

**Study of the arbuscular mycorrhizal fungi (AMF) associated
with the Azorean endemic woody plant *Picconia azorica* (Tutin)
Knobl. and their potential application on sustainable
restoration programs**

Tese de Doutoramento

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DECLARATION

The present dissertation includes results that have been published in collaboration with other researchers.

For the due effects of the provisions of paragraph 2 of article 8 of Decree-Law 388/70, the author of this dissertation states that she intervened in the conception and execution of the experimental work, in the interpretation of the results and in the writing of the manuscript: **Communities of arbuscular mycorrhizal fungi under *Picconia azorica* in native forests of Azores** published in Symbiosis, vol. 71, N. º 3, 2017 (DOI: 10.1007/s13199-017-0487-2).

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RESUMO

Os fungos micorrízicos arbusculares (FMA), simbioses obrigatórios pertencentes ao filo Glomeromycota, formam relações mutualistas com cerca de 90% das espécies de plantas terrestres. Estes fungos fornecem água e minerais à planta e em troca a planta fornece produtos da fotossíntese. As comunidades de FMA presentes na rizosfera da planta endêmica *Picconia azorica*, de duas ilhas do arquipélago dos Açores, foram estudadas com recurso a métodos morfológicos e moleculares. Os esporos de FMA isolados do solo foram morfológicamente classificados em 46 morfotipos. A caracterização molecular dos esporos foi efetuada por sequenciação de um fragmento de rADN com cerca de 1,5 kb (SSU-ITS-LSU) e resultou na obtenção de 125 sequências. Após análise filogenética estas sequências foram alocadas a 18 filotipos, as quais mostraram pertencer às famílias *Acaulosporaceae*, *Archaeosporaceae*, *Claroideoglomeraceae*, *Gigasporaceae* e *Glomeraceae*. Oito destas sequências não agruparam com nenhuma sequência previamente conhecida, o que sugere que correspondem a espécies que ainda não foram molecularmente identificadas ou que constituem novas espécies para a ciência. A análise da ecologia revelou que existem diferenças na comunidade de FMA entre as ilhas Terceira e São Miguel. A comunidade de FMA no solo da ilha Terceira é dominada por elementos das famílias *Acaulosporaceae* e *Glomeraceae*, enquanto, que em São Miguel é dominada pelas famílias *Glomeraceae* e *Gigasporaceae*. A análise microscópica das raízes de *P. azorica* revelou que as amostras se encontravam altamente colonizadas (89 – 99%) pelas diferentes estruturas do fungo (hifas, arbúsculos e vesículas), permitindo assim considerar a planta como sendo micotrófica e altamente dependente de FMA. A identificação dos fungos presentes nas raízes de *P. azorica* foi efetuada com base no fragmento de 1.5 kb do rADN e com recurso ao método de restrição terminal de fragmentos polimórficos (T-RFLP) conhecidos, no programa TRAMPR. A análise identificou 22 filotipos pertencentes às famílias *Acaulosporaceae*, *Claroideoglomeraceae*, *Diversisporaceae*, *Gigasporaceae* e *Glomeraceae*. Verificou-se que a espécie *Acaulospora brasiliensis* estava presente nas raízes de todas as plantas analisadas e que a família *Glomeraceae* foi a família mais representada com a

identificação de 13 filotipos. Esta análise revelou ainda que em ambas as ilhas na colonização das raízes dominavam espécies da família *Glomeraceae*, sendo que nas raízes da ilha Terceira a presença de *Acaulosporaceae* foi mais frequente, enquanto, que nenhum membro da família *Claroideoglomeraceae* foi identificado em amostras de raízes de São Miguel. Os resultados destes estudos sugerem que as comunidades de FMA nas florestas nativas dos Açores são determinadas por fatores históricos e geográficos, bem como pelas propriedades químicas do solo e pelas condições ambientais. Para o estudo da capacidade de colonização de plantas micropropagadas pelas espécies *Acaulospora laevis* e *Acaulospora koskei* foram isolados e sequenciados segmentos de quatro genes de *P. azorica* homólogos a outros previamente relacionados à simbiose e foi analisada a expressão destes genes por qRT-PCR durante os estágios iniciais da simbiose com FMA. Nestes ensaios a indução da expressão por *Ac. laevis* revelou ser superior à obtida com *Ac. koskei*. A influência do FMA *Funneliformis mosseae* sobre o desenvolvimento de plantas de *P. azorica* micropropagadas foi estudada. Ao fim de 8 meses de tratamento as plantas inoculadas com o FMA revelaram-se maiores e mais robustas que as plantas não inoculadas. Esta é a primeira vez em que se estuda a condição de FMA presentes na rizosfera de *P. azorica* nas florestas nativas Açorianas. A *P. azorica* demonstrou elevada dependência pelos FMA o que revela sintonia com o facto de estes FMA terem revelado potencial para serem aplicados como promotores do sucesso na utilização de plantas micropropagadas em programas de conservação e restauração de florestas nativas no arquipélago dos Açores.

Palavras-chave: fungos micorrízicos arbusculares; *Picconia azorica*; florestas nativas dos Açores; identificação molecular de FMA; diversidade; colonização de raízes; interação FMA-*P. azorica*.

ABSTRACT

Arbuscular mycorrhizal fungi (AMF) are obligate symbionts, belonging to the phylum Glomeromycota that form mutualistic symbioses with about 90% of land plant species. They provide the host plant with mineral nutrients and water, in exchange for photosynthetic products. The communities of AMF present in the rhizosphere of endemic tree *Picconia azorica* from two Azorean islands, Terceira and São Miguel, were assessed by morphological and molecular methods. AMF spores isolated from the soil were morphologically classified in 46 distinguishable morphotypes. Molecular characterisation of a c. 1.5 kb rDNA fragment, comprising SSU-ITS-LSU, resulted in 125 AMF sequences. Phylogenetic analyses assigned the sequences to 18 phylotypes, belonging to the families *Acaulosporaceae*, *Archaeosporaceae*, *Claroideoglomeraceae*, *Gigasporaceae* and *Glomeraceae*. Also, 8 phylotypes did not cluster with sequences of any described species implying that they are either species that have not yet been identified or are new to science. Ecological analyses revealed differences in AMF community composition between islands. In Terceira, the rhizospheric soil was dominated by species belonging to *Acaulosporaceae* and *Glomeraceae*, while São Miguel was dominated by *Glomeraceae* and *Gigasporaceae*. Microscopic survey of *P. azorica* root samples showed that all studied samples were highly colonised (89 – 99%) by AMF structures (hyphae, arbuscules and vesicles) which allowed us to classify the endemic plant as mycotrophic and highly dependent on AMF. The identity and diversity of AMF colonising the roots of *P. azorica* were assessed by terminal restriction fragment length polymorphism (T-RFLP) of the c. 1.5 Kb rDNA fragment. Database terminal restriction fragment length polymorphism (T-RFLP) approach in conjunction with TRAMPR package was used to identify the AMF community present in the roots. By that were identified 22 phylotypes belonging to *Acaulosporaceae*, *Claroideoglomeraceae*, *Diversisporaceae*, *Gigasporaceae* and *Glomeraceae*. *Acaulospora brasiliensis* was present in all studied roots of all sampled plants and *Glomeraceae* showed to be the more diverse family with 13 phylotypes identified. It was also observed differences in AMF community composition between islands. Although in both islands root colonization was dominated by species belonging to *Glomeraceae*, in Terceira the presence of *Acaulosporaceae* was more frequent, while

no *Claroideoglomeraceae* were identified in São Miguel root samples. All results suggest that AMF communities in native forest of Azores are determined by geographic and historical factors as well as soil chemical properties and environmental conditions. The ability of *Acaulospora laevis* and *Acaulospora koskei* to colonise micropropagated plants of *P. azorica* was tested by isolating and sequencing of partial sequences of four *P. azorica* symbiosis-related genes and analyses of gene expression by qRT-PCR during early stages of AMF symbiosis. This revealed that both AMF species varied in their capacity to induce the early stages of the plant symbiotic response, in which *Ac. laevis* showed superior to *Ac. koskei*. The influence of *Funneliformis mosseae* on the growth of micropropagated *P. azorica* was investigated. After eight months of growth, inoculated plants showed to be bigger and more robust than non-inoculated plants. This is the first time that AMF status in the rhizosphere of *P. azorica* in native forest of the Azores was investigated. *P. azorica* showed to be highly dependent on AMF which is in line with the showed potential of AMF application as promoters of plant establishment in conservation and restoration of native forests of the Azores archipelago.

Key-words: arbuscular mycorrhizal fungi; *Picconia azorica*; native forests of Azores; AMF molecular identification; diversity; root colonization; AMF-*P. azorica* interaction.

LIST OF ABBREVIATIONS (SI units not included)

AM	Arbuscular Mycorrhiza
AMF	Arbuscular Mycorrhizal Fungi
Att	Attempt
BLAST	Basic Local Alignment Search Tool
BS	Bootstrap
c.	Approximate
CA	Correspondence analysis
CBA – UAc	Centro Biotecnologia dos Açores – Universidade dos Açores
CTAB	Cetyltrimethylammonium Bromide
DNA	Desoxiribonucleic Acid
dNTP	Deoxyribonucleosides triphosphates
EDTA	Ethylenediamine-tetracetic acid
FAM	Fluorochrome (Carboxyfluorescein)
INVAM	International Culture Collection of Arbuscular and Vesicular-Arbuscular Endomycorrhizal Fungi
ERM	Extraradical mycelium
IRM	Intraradical mycelium
ITS	Internal Transcribed Spacer
LSU	Large Subunit
NCBI	National Centre for Biotechnology Information
M	Minimal medium
mRNA	Messenger RNA
MSR	Modified Strullu-Romand medium
OTU	Operational Taxonomic Unit
PCR	Polymerase Chain Reaction
PPA	Pre-penetration apparatus
PVLG	Polyvinyl alcohol lacto-glycerol
rDNA	Ribossomal DNA
RFLP	Restriction Fragment Length Polymorphism
RT	Reverse-transcription
SG	Lombo Gordo
SR	Ribeira Quente
SSU	Small subunit
TAE	Tris-acetate-EDTA
TB	Terra Brava
Tris	Tris-(hydroxymethyl)-aminomethane
T-RFLP	Terminal Restriction Fragment Length Polymorphism
TS	Serreta
U	Unit

UV	Ultra Violet
V	Volts
VIC	Fluorochrome
WM	White medium

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1. GENERAL INTRODUCTION

The Azores archipelago has singular ecosystems with native forests – Laurissilva which is a unique reservoir of biodiversity and covers less than 10% of the total area (Guimarães and Olmeda, 2008). However, these native forests had been mostly destroyed since human occupation (Martins, 1993; Borges *et al.*, 2008; Triantis *et al.*, 2010), due to expansion of agricultural land, forestation, mostly with *Cryptomeria japonica*, competition by alien species and isolation of populations (Silva *et al.*, 2009).

Picconia azorica (Tutin) Knobl. (*Oleaceae*) is an Azorean endemic plant that was overexploited for furniture construction and religious sanctuary, which led to become almost extinct in some islands (Martins *et al.*, 2011). Several propagation techniques have been proposed to be used in the restoration of natural populations of *P. azorica*. Also, several studies have demonstrated that introduction of target indigenous species of plants associated with a managed community of microbial symbionts is a successful biotechnological tool to aid the recovery of endangered populations, in which inoculation with indigenous arbuscular mycorrhizal fungi not only enhanced the establishment of key plant species but also increase soil fertility and quality.

1.1. *Picconia azorica*

P. azorica, commonly named “pau-branco”, is one of only two existent species in the genus *Picconia*, the other being *Picconia excelsa* (Aiton) DC, which is found on the Canary Islands (Spain) and the Madeira Islands (Portugal). Given its phylogeny, genetics and geography, this is a relevant taxon that should be considered a true relict (Vargas 2007).

P. azorica is an evergreen shrub or small tree growing up to 8 m tall, with simple, lanceolate to ovate, opposite leaves about 6 cm long with prominent midrib and inconspicuous lateral veins (Sjögren, 2001; Martins *et al.*, 2011). It flowers from March to July with small white flowers in axillary clusters (Fig. 1a). Its fruits are dark blue drupes about 1.5 cm long (Fig. 1b), which become ripe from July to the end of October. It is common to observe overlapping flowering and fructification, with flowers and