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Fungus used for germination is supplanted after reintroduction of *Hadrolaelia jongheana* (Orchidaceae). Fungo usado para germinação é suplantado após reintrodução de *Hadrolaelia jongheana* (Orchidaceae).

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Abstract

The great diversity in colors and forms become the orchids a business with high economic value. The habitat fragmentation contributes to the extinction of orchids. Inoculation of orchid with mycorrhizal fungi for seedlings can guarantee the success of reintroduction. For this purpose, seeds of *Hadrolaelia jongheana* were germinated using an isolate of *Tulasnella* sp. Seedlings were transferred to the natural field. Roots samples were collected before re-introduction, and 120th and 240th days. The diversity of mycorrhizal fungi was performed by ITS-PCR-DGGE. The ecological succession occurred in the field and the diversity was higher after 240th d. This work comprises the first study using tropical orchids for reintroduction for approaching to ecological aspects of mycorrhizal fungi association in Brazil with conservation purposes.
Keywords: Mycorrhizal. Endangered orchid. Hotspot. *Tulasnella*. Tropical orchid.

Resumo

A grande diversidade de cores e formatos tornam as orquídeas um negócio com alto valor econômico. A fragmentação de habitats contribui para a sua extinção. A inoculação de orquídeas com fungos micorrízicos para a propagação, pode garantir o sucesso da reintrodução. Para essa proposta, sementes de *Hadrolaelia jongheana* foram germinadas usando um isolado de *Tulasnella* sp. Mudas foram transferidas para o ambiente natural, amostras de raízes foram coletadas antes e depois de 120 e 240 dias da reintrodução. A diversidade de fungos micorrízicos foi avaliada por ITS-PCR-DGGE. A sucessão ecológica foi observada no ambiente natural com maior diversidade aos 240 dias. Esse é o primeiro estudo com fins de conservação com o uso de reintrodução de orquídeas tropicais micorrizadas e foco em aspectos ecológicos.
Palavras-chave: Micorríza. Orquídeas em risco. Hotspot. *Tulasnella*. Orquídea tropical.

Introduction

Over 27,000 species of orchids have been described worldwide (GOVAERTS, 2016). In Brazil it has been described 2,419 species of orchid, of which 1,620 species are endemic (NETO et al., 2013).

Over the last ten years the official list of species of Brazilian Endangered Flora has increased 10% for species of orchids (BRASIL, 2008, 2014). Most recently, 439 species were evaluated of which 169 were considered endangered (NETO et., 2013). The main cause of orchid species loss is due to both direct human action and to habitat destruction and fragmentation of ecosystems (FÁVARO, 2012), which indirectly leads to a decrease in pollinators, essential for the orchid's life cycle (REITER et al., 2016) and including illegal logging (CHUGH, GUHA, RAO, 2009).

Other important factor contributing to increase of orchids in risk of extinction is their high commercial value (POPOVA et al., 2016). The orchid commerce moves annually millions of dollars (CHUGH, GUHA, RAO, 2009), and the over-collecting is a common practice to increase the collections with commercial value, resulting in the decrease of established populations in their habitat, where 62% for all known orchids are cited as endangered (ADHIKARI, FISCHER, FISCHER, 2016; ZHANG et al., 2015). Thus, some strategies for biodiversity conservation, like seed collections, germplasm bank and reintroduction essays in natural areas of high extraction has been suggested (SEATON, PRITCHARD, MARKS, 2015).

Brazil is recognized for its biological richness, and the world's attention turns towards its loss of the biodiversity (MITTERMEIER et al., 2005). Only 8% of the original Atlantic Forest in Brazil remains, comprising 123,400 Km^2 with more than 8,000 endemic plant species, so this biome is defined like hotspot of biodiversity (RODRIGUES, 2010), making it one of the most threatened biomes of the world. *Hadrolaelia jongheana* is an endangered orchid's species that has been considerably decreased in the Atlantic Forest.

An important aspect of conservation is to maintain the natural conditions of orchid habitat to complete its life cycle (RASMUSSEN, RASMUSSEN, 2007), since orchids depend on the association with mycorrhizal fungi present in environment to germinate and for the maintenance of the basal metabolism (BOLDRINI et al., 2010; LÓPEZ-CHÁVEZ et al., 2016). Furthermore, to some orchids species the mycorrhizal association require an interaction with specific fungi (DEARNALEY, MARTOS, SELOSSE, 2012; RASMUSSEN, 2002; YAM et al., 2010).

The orchids can be associated with a wide range of fungi, including endophytic ones, especially those belonging to Ascomycota and Basidiomycota (OLIVEIRA et al., 2014). The most common mycorrhizal fungus associated with epiphytic orchid has been the polyphyletic group rhizoctonia (MARTOS, FLORENT et al., 2012), which have a wide geographical distribution (BOLDRINI et al., 2010). Rhizoctonia-like includes taxa from Tulasnellaceae (FREITAS et al., 2020), Sebacinales and Ceratobasidiaceae, and have a positive phylogenetic approach than terrestrial ones (see more taxa in JIANG et al., 2015). These group rarely form sexual spores (BONNARDEAUX et al., 2007), becoming its strategy of dispersal a mystery.

For orchid's conservation, mainly epiphytic ones, effective actions are necessary for the recovery populations (OLIVEIRA et al., 2014). Most crucial to this endeavor is the maintenance of conditions required for orchids to complete their life cycle in their natural environment (RASMUSSEN, RASMUSSEN, 2007).

Studies generating data for being used to support orchid conservations plans and to highlights the efficiency of the artificially introduced fungi are important. Therefore, the reintroduction of *H. jongheana* plantlets previously colonized with *Tulasnella* sp isolates into the Atlantic Forest fragments can more efficiently withstand the environmental changes undergone, as well as allow the succession of fungal colonization by mycorrhizal fungi species common and adapted to local conditions. Thus, the objective of the present study was to evaluate the changes in the community of orchid mycorrhizal fungi and the development of *H. jongheana* plantlets inoculated with mycorrhizal *Tulasnella* sp. after reintroduction in an Atlantic Forest fragment.

Material and methods

Experimental design

Seedlings of *H. jongheana* obtained by symbiotic propagation (BOCAYUVA, 2012) with *Tulasnella* isolate M65 (PEREIRA et al., 2005) were acclimatized in pine bark under greenhouse conditions. Thirty-two seedlings were reintroduced into the natural environment, and eight were kept in greenhouse.

Reintroduction took place in an Atlantic Forest fragment surrounding the Serra do Brigadeiro State Park (PESB), near the city of Muriaé (S 20 ° 53', W 42 ° 32'), in the state of Minas Gerais (Fig. 1A). The fragment used for reintroduction is surrounded by pasture with the presence of cattle to produce meat and milk.

The area has an average altitude of 1,050 m and is located between two river basins (Rio Doce and Rio Paraíba do Sul), surround by inselbergs. The elevation of the region creates a recurring fog throughout the year, which produces humidity of 80%, even in the dry season. The study location, therefore, is located in an area of transition between ombrophilous and semideciduous forest (VELOSO, FILHO, LIMA, 1991).

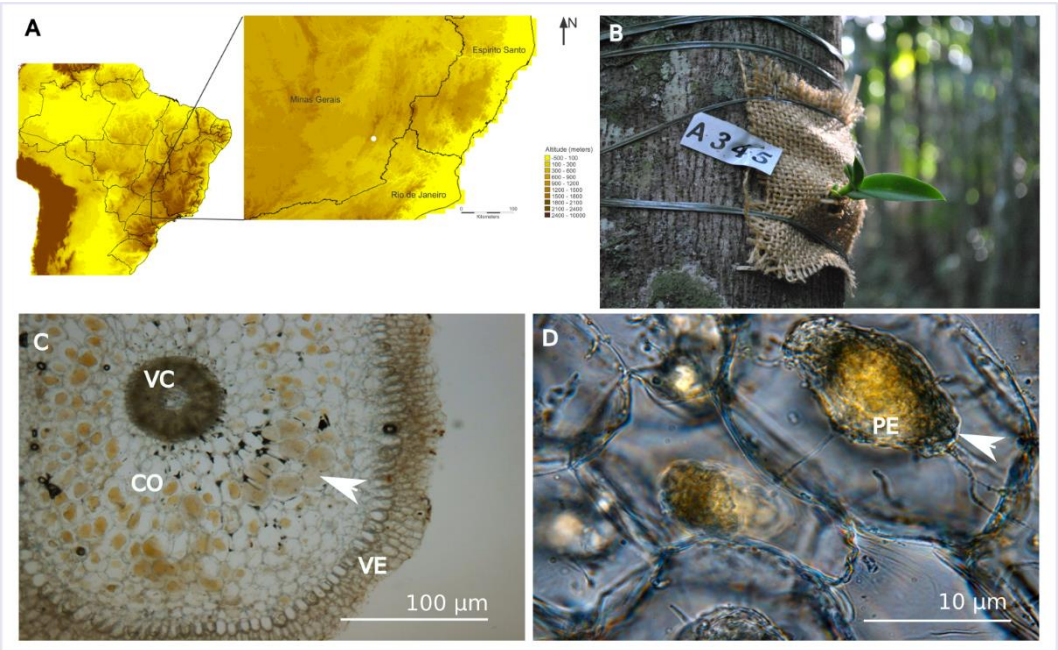

Fig. 1 - Reintroduction of *Hadrolaelia jongheana*. A) Area of reintroduction in (○) Muriaé, (S 20 ° 53', W 42 ° 32'), Minas Gerais State, Brazil. B) Seedlings reintroduction in field using reed screen with sphagnum substrate and fixed to the phorophyte; C) Transversal cut of orchid root showing pelotons (arrow): D) detail of peloton (arrows) in the cortical cell. (PE) pelotons; (VE) velame; (CO) cortex; (VC) conductors' vessel.

The reintroduction site is approximately 15 m from a small river, thereby further favoring humidity. The reintroduction was done at the end of the rainy season, however the dry season was atypical with sporadic rains, during all experimental time the rainfall accumulation was 1,363 mm, and temperature was 16.8 °C to 24.5 °C (INMET, 2020).

Eight phorophytes were chosen and labeled from the absence of other species of epiphytic orchids. The average plant species density of the PESB is 642 individuals per ha^{-1} (FÁVARO, 2012) and the distance between the phorophytes used varied between 1 and 52 m. The height of the phorophytes varied between 5 and 25 m, the DBH (diameter at breast height) between 6 and 55 cm. The phorophytes were located between 7 and 40 m from the present stream and 2 to 54 m from the edge of the fragment. All phorophytes were shaded by the canopy formed by the presence of other larger plant species.

Four seedlings were fixed to each phorophyte at a height of 1.6 to 2.0 m. In reintroduction sites when other epiphytes were present, it was manually removed. Seedlings were placed in a 10 x 10 cm reed screen with 70 g of sphagnum substrate for moisture retention, carbon source for the mycorrhizal fungi associated with orchids roots and fixed to the phorophyte with the aid of a synthetic plastic rope (Figure 1B).

Eight seedlings were kept in benches of seedlings that allowed the free flow of excess water used in regular irrigation without direct contact with soil in partially shady greenhouse, with 60% brightness retention, and watered regularly. The greenhouse used had superior protection structure and lateral adverse weather conditions such as rainfall in addition to temperature control.

Orchid root samples were randomly collected, 5-7 cm length, from 13 orchids before reintroduction, and four and five samples of roots of orchids were sampled in the greenhouse for 120^{th} and 240^{th} days, respectively. Root fragments of five orchids were collected 120^{th} and 240^{th} d after reintroduction, 5-7 cm length. Under stereomicroscopic the presence of mycorrhizal colonization was confirmed by the presence of hyphae or pelotons, in the root cortical cells (Fig. 1C and D).

Molecular characterization of fungi associated with the roots of *C. jongheana*

The extraction of total DNA from orchid roots fragments containing pelotons were performed using the NucleoSpin® Soil Kit (Macherey-Nagel) follow the manufacturer's recommendations. DNA integrity was confirmed on 0.8% agarose gel stained in ethidium bromide solution (0. 5 μg mL^{-1}) under UV light in photodocumentator L-Pix-CHemi (Loccus, São Paulo, SP, Brasil).

The total DNA samples obtained were used as templates in PCR reactions composed of 1x Colorless GoTaq ™ buffer, $MgCl_2$ [1.5 mM], dNTP [200μM], Go Taq DNA Polymerase ™ (Promega, Madison, USA) [1.25U] and primers Specific [0.2μM].

In order to amplify the regions corresponding to the transcribed internal spacer region (ITS) including the 5.8S region of fungi in general the primers ITS1F: 5'-CTTGGTCATTTAGAGGAAGTAA-3' (GARDES; BRUNS, 1993) and ITS4: 5'-TCCTCCCGCTTATTGATATGC-3' (WHITE et al., 1990) having amplicons of approximately 600 bp. For the detection of the fungus used as the starting inoculum the pair of primers specific for the family Tulasnellaceae, ITS1: 5'-TCCGTAGGTGAACCTGCGG-3' (WHITE et al., 1990) and

ITS4tul: 5'-CCGCCAGATTCACACATTGA-3' (TAYLOR, MCCORMICK, 2008) with average size of 600 bp, were used.

For the pair of ITS1F and ITS4 primers, PCR steps were: 95 °C for 2 min for initial denaturation, followed by 39 cycles at 95 °C for 1 min for denaturation, 50 °C for 1 min for primer annealing, 72 °C for 1 min in the polymerization step and the final extension at 72 °C for 10 min. For the ITS1 and ITS-4Tul primers the temperatures respectively follow 96 ºC for 2 min, thus, 39 cycles of 94 ºC for 30 s, 54 ºC for 40s 72 ºC for 1 min and 72 ºC for 6 min for final extension. PCR product confirmation was performed by 1.5% agarose gel electrophoresis, stained in ethidium bromide solution and observed under UV light.

Composite sample were assembled with all samples of the same time, before reintroduction, 120^{th} and 240^{th} for greenhouse and field. Each sample before to amplification with ITS1F and ITS4 was appropriately homogenized and 5 µL aliquots of each reaction containing respective amplicons were mixed and used. In total, five composite samples were assembled, each sample was fractionated into three tubes and each tube subjected to a new round of amplification with primers ITS1GC and ITS4.

DGGE

Each composite sample from second PCR amplicons from ITS1GC and ITS4 were analyzed by Denaturing Gel Electrophoresis (DGGE) utilizing a DCode™ model machine system (Bio-Rad, California USA). The polyacrylamide gel used contained 8% (w:v) acrylamide:bisacrylamide (37.5:1) in Tris-acetate-EDTA (TAE) 1X (Tris/acetic acid/EDTA, pH 8.0). A linear denaturing gradient was formed with the aid of the trainer Hoefer gradient SG50 (Amersham Biosciences) and the mixture of two stock solutions of polyacrylamide to obtain a final gradient ranging from 30 to 50% that was used as recommended by Tao et al. (2008). The condition of 100% of the denaturing agents consisted of urea 7 mol L^{-1} (Sigma, Cat # U5378) and 40% formamide (v:v) (Sigma, Cat # F9037) and another solution was created without these compounds. The DGGE analysis were performed in 1X TAE buffer at a constant temperature of 60°C at 80 V for a period of 10 min, followed by 60 V for 20 h. The gels had a thickness of 0.75 mm and dimensions of 16 x 16 cm and were stained, after completion of electrophoresis, for 30-40 min in solution of 1X SYBR Gold® (Sigma-Aldrich) according to the manufacturer's recommendations. The images of the gels were observed under UV light and were then captured and digitized using a photodocumentation imaging system (Loccus Biotecnologic L-Pix Chemi).

Images were analyzed in Bionumerics® Software version 5.10 allowing the construction of dendrograms from the level of similarity of the samples obtained from the fragment of *H. jongheana* root.

Statistical analysis, diversity index and artificial neural networks

The tests were performed in software R v. 3.56.3 (R CORE TEAM, 2020) application with the χ^2 test of Pearson, to see if there is a relationship between the presence of general fungi and the Tulasnellaceae family and if the survival rate is significance or not.

To evaluate the factors that could influence the ecological succession, a principle component analysis (PCA) was performed in software PAST v. 4.06 (HAMMER, HARPER, RYAN, 2001). Results from DGGE were analyzed with the maximum and minimum temperatures and precipitation for the month of collection from INMET data (2020).

The diversity was measured by the Shannon-Weaver index in PAST software v. 3.14 (HAMMER, HARPER, RYAN, 2001) and the contents were subjected to analysis of variance (ANOVA) and the averages compared by LSD test with Bonferroni protection test at 0.05 probability in ExpDes package in software R v. 3.5.3 (R CORE TEAM, 2020).

Artificial neural networks (ANN) have based in artificial intelligence. For that, using, percentage matrices of the Bionumerics software obtained from DGGE gels. These data were then analyzed by Gephi v. 0.9.1 software (BASTIAN, HEYMANN, JACOMY, 2009). The distance among the dots represented the correlation distance, and the wider is the line thickness shows that the correlation is stronger.

Results

During the experimental period, at 120th and 240th d, a significant seedlings survival rate was observed (Pearson's χ^2, *p-value = 0.0068*). All eight orchids seedling of *H. jongheana* maintained under greenhouse survived throughout the study period. Among 32 reintroduced seedlings, 23 survived the first 120th d and all of them remained alive until 240th d.

All sampled roots of *H. jongheana* were colonized by fungi (Fig. 1C and 1D). However, there was a change in the proportion of amplicons corresponding to Tulasnellaceae in seedlings kept in both condition, greenhouse and reintroduced (Fig. 2B). Furthermore, Tulasnellaceae was not observed in seedlings under field conditions just in 120th (Fig. 2B).

Three groups are forming in function of time (Fig. 2A). The first group is the seedlings before reintroduction and that in the field for 120th. The second group is formed by those in the field for 240th days, and the third one is comprised by the seedlings kept in the greenhouse for 120th and 240th days after reintroduction.

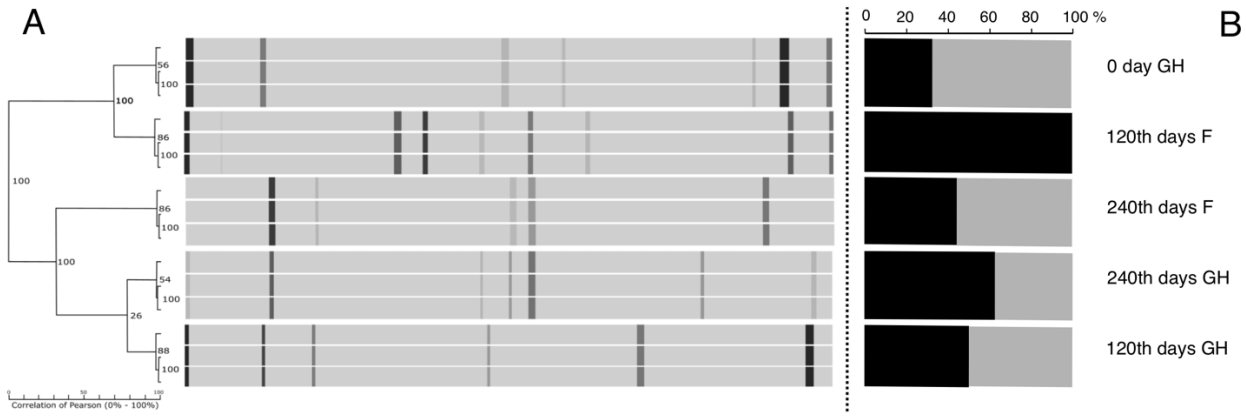

Fig. 2 - A) Pearson correlation dendrogram of fungi present in root samples in seedlings of *Hadrolaelia jongheana*, in greenhouse (GH) and reintroduction in field (F). Values represent the arms probability value for the branch, B) Proportion of mycorrhizal fungi in association with *H. jongheana* seedlings. Before reintroduction (zero day), 120th and 240th days GH and F. The grey color is the prevalence of the presence of amplicons for Tulasnellaceae obtained with the ITS1 / ITS4Tul primers and black for fungi in general with ITS1F / ITS4.

The environmental variables explain 60% and 23.2% of the fungal composition of the variability of the fungi associated to *H. jongheana* roots (Fig. 3). The maximum temperature was the main environmental variable influencing the fungal community at 240th days in field. All samples are distributed separately showing the changing over the time.

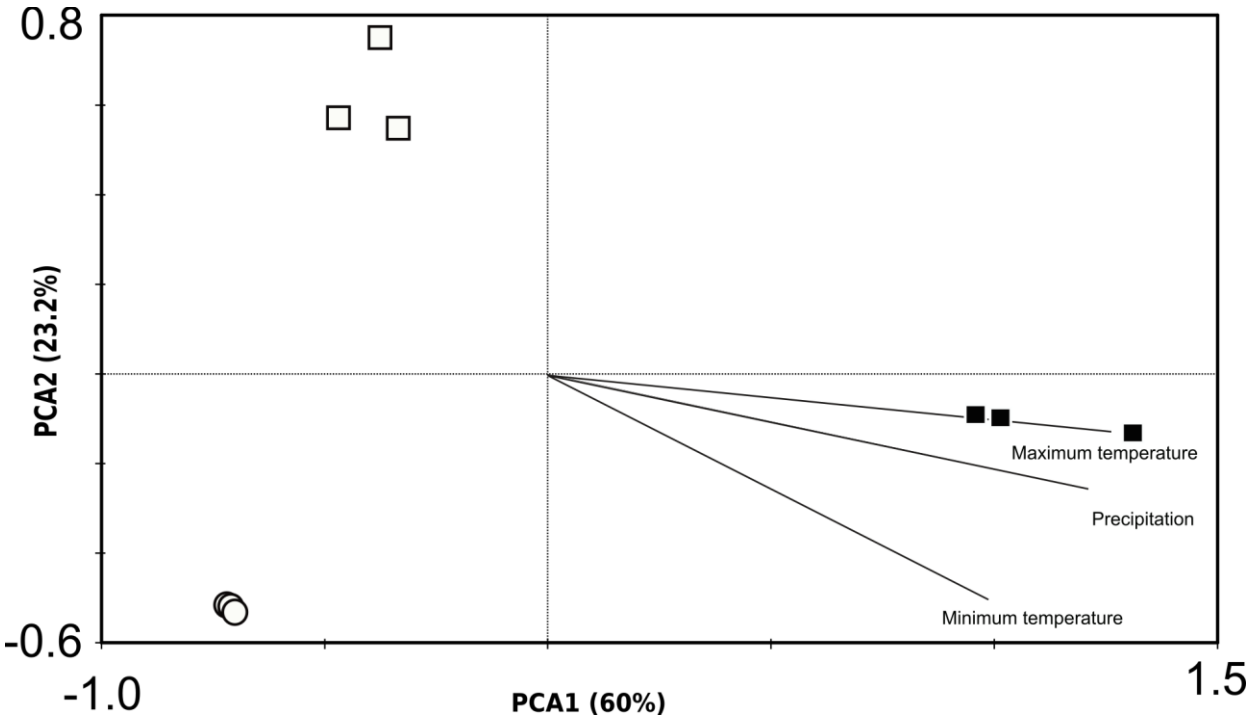

Fig. 3 - Principal component analysis (PCA) for *Hadrolaelia jongheana* plants reintroductions, A) o - field 0 day; □ - field 120th days; ■ - field 240th days and including environmental variables: maximum and minimum temperature and rainfall.

The Shannon index varied in function of the time of evaluation and growing condition (*p> 0.001* - Table 1). The index diversity is like the proportion of fungi, which is high over the time (Fig. 2B and table 1) with exception of 240th days under environmental conditions.

Table 1 – Shannon index (*H*') of the reintroduction seedlings

Time	*H*' Media (group)
240th days - Field	2.438 (a)
120th days - Field	1.734 (b)
0 day – Green House	1.391 (c)

The means followed by the same letter do not differ a 5% probability by LSD test with Bonferroni protection test.

ANN analysis revealed that reintroduced orchids have more similarity to the fungal profile than orchids before reintroduction. There is an amplicon that strongly connects orchids before and

after 120th d reintroduction but does not connect the samples before and after 120th and 240th d after reintroduction. No connection of the fungi was detected before and after 120th d of the reintroduction (Fig. 4).

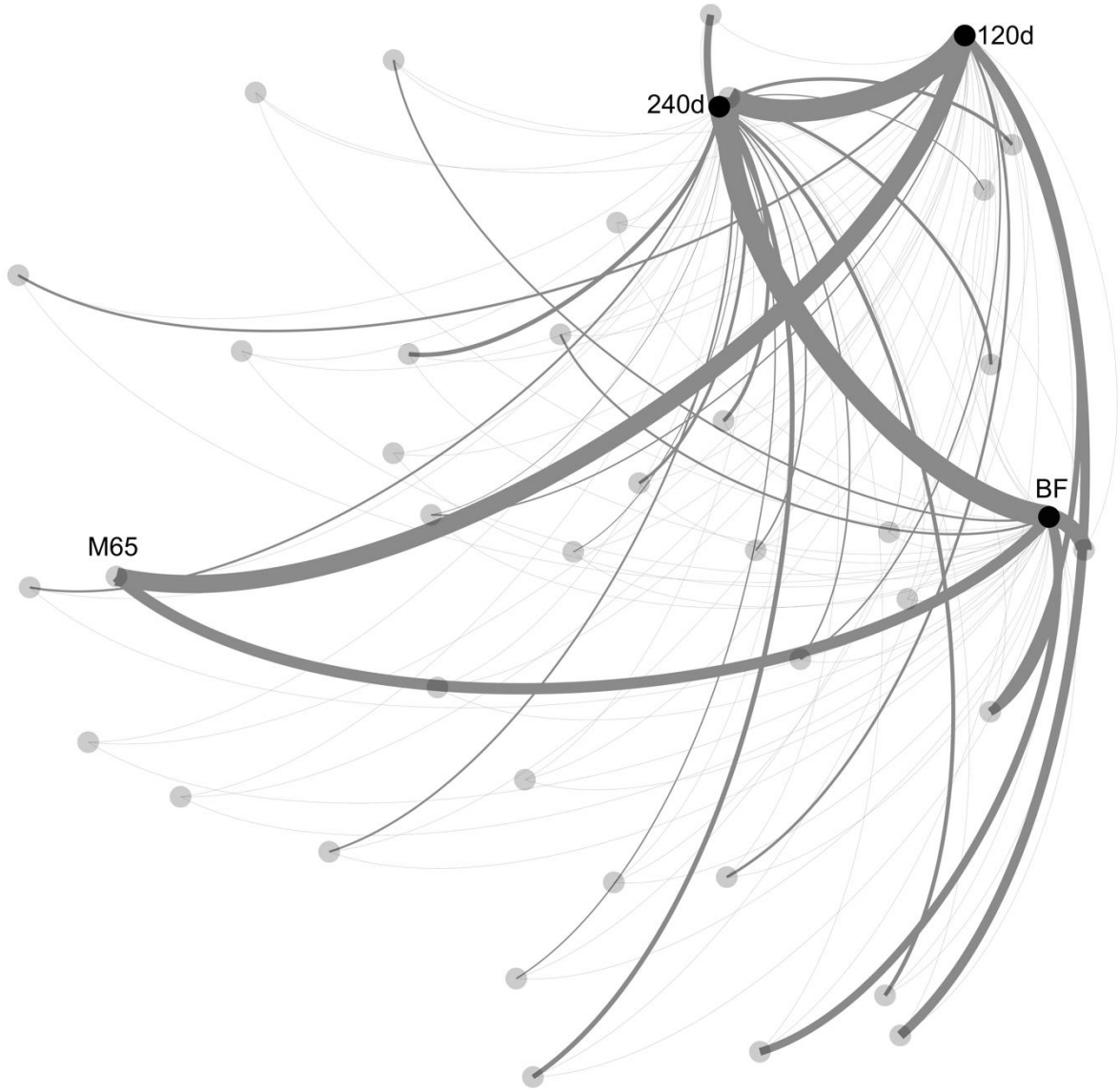

Fig. 4 - Artificial neural networks (ANN) from DGGE analysis revels the difference of fungi diversity profile among the experimental time, the fungi before reintroduction were replaced after 120thd of reintroduction. The gray dots represent a different amplicon BR: Before reintroduction, 120d: 120th and 240d: 240th days after reintroduction of *Hadrolaelia jongheana* seedlings in the natural environment. M65: fungi used for germination of microseed of *H. jongheana.*

Discussion

The survival of 100% of the seedlings in the greenhouse was probably due to the favorable conditions (humidity and temperature) for development, while 72% of the seedlings in the field survived. After five years of being introduced, Yam et al. (2010) found the survival of five native and endangered orchid species varying from 10 to 95%, while Zettler et al. (2007) found 100% survival of the mycorrhized epiphytic *Epidendrum nocturnum*. Therefore, environment factors can interfere in the survival of seedlings in the field and mycorrhization could be a key factor for survival success.

The elevation and composition of the floral community at the reintroduction site may have influenced the survival results of orchid individuals after reintroduction (SANCHEZ et al., 2013). The long, dry summer during the 240 d of the experiment reduced the reintroduced population to almost 30%, as has been reported in other studies (PARTHIBHAN, SENTHIL KUMAR, RAO, 2015). Other factors, such as moisture and the availability of mycorrhizal fungi in dry regions (PHILLIPS et al., 2014) lead to a reduction in the survival of adult individuals.

The characteristics of the forest fragments used, and the choice of reintroduction site, were also crucial since a higher mortality rate was found with phorophytes experiencing an edge effect (less than 15 meters from the edge). We can observe that the seedlings mortality at the field occurred until 120 d, after this time the survival was 100%. This suggests that microhabitat homeostasis is important to the survival of orchid seedlings, since the seedlings in fragments exposed to an edge effect experienced changes in their conditions such as light, temperature, humidity, among others (NASCIMENTO, LAURANCE, 2006). Therefore, species that occur in low density, such as the species of orchid in this study, suffer from the direct action of the changes caused by the edge effect, resulting in a lower survival rate for plants placed in that area. Environmental variables not addressed in this study, such as temperature, humidity and others, may be determinants of mortality.

All seedlings were colonized, in all evaluated times, showing the high dependence of this epiphytic orchid requirement for the interaction, maybe to optimize the process for obtaining nutrients, since there is no direct contact with soil, generally the main source of nutrients for plants (MARTOS et al., 2012). Thus, successful in orchids establishment of habitats depends not only on abiotic conditions but also biotic factors, including interactions with organisms (HUNDERA et al., 2013; PHILLIPS et al., 2014). It was observed a decrease in the Tulasnellaceae family proportion over the time, both those maintained in greenhouse and field (Fig. 2B). However, the presence of other fungi, such as endophytic ones (OLIVEIRA et al., 2014), explain and suggests that there is an dynamic interaction orchid-microorganisms and microbial succession is common.

Even the presence of pelotons was observed after 120th days, in reintroduced seedlings, no Tulasnellaceae was detected (Fig. 1C and D). This may be due to the total or partial elimination of representatives of this family in this time. In this period we observed the lowest rainfall, about 20 mm/month, that may cause desiccation of root tissue, since relative humidity decreasing is correlate to the epiphytic orchids low density or richness (HUNDERA et al., 2013). Tulasnellaceae seems to be affected positively by the pluviometric index, since at 240th day this index was 211 mm, and this group of fungi was observed again. Other aspects that should be highlighted is that the combination of primers ITS1 and ITS4Tul is not able to detect some Tulasnellaceae species (OLIVEIRA et al., 2014; TAYLOR, MCCORMICK, 2008).

The occurrence of Tulasnellaceae decreased over the time (Fig. 2B), even at greenhouse, probably is a natural process of succession by other fungi, with consequent change in their diversity.

This changing is also observed by the DGGE analysis (Fig. 2A), where a group was formed by the times of 120th and 240th days. The isolate M65 was efficient for the seeds germination of the studied orchid (BOCAYUVA, 2012), however, it is no longer dominant at 120th days, both in the greenhouse and in the field, suggesting that the M65 had a low competition capacity in natural conditions.

There was a positive relationship between the community of fungi associated with the roots of *H. jongheana* and the temperature, especially the maximum (Fig. 3), corroborating with Krömer et al. (2013), who pointed out the importance of temperature on the populations of orchids.

The diversity of fungi measured by the Shannon index showed that this value increases in the field over the time, may be because this reintroduced site is the area of natural occurrence of this orchid species, presenting environmental conditions for meet again the natural fungi community, contributing to increase in the diversity index.

The ANN indicates that soon after the reintroduction, mycorrhizal fungi introduced during germination were replaced by the native fungi of the reintroduction site. This is expected, since local fungi are more adapted to environmental conditions than those used for the germination of orchids.

We suggest that the amplicon detected that strongly connects the orchids before and after 120thd of the reintroduction is the fungus M65, used in the germination of the seeds used in the present study. However, this fungus, highly efficient in germination and possibly requiring a high energy demand, was possibly replaced by others, showing no connection with the orchid samples after 240thd of reintroduction.

The lack of connection before and after 120thd of reintroduction indicates that fungal substitution occurred rapidly, however the connection between before and after 240thd reintroduced may indicate that the fungi profile has stabilized, and the fungi prior to reintroduction could be detected. This event indicates that there was competition of the fungi in the process of colonization of the orchids soon after the reintroduction, in which the inoculum M65 was replaced, but other fungi were not replaced, although they underwent selective pressure of the native fungi, these initial fungi were able to maintain established in reintroduced individuals.

Symbiotic propagation techniques are well established (ZETTLER et al., 2007), however information on the handling of orchids with conservation purposes are scarce. Therefore, for the success of reintroduction, it is important to know the main factors that influence on the establishment and persistence in natural conditions. Those information is also of strategically important to conservation purposes (SMITH, JAMES, MCLEAN, 2010). Although succession occurs, reintroduction of *H. jongheana* was successful, showing that the use of seedling associated to mycorrhizal fungi is very important.

This work comprises the first study using tropical orchids, approaching to ecological aspects of mycorrhizal fungi association in Brazil with conservation purposes. We have demonstrated a successful method for the reintroduction of epiphytic orchids. We observed that the fungi used during the development of the plant from germination to seedling are replaced by natural fungi of the environment, causing these new populations of orchids to remain in the place of reintroduction. In this way conservation plans of orchids with the reintroduction of orchid seedlings produced in a controlled environment can be adopted for the conservation of declining populations, without the need for other actions for the development of new populations. Aspects that may be investigated in the future, such as the seasonality and longer monitoring time of reintroduced seedlings of epiphytic orchids may provide more information on reintroduction.

Conclusions

- *Hadrolaelia jongheana* previously colonized by the fungus *Tulasnella* was successful in the process of reintroduction, creating new methodologies for application in the propagation of species threatened with extinction.
- Changing in the fungi profile occurs in *H. jongheana* orchid in both, greenhouse and field. Being the temperature one of the environment variable that promote this changing.
- The reintroduction of *H. jongheana* in naturally occurring area increase the diversity of fungi associated its roots.
- The fungus M65 used in microseed orchid germination is replaced by other natives ones after 240thd of reintroduction.

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Compliance with ethical standards

Ethical approval this article does not contain any studies with human participants or animals performed by any of the authors.

Conflicts of interest

The authors declare that they have no conflict of interests.

Authors' contributions

C.A.V. and M.C.M.K designed the study. Material preparation and data collection were performed by C.A.V., M.F.B., T. G. R. V. and E.F.S.F. Analyses were performed by C.A.V., T.G.R.V and B.C.M. The first version of the manuscript was written by C.A.V. and was revised by M.C.M.K. All authors commented on previous versions of the manuscript and approved the final version.

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