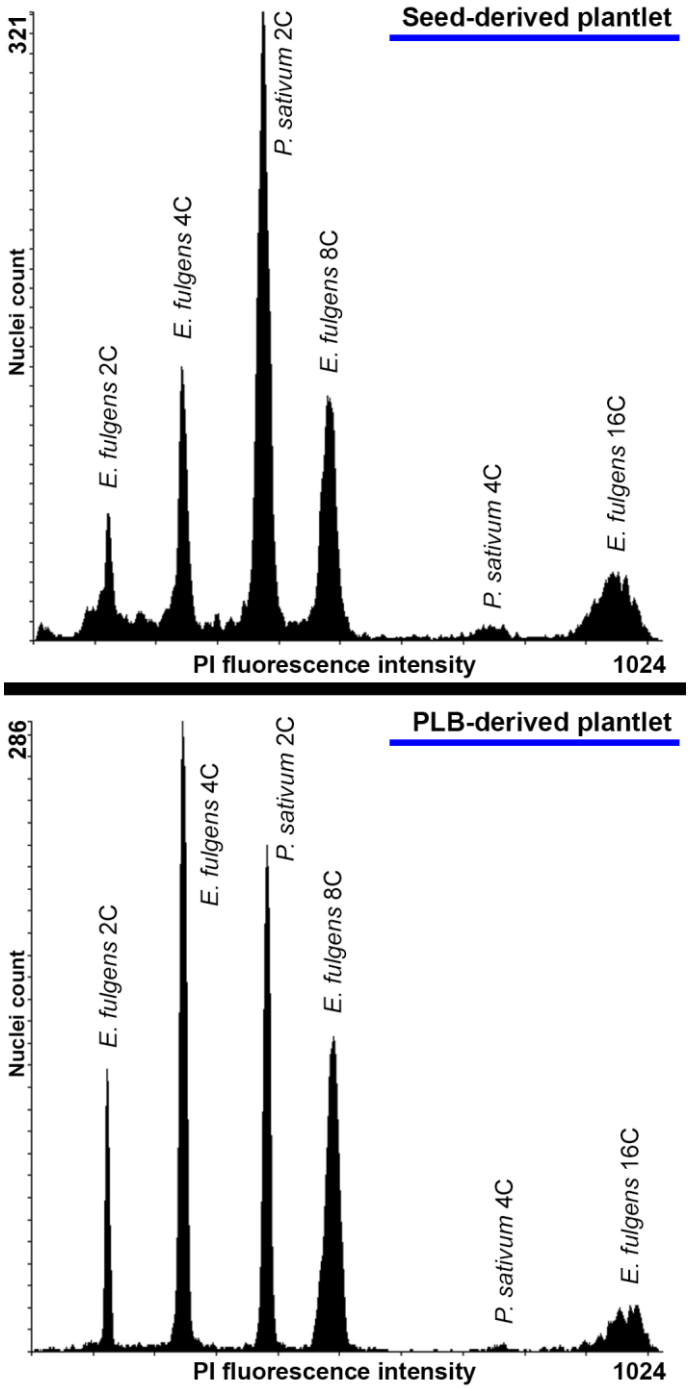

Figure 8: Cytogenetic stability of PLB-regenerated plantlets from leaf explants. Histograms of relative fluorescence intensity (linear scale) obtained after the analysis of propidium iodide-stained nuclei isolated from leaf tips of *Epidendrum fulgens in vitro* plantlets using *Pisum sativum* 'Ctirad' as internal reference standard.

DISCUSSION

ENDOPOLYPLOIDY IN *E. FULGENS* ORGANS AND TISSUES

The results of the present study showed endopolyploidy is present in all tissues from *Epidendrum fulgens*, which is a diploid orchid species (2n = 2x = 24) (ASSIS *et*

al., 2013; FELIX; GUERRA, 2010). This is the first report of endopolyploidy occurrence in its somatic tissues.

This result is not unexpected, as endopolyploidy is a common feature in plants (BAROW; MEISTER, 2003). Endopolyploidy varies according to botanical family and organ type (BAROW; JOVTCHEV, 2007). It is present even in the model plant *Arabidopsis thaliana* roots (BHOSALE *et al.*, 2018) and in common crop plants as maize (LI *et al.*, 2019) and potato (LAIMBEER *et al.*, 2017).

Different from many other studies, which are focused on specific tissues, our data provides a detailed map of the endopolyploidy in virtually all somatic tissues of *E. fulgens*. Flow cytometry analysis of pollinia resulted on histograms with 1C and 2C peaks of fluorescence intensity. The 1C peak correspond to the haploid vegetative nuclei from pollen grains, while the 2C peak is relative to generative nuclei in 2C state and 2C nuclei from surrounding somatic tissue (TRÁVNÍČEK *et al.*, 2015). The 2C nuclei peaks recorded may also represent diploid pollen nuclei, as they are quite frequent in orchids (TEOH, 1984), or by grouped 1C nuclei that were not correctly isolated during sample preparation. The last explanation is less probable, since no triads or tetrads were recorded on the FC histograms, which leads us to the conclusion that *E. fulgens* may produce a high number of diploid pollen.

The analysis of pollinia serve as an accurate start point to define the correct positions of the other cytotypes and to undoubtedly estimate the DNA C-value, as 2C peaks may be absent in many somatic tissues, leading to incorrect C-value calculations (KRON, 2015; TRÁVNÍČEK *et al.*, 2015).

Our results showed that the base of protocorms presented the highest proportion of cytotypes with greater ploidy levels. This feature was also observed in *Phalaenopsis* protocorms by flow cytometry (CHEN, W. H.; TANG; KAO, 2009) and by microscopic analysis of DAPI-stained nuclei (JEAN *et al.*, 2011).

A known effect of endopolyploidy is the increase in cell size (REJLOVÁ *et al.*, 2019). Orchid embryos have larger cells at the basal end (YEUNG, EDWARD C., 2017). During symbiotic germination, only the basal portion of protocorms are colonized by mycorrhizal fungi hyphae (CHEN, J. *et al.*, 2014). The colonization of the embryo cells leads to nuclear hypertrophy (PETERSON; UETAKE; ZELMER, 1998). The increase in cell size is perhaps one of the explanations for the higher cycle value of protocorm base, considering that such long term coevolutionary morphological

alterations are also maintained in asymbiotic germination (YEUNG, E. C.; LI; LEE, 2019).

Root tips were the second most endopolyploid tissue (Figure 2, Table 1). Curiously, root tips were used for the definition of the diploid status of *E. fulgens* (ASSIS *et al.*, 2013; FELIX; GUERRA, 2010). As chromosomes counts are performed with metaphase cells found in meristems, possibly, the endocycles occur in more specialized root tissues, as it was meticulously detailed in the roots of *Arabidopsis* by Bhosale *et al.* (2018).

We detected differences in the cycle value between leaf tips and leaf bases. Surprisingly, leaf tips, which contain more aged and differentiated cells in comparison with leaf bases, contained a lower proportion of 16C than leaf bases (Figure 2). The same endopolyploid pattern was already reported in leaves of *Spathoglottis plicata* (YANG; LOH, 2004), though.

Petals and labella have different patterns of endopolyploidy. That is remarkable, as the labellum is a modified petal or tepal (MONDRAGÓN-PALOMINO; THEISSEN, 2009). Labella are much more complex (morphologically) than regular petals, because they are involved in pollinator's attraction. In *Ophrys* orchids, endoreduplication and partial endoreduplication detected in the labella were correlated with cell size and complexity (BATEMAN *et al.*, 2018).

GENOME SIZE ESTIMATION

The small variation of the genome size estimation of *E. fulgens* using the 2C peaks from different explants is an indication of accuracy. Furthermore, the fluorescence relations between the peaks were always ≈2.0, which means the species has a conventional type of endoreplication.

The C-value of 1.49 calculated for *E. fulgens* is within the range for the family, which is quite large, varying from 0.38 to 44.84 pg (LEITCH *et al.,* 2019). There are only two *Epidendrum* spp. with known C-values: *E. steinbachii*, with 1.50 pg (JONES; KUEHNLE; ARUMUGANATHAN, 1998) and *E. rigidum,* with 1.21 pg (TRÁVNÍČEK *et al.*, 2015).

PLB INDUCTION AND PLANTLET REGENERATION

As far as we are aware, there is only one study reporting the successful micropropagation of orchids using flower parts as explants. According to Santana and Chaparro (1999), it was possible to obtain PLB from *Oncidium* flower buds. Teixeira da Silva and Giang (2014) tried to induce PLB from different *Phalaenopsis* flower parts. The authors were able to obtain some explant swelling and even callus, but no organogenesis or PLB induction.

One of the reasons for using flower parts as explants in the present study, besides the difference in endopolyploidy, is the high availability of explants and the lower contamination rates. For the pollinia, the main reason was to obtain haploid cultures for subsequent homozygote development, which could be useful for genetic improvement. There are no studies exploring this biotechnology for orchids, although it is commonly used for many other plants.

The regeneration of orchids from leaf explants is relatively common. The leaf basal region showed the highest morphogenetic response rates in *Phalaenopsis* (GOW; CHEN; CHANG, 2009) and bromeliads (ALVES; DAL VESCO; GUERRA, 2006; DAL VESCO *et al.*, 2011; SCHERER *et al.*, 2013). However, for *Oncidium* orchids, PLB were more frequently induced at the leaf tip (CHEN, J-T; CHANG, 2001; CHUNG, H.-H.; CHEN; CHANG, 2005). Apparently, the morphogenetic response is species specific.

Our results show that 10 µM TDZ is the most efficient concentration for PLB induction in *E. fulgens* leaf explants. Many other studies concluded that TDZ is an efficient PGR for PLB induction in many different orchid species and hybrids (CHEN, J-T; CHANG, 2004, 2001; CHUNG, H-H.; CHEN; CHANG, 2005; PARK *et al.*, 2002; PARK; MURTHY; PAEK, 2003), including in *Epidendrum radicans* (CHEN, L-R.; CHEN; CHANG, 2002), which is closely related with *E. fulgens.* The preeminence of TDZ and other cytokinins over other PGR for PLB induction in orchids was recently reviewed in detail by Cardoso *et al.* (2020). TDZ is chemically different from auxin and cytokinin, although it displays response-like activities from both (GUO *et al.*, 2011). Recent studies suggest that its mechanism of action is far more complicated than that (DINANI *et al.*, 2018).

CYTOGENETIC STABILITY OF PLB-REGENERATED PLANTLETS

According to the results from the present study, plantlets regenerated from leaf explants had the same ploidy level of control plantlets obtained from seeds. These results do not fully reject the hypothesis that polyploid plants can be obtained from polysomatic explants. The sample size is important to give robust support to the evidences of this study.

The result obtained can be seen either positively, as the protocol can be used for stable cloning of genetic superior plants for micropropagation purposes, or negatively, as polyploid plant obtainment will perhaps need a more complex protocol, possibly with the use of chemicals, as colchicine. One factor that must be considered is that the use of PGR can also lead to ploidy alterations, as it is known for 2,4-D (BAIRU; AREMU; VAN STADEN, 2011), for example. However, although many somaclonal variations are reported in micropropagated plants using TDZ, ploidy alterations are not among them (DEWIR *et al.*, 2018).

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Capítulo 3 - Molecular identification of fungi isolated from roots and symbiotic protocorms of *Epidendrum fulgens* Brongn. (Orchidaceae) and their potential for symbiotic seed germination

ABSTRACT

Epidendrum fulgens is a terrestrial orchid native from the Atlantic rainforest of Brazil. The genetic structure, ecological distribution and phylogeography of natural populations of *E. fulgens* were studied, but nothing is known about its relations with microorganisms. In the present study, we isolated and identified mycorrhizal and endophytic fungi associated with roots and symbiotic protocorms of *E. fulgens.* The ITS region of the nuclear rDNA was used for the molecular identification of the isolates using phylogenetic analysis and probabilistic taxonomic placement. A total of 14 isolates were obtained, corresponding to 7 different molecular operational taxonomic units (MOTUs). The ITS sequences from the isolates were highly different from known sequences deposited in the GenBank database. Only one isolate was identified at the species level, showing that *E. fulgens* associates with many undescribed fungal taxa. Three MOTUs were assigned to known mycorrhizal fungi from the Cantharellales and Sebacinales within Basidiomycota. The other four MOTUs were assigned to fungi from the Xylariales, Chaetothyriales, and Pleosporales within Ascomycota. The results demonstrate that *E. fulgens* is a generalist in relation to its association with fungi. The symbiotic plasticity of *E. fulgens* can in part explain the still high occurrence of this plant in many different habitats and its capacity to colonize different substrates. Three different mycorrhizal fungal species are shown to be associated with *E. fulgens* in different stages of its life cycle, which has implications for conservational purposes, as the plant might need different fungal species for seed germination and for further development. The isolates obtained in this study may be used to produce mycorrhized plants for environmental reintroduction and to produce symbiotic seedlings for horticultural purposes. Furthermore, the results of this study are valuable to better understand the synecology of *E. fulgens* and to give support to future studies focusing on its conservation.

Key-words: Dark septate endophytes; mycorrhiza; peloton; Cantharellales; Sebacinales.

INTRODUCTION

One of the most striking features of the orchid family is the production of fruits containing many tiny seeds without endosperm (ARDITTI; GHANI, 2000), which leads to an obligatory mycoheterotrophic stage for the seed germination and protocorm development (RASMUSSEN; RASMUSSEN, 2014). Since orchids are dependent on the mycorrhizal association for germination and plant establishment, it is expected that differences in fungi community can mediate the spatial distribution and coexistence of orchid populations (JACQUEMYN *et al.*, 2014). Therefore, it is important to know the relation between the occurrence of orchids in landscape and the presence and abundance of fungal species (MCCORMICK; JACQUEMYN, 2014).

Most of the fungi associated with orchids are not obligatory symbionts, but rather free living saprophytes (SMITH; READ, 2008). Unlike arbuscular mycorrhizal fungi, the orchid mycorrhiza comprise a polyphyletic group of species from the Ascomycota and Basidiomycota phyla (DICKIE; JOHN, 2017). Initially classified as belonging to the former *Rhizoctonia* group, due to their micromorphological characteristics, the mycorrhiza fungi from orchids are now known to comprise more than 23 distinct fungi genera, including saprotrophs, ectomycorrhizae and also parasites and plant pathogens (DEARNALEY; MARTOS; SELOSSE, 2012). Molecular phylogenetic studies reveal that the number of fungi species forming orchid mycorrhiza can be as higher as the number of orchid species (HEIJDEN *et al.*, 2015).

The specificity of mycorrhizal interactions has been a frequent issue due to its importance for the development of conservation programs (DEARNALEY; MARTOS; SELOSSE, 2012). Some orchid species can develop better when symbiotically cultivated *in vitro* with their specific mycorrhizal fungi (PEREIRA *et al.*, 2011), while others can be grown with fungi isolated from orchids of a different genus (HOSSAIN *et al.*, 2013). Some orchids have extremely limited spatial distribution, while their mycorrhizal fungi is continentally dispersed (DAVIS *et al.*, 2015).

Epidendrum fulgens is an abundant and widespread terrestrial orchid that occurs in shrubby sand dunes and rock outcrops (SUJII; COZZOLINO; PINHEIRO, 2019). This vegetation type represents a harsh environment formed by marine deposits between the sea and the Atlantic rainforest mountain chains in Brazil, where plants are subjected to constant winds, floods, drought, high salinity and low nutrient levels

(SCARANO, 2002). Populations of *E. fulgens* were extensively studied in relation to their ecological distribution (SOUZA ROCHA; LUIZ WAECHTER, 2010), phylogeography (PINHEIRO *et al.*, 2011) and genetic structure (SUJII; COZZOLINO; PINHEIRO, 2019), however, nothing is known about their symbiotic relations with microorganisms.

The objective of this work was to isolate and identify mycorrhizal and endophytic fungi associated with roots and symbiotic protocorms of *E. fulgens.* Specifically, we used nuclear rDNA sequences obtained from the fungi isolated from *E. fulgens* to address the following questions: (1) What is the most suitable explant source for the isolation of fungi? (2) are the fungi associated with roots the same as for symbiotic protocorms? (3) are these fungi suitable for symbiotic seed germination? (4) what can the molecular data tell us about the synecology of *E. fulgens*? Considering the highly variable habitats of natural occurrence of this orchid species, we also expect to obtain a high molecular diversity of fungi species associated with it. We hypothesize that the fungi can be more easily isolated and are more likely to be mycorrhizal when obtained from symbiotic protocorms than from adult plant roots. We discuss the obtained data in the light of its biodiversity significance and ecological implications and we approach their importance for conservational and horticultural purposes.

MATERIAL AND METHODS

SAMPLING MATERIAL

Three natural populations of *Epidendrum fulgens* Brongn (Orchidaceae) were sampled in the Restinga vegetation of the Atlantic rainforest in Florianópolis, southern Brazil. Twelve vegetative off-shoots, produced in the floral stalks, were carefully removed from healthy plants of each population and transplanted to 2 L pots containing a mix of autoclaved sand and commercial substrate (Tropstrato HT®) in a 1:1 ratio. These materials were used to form the germplasm collection (GC) of the Laboratory of Plant Physiology and Genetics (LFDGV) from the Federal University of Santa Catarina (UFSC), in Florianópolis, Brazil. The potted off-shoots were kept in a greenhouse with 50% shade and were watered daily. After the observation of serendipitous seed germination occurring in the greenhouse, seeds were sown at the base of potted plants in order to obtain a high number of symbiotic protocorms for analyses. Seeds were

harvested from mature pods obtained by hand-pollinated flowers. Fruits were surface sterilized with 70%ethanol for 5 min and 1% NaClO for 10 min. After five months, protocorms at initial stage of development, with first emerging leaf and root, were collected and used for microscopic annotations and fungi isolation, as described below.

OBSERVATION OF FUNGAL STRUCTURES INSIDE PROTOCORMS AND ROOTS

Symbiotic protocorms and roots were collected from the GC and analyzed for the presence of mycorrhizal fungi using scanning electron microscopy. They were fixed in glutaraldehyde (2.5%) with a sodium phosphate buffer (0.1 M, pH 7.2) for 24 h and dehydrated in ethanol series (20%, 40%, 60%, 70%, 80%, 90% and 100%) for 30 min each, followed by critical point method drying in liquid carbon dioxide (EM CPD 030/Leica, Germany). Roots from protocorms were cross sectioned in 0.5 mm layers, while protocorms were longitudinally cut in half or kept intact. Samples were then fixed on aluminum stubs with double-sided sticky carbon tape, coated with gold (EM SCD 500/Leica, Germany) and examined in a scanning electron microscope (JEOL, JSM-6390LV) at different resolutions and magnifications. After confirmation of the presence of mycorrhizal pelotons in the explants, the fungi isolation was performed.

ISOLATION AND CULTIVATION OF THE FUNGI

The fungi were isolated from roots of adult plants, from symbiotic protocorms and from symbiotic protocorm roots, obtained as previously described. Root segments from adult plants (≈5 cm) and symbiotic protocorms with emerging first leaf and root were surface sterilized with 70% ethanol for 1 min and with 0.5% sodium hypochlorite for 5 min, then washed three times with sterile distilled water. The roots were cut in 2 cm long segments and the velamen was removed for exposing the root cortex and to allow the observation of fungi pelotons under a stereomicroscope. Single pelotons or portions of the root cortex containing pelotons were dissected with a sterile needle and inoculated onto PDA medium supplemented with 100 mg L^{-1} streptomycin. The surface sterilized symbiotic protocorms were longitudinally cut and inoculated with the internal tissues in contact with the culture medium. The inoculated material was maintained in the dark at 25°C.

Petri dishes were daily checked under an inverted microscope for the presence of fungi hyphae emerging from the inoculated material. The fungi hyphae with

rhizoctonia-like characteristics, i.e., slow growth rate, presence of septate hyphae with 90° angle branches, and production of sclerotia and monilioid cells (CURRAH and ZELMER, 1992) were selected and transferred to new Petri dishes with fresh PDA medium. Colonies were subcultivated every four weeks in fresh PDA medium.

DNA EXTRACTION, PCR CLONING, SEQUENCING AND PHYLOGENETIC ANALYSIS

About 50 mg of fresh mycelia from each fungi isolate were scratched from the surface of the colonies and used for DNA extraction with the CTAB protocol (DOYLE and DOYLE, 1990). The DNA was quantified in a NanoDrop® spectrophotometer (Thermo Fisher Scientific) and the quality was evaluated under UV light in 0.8% agarose gel stained with GelRed™ (Biotium).

The DNA was appropriately diluted and the ITS1+5.8S+ITS2 was amplified using the primers ITS1F (GARDES; BRUNS, 1993) and ITS4 (WHITE *et al.*, 1990). The PCR reactions were performed according to Oliveira *et al.* (2014). The resulting products were purified by precipitation in 20% polyethylene glycol 8000 and 2.5 M NaCl solution and washed in 80% ethanol. The amplification was checked with in 1.5% agarose gel stained with GelRed™ (Biotium) under UV light. The fragments were sequenced in both directions using the BigDye terminator kit (Applied Biosystems, Foster City, CA, USA) following the manufacturer's instructions. Products from the sequencing reaction were purified with 2 µL of EDTA (125 mM, pH 8), 2 µL of sodium acetate (3 M) and 50 µL of ethanol 100% for 15 min. Sequencing of purified fragments were done by the Sanger method (SANGER; NICKLEN; COULSON, 1977) using an ABI3500XL Genetic Analyzer (Applied Biosystems), following the manufacturer's instructions.

Electropherograms of forward and reverse sequences were manually edited with CLC Workbench software. Consensus sequences of ITS1+5.8S+ITS2 were generated and deposited on GenBank database under the accession numbers MT192312-MT192322.

A BLASTn search against the NCBI database (http://www.ncbi.nlm.nih.gov) was used to check the sequences identity. Only curated sequences obtained from type materials were considered, and the algorithm word size was reduced to 7. Sequences were aligned with MUSCLE (EDGAR, 2004) and adjustments were performed using

MEGA7 v7.0.9 software. A matrix of pairwise distance among sequences was computed in the R environment (R core team, 2019), using functions from the “APE” package v 5.3 (PARADIS; SCHLIEP, 2019), with the TN93 model of DNA evolution (TAMURA; NEI, 1993). In order to define molecular operational taxonomic units (MOTUs), the sequences from the distance matrix were clustered with the “spider” package (BROWN *et al.*, 2012), using similarity threshold values of 99.6% (VU *et al.*, 2019) and 97% (NILSSON *et. al,* 2008) for species delimitation.

A phylogenetic tree was constructed using a maximum likelihood (ML) approach in RAxML-HPC2 v8.2.12 (STAMATAKIS, 2014) with combined rapid bootstrapping, using the GTR+GAMMA model of nucleotide substitution, as implemented on the CIPRES Science Gateway v.3.3 (http://www.phylo.org/sub_sections/portal/). The best-fit model for nucleotide substitution was previously defined with jModeltest software (DARRIBA *et al.*, 2012). Based on the probabilistic taxonomic placement the isolates at the phylum level, we selected an outgroup (*Glomus proliferum* – Accession no. FM992401), because it is within the phylogenetically closest phylum, the Mucoromycota. The phylogenetic tree with the best score was illustrated using FigTree v1.4.4.

A probabilistic taxonomic placement was also used in order to identify the isolates. For that, we used PROTAX software (ABARENKOV *et al.*, 2018), as implemented in the PlutoF web workbench (ABARENKOV *et al.*, 2010), on the UNITE database (NILSSON *et al.*, 2019). PROTAX is based on a Bayesian multinomial regression model and uses a reference sequence database and a taxonomic tree structure to estimate the probability with which a sample sequence can be placed in a given taxon, considering also species for which no reference sequences are available (SOMERVUO *et al.*, 2016). Identification was considered as “plausible” when the probability of taxonomic placement was >50% and “reliable” when >90%, as in Somervuo *et al.* (2017).

SYMBIOTIC SEED GERMINATION

In order to test the capacity of the fungal isolates to promote orchid seed germination, *in vitro* seed germination experiments were performed using representative isolates of the MOTUs defined by the clustering analysis.

Seeds were harvested from fruits of *E. fulgens* obtained from hand-pollination, disinfested with 0.5% NaClO for 10 min and washed three times in sterile distilled water. Thereafter, they were inoculated in 90 mm diameter Petri dishes containing agar/oat medium (4 g L^{-1} oatmeal flour; 7 g L^{-1} agar), either with or without a 1 cm^2 fungus inoculum plug at the center of the plate. The fungi used were previously cultivated for 4 weeks in fresh PDA medium and the inoculum plugs were obtained from the active growing hyphae from the colony margins using a cork borer. For comparison with asymbiotic germination, Petri dishes containing MS media (MURASHIGE; SKOOG, 1962) without fungus inoculum were used. Dishes were kept at 25°C, and 16h photoperiod. Each Petri dish was considered as a repetition, with a mean of 217 ± 39 seeds per plate. A minimum of three repetitions per treatment were performed.

Data were collected 12 weeks after sowing by inspecting each plate under a stereomicroscope. All seeds from each plate were accessed and a score from N0 (no germinated) to N5 (development of first leaf and root) was attributed according to protocorms developmental stage, and a growth index (GI) was calculated from the developmental scores as described by (OTERO; ACKERMAN; BAYMAN, 2004). Percentage and categorical data were compared with confidence intervals and GI means were compared with ANOVA and Tukey post-hoc test (p=0.05).

RESULTS

SCANNING ELECTRON MICROSCOPY

The presence of fungi with *rhizoctonia-like* characteristics was observed both on the outer surface of symbiotic protocorms and inside the cells of protocorms and protocorm roots. Fungi mycelia were abundantly observed on the basal region of protocorms, while close to the apical meristem they were inconspicuous (Fig 1a).

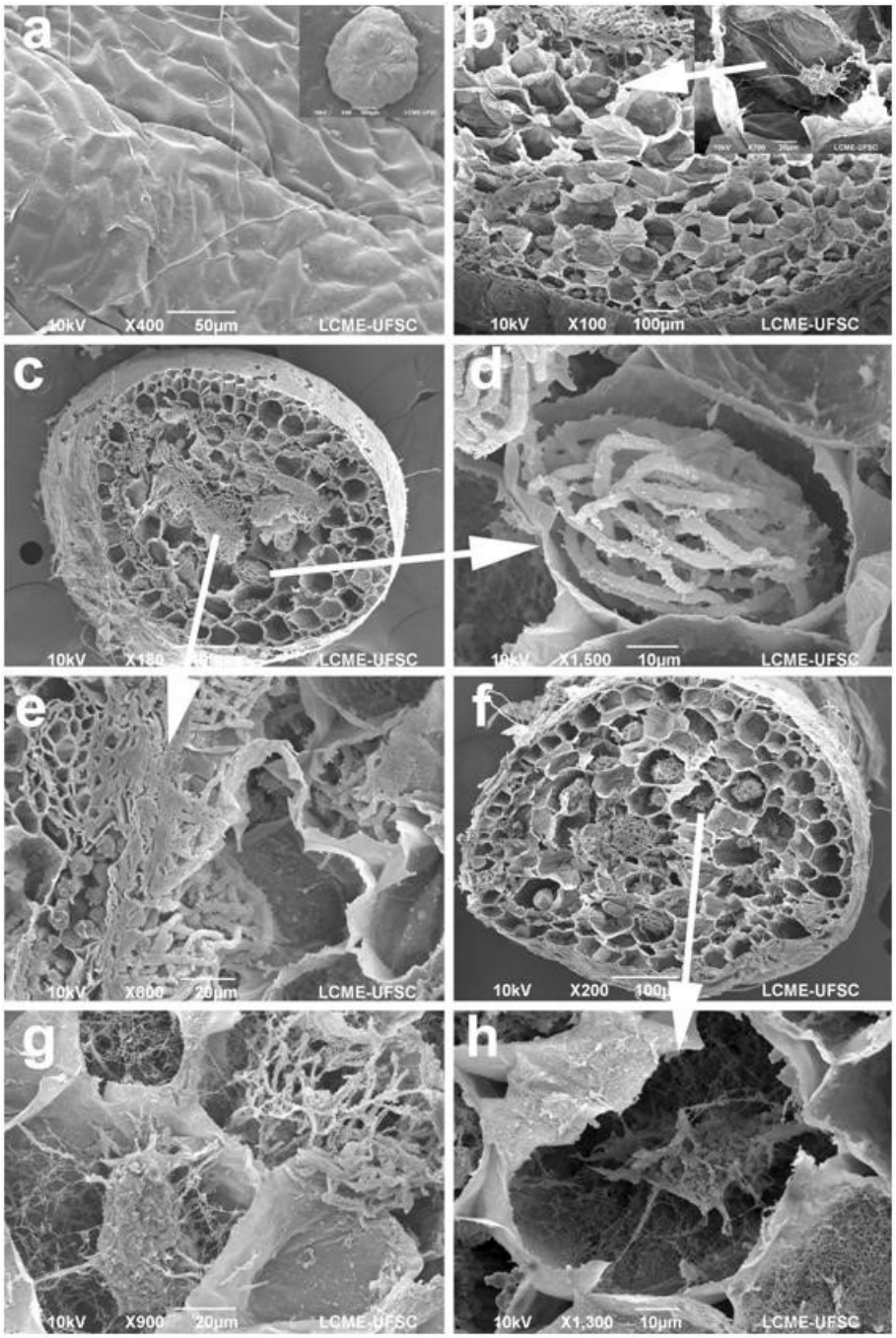

Figure 1: Scanning electron micrographs of the formation of orchid mycorrhizal structures in *Epidendrum fulgens*. a) A view from the top surface of symbiotic protocorms showing running hyphae with rhizoctonia-like characteristics; b) Hyphal coils (pelotons) in cortical cell of symbiotic protocorm; c-e) Transverse section of symbiotic protocorm root showing pelotons in different developmental stages. Note that compact pelotons are very close to the central cylinder; d) Pelotons with active hyphae colonizing entire cell; e) Compact and degraded pelotons surrounded by callose; f-h) Details of aged pelotons in the root of symbiotic protocorm.

The hyphae were eventually observed penetrating the epidermal cells of protocorms. In the cortex cells of protocorms and protocorm roots, active and degraded pelotons were observed (Fig 1c-e). Structures, apparently from fungal origin, but that do not resemble pelotons, were also observed (Fig 1f-h).

ISOLATION AND CULTIVATION OF THE FUNGI

The daily observation of explants with an inverted microscope was essential for accurately select the mycelia with *rhizoctonia*-like characteristics. The first emerging hyphae were observed 24-48h after explant inoculation. A total of 14 isolates were obtained: 4 of them isolated from adult plant roots, 2 from protocorm roots, and 8 from the cortex of protocorms (Table 1).

PHYLOGENETIC ANALYSIS

We generated 14 new sequences from the isolates. Clustering analysis of the distance matrix depicted seven different MOTUs out of the 14 isolates (Table 1). Isolates P11, P13, P14 and P16, obtained from protocorms, were shown to be identical to isolates P4R and S2, obtained from protocorm roots and adult plant roots, respectively. Isolates P8, P12 and P15, obtained from protocorms, were also 100% identical to each other. All the other isolates corresponded individually to different MOTUs.

Table 1: MOTU´s definition and details of fungal isolates obtained in the present study with respective GenBank accession numbers, sequence length, name of strain and isolation source.

Isolation source	Strain	Accession number	Sequence length	MOTU definition (99.6% cut value)	MOTU definition (97% cut value)
Protocorm	P2	MT192320	594 bp	1	1
	P8	MT649482	916 bp	2	2
	P12	MT649481	924 bp		
	P15	MT649480	880 bp		
	P11	MT192317	535 bp	3	3
	P13	MT192316	575 bp		
	P14	MT192315	453 bp		
	P16	MT192314	555 bp		
Root (adult plants)	S2	MT192312	491 bp		
	R1	MT192313	448 bp	4	4
	CP	MT192322	557 bp	5	5
	DMGRG	MT192321	507 bp	6	6
Root (protocorms)	P4R	MT192319	606 bp	3	3
	P9R	MT192318	529 bp	7	7

The percentage of sequence identity varied substantially (85.63–99.63%) among the different isolates, and the most similar sequences from type species in GenBank, according to the BLASTn results (Table 2).

Table 2: Results from BLASTn of ITS1+5.8S+ITS2 representative sequences from the MOTUs defined by the clustering analysis of sequences from isolates obtained from fungi isolated from roots and cortex of protocorms and from roots of adult plants of *Epidendrum fulgens* (see table 1 for MOTUs definition). The most similar sequences in GenBank are shown for each MOTU followed by their accession numbers, BLASTn statistics and respective references. Only sequences from type materials were considered.

MOTU	Closest match in Genbank (Accession number)	Identity	Query Cover	E value	Score	Reference
1	*Curvularia affinis* (HG778981)	97.75%	96%	0.0	976	Madrid *et al.* (2014)
2	*Serendipita herbamans* (NR_144842)	85.63%	72%	9^{-102}	425	Riess *et al.* (2014)
3	*Tulasnella cumulopuntioides* (NR_160570)	94.77%	100%	0.0	833	Fujimori *et al.* (2019)
4	*Tulasnella ellipsoidea* (LC175314)	86.05%	95%	3^{-139}	503	Fujimori *et al.* (2019)
5	*Exophiala oligosperma* (KF928422)	99.63%	97%	0.0	969	Attili-Angelis *et al.* (2013)
6	*Xylaria karyophthora* (KY564220)	95.75%	97%	0.0	795	Husbands *et al.* (2018)
7	*Cryptodiscus incolor* (NR_121357)	83.93%	98%	1^{-151}	544	Schoch *et al.* (2014)

Table 3: Probabilistic taxonomic placement of MOTUs defined by the clustering analysis. The probabilities are given for each taxonomic level.

MOTU	PROTAX taxonomic probabilities						
	Phylum	Class	Order	Family	Genus	Species	ID
1	Ascomycota 100%	Dothideomycetes 99.9%	Pleosporales 99.9%	Pleosporaceae 99.6%	*Curvularia* 94.3%	*affinis* 24.7%	*Curvularia* sp.
2	Basidiomycota 99.3%	Agaricomycetes 98.8%	Sebacinales 91.3%	Sebacinaceae 79.8%	unknown 67.6%	unknown 8.35%	*Sebacinales* sp.
3	Ascomycota 100%	Eurotiomycetes 99.8%	Chaetothyriales 99.8%	Herpotrichiellaceae 99.7%	*Exophiala* *99.0%*	*oligosperma* *80.8%*	*Exophiala oligosperma*
4	Ascomycota 100%	Sordariomycetes 99.8%	Xylariales 99.8%	Xilariaceae 99.7%	*Xylaria* 88.4%	unknown 88.4%	*Xylaria* sp.
5	Basidiomycota 100%	Agaricomycetes 99.9%	Cantharellales 99.7%	Tulasnellaceae 99.4%	*Tulasnella* 88.1%	unknown 88.1%	*Tulasnella* sp.1
6	Basidiomycota 99.9%	Agaricomycetes 99.8%	Cantharellales 99.5%	Tulasnellaceae 99.0%	*Tulasnella* 68.2%	unknown 68.2%	*Tulasnella* sp.2
7	Ascomycota 80.3%	Lecanoromycetes 37.9%	Ostropales 37.2%	Stictidaceae 34.1%	Cryptodiscus 23.1%	unknown 23.1%	Ascomycota sp.

Note: Data obtained with PROTAX software (ABARENKOV et al., 2018). ID column is the final identification considering only reliably placed taxonomic levels (≥ 90%).

The ML phylogenetic tree presented branches with medium to high support values (Fig 2). The probabilistic taxonomic placement was in accordance with the topology and support values of the phylogenetic tree (Table 2). From the seven MOTUs defined in the clustering analysis, only *Exophiala oligosperma* could be identified at the species level using the probabilistic taxonomic placement. *Curvularia* sp. was identified at the genus level. Other three MOTU´s were identified plausibly at the genus level, namely *Tulasnella sp*.1, *Tulasnella sp*.2 and *Xylaria* sp. One MOTU was reliably attributed to the Sebacinales and another could only be identified as a plausible Ascomycota.

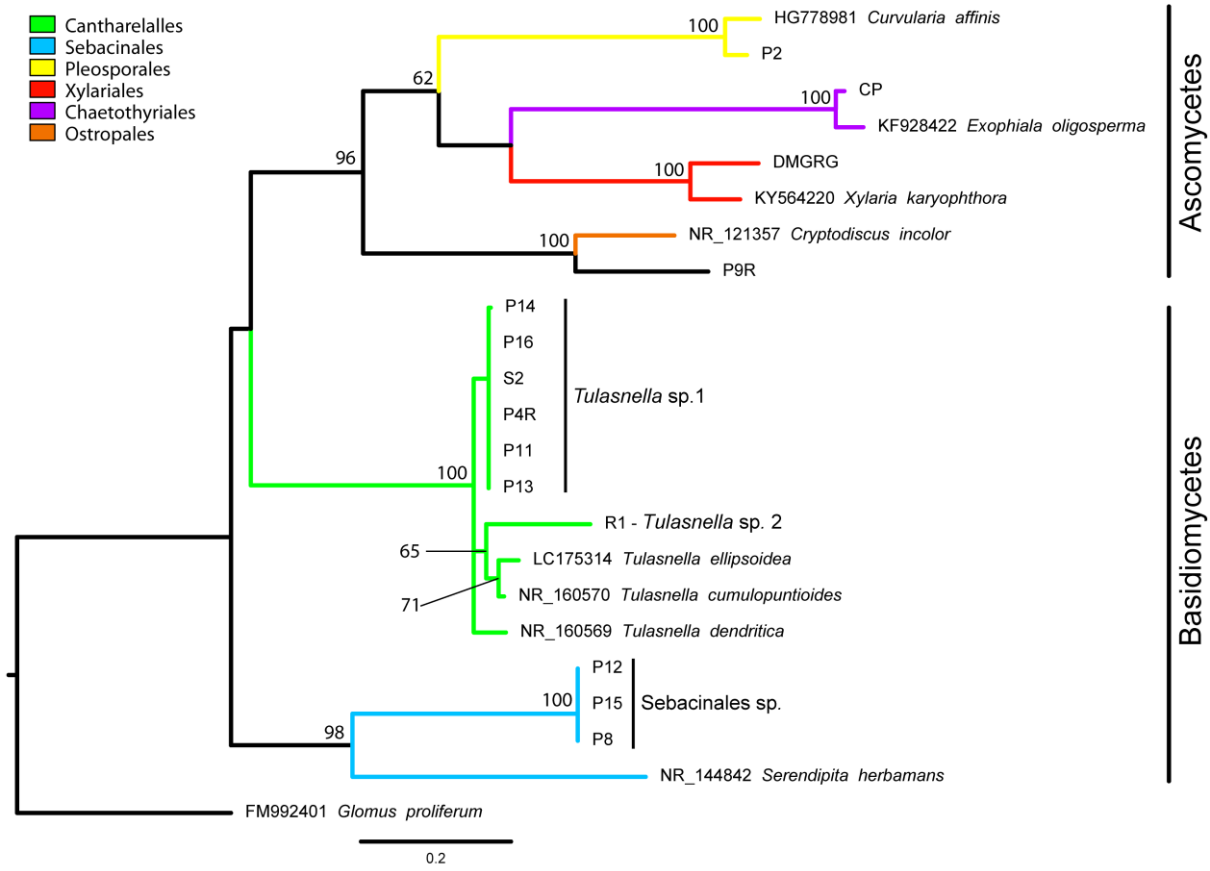

Figure 2: Maximum likelihood phylogenetic tree of mycorrhizal and endophytic fungi isolated from *Epidendrum fulgens* and their closest BLASTn matches from type sequences in GenBank. Numbers next to branch nodes indicate bootstrap support values (only scores above 60 are shown). Ascomycetes and Basidiomycetes specific branches are indicated. Fungal orders identified by probabilistic taxonomic placement (>90%) are indicated by branch colors. *Glomus proliferum* was used as an outgroup.

Although three out of four ascomycetes from this study were obtained from roots of adult plants, one of them was obtained from symbiotic protocorms, which increases the probability of its endophytic status. We also performed a meticulous sterilization of the explants and removed the velamen, decreasing the possibility of obtaining fungi from non-living tissues. From the 30 root explants from adult plants inoculated *in vitro*, four resulted in the successful isolation of fungi, while the others remained aseptic. Therefore, symbiotic protocorms were more suitable than roots of adult plants for the isolation of mycorrhizal fungi. From the 25 total protocorms used for isolation, eight isolates were obtained and only one of them was not mycorrhizal. On the other hand, only two mycorrhizal isolates were obtained from roots of adult plants.

SYMBIOTIC SEED GERMINATION

From the four ascomycetes isolates obtained in this study, only the *Curvularia* sp. was used for the symbiotic seed germination experiments. However, *Curvularia* sp.

overgrew the seeds and did not promoted germination. Therefore, we present only data of symbiotic seed germination using the three basidiomycetes isolates, namely *Tulasnella* sp.1, *Tulasnella* sp.2 and Sebacinales sp., as well as from the asymbiotic treatment with MS medium.

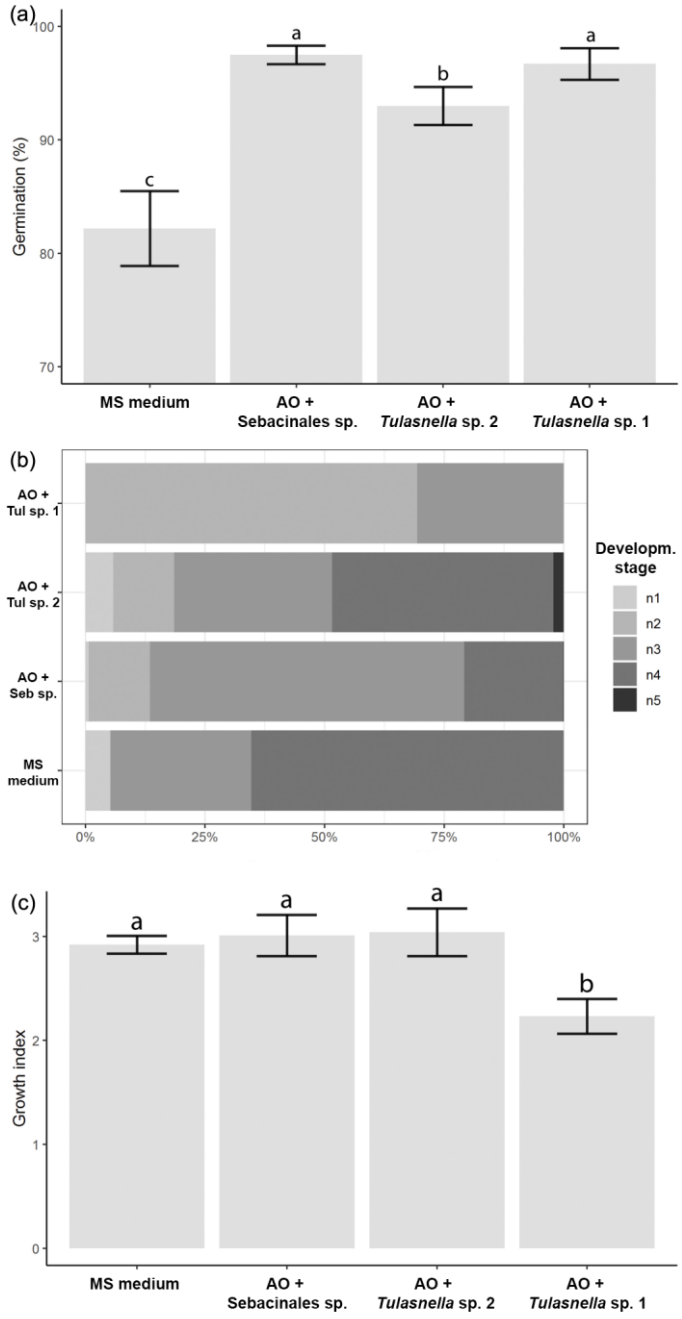

Figure 3: Seed germination and development of protocorms of *Epidendrum fulgens* in symbiotic association with different fungi isolates, 12 weeks after sowing. a) Percentage of germination; b) Percentage of protocorms in each stage of development; c) Growth index (GI) of protocorms. Error bars are confidence intervals (p=0.05). Different letters above columns represent significant differences according to Tukey test (p≤0.05).

The three isolates promoted seed germination and protocorm development. Seeds did not germinate in AO medium without fungus inoculum. Germination rate was significantly higher ($p \geq 0.05$) for the three isolates in comparison with MS medium (Fig 3a). The Sebacinales sp. isolate promoted the highest germination rate (97.5%), while 93% of the seeds germinated with the *Tulasnella* sp.2, and only 82.2% in MS medium. Only protocorms in contact with the *Tulasnella* sp.2 reached developmental stage 5, when there is the first adventitious root emission (Fig 3b). The GI, on the other hand, was significantly lower for the *Tulasnella* sp.1 isolate and did not vary significantly between the other treatments (Fig 3c). Symbiotic protocorms developed numerous rhizoids just after emerging from seed coat. They were mainly concentrated at the protocorm base. Such structures were not observed on asymbiotic protocorms.

DISCUSSION

The observation of intact and collapsed pelotons in protocorms and protocorm roots of *E. fulgens* suggests there was an active symbiotic relation between partners , as peloton formation is an important criterion for orchid mycorrhiza definition (RASMUSSEN, 2002). They are formed by hyphal coils that will further collapse due to plant digestion (DEARNALEY; MARTOS; SELOSSE, 2012), although both intact and collapsed protocorms provide nutrients for the host (KUGA; SAKAMOTO; YURIMOTO, 2014). After the peloton is degraded by the protocorm cells, collapsed hyphae form a central clamp that is surrounded by callose (RICHARDSON; PETERSON; CURRAH, 1992), which explains the dense hyphal coils observed (Fig. 1e).

It is also possible that some intracellular structures observed may have been formed by non-mycorrhizal fungi. The intracellular colonization of some dark septate endophytes (DSE), for example, can lead to the formation of clusters of inflated thick-walled cells, sclerotial bodies and, occasionally, even structures that resemble ectomycorrhizae or pelotons from orchid mycorrhizae (JUMPPONEN, ARI; TRAPPE, 1998). As single pelotons can be formed by more than one fungal species (KRISTIANSEN *et al.*, 2001; ZHU *et al.*, 2008), it is doubtful to attribute such structures to only one species based only on microscopic observations. Co-cultivation

experiments, with single fungal isolates, are probably the best way for answering this question.

Macromorphological characteristics of colonies are poor markers for taxonomic placement, as the same fungi species can sometimes present different macromorphological features according to differences in incubation temperatures and pH of the culture media (RAY; CRAVEN, 2016). The comparison of fungi DNA sequences allows the discovery of many cryptic species, and reveal that even traditionally used phenotypic characters are nowadays recognized as rather the result of convergent evolutionary process that do not automatically forecast relatedness (SCHOCH *et al.*, 2014).

In the present study we revealed a diversity of endophytic and mycorrhizal fungi associated with *E. fulgens* protocorms and adult plants based on molecular data. Sequences from 10 isolates can be assigned to already known orchid mycorrhizal fungi genera from the Basidiomycota phylum. Surprisingly, the fungi isolates identified as mycorrhizal in the present study belong to different fungal orders according to the host tissue of origin. While isolates obtained from roots, from both adult plants and protocorms, belonged to the *Tulasnella* genus (Cantharellales, Agaricomycotina) the isolates obtained from protocorm cortex were also from the order Sebacinales. This evidence indicates that protocorms and adult plants of *E. fulgens* associate with different mycorrhizal fungi. Orchids may switch their mycorrhizal fungal partners during their life cycle and some seeds fail to germinate with fungi isolated from adult plants (DEARNALEY, 2007; RASMUSSEN *et al.*, 2015), as in *Platanthera praeclara* (SHARMA *et al.*, 2003). In other cases, as in *Arundina graminifolia,* fungi isolated from protocorms are more efficient to promote seed germination than those obtained from adult plants (MENG *et al.*, 2019).

'Epulorhiza' is one of the most common genera known to form mycorrhiza with terrestrial orchid species (CURRAH; ZETTLER; MCINNIS, 1997). The use of the name '*Epulorhiza'* is no longer recommended (DEARNALEY; MARTOS; SELOSSE, 2012), and after the concept "one fungus = one name", it has become a synonym of *Tulasnella* (OBERWINKLER; CRUZ; SUÁREZ, 2017). *Tulasnella* spp. were already isolated from many orchid species, including *Epidendrum secundum* (PEREIRA *et al.*, 2014). For this orchid, it was demonstrated that when the orchid seeds and the isolates came from the same population, the protocorms developed faster (PEREIRA *et al.*, 2011).

In the present study we isolated *Tulasnella* sp.1 from both protocorms and adult plants (Table 2), which reveals that *E. fulgens* adult plants can still harbor the same fungus used for seed germination. On the other hand, *Tulasnella* sp.2 was only isolated from roots of adult plants, while the Sebacinales sp. was only obtained from protocorms. Nevertheless, the *Tulasnella* sp.1 and 2 and the Sebacinales sp. promoted seed germination and fast development of protocorms. These results indicate that *E. fulgens* is versatile in relation to its symbiotic relationships with fungi, using different species both for seed germination and during its adult life. We suggest that further studies should be carried out using a larger sample to better understand the extent of this symbiotic plasticity.

The P8, P12 and P15 isolates were attributed to the Sebacinaceae family according to the probabilistic taxonomic placement using the PROTAX software (p= 91.3%). However, the sequences from these isolates have the 5.8S signatures that attribute them to the Serendipitaceae family, according to Oberwinkler *et al.* (2014). We consider that this is an inaccurate result and that it was caused because PROTAX uses the classification system from the Index Fungorum, in which the Serendipitaceae family was wrongly assigned to the Auriculares order.

Until the early 2000 only taxonomists were interested in the Sebacinales order (WEISS *et al.*, 2016). This has changed drastically when two species have received a lot of attention: *Serendipita indica* (formerly *Piriformospora indica*) and *Serendipita vermifera* (BOKATI; CRAVEN, 2016). Their importance relies on the fact that they can interact with a vast number of host plants, promoting many positive effects, as increased productivity and resistance to biotic and abiotic stresses (OELMÜLLER *et al.*, 2009; FRANKEN, 2012; GILL *et al.*, 2016; RAY; CRAVEN, 2016). Although environmental DNA sequences from Serendipitaceae where already obtained from orchids in Ecuador (SUÁREZ *et al.*, 2009) and in Brazil (OLIVEIRA *et al.*, 2014), there are no species described for the American continent.

The other isolates were attributed to the Ascomycota phylum, most of them from the group of the dark DSE. DSE are conidial or sterile septate fungi, with distribution from the tropics to arctic regions, which were already reported to associate with several hundred plant taxa, including orchids (JUMPPONEN; TRAPPE, 1998). They are common root endophytes and several beneficial aspects of their interaction with host plants are known, also leading to the question of whether its interaction with plants

may also be considered as a mycorrhizal symbiosis (JUMPPONEN, 2001). In this study, DSE were isolated possibly due to the selection of hyphae with *rhizoctonia-like* characteristics, as they are not initially melanized.

A diversity of ascomycetes isolated from roots of tropical orchids in Ecuador were considered as non-endophytic because they could have originated from the velamen of roots that were only partially removed after moderate surface sterilization (HERRERA; SUÁREZ; KOTTKE, 2010). Appropriate surface sterilization of explants reduce the contamination from bacteria as well as from faster-growing ascomycetes (DEARNALEY; MARTOS; SELOSSE, 2012).

Within the ascomycetes, Chaetothyriales was the most frequently order registered from roots of 77 epiphytic orchids from the Reunion Island (MARTOS *et al.*, 2012). The genus *Exophiala* is mostly studied as an important opportunistic etiologic agent of human diseases, although it is frequently isolated from natural environments as in soil, water, air, rhizosphere and plant tissues (MACIÁ-VICENTE; GLYNOU; PIEPENBRING, 2016). Apparently, *Exophiala* spp. are frequently sampled from halophytes (KHALMURATOVA *et al.*, 2015), as is also the case of the present study. They are also reported as endophytes from mangrove species (LI *et al.*, 2016) and marine algae (FLEWELLING *et al.*, 2015). They are reported as orchid endophytes (OGURA-TSUJITA; YUKAWA, 2008; SOMMER *et al.*, 2012; TAO *et al.*, 2008), including in commercially grown *Phalaenopsis* (HUANG *et al.*, 2014).

Some *Curvularia* spp. confer positive effects to plants. *Curvularia geniculata* increased the growth of *Cajanus cajan* through phosphate solubilization (PRIYADHARSINI; MUTHUKUMAR, 2017), which was also observed from *Curvularia lunata* isolated from orchids (SAHOO, 2018). *Curvularia protuberata* isolated from roots of a grass that inhabits geothermal soils conferred heat tolerance to host plants (REDMAN *et al.*, 2002) and also to some important agricultural plants (REDMAN; RODRIGUEZ; HENSON, 2003), which was later attributed to a three-way symbiosis including a fungal virus (MÁRQUEZ *et al.*, 2007). Whether the *Curvularia* species obtained in the present study could or could not result in heat tolerance to *E. fulgens* plants or protocorms is an interesting question, especially considering the characteristics of the Restinga vegetation, where temperatures of the sand can reach 70 °C in the summer (SCARANO, 2002). However, our results demonstrated that the

Curvularia sp. isolated from symbiotic protocorms failed to promote seed germination, at least under *in vitro* conditions.

In summary, the results of the present study demonstrate that *E. fulgens* is a generalist in relation to its association with fungal symbionts, most of them undescribed. Only one isolate could be identified to the species level, which suggests a high diversity of fungi associated with *E. fulgens.* The symbiotic plasticity of *E. fulgens* can in part explain the still high occurrence of this plant in many different habitats and its capacity to colonize different substrates. Whether the occurrence of *E. fulgens* in different landscapes is restricted to the availability of fungal inoculum is yet to be evaluated. However, at least three different mycorrhizal fungal species are shown to be associated with *E. fulgens* plants in different stages of its life cycle. This fact has implications for conservational and horticultural purposes, as the plant has high ornamental value and its economical use as a pot plant was already proposed (MÜLLER, 2011). The diversity of fungi associated with *E. fulgens* must be considered for future studies dealing with its conservation, as the plant might need different fungal species for seed germination and for further development. The isolates obtained in this study may be used to produce mycorrhized plants for reintroduction in natural populations. They can also be used to produce symbiotic seedlings for the ornamental market with advantages over the asymbiotic methods, as mycorrhized orchids are more adapted to high light environments (LEE *et al.*, 2014) and present better growth rates and more resistance to bacterial soft rot disease (WU; HUANG; CHANG, 2011).

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Capítulo 4 - *Serendipita restingae* sp. nov. (Sebacinales): an orchid mycorrhizal agaricomycete with wide host range

ABSTRACT

The Serendipitaceae family was described in 2016 to accommodate the Sebacinales 'group B' clade, which contains peculiar species of cultivable endophytes involved in symbiotic associations with a wide range of plant species. Here we describe a new *Serendipita* species isolated from protocorms of the terrestrial orchid *Epidendrum fulgens* in Brazil. This species is described based on phylogenetic analysis, genome size estimation and on its microscopic and ultrastructural features in pure culture and in association with the host´s protocorms. Its capacity to promote the germination of *E. fulgens* seeds and to associate with roots of other plant species was also investigated. *Serendipita restingae* sp. nov. is closely related to the cultivated *Serendipita* sp. MAFF305841, from which it differs by 14.2% in the ITS region and by 6.5% in the LSU region. It is morphologically distinguished from other Serendipita spp. by the production in water-agar medium of microsclerotia formed of non-monilioid hyphae. Serendipita restingae promoted the germination of *E. fulgens* seeds, forming typical mycorrhizal pelotons within protocorm cells. It was also able to colonize the roots of *Arabidopsis thaliana*, maize, soybean and tomato under *in vitro* conditions. *Arabidopsis* plants grown in association with *S. restingae* increased their biomass more than 4-fold. Serendipita restingae is the first Serendipitaceae species described for the Americas. Considering its growth-promoting capacity and wide host range, it has a promising future as model endophyte for studies, to replicate or enlarge studies on the related *Serendipita indica*, and as a valuable source of inoculants research for agriculture.

Keywords: Serendipitaceae; symbiotic; flow cytometry; genome size; new species; microsclerotia

INTRODUCTION

The Sebacinales are a monophyletic order of Basidiomycetes involved in a broad range of symbiotic associations with the roots of land plants (Weiß et al. 2004; Oberwinkler et al. 2013; Weiß et al. 2016). They likely originated 300-400 million years ago (He et al. 2019) and are globally distributed (Garnica et al. 2016), from agricultural

to pristine ecosystems (Setaro and Kron 2011; Riess et al. 2014). While most mycorrhizal basidiomycetes form ectomycorrhiza (Garnica et al. 2016), the species within the Sebacinales establish remarkably flexible associations (Selosse et al. 2007). They can also be found as symptomless endophytes (Wilson 1995) within the roots of many plant species or as free-living saprotrophs (Selosse et al. 2009; Garnica et al. 2016; Weiß et al. 2016).

The order is phylogenetically divided into two families: the Sebacinaceae, which often form macroscopically visible basidiomes, and the Serendipitaceae, in which only anamorphic species were studied in detail hitherto (Weiß et al. 2004; Weiß et al. 2016). These two families also have key differences in ecology and biotechnological potential (Weiß et al. 2004; Oberwinkler et al. 2014). While the first contains species described as root endophytes, ectomycorrhizal or orchid mycorrhizae restricted to fully or partially heterotrophic orchids (Weiß et al. 2016), the second family has a much wider mycorrhizal spectrum, including ericoid (Selosse et al. 2007; Vohník et al. 2016), ectendomycorrhizas from the cavendishioid (Setaro et al. 2006), pyroloid (Setaro et al. 2011), arbutoid (Hashimoto et al. 2012) and jungermannioid (Kottke et al. 2003) types, and orchid mycorrhiza (Warcup 1981; Warcup 1988; Suárez et al. 2008; Yagame and Yamato 2008); finally they also occur as symptomless endophytes (Selosse et al. 2009; Riess et al. 2014; Venneman et al. 2017).

Perhaps less anchored in biotrophy than the uncultivable Sebacinaceae, Serendipitaceae maintained genes responsible for saprotrophy in parallel with the evolution of biotrophic ability (Zuccaro et al. 2011). This enables not only their axenic culture, but also allows an outstanding capacity to colonize the roots of a wide range of mono- and dicotyledonous plants as endophytes, while promoting many beneficial effects to their hosts (Lahrmann and Zuccaro 2012). This makes them unique models for the study of mutualistic interactions with plants and excellent biotechnological tools for sustainable agriculture (Deshmukh et al. 2006). For example, the colonization of switchgrass roots by *Serendipita vermifera* can considerably increase the host´s shoot and root biomass (Ghimire and Craven 2011), an increase that is superior to that obtained by years of switchgrass genetic improvement efforts (Ray et al. 2015).

Although many environmental DNA sequences available in GenBank are assigned to the Serendipitaceae, only four species have been described to date. *Serendipita indica* was isolated from desert soil samples in India under the name

Piriformospora indica (Verma et al. 1998). *Serendipita vermifera* was first isolated in Germany (Oberwinkler, 1964) and later from the roots of Australian terrestrial orchids (Warcup and Talbot 1967), but it seems that this name is now given to a wide range of different *Serendipita* spp. in the literature, and reflects a species complex (Weiß et al. 2016). *Serendipita williamsii* (Basiewicz et al. 2012), which is closely related to *S. indica,* was also isolated from arbuscular mycorrhiza spores. More recently, *S. herbamans* was isolated from the roots of a Polygonaceae in Europe where it grew as an endophyte (Riess et al. 2014).

Research in progress in Brazil is focusing on the biotechnological applications of Sebacinales in agriculture. Studies are being carried out with pineapple (Cruz et al. 2015) and rice (Bertolazi et al. 2019) using non-native strains. The use of inoculants from native strains has the advantage of not only avoiding global transport of inoculum, which may threaten local biodiversity and soil functions (Schwartz et al. 2006; Hart et al. 2018), but also because they tend to be more competitive and adapted to local conditions (Weiß et al. 2016), including infrequent or extreme events.

Epidendrum fulgens is an abundant and widespread terrestrial neotropical orchid that occurs in shrubby sand dunes and rock outcrops of the Atlantic rainforest in Brazil (Sujii et al. 2019). This orchid occurs in a harsh and stressing environment formed by marine deposits between the sea and the Atlantic rainforest mountain chains in Brazil, where plants are subjected to constant winds, floods, drought, high salinity and low nutrient levels (Scarano 2002).

This study reports morphological and molecular characteristics of a novel species of *Serendipita* isolated from germinations of *E. fulgens.* In this species as in other orchids, the small reserveless seeds germinate into an undifferentiated seedling called a protocorm supported by the fungus (Rasmussen 1995; Dearnaley et al. 2017). In addition, we provide experimental evidence of the growth-promoting capacity of this newly described fungal species in different plant species through endophytic root symbiosis.

MATERIAL AND METHODS

ISOLATION AND CULTIVATION OF THE FUNGUS ENDOPHYTE

The fungus was isolated from symbiotic protocorms of *Epidendrum fulgens* Brongn growing over the roots and pot substrate of greenhouse *E. fulgens* germplasm plants, at the Federal University of Santa Catarina (Florianópolis, Brazil). These plants were collected without their original substrate from natural populations in Florianópolis, Brazil (27°37'27.9"S and 48°27'25.4"W), in the Restinga vegetation (Araujo 1992), and were maintained for 3 to 8 years in greenhouse conditions. After serendipitous observations of naturally occurring symbiotic germination in the greenhouse, a few potted plants were separated and *E. fulgens* seeds were sown at their base. After 4-6 months, young symbiotic protocorms were collected and surface sterilized with 70% ethanol for 1 min and 0.5 % sodium hypochlorite for 5 min, followed by three rinses in sterile distilled water. They were longitudinally cut and deposited in Petri dishes containing PDA medium supplemented with 100 mg L^{-1} streptomycin and incubated in the dark at 25 °C. The plates were checked daily under an inverted microscope for the emergence of fungal hyphae. The tips of hyphae were then selected and transferred to new Petri dishes with fresh PDA medium.

For culture before DNA extraction, fungal isolates were transferred to glass flasks containing 100 mL of potato dextrose broth and maintained under constant agitation at 100 RPM for 2 weeks. The mycelia were harvested, filtered through cheesecloth and rinsed several times with sterile distilled water to remove culture media.

DNA EXTRACTION, PCR CLONING, SEQUENCING, AND PHYLOGENETIC ANALYSIS

About 50 mg of fresh mycelia from the fungal isolate was ground in a Precellys® homogenizer and the total DNA was isolated using the CTAB protocol (Doyle and Doyle 1990). The isolated DNA was diluted and the ITS and D1/D2 regions of the nuclear rDNA were amplified with the primers ITS1F (Garden and Bruns 1993) and NL4 (O'Donnell 1993). The PCR reaction was carried out in a 20 µL final volume containing 50 ng of template DNA, 0.2 µM of each primer, 0.2 mM dNTPs, 1x PCR buffer, 0.2 mg mL^{-1} bovine serum albumin, and 2.5 mM $MgCl_2$. Thermal cycling parameters were an initial denaturation step at 94 °C for 2 min followed by 35 cycles

of denaturing at 94 °C for 1 min, annealing at 58 °C for 30 s and extension at 72 °C for 1 min, plus a final extension at 72 °C for 7 min. The resulting PCR products were checked by electrophoresis on a 1.5% agarose gel stained with GelRed® (Biotium™) and visualized through UV light. The PCR products were purified by precipitation with 1 volume of a solution of 20% polyethylene glycol 8000 and 2.5 M NaCl. Purified PCR products were sequenced in both directions with the BigDye™ Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems Foster City, CA, USA) on an ABI 3500xl genetic analyzer (Applied Biosystems). Sequence chromatograms were assembled and manually edited using CLC Genomics Workbench (Qiagen Bioinformatics; http://www.qiagenbioinformatics.com). The newly acquired DNA sequence was deposited in GenBank under accession number MN595219.

A Blast search against the NCBI database (http://www.ncbi.nlm.nih.gov) was used to check the sequence similarities. A phylogenetic hypothesis based on the ITS plus D1/D2 regions of the nuclear 28S rDNA was made using the newly obtained sequence and 35 GenBank accessions spanning the ITS and D1/D2 regions from taxa representative of Sebacinaceae and Serendipitaceae. The matrix produced by Riess et al. (2014) and sequences from the Sebacinaceae by Oberwinkler et al. (2014) were used as references. Sequences were aligned with MUSCLE (Edgar 2004) and minimal manual adjustments were performed using MEGA7 v7.0.9 software. Four sequences were removed because they lacked partial ITS1 or ITS2 regions, leaving 33 sequences in the final data set. The final alignment length was 1685 bp and was deposited in TreeBase (http://purl.org/phylo/treebase/phylows/study/TB2:S26366).

Phylogenetic analyses were performed using a Maximum Likelihood (ML) approach in RAxML-HPC2 v8.2.12 software (Stamatakis 2014) with combined rapid bootstrapping and 1000 runs, using the GTRCAT model for DNA substitution. We also performed a Bayesian Markov Chain Monte Carlo (MCMC) analysis in MRBAYES 3.2.7a (Ronquist and Huelsenbeck 2003) using GAMMA distribution within sites, with two replicates and four heated Markov chains. Jmodeltest2 software (Darriba et al. 2012) was used to select the best-fit model of nucleotide substitution. In each run, 200,000 sample generations were used. The first 50,000 were discarded and one tree every 100 generations was pooled and used to compute the final majority-rule consensus tree to estimate branch support. All phylogenetic analyses were performed

on the CIPRES Science Gateway v.3.3 (http://www.phylo.org/sub_sections/portal/). The phylogenetic trees with the best scores were illustrated using FigTree v1.4.4.

MORPHOLOGICAL STUDIES

Microscopic observations were made from fresh and fixed culture samples and symbiotic protocorms. To morphologically describe the fungal isolate and its interaction with the host plant we used light microscopy, transmission electron microscopy (TEM) and scanning electron microscopy (SEM). For TEM and SEM, symbiotic protocorms of *E. fulgens* at the late stage of development, with emerging first leaf, as well as 0.5 cm^2 plugs of one-month-old colonies growing on PDA, were fixed in 2.5 glutaraldehyde-paraformaldehyde in sodium cacodylate buffer (0.1 M, pH 7.2) for 48 h (Karnovsky 1965). For SEM, the fixed samples were dehydrated in ethanol series (20%, 40%, 60%, 70%, 80%, 90%, and 100%) for 30 min each, followed by critical point drying in liquid carbon dioxide (EM CPD 030/Leica, Germany). Dried samples were longitudinally sectioned, mounted over aluminum stubs with double-sided sticky carbon tape, coated with gold (EM SCD 500/Leica, Germany) and examined under a JEOL, JSM-6390LV scanning electron microscope (LCME-UFSC). For TEM examinations, we used the methodology described by Suárez et al. (2006), and sections were examined under a JEOL JEM1011 (JEOL, Inc., Peabody, MA) transmission electron microscope of the Central Laboratory of Electron Microscopy at the Federal University of Santa Catarina (LCME/UFSC, Florianópolis, Brazil).

To count the number of nuclei in hyphae, colonies were grown in 300 mL glass flasks with 30 mL of PDA medium and sterile microscopic cover slides were perpendicularly placed on the media surface to allow colonization of the slides. The colonized slides were removed in a flow chamber, and fixed and stained for microscopic observations according to the methodology described by Wilson (1992).

GENOME SIZE ESTIMATION

Flow cytometry was used for nuclear genome size estimation. Initially, we used *Solanum lycopersicum* ‘Stupické’ (2C = 1.96 pg DNA) as an internal reference standard to estimate the genome size of *Arabidopsis thaliana* ‘Col-0’. Seeds of the reference standard were kindly provided by Dr. Jaroslav Doležel of the Institute of Experimental Botany of the Czech Academy of Sciences. Then, we used *A. thaliana*

(2C= 0.32 pg) as an internal reference standard for the genome size estimation of the fungal isolate. That was necessary because genome size differences between *S. lycopersicum* and the fungal isolate were too large to allow flow cytometry analysis using internal standardization, i.e., simultaneous isolation, staining and analysis of the sample and the reference standard. Internal standardization is recommended to avoid errors due to instrument drift and variation during the sample preparation and staining (Doležel and Bartoš 2005).

Nuclei from the leaves of the reference standard (≈25 mg) and the mycelia of the fungal isolate (≈5 mg) were simultaneously extracted by chopping with a razor blade (Galbraith et al. 1983) on 2 mL ice-cold Otto I buffer (Otto 1990) containing 0.1M citric acid (Merck) and 0.5% Tween 20 (Synth). The nuclei suspension was filtered through a 40 µm nylon mesh (BD Falcon) and centrifuged at 150g for 5 min. The supernatant was removed with a pipette and the pellet was resuspended after the addition of 100 µL of fresh ice-cold Otto I buffer. For nuclei staining, the suspension (200 µL) was incubated in the dark for 30 min after the addition of 500 µL of Otto II buffer (Otto 1990) supplemented with 50 µg mL^{-1} of propidium iodide (PI; Sigma-Aldrich) and RNase (Sigma-Aldrich). Fluorescence intensity was measured with a BD FACSCanto™ II flow cytometer from the LAMEB/UFSC, equipped with an argon laser (488 nm) used for PI excitation. The position of each peak, from fungus and reference standard, was settled by analyzing a first run with each sample separately. The G1 peaks were assigned to a specific channel and the equipment voltage and gain were kept constant throughout the analyses. Eight independent replicates were performed and at least 40,000 G1 nuclei from the fungus sample were analyzed for each replicate.

Flowing software 2.5.1 was used to process the data. First, we analyzed dot-plots of fluorescence intensity on a linear scale vs. forward scatter light in a logarithmic scale. A polygonal region including all PI stained nuclei was created on dot-plots from which gated histograms of fluorescence intensity in linear scale were created. Linear regions were created on histograms to gate and obtain descriptive statistics of only intact nuclei.

The sample genome size was calculated by multiplying the fluorescence intensity ratio between the G1 cell cycle peaks of the fungus and the reference standard by the DNA 2C-value of the reference standard (Doležel and Bartoš 2005).

To convert DNA content in picograms (pg) to base pairs (bp), we considered that 1 pg = 0.978 x 10^9 bp (Doležel et al. 2003).

SYMBIOTIC SEED GERMINATION

In order to test whether the isolated fungus was able to promote seed germination, we performed an *in vitro* assay. Seeds were harvested from one mature fruit of *E. fulgens*, disinfected with 0.5% NaClO for 10 min and washed three times in sterile distilled water. Thereafter, they were inoculated in Petri dishes containing agar/oat medium (4 g L^{-1} oatmeal flour; 7 g L^{-1} agar), with a 1 cm^2 plug of the fungal isolate inoculum at the center of the plate. The fungus had previously been cultivated for 4 weeks on PDA medium and the inoculum plugs were obtained from the active growing hyphae from colony margins. For comparison, Petri dishes containing MS media (Murashige and Skoog 1962) without fungal inoculum were used as control asymbiotic germination. Dishes were kept at 25 °C, in a 16-h photoperiod.

INTERACTION WITH OTHER PLANT SPECIES

To test the interaction capacity of the fungal isolate, we performed inoculation assays on species from other plant families. We chose *A. thaliana* because it is a model plant that could help future interaction mechanism studies. We also used *Zea mays, Glycine max,* and *S. lycopersicum* because of their agricultural importance. For *A. thaliana* (Col-0), experiments were performed according to method 1 described by Johnson et al. (2011), with modifications. Briefly, *A. thaliana* seeds were surface sterilized in 1% NaClO for 7 min., washed in sterile distilled water and inoculated in Petri dishes containing 25 mL of half-strength MS medium (Murashige and Skoog 1962), supplemented with 1% sucrose. Petri dishes were kept in the dark at 5 °C for 7 days to ensure homogenous germination and were then transferred to a growth chamber at 23 °C with a 16-h light photoperiod and kept in this condition for 10-12 days. Homogenous seedlings were selected and used for inoculation experiments. Polypropylene dishes of 8 cm width and 4.5 cm height were poured with 25 mL PNM medium, and a sterile 70 µm nylon mesh was placed on the medium surface. The dishes were inoculated by placing a 0.5 cm^2 PDA medium inoculum plug from a 4-week-old fungal colony on the center of the nylon mesh. For mock treatment, plugs from fresh non-inoculated PDA medium were used. Two seedlings were placed on the

nylon mesh in each dish and were kept at 23 °C with a 16-h photoperiod. A total of 26 dishes per treatment were used (inoculated or mock treatment). Two weeks after inoculation, plants were harvested and dried at 50 °C until they reached a stable weight, for dry mass measurement. To evaluate root colonization, 1 cm long root segments were cleared in 10 % KOH overnight, rinsed in water, acidified for 3 min in 1 % HCl, stained in 0.05% trypan blue in lactophenol solution (lactic acid/phenol/glycerol/distilled water in a 1:1:2:1 mixing ratio), mounted on microscopic slides and observed with a light microscope.

To evaluate the capacity of the fungus to infect roots of maize, soybean, and tomato, the seeds were surface sterilized with 1% NaClO for 10 min, washed with sterile distilled water and inoculated in test tubes containing 10 mL of PNM medium (Johnson et al. 2011) with a 0.25 cm^2 fungal inoculum plug. After 2 weeks in the dark at 25 °C, 1 cm long root segments were harvested from 6 seedlings of each species to evaluate root colonization using the same methodology as that described for *A. thaliana*.

RESULTS

PHYLOGENETIC POSITION OF THE ISOLATE

Three different attempts at fungal isolation were made, from which fourteen fungal isolates were obtained, including from the roots of adult plants. According to the phylogenetic analysis of the ITS region (data not shown), these isolates belonged to the Cantharellales and Sebacinales orders within the Basidiomycota, and to the Pleosporales, Xylariales, Chaetothyriales and Ostropales orders within the Ascomycota. Three isolates obtained from protocorms, and assigned to the Sebacinales order, presented homogenous characteristics in culture. The ITS and D1/D2 regions of the 28S rDNA sequences from these three isolates were 100% similar to each other. Therefore, an ex-type strain (Brenae2610) was randomly chosen to perform the morphological, phylogenetic and inoculation analyses. We looked for similar sequences using the BLAST search against the NCBI database using sequences from the ITS region, which is the universal barcode locus for fungi (Schoch et al. 2012), and the D1/D2 regions of the 28S rDNA, which is the standard region for Sebacinales phylogenetic studies (Weiß et al. 2004). Sequences retrieved from

GenBank shared 88.27-89.25% identity with our sequence and they corresponded mostly to environmental DNA *Serendipita* spp. sequences issuing from orchid mycorrhizae and from mycothalli of the hepatic *Aneura* (Table S1). This result suggested that our sequence belongs to a species that was neither morphologically nor molecularly described.

The newly generated sequence contains two characteristic introns on the end of the 18S gene: downstream from the end of 18S /beginning of ITS1, a small intron of 35 bp length, from positions 37 to 72; and another one of 228 bp length, from positions 81 to 309. According to the AIC criterion, the alignment best-fit evolutionary model for nucleotide substitution was GTR+I+G. The phylogenetic tree inferred from the concatenated ITS+D1/D2 regions of the 28S rDNA sequences presented clades with high support values. The ML and Bayesian analyses yielded trees with the same topology and similar node support values. The newly isolated fungus clusters together with species within the Serendipitaceae family in the order Sebacinales form a highly supported terminal lineage (Fig. 1). It is closely related to *Serendipita* sp. MAFF 305841(KF061292), but it differs from it by 98 bp in the ITS region (14.22%) and by 44 bp in the D1/D2 regions of the 28S rDNA (6.51%). Thus, this isolate represents a new species in the Serendipitaceae that we describe as a new species of the *Serendipita* genus.

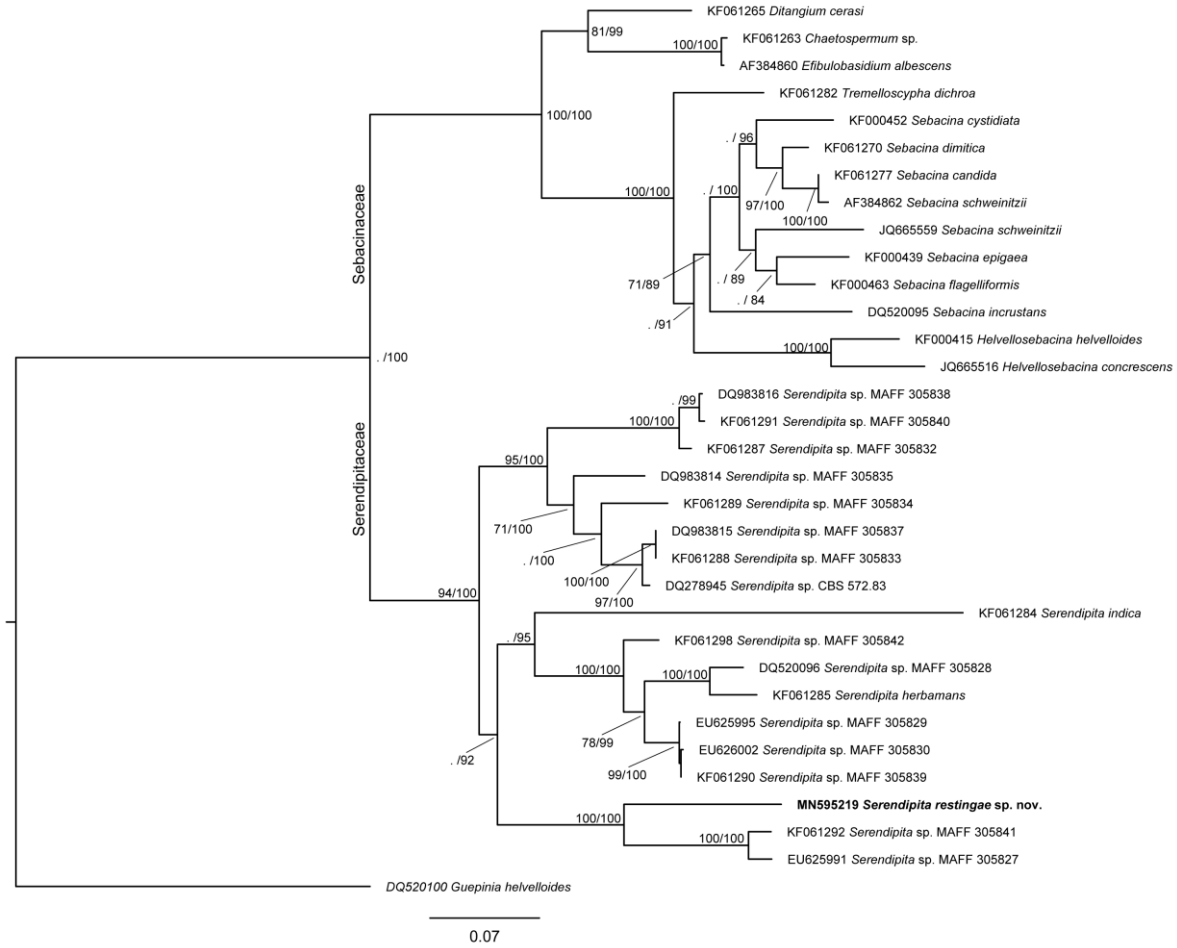


Figure 1: Midpoint rooted ML tree obtained from ITS+D1/D2 regions of 28S rDNA (1685 bp length alignment) showing the placement of *Serendipita restingae* sp. nov. within the Sebacinales. Numbers on nodes are ML bootstrap support values (≥ 70% are shown) based on 1000 replicates / Bayesian estimates of posterior probabilities in percent (≥ 80% are shown) inferred with MRBAYES. *Guepinia helvelloides* (Auriculares) was used as an outgroup. Note that the name *Serendipita sp.* is given to a wide range of different *Serendipita* spp. in the literature, commonly referred to as *Serendipita vermifera* (Weiß et al. 2016). See table S2 for a complete description of GenBank accessions used in the analysis.

TAXONOMY

Serendipita restingae Y. Fritsche, Selosse, Guerra**, sp. nov. –** Figs. 1, 2 and Fig S1.

MycoBank no.: MB 835677

GenBank accession number MN595219

Diagnosis: The anamorphic *Serendipita restingae* can be distinguished from other *Serendipita* species as they produce microsclerotia formed from non-monilioid hyphae on water-agar medium (Fig 2d-e). The following diagnostic nucleotide characters can be used to distinguish other Serendipitaceae species from *S. restingae,* respectively: **ITS1**: upstream from the end of 18S at positions 65(T:C), 148(T/C/G:A), 178(G/A:T).

ITS2: upstream from the end of 5.8S at positions 16(A/C/–:G), 48(G/T/C:A), 49(T/C:A), 59(T/C:A), 71(T:C), 78(T/C:G), 79(G:A), 80(T/C:G), 160(T/C/G:–), 161(T/C/A:–).
28S: upstream from the end of ITS2 at positions 125(G:T), 137(C/T:A), 166(C/T:–), 239(A/C:G), 250(G:A), 446(T/G:C), 452(T:C), 538(T/C:–), 539(G/A:–), 547(T/C:–), 548(A/G:–).

Typus: Brazil, Santa Catarina state, Florianópolis (27°34'58.6"S 48°30'18.7"W), at sea level, from *Epidendrum fulgens* protocorms, in Sept 2018, collected and isolated by Yohan Fritsche (Holotype SR2619, preserved in a metabolically inactive state at the LFDGV Collection from the Federal University of Santa Catarina, Florianópolis SC, Brazil - 27°34'56.3"S 48°30'21.7"W). Ex-type culture Brenae2610, deposited at the LFDGV Collection.

Host: *S. restingae* was isolated from symbiotic protocorms of *Epidendrum fulgens* Brongn. (Orchidaceae).

Description: The fungal colony grows 0.19 ± 0.01 mm day^{-1} on PDA medium at 25 °C in the dark. Cultures are white to cream in color and the growth habit is zonate, with submerged edges and an intermediate zone of aerial mycelia, mostly composed of monilioid hyphae (Fig 2a-b). Mycelia are composed of hyaline, irregularly septate and thin-walled hyphae, with a diameter of (0.9)1.4–2(2.4) µm, lacking clamp connections. Hyphal coils were frequently observed (Fig 2f). Hyphae is multinucleate (Fig 2g), although, presumably due to irregular septation, the number of nuclei per cell is quite variable. On the surface of the water-agar medium, after two months of culture, either in the dark or under indirect light, microsclerotia (72 ± 36 µm) composed of non-monilioid hyphae are abundantly formed (Fig 2d-e). The fungus produces abundant thin-walled monilioid hyphae, in chains of up to 14 cells, after about four weeks of culture in a variety of synthetic culture media (Fig 2c). Thick-walled chlamydospores (ripe monilioid cells) were globose to sub-globose and (5)7–10(11) × (5)7–10(13) µm. Sexual structures were not observed. The ultrastructure analysis of septal pores showed they consisted of dolipores with straight/flat imperforate parenthesomes, composed of two outer electron-dense layers surrounding an inner less electron-dense

lumen (Fig 2h), as typical in Sebacinales. Hyphal cell walls are 47 ± 10 nm thick, while chlamydospore wall thickness is 0.8 ± 0.2 µm.

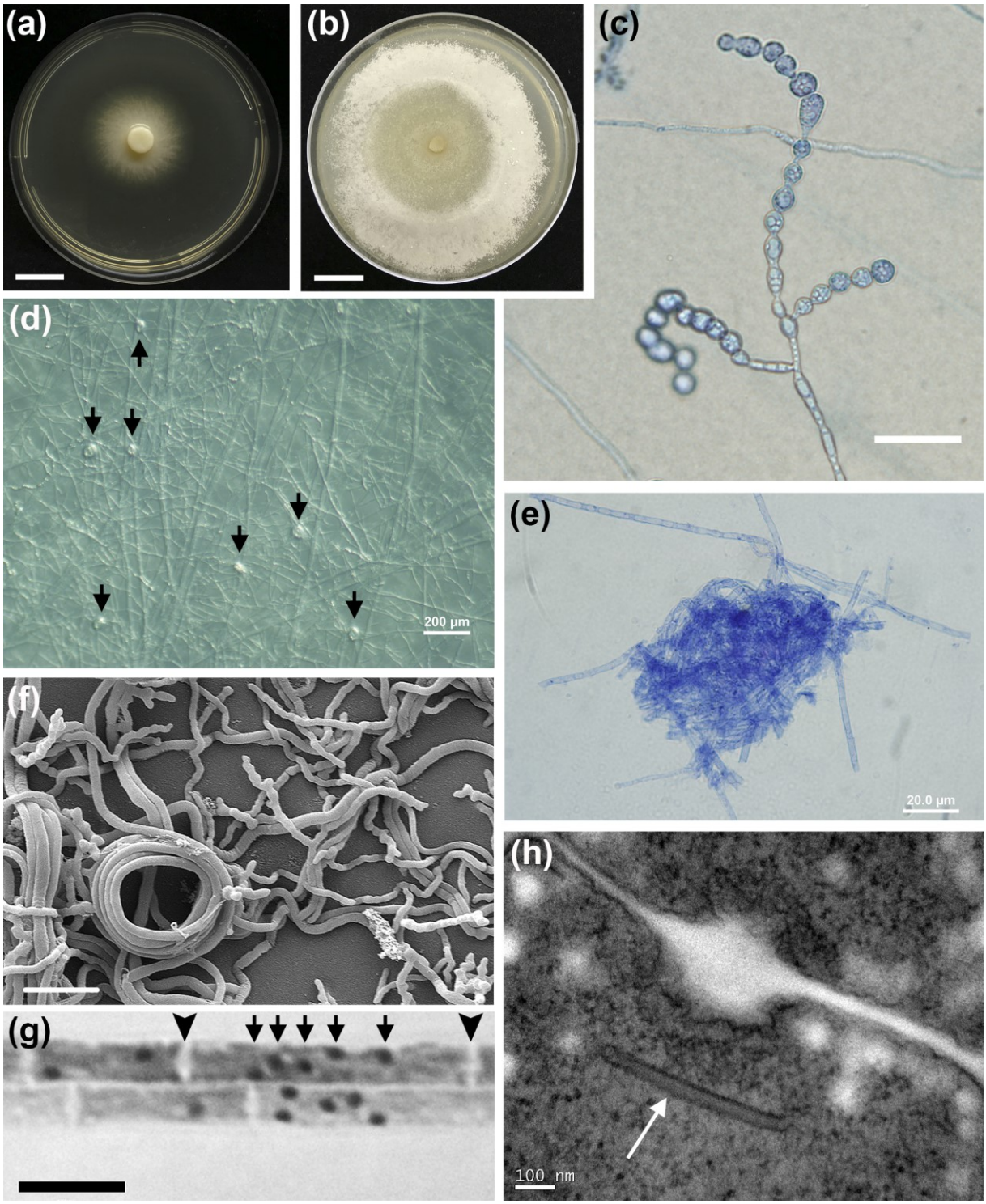


Figure 2: Morphological features of Serendipita restingae sp. nov. a-b) Ex-type Brenae2610 colonies on PDA after 6 and 40 days, respectively bar = 1.8 cm; c) Monilioid hyphae stained with 0.05% trypan blue, bar = 25 µm; d) Microsclerotia (arrows) on the surface of agar-water medium after 2 months of culture; e) Detail of a microsclerotium stained with 0.05% trypan blue. Note that it is not formed from monilioid hyphae; f) Scanning electron micrograph of hyphae on the agar surface showing typical hyphal coils, bar = 10 µm; g) Giemsa-stained hyphae showing multinucleate cells. Arrows point to nuclei and

arrowheads point to septa, bar = 5 µm; h) Transmission electron micrograph of a side of the dolipore septum with a flat and imperforate parenthesome (arrow).

Geographic distribution: *S. restingae* is described from a strain in culture. Its actual geographic distribution is yet to be defined.

Etymology: Named after the habitat of *Epidendrum fulgens* orchids, the sand dune vegetation within the seashore along the coastal range of the Brazilian Atlantic Rainforest, known as Restinga (Araujo 1992; Scarano 2002).

GENOME SIZE

The genome size of *A. thaliana* was estimated using *S. lycopersicum* as an internal reference standard (i.e. the calibration step necessary for further use of *A. thaliana* as an internal reference standard for fungal genome size estimation) (Fig. S1a). The haploid genome size of *A. thaliana* was estimated as 0.16 pg of DNA. With this procedure, we were further able to use it as an internal reference standard for the estimation of *S. restingae* genome size.

The coefficients of variation (CV) from G1 cell cycle nuclei fluorescence peaks of the fungal sample and the internal reference standard were, on average, 9.7% and 4.6% respectively. The genome size estimation between the eight replicates varied by less than 1.3%. In total, the fluorescence of >437 thousand nuclei of the G1 peaks of the fungal sample was measured to calculate the genome size, which was estimated as 0.0369 ± 0.0005 pg or 36.10 ± 0.48 bp (Fig. S1b).

GERMINATION OF *E. FULGENS* SEEDS

The germination rate was significantly higher for symbiotic germination in comparison with asymbiotic MS medium. 97.5% (± 0.8%, confidence interval) of seeds germinated on AO medium with *S. restingae*, while only 82.5% (± 3.3%) germinated asymbiotically on MS medium. No seed germinated on AO medium in the absence of fungal inoculum. Symbiotic protocorms developed numerous rhizoids just after emerging from the seed coat. They were mainly concentrated at the protocorm base (Fig. 3b). Such structures were not observed on asymbiotic protocorms (Fig. 3a).

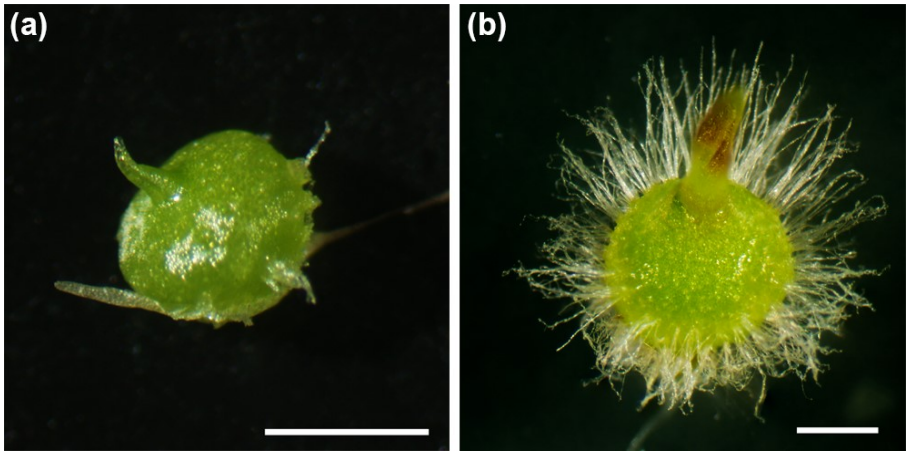


Figure 3: Protocorms of *E. fulgens* four weeks after seed sowing. a) Asymbiotic protocorm on MS medium. b) Symbiotic protocorm on agar-oat medium inoculated with *Serendipita restingae*. Note the presence of numerous rhizoids in symbiotic protocorms. Bars = 1 mm.

Typical pelotons were observed at the basal cells of symbiotic protocorms (Fig. 4b). Ultrastructural analysis of symbiotic protocorms showed a sebacinoid hypha penetrating a rhizoid cell wall (Fig. 4c). The hyphae further invaded the cortical cells and were observed passing from one cortical cell to another, leading to the formation of host cell wall appositions (Fig. 4d). The hyphal diameter was narrowed at the penetration sites (Fig. 4c-d). No sign of damage was observed in plant host cells. Hyphae intensively colonized living cortical cells, and typical sebacinoid dolipore septa were observed (Fig. 4e-f).

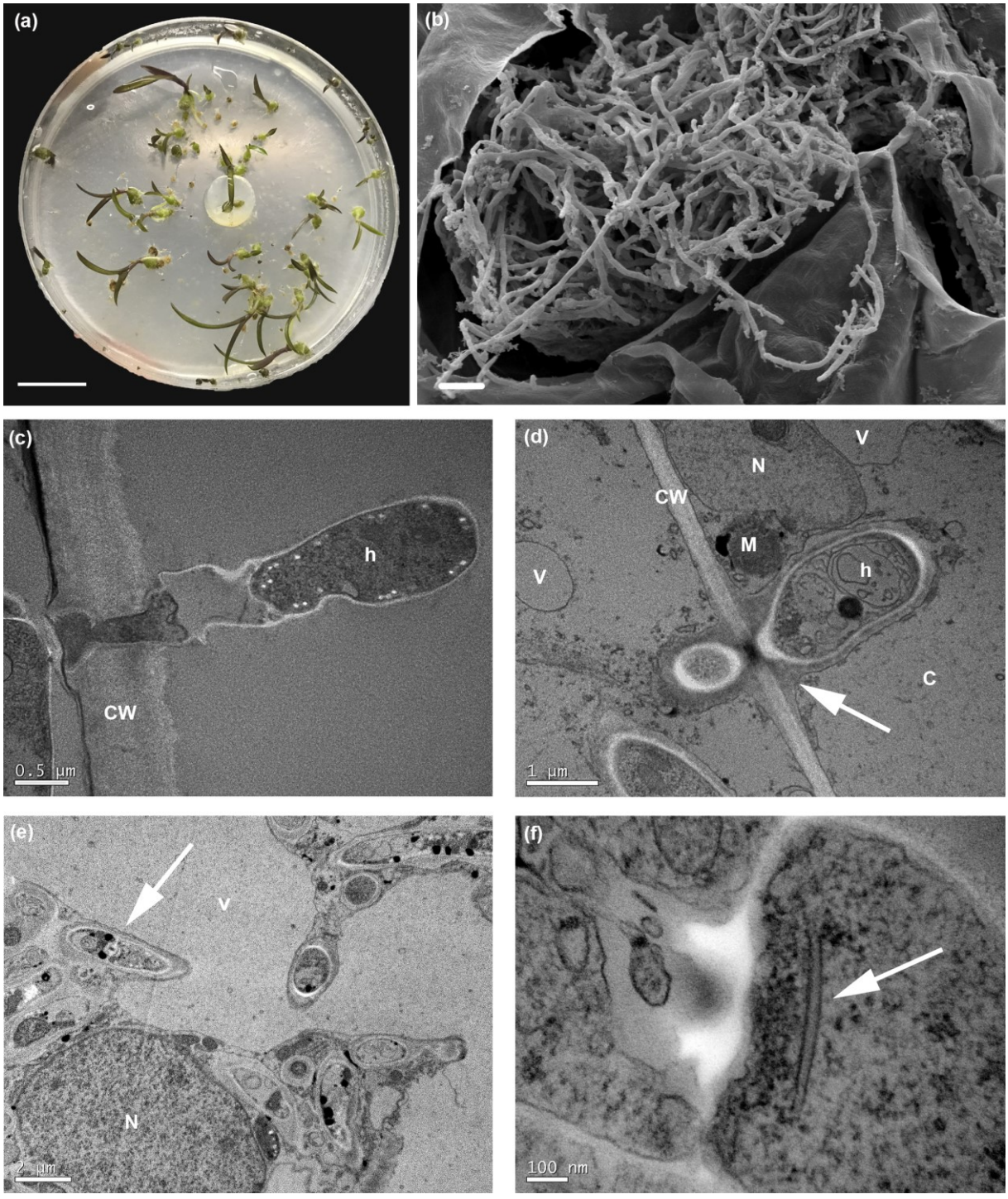


Figure 4: Interaction between *S. restingae* sp. nov. and the orchid *E. fulgens*. a) A petri dish with agar-oat medium inoculated with *S. restingae* allowed the symbiotic germination of *E. fulgens* seeds and the complete development of plantlets with leaves and roots after 16 weeks, bar 1.8 cm; b) Scanning electron micrograph of a typical orchid mycorrhiza peloton inside a protocorm cortical cell, bar 10 µm; c-f) Transmission electron micrographs showing ultrastructural features of the mycorrhizal interaction; c) Hyphae penetrating a rhizoid cell. d) Hyphae passing from one cortical cell to another, and the formation of a host cell wall apposition (arrow); e) Overview of a colonized cortical cell showing a hypha with dolipore (arrow); f) Detail of the dolipore with imperforate parenthesome (arrow). Abbreviations: *N,* plant cell nucleus; *CW,* plant cell wall; *V,* plant cell vacuole; *C,* plant cell cytoplasm; *M,* plant cell mitochondria; *h,* hyphae.

It was possible to obtain complete plantlets, with more than one root and fully developed leaves after 4 months on AO medium inoculated with the fungus, without any sub-cultivation (Fig. 4a).

INOCULATION WITH *ARABIDOPSIS THALIANA*

Serendipita restingae was able to colonize *A. thaliana* roots *in vitro* under experimental conditions. Colonization of host cells was confirmed by examination of cleared and stained roots (Fig. 5c). Hyphae were abundant throughout the entire root surface, from distal to proximal regions, where abundant production of monilioid cells occurred. Intense colonization of outer cortical cells and root hairs was observed, also with intense monilioid cell production. No coiled hyphae were observed inside the root tissue, although typical hyphal coils, also observed on the agar surface, were frequent on the root surface.

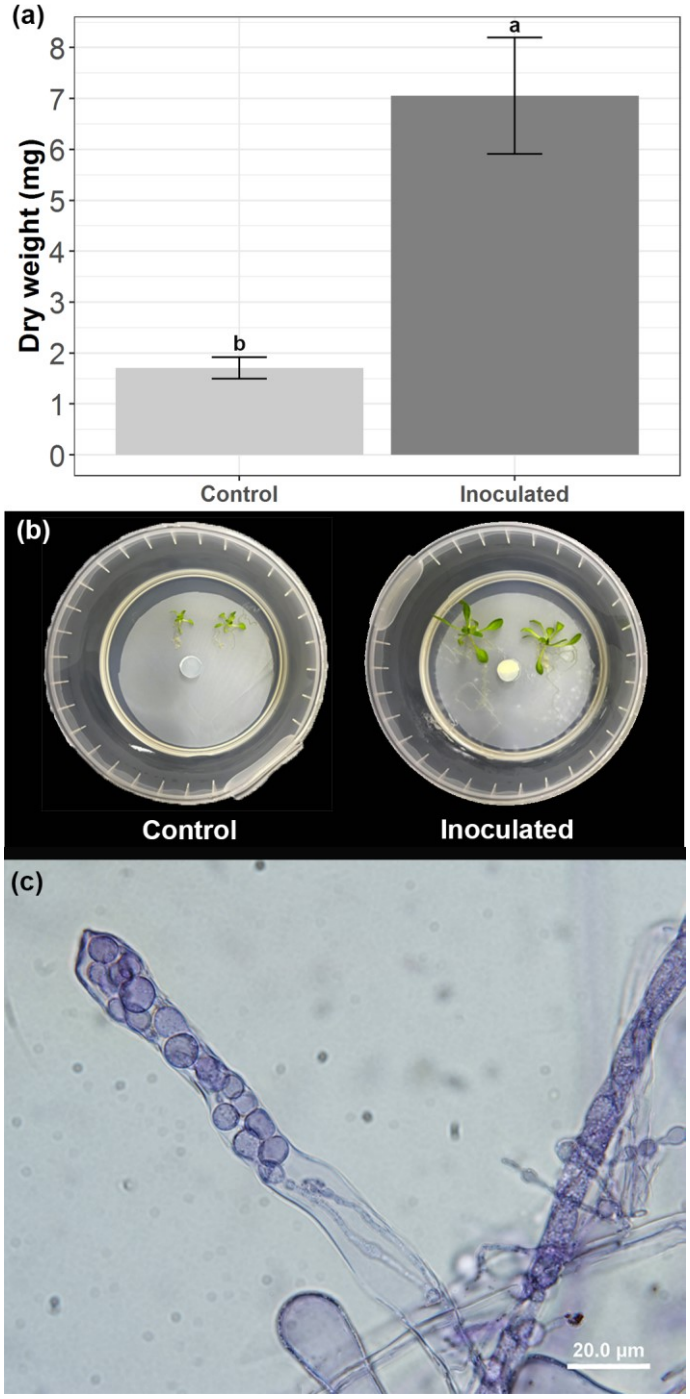


Figure 5: *In vitro* inoculation experiment between *Serendipita restingae* sp. nov. and *Arabidopsis thaliana.* a) Total dry weight (roots and shoots) and phenotypes of *Arabidopsis* plants 14 days after inoculation in PMN medium at 23 °C with a 16-h photoperiod, either with (inoculated) or without (control) a 0.5 cm^2 *S. restingae* inoculum plug (means of 26 replicates with two plants per dish). Error bars are confidence intervals, and different letters indicate significant differences according to the F-test (p = 0.001). b) The phenotypes of the inoculated and non-inoculated (control) plants. c) Detail of an *A. thaliana* root hair fully colonized by *S. restingae* sp. nov. monilioid hyphae.

Experimental data suggest strongly beneficial effects of the interaction between *S. restingae* and *A. thaliana.* Both roots and shoots have visually developed better in the presence of the fungi (Fig. 5 b). Significant differences in dry weight between

inoculated and non-inoculated plants were observed. The dry weight of entire plants was more than 4-fold higher in seedlings co-cultivated with *S. restingae* (Fig. 5a).

INTERACTION WITH MAIZE, SOYBEAN, AND TOMATO

Hyphae were also able to colonize maize, soybean and tomato roots *in vitro*, although we did not evaluate their effects on plant growth. On maize root surface, noticeable amounts of branched, coiled and monilioid hyphae were observed, while over tomato roots the hyphae were less abundant. Hyphae over the soybean root surface were abundant on the root tip, while on the proximal root region hyphae were inconspicuous.

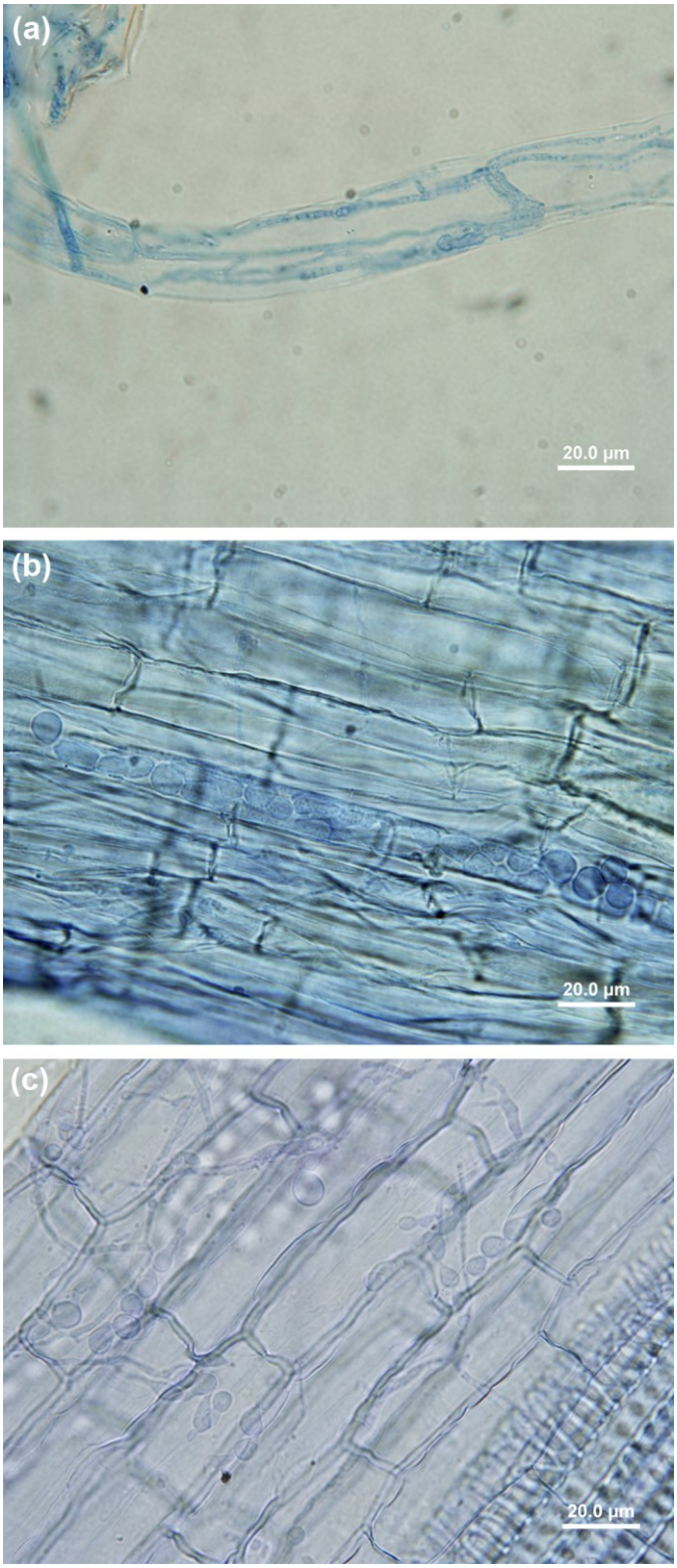


Figure 6: *Serendipita restingae* sp. nov. colonizing roots of different crop plant species. a) hyphae inside a maize root hair; b) monilioid hyphae in the root cortex of soybean; c) monilioid hyphae on the root cortex of tomato.

Cleared and stained roots examined under the microscope showed the presence of hyphae colonizing the roots of the three species, although colonization

was more intense in maize roots, where even root hairs were fully colonized by monilioid hyphae (Fig. 6a), and less intense in tomato and soybean (Fig. 6b-c).

DISCUSSION

A NEW SERENDIPITACEAE SPECIES

After the pioneering work by Warcup (Warcup and Talbot 1967; Warcup 1981; Warcup 1988), fungal strains from the Serendipitaceae have been more frequently isolated over the last few years. Vohník et al. (2016) isolated a Serendipitaceae fungus from Ericaceae roots, in Norway, that survived for years in pure culture, although Serendipitaceae associated with such a host are notoriously difficult to isolate (Berch et al. 2002). A strain attributed to the *S. vermifera* species complex was isolated from poplar (Salicaceae) roots in France (Lacercat-Didier et al. 2016). Using sudangrass roots as traps, Venneman et al. (2017) were able to obtain 51 axenic cultures in the Democratic Republic of Congo, which were closely related to *S. indica* and *S. williamsii*. Novotná and Suárez (2018) isolated *Serendipita sp.* from roots of *Stanhopea connata* (Orchidaceae) in southern Ecuador, which is also closely related to *S. indica,* according to its sequence. Although environmental DNA sequences from Serendipitaceae have been retrieved from American orchids (Suárez et al. 2009; Oliveira et al. 2014) and Ericaceae (Setaro et al. 2006; Selosse et al. 2007), *S. restingae* is the first *Serendipita* species isolated and described for the American continent.

Phylogenetic analyses resolved *S. restingae* in a monophyletic clade that also included the strains MAFF 305841 and MAFF 305827 (GenBank accessions KF061292 and EU625991, respectively) of the *S. vermifera* species complex (Fig. 1). Like these two strains, *S. restingae* also contains two characteristic introns on the end of the 18S gene.

The *Serendipita* genus proposed by Roberts (1993) was lately accepted by the mycological community (Kirk et al. 2013). Although Warcup (1988) suspected his *S. vermifera* strains could be a complex of species, the first molecular evidence came from 28S rDNA phylogenetic analysis (Weiß et al. 2004). Later, Basiewicz et al. (2012) provided physiological evidence for this species complex and, more recently, Riess et al. (2014) added ITS sequences to resolve its phylogeny, which also rendered a polyphyletic group. Therefore, Warcup´s *S. vermifera* strains are currently considered

a complex of still undescribed species and should not be designated as *S. vermifera* any more (Weiß et al. 2016). Our description of *S. restingae* makes it formally paraphyletic and calls for more efforts in taxonomic analysis of Serendipitaceae.

Molecular phylogenetic studies showed that morphology is a poor marker to distinguish monophyletic groups in the Sebacinales (Weiß et al. 2016), and that the use of structural characters is practically irrelevant for species-level delimitation (Oberwinkler et al. 2013). For molecular species delimitation within the Sebacinales, a 1% LSU threshold distance was shown to correspond to the widely used 3% ITS dissimilarity (Setaro et al. 2011). The dissimilarity index in our study vastly exceeds these threshold values for both markers.

FEATURES OF *S. RESTINGAE*

Besides molecular differences, *S. restingae* is the first Sebacinales species reported to produce microsclerotia in culture medium. As far as we are aware, the microsclerotia produced by *S. restingae* are among the smallest known to science. The 100 µm microsclerotia of the plant pathogen *Macrophomina phaseolina* are the smallest (Smith et al. 2015; Money 2016), but are larger than these of *S. restingae.* These structures may serve as a diagnostic feature for the species, have never been reported for any other Serendipitaceae species so far, and deserve to be studied in natural conditions. This feature can also be favorable for the production of inoculants, since these structures may enable the fungus to survive periods of adverse conditions, which are too severe for the regular mycelium (Townsend and Willetts 1954). They can also be a source for the future study of secondary compounds, which are often produced in sclerotia (Smith et al. 2015). Among orchid mycorrhizal fungi, sclerotia formed by non-monilioid hyphae were previously reported for '*Epulorhiza' amonilioides* = *Tulasnella amonilioides* (Almeida et al. 2014), from the Cantharellales order.

Monilioid cells are reported for all described *Serendipita* spp. (Warcup and Talbot 1967; Verma et al. 1998; Basiewicz et al. 2012; Riess et al. 2014). Although differences in the size and number of monilioid cells produced by *S. restingae* could also be highlighted, such phenotypic characteristics are highly variable and are influenced by environmental and cultural factors, so that their use as diagnostic features should be treated with caution (Jeewon and Hyde 2016). The monilioid cells observed in cultures of *S. restingae* are initially thin-walled and, with maturation, they

become thick-walled and could be considered as chlamydospores (Milligan and Williams 1987).

The number of nuclei per cell compartment is also unsuited to distinguishing *Serendipita* species, as e.g. *S. williamsii* and *S. indica* (Basiewicz et al. 2012), although Verma et al. (1998) also observed many cells with more than one nucleus when they described *S. indica*. According to Milligan and Williams (1988), *S. vermifera* is multinucleate, although there is no information for either of Warcup´s Serendipitaceae strains, or for the *S. vermifera sensu* Oberwinkler. For *S. herbamans*, the number of nuclei was not provided (Riess et al. 2014).

S. restingae dolipore ultrastructure resembles that of *S. indica* (Verma et al. 1998) and *S. williamsii* (Williams and Thilo 1989), with a flange composed of electron-translucent material. This feature is in contrast to the electron-dense flanges normally observed in other endophytic sebacinoid fungi (Kottke et al. 2003; Setaro et al. 2006; Selosse et al. 2007; Riess et al. 2014).

Scanning electron microscopy clearly shows the presence of pelotons in the protocorm cortical cells (Fig. 4b). Peloton formation is a crucial criterion for the recognition of orchid symbionts (Rasmussen 2002; Rasmussen et al. 2015). These structures are the site of nutrient exchange between orchids and fungi and are recognized as typical of orchid mycorrhiza (Dearnaley et al. 2017). The nutrient exchange between orchid and fungi symbionts occurs in intact pelotons, but a considerable amount of carbon and nitrogen is released to host cells after peloton lysis (Kuga et al. 2014; see Selosse 2014 for a discussion).

Our study provides an accurate estimation of the genome size of *S. restingae.* Our estimation of *A. thaliana* using *S. lycopersicum* as an internal reference standard resulted in a C-value of 0.16 pg DNA, which fits exactly the value of previous measurements (e.g. Bennet et al. 2003; Tavares et al. 2014). As there are no stable and well-characterized fungal DNA standards for flow cytometry (D'Hondt et al. 2011), this step was required to avoid errors due to nonlinearity and to allow internal standardization (Doležel and Greilhuber 2010).

Although the genome size data available from other Sebacinales were obtained by different methods, our results are in accordance with these previous estimations. The genome size of *S. indica* was estimated to range from 15.3 to 24.97 Mb (Zuccaro et al. 2009; 2011). Basiewicz et al. (2012) estimated the genome size of *S. williamsii*

and *S. vermifera* (stains MAFF 305828, 305830 and 305842) to be, respectively, 22 Mb and 21-26 Mb. More recently, the complete genome sequencing of *S. vermifera* MAFF305830 revealed a 38.1 Mb genome size (Kohler et al. 2015).

AN ENDOPHYTIC FUNGUS WITH A WIDE RANGE OF HOSTS

One interesting feature of some *Serendipita* spp. is their capacity to colonize living and dead cells of plant hosts. In barley roots, *S. indica* colonizes living cortical cells, establishing a biotrophic interaction, but its proliferation requires host cell death in some cells (Deshmukh et al. 2006). Although this interaction resembles hemibiotrophy, there is no evidence of massive host cell death and, instead of detrimental effects, the plants show growth promotion and increased resistance to biotic and abiotic stresses (Lahrmann and Zuccaro 2012). *Serendipita indica* can thus be seen as biotrophic at tissue level: considering *S. restingae*, it is more difficult to draw a conclusion regarding the colonization pattern from our observations, since we were not able to observe whether colonized cells are alive or not. Future analyses may answer that question, but at least we did not evidence massive cell death in response to colonization.

In the present study, beyond confirming its association with the orchid as a mycorrhizal fungus at the germination stage, we demonstrate that the capacity of *S. restingae* to associate with *Arabidopsis* roots is notable, since both organisms have evolved in completely different habitats. Nevertheless, considering that other *Serendipita spp.* are capable of colonizing *Arabidopsis* roots (Peškan-Berghöfer et al. 2004; Basiewicz et al. 2012; Riess et al. 2014; Ray and Craven 2016), this result was already expected for *S. restingae*. Sebacinales were also shown to associate with *Arabidopsis* under natural conditions (Weiß et al. 2011) as well as with Brassicaceae in general (Selosse et al. 2009). *Serendipita indica* can even transfer P to *A. thaliana* (Bakshi et al. 2015). In addition to being an excellent model for future studies, due to the great availability of mutants, the positive interaction between *S. restingae* and *Arabidopsis* suggests it has potential as a source of inoculants for other Brassicaceae.

A. thaliana plants inoculated with *S. indica* were found to upregulate defense-related genes, which resulted in higher resistance of the plants to pathogens and better drought tolerance (Sherameti et al. 2008), which was also recently demonstrated for maize (Abdelaziz et al. 2017). Serendipitaceae endophytes were also detected in the

roots of maize collected in the field (Weiß et al. 2011). *Serendipita herbamans* was detected in the roots of many crop plants collected in crop fields in Germany, including wheat, barley, triticale, sunflower, pea as well as forage crops as *Lolium perenne, Trifolium pratense* and *Trifolium repens* (Riess et al. 2014). These results show that the interaction between Sebacinales and domesticated plants may not be restricted to *in vitro* artificial conditions, but are rather common in domesticated landscapes, and this offers promising perspectives for experimental research and inoculum development.

In soybean, *S. indica* inoculation improved plant growth and nutrient acquisition, and promoted synergistic interactions with rhizobia (Bajaj et al. 2018). Tomato plants inoculated with *S. indica, S. vermifera, S. williamsii* and *S. herbamans* showed resistance to *Fusarium oxysporum* f. sp. *lycopersici* (Sefloo et al. 2019). Hydroponic tomato plants were also successfully inoculated with *S. indica*, which increased fruit production by up to 100% (Fakhro et al. 2010). Tomato plants inoculated with *S. indica* presented a 26% reduction in the disease index caused by the tomato yellow leaf curl virus (Wang et al. 2015).

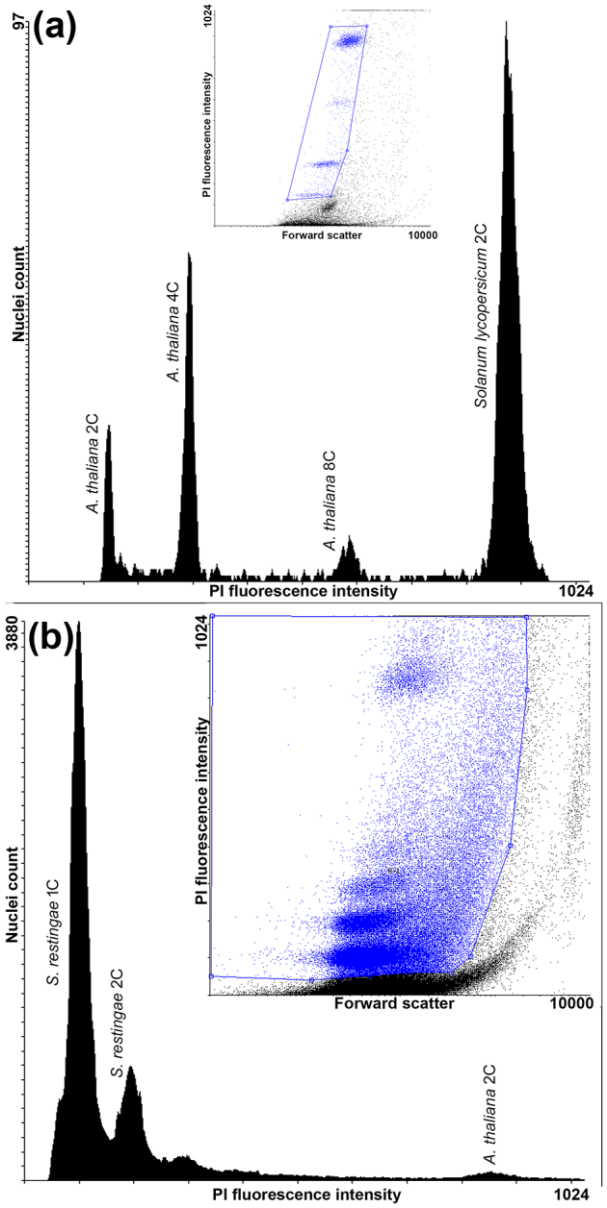
Here, our objective in relation to crop plants was only to demonstrate whether *S. restingae* was able to infect the roots of these plants. However, our results show that it may be a fruitful endophyte for inoculation experiments on domesticated plant species. Future experiments will evaluate the resulting effects of this interaction.

A CONTRIBUTION TO THE DESCRIPTION OF THE OVERLOOKED FUNGAL RESTINGA BIODIVERSITY

The description of *S. restingae* sp. nov. and its orchid mycorrhizal status is a new contribution to unveiling the promising fungal diversity of the Restinga ecosystem of the Atlantic rainforest of Brazil. Considering the biodiversity hotspot status of this biome, we can reasonably imagine an immense diversity remains yet to be discovered. In addition, the root endophytic ability of this species considerably improves plant growth in *Arabidopsis*, and its capacity to associate with the roots of important crop species opens up a whole new avenue of future agronomic research.

We consider that, in the future, more studies should focus on phylogeography and diversity of the Sebacinales in the Restinga ecosystem and more generally in South America. Many orchids occur in sympatry in this ecosystem and, whether they share

mycorrhizal fungi or have specific associations, identifying their mycorrhizal fungi is an important issue for future study. Indeed, looking at orchid mycorrhizal fungi may help identify many species that also have endophytic abilities (Girlanda et al. 2011; Selosse and Martos 2014). We also consider it important to investigate whether *S. restingae* has dual abilities in nature, i.e. is both orchid mycorrhizal and endophytic with other plant species from the same habitat. Future studies using metabarcoding approaches could also be useful, especially to understand the distribution of *S. restingae* in soil, independently of their symbiont hosts.



Supplementary figure 1 (Fig S1): Flow cytometric representative histograms of relative fluorescence intensities of propidium iodide-stained nuclei isolated from: a) fresh leaves of *Arabidopsis thaliana* and *Solanum lycopersicum* cv. Stupické as an internal DNA reference standard; b) mycelium of *Serendipita*

restingae and fresh leaves of *Arabidopsis thaliana* as an internal DNA reference standard. In each panel a small size image of the dot-plot and the gated regions created to generate the histograms is shown.

Table S1: Closest matches in GenBank by BLAST analysis of Serendipita restingae ITS + D1/D2 nc 28S rDNA sequence (accession no. MN595219)

Description	Source	Accession no.	Identity	Reference
Uncultured Sebacinales	*Angraecum cadetii* (Orchidaceae)	JF691222	89.25%	Martos et al. (2012)
Uncultured Sebacina	*Ricardia palmata* (Aneuraceae)	EU909227	88.68%	Krause et al. (2011)
Uncultured Sebacina	*Ricardia palmata* (Aneuraceae)	EU909228	88.44%	Krause et al. (2011)
Uncultured Sebacinales	*Neottia cordata* (Orchidaceae)	KJ188491	88.78%	Těšitelová et al. (2015)
Uncultured Sebacinales	*Neottia ovata* (Orchidaceae)	KJ188542	88.62%	Těšitelová et al. (2015)
Serendipita sp. MAFF 305827	*Eriochilus cucullatus* (Orchidaceae)	EU625991	88.27%	Warcup (1988)
Uncultured Sebacinales	*Neottia ovata* (Orchidaceae)	KJ188487	88.79%	Těšitelová et al. (2015)
Uncultured Sebacina	*Ricardia palmata (Aneuraceae)*	EU909229	88.30%	Krause et al. (2011)
Uncultured Sebacinales	*Neottia ovata* (Orchidaceae)	KJ188464	88.74%	Těšitelová et al. (2015)
Uncultured Sebacinales	*Neottia cordata* (Orchidaceae)	KJ188488	88.62%	Těšitelová et al. (2015)
Uncultured Sebacina	*Ricardia palmata* (Aneuraceae)	EU909224	88.68%	Krause et al. (2011)

Note. All retrieved sequences, with the exception of EU625991, are from environmental sequences, i.e. from non-isolated fungi. The sequence EU625991 was included in the phylogenetic analysis (Fig. 1)

Table S2: Details of sequences used for phylogenetic analysis

Family	Species	Authorities and reference	GenBank accession	Herbarium voucher	Host / substrate	Strain name / no.	Collected / isolated by	Country	Reference
Sebacinaceae	*Ditangium cerasi*	**(Schumach.) Costantin & L.M. Dufour**, *Nouv. Fl. Champ.* , Edn 1 (Paris): 187 (1891)	KF061265	TUB 020203	on *Prunus avium* trunk	FO 42814	R. Kautt	Germany	Oberwinkler et al. (2014)
	Chaetospermum sp.	**Sacc.**, *Syll. fung.* (Abellini) **10**: 706 (1892)	KF061263	TUB 020201	on *Fallopia japonica* litter		P. Welt	Germany	Oberwinkler et al. (2014)
	Efibulobasidium albescens	**(Sacc. & Malbr.) K. Wells**, *Mycologia* **67**(1): 149 (1975)	AF384860						Oberwinkler et al. (2014)
	Tremelloscypha dichroa	**(Lloyd) Oberw., Garnica & K. Riess**	KF061282		on dead deciduous wood	45376	L. Ryvarden	Belize	Oberwinkler et al. (2014)
	Sebacina cystidiata	**Oberw., Garnica & K. Riess**, in Oberwinkler, Riess, Bauer & Garnica, *Mycol. Progr.* **13**(3): 468 (2014)	KF000452	TUB 020024	on dead wood, under *Abies alba, Corylus avellana, Fagus sylvatica, Quercus robur* and *Quercus rubra*	TÜ-02c	S. Garnica	Germany	Oberwinkler et al. (2014)
	Sebacina dimitica	**Oberw.**, *Ber. bayer. bot. Ges.* **36**: 53 (1963)	KF061270	TUB 020207	under *Picea abies*	OJ-3e	K. Riess	Germany	Oberwinkler et al. (2014)
	Sebacina candida	**L.S. Olive**, *Bull. Torrey bot. Club* **85**: 21 (1958)	KF061277	TUB 020330	on soil, under *Monotropa uniflora*		S. Setaro	USA	Oberwinkler et al. (2014)
	Sebacina schweinitzii	**(Peck) Oberw.**, in Kirschner, Oberwinkler & Hofmann, *Nova Hedwigia* **105**(3-4): 337 (2017)	AF384862						Oberwinkler et al. (2014)
	Sebacina schweinitzii	**(Peck) Oberw.**, in Kirschner, Oberwinkler & Hofmann, *Nova Hedwigia* **105**(3-4): 337 (2017)	JQ665559						Oberwinkler et al. (2014)
	Sebacina epigaea	**(Berk. & Broome) Bourdot & Galzin**, *Hyménomyc. de France* (Sceaux): 39 (1928) [1927]	KF000439	TUB 020010	on dead wood, under *Picea abies*	AT-35	K. Riess	Germany	Oberwinkler et al. (2014)
	Sebacina flagelliformis	**Oberw., Garnica & K. Riess**, in Oberwinkler, Riess, Bauer & Garnica, *Mycol. Progr.* **13**(3): 468 (2014)	KF000463	TUB 020035	on dead wood, under *Abies alba* and *Fagus sylvatica*	TÜ-08a	S. Garnica	Germany	Oberwinkler et al. (2014)
	Sebacina incrustans	**(Pers.) Tul. & C. Tul.**, *Annls Sci. Nat.* , Bot., sér. 5 **15**: 225 (1871)	DQ520095						Oberwinkler et al. (2014)
	Helvellosebacina helvelloides	**(Schwein.) Oberw., Garnica & K. Riess**, in Oberwinkler, Riess, Bauer & Garnica, *Mycol. Progr.* **13**(3): 467 (2014)	KF000415	TUB 019983	on soil, under *Picea abies*	AT-07	K. Riess	Austria	Oberwinkler et al. (2014)
	Helvellosebacina concrescens	**(Schwein.) Oberw., Garnica & K. Riess**, in Oberwinkler, Riess, Bauer & Garnica, *Mycol. Progr.* **13**(3): 467 (2014)	JQ665516						Oberwinkler et al. (2014)
Serendipitaceae	*Serendipita sp.*	**P. Roberts**, *Mycol. Res.* **97**(4): 474 (1993)	DQ983816	MAFF 305838	*Caladenia tesselata* (Orchidaceae)	0907	J.H. Warcup	Australia	Warcup (1988)
	Serendipita sp.	**P. Roberts**, *Mycol. Res.* **97**(4): 474 (1993)	KF061291	MAFF 305840	*Caladenia patersonii* (Orchidaceae)	0915	J.H. Warcup	Australia	Warcup (1988)
	Serendipita sp.	**P. Roberts**, *Mycol. Res.* **97**(4): 474 (1993)	KF061287	MAFF 305832	*Caladenia reticulata* (Orchidaceae)	0748	J.H. Warcup	Australia	Warcup (1988)
	Serendipita sp.	**P. Roberts**, *Mycol. Res.* **97**(4): 474 (1993)	DQ983814	MAFF 305835	*Caladenia catenata* (Orchidaceae)	0770	J.H. Warcup	Australia	Warcup (1988)
	Serendipita sp.	**P. Roberts**, *Mycol. Res.* **97**(4): 474 (1993)	KF061289	MAFF 305834	*Glossodia minor* (Orchidaceae)	0768	J.H. Warcup	Australia	Warcup (1988)
	Serendipita sp.	**P. Roberts**, *Mycol. Res.* **97**(4): 474 (1993)	DQ983815	MAFF 305837	*Caladenia dilatata* (Orchidaceae)	0846	J.H. Warcup	Australia	Warcup (1988)
	Serendipita sp.	**P. Roberts**, *Mycol. Res.* **97**(4): 474 (1993)	KF061288	MAFF 305833	*Caladenia dilatata* (Orchidaceae)	0750	J.H. Warcup	Australia	Warcup (1988)
	Serendipita sp.	**P. Roberts**, *Mycol. Res.* **97**(4): 474 (1993)	DQ278945	CBS 572.83	*Caladenia dilatata* (Orchidaceae)	0750	J.H. Warcup	Australia	Warcup (1988)
	Serendipita sp.	**P. Roberts**, *Mycol. Res.* **97**(4): 474 (1993)	KF061298	MAFF 305842	*Microtis unifolia* (Orchidaceae)	0977	J.H. Warcup	Australia	Warcup (1988)
	Serendipita sp.	**P. Roberts**, *Mycol. Res.* **97**(4): 474 (1993)	DQ520096	MAFF 305828	*Eriochilus cucullatus* (Orchidaceae)	0693	J.H. Warcup	Australia	Warcup (1988)
	Serendipita sp.	**P. Roberts**, *Mycol. Res.* **97**(4): 474 (1993)	EU625995	MAFF 305829	*Eriochilus scaber* (Orchidaceae)	0714	J.H. Warcup	Australia	Warcup (1988)
	Serendipita sp.	**P. Roberts**, *Mycol. Res.* **97**(4): 474 (1993)	EU626002	MAFF 305830	*Cyrtostylis reniformis* (Orchidaceae)	0723	J.H. Warcup	Australia	Warcup (1988)
	Serendipita sp.	**P. Roberts**, *Mycol. Res.* **97**(4): 474 (1993)	KF061290	MAFF 305839	*Phyllanthus calycinus* (Orchidaceae)	0914	J.H. Warcup	Australia	Warcup (1988)
	Serendipita sp.	**P. Roberts**, *Mycol. Res.* **97**(4): 474 (1993)	KF061292	MAFF 305841	*Microtis rara* (Orchidaceae)	0963	J.H. Warcup	Australia	Warcup (1988)
	Serendipita sp.	**P. Roberts**, *Mycol. Res.* **97**(4): 474 (1993)	EU625991	MAFF 305827	*Eriochilus cucullatus* (Orchidaceae)	0140	J.H. Warcup	Australia	Warcup (1988)
	Serendipita indica	**(Sav. Verma, Aj. Varma, Rexer, G. Kost & P. Franken) M. Weiss, F. Waller, Zuccaro & Selosse**, *New Phytol.* **211**(1): 22 (2016)	KF061284	DSM11827	Spore of *Glomus mosseae*		S. Verma et al	India	Verma et al. (1998)
	Serendipita herbamans	**K. Riess, Oberw. & Garnica**, *PLoS ONE* **9**(4): e94676, 8 (2014)	KF061285	DSM27534	*Bistorta vivipara* (Polygonaceae)		K. Riess, S. Garnica	Germany	Riess et al. (2014)
	Serendipita restingae sp. nov.	**Y. Fritsche, Selosse & Guerra**	MN595219	SR2610	Protocorms of *Epidendrum fulgens*	Brenae SR2619	Y. Fritsche	Brazil	This study
Incertae sedis	*Guepinia helvelloides*	**(DC.) Fr.**, *Elench. fung.* (Greifswald) **2**: 30 (1828)	DQ520100			AFTOL-ID 1680			

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CONSIDERAÇÕES FINAIS E PERSPECTIVAS

Os resultados obtidos com a presente tese superaram as expectativas. Foram geradas informações relevantes que permitirão a propagação, o melhoramento genético e a conservação de *E. fulgens.* Mostrou-se que simples modificações físicas no ambiente *in vitro* levam a mudanças consideráveis na velocidade de desenvolvimento, na produtividade e na qualidade das mudas obtidas através da germinação assimbiótica de sementes de *E. fulgens*. Comprovou-se que os espectros de luz e a ventilação da atmosfera *in vitro* afetam diferentemente cada estágio de cultivo. Com o protocolo desenvolvido é possível obter ganhos consideráveis de produtividade e na qualidade das mudas obtidas com uma redução significativa dos custos de produção. Estudos futuros devem abordar também as etapas posteriores à aclimatização, uma vez que as diferenças observadas nas plântulas *in vitro* podem estender-se durante a fase *ex vitro*, o que pode ser importante em condições reais de cultivo comercial, onde as plantas são mais sujeitas à ação de microrganismos patogênicos ou a condições não ideais de cultivo.

Os estudos da micropropagação de *E. fulgens* com o uso de diferentes explantes após um estudo detalhado dos padrões de ploidia, apresentados no capítulo 2, permitiram a geração de inúmeras informações relevantes. Primeiramente, mostramos pela primeira vez que o fenômeno da endopoliploidia é presente em todas as partes da planta, mas que os diferentes citótipos ocorrem em proporções variáveis. Testamos virtualmente todos os explantes disponíveis para a indução de estruturas semelhantes à protocormos (ESP) e mostramos que estes possuem diferentes taxas de resposta. As peças florais, como pétalas e labelo apresentaram altas taxas de oxidação e não se mostraram adequadas como explantes para a indução de ESP. As polínias germinaram facilmente, mas não deram origens a culturas com potencial regenerativo. Bases de protocormos são altamente responsivas e folhas possuem uma resposta menor, ao passo que ápices radiculares são explantes recalcitrantes à indução de ESP. Um protocolo completo com excelentes resultados de desempenho foi desenvolvido, o que permite a propagação em massa de genótipos superiores com estabilidade citogenética dos regenerantes. A investigação sobre a estabilidade citogenética das plantas deve ser mais aprofundada em estudos futuros, com uma ampliação substancial do número de regenerantes amostrados.

Os resultados do isolamento de fungos são, provavelmente, os mais promissores. Os dados obtidos sugerem a necessidade de um aprofundamento desta pesquisa. A evidência de diferentes fungos associados às plantas de acordo com o ciclo de vida definitivamente precisa ser minuciosamente investigada. A amostragem de plantas em populações naturais também precisa ser realizada, buscando definir a distribuição geográfica de *S. restingae.*

Como a principal proposta deste estudo foi trazer à luz um recurso genético negligenciado e fornecer todo o conhecimento necessário apara a sua exploração sustentável, considera-se que o conhecimento científico gerado com esta tese permite exatamente isso, pois fornece um caminho para garantir tanto seu uso como sua conservação.