In vitro plantlet regeneration from mature zygotic embryos of *Pinus pinea* L.: overcoming the rooting problems

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I hear and I forget
I see and I remember
I do and I understand

Confucius (551 A.D. – 479 A.D.)

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Resumo

Regeneração *in vitro* de plântulas de embriões zigóticos maduros de *Pinus pinea* L.: superando os problemas de enraizamento

Na natureza, as plantas e os microrganismos estabelecem associações de várias ordens. Nas culturas *in vitro* de plantas, mesmo as associações favoráveis com microrganismos foram, durante muitos anos, consideradas como contaminantes. Só mais tarde, as vantagens da inoculação *in vitro* (co-cultura) foram demonstradas e as técnicas de biotização (bacterização ou micorrização) usadas com o objetivo de melhorar as condições de crescimento *in vitro*.

As dificuldades do enraizamento *in vitro* de uma das espécies mais importantes da floresta mediterrânica portuguesa, *Pinus pinea* L., conduziu à escolha deste sistema biológico, como matéria de estudo para a tese. Neste estudo, foram utilizados fungos ectomicorrízicos para otimizar a fase de enraizamento de plantas de *Pinus pinea* L. micropropagadas via organogénese. A introdução de ectomicorrízas no processo de micropropagação reativou o crescimento das raízes e induziu a melhoria dos vários parâmetros do sistema radicular adventício conduzindo a uma menor perda de plantas durante a aclimatização. Com efeito, a micorrização melhorou a funcionalidade das raízes, facilitando a absorção de nutrientes e de água.

Neste trabalho, efetuou-se também uma extensiva caracterização morfológica e molecular das ectomicorrízas associadas a *P. pinea*. Das várias co-culturas testadas, selecionou-se a interação *Pisolithus arhizus/P. pinea* para estudar os sinais bioquímicos pré-simbióticos estabelecidos durante as etapas iniciais da co-cultura *in vitro*. Para possibilitar este estudo foi desenvolvido um novo sistema de co-cultura, o qual já está patenteado. Os resultados indicam que a presença de compostos fenólicos, nomeadamente o ácido *o*-coumarico, poderão ser importantes mediadores na interação fungo/planta.

Abstract

In nature, plants and microorganisms establish symbiotic associations of various orders. However, for many years such associations were deemed unnecessary in *in vitro* cultures because the culture medium provides ample amounts of nutrients and plant growth regulators to a growing plant. Only recently, the benefits of biotization (bacterization or mycorrhization) of plants regenerated *in vitro* were demonstrated by improvements in their growth and vigor.

Pinus pinea L., which is of one of the most important species of Portuguese Mediterranean forests, can be regenerated *in vitro* from embryo cotyledons but the growth of adventitious roots induced in shoots ceases shortly after their formation. Overcoming this particular biological impediment was the study subject of the thesis. In this study, ectomycorrhizal fungi were used to improve adventitious rooting of *Pinus pinea* L. plants micropropagated through organogenesis. The introduction of ectomycorrhizae during the micropropagation process reactivated the root growth and improved several root characteristics leading to a reduced loss of plants during acclimatization. In fact, the mycorrhization enhanced root functionality facilitating the absorption of nutrients and water.

In this work, an extensive characterization of morphological and molecular ectomycorrhizae associated with *P. pinea* was also undertaken. Of the several fungus species tested, the interaction of *Pisolithus arhizus/P. pinea* was selected for studying the pre-symbiotic biochemical signals established during the initial stages of co-culture *in vitro*. To facilitate this study, a novel co-culture system was developed which has been patented. The results indicate that the phenolic compounds, in particular the *o*-coumaric acid ester might be important mediators in the interaction between the fungus and stone pine.

Abbreviations

AC - activated charcoal
AM - arbuscular mycorrhizal
ARF - adventitious root formation
BAF - biotin-aneurin-folic acid agar
BAP - benzylamino purine
CW - cool white light
DCR medium - Gupta and Durzan (1985)
DM - direct measurement;
DNA - deoxyribonucleic acid
ECM - ectomycorrhiza
GD medium - Gresshoff and Doy (1972)
GL - gro-lux
HEPES buffer - N-2 Hydroxyenthyl piperazine-N´-2-ethane sulfonic acid
HPLC-UV - high-performance liquid chromatography
IAA - indole-3-acetic acid
IM - indirect measurement
ITS - internal transcribed spacer
LC-DAD-MS Liquid chromatography - Diode array detector - Mass spectrometry
LP - medium Quorin and Lepoivre (1977)
LSU - large subunit
MMN - modified Melin-Norkrans medium (Marx 1969)
MS medium - Murashige and Skoog (1962)
NAA - naphthalene acetic acid
NCBI - National Center for Biotechnology Information
PBS - phosphate buffer saline
PCR - polymerase chain reaction
PGRs - plant growth regulators
PPF - photosynthetic photon flux
rDNA - ribosomal genes
rRNA - ribosomal RNA
RT- retention time
RTD - root tissue density
SH medium - Schenk and Hildebrandt (1972)
SRA - specific root area
SRL - specific root length

SS - standard solutions

SSU - small subunit

UC - unknown compounds

UPGMA - unweighted pair group method with arithmetic average

WS - work solutions

WPM - woody plant medium - Lloyd and McCown (1981)

WPMRI - woody plant medium root induction

WPMRE - woody plant medium root expression

WPMS - woody plant medium solid phase

WPML - woody plant medium liquid phase

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Thesis Publications

Ragonezi C, Klimaszewska K, Castro MR, Lima M, Oliveira P, Zavattieri A (2010) Adventitious rooting of conifers: Influence of physical and chemical factors. Trees - Structure and Function 24(6):975-992 doi:10.1007/s00468-010-0488-8.

Ragonezi C, Castro MR, Klimaszewska K, Lima M, Zavattieri A (2010) Influence of light quality and intensity on adventitious root formation in microshoots of *Pinus pinea* L. Acta Hort. (ISHS) 865:287-291 http://www.actahort.org/books/865/865_38.htm.

Castro MR, Ragonezi C, Klimaszewska K, Lima M, Oliveira P, Zavattieri A (2010) Mycorrhiza-like structures in rooted microshoots of *Pinus pinea* L. Acta Hort. (ISHS) 865:179-185 http://www.actahort.org/books/865/865_22.htm.

Ragonezi C, Caldeira AT, Martins MR, Salvador C, Santos-Silva C, Ganhão E, Klimaszewska K, Zavattieri A (2012) Molecular approach to characterize ectomycorrhizae fungi from Mediterranean pine stands in Portugal. Manuscript accepted for publication at the Brazilian Journal of Microbiology. ISSN 1517-8382.

Ragonezi C, Caldeira AT, Martins MR, Dias LS, Santos-Silva C, Ganhão E, Miralto O, Pereira I, Louro R, Klimaszewska K, Zavattieri A (2012) *Pisolithus arhizus* (Scop.) Rauschert improves growth of adventitious roots and acclimatization on *in vitro* regenerated plantlets of *Pinus pinea* L. Propagation of Ornamental Plants 12:139-147.

Ragonezi C, Teixeira D, Caldeira AT, Martins MR, Santos-Silva C, Ganhão E, Klimaszewska K, Zavattieri A (2012) *O*-coumaric acid ester, a potential signaling molecule detected during early *in vitro* co-culture between *Pinus pinea* L. plantlets and the ectomycorrhizal fungus *Pisolithus arhizus* (Scop.) Rauschert. Manuscript submitted.

Patent

Castro MR, Ragonezi C, Oliveira P, Zavattieri A (2010) Patent - System and method for *in vitro* culture of plants for analysis of metabolites released by the root system Nº 105239. Patent approved by the National Institute of Industrial Property (INPI).

Other publications related with the thesis are in the annexes.

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Chapter 1

General Introduction

The general introduction describes the taxonomy and biology of pines in order to provide the context for stone pine (*Pinus pinea* L.) as the subject of this research project. This is followed by a description of the problems related to *in vitro* adventitious rooting of conifers, as well as stone pine, and the original technique of *in vitro* biotization with ectomycorrhizal fungi. In addition, analysis of molecular and biochemical methods used to characterize the fungal species and to better understand the role of the signaling factors that are involved in the interaction are presented.

1. Plant material (*Pinus pinea* L.)

Conifers are the largest and most economically important group of Gymnosperms (Whetten 2001). This group is present in all continents (exceptions are the Arctic zone and the Antarctic) and consists of dominant species widely distributed most in boreal forest ecosystems and even in some tropical environments (Gernandt et al. 2011, Neale and Kremer 2011). Within this group there is the world's oldest known tree (*Pinus longaeva*) and the world's tallest tree (*Sequoiadendron giganteum*).

Conifers include six to eight families, with a total of 65-70 genera and 600-630 species (Catalogue of Life: 2007 - Conifer database). The most distinct families are shown in the phylogenetic diagram (Fig. 1). For a classification by linear sequence based on molecular data see the work of Christenhusz et al (2011).

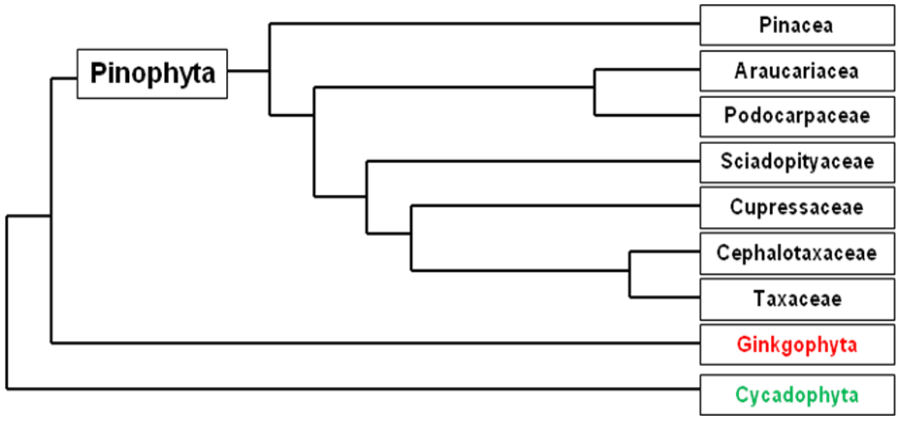


Fig. 1 - A schematic representation of the Pinophyta phylogeny (from Quinn and Price 2003).

The earliest conifer fossils are found in the Upper Carboniferous (Stewart and Rothwell 1993, Farjon 2008) and many of the extant families can be recognized by the late Triassic or early Jurassic (Singh 2006). Major fossil orders of conifers or conifer-like plants include the Cordaitales, Vojnovskyales, Voltziales and in some cases the Czekanowskiales.

Examples of contemporary conifers include: Pines (*Pinus* spp.), Spruces (*Picea* spp.), Cowtail Pine (*Cephalotaxus* spp.), Cypress Pine (*Callitris* spp.), Firs (*Abies* spp.), Larches (*Larix* spp.), Bald Cypresses (*Taxodium* spp.), Yellowwood (*Podocarpus* spp.), Yews (*Taxus* spp.), Arbor vitae (*Thuja* spp.), Junipers (*Juniperus* spp.), Cedars (*Cedrus* spp.), Douglas-firs (Pseudotsuga spp.) and Golden Larch (Pseudolarix spp.), among others.

Pinus is the largest extant genus of the Pinaceae family (Fig. 2) naturally occurring almost exclusively in the Northern Hemisphere, but introduced and widely naturalized in both hemispheres (Procheş et al. 2012) with over 100 widely recognized species (Richardson 1998, Farjon 2001, Alves-Freitas et al. 2011). Stone pine (*Pinus pinea* L.) is an economically important conifer distributed across the Mediterranean Basin (Fig. 3). In Portugal it is cultivated mainly for the production of nut seeds (pinion) for the food consumer market.

Taxonomic Hierarchy

Kingdom	Plantae – Planta, Vegetal, plants, plantes
Subkingdom	Viridaeplantae – green plants
Infrakingdom	Streptophyta – land plants
Division	Tracheophyta – vascular plants, Tracheophytes
Subdivision	Spermatophytina – Spermatophytes, seed plants, Phanérogames
Infradivision	Gymnospermae – Gymnosperms, Gymnospermes, Gimnosperma
Class	Pinopsida – conifers
Order	Pinales – pines
Family	Pinaceae – pines
Genus	Pinus L. – pines
Species	*Pinus pinea* L. – Italian stone pine

Fig. 2 - Taxonomic hierarchy of *Pinus pinea* L. (from Integrated Taxonomic Information System (ITIS), 2012).

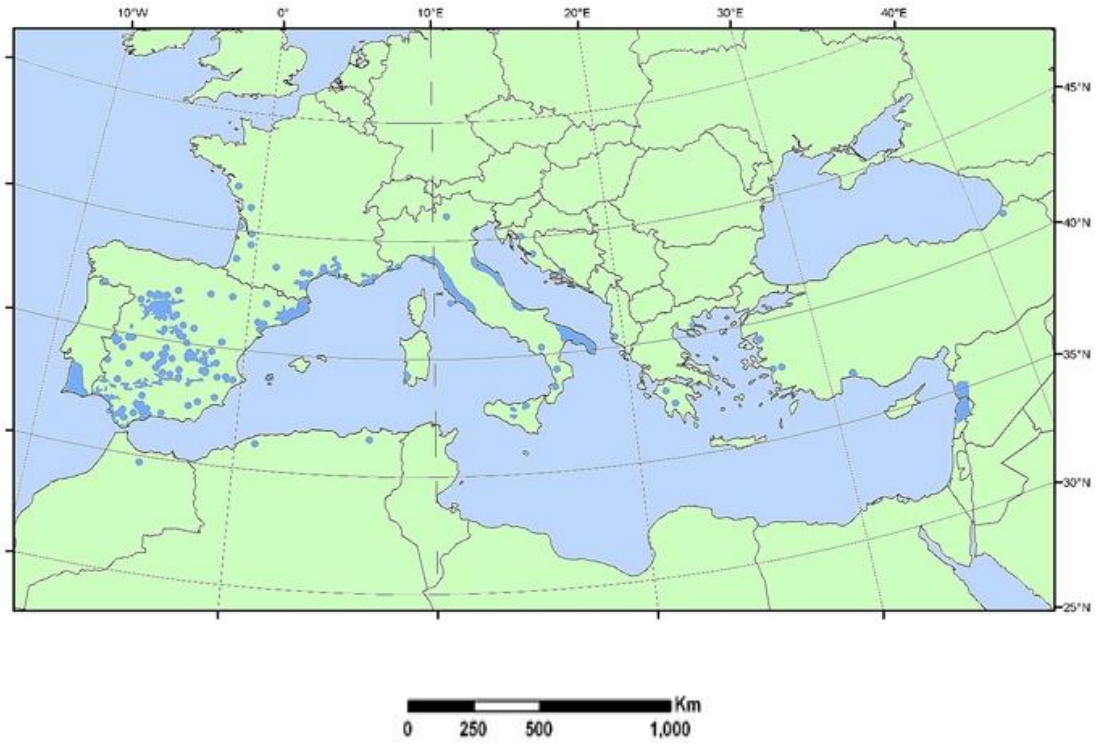


Fig. 3 - The distribution map of *Pinus pinea* L. throughout the Mediterranean Basin and adjacent zones (from Euforgen.org).

The total area covered by stone pine is around 380,000 ha (Varol and Tel 2010). The Iberian Peninsula accounts for approximately 75% of *Pinus pinea* stands, the largest area occurring in Spain with an area of 464,000 ha (BDN 2008), followed by Portugal with an area of 130,300 ha (IFN5 2010).

Stone pine trees can reach a maximum height of 25 meters, and the trunk is often short and slightly sinuous. The canopy has a horizontal spread and ascending branches that gives their adult crown a characteristic umbrella-like shape (Fig. 4). From this feature is derived one of the most common names in English: umbrella pine. Other common names that occur are: *pinheiro manso* (Portuguese); *pino piñonero*, *pino manso*, *pino doncel*, (Spanish); *pin parasol*, *pin pignon* (French) and *pino domestico* (Italian).

Fig. 4 - *Pinus pinea* L. trees in a typical pine stand in Alcácer do Sal district, Portugal.

Some *P. pinea* tree characteristics like glabrous twigs and green needles that occur in fascicles of two can be found in typical pine stands. Like other pine species, *P. pinea* is monoecious, although fecundation rarely occurs within the same tree since, in most cases, the male gametes and female cones are formed in different branching systems and maturation is sometimes not coincident on the same tree. Pollen grains are mainly transported by wind (anemophilous pollination) and fecundation takes place 2 years after pollination (Singh 2006). Cones reach maturity after 3 years and are 8-14 cm in length (Fig. 5a). Figure 5b shows a typical *P. pinea* seed without the seed-coat. They are heavy and mostly dispersed by small mammals (Fady et al. 2004). Seed production

commences after 12 to 18 years depending whether tree occurrence is in isolation or in stands (Moussouris and Regalo 1999).

Fig. 5 - Mature cone of a *P. pinea* tree (a) and seed without the seed-coat (b).

P. pinea occurs either in arid inland or coastal areas affected by salinity stress (Correia et al. 2010), but also grows in harsh environments. This species is highly sensitive to climate variants (De Luis et al. 2009b) and faces regeneration problems in many localities.

Prior to the anthropogenic range expansions of the last few thousand years, stone pine was probably confined to the Iberian Peninsula, since is the only area that can be found away from ancient trade routes (Rikli 1943). There still remains a great deal of controversy regarding the natural expansion and origin of this species. Richardson (1998) stated that it is “impossible to determine its natural range". Agrimi and Ciancio (1994) have located in the western Mediterranean zone the origin of *P. pinea,* despite the fact that some theories hold that the species is native not only to the western Mediterranean but also to the eastern Mediterranean Basin (Barbéro et al. 1998). *P. pinea* was extensively planted around the Mediterranean Sea throughout history by the Etruscans, the Greeks, the Romans and the Arabs (Fady et al. 2004, Evaristo et al. 2007).

Although it has been cultivated since the Roman period for timber (for construction and ship-building), the most economically important product derived from the tree is its seed, the pine nut. World production of pine nuts is about 20,000 tons/year (Nergiz and Donmez 2004). The main countries where pine nuts are traditionally produced and consumed are Portugal, Spain, Italy, Tunisia and Turkey.

Concerning seed production, in the case of *P. pinea*, as a nut-producing tree, seed yield is the main clonal selection criterion (Mutke et al. 2005). Due to the rise of traditional agriculture and long-distance trading, this species could have experienced a

further reduction in its genetic diversity, which may be explained by the recent expansion of the species or low mutation rates (Vendramin et al. 2007). Among widely distributed sexually-reproducing trees, stone pine may be considered an exception due to its low level of genetic diversity. Nevertheless, it should be emphasized that whereas some species can survive in harsh environments, they do not represent the norm (Godt and Hamrick 1997). An understanding of the current adaptive diversity of *P. pinea* is a prerequisite for outlining its potential distribution and the consequences that it may face from environmental changes in the future.

The economic exploitation of pine nuts and the existence of great potential for *P. pinea* improvement are some of the reasons for the development of genetic breeding programs, based on the identification of excellent genotypes by establishing clonal banks with different origins (Alonso et al. 2006). The implementation of *in situ* conservation networks, as is the case with many other forest trees around the world, is essential. Despite the fact that the rate of survival of *P. pinea* in forest fires is considerably higher than that of other Mediterranean pines (Rodrigo et al. 2004), wild fires and overgrazing are still the greatest risks faced by stone pine forests. Fire protection and preventive measures to reduce these risks should also be introduced for the conservation of this typical Mediterranean pine (Fady et al. 2004).

According to the US Food and Drug Administration, there is a growing worldwide market for pine nuts, due to the fact that consumption reduces the risk of coronary heart disease, which is attributed to the high linoleic acid content (Nergiz and Donmez 2004). Also, the seeds have a high nutritional content, being particularly rich in proteins and vitamins (Savage 2001), and are thus considered a food supplement. In addition to their nutritional value, stone pine nuts have been considered as an aphrodisiac in ancient times, by Roman poets and Greek physicians (Moussouris and Regalo 1999).

Other products of economic value include resin, bark (for tannin extraction), and pine cone shells (for fuel) (Khaldi et al. 2011). *P. pinea* is also cultivated for environmental protection: ecological restoration (afforestation of coastal areas and dunes), protection of agricultural crops (Fady et al. 2004), can potentially help mitigate desertification problems (Correia et al. 2010), provide food and shelter for local wildlife (Montero et al. 2004), and is currently considered as a viable alternative for use for abandoned farmland (Cortizo et al. 2009). It is also an aesthetically attractive tree which is planted in parks and gardens throughout the world. It has been successfully introduced in North Africa as well as Argentina, Australia, South Africa and the USA.

The great importance of the species derives from its environmental, aesthetic, and soil-conservation uses, its high economic value and its ability to survive intense fire damage. Due to its importance and that of applications, interest in the species from forest managers and researchers has increased considerably.

2. In vitro propagation

In vitro propagation, most frequently termed micropropagation, refers to the application of plant-cell and tissue-culture technology in order to improve mainly the performance of important agriculture crop species. The growth of commercial micropropagation as an industry occurred during the 1970s and 1980s (Ahamed et al. 2001) and generated a great deal of excitement among researchers, mostly due to cell totipotency through the regeneration of entire plants from single cells (Metivier 2000, Razdan 2003, Iliev et al. 2010).

Plant tissue culture, also known as *in vitro* culture is the science of growing plant cells, tissue or organs isolated from the mother plant on artificial culture media (George et al. 2008). By manipulating the culture medium composition, the plant cells transferred to the nutrient medium will start to divide and produce cell masses that will eventually produce new plants, often in large numbers (Klimaszewska et al. 2011).

The *in vitro* growth and development of a plant is determined by a number of complex factors: (a) the genetic structure of the plant (b) nutrients: water, sugars, macro- and micro-elements (c) physical growth factors: light, temperature, pH, O_2 and CO_2 concentrations, and (d) certain organic substances: plant growth regulators (PGRs) and vitamins.

Several techniques for *in vitro* plant propagation have been developed, for example, the induction of axillary and adventitious shoots, the culture of isolated meristems and plant regeneration by means of organogenesis and/or somatic embryogenesis. These different plant-tissues culture techniques can present advantages in comparison with traditional methods of agronomic, horticultural and forestry species propagation (Iliev et al. 2010). Some of these benefits include: (1) consistent production of the same genotypes over time, (2) greater genetic gains, (3) flexibility for the rapid deployment of suitable clones given changing breeding goals and/or environmental conditions, and (4) the capability for managing genetic diversity and genetic gain in plantation forestry (Park et al. 1998). Furthermore, the benefits produce plants that require fewer pesticides and which are in some cases pathogen-free (Ahamed et al. 2001).

The propagation of superior mature individuals is an effective way of achieving genetic gain by exploiting genetic variance (dominance, additive and epistatic) within a given generation without the need for proceeding through long breeding cycles (Ahuja 1993). Consequently, the regeneration of mature trees, either singly, or as part of conventional breeding programmes, could provide a powerful instrument for improving forestry management (Cortizo et al. 2009), and it is an increasingly essential tool for all propagation programs.

In the case of trees, conventional breeding is not as straightforward since in some cases they have long life cycles, are self-incompatible and very slow to mature (Merkle and Dean 2000, Campbell et al. 2003). Maturation is an important issue since it induces changes in meristem behavior, thus reducing the propagation potential of forest trees; also, the economic value of a tree is better assessed after it reaches maturity (Greenwood 1995, von Aderkas and Bonga 2000, Mitchell et al. 2004).

3. *In vitro* propagation in conifers

Conifer cultures are generally initiated from immature or mature zygotic embryos. The uses of germinating embryos cotyledons or primordial shoots excised from seedlings or trees are less frequent (Bonga et al. 2009). In previous, most efforts were focused on propagation by first inducing adventitious shoot formation, primarily from cotyledons, and then roots by means of organogenesis. Success on a commercial scale has been limited to a few species, for example radiata pine (*Pinus radiata*) (Bhowmik and Matsuiz 2001, Bonga et al. 2003). Developed over the last two decades, somatic embryogenesis is considered a more effective method (Bonga et al. 2003), which is different from organogenesis because organs, such as shoots and roots, have one primary pole or growing point, whereas embryos are bipolar structures with both shoot and root meristems (Bhowmik and Matsuiz 2001, Preece 2003).

Distinct steps in organogenesis include: (1) establishment or bud induction, or both; (2) bud and shoot development and multiplication; (3) rooting of developed shoots; and (4) hardening of plantlets (Saborio et al. 1997). In the case of somatic embryogenesis, the method can produce any number of zygotic-like somatic embryos and plants from one seed, thus providing a standard for mass clonal propagation (Klimaszewska et al. 2007). Although somatic embryogenesis technology has worked well with many conifer species using zygotic embryos as starting material, attempts to achieve the same result with adult conifers have failed (Klimaszewska et al. 2011).

Since the genetic potential for features (height growth, branching and crown shape) cannot be evaluated until the tree is fully-grown, it is important to develop methods for propagating conifers in the mature stage (von Aderkas and Bonga 2000). In the last 25 years there have been several reports regarding the *in vitro* culture of conifers (Horgan 1987, Ewald 1998, Chang et al. 2001, Parasharami et al. 2003, Prehn et al. 2003, 2003, Oliveira et al. 2003, Renau-Morata et al. 2005, Alonso et al. 2006, Zavattieri et al. 2009, Salaj et al. 2007, Bonga et al. 2010).

Within the *Pinus* genus, successful micropropagation of *explants i*s reported for a number of species such as *P. taeda* (Mott and Amerson 1981), *P. pinaster* (Dumas and Monteuuis 1995), *P. ayacahuite* (Saborio et al. 1997), *P. nigra* (Özkurt et al. 2008), *P. sylvestris* (De Diego et al. 2009), *P. maximartinezii* (Robledo et al. 2009), *P. radiata* (Zhang et al. 2009), *P. massoniana* (Zhu et al. 2010), *P. kesiya* (Choudhury and Kumaria 2010), and *P. peuce* (Stojičić et al. 2012).

Micropropagation of *P. pinea* via organogenesis has been reported during the last few decades. The most common method, it is based on the induction of shoot buds from cotyledonary explants dissected from mature seeds and in most cases, cultured in media supplemented with some varieties of PGRs (see point 9)

4. *In vitro* rooting

Conifers have a major role to play in reforestation strategies, and despite the studies that have been published, current research on their vegetative propagation has not been satisfactorily investigated. One of the most common problems encountered in the micropropagation of conifers is the reduction in the ability of cuttings to root (Cortizo et al. 2009, Zhang et al. 2010). This problem is mainly related with maturation, which affects reproductive competence, morphology, and growth rate (Greenwood and Hutchison 1993, Libby and Ahuja 1993, Mitchell et al. 2004).

The rate of spontaneous rooting is generally low in micropropagated cuttings in conifers (Burns et al. 1991, Budimir and Vujicic 1992, Normand et al. 1996, Stojičić and Budimir 2004, Ewald 2007a). In most conifer micropropagation protocols, rooting treatment is required in order to increase the rooting rate (Ragonezi et al 2010b).

5. Adventitious root formation

Esau (1953) defined the term "adventitious root" as a root that arises on an already lateralized root axis or at a place on the plant that is not itself a root (e.g. on a shoot).

These roots may occur naturally from stem tissue or may be induced by different conditions: a stressful environment, mechanical damage or the tissue-culture regeneration of shoots (Li et al. 2009). Adventitious root formation (ARF) is essential for plant growth regulation and development since it is a key step in the vegetative propagation of woody or horticultural species (Hess 1994, Sorin et al. 2006). The inability to induce ARF in conventional cuttings or tissue culture is a major limiting factor when cloning plants for genetic improvement and/or commercial applications (Metivier 2000).

ARF is a complex developmental process that consists of three successive but interdependent physiological phases: induction, initiation and expression. Each of these phases has different requirements (Li et al. 2009). The chemical and physical factors that affect rooting include: PGR´s, nutrients (mainly the carbohydrate source), temperature and light (Ragonezi et al. 2010b).

An efficient rooting treatment can lead to a high rate of rooting and higher quality of the root system (Ragonezi et al. 2010b). Root number, length, and the absence of callus at the base of the shoot could have an influence on plant behavior in the *ex vitro* phase (De Klerk et al. 1999, Hartmann et al. 2002).

In the past few years a number of studies have been published describing attempts to improve rates of *in vitro* rooting in many species: *Pinus armandii* var. *amamiana* (Ishii et al. 2007); *Larix* sp. (Ewald 2007a); *Taxus baccata* L. (Ewald 2007b); *Juniperus phoenicea* (Loureiro et al. 2007); *Pinus pinea* L. (Zavattieri et al. 2009, Ragonezi et al. 2010a); *Pyrus communis* L. (Sun et al. 2009); *Teucrium fruticans* L. (Frabetti et al. 2009); *Olea europea* L. (Padilla et al. 2009); *Quercus rubra* L. (Vengadesan et al. 2009a); *Citrus sinensis* L. Osbeck × *Poncirus trifoliata* L. Raf. (Montoliu et al. 2010); and *Pistacia vera* L. (Benmahioul et al. 2012).

Also, some efforts using biotization procedures (for definition of this, see point 7 of this chapter) to overcome *in vitro* rooting and improve transplantation survival rates can be cited in different works and for different species: Ragonezi et al. (2012) with *P. pinea* and *P. arhizus*; Normand et al. (1996) used *Hebeloma cylindrosporum* in the rooting and acclimatization phases of *P. sylvestris*; also with this pine species, Niemi et al. (2000) associated with *Pisolithus tinctorius*; Sarmast et al. (2012) evaluated the application of *Agrobacterium rhizogenes* with *Araucaria excelsa* R. Br. var. *glauca*; in Gosal et al. (2008) *Populus deltoides* plantlets were biotized during hardening with *Piriformospora indica* and *Pseudomonas fluorescens*; Chittora et al. (2010) working

with micropropagated *Terminalia bellerica* Roxb plants inoculated with *Piriformospora indica* during *ex vitro* acclimatization; Vettori et al. (2010) analyzed the effect of *Azospirillum brasilense* Sp245 on the micropropagation of three fruit rootstocks: *Prunus cerasifera*×*P*. *spinosa*, *Prunus persica* × *P. amigdalus*, and MM 106 apple (Northen Spy×M1); Martins (2008) worked on micropropagated *Castanea sativa* plants and *Pisolithus tinctorius*.

Certain combinations of ectomycorrhizal fungi and proper procedures of co-culture could be exploited for the *in vitro* root development improvement of stone pine or any other target species. Based on taxonomic and ecological extrapolation, an estimated 86% of terrestrial plant species acquire mineral nutrients via mycorrhizal root symbionts (Brundrett 2009). Pinaceae is considered the oldest extant plant family that is associated with ectomycorrhizal fungi (Hibbett and Matheny 2009).

6. Mycorrhizae

Since the term symbiosis was introduced by De Bary in 1879, the importance of associations among the numerous organisms has increased. Mycorrhizae are symbiotic structures formed between plant roots and fungi. Plants provide photosynthetically-fixed carbon and a habitat for the fungi, whereas mycobionts provide dissolved- and organically-bound nutrients (Smith and Read 2008). Fungal activity represents an important element of active biomass in forest ecology, since establishment, survival and decomposition in the forest trees dynamics are largely dependent on these organisms (Rosling 2003).

Seven distinct types of mycorrhizae are recognized, but several of them are very similar (Brundrett 2002). Endomycorrhizae and Vesicular-arbuscular mycorrhizae (AM) are the most widespread types. Ectomycorrhizae (ECM) occurs in certain families of Gymnosperms and Dicotyledons and in one Monocotyledon genus (Brundrett 2009). The remaining types of mycorrhizae are restricted to specific plant families. The main differences between a non-mycorrhizal root and a colonized root by either endo- or ectomycorrhizal fungi can be found in Figure 6.

Brundrett (2002) distinguished the various stages in the beginning of the association:

1 Fungi attracted by exudates proliferate on the surface of plants;
2 Fungi develop mechanisms for penetrating living plants without causing harm;
3 The space within living plants becomes an important habitat for these endophytes; providing them with shelter from adverse soil conditions, parasitism and predation;

4 Fungi become dependent on the host for energy;

5 Absorptive hyphae within plants increase their surface area and permeability.

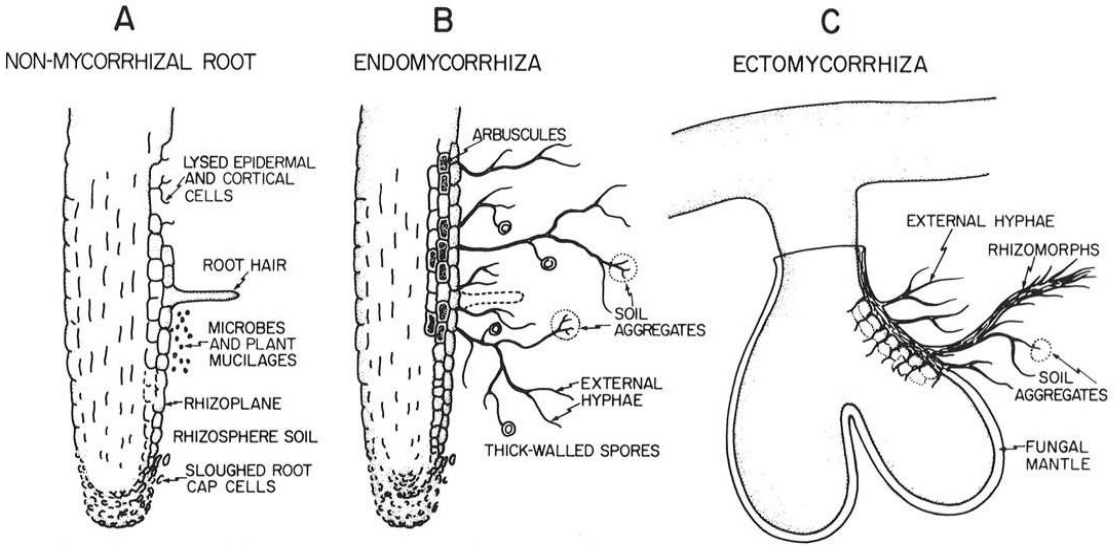


Fig. 6 - Morphological changes in roots as they become mycorrhizal and the effects of those changes on the development of a mycorrhizosphere. A - Generalized non-mycorrhizal root with root hairs and indicated sources of organic materials available as substrates for rhizosphere microorganisms. B - Endomycorrhiza with indicated morphological changes such as reduced tissue sloughing, lack of root hairs, presence of external hyphae, thick-walled spores, and associated soil aggregates; no obvious change in surface area. C - Ectomycorrhiza indicating dramatic morphological changes such as development of a fungal mantle plus extensive external hyphae and rhizomorphs and associated soil aggregates, loss of root hairs, and greatly increased branching and surface area (from Linderman 1988).

The main role of this association is the acquisition of nutrients by exploring the soil volume around the host with the aid of the hyphae, which are more responsive and more extensive than the roots themselves (Van der Heijden et al. 2006b, Requena et al. 2007, Turk et al. 2008, Smith and Smith 2011, Cairney 2011, Kiers et al. 2011). Mycorrhizal colonization tends to be reduced when nutrient availability is high (Kiers and van der Heijden 2006) and may become inactive or be lost by attrition. Also, they may experience cycles of dormancy and activity, which is a common feature in the perennial root system of trees (Kottke and Oberwinkler 1986).

6.1 Ectomycorrhizae

Within ectomycorrhizal symbioses, the host (plant roots) and the symbiont (ECM fungi) function collectively as an entity. The development of ECM in plants frequently allows them to establish in habitats that neither symbiont may be able to occupy independently (Nehls et al. 2000). ECM fungi include at least 6000 species (Hibbett et al. 2000) and involve economically important woody plant families and many lineages

of fungi that generally belonging to Basidiomycota (agarics, bolets) and Ascomycota (truffles) (Brundrett 2009, Tedersoo et al. 2010a).

ECM symbioses are restricted to <5% of terrestrial plant species (Landeweert et al. 2001) and are ubiquitous in the Pinaceae family (Le Page et al. 1997). This mutualistic relationship among ECM fungi grants conifers an ecological advantage for surviving harsh conditions where climate is strongly seasonal and soils are nutrient-poor (Castro et al. 2010). Other families that are associated with ECM fungi include Fagaceae, Myrtaceae Dipterocarpaceae (Brundrett 2002, Smith and Read 2008, Bonfante and Genre 2010).

For mycorrhizated plants the modifications in both cell organization and physiological and morphological facets are associated with numerous benefits. The fungus improves plant nutrient uptake by means of rhizosphere exploitation and in return it receives carbohydrates that are essential for completion of the fungal life cycle (Bonfante and Anca 2009). The symbiont may also alleviate the environmental stress caused by chemicals, herbivory, pathogens, fire or drought (Smith and Read 2008), and as biofertilizers they may counteract fertilization excess and thus promote sustainable agriculture (Bonfante and Genre 2010).

Ectomycorrhizae are characterized mainly by the presence of a fungal sheath (mantle), which adheres to the root exterior and forms a hyphae structure (Ammarellou and Saremi 2008). The fungus mycelium is connected to the extramatrical hyphae that explore the substrate, and is responsible for nutrition and water uptake (Barker et al. 1998). One of the most striking features of the ECM root is the Hartig net which extends into the root, penetrating between epidermal and cortical cells. The Hartig net forms a crossing point from the inner zone of the mantle through which the symbionts exchange materials (Barker et al. 1998, Bending et al. 2006, Frey-Klett et al. 2007). This is the primary zone for nutrient transfer in the association (Burgess et al. 1994, Dell et al. 1994, Brundrett 2004). In Figure 7 there is a schematic representation of ectomycorrhizal development events.

6.2 Ectomycorrhiza-like structures

As mentioned before, development between the ECM fungi and the host plant involves many changes in root physiology and morphology. Both root elongation and root hair formation are suppressed and short lateral roots go through dichotomous branching that generally culminates in the formation of coralloid structures. In conifers, externally

supplied fungal exudates, extracts, or synthetic auxins can partially mimic the effect of mycelium in inducing root proliferation and dichotomous branching of lateral roots (Smith and Read 2008). Normally in the *Pinus* genus, ECM roots are usually dichotomously branched, as described by Agerer (1987-2002), and in some cases extensive dichotomous and coralloid branching of lateral roots can occur without fungal intervention, and this kind of formation is known as an ectomycorrhiza-like structure (Castro et al. 2010).

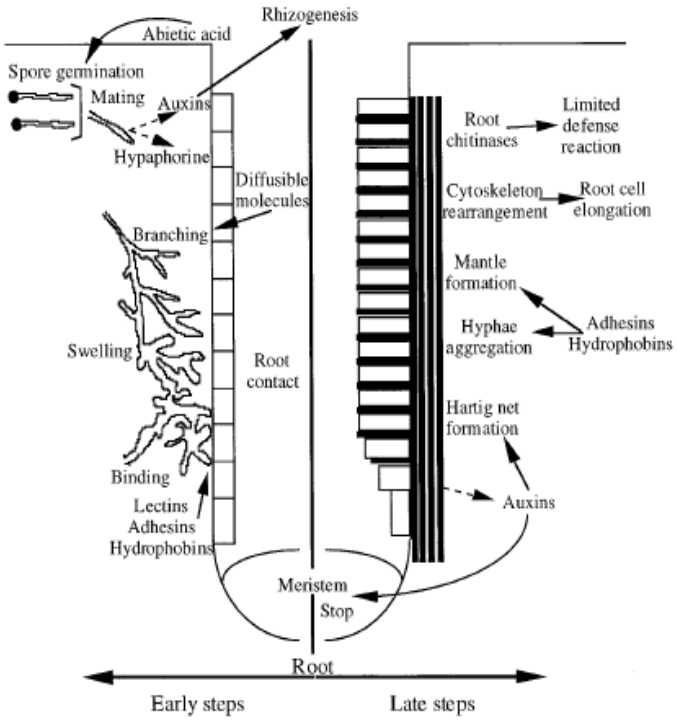


Fig. 7 - Schematic representation of ectomycorrhizal development. Morphological events taking place during early (left) and late (right) stages of Ectomycorrhizal formation are indicated (from Baker et al. 1998).

Some research has been carried out on the role of the plant hormones and fungal/root exudates in the production of ECM or ECM-like structures in pine (Slankis 1973, Faye et al. 1980, Rupp and Mudge 1985, Gogala 1991, Castro et al. 2010). Faye et al. (1980), studying *P. pinaster*, demonstrated that the host plant genome contains all the genetic information required to form an ECM-like organ. In the work of Castro et al. (2010), ARF regenerated by *P. pinea* microshoots as well as axenic embryo root cultures developed ECM-like structures. It was demonstrated that these structures appeared in all experimental settings tested, with the frequency of dichotomous branching increasing with the reduction of macronutrients in the medium and also in cultures that spent more than one month on the same co-culture medium (which is similar to drought conditions in nature). These findings support those of Kaska et al. (1999), in which nutrient-limiting conditions were required for the formation of both

spontaneous and chemically-induced dichotomous and coralloid branching in three different species of pine: *P. taeda*, *P*. *halepensis* and *P*. *muricata*. A combined exogenous application of IAA and ethylene induced root responses equivalent to the presence of truffle mycelium in both host plant (*Cistus incanus*) and the non-host plant (*Arabidopsis thaliana)* (Splivallo et al. 2009)

PGRs supplied by the ECM fungi have an effect on the thickness, extension and branching of roots, and, when applied experimentally, can induce similar root morphologies as in the absence of fungus (Kaska et al. 1999, Barker and Tagu 2000). By analyzing the anatomical aspects of the ECM-like structures, the similarity between extensive dichotomous branching of lateral roots that grow without fungal presence and those roots derived from the fungal inoculation can be observed. Due to this association, it may be difficult to diagnose ECM roots without histological and anatomical studies. Many theories may be developed on the formation of ECM-like structures and the most evident of them are, nutrient limiting, genetic information and chemically induced roots. Nevertheless, this topic has not been sufficiently investigated and needs to be further examined.

6.3 - Molecular characterization of mycorrhizal fungi

The identification of ECM fungi species is a difficult task. Traditionally, ectomycorrhizae have been identified by using colour, shape and other macroscopic features (Agerer 1987-2002). On the other hand, many fungi species have not been described by morphological methods and are rarely identified using only morphological techniques (Iotti and Zambonelli 2006). Identification remains largely dependent on initial analysis based on morphotyping, and a high level of skill is required by the analyst (e.g. personal experience, ability, rough analysis) (Rosling 2003).

The most reliable approach to the study of ectomycorrhizal community composition is a combination of morphological and molecular identification techniques (Gamper et al. 2009, Walbert et al. 2010, Bahram et al. 2011, Zambonelli et al. 2012).

In the 1990s, a revolution in molecular tools addressed the cultivability issue and substantially enhanced the identification of ECM fungi *in situ* (Gardes et al. 1991b, Egger 1995, Horton and Bruns 2001, Anderson and Cairney 2004). With the application of these techniques, many common yet unidentified ECM fungi could be taxonomically assigned (Vrålstad et al. 2000, Kõljalg et al. 2002). Since this molecular

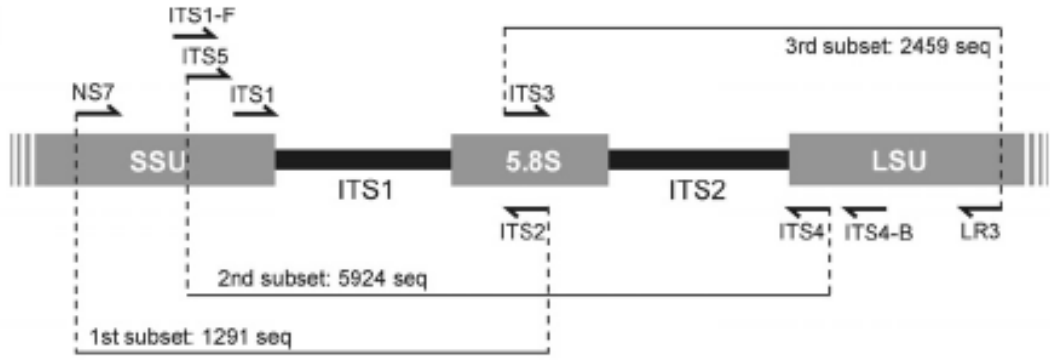
revolution, the reliability of the identification of ECM fungi species has been greatly improved through taxonomic analysis, phylogenetic relationships within the major groups of mycorrhizal fungi and the use of deoxyribonucleic acid (DNA) sequence databases (Horton and Bruns 2001). In recent years, a variety of DNA-based methods have been developed in order to identify ECM fungi (Landeweert et al. 2003a, Reich et al. 2009, Bonito et al. 2011) and also to verify the genetic variation inside a specific group (Alves et al. 2007, Caldeira et al. 2009).

The most important methodological advance in the study of ECM communities has been the application of polymerase chain reaction (PCR) based techniques (Mullis and Faloona 1987, Gardes et al. 1991b, Lanfranco et al. 1998, Landeweert et al 2003a, Morris et al. 2008). PCR-based techniques targeting ribosomal DNA regions are widely employed because of their specificity and sensitivity (Iotti and Zambonelli 2006). These regions combine the advantages of high copy number, highly conserved sequence tracks that may serve as sites for primer design, and variable regions between the priming sites (Horton and Bruns 2001).

Most molecular studies of ECM fungi involve analyses of the internal transcribed spacer (ITS) region. This nuclear region, which is well known in different fields of molecular biology and fungal systematics, lies between the small subunit (SSU) and the large subunit (LSU) ribosomal RNA (rRNA) genes and contains two non-coding spacer regions separated by the 5.8S rRNA gene (Fig. 8a,b). In fungi, it is typically about 650-900 bp in size, including the 5.8S gene. It is usually amplified by either the universal primer pair ITS1 and ITS4 (White et al. 1990, Gardes et al. 1991b, Gardes and Bruns 1993) or even with the pair ITS1 and ITS5 which has a slightly larger fragment.

The similarity of sequence of the ITS region is widely used in taxonomy and molecular phylogeny studies for identifying and distinguishing species, due to the high degree of variation between closely related species (Baldwin et al. 1995, Gomes et al. 2002, Kanchanaprayudh et al. 2003, Mello et al. 2006, Rajaratnam and Thiagarajan 2012). Several studies also use short standardized DNA regions namely “barcodes” for identifying biological material (Chase and Fay 2009). Molecular DNA barcoding of fungi identification has, during the last 15 to 20 years, become an essential part of fungal ecology research and has provided new insights into the diversity and ecology of many different groups of fungi (Horton and Bruns 2001, Anderson and Cairney 2004, Seiffert 2009). At present, metagenomic approaches and pyrosequencing are promising tools in the identification of microbial communities (Uroz et al. 2012, Tedersoo et al. 2010b).

a



b

Primer	Author	Primer sequence	Position
Forward primers			
NS7	White et al. 1990	GAGGCAATAACAGGTCTGATGC	1403-1426
ITS1-F	Gardes and Bruns 1993	CTTGGTCATTTAGAGGAAGTAA	1723-1744
ITS5	White et al. 1990	GGAAGTAAAAGTCGTAACAAGG	1737-1758
ITS1	White et al. 1990	TCCGTAAGGTGAACCTGCGG	1761-1779
ITS3	White et al. 1990	GCATCGATGAAGAACGCAGC	2024-2045
Reverse primers			
ITS2	White et al. 1990	GCTGCGTTCTTCATCGATGC	2024-2043
ITS4	White et al. 1990	TCCTCCGCTTATTCATATGC	2390-2409
ITS4-B	Gardes and Bruns 1993	CAGGAGACTTGTACACGGTCCAG	2526-2548
LR3	Vilgalys and Gonzalez 1990	CCGTGTTTCAAGACGGG	3029-3045

Fig. 8 - Commonly used primers used for amplifying parts of the entirety of the ITS region. a) Relative position of the primers, design of the subsets and number of sequences in each subset. b) Primer sequences, references and position of the primer sequence according to a reference sequence of *Serpula himantioides* (AM946630) stretching the entire nrDNA repeat (from Bellemain 2010).

7. Biotization

Biotization could be defined as the use of beneficial microbial inoculants(s) in *in vitro* cultivated plant material for promoting developmental and physiological changes that may: enhance growth, induce plant disease resistance for dealing with biotic or/and abiotic stress, and among other benefits for producing useful compounds.

The *in vitro* culture conditions result in plantlets with altered morphology, anatomy and physiology, which may be susceptible to biotic and abiotic stresses in acclimatization (Vestberg et al. 2004, Chandra et al. 2010); also this phase, this can make the cost-intensive process higher in plantlet production. The transplantation stage continues to be a major bottleneck in the micropropagation of many plants (Saadat and Heenerty 2001, Hazarika 2003). As a result, significant losses can occur during the

acclimatization phase due to the combined effect of nutritional and environmental stress to microplants (Grunewaldt-Stoecker 1997, Swain et al. 2010). In particular, fresh-rooted plants frequently demonstrate a delicate and inefficient root system, stomata with vulnerable closure mechanisms, lower cuticle production and water stress followed by death (Vettori et al. 2010).

During recent years, numerous studies have focused on the application of colonizing microorganisms (e.g. rhizobacteria and mycorrhizal fungi) in rooted plants. They have made it possible to increase the adaptive capacity and consequently the survival rate of micropropagated plants in the acclimatization phase due to their ability to induce plant resistance to different forms of environmental stress (Vettori et al. 2010, Ragonezi et al. 2012).

The following are examples of biotization between several plant species and microbial inoculants: Gay et al. (1992) used ECM fungi as a tool for enhancing the rooting of micropropagated cuttings of *Pinus halepensis*; Martins et al. (1996) also reported *Amanita muscaria, Laccaria laccata, Piloderma croceum* and *Pisolithus tinctorius* as useful in the acclimatization of micropropagated plantlets of *Castanea sativa*. Grange et al. (1997) studied the effect of different genotypes of *Hebeloma cylindrosporum* on *in vitro* rooting of micropropagated cuttings of *Prunus avium* and *P. cerasus. Paxillus involutus* with seedlings of *Pinus sylvestris* was evaluated by Rudawska and Kieliszewska (1997). Reddy and Satyanarayana (1998) screened ECM fungi, *Cenococcum geophilum*, *Laccaria laccata, Paxillus involutus* and two isolates of *Pisolithus tinctorius*, in order to inoculate micropropagated plantlets of *Populus deltoids*. Also, *P. tinctorius* was used in *Q. suber* woods (Díez et al. 2000). In the work of Senthilkumar et al. (2008), *Oryza sativa* L. was inoculated with rhizobia; *Glomus deserticola* was used in *Prunus avium* (Lovato et al. 2006); *Glomus etunicatum* in the inoculation of *Curcuma zedoaria* (Miachir et al. 2004); strawberry and banana microplants were inoculated with arbuscular mycorrhizae respectively (Murphy et al. 1997, Mandhare and Suryawanshi 2005).

Nonpathogenic organisms in plant tissue culture in order to improve the micropropagation system can be exploited once the conditions in the co-culture are optimized. Such microorganisms would not only act as inducers of the responses against stress resistance but may also occupy microsites on the host plants, making them unavailable to pathogens (Nowak 1998).

Although the biotization technique is of the greatest importance for the growth, improvement and development of the micropropagated plantlets, some disadvantages in the establishment of the association may be highlighted, such as inoculum contamination and unexpected behavior of the symbiont and the host under *in vitro* conditions.

In many cases, the colonization process among host-microorganism interactions is initiated before actual physical contact (Harrison 2005). The initial stages of mycorrhizal colonization events are dependent both on fungal growth and rhizospheric signals (Martin et al. 2001, Badri et al. 2009), mainly through root exudates that mediate the interactions with neighboring plants and microorganisms (Weir et al. 2004, Bais et al. 2006, Broeckling et al. 2008). Current research into ECM development and functioning is aimed at understanding this plant-microbe interaction and the developmental and physiological processes that underly the colonization and morphogenesis modifications.

8. Signalling

Signaling is a crucial step in any host/symbiont interaction. During all the stages (pre-symbiotic, physical contact and mycorrhization) the signals exchanged between plant and fungi are fundamental. Some aspects, for example the rhizosphere monitorization, the research of the chemical signals and following the evolution of the interaction are extremely important.

As with many host-microbe interactions, it is possible to describe the beginning of the colonization process with signaling (pre-symbiotic stage) between the two partners progressing to the symbiosis stage. The pre-symbiotic phase of the interaction culminates in a physical encounter between symbionts, by means of the adhesion of the fungus to the root surface (Barker et al. 1998, Pandey 2002, Bonfante and Genre 2010). These signals lead to a complex development of specific structures in both the plant and the fungus, observable by morphological transformations. Signaling is a critical constituent of any symbiosis; nevertheless, very little is known about the nature of these signals and how this process is instigated.

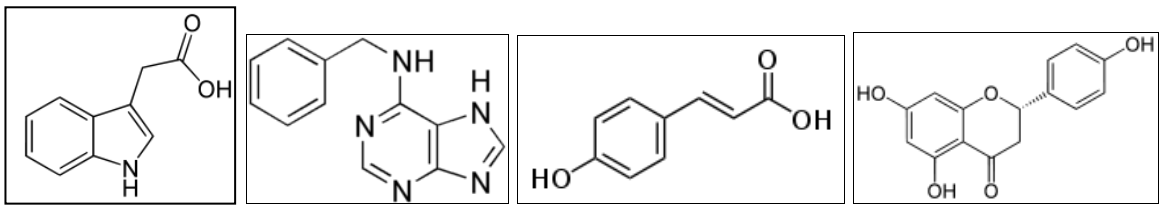
Mycorrhizal fungi are able to achieve an intimate association with their host. The fungus must deal with host protection mechanisms and be able to initiate nutrient transfer across the root-fungus interface (Reis et al. 2011). In this way, intense cell activity occurs both before and after physical contact between partners. Up- and down-regulation of gene expression is a major mechanism for controlling ectomycorrhizal

symbiosis development and functioning (Martin 2007). Signaling evidence of fungal origin may be involved in this process and may be different from the signals involved before the fungus penetration into the root tissue (Podila 2002).

Communication in the rhizosphere is essential for many interactions and their corresponding evolution. Identification of the molecules that participate in this colonization at the developmental and physiological level is extremely important, especially for understanding the host-symbiont interaction (Baptista et al. 2011, Felten et al. 2012). According to Martin et al. (2001), molecules that control the interactions can be classified as follows:

• Tropism of hyphae for host tissues (rhizospheric signals);
• Attachment and invasion of host tissues by hyphae (adhesins, hydrolases);
• Induction of organogenetic programs in both fungal and root cells (hormones and secondary signals);
• Facilitating survival of the mycobiont, despite plant defense responses;
• Coordinating strategies for exchanging carbon and other metabolites for *in planta* colonization and for balancing growth of the soil fungal web with its role in gathering minerals from the soil.

The early plant host signals secreted into the rhizospheric include auxins, flavonoids, cytokinins, alkaloids, strigolactones and other metabolites. Recently, plant phenolic compounds (*p*-coumaric acid, coumarin, naringenin and other flavonoids) have also been cited as potential signalling candidates during mycorrhizal formation (Lynn and Chang 1990, Mandal et al. 2010, Bonfante and Genre 2010, Amalesh et al. 2011, Plett and Martin 2012, Hassan and Mathesius 2012). Some of the chemical structures of these compounds appear in Figures 9 and 10. These substances may be involved in the positive responses between the host and the symbiont.



A **B** **C** **D**

Fig. 9 - Chemical structures of signalling compounds secreted into the rhizosphere by the plant host. A: Indole-3-acetic acid, B: 6-Benzylaminopurine, C: Coumaric acid and D: Naringenin.

Martin et al. (2001) illustrate some of the responses between partners in the signalling compounds between *Eucalyptus globulus* and *Pisolithus* sp. inoculation in Figure 10.

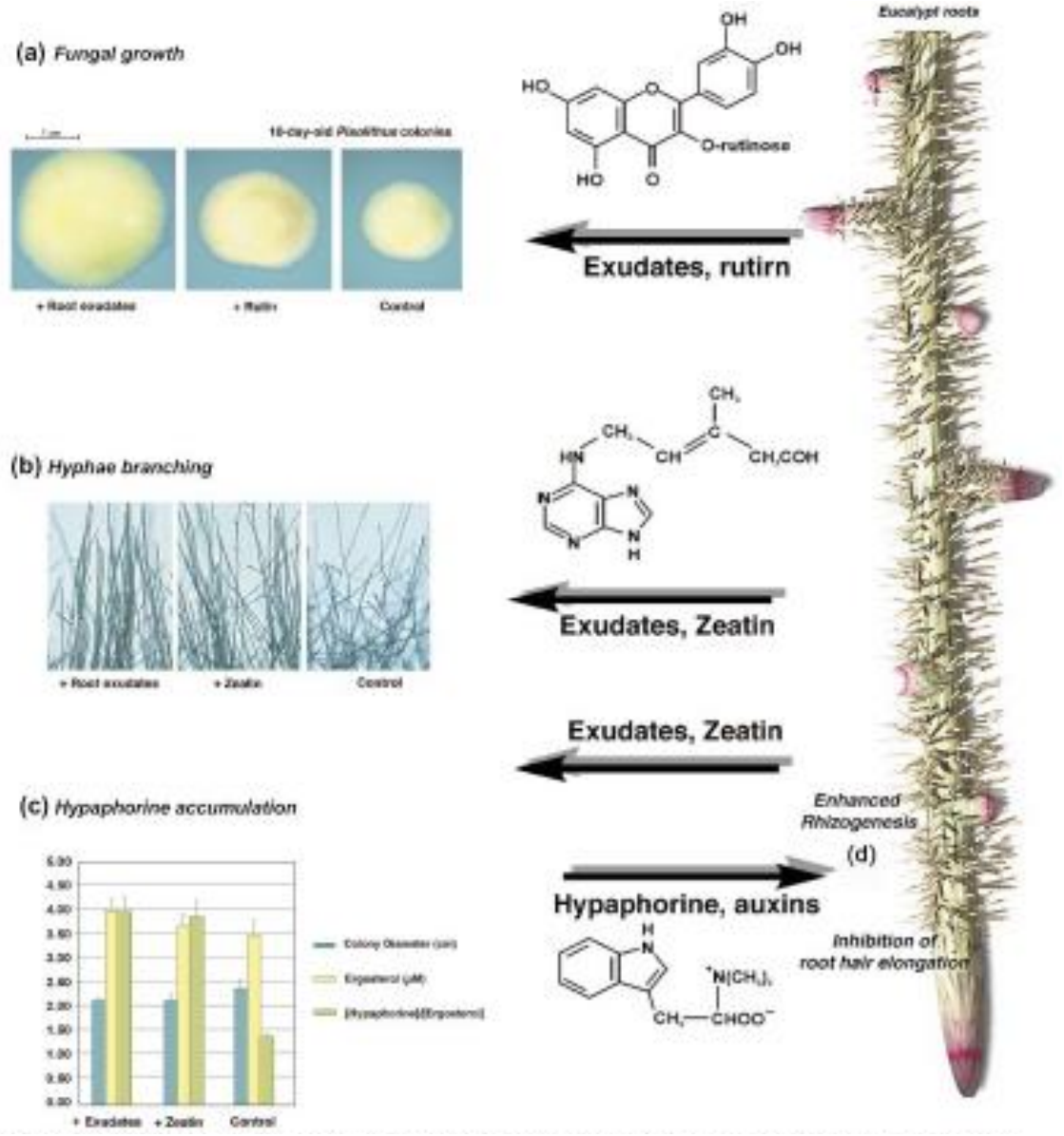


Fig. 10 - The molecular cross-talk taking place in the rhizosphere of *Eucalyptus globulus* colonized by *Pisolithus*. Root exudates alter the morphology of the mycelium of *Pisolithus*. These morphological changes are induced by the flavonol rutin, which stimulates fungal growth, expressed as colony diameter (a). Low concentrations of zeatin modify the hyphae branching angle (b) and the accumulation of the tryptophan betaine, hypaphorine (c). On the other hand, *Pisolithus*-secreted hypaphorine and indole-3-acetic (IAA) trigger morphological changes (i.e. arrest of root hair elongation and stimulation of short root formation) in the root system (d). (Béguiristain and Lapeyrie 1997) (from Martin et al. 2001).

Like some of the substances shown in Figure 10, the several rhizospheric signals exchanged between the symbionts and the molecular communication are responsible for important morphological changes (Martin et al. 2007, Dahm and Golińska 2011). Auxins serve as extremely potent morphogenetic signals for root systems. At low

concentrations, they increase root growth and stimulate the formation of new meristems and lateral roots (Tagu et al. 2002). Splivallo et al. (2009) reported that IAA and ethylene produced by truffles (*Tuber borchii* and *Tuber melanopsorum*) act together on plant roots inducing changes such as root shortening, increased branching, and root hair elongation. In the case of Pinaceae ECM, dichotomous branching of short roots occurs, sometimes resulting in the formation of coralloid structures made up of the assemblage of numerous root branching (Barker and Tagu 2000). For the arbuscular mycorrhizae host interaction, abundant data are available on the effect of flavonoids on hyphal growth, hyphal differentiation and root colonization (Vierheilig et al. 1998, Scervino 2005a, Steinkellner et al. 2007). Also strigolactones have been identified as the AM fungi hyphal branching factor in root exudates of the AM host plant *Lotus japonicus* (Akiyama et al. 2005).

9. Background to the Doctoral Dissertation and Aims

The stone pine is one of the oldest forest resources used in Portugal. The tree is exploited in multiple ways, and main products are wood, resin and pinions. The first two have diminished in importance nowadays, especially when compared with the great economic value of the fruit, the pinion. In 1988, an outline of a breeding program, was presented whose main objective was to genetically improve the quality and quantity of pinions (Barreira and Alpuim 1988). Since then, various actions have been carried out as part of integrated ID projects, in particular PAMAF 2090 “Improvement of *P. pinea* L. for the production of edible seeds in Southern Portugal” and PIDDAC 212 “Improvement of *Pinus pinea* L. for pinion production”, both under the auspices of the National Forestry Station. At an early stage of the implementation of these breeding programs Provenance Regions were delimited, serving for the purposes of basic identification and selection for the reproductive material in accordance with the rules for the certification of seeds. At the same time, selected stands, good producers of pinion, were subsequently registered in the National Catalog of Basic Materials (CNMB) (http: www.dgrf.min-agricultura.pt). The selection of good pinion-producing trees (plus trees), sited in the above-mentioned zones, was the starting point for the establishment of new areas as well as the installation of clonal orchards producing seeds and grafting material.

Two different approaches for increasing selected material were studied as part of PAMAF 2090: grafting and micropropagation. The vegetative propagation of selected “plus trees” was based on genetic research on the heritability of relevant characters to obtain maximal gains in pinion production, such as cone weight, the number of seeds per cone and seed length. These characteristics are in accordance with Alpuim and Rocha (1994) maternal inheritance (0.81, 0.81 and 0.55, respectively), justifying the use of good mother plant producers as plant material for vegetative propagation to achieve plantations with the greatest commercial value. Nevertheless, clonal propagation by means of grafting and cutting is an arduous task, and therefore not ideal for the large-scale multiplication of elite cultivars. Micropropagation of conifers has been shown to be feasible in other conifers, and for this reason in 1997 micropropagation studies funded by the PAMAF 2090 began at the Breeding and Biotechnology Laboratory of ICAAM (LMBV), University of Évora (a partner in the project) in Portugal.

The first attempts at *P. pinea* L. *in vitro* culture were carried out in the 1990s (Diamantoglou et al. 1990). Due to the economic and ecological interest of this species

in the Mediterranean basin, and as soon as successful protocols for other conifers organogenesis had been established, different groups throughout the region began their own *in vitro* cultivation.

During the 1990s, organogenesis from excised immature cotyledons was initiated in Spain (García-Ferriz et al. 1994). In Florence, in Italy, Capuana and Gianini (1995) established *in vitro* propagation from mature cotyledons collected from open pollinated trees in natural pine stands. Also, at the ENEA Research Center near Rome, complete plants were obtained from adventitious buds induced in isolated cotyledons (González et al. 1998).

These research efforts differed with respect to several culture factors, including basal medium, growth regulator concentration and exposure time, and environmental conditions. As an example, the basal media used in each micropropagation of stone pine was variable (DCR; MS; SH, GD; LP * see medium name in abbreviations). The different research actions differed with regard to many of these factors; however, the use of embryo explants from cotyledons (mature or immature seed source) such as the initial plant material and *N*6-Benzylaminopurine (BAP) in the induction of adventitious shoots were common to all protocols. Generally, in conifers the shoots are produced by direct organogenesis at the surface of cotyledons without callus formation between 6-10 weeks after the beginning of the culture.

In Portugal, in the work of Zavattieri A, Figueira S, Cavaleiro C, Peixe A (unpublished), two different approaches have been tested: axillary bud induction in one year mother plants (different treatments that include foliar cytokinins application and decapitation of terminal apices) and direct organogenesis from complete mature embryos or from excised cotyledons from mature seeds in accordance with Dantas (1995). The best results obtained were between 50-215 shoots per cotyledon (unpublished). Ordás et al. 2007 reported a total of 200 elongated shoots derived from one seed after 22 weeks in culture using modified ½ LPC (medium modified by Humara et al. 1999). In the works of Medina (1996) and Teles Ameixa (1997), best results were obtained in GD medium supplemented with 1,5 mg/l BAP. On average 6.6 new pine shoots were produced from each shoot after 3 sub-cultures (84 days). González et al. (1998) reported a maximum number of shoots obtained from cotyledons explants in ½ LP medium supplemented with 1 mg//l BAP with 44% shoot survival and the shoot elongation in the same medium with 0.5% activated charcoal (AC) (multiplication rate was not shown). In Alonso et al. (2006), the isolated shoots derived from cotyledons were successively subcultured on ½ LP with AC (LPC) or BA and IBA for 15 days and transferred back to ½ LPC medium

for 30 days. An average of 4.64 multiplication rate and 97.4% of shoots higher than 10mm were obtained after 4 subcultures (26 weeks).

Major improvements were carried out from 2000 to 2010 in micropropagation protocols in order to increase the number of shoots produced per cotyledon in the Iberian Peninsula. Two groups were particularly involved in this, led by Prof. Ordás in Spain (Valdés et al. 2001, Moncaleán et al. 2003, Moncalean et al. 2005, Alonso et al. 2006, Cortizo et al. 2009) and Prof. Zavattieri in Portugal, whose focus is mainly on solving problems during the rooting phase (Oliveira et al. 2003, Zavattieri et al. 2009, Ragonezi 2010a). Outside the Iberian Peninsula, Sul and Korban (2004) investigated the effect of salt formulation, carbon source, cytokinins, and auxins in shoot organogenesis from cotyledons.

Acceptable multiplication rates were obtained for *Pinus pinea* L. and the findings published in a number of works mentioned in the bibliography. Continuous research efforts led the team headed by Prof. Zavattieri to select the most suitable protocol for shoots induction from mature cotyledons: WPM media supplemented with 5 mg/l BAP, 2% sucrose, 0.70% Difco-Bacto agar (Difco®) and 25/19ºC day night temperatures, 16h photoperiod of cool-white fluorescent light at 80 µmol m^{-2} s^{-1}. For elongation the medium also consisted of WPM with 100 mg/l of myo-inositol, 2% sucrose 0.65% agar-agar (Merk®) and 2% AC. In the case of multiplication, the medium was the same as in the elongation protocol, without AC and supplemented with 0.1 mg/L of NAA and 4mg/L of BAP. When microshoots reached at least 2 cm length they were transferred to the rooting medium. This was the protocol in use in the laboratory at the beginning of this thesis.

Even when micropropagation protocols for stone pine culture initiation, maintenance and multiplication had been established, the number of rooted shoots was of low frequency (as frequently observed in other conifers) and had a short growth span. Much effort was made to overcome a bottleneck in the *in vitro* adventitious root formation (ARF). Significant progress was made with different combinations of carbon source, light and temperature during the induction and expression phases of the ARF (Zavattieri et al. 2009).

Even when these results seemed quite promising, the adventitious roots formed stopped growing which complicated the process or made it impossible to transfer pine plants to *ex vitro* conditions. On the other hand, in parallel research, the LMBV group demonstrated that some fungi could help to overcome this difficulty: a co-culture double

phase solid system was devised to rescue halted root growth, with successful transition to acclimatization stages and outplanting. From a random sample of 12 fungi derived from soil samples from the pine stand of Mata de Valverde (Alcácer do Sal) at least 9 had positive effects (Oliveira et al. 2003). These results opened a new interesting field in the study of the beneficial effect of microorganisms in overcoming the rooting phase and improving acclimatization. In 2007 the Foundation for Science and Technology (FCT) funded the PTDC/AGR-CFL/71437/2006 project *"Analysis and mastering* of root growth signalling by ectomycorrhizal fungi in *Pinus pinea* L." coordinated by Prof. Amely Zavattieri (LMBV) for the continuation of biotization research involving stone pine and ectomycorrhizal fungi.

The project included a doctoral thesis with the following aims:

- to improve the rooting phase of *Pinus pinea* L.;

- to use *in vitro* co-culture (biotization) to solve *in vitro* rooting problems and increase acclimatization survival;

- to characterize the fungus-root interactions that enable the development of roots;

- to evaluate the causal relationships between sustained rooting *in vitro* and the subsequent performance of inoculated plants;

- to properly characterize the different species of ECM fungi collected in pine stands during all stages of biotization;

- to identify the primary factors controlling the development of the symbiosis and signalling mediators between ECM fungi and *Pinus pinea* roots.

This doctoral thesis focuses mainly on overcoming adventitious rooting problems in microshoots of *Pinus pinea* L. In this study, a new insight into the innovative biotization technique used in the micropropagation emphasises the potential for using ECM fungi to promote root growth. The identification of signalling mediators between ECM fungi and stone pine roots was facilitated by a rigorous molecular protocol developed to characterize the inoculated fungi.

10. Outline of the Doctoral Dissertation

This doctoral thesis was designed to be based on a number of papers that have been submitted or accepted for publication, or published, and that will provide an account of the research that underpins this thesis. Firstly, there is a general introduction (Chapter 1) and details of extensive bibliographical research are provided regarding the different subjects of the thesis. All the publications presented (Chapters 2-8) were written to stand alone, therefore, the reader may find some repetition in parts of the manuscripts, especially in the introduction and method sections. This results from the common use of the sample, instruments and procedures in the set of publications. All publications are linked to the rationale of the thesis and there is a logical sequence to them. To conclude, final considerations and future perspectives are presented (Chapter 9), comprising all issues addressed in the thesis. This doctoral thesis was organized on the basis of a clear research rationale; all the articles are linked so that the main aim is achieved.

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Chapter 2

Adventitious Rooting of Conifers: Influence of Physical and Chemical Factors

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REVIEW

Adventitious rooting of conifers: influence of physical and chemical factors

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Abstract In conifers, vegetative propagation of superior genotypes is the most direct means for making large genetic gains, because it allows a large proportion of genetic diversity to be captured in a single cycle of selection. There are two aims of vegetative propagation, namely large-scale multiplication of select genotypes and production of large numbers of plants from scarce and costly seed that originates from controlled seed orchard pollinations. This can be achieved, in some species, either through rooted cuttings or rooted microshoots, the latter regenerated through tissue culture in vitro. Thus far, both strategies have been used but often achieved limited success mainly because of difficult and inefficient rooting process. In this overview of technology, we focus on the progress in defining the physical and chemical factors that help the conifer cuttings and microshoots to develop adventitious roots. These factors include plant growth regulators, carbohydrates, light quality, temperature and rooting substrates/media as major variables for development of reliable adventitious rooting protocols for different conifer species.

Keywords Cuttings · Gymnosperms · In vitro culture · Micropropagation · Microshoots

Abbreviations

ACC	1-Aminocyclopropane-1-carboxylic acid
AOA	Aminooxyacetic acid
ARF	Adventitious root formation
AVG	Aminoethoxyvinylglycine
BA	6-Benzyladenine
cGMP	Cyclic guanosine monophosphate
CW	Cool white light
DCR	Gupta and Durzan (1985)
GA_3	Gibberellic acid
GD medium	Gresshoff and Doy (1972)
GL	Growth-lux
IAA	Indole-3-acetic acid
IBA	Indole-3-butyric acid
L9 medium	Ewald (2007b)
LP medium	Quorin and Lepoivre (1977)
MAPK	Mitogen-activated protein kinase
MS medium	Murashige and Skoog (1962)
NAA	Naphthalene acetic acid
PGR	Plant growth regulator
PPFD	Photosynthetic photon flux densities
PS medium	Pinus strobus medium-Tang and Newton (2005a)
PBZ	Paclobutrazol
RD	Red-rich daylight
RIM medium	Abo El-Nil (1982)
RW medium	Risser and White (1964)
SH medium	Schenk and Hildebrandt (1972)

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STS	Silverthiosulfate
TE medium	Tang et al. (1998)
TDZ	Thidiazuron
WPM medium	Lloyd and McCown (1981)
WW	Warm white

Introduction

Vegetative propagation of trees has been a useful tool in traditional tree improvement and holds important prospects for reforestation (Libby 1986). It provides the possibility for multiplication of select superior trees with favorable genetic combination and to produce genetically homogenous plant material that will grow predictably and uniformly. In addition, improved efficiency in management and finished product utilization may also be achieved (Sutton 2002).

Conifers (cone-bearing trees) are the best known and most important economically among gymnosperms, covering approximately 60% of the forested areas of the world, and are mostly used for the production of softwood lumber, pulp and paper (Wenger 1984). Conifers comprise eight families, 68 genera and 629 species (Farjon 1998) including pines (*Pinus* spp.), spruces (*Picea* spp.), cowtail pine (*Cephalotaxus* spp.), cypress pine (*Callitris* spp.), firs (*Abies* spp.), larches (*Larix* spp.), bald cypresses (*Taxodium* spp.), yellowwood (*Podocarpus* spp.), yews (*Taxus* spp.), arbor vitae (*Thuja* spp.) and junipers (*Juniperus* spp.) (Farjon 1998).

In spite of the major role conifers are bound to play in reforestation strategies, current research on their vegetative propagation is not sufficiently developed (Sutton 2002). In part, this is due to the slow progress in propagation methods, mainly because of rooting problems associated with the tree maturation phase, an age-related developmental process that affects reproductive competence, morphology, and growth rate (Greenwood and Hutchison 1993). This notwithstanding, commercial scale propagation through rooted cuttings of young trees has been reported for radiata pine (*Pinus radiata* D. Don.), Norway spruce (*Picea abies* [L.] Karst.), Sitka spruce (*Picea sitchensis* [Bong.] Carr.), black spruce (*Picea mariana* [Mill.] B.S.P.) and sugi (*Cryptomeria japonica* D. Don) (Menzies et al. 2001). For a few economically important forest conifer species, an alternative vegetative, large-scale in vitro propagation technology has been developed, called somatic embryogenesis that utilizes mature or immature seed embryos as starting explants (reviewed by Klimaszewska et al. 2007). The advantages of somatic embryogenesis over rooted cuttings are: unlimited number of clonal somatic plants that can be produced from a single seed embryo (without a need for a separate step involving adventitious rooting) and the possibility of long-term storage of a given genotype in liquid nitrogen (cryopreservation). For example, in Norway spruce, both means of vegetative propagation, namely somatic embryogenesis and rooted cuttings of donor somatic trees, are being combined for clonal selection and commercial production of genetically superior seed families (Lamhamedi and Tousignant 2008).

Adventitious roots are post-embryonic roots that arise from the stem and leaves and from non-pericycle tissues in old roots. These roots may form naturally from stem tissue or may be induced by stressful environmental conditions, by mechanical damage or following tissue culture regeneration of shoots (Li et al. 2009). Adventitious root formation (ARF) is a critical step in vegetative propagation. An efficient rooting treatment can lead to a high percentage of rooting and a higher quality of the root system (De Klerk et al. 1997). Quality involves root number and length, and the absence of callus at the base of a shoot, all of which influence the performance of the plants after transfer to soil (Mohammed and Vidaver 1990). Many factors, during the rooting phases, can cause poor quality of the shoots at the time of planting, thus affecting growth (De Klerk et al. 1999; Hartmann et al. 2002; Mohammed and Vidaver 1990).

Adventitious rooting is a complex developmental process that consists of three successive but interdependent physiological phases: induction, initiation and expression, and each of these phases have different requirements. The induction phase comprises molecular and biochemical events without visible changes. The initiation phase is characterized by cell divisions and root primordia organization. The expression phase is characterized by intra-stem growth of root primordia and root emergence (Li et al. 2009). The chemical and physical factors that affect rooting include plant growth regulators (PGRs) (Wiesman et al. 1989; Davis and Haissig 1990), nutrients (the carbohydrate source foremost) (Wiesman and Lavee 1995), temperature and light (Haissig 1990; Corrêa and Fett-Neto 2004). Increasing body of knowledge on ARF pathway activation is generated from research on angiosperms. Recently, it has been discovered that nitrate, both a nitrogen source and a signal molecule, is transported by the NRT1.1 nitrate transporter and the transduction of nitrate signal is associated with a modification of auxin transport (Krouk et al. 2010). Thus, the NRT1.1 represses lateral root growth in *Arabidopsis* at low nitrate concentration by promoting auxin transport out of these roots. In mung bean, the adventitious root induction phase was regulated by a complex set of cellular messengers, among which some were activated by hydrogen peroxide, nitric oxide and

calcium (Li and Xue 2010). However, the signaling network responsible for root development has not been discovered yet.

In tissue culture, plant regeneration can be achieved either through the development of axillary shoot buds or through adventitious shoot formation, most frequently from callus. In both cases, the microshoots have to be rooted through adventitious rooting. The process of in vitro organ regeneration from the explants occurs through an apparent reversal of cell differentiation and acquisition of meristematic characteristics (Gahan 2007). Because many environmental and endogenous factors regulate rooting, some aspects of this regulation can be exploited to control rooting in vitro, through the application of chemicals, light and/or temperature control, or biotization. Different types of chemicals may be applied: PGRs to promote cell dedifferentiation, trigger the initial meristematic activity (Wiesman et al. 1989; Davis and Haissig 1990) and to promote the elongation and development of formed roots (Hartmann et al. 2002; Wiesman et al. 1989); nutrients to promote growth of the new roots (Wiesman and Lavee 1995); and protecting agents, such as biocides, to help protect against pathogens during the entire rooting period if carried out in a non-sterile substrate (Henrique et al. 2006).

Adventitious rooting in conifers has long been discussed, but the available information is fragmentary and circumstantial. Since the extensive articles by Gaspar and Coumans (1987) and Mohammed and Vidaver (1988) appeared over 20 years ago, no other review on root production and plantlet development in conifers has been published. Some aspects, widely discussed in those reviews, such as biological factors, root morphogenesis, genetic stability and acclimatization, are not covered in the present review. Instead, we focused on the progress in defining the chemical and physical factors that help the conifer cuttings and in vitro regenerated microshoots to develop adventitious roots. We compiled (mostly in the tabular form) the most successful, for a given conifer species, protocol/s of chemical treatments and physical factors that promoted adventitious rooting in both propagation systems.

Rooting of conifer cuttings

Four discrete stages of adventitious root formation in cuttings can be distinguished (Hamann 1998): (1) proliferation of cells at the base of the cutting, (2) differentiation of wound vascular tissue and periderm, (3) dedifferentiation of a zone near the wound cambium and wound phloem to form a root initial, and (4) formation of a root meristem. To obtain high-quality young plants in the shortest possible time, cuttings must root quickly and abundantly. Cuttings must also be able to produce lateral branching and grow fast after rooting (Moe and Andersen 1988). Propagation by cuttings has long been established in many conifer species. Ritchie (1991) calculated that more than 65 million rooted conifer cuttings were already produced around the world, and that half of this production was for sugi (*C. japonica*) in Japan, at least 10 million for radiata pine (*P. radiata*) in Australia and New Zealand, and about 21 million for Norway spruce (*P. abies*), Sitka spruce (*P. sitchensis*) and black spruce (*P. mariana*) in Canada, Scandinavia and the British Isles together.

In conifers, in addition to chemical and physical rooting treatments, the success of propagation by cuttings depends on a variety of other factors that include cutting collection time and season, cutting size, whether the needles are kept or not, condition and age of the source plant, plant nutritional condition, pruning treatments, and type and health of the cuttings at collection (Silva 1985). The role of donor plant growing conditions has long been recognized as important in influencing the rooting capacity of cuttings (Hartmann and Kester 1983; Moe and Andersen 1988).

Plant growth regulators

Auxins

For many decades, IBA has been applied to different plant species to induce adventitious roots, and conifers follow the rule. Nordstrom et al. (1991) attributed this preference, relative to IAA, to the higher stability of IBA. On the other hand, in many conifers the cuttings respond well to a pulse treatment with NAA. The combinations of various types of PGRs, the concentrations and application are extensive and are summarized in Table 1. Although IBA promoted rooting of cuttings in most of the conifers, in Pinus spp. NAA was also used at concentrations that varied between 1.6 and 2.7 mM. IBA was most frequently used at 24.6 or 49 μM mixed with talc or in water solution, and usually involved a quick dip or pulse treatment of the cut surfaces, with or without additional wounding, and was followed by transfer of the cuttings to substrates or to water nutrient solutions for rooting. In most cases, mixtures of sand, perlite and/or vermiculite were used in the substrates without any particular preference. The highest mean rooting percentage obtained in various experiments was 86% (Table 1).

Polyamines

Polyamines are generally considered to be growth regulators that are implicated in a range of developmental processes (Martin-Tanguy 2001; Kaur-Sawhney et al. 2003; Couée et al. 2004). It has been reported that the inhibition

Table 1 Treatments and growth conditions applied in two phases of rooting of conifer cuttings

Conifer species	Plant material	Root induction	Root growth	Environmental conditions	Rooting	References
Abies fraseri	Cuttings	IBA at 4 mM, 3 s dip	Horticultural perlite:peat 3:2 (v/v)	26.1 ± 2/20.5 ± 2 C day/night	31%	Rosier et al. (2004a, b)
Cedrus deodara	Cuttings	IBA at 5,000 ppm in talc or NAA at 10,000 ppm with activated charcoal, both with 1% captan and 1% sucrose	Unknown	Unknown	69%	Shamet and Bhardwaj (1995)
Cedrus deodara 'Shalimar'	Cuttings collected in late fall to early winter	IBA at 5,000 ppm, quick dip	Sand:perlite	Bottom heat maintained at 24 C. Greenhouse, 120 days under intermittent mist	67%	Nicholson (1984)
Chamaecyparis lawsoniana Parl.	Apical cuttings 15 cm long	IBA at 10,000 ppm for 5 s	Vermiculite	Not mentioned	99%	Stumpf et al. (1999)
Cupressus dupreziana Camus	Cuttings collected in early winter	IBA at 500-1,000 ppm, 24 h soaking in a solution	Coarse sand:perlite (1:1, v/v)	Bottom heat intermittent mist with ambient temperature of 15.5-23.8 C	90%	Nicholson et al. (1999)
Cupressocyparis leylandii Dallim. and A. B. Jackson	Callused cuttings, with a callus size of c. 1 cm diameter	IBA at 10,000 ppm and double wounding	Sterilized pumice (particle size 1-15 mm) and one part Southland peat (v/v).	Tunnel house, less than 25 C. Sun, tunnel shading of c. 40-50%.	43%	De Silva et al. (2005)
Juniperus scopulorum ''Skyrocket''	Cuttings	K-IBA in 0.9% talc powder	Peat:perlite (2:1) medium under low polytunnels, no mist greenhouse	Unknown	96%	Bielenin (2003)
Larix x eurolepis (European x Japanese larch)	Cuttings	IBA at 0.5%in talc powder including a fungicide, dipped in the solution	Peat:compost:pouzzolane (2:1:3) under greenhouse conditions	Unknown	87%	Pâques and Cornu (1991)
Picea sitchensis (Bong.) Carr.	Cuttings	IBA at 10^{-6} to 10^{-5} M, water solution	PGRs were prepared as aqueous solutions and no nutrients were used	20 C, 16 h L, 70-90 LI, CW fluorescent lamps	70%, 20 days	Selby et al. (1992)
Pinus banksiana	Cuttings	NAA at 5.4 mM, pulse treatment for 10 s and then 1:1 (v/v) forestry mix/ vermiculite	Conventional polyethylene-covered greenhouse (poly-house)	10-30 C, 16 h L, 1,500 (sunny) 350 LI (cloudy)	87%	Browne et al. (2000)
Pinus banksiana	Cuttings	IBA at 25 mM in 100% ethanol, brief wetting of the cut basal surface of the cuttings	Intermittent water-mist in a sand-perlite substrate	750-900 LI (sunny) to 200 LI (cloudy)	94%, 25 days	Haissig (1990)
Pinus banksiana	Cuttings (central axis, 3 months)	NAA at 5.4 mM, pulse treatment for 10 s and then 1:1 (v/v) Forestry mix/ vermiculite	Phosphoglucoisomerase multipots (PGI-45, 110 ml, Plastiques Gagnon, Que.) filled with medium (1:1 v/v forestry mix/ vermiculite)	20-28/14 to18 C day/night, 16 h L and 350-700 (sunny) to 150-270 LI (cloudy), 500-W high-pressure sodium lamps	95%	Browne et al. (1996)
Pinus caribaea var. *hondurensis* Morelet	Cuttings	IBA at 19.7 mM, immersion in a gel solution for 2 s	50% carbonized rice hulls and 50% vermiculite	Intermittent mist system in an enclosed polyethylene propagation house	95%	Henrique et al. (2006)
Pinus contorta	Cuttings (hypocotyl)	IBA at 1.23 mM pulse treatment for 6h and then in Hoagland nutrient solution	Brief rinse in water and culture in Hoagland solution	22 C and 200 LI, fluorescent tubes (Philips TLD 58 W/84) and incandescent light	100%	Lindroth et al. (2001)

Table 1 continued

Conifer species	Plant material	Root induction	Root growth	Environmental conditions	Rooting	References
Pinus elliottii var. *elliottii* x *P. caribaea* var. *hondurensis*	Cuttings (lateral tip)	Pasteurized coarse perlite:pine bark peat 1:1:1 (v/v/v), with additions of 0.5 kg Micromax m^{-3} (Granular by Scotts Australia) and 2.5 kg Osmocote m^{-3} (Low Start 5-6 month by Scotts Australia)	Controlled environment glasshouse with the appropriate temperature treatment	25 C, 11.5-13 h L, 70% of natural daylight irradiance (about 2,300-2500 micro-Einstein's in SE QLD)	83%, 12 weeks	Rasmussen et al. (2009)
Pinus radiata	Derooted seedling cuttings	IBA at 44.3 µM in 1/2 MS, 0.8% agar, 2% sucrose, for 10 d	Plant growth room	22 C with continuous lighting at 80 LI	95%	Li and Leung (2000)
Pinus sylvestris	Cuttings	IBA at 4,000 ppm dipped for 10 s in a solution of 95% ethanol and the PGR	Inserted in 90 ml containers filled with 60% peat and 40% perlite	25 C and then 20 C	54.4%	Hogberg (2005)
Pinus strobus L.	Cuttings	NAA at 1.6 mM, 5 min pulse treatment	Moist silica:sand	24 C, 16 h L, 40 LI CW fluorescent tubes	97%, 4 weeks	Goldfarb et al. (1998)
Pinus taeda L.	Cuttings (seedlings and hedged donor plants)	Unknown	Perlite:vermiculite 1:1 (v/v)	20-27 C, 14 h L, CW fluorescent tubes	80%	Hamann (1998)
Pinus taeda L.	Cuttings (hypocotyl)	IBA at 10 µM, pulse treatment then transferred to distilled water for rooting	Unknown	27/20 C day/night, 16 h L, 100 LI, CW fluorescent tubes	82%, 15-30 days	Diaz-Sala et al. (1996)
Pinus taeda	Cuttings	NAA at 2.7 M, 5 min pulse as described by Diaz-Sala et al. (1996)	Unknown	16 h L, 90 LI, CW fluorescent tubes	Greater than 80%, 30 days	Greenwood et al. (2001)
Pinus taeda	Cuttings (hypocotyl)	IBA 10 µM in distilled water was stuck through holes in with styrofoamrafts (Hansen and Ernstsen 1982) and floated in trays made of PVC	The IBA solution was replaced with distilled water after 11 days	27/20 C day/night, 100 LI, fluorescent and incandescent lamps	94%, \25 days	Greenwood and Weir (1994)
Pinus virginiana	Cuttings (open-pollinated progeny)	IBA or NAA at 6 mM, applied for 3 s	Horticultural perlite:peat 3:2 (v/v)	25.5 ± 2/20.0 ± 2 C day/night temperatures	47% (semi-hard woody)	Rosier et al. (2004a, b)
Pseudotsuga menziesii (Mirb.) Franco	Cuttings, 15 cm long	IBA at 12.3-123 mM or NAA at 2.5-7.5 mM dipped for 10 s	Peat moss:fine sand 2:1 (v/v)	Rooting medium heated to 21 C and intermittent mist. Natural light in a conventional glasshouse	68%	Copes and Mandel (2000)
Thuja occidentalis ´Smaragd´	Cuttings	K-IBA 0.6% in talc powder, dipped	Peat:perlite 2:1 (v/v) under low polytunnels greenhouse without misting	Unknown	100%	Bielenin (2003)

LI light intensity in µmol m^{-2} s^{-1}

L photoperiod

of polyamine synthesis blocks the mitotic cell cycle by blocking the transition between G1 and S phase where increased levels of spermidine and spermine have been found (Couée et al. 2004). Both Martin-Tanguy and Carré (1993) and Tarenghi et al. (1995) hypothesized that the endogenous concentrations of polyamines might be growth limiting based on observations of the developmental stimulation of higher plants. A direct relationship between high polyamine content, such as putrescine and spermine, and the onset of ARF has been demonstrated, which accentuates the possible participation of these substances in the general cellular processes of division and differentiation in the rooting process (Couée et al. 2004; Martinez-Pastur et al. 2007). Polyamine metabolism has also been pointed as responsive to environmental circumstances, therefore playing an important role in the relations between plant and external conditions (Couée et al. 2004; Tang and Newton 2005b).

Tang and Newton (2005b) tested the influence of polyamines on the overall rooting frequency of *Pinus virginiana*. In their trials, the administration of 0.001 mM putrecine or spermidine in the NAA supplemented medium resulted in a 25% increase of rooting frequency, whereas 0.001 mM spermine caused a 6.7% decrease of rooting frequency.

Ethylene

The information concerning ARF in cuttings of conifers and ethylene is limited to a few articles. In general, Ethrel (a commercial formulation of the slow-release ethylene compound ethephon) promoted rooting and root growth in Engelmann spruce 2 weeks after planting (Scagel and Linderman 2000). Ethrel induced changes in root initiation of cuttings of Douglas fir [*Pseudotsuga menziesii* (Mirb.) Franco] 2 weeks after application and these changes were positively correlated with subsequent increases in shoot growth. In these studies, the results indicated that exogenously applied Ethrel influenced root initiation indirectly by increasing levels of free IAA at the rooting site (Scagel et al. 2000). Ethrel was also reported to increase IAA conjugates in roots of Engelmann spruce (*Picea engelmannii* Parry ex Engelm.), lodgepole pine (*Pinus contorta* Dougl.) and Douglas fir. On the other hand, Bollmark and Eliasson (1990) concluded that the enhanced rooting of Norway spruce (*P. abies*) hypocotyl cuttings, promoted by the treatment with ACC or Ethrel, was attributed to the ethylene-mediated acceleration of the breakdown of cytokinins.

When cuttings of Japanese black pine (*Pinus thunbergii* Parl.) were soaked for 10 min in Ethrel solution (69.2 µM ethephon) 24 h prior to soaking in Oxyberon (19.7 mM IBA solution), a significantly higher rooting ability was observed compared with the controls without the pre-treatment with Ethrel (Mori, Miyahara, Tsutisumi, Kondo, unpublished). Similarly in *P. abies* L. (Karst), the hypocotyl cuttings produced 64 adventitious roots after 28-days treatment with 0.1 µM Ethephon compared with 22 roots in untreated controls and with two roots after treatment with the ethylene inhibitor $CoCl_2$ at 10 µM (Wang and Pan 2006).

Ethylene inhibitors

Several ethylene inhibitors, compiled by Kumar et al. (1998), inhibit both ethylene biosynthesis or ethylene actions in a plant. Among the most commonly used are aminoethoxyvinylglycine (AVG) and aminooxyacetic acid (AOA), both inhibiting 1-aminocyclopropane-1-carboxylic acid (ACC) synthase and cobalt ions that inhibit the conversion of ACC to ethylene (Biddington 1992). Another inhibitor widely used is the silver ion, either as nitrate or as more mobile thiosulphate (STS), which excels in ethylene action inhibition (Beyer 1976). Inhibition of ethylene may lead to a lower number of adventitious roots, decrease of response to endogenous and exogenous auxins and reduced root hair formation (Clark et al. 1999). Ethylene itself has been reported to have no effect or even inhibit rooting depending on its concentration and genotypes, and therefore its role is still disputed (Mudge 1988). Inhibitors, when at high concentrations can promote stress, resulting in the synthesis of ethylene and root formation, thus defeating the purpose of their use. De Klerk et al. (1999) reported that STS may induce ethylene formation because silver is a heavy metal and damages the tissue. When STS was added along with auxins, the appearance of the rooted microcuttings at the time of transplanting was strongly improved (De Klerk et al. 1999).

As described by Kumar et al. (1998), ethylene inhibitors work within specific concentrations. The use of these substances under or above the recommended levels, might not have an inhibiting effect as desirable, or might promote the ethylene synthesis due to tissue damage (De Klerk et al. 1999). Also, there is not much work relating conifers with ethylene inhibiting substances, since the actual major scientific goal is to promote and improve conifer rooting and not the opposite. Nonetheless, such a study is fundamental to fully comprehend the role of ethylene in conifer ARF.

Plant growth retardants

Plant growth retardants are organic compounds that retard cell division and cell elongation (Arteca 1995). A number of growth retardants and inhibitors have been tested for their ability to influence rooting of cuttings. They are responsible for antagonizing the activity or inhibiting the

synthesis of gibberellins, which normally inhibit rooting (Hartmann and Kester 1983; Davis et al. 1988).

Henrique et al. (2006) investigated the effect of different levels of auxins (NAA, IBA) and gibberellin synthesis inhibitor (PBZ) on the rooting of 4-6 cm long shoots obtained from cuttings of *Pinus caribaea* var. *hondurensis* Morelet. Sixty days after planting, the IBA-treated cuttings rooted at a higher frequency than those treated with NAA, but IBA applied together with PBZ was the most effective treatment. However, daminozide (currently used as a growth retardant for many plants) inhibited rooting of *Chamaecyparis obutusa* seedling cuttings after spray application of 2,000 ppm wettable solution (Shigehiro 2006).

Carbohydrates

Non-structural carbohydrates usually accumulate in needle fascicles during propagation, sometimes after an initial decrease. However, concentrations of specific carbohydrates such as sucrose or glucose in needle fascicles may not be uniform among tissues or with time during propagation (Veierskov 1988). Perhaps, the changing concentrations of specific carbohydrates in cuttings during propagation are linked to the direct control of ARF, for instance, because auxin treatments often concomitantly promote adventitious rooting and modify concentrations of individual carbohydrates within specific regions of cuttings during propagation (Haissig 1990).

Nevertheless, in a study with Sitka spruce cuttings, little correlation between rooting and concentration of sugars in stems and foliage was found (Van den Driessche 1983). It is possible that interaction between carbohydrates and hormones, nitrogen and carbon ratios, light and carbohydrate and also temperature pre-treatments, as well as the carbohydrates status of the mother plants, make the comparison of results from different studies difficult.

Light

Roots of *P. radiata* cuttings from seedlings had the highest dry weight under high PPFD, which had a red to far-red ratio similar to daylight (Wenger 1984). In other experiments with *P. radiata*, the best rooting conditions for short shoots were: treatment with 50 ppm IBA for 24 h, then planting at 20-25 C under a 12-h photoperiod (as compared to 18-h photoperiod or continuous illumination). The needle fascicles rooted better if they were collected during winter or early spring, suggesting a direct influence of short days (Kummerow 1966).

Both McClelland et al. (1990) and Corrêa et al. (2005) have stressed the need for increased efficiency of the process and that rooting should be carried out in the dark for the first few days. However, rooting can be influenced by light intensities, as reported by Kunneman and Ruesink (1997) who showed better responses to 66-83 µmol m^{-2} s^{-1} in a few *Juniperus cultivars*, while 27-37 µmol m^{-2} s^{-1} were more suitable for the *Chamaecyparis* and *Cupressocyparis cultivars.*

Temperature

A temperature range between 18 and 27ºC is commonly used during rooting of the cuttings. The effect of artificial light, CO_2 and temperature on rooting in ornamental cultivars of *Chamaecyparis*, *Cupressocyparis* and *Juniperus* was studied by Kunneman and Ruesink (1997). These authors demonstrated that rooting was best at a constant temperature of 23ºC, compared with 17 and 29ºC. On the other hand, for *Pinus taeda* L., the best rooting of cuttings (seedlings and hedged donor plants) (80%) was obtained by maintaining the air temperature at approximately 27ºC during the day (14-h photoperiod) and 20ºC at night (Hamann 1998), and the temperature of the rooting medium was kept at 25ºC using a root zone heating system.

Cedrus spp., in general, are difficult to root, for example *Cedrus libani* A. Rich. is considered almost impossible to propagate by cuttings; however, *Cedrus deodara* (Roxb.) G.Don 'Shalimar' can be rooted to 67% if cuttings are collected in late fall to early winter and, after a quick dip in IBA solution, placed in a sand-perlite medium maintained at 24ºC with bottom heat (Nicholson 1984 cited by Pijut 2000). In Table 1, other examples of temperature treatments for adventitious root formation in conifer cuttings are listed.

Substrates for rooting

The blends of propagation substrates should create suitable air and drainage characteristics and remain moist, but not waterlogged during the period of time that roots are initiated. Different substrates can be used to promote rooting in conifer cuttings, the most common being vermiculite, perlite or a combination of both. However, the requirements of various species can be very different. Davidescu et al. (2003) found that propagation by cuttings of *Thuja occidentalis* L. 'Columna' and *T. occidentalis* 'Danica' was best in peat substrate in August. Six substrates were used in *Picea* cuttings by Mazăre et al. (2007): sand, perlite, peat, sand with perlite, sand with peat and perlite with peat, all at 1:1. The rooted cuttings were at a higher proportion in sand with peat, but increased rooting index (13% as visually established based on the number of primary and secondary roots) was obtained in perlite with peat.

Rooting of loblolly pine (*P. taeda*) cuttings has been extensively studied, but the description of substrates used

Table 2 Treatments and growth conditions applied in two phases of rooting of conifer microshoots in vitro

Conifer species	Plant material	Root induction	Root growth	Environmental conditions	Rooting (%)	References
Juniperus oxycedrus	Shoots (terminal shoots from lateral branches—5 cm length)	NAA, IAA or IBA separately, or in combination in solidified medium B (SH macronutrients supplemented with 3% sucrose, 0.7% agar) for 30 days	The same medium without auxins	26 ± 2 C, 16 h L, 80 LI, Gro-lux (F36W/ GRO)	None of the auxins or auxin combinations tested promoted satisfactory rooting. The frequency of rooted shoots ranged from 7 to 10%	Gómez and Segura (1994)
Juniperus phoenicea	Shoots 2-3 cm long (from axillary buds)	IBA at 2.4 µM, 5 min dipping and cultured on medium without PGRs	Plantlets with 2 cm long roots were transferred to pots with sterilized mixture of peat:perlite 3:2 (v/v)	22 ± 1 C, 16 h L, 400 LI, OSRAM (Munich, Germany) L36W/21lamps	40%	Loureiro et al. (2007)
Larix sp.	Shoots (newly formed shoot tips, approximately 3-4 cm long and without any visible bud primordia)	NAA at 10.7 µM in L9 medium for 2 weeks	After 2 weeks, the induced shoots were transferred directly into Jiffy-7 peat pellets (ø 42 mm) saturated with water	17 C and 16 h L, white light (OSRAM L58W/31-830)	100%	Ewald (2007a)
Picea sitchensis	Adventitious shoots (from cotyledons)	PGR-free ½ MS, 0.7% agar	Transfer to a mixture of equal volumes of Levington M3 compost (Fisons, Ipswich, UK), perlite and vermiculite (William Sinclair Horticulture Ltd., Lincoln, UK) in 9 cm diameter plastic pots	20 ± 1 C, 16 h L, 1.5 LI, CW fluorescent lamps	84%, 22 weeks	Drake et al. (1997)
Picea chihuahuana	Adventitious shoots (from embryos)	IBA at 14.8 or 24.6 µM in ½ SH liquid medium, placed vertically on filter paper for 48 h	Fresh liquid medium (1/2 SH) without PGRs	26 ± 2 C, 16 h L, 46-48 LI	±8.5%	López-Escamilla et al. (2000)
Pinus armandii var. *amamiana*	Adventitious shoots (from embryos)	IBA at 4.9-14.8 µM in RIM medium	Transfer to pots with florialite containing 0.1% hyponex for 2 weeks under 100% humidity	25 C, 16 h L, 70 LI, fluorescent light	Unknown	Ishii et al. (2007)
Pinus ayacahuite	Adventitious shoots (from zygotic embryos)	NAA at 100 µM solution for 8 h	Transfer to ½ GD medium PGR-free with 0.05% activated charcoal, 30 mM sucrose and 1% agar	25 ± 1 C, 16 h L, 60-80 LI, Sylvania Gro-Lux F40T12 Gro-WS lights	40%	Saborio et al. (1997)
Pinus contorta	Adventitious shoots (from embryos)	IBA at 1.23 mM for 6 h and then liquid medium	12 weeks later potted in mineral wool	Unknown	70%	Hogberg et al. (2005)
Pinus contorta	Adventitious shoots (from embryos)	IBA at 1.23 mM, pulse treated for 6 h	Cultured in liquid mineral nutrient solution, composed according to the nutrient requirements of P. sylvestris given by Ingestad (1979) with N concentration at 4.5 mM	20 C, 110 LI, fluorescent light supplemented with incandescent light	77%	Flygh et al. (1998)
Pinus heldreichii	Adventitious shoots (from embryos)	IBA at 1 mM, pulse treatment for 2 or 5 h, afterward transferred to ½ GD with 2% sucrose	Transfer to a greenhouse	25 ± 2 C, 8 h L, 47 LI, white fluorescent tubes	14%, for up to 18 weeks	Stojicic et al. (1999)

Table 2 continued

Conifer species	Plant material	Root induction	Root growth	Environmental conditions	Rooting (%)	References
Pinus brutia x *Pinus halepensis*	Shoots (from fascicle buds-clone A and B)	IBA at 2.46 µM plus NAA at 2.7 µM plus 0.65% agar (Sigma) plus 1.5% sucrose	Transfer after 7 days to a mixture of peat:perlite 1:1 (v/v)	14-19 C, 18 h L, 65-70 LI, high-pressure lamps (HPI/T, SON, 400 W)	84% clone A, 32% clone B, 10-16 weeks	Scaltsoyiannes et al. (1994)
Pinus canariensis	Adventitious shoots (from adventitious buds induced from cotyledon explants)	IBA at 1 mM, 4 h liquid pulse treatment	Transfer to peat:vermiculite, 1:1 (v/v)	Unknown	83%	Martínez Pulido et al. (1990)
Pinus eldarica	Adventitious shoots (from adventitious buds induced from cotyledon explants)	BA at 0.22 µM, IBA at 10 µM and NAA at 5 µM in ½SH medium	½ SH medium with 1% activated charcoal. Vermiculite:perlite:peat 2:2:1 (v/v) in plastic bags to maintain high humidity for 4 weeks	16 h L, 26/18 C day/night, 250 LI	78%	Sen et al. (1994)
Pinus massoniana L.	Adventitious shoots (from adventitious buds induced from mature embryos)	IBA at 9.8 µM, BA at 2.2 µM and 2% sucrose in ½ GD medium for 1 week	Subculture onto ½ GD medium with IBA at 0.98 lM and BA at 2.2 lM for 4 weeks. Then, transfer to pots filled with a mixture of vermiculite and perlite 3:1 (v/v)	25 ± 2 C, 14 h L, 80 LI, CW fluorescent tubes	70%	Zhang et al. (2006)
Pinus elliotti	Adventitious shoots (induced from callus)	IAA at 1 µM and IBA at 1 µM	Perlite:peat moss:vermiculite 1:1:1 (v/v/v)	23 C, 16 h L, 100 LI, CW fluorescent tubes	26-35%	Tang and Newton (2007)
Pinus kesiya	Adventitious shoots (from 2 to 3 week old seedling explants)	NAA at 16.1 µM in GD medium for 24 or 120 h	Subculture to the same medium without PGR	25 ± 1 C, 16 h L, 50-70 LI, CW fluorescent and incandescent lamps	67%	Nandwani et al. (2001)
Pinus pinaster	Adventitious shoots (from adventitious buds induced from embryos)	NAA at 5.4 µM in induction medium (RW macroelements, ½ MS microelements, 50 mg l^{-1} myo-inositol, 2 mg l^{-1} glycine, 1 mg l^{-1} thiamine, 1 mg l^{-1} pyridoxine, 1 mg l^{-1} nicotinic acid, 1% sucrose)	Same medium without PGRs with 3% sucrose. Then, transfer to a sterile peat:vermiculite, 1:1 (v/v)	25 ± 1 C, 16 h L, 80 ± 5 LI	86%, 3 weeks	Álvarez et al. (2009)
Pinus pinaster	Shoots (from axillary buds)	NAA at 10^{-6} M for the first 16 days in basal nutrient medium with nitrate at 3.3 mM and glutamine at 2 mM	Unknown	Unknown	92%, 30 days	Faye et al. (1989)
Pinus pinea L.	Adventitious shoots (from cotyledons)	IBA at 10 µM in DCR (½ macroelements) with 3% sucrose for 10 days	Peat:sand:perlite (2:1:1 v/v) mixture	23 ± 1 C, 16 h L, 80-100 LI	34%	Capuana and Giannini (1995)

Table 2 continued

Conifer species	Plant material	Root induction	Root growth	Environmental conditions	Rooting (%)	References
Pinus pinea L.	Adventitious shoots (from embryos)	NAA at 10 µM in ½ LPC (LP medium with 0.5% (w/v) of activated charcoal), 20% glucose and 0.8% Roko-Agar	The same medium without PGRs and then transferred to sterile peat:perlite 1:4 (v/v)	1 week at 19 C dark, 2 weeks at 19 C, 16 L, 100 LI and then 21 C 16 h L, 100 LI, white fluorescent tubes	68%, 3-6 weeks	Alonso et al. (2006); Ordás et al. (1999)
Pinus pinea L.	Adventitious shoots (from cotyledons)	NAA at 0.05 µM in ½ MS medium	Unknown	23 C, 16 h L, 60-70 LI, CW fluorescent tubes	15-20%	Sul and Korban (2004)
Pinus pinea L.	Adventitious shoots (from cotyledons)	NAA at 10.7 µM in ½ WPM macroelements with 0.117 M glucose and 0.8% agar	The same without PGR and with 58.4 mM glucose	1 week at 19 C dark, 2 weeks at 19 C, 16 h L, 90 LI and then 21 C, 16 h L and 90 LI, CW fluorescent tubes	70%, 4 weeks	Ragonezi et al. (2010)
Pinus pinea L.	Adventitious microshoots (from cotyledons)	NAA at 10.7 µM with 0.117 of sucrose or 0.117 M of glucose in WPM gelled with 0.65% Difco Bacto-agar	The same without PGR and with 58.4 mM sucrose	Induction for 2 weeks and then for expression 25/19 C day/night 16 h L	Average 53% when 0.117 M of glucose was used in the medium	Zavattieri et al. (2009)
Pinus radiata	Shoots (from isolated meristems)	IBA at 8.2 mM and NAA at 5.4 mM in 5% National Midesa agar for 5 d	Transfer to ½ LP medium supplemented with 10% sucrose	24 ± 2 C, 16 h L, 20-30 LI, CW fluorescent tubes	28%	Prehn et al. (2003)
Pinus radiata	Shoots (seedlings from a mixed population of open-pollinated seed)	NAA, IBA and BAP at 2.7, 5.0 and 0.11 µM, respectively, for 10 days in SH macro- and micro-salts, 3% sucrose	Transfer to ½ SH medium with 1% sucrose and without PGR	23 ± 2 C, 16 h L 80 LI CW—fluorescence tubes	43%	Schestibratov et al. (2003)
Pinus roxburghii Sarg	Shoots (from axillary buds)	BA at 10 µM in MS medium with 2% sucrose and 0.6-0.8% agar	Transfer to semisolid (0.6% agar) and liquid ½ MS with filter paper bridges for elongation of roots. The rooted plantlets were washed thoroughly and transferred to liquid ¼ MS medium containing 1% sucrose and absorbent cotton	25 ± 2 C, 16 h L, 30 LI, CW fluorescent tubes	97%	Kalia et al. (2007)
Pinus sylvestris	Shoots (from axillary buds)	NAA at 53.8 µM in 0.6% water agar. Shoots were placed for 24 h	Transfer to l/2-strength basal medium (1/8-strength MS medium as modified by Cheng (1975), supplemented with 3% sucrose, 1% Difco Bacto-agar) with 1% sucrose and 1% agar	26 ± 2 C, 16 h L, 107-240 LI, Sylvania Gro-Lux and fluorescent LV 20	64%	Zel et al. (1988)

Table 2 continued

Conifer species	Plant material	Root induction	Root growth	Environmental conditions	Rooting (%)	References
Pinus sylvestris	Adventitious shoots (from zygotic embryos)	NAA at 0.67 mM in 1/6 MS medium for 24 h	The same medium without PGR	Unknown	33%	Sonia Tsai and Huang (1985)
Pinus sylvestris	Adventitious shoots (from cotyledons)	NAA at 2.7 µM ½ GD medium ½ micronutrients, ½ macronutrients, and ½ organics of those in GD medium 1% agar	The same medium without PGR for 4 week	Unknown	6%	Häggman et al. (1996)
Pinus strobus L.	Adventitious shoots (from zygotic embryos)	IAA at 0.01 mM and IBA at 0.01 mM in PS medium	Perlite:peat moss:vermiculite (1:1:1 v/v) in a greenhouse	24 C, 16 h L, 50 LI, CW fluorescent tubes	36%, 6 weeks	Tang and Newton (2005a, b)
Pinus taeda L.	Adventitious shoots (from zygotic embryos)	IBA at 2.46 µM, GA_3 at 1.44 µM and BA at 4.43 µM in TE medium	Vermiculite:commercial compost 3:1 (v/v)	25 C, 16 h L, 100 LI, CW fluorescent tubes	Unknown	Tang and Guo (2001)
Pinus virginiana Mill.	Adventitious shoots (from zygotic embryos)	NAA at 0.05 µM in TE medium for 6 weeks	Established in soil in a greenhouse	24 C, 16 h L, 50 LI, CW fluorescent tubes	18%	Tang et al. (2004)
Pseudotsuga menziesii (Mirb.) Franco	Adventitious shoots (from cotyledons)	NAA at 10.7 µM in ½ DCR with 1% sucrose for 6 days (2 days in darkness and 4 days in light)	The same without PGR (only in light)	Unknown	40%, 4 weeks	Hutzell and Durzan (1993)
Sequoia sempervirens (Lamb.) Endl	Shoots (from axillary buds)	IBA at 12.3 µM in ½ MS medium with 2% sucrose, 0.75% Difco agar, for 3 months	The same without PGR	22 ± 3 C, 9 h L, 60 LI, CW fluorescent tubes	61%	Blazkova et al. (1997)
Taxus meirei	Shoots (from steckling)	IBA at 12.5 µM ½ MS medium supplemented with 20 g l^{-1} sucrose for 3 months	The same without PGR	24 ± 1 C, 16 h L, 45 LI, CW fluorescent tubes	55%	Chang et al. (2001)
Taxus brevifolia	Adventitious shoots (from zygotic embryos)	Treated with ABT rooting powder (ABT Research Center, Beijing, China)	Transfer rooted shoots to 2-inch pots of plant growth medium (vermiculite: perlite 1:1 (v/v), J. Mollenma Co., Grand Rapids, MI, USA)	26 C, 16 h L, 80 LI	58%	Chee (1995)
Taxus baccata L.	Shoots (from closed buds or shoot tips)	IBA at 9.8 µM, spermidine at 6.88 mM and TDZ at 4.5 mM in 1/3 L9 medium with 0.5% sucrose	JIFFY 7 peat pellets saturated with water	15-17 C, 16 h L, 30 LI, white light radiation	Unknown	Ewald (2007b)
Thuja occidentalis L.	Adventitious shoots (from zygotic embryos)	IBA at 25 mM in 1/3 MS, 3% sucrose and 0.7% agar	Transfer to autoclaved Redi-Earth (W.R. Grace & Co., Ontario)	20 C, 16 h L, 30-40 LI	60%, 3-4 weeks	Harry et al. (1987)

LI light intensity in µmol m^{-2} s^{-1}

L photoperiod

was not always included. Hamann (1998), working with this species, used a substrate consisting of equal parts of perlite and coarse vermiculite, to a depth of 1.5 ± 2 cm and obtained 80% rooting. In Brazil, with the same species, the cuttings were placed in plastic tubes containing Mec-plant (substrate composed of biostabilized pine bark) overlaid with vermiculite (Alcantara et al. 2007). Table 1 shows a compilation of different substrates applied to conifer cuttings.

In vitro rooting of conifer microshoots

Many basic studies on rooting are now carried out in vitro. Using seedling explants and in some cases also explants from mature trees, it has become current, in some species, to produce rooted micropropagated shoots (microcuttings) by in vitro organogenesis (Niemi et al. 2004). Tissue culture method facilitates administration of PGRs and other compounds and avoids microbial degradation of applied compounds (De Klerk et al. 1999). According to many workers, further research is required on the influence of factors such as donor plant age, genotype and type of explant, microcutting quality, auxin treatment, root system and environmental conditions on rooting and acclimatization (Fett-Neto et al. 2001; Greenwood et al. 2001; Bielenin 2003; Henrique et al. 2006). Adventitious rooting of microshoots is characterized by the same four phases as rooting of cuttings (see above).

Rooting medium

The success of plant tissue culture as a means of plant propagation is greatly influenced by the composition of the culture medium. In vitro rooting of conifer microshoots usually occurs in gelled nutrient media (mostly agar- and gellan-gum based) as substrate. This ensures the consistent distribution of PGRs, macro- and micronutrients, and also provides a better contact between shoots and substrate, resulting in more synchronous rooting (Mohammed and Vidaver 1990). However, the quality of produced roots is not always satisfactory. Gelled media probably obstruct gas exchange and inhibit the development of the vascular system in roots, as well as the production of root hairs (Skolmen and Mapes 1978). Culture media and physical supports currently used for rooting of conifer shoots are listed in Table 2. Nutrients are usually reduced to half the strength of that used for shoot production (Blazkova et al. 1997). In general, it has been reported that lower concentration of salts in the culture medium, particularly nitrogen, seems to favor the adventitious rooting of cuttings (Ordás et al. 1985). In our experience, reducing the WPM macronutrients to half strength increases significantly the percentage of rooted microshoots of *Pinus pinea* L. (Ragonezi et al. 2010).

Plant growth regulators

In vitro organogenesis is a complex series of events that a cell or groups of cells undergo in response to external/internal stimuli such as phytohormones. According to Thorpe (1980), organogenesis is a developmental process that comprises (a) attainment of competence or pre-induction phase, (b) induction or determination phase, and (c) expression phase or post-initiation phase. Cell/tissue responses to form adventitious roots may be different according to species, physiological status of the explants, the phase of the rooting process, and the interaction of the chemical and physical factors of the culture. Table 2 summarizes the available information in the scientific literature, including species, rooting induction treatments, culture media, physical conditions, light regimes and rooting percentages.

Most frequently, the treatments involved IBA (13 references) or NAA (15 references). For five species, a mixture of IBA either with NAA or IAA was used. On the other hand, one research report cited 97% rooted shoots of *Pinus roxburghii* when 10 µM BA was applied before transfer to a liquid medium for root expression. In one experiment, *Taxus brevifolia* treated with ABT rooting powder (developed by ABT Research and Development of Chinese Academy of Forestry) produced roots in 58% of the microshoots derived from cotyledon explants. In one case, it was possible to root 84% of the microshoots of *P. sitchensis* derived from cotyledons without any application of PGRs and by rooting directly in a substrate mix. IBA was applied at concentrations that ranged from 1 to 25 µM, most often between 2.5 and 14.8 µM, and only in one study IBA was applied at 25 µM. NAA concentrations varied between as low as 0.05 and 100 µM, with 10.7 µM being the most commonly used. However, the best results were obtained with NAA at concentrations higher than 50 µM, while low concentrations gave poor rooting percentages. The physical support for the shoots during the root expression phase was either a substrate (16 references) or a culture medium without PGRs (18 references).

Carbohydrates

Sucrose is commonly used in tissue culture media because it is the main sugar translocated in the phloem of many plants. However, other carbohydrates such as glucose and fructose have been also used to improve organogenesis (Faye et al. 1989; Ordás et al. 1999; Zavattieri et al. 2009). The exogenous sucrose (in the presence or absence of auxin) is beneficial for the rooting of most herbaceous and

woody plants (Haissig 1982). Generally, enrichment with sucrose improves rooting, but this has its limits, as sucrose at high concentrations tends to have negative effects, especially during the root expression phase. A negative interaction between carbohydrates and light could emerge at such high concentrations, either through transformation of added sugars into soluble and storage forms, or through altered nitrogen/sucrose or auxin/sucrose ratios (Moncousin 1991).

In conifers, the data on the influence of carbohydrates in adventitious rooting are limited. Zavattieri et al. (2009) made a direct comparison between different carbon sources (sucrose or glucose at different concentrations) for the induction and expression phases of the adventitious roots in microshoots of *Pinus pinea*. An increased number of roots per shoot and an accelerated root formation were consistently obtained using glucose. However, there were no differences in the overall frequency of rooting. Light (16-h photoperiod, 25/19 ºC day/night) and less sugar were beneficial for the ensuing root expression phase. Large differences in the ability to form roots were observed among clones with the rooting percentages ranging between 0 and over 75%. Other examples can be found in Table 2.

Light

Plants grown in vitro have been in most cases subjected to fluorescent lamps. These fluorescent lamps have a broad emission peak in the yellow-red region of the spectrum with different spectral emissions and wavelengths from 350 to 750 nm. However, little attention has been given to the wavelength specificity and its effect on organogenesis, especially in ARF.

Broad-spectrum CW light is often used in rooting studies conducted in vitro (Flygh et al. 1998; Stojičić et al. 1999; Zhang et al. 2006; Ishii et al. 2007; Tang and Newton 2007). Different types of light sources such as Growth-lux (GL) (Gómez and Segura 1994), high-pressure lamps (Scaltsoyiannes et al. 1994) and their combinations such as fluorescent light supplemented with incandescent light (Flygh et al. 1998) are also applied. Different light quality influenced the rooting frequencies according to the PPFD used. Under the intensity of 90 µmol m^{-2} s^{-1} from CW lamps, 70% microshoots of *P. pinea* rooted; however, under GL lamps, with the same intensity, rooting was <50% (Ragonezi et al. 2010).

In the case of shoots of Sitka spruce (*P. sitchensis*), the rooting frequency was high (84%) when cultivated under low illumination (1.5 µmol m^{-2} s^{-1}) (Drake et al. 1997), but in *Juniperus phoenicea* 40% of rooting was achieved under light intensity of 400 µmol m^{-2} s^{-1}. The majority of studies relating to ARF applied light intensities that varied from 60 to 80 µmol m^{-2} s^{-1} (Gómez and Segura 1994; Scaltsoyiannes et al. 1994; Nandwani et al. 2001; Zhang et al. 2006; Ishii et al. 2007). The influence of light (quality) on ARF in shoots of other species is listed in Table 2.

Photoperiod

A photoperiod of 16 h is generally used for in vitro rooting of most conifers (Anderson and Ievinsh 2002; Parasharami et al. 2003). However, different photoperiodism requirements to induce ARF have been published. As an example Burkhart and Meyer (1991), while testing the effect of GA inhibitors to promote in vitro rooting of axillary shoots of white pine (*Pinus strobus* L.), obtained 43% rooted shoots with a pulse treatment of NAA under a long (18 h) photoperiod with CW lamps at 50 µmol m^{-2} s^{-1}. In Picea glauca (Moench) Voss, a higher percentage of shoots rooted under continuous light (Campbell and Durzan 1975).

Temperature

Most rooting protocols reported temperatures in the range of 23-27 C (Table 2). Apart from the example of *T. occidentalis* shoots that rooted at 60 and 10% at 25 and 20°C, respectively (Harry et al. 1987), the lower temperature ranges tended to be beneficial in the induction of roots from shoots of many other gymnosperms. *Picea glauca* rooting was greater at 20/18°C day/night temperature regimes, compared with 24/18, 20/15 or 25/25°C (Rumary and Thorpe 1984); in Douglas fir, 19°C promoted rooting and normal plants, while at 24°C few roots formed along with callus at the stem/root junction, causing discontinuity in the vascular system (Cheng 1977). In white pine (*P. strobus*), the highest rooting frequency was obtained after elongated shoots were treated at 4°C for 4 weeks (Tang and Newton 2005a). The positive effect of combining low temperature and darkness in the induction phase (for the first 2 weeks) was observed in *P. pinea* L., which rooted at a higher percentage at 19°C compared with 25°C (Ragonezi et al. 2010). This is in agreement with the results reported for *P. menziesii* (Cheng and Voqui 1977) and *P. radiata* (Smith 1986). A possible explanation for the effect of low temperature and darkness in promoting ARF was given by Hartmann et al. (2002). They noted that under these physical environmental conditions, fewer cell wall deposits, less vascular tissue and thinner walls might have facilitated the movements of exogenous PGRs to regeneration sites. In loblolly pine (*P. taeda*), Hutchison et al. (1999) observed that during the first 2 days of the rooting process the cambium layer of the hypocotyls dedifferentiated into parenchyma cells in both hypocotyls and epicotyls. Since dedifferentiation

is a part of the regeneration process (Christianson and Warnick 1983), a higher proportion of already undifferentiated cells may improve shoot or root organogenesis. A possible effect of low temperature and darkness could be explained by their influence on auxin metabolism in relation to rooting through modification of peroxidase activities and formation of endogenous phenolic compounds (Druart et al. 1982).

Conclusions

Although difficult to unify, the research results covered in this review highlight some tentative suggestions to explore the physicochemical variables in experimental rooting of conifers, as a guideline for development of more effective conditions for each species. This review also shows the difficulty in establishing correlations between species, PGR concentrations and treatments or any other of the variables cited.

However, an ongoing research on elucidating important aspects of ARF signaling network in angiosperms should eventually provide a better understanding of the process and aid in developing efficient rooting protocols. Whether the same or similar molecular events will be identified in conifers, the evolutionary and physiologically different organisms, remains unknown. A study undertaken by Brinker et al. (2004) in *P. contorta* showed that the transcription level of 200 genes changed from root induction to development suggesting a complex network of interactions in this conifer species.

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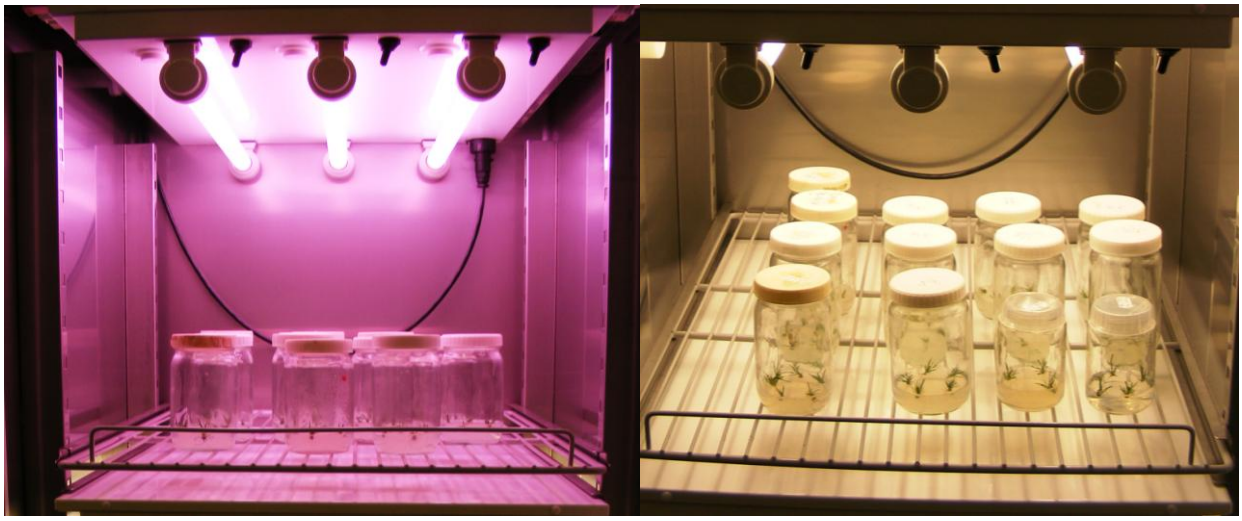

Chapter 3

Influence of Light Quality and Intensity on Adventitious Root Formation in Microshoots of *Pinus pinea* L.

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Ragonezi C, Castro MR, Klimaszewska K, Lima M, Zavattieri A (2010) Influence of light quality and intensity on adventitious root formation in microshoots of *Pinus pinea* L. Proceedings of the IV International Symposium on Acclimatization and Establishment of Micropropagated Plants. Acta Horticulturae 865:287-291.
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Influence of Light Quality and Intensity on Adventitious Root Formation in Microshoots of *Pinus pinea* L.

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Keywords: stone pine, micropropagation, Gro-lux lamps (GL), Cool-white lamps (CW)

Abstract

In the present study of *Pinus pinea* L., further improvement of microshoot rooting was achieved by applying Cool-white light at increased intensity from 60 to 90 µmol m^{-2} s^{-1}. In contrast, light provided by Gro-lux lamps promoted rooting of the microshoots at the same frequency regardless of its intensity. Majority of microshoots (70.4%) grown under Cool-white lamps at the intensity of 90 µmol m^{-2} s^{-1} were also significantly taller when compared with those from other tested treatments.

INTRODUCTION

The light-mediated changes in plant growth and development are referred to as photomorphogenesis, and plants have extraordinary versatility to perceive different light signals in different developmental contexts. Different groups of photoreceptors of the photosensory systems regulate plant development, namely cryptochromes, phototropins and phytochromes each of them monitoring different wavelengths regions of the spectrum (Quail, 2002). Plants use phytochrome to detect and respond to red and far-red wavelengths and cryptochromes were the first blue light receptors isolated and characterized. Light can also modify the efficacy of plant growth regulators (PGRs) as well as affect the endogenous hormone balance. Auxin plays a central role in the determination of rooting capacity, and light conditions are known to affect auxin metabolism and tissue receptivity (Reid et al., 1991). From an applied point of view, plant morphogenesis may be influenced by the correct choice of lamps and filters (Fuerakranz et al., 1990). For example, red-light improved rooting percentage and root numbers in shoots of two genotypes of grape propagated in vitro (Poudel et al., 2008). However, little is known about the effect of green and yellow lights, which seem to be involved in the regulation of in vitro plant development (Loreti et al., 1991).

The purpose of this work was to establish if light quality and intensity, within the visible range, influence root growth and development of *Pinus pinea* microshoots with the aim of enhancing the present rooting protocol achieved with Cool-white (CW) lamps.

MATERIAL AND METHODS

Plant Material

Cotyledons from non-germinated embryos of stone pine (*Pinus pinea* L.) were used as explants. The seed coat was cracked with a nut cracker and discarded. The remainder megagametophytes were surface sterilized by immersion in 70% (v/v) ethyl alcohol for 2 minutes followed by three rinses in sterile bi-distilled water. They were then disinfected with sodium hypochlorite 10% (v/v) (commercial bleach with 5% free chlorine) for 25 minutes followed by four rinses in sterile bi -distilled water. All of the following steps were carried out under aseptic conditions. An embryo was excised from the megagametophyte by making a longitudinal incision with a scalpel and by gently pulling the edges of the cleft with two forceps. Finally, the cotyledons were excised from

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the embryo axes with a cut at their bases.

Microshoot Induction from Embryo Cotyledons

The whole set of cotyledons of each of 20 excised embryos was cultured separately in a Petri-dish (9 x 1.5 cm) containing WPM (McCown and Lloyd, 1981) medium supplemented with 5 mg/L of benzylamino purine (BAP) for shoot organogenesis. After a month, the explants with shoot buds were transferred to a fresh PGR-free medium with 2 g/L of activated charcoal (AC) to promote shoot elongation.

Rooting of Microshoots

Seven, eleven, and six clonal shoots were tested at 60, 90, and 110 µmol m^{-2} s^{-1}, respectively. In all experiments, shoots 2 cm in height were transferred to WPM with half concentration of the macronutrients, 0.65% Difco Bacto-Agar and different carbon sources (see below), and adjusted to pH 5.8 before autoclaving. For root induction, which lasted two weeks, the medium was supplemented with 10.7 µM naphthalene acetic acid (NAA) (Oliveira et al., 2003), and 0.12 M glucose (WPMRI). The cultures were kept for two weeks in a growth chamber; the first week in darkness. During the 2nd week of induction (under 16 h photoperiod and constant temperature of 19ºC) and during root expression phase, two light sources were used: Sylvania Gro-lux lamps 18W (GL) and Philips Cool-white lamps 18W (CW) at different photosynthetic photon flux (PPF – see above) depending on the experiment. In all experiments root expression medium was WPM consisting of half concentration of the macronutrients, without PGRs and with 0.058 M glucose (WPMRE) at 16 h photoperiod and 24/19ºC day/night temperatures. The root emergence was monitored for six weeks.

Light Sources Spectra

Gro- lux: The color tone of GL light is violet; this is the result of the combination of blue and red wave lengths. Cool-white: Broad-spectrum CW light lamps supply blue, yellow, and green light but very little far red light (Fig. 1). Light intensity was measured in the middle of the culture flask with the quantum sensor (Skye Instruments Ltd., UK SKP 200).

Evaluated Parameters and Statistical Analyses

Rooting percentages and the number of roots produced over time for each light quality and intensity were monitored. The percentages of rooted microshoots were compared by the analysis of variance (ANOVA) for clones and light sources using Statistica six Sigma. Means were compared by Duncan's Range test.

RESULTS

P. pinea microshoots rooted at 40% under CW lamps and at 47.1% under GL lamps at 60 µmol m^{-2} s^{-1} (Fig. 2, Experiment 1). In order to test if increased to 90 µmol m^{-2} s^{-1} light intensity from both types of lamps would influence the rooting response; one additional lamp was added in each tissue culture chamber (Experiment 2). Since there were no statistical differences among clones in the first experiment, the data analysis in experiment 2 considered only light quality and light intensity. In the latter experiment 70.4% microshoots rooted under CW and 51% under GL lamps (Fig. 2). There was a significant difference in the rooting percentage under CW lamps at 60 versus 90 µmol m^{-2} s^{-1}, but no difference was observed at the two intensities for GL lamps (Fig. 2, Table 1). This indicated that the *P. pinea* microshoots were not sensitive to variation in the photosynthetic photon flux (PPF) from GL lamps with respect to rooting response. When the light was increased to 110 µmol m^{-2} s^{-1} , reduction of the rooting percentages was observed for both lamp types, particularly for GL (Fig. 2).

The number of roots produced per week increased exponentially during two weeks on WPMRE medium under both types of light (Fig. 3).

DISSCUSION

During the first week of the rizhogenic process (induction) the combination of 0.12 M glucose with 10.7 µM NAA in the medium, and continuous darkness were the most favorable conditions compared with previously tested ones (unpublished results).

Rooting percentage was influenced by different light quality according to the PPF used. The fact that similar rooting percentages were obtained for GL lamps at 60 and 90 µmol m^{-2} s^{-1} indicated that an increase in light intensity did not stimulate rooting. In contrast, increased intensity of CW light increased the rooting percentage by more than 30%. This is an interesting result from the practical point of view because without any change in the tissue culture protocol, except for one additional CW lamp in the culture chamber, it was possible to obtain an increased number of rooted stone pine plantlets. However, at a higher light intensity (110 µmol m^{-2} s^{-1}) ARF was inhibited under both lamps indicating that optimal conditions were exceeded.

Comparing both fluorescent lamps spectra, GL lamps emit blue and red waves and CW more yellow and green. These differences in the spectra could explain the results of this study, which are consistent with the previous observations that phytochrome was mainly involved in root formation (Tyburski and Tretyn, 1999) and that blue light inhibited photomorphogenesis (Seibert et al., 1975). The yellow component of CW lamps might have enhanced adventitious rooting in *P. pinea* similarly to the results of Fuerakranz et al. (1990). The authors reported that yellow light and CW lamps were superior for rooting of *Prunus serotina* compared with red and blue lights alone**.**

Maximum rooting that occurred after approximately 15 days in the expression medium and was consistent in all the treatments, indicated that the time to trigger microshoot response and growth of new roots were independent from the light quality, and that this parameter was determined by other physiological factors.

ACKNOWLEDGEMENT

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Table

Table 1. Duncan test for variable rooting percentage.

	Light quality	Intensity	GL 60, 47143	GL 90, 45714	GL 110, 05000	CW 60, 42643	CW 90, 71571	CW 110, 16667
1	GL	60		0,8900	0,0005*	0,6830	0,0229*	0,0089*
2	GL	90			0,0007*	0,7663	0,0215*	0,0103*
3	GL	110				0,0012*	0,0000*	0,2629
4	CW	60					0,0127*	0,0161*
5	CW	90						0,0000*
6	CW	110						

Figures

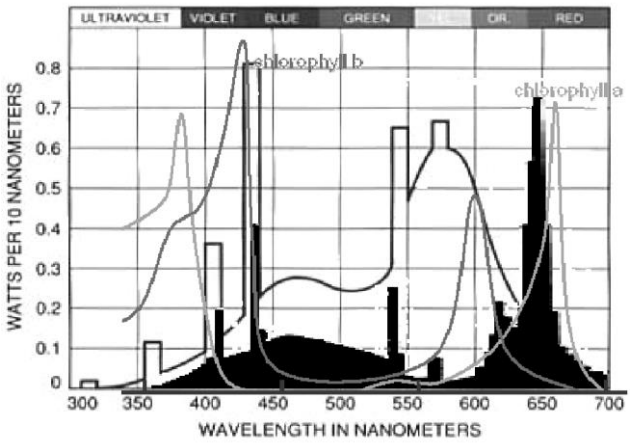

Fig. 1. Spectra of Sylvania Gro-lux lamps (white) and Philips Cool-white lamps (black).

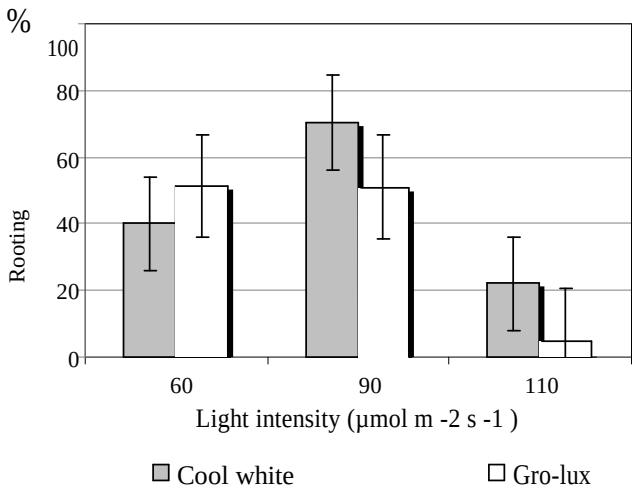

Fig. 2. Rooting of *P. pinea* microshoots under different light quality and intensity. See significance in the Duncan Range test above.

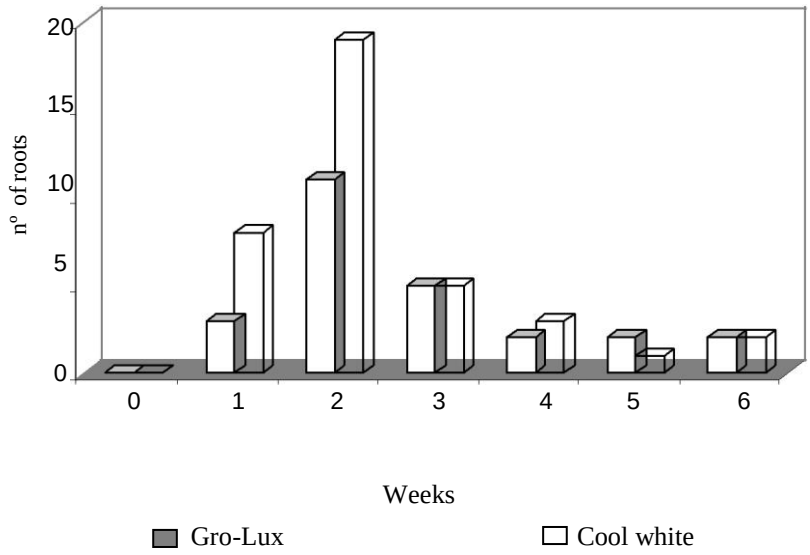

Fig. 3. Number of roots produced per week considering all microshoots for each light treatment under Cool-white and Gro-lux lamps at 60 µmol m^{-2} s^{-1}.

Chapter 4

Mycorrhiza-like Structures in Rooted Microshoots of *Pinus pinea* L.

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Mycorrhiza-like Structures in Rooted Microshoots of *Pinus pinea* L.

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Keywords: ectomycorrhizae, stone pine, rhizotron, adventitious roots, acclimation, biotization

Abstract

***Pinus pinea* L. (stone pine) is one of the major plantation species in Iberian Peninsula, being Portugal the largest edible seed producer in the world. The induction and improvement of in vitro rhizogenesis of microshoots of *Pinus pinea* was developed in our laboratory using a co-culture system with ECM fungi. In the acclimation phase in mixed substrates, or in rhizotrons, anatomical and morphological studies were done to observe the evolution of the root system in microshoots from the co-culture system vs. control plants. Extensive dichotomous and coralloid branching of lateral roots occurred spontaneously in inoculated and control plants as well. Moreover, similar branching occurred in liquid culture of excised seedling roots without the presence of ECM fungi. The striking similarity of these organs with pine ectomycorrhizas prompted their anatomical analysis; however the presence of Hartig net was not confirmed. These results suggested that the development of ECM-like structures might have occurred spontaneously.**

INTRODUCTION

Mycorrhizas are symbiotic structures formed between plant roots and fungi that act as an extension of absorption system, where the fungal partner obtains photosynthetic sugars from the host plant while, in return, the plant receives mineral nutrients from the fungus (Smith and Read, 1997). Ectomycorrhizas (ECM) are the main absorption organs in conifers, and the exchange of nutrients occurs in a specific structure that is formed between the fungal hyphae and the outer root layers. This structure is known as the Hartig net, which is formed by the hyphae penetrating from the surrounding mantle into the root apoplast (Smith and Read, 1997; Brundrett et al., 1996). This mutualistic relationship with fungi grants conifers an ecological advantage to withstand the harsh living conditions.

Stone pine is an extremely appreciated edible nut producer and one of the major plantation species in the Iberian Peninsula. Achieving its clonal propagation is a major goal in the biotechnological development for this species, but has met overwhelming difficulties. The complex interactions between pines and their ECM partners suggest that they might be capable of overcoming such difficulties. The objective of our work was to ameliorate the in vitro adventitious rooting (which does not develop well in the agar cultures) by co-culturing with ECM fungi collected from Stone pine stands (Oliveira et al., 2003). It was also to characterize the fungi-root interactions that enable the development of roots in microshoots and to identify the signalling mediators between ectomycorrhizal fungi and stone pine roots. These potentially new insights into the interactions that take place in the pine rhizosphere could allow us to develop an axenic system that mimics the promoting effect of ECM fungi.

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MATERIALS AND METHODS

Plant Material

Mature seeds of stone pine were obtained in March 2007 from selected 'plus' trees (Alcácer do Sal Region, Portugal) and were stored in a cold chamber at 4°C until used.

Shoot Organogenesis

For the description of shoot organogenesis, see Oliveira et al. (2003) and Ragonezi et al. (2008).

Root Organogenesis

Microshoots were placed in rooting medium. The media used were WPMRI and WPMRE (woody plant medium root induction and expression, respectively) (Ragonezi et al., 2008).

Fungi

Fungi were isolated from single ectomycorrhizas as described by Oliveira et al. (2003) collected at the same location as the cones used for obtaining mature seeds for organogenesis experiments. The fungi were maintained in pure culture using standard procedures (Brundrett et al., 1996). Sixty-five independent isolates were obtained (unpublished results) and screened for their effect on root growth, many of which are included in the present study.

In Vitro Co-Culture

Following the rooting induction and expression phase, the individuals that showed root development were transferred to the double-layer medium and, after a brief period of adaptation to the medium, inoculated with selected fungi, with matching controls to monitor root growth in the absence of inoculation (Oliveira et al., 2003).

Acclimation

The plantlets were transferred to vermiculite for two weeks and then transferred two different substrate systems to observe the development of the root system: mixed substrate in containers, or peat in a rhizotron (Finlay and Read, 1986). During acclimation plants grew in a growth chamber at 25/19°C day/night temperatures, with 16 h photoperiod (270 $\mu mol\ s^{-1}\ m^{-2}$) for 10 weeks. The relative humidity of the growth chamber started at 80% and gradually decreased to 60%. Plants were watered as required with alternating sterile water and liquid WPM (macronutrients only).

Rhizotron

Rhizotrons allow the visualization of root development whenever desired without disturbing the normal functions of the plants. Basically, they are made of two acrylic plates, 20 x 20 cm each, with interval made by 5 mm spacers and filled with turf, in order to support and feed the plants, as suggested by Finlay and Read (1986) with some adaptations from Bending and Read (1996). The root growth was observed thereafter for 4 to 6 weeks, the time required for most of the plants to explore the available space in the rhizotron. Rhizotrons were very useful for the identification of target structures, as well as other symbiotic features that could then be extracted for further studies with minimal disturbance to the rest of the root architecture.

Axenic Root Cultures

Root segments of 2 cm long obtained from germinated seeds were excised and were cultured in liquid medium in an orbital shaker (125 rpm) according to Kaska et al. (1999) for 3 to 4 weeks. Afterward, the roots were photographed and used for histological comparison.

Root Microscopy

Two different methods were used to study root anatomy: 1) hand sections of root segments were obtained according to Brundrett (2008): segments were placed between pieces of laboratory Parafilm® and cut as thin as possible using a razor blade under a Zoom Stereo Research Microscope 7-70X Olympus SZH10. Structural details of the root anatomy and in some cases of the Hartig-net of ECM (when found) were observed under Light Microscope with image acquisition device Olympus CX -40 and photographs were taken with a Canon Power-Shot A630 camera. 2) Dichotomous and short roots were fixed in FAA, dehydrated in an aqueous series of ethanol (70, 80, 95, 100%), clarified in xylol, embedded in paraffin and cut with a rotary microtome (8–10 µm).The sections were stained with toluidine blue, mounted in entellan acid and observed under light microscope (Giomaro et al., 2000).

RESULTS AND DISCUSSION

Adventitious roots regenerated by stone pine microshoots as well as axenic embryo root cultures developed mycorrhizal- like (dichotomous) root laterals even without fungal infection. This is not exceptional and was observed for intact plants as well as for excised roots. In the former, these structures appeared in all experimental settings tested, with the frequency of dichotomous branching increasing as a consequence of a reduction of macro-nutrients in the medium (compared with complete macronutrients) and also in cultures that spent more than one month on the same co-culture medium. These results suggested that without any fungus interaction, some stone pine genotypes responded to less favourable in vitro growing conditions (i.e., lower nutrient concentration and/or lower water availability) by producing mycorrhizal coralloid-like structures. Also coralloid structures appeared as a consequence of applied naphthalene acetic acid (NAA) in the induction medium (Fig. 1b).

On the other hand, under in vitro co-culture (pine-fungus) there was a great variability in the plant response. Some combinations of pine clones with specific fungi showed dichotomous branching while others did not (Figs. 2a, b).

After a few months in substrate, at the moment of transferring to larger containers, dichotomous ectomycorrhizal-like root tips were detected for several inocula, each producing a different rootlet type, indicative of specificity in the development of these structures as a function of the provided inoculum. At least in these earlier stages, non-inoculated controls were devoid of such structures (Fig. 3). Rhizotron-contained plants did not show any such developments. A careful examination of all ECM- like morphotypes failed to reveal Hartig net anatomical features, thus not confirming the symbiotic nature of these structures. The mycorrhizal potential of the fungal inocula is still under investigation, thus not allowing to determine whether that is due to the taxonomical identity of the fungi or the mimicry of dichotomous development by ECM fungi which might start a symbiotic relationship but failed to persist in that condition.

Some plants presented a great number of monopodial short roots with root hairs; others dichotomous branching without hairs and also dichotomy with hairs (Figs. 4a, b, c). Only through the histological observations it was possible to distinguish between true mycorrhizal symbioses and mycorrhizal-like structures as well as ectoendomycorrhizae, the latter not correlating with a particular co-culture inoculum, thence probably from contamination with E-type propagules (Figs. 5a, b).

Axenic root cultures showed profuse dichotomous branching similar to those of in vitro cultured plants (Fig. 6).

Many environmental or cultural conditions could have influenced the capacity of some stone pine clones to produce mycorrhizal-like structures. At the current stage of our study it is impossible to know if there is a correlation between pine clones and their propensity to form mycorrhizal-like structures. Responses to the inocula used in the co-culture (either the resumed growth of roots previously formed as a consequence of plant growth regulators treatment, or dichotomous branching) was not consistently correlated with the induction of mycorrhizal symbiosis in the acclimation phase. Nevertheless, the

characterization of biochemical signals that are likely mediating these effects will continue to be pursued, in order to understand the possible physiological implications for the plants from the development of these structures.

CONCLUSIONS

- This is the first report on the abundant mycorrhizal-like structures in stone pine roots that were produced by axenic cultures, in in vitro-cultures and in subsequent acclimation phase in mixed substrates.
- There was a strong similarity between extensive dichotomous and coralloid branching of lateral roots that grew spontaneously in stone pine with those derived from fungal inoculation.
- Due to this similarity it may be difficult to diagnose ectomycorrhizas without confirmation of the ECM status by histological analysis.
- Since this response appeared to be 'genotype dependent' more studies will be needed to establish correlation between stone pine clones and the root system morphology.
- The biochemical studies that are being carried out presently on the co-cultured roots could elucidate the nature of the compounds that cause a highly effective adventitious rooting in the presence of certain fungi and afterwards in the acclimation phase.

ACKNOWLEDGEMENTS

This work was supported by FCT Portugal: PTDC/AGR-CFL/71437/2006 Analysis and Mastering of Root Growth Signalling by Ectomycorrhizal Fungi on *Pinus pinea* Microshoot Cultures.

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Figures

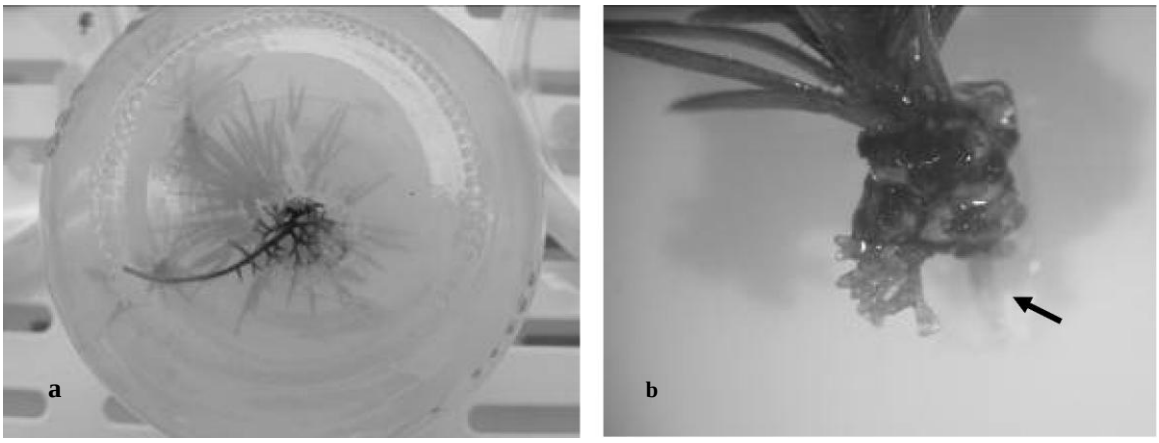

Fig. 1. (a) After more than one month without transferring to new medium, some genotypes developed coralloid mycorrhizal-like structures. (b) Coralloid structures also appeared as a consequence of NAA in the rooting induction medium. The arrow indicates a normal root type induced by auxin treatment.

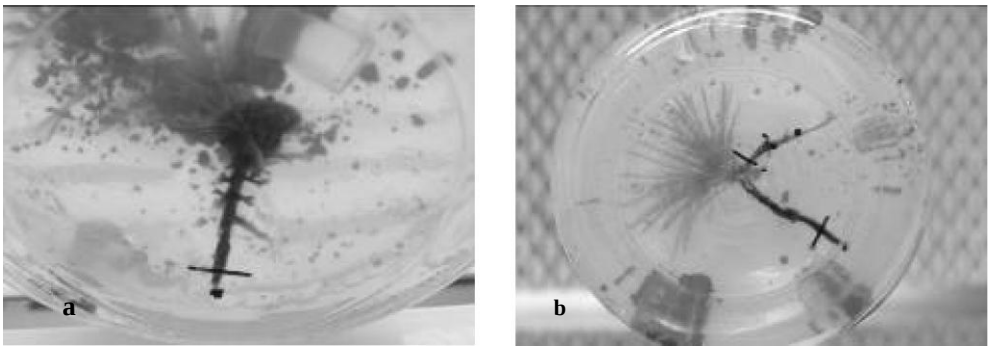

Fig. 2. (a) Differential responses in the in vitro adventitious root formation in co-culture system. Root with dichotomous branching. (b) Normal in vitro growth of the root system. Mycelium could be seen growing on the surface of the culture medium in both culture vessels.

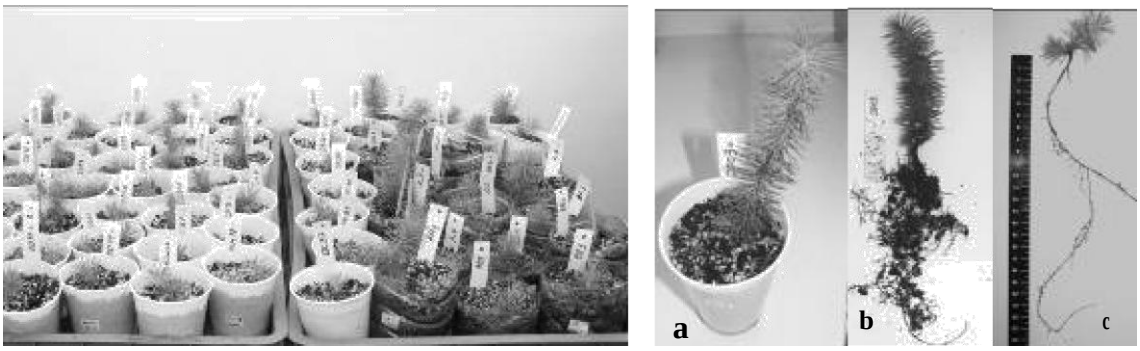

Fig. 3. Left: Rooted plants in the acclimation phase. Plants after 4 months in the acclimation phase. (a) Inoculated pine plant, identified as DD03, shows a good development of the aerial part and (b) also compact and dichotomous branched root system. (c) Control plant, not inoculated with less aerial part development and a linear root system 23 cm long.

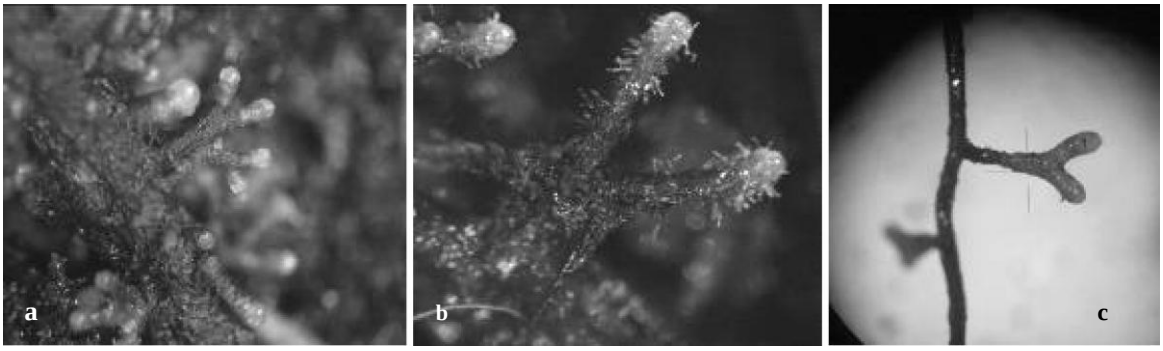

Fig. 4. (a) Different anatomical structures of the roots in plants derived from co-culture after 4 month in the acclimation phase (pine clones/different fungi isolates). Extensively dichotomous branching without hairs, (b) dichotomous short root with hairs and (c) ectomycorrhizal-like structure.

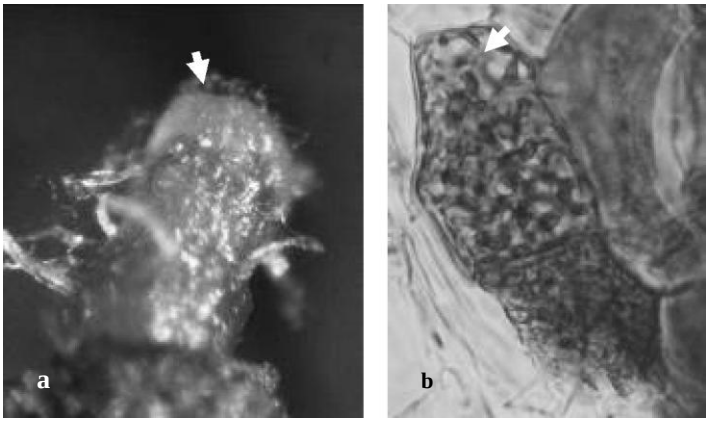

Fig. 5. (a) Short root covered with mycelia. (b) Longitudinal section shows the intracellular hyphae.

Fig. 6. Dichotomous branching observed in the liquid axenic root cultures after one month of growth.

Chapter 5

Molecular Approach to Characterize Ectomycorrhizae Fungi from Mediterranean Pine Stands in Portugal

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Molecular approach to characterize ectomycorrhizae fungi from Mediterranean pine stands in Portugal

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Abstract

Stone pine (*Pinus pinea* L.), like other conifers, forms ectomycorrhizas (ECM), which have beneficial impact on plant growth in natural environments and forest ecosystems. An *in vitro* co-culture of stone pine microshoots with pure mycelia of isolated ECM sporocarps was used to overcome the root growth cessation not only *in vitro* but also to improve root development during acclimation phase. *Pisolithus arhizus* (Scop.) Rauschert and *Lactarius deliciosus* (L. ex Fr.) S.F. Gray fungi, were collected, pure cultured and used in *in vitro* co-culture with stone pine microshoots. Samples of *P. arhizus* and *L. deliciosus* for the *in vitro* co-cultures were collected from the pine stands southwest Portugal. The *in-situ* characterization was based on their morphotypes. To confirm the identity of the collected material, ITS amplification was applied using the pure cultures derived from the sporocarps. Additionally, a molecular profile using PCR based genomic fingerprinting comparison was executed with other genera of Basidiomycetes and Ascomycetes. Our results showed the effectiveness of the techniques used to amplify DNA polymorphic sequences, which enhances the characterization of the genetic profile of ECM fungi and also provides an option to verify the fungus identity at any stage of plant mycorrhization.

Key words - *Pisolithus arhizus*, *Lactarius deliciosus, Pinus pinea,* M13-PCR, ITS

Introduction

Ectomycorrhizal fungi (ECM) are major components of the soil fungal communities in most forests around the world and, are ecologically and economically important (Mello *et al*., 2006). Plants in Betulaceae, Pinaceae and Fagaceae families are obligate ECM (Smith and Read, 1997). ECM fungi include species from multiple families in the Basidiomycetes, Ascomycetes and some from the Zygomycetes (Bruns *et al*., 2002). Globally, as many as 10,000 fungus species and 8000 plant species maybe involved in ECM associations (Taylor and Alexander, 2005).

Development and growth of pine (*Pinus* spp.) roots are regulated in nature by ECM (Smith and Read, 1997). Inoculation with specific fungi can enhance pine root formation and/or subsequent root branching of cuttings (Normand *et al*., 1996; Karabaghli *et al*., 1998; Niemi *et al*., 2000). Some research results demonstrated the potential for use of ECM fungi in the vegetative propagation of conifers (Gay, 1990; Niemi *et al*., 2005) and during *in vitro* rooting of pine shoots (Zavattieri *et al*., 2009; Ragonezi *et al*., 20010a). Inoculations enhanced plant performance and contributed to alleviation of stress related with acclimation in a nursery and the subsequent growth in the field.

Stone pine (*Pinus pinea* L.) is one of the most important pines economically (due to the valued edible nut production) in the Mediterranean basin and it forms ectomycorrhizas. Rincón *et al*., (1999) reported that at least eight genera of ECM were associated with *P. pinea* seedlings in the nursery (*Amanita*, *Hebeloma*, *Laccaria*, *Lactarius*, *Pisolithus*, *Rhizopogon*, *Scleroderma*, and *Suillus*). Two species of fungi are commonly used for inoculation in controlled mycorrhization programs associated with *P. pinea*: *Pisolithus arhizus* (Scop.) Rauschert, (Marx *et al*., 1982; Burgess *et al*., 1995) a cosmopolitan fungus which grows in warm temperate regions of the world and is easy to propagate *in vitro* (Marx *et al*., 1982; Cline *et al*., 1987) and *Lactarius deliciosus* (L. ex Fr.) S.F. Gray, typically a Basidiomycetes which produces high commercially valuable edible fruiting bodies (Singer, 1986; Hutchison, 1999; FAO, 2004; Hortal *et al*., 2006).

In nature, and also in controlled inoculations, genetically distinct mycelia of the same ECM species were found on the root system of a single tree (Guidot *et al*., 1999). This was also demonstrated by others studies with *Pinus banksiana* (De La Bastide *et al*., 1995) and *Pinus pinaster* (Gryta *et al*., 1997). Even in cases where the *in vitro* inoculation was controlled, genetic diversity has been found in *ex vitro* phases caused

by the lack of effective sterilization of the mixed substrates, contamination from the environment in the growth chamber and in some cases from the irrigation source. On the other hand, ECM fungi are relatively selective of host plant species (Allen *et al*., 1995) and host responses could be partially attributable to variation between different fungus taxa and strains. For all these reasons, accurate characterization and identification of the ECM fungi are fundamental requirements for *in vivo* or *in vitro* mycorrhization programs.

The traditional method of fungal identification by colour, shape and other macroscopic features and microscopic characteristics (Agerer, 1987-2002) could be applied only to a limited number of fungal species (Iotti and Zambonelli, 2006).Neverthless, today a wide range of molecular techniques can be used to distinguish DNA sequence for the identification of ECM fungi (Gardes *et al*.; 1991a, 1991b; Henrion *et al*., 1992; Hortal *et al*., 2006) and also to verify the genetic variation within a specific group (Alves *et al.,* 2007; Caldeira *et al*., 2009).

Amplification of the internal transcribed spacer (ITS) regions in the ribosomal genes (rDNA) usually reveals interspecific variations (Bruns *et al*., 1991, Gomes *et al.,* 2000, Horton 2002). This region has four primary advantages over other regions: 1 - it is multicopy, so the amount of sample material needed for successful amplification is low; 2 - it has well-conserved fungal specific priming sites directly adjacent to multiple highly variable regions; 3 - there are many sequences already available for comparison, which facilitates the identification of unknown samples; and 4 - it correlates well with morphologically defined species in many groups (Smith *et al*., 2007).

Genetic profiles and polymorphic sequences on the other hand, are important tools for rapid and effective characterization of ECM species (Caldeira *et al*., 2009). The polymerase chain reaction (PCR) based genomic fingerprinting is a good alternative to methods that rely on specifically targeted primers. This technique, which analyzes the whole genome, has been shown to be relatively robust and discriminatory (Alves *et al*., 2007). PCR fingerprinting is also used in the study of genetic variability in yeast and filamentous fungi (Godoy *et al.,* 2004; Alves *et al.,* 2007; Lopes *et al.,* 2007).

The goals of the present study were, first to identify ECM fungi associated with stone pine stands through PCR amplification of the ITS region of the ribosomal genes and to use them in *in vitro* mycorrhization experiments. Second goal was to test the applicability of the M13-PCR fingerprinting methodology for monitoring different species

of Basidiomycetes and Ascomycetes which can be found in association between *P. pinea* and ECM fungi.

Material and methods

Collection of mushrooms from stone pine (*Pinus pinea* L.) stand

Fruiting bodies of *Pisolithus arhizus* (Scop.) Rauschert and *Lactarius deliciosus* (L. ex Fr.) S.F. Gray were collected from a pure stand of stone pine (N 38° 25'; W 7° 56') in January of 2010. Morphological identification was done *in situ* at the collection time. Specimens were stored at 4ºC prior to sterilization and isolation procedures. Voucher specimens of *Pisolithus arhizus* and *Lactarius deliciosus* were deposited at Évora University Herbarium with the numbers UEVH-FUNGI 2001610 and UEVH-FUNGI 2001712, respectively.

Mycelia isolation and fungal cultures

For the asepsis, the fruiting bodies were cut into large pieces, placed in running water for 10 min and then in 70% ethanol for 2 min. Then, pieces were rinsed with sterile distilled water in a laminar flow unit, placed in 20% (v/v) commercial bleach (≤ 5% active chlorine) for 10 min and rinsed four times with sterile water. The larger pieces were then cut in smaller pieces (50 mm^3) for growth and subsequently DNA extraction or were stored at -20ºC. Isolates were cultured in Hagen medium (Modess, 1941). The formulation of modified Hagen per liter was: KH_2PO_4 0.5 g, NH_4CL 0.5 g, $MgSO_4$ $7H_2O$ 0.5 g, $FeCL_3$ (1%) 0.5 ml, glucose 5 g, malt extract 5 g, thiamine HCL 50 µg and agar 15 gand the pH was adjusted to 4.5-5.0. With the purpose to avoid the contamination by bacteria, 100 mg ml^{-1} of Rifampicin (Sigma-Aldrich®) was added to the media after cooling. Pieces of sporocarps were kept in petri dishes Hagen medium, grown at 25ºC in the dark and sub-cultured at weekly intervals. Isolates have been growing in Hagen slants for 14 days at 25ºC and stored at 4ºC. Fungal isolates of *Pisolithus arhizus* and *Lactarius deliciosus* were deposited in the Culture Collection of the Biotechnology Laboratory of University of Évora and preserved at −80°C in cryovials containing 10% glycerol.

DNA extraction

The extraction of the genomic DNA from the smaller fragments of sporocarps and from the mycelia (after 14 days of culture) was performed using the modified microsphere method (Martins, 2004; Guimarães *et al.,* 2011). The quality and quantity of the obtained DNA was evaluated by agarose gel.

ITS region amplification and sequencing

The region containing partial portions of the small subunit (18S), both internal transcribed spacers (ITS) and the 5.8S of the rDNA repeat unit was amplified using the oligonucleotides primers ITS5 (5'-GGAAGTAAAAGTCGTAACAAGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') (Gardes and Bruns, 1993). PCR reactions were carried out on a MyCycler Thermal Cycler (BIO-RAD) and consisted of initial denaturing at 95ºC for 3 min followed by 30 cycles at 92ºC each 30 s, 55ºC for 30 s, and 72ºC for 1 min. The reaction was completed by a 10-min extension at 72ºC. PCR products were analyzed by agarose gel (1%) electrophoresis, purified with the NucleoSpin Extract II Kit (Macherey-Nagel) and sequenced by capillary electrophoresis using the ABI PRISM 3730 xl sequencer (Applied Biosystems) with the Kit BDT v1.1 (Applied Biosystems).

M13-PCR amplification

The M13 primer (5'- GAGGGTGGCGGTTCT-3') was used for the PCR. The PCR conditions consisted of an initial denaturing step of 5 min at 94 °C followed by 40 cycles of 1 min at 94 °C, 1 min at 50 °C and 2 min at 72 °C. The reaction was completed with a final extension at 72 °C for 5 min and then cooled at 4 °C. A sample of each PCR reaction product was electrophoresed in a 1.5% agarose gel and visualized, by staining with ethidium bromide, in a UV transilluminator (BIO-RAD). To evaluate the reproducibility of the assay, each sample has been analyzed in at least three independent PCR reactions. A negative control (without DNA template) has been included in every run. Subsequently, DNA sequence analysis was employed for confirmation of the fingerprint technique characterization.

Data analysis

The nucleotide sequences of the ITS region were aligned with those of related fungal species retrieved from the GenBank (National Center for Biotechnology Information - NCBI) databases for the homology analysis using the BLASTN 2.2.25+ program. The phylogenetic relationships between different species were inferred after multiple alignments using CLUSTAL W (Thompson *et al*., 1994). The distances of the DNA arrays were calculated with the option of Jukes-Cantor and from these matrixes, using the Neighbor-Joining method, the phylogenetic tree was constructed, using the program Mega 5 (Tamura, 2011).

For the M13-PCR analysis, the phylogenetic tree was generated by the Unweighted Pair Group Method with arithmetic Average (UPGMA), through the use of the Dice coefficient of similarity using Quantity One 1-D Analysis software (BIO-RAD).

Results and discussion

Collection of fruiting bodies from stone pine stand

Representative voucher specimens of *Pisolithus arhizus* and *Lactarius deliciosus* fruiting bodies are shown in Fig. 1a and Fig. 2a. Based on preliminary tests, we have selected Hagen medium as the most suitable for isolation and growth of the mycelia from sporocarps. The cultured mycelia were characterized by yellowish-ochraceous with paler margin in the case of *P. arhizus* (Fig. 1b) and pinkish with paler margin for *L. deliciosus* (Fig. 2b). The microscopic features showed the secondary mycelia at the septa of a Basidiomycota hypha (Fig. 1c e 2c).

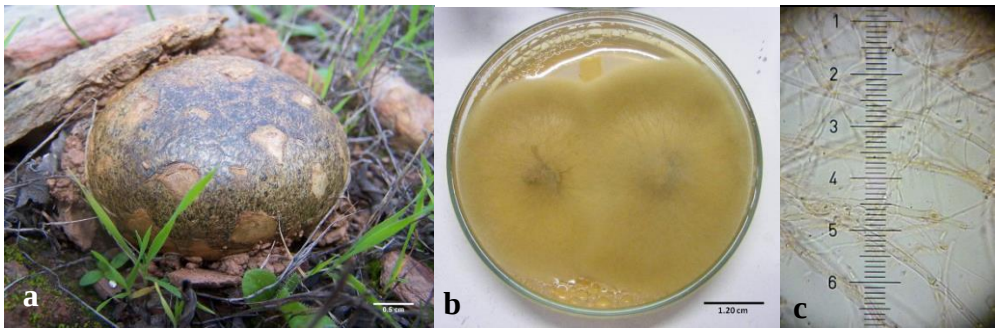

Fig. 1 - *Pisolithus arhizus* sporocarp collected in a stone pine stand selected for *Pinus pinea*-ECM associations study (a). The mycelia cultured in Hagen medium (b). Secondary mycelia. Each interval 2.5 µm (c).

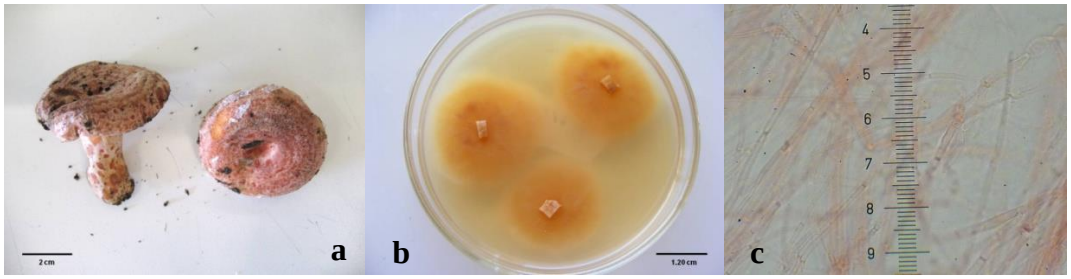

Fig. 2 - *Lactarius deliciosus* sporocarps collected in a pine stand selected for *Pinus pinea*-ECM associations study (a). The mycelia cultured in Hagen medium (b). Secondary mycelia. Each interval 2.5 µm (c).

Fresh mycelia of each culture were used to inoculate stone pine microshoots at the rooting phase (Fig. 3). The mycorrhization of the plants were confirmed in the acclimatization phase (Fig. 4a and b) revealing the typical ECM structure. The ECM fungi presence was monitored and confirmed during the mycorrhization process, by

applying two molecular complementary approaches: ITS sequencing and M13-PCR amplification.

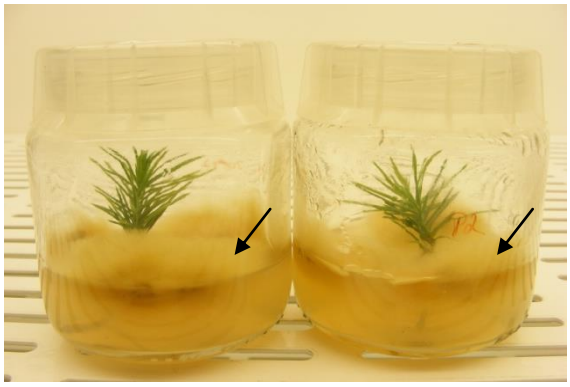

Fig. 3 - *In vitro* co-culture of *Pinus pinea* and *P. arhizus* mycelium (arrows).

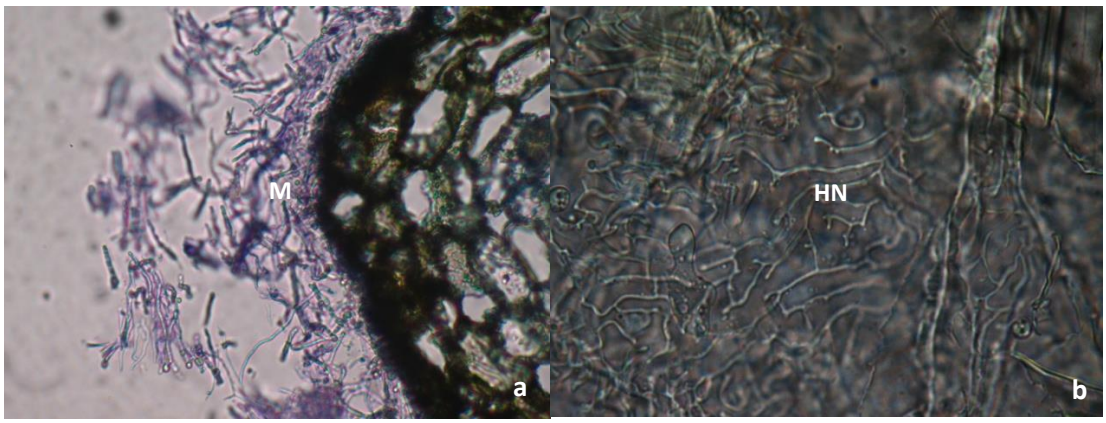

Fig. 4 - Cryostat transversal root section of the colonized by *P. arhizus* pine root showing the mantle hyphae (M) (100x); Scale bar 20 µm (a). Details of the transversal section showing well-differentiated Hartig-net (HN) in cortical cells (1250x); Scale bar 7.5 µm (b).

Species identification of *P. arhizus* and *L. deliciosus*

In the past, the most common approach to evaluate fungal biodiversity in various plants/ systems has been the sample collection, fungal isolation and identification based on classical methods (Genilloud *et al.,* 1994; De Jager *et al.,* 2001; Moreira *et al.,* 2001; Schmit and Lodge, 2005), but nowadays several molecular techniques can be used namely for ECM fungi identification (Rosling, 2003; Leake *et al.,* 2004; Hortal *et al.,* 2006; Caldeira *et al.,* 2009).

Amplification of the ITS region is a common approach in molecular identification strategies (Hortal *et al.,* 2006; Alves *et al.,* 2007). PCR products of ITS4/ITS5 primers, corresponding to the ITS1, 5.8S and ITS2 regions of the rDNA were approximately 644 bp and 400 bp obtained from dikariontic isolates from *Pisolithus sp*. P1001 and *Lactarius deliciosus* UEZB1 respectively. Sequence alignments of *P. arhizus* showed

identities that ranged from 99-100% among isolates belonging to *P. arhizus.* Sequences were aligned at the NCBI for isolates corresponded to other *Pisolithus spp.* In the case of *L. deliciosus* the homology was over 99%. The most similar sequences of *P. arhizus* and *L. deliciosus* are shown in Table 1. The phylogenetic tree (Fig. 5) was obtained from the alignment of these sequences. We identified two different clusters, *Pisolithus* sp. P1001 and *L. deliciosus* isolate UEZB1 (Fig. 5). Multiple alignment of *Pisolithus sp.* cluster corresponded to a partial sequence of 18S RNA gene and ITS1, 5.8S ribosomal RNA gene and ITS2, and partial sequence of 28S RNA ribosomal region. *L. deliciosus* UEZB1 corresponded to partial sequence of ITS1, 5.8S ribosomal RNA gene, ITS2 and partial sequence of 28S ribosomal RNA. Both sequences were published in GenBank with accession number HQ896485 and JQ066791, respectively.

Table 1. ITS rDNA homology from fungal strains used in the phylogenetic tree construction. The nucleotide sequences of the ITS region were aligned with those of related fungal species retrieved from the NCBI databases (www.ncbi.nlm.nih.gov).

Strains	**Identification nº (NCBI)**	**Homology**
Pisolithus sp. KH-NC09	gb|GQ429212.1	99%
Pisolithus microcarpus	emb|AM084706	100%
Pisolithus tinctorius R15	gb|AF374695	99%
Pisolithus microcarpus VIC30598	gb|HQ693097	100%
Lactarius deliciosus H:6002989	gb|GU373514.1	100%
Lactarius sp. isolate cm130.ps	gb|EU668299.1	100%
Lactarius deliciosus isolate CSUFTXY7	gb|HQ635086.1	100%
Lactarius deliciosus LDTA30	gb|FJ858745.1	100%

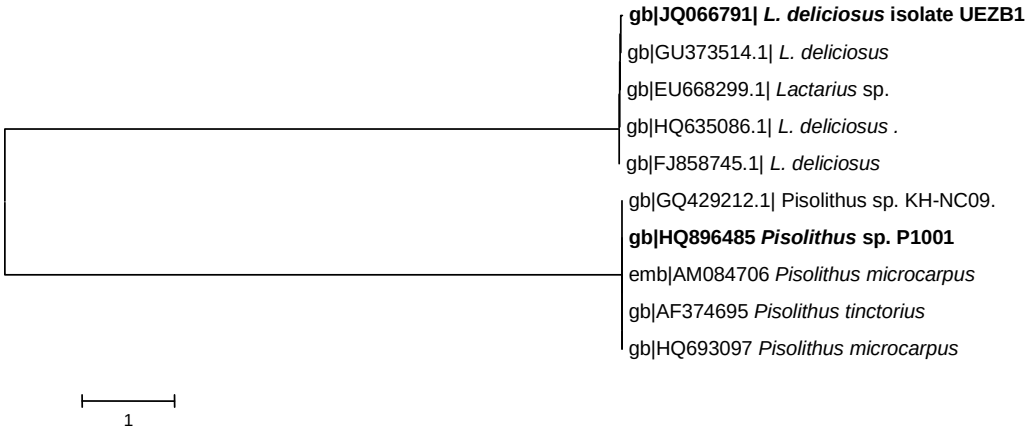

Fig. 5 - Phylogeny tree based on the ITS sequence

Intraspecies identification by M13-PCR

The amplification using the M13 primer has generated a profile with 7-14 DNA fragments ranging from 100 to 2700 bp in the Basidiomycetes sporocarps species (*P. arhizus*, *L. deliciosus* and *R. roseolus*), *Pisolithus sp.* isolated P1001 and select Ascomycetes (*P. brevicompactum*, *A. niger*, *Cladosporium sp*.1, *and F. oxysporum*). These Ascomycetes species could live in association with ECM fungi and were commonly found in the isolation process. Reproducibility of the M13-PCR fingerprinting techniques was checked by comparing the banding profiles resulting from independent extractions and amplifications of the same fungi strain. The different samples have generated distinct patterns in the electrophoresis analysis (Fig.6).

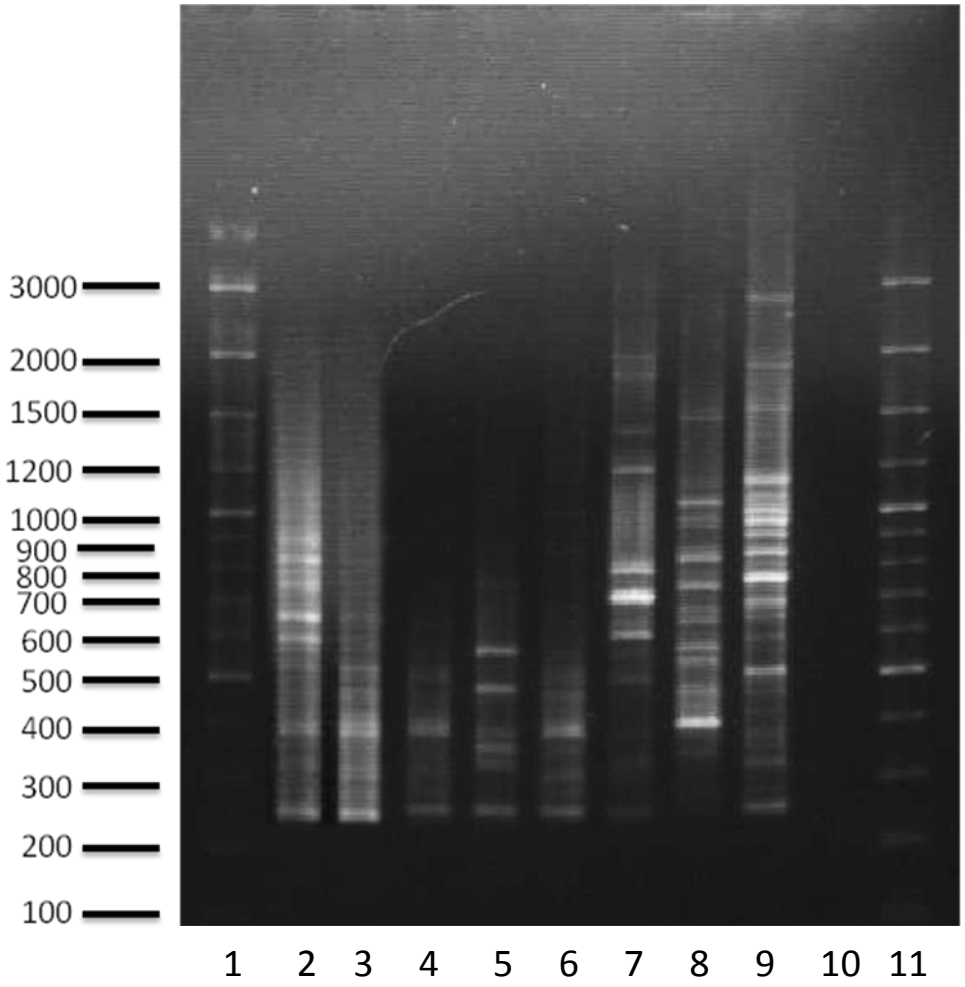

Fig 6 - Fingerprinting patterns obtained from obtained by amplification of genomic DNA. Lanes: 1 and 11 DNA molecular ladder 100 bp plus (Fermentas), 2: *Pisolithus arhizus* culture, 3: *Pisolithus arhizus* sporocarps, 4: *Lactarius deliciosus* sporocarps, 5: *Rhizopogon roseolus* sporocarps, 6: *Penicillium brevicompactum* sporocarps, 7: *Aspergillus niger* sporocarps 8: *Cladosporium sp*., 9: *Fusarium oxysporum* sporocarps, 10: Control.

The M13-PCR band profile generated in each fingerprint varied according to the species included in this study: 10 fragments ranging from 200-900 bp for *P. arhizus,* 7 fragments for *L. deliciosus* (from 200-550 bp) and 9 fragments for *R. roseolus* (from 200-750 bp). The Ascomycetes, *P. brevicompactum* presented 9 fragments (from 200-1100 bp), *A. niger* 10 fragments (from 200-1850 bp), *Cladosporium* sp.1 presented 13 fragments (from 200-1850 bp) and *F. oxysporum* 14 fragments (from 200- 2700 bp). The Ascomycetes group presented a higher DNA fragments (ranging from 950-2700 bp) than the Basidiomycetes, with fragments from 150-900 bp. Figure 7 shows a phylogenetic tree based on M13-PCR fingerprinting. In the analysis of the phylogenetic tree, *P. arhizus* formed a cluster of 41% similarity with *R. roseolus*, which forms a cluster with *L. deliciosus* with a homology of 34%. This approach also allowed the distinguishing between Basidiomycetes and Ascomycetes group, which formed a cluster with 15% of similarity for *P. brevicompactum*, *8% for A. niger, 5%* for *F. oxysporum and 2% for Cladosporium* sp.1.

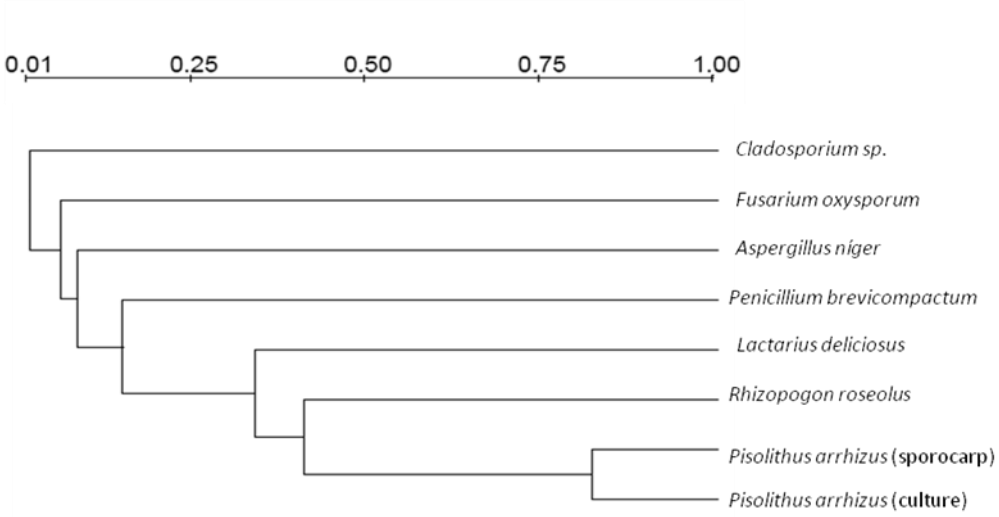

Fig. 7 - Phylogenetic tree analysis based on the PCR fingerprinting patterns for different species of Basidiomycetes and Ascomycetes. To evaluate the reproducibility of the assay, each sample has been analyzed in at least three independent PCR reactions. The distance values between branches are reported as percentage of similarity (0-100%).

These results demonstrated that M13-PCR discriminated among between species and taxonomic groups. Based on the specific PCR fingerprints and the high interspecies variation of these banding patterns, a clear distinction among all species was feasible. M13-PCR highlighted differentiation at the species and strain level (Caldeira *et al.,* 2009). In this study, the M13-PCR approach was a rapid method to amplify DNA polymorphic sequences, with a high level of similarity for the same species, which

enhances the characterization of the genetic profile of sporocarps such as *L. deliciosus*, *P. arhizus* and *R*. *roseolus*.

The advantages of this DNA amplification method are the technique simplicity, universal availability of PCR primers, reproducibility and amenability to the computer database analysis. Using only a single primer M13-PCR, it was possible to achieve high levels of resolution. This makes the procedure much faster and easier, and greatly reduces the cost (Alves *et al.,* 2009). Hence, PCR fingerprinting offers a simple and reliable alternative method to resolve taxonomic problems and to "label" strains of filamentous fungi (Meyer *et al*., 1991).

Conclusions

Results of this study demonstrate that the combined use of sequence analysis of the ITS regions of the rDNA and the PCR fingerprinting technique can be successfully applied as an excellent tool to examine the species collected in the field associated with *Pinus pinea* and also as a methodology to monitor the fungi species involved in all the steps in a mycorrhization program. The applied molecular techniques accurately characterized field collected sporocarps and confirmed the presence of the fungus in inoculated plants.

Owing to its low cost and rapidity, the M13-PCR has a wide application in applied mycology as was demonstrated in this study. Also, we confirmed that the M13-PCR technique has a high level of reproducibility because the fungal samples amplified in independent PCRs displayed similar banding pattern profiles.

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Chapter 6

Pisolithus arhizus (Scop.) Rauschert Improves Growth of Adventitious Roots and Acclimatization on *in vitro* Regenerated Plantlets of *Pinus pinea* L.

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PISOLITHUS ARHIZUS (SCOP.) RAUSCHERT IMPROVES GROWTH OF ADVENTITIOUS ROOTS AND ACCLIMATIZATION OF *IN VITRO* REGENERATED PLANTLETS OF *PINUS PINEA* L.

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Abstract

Stone pine (*Pinus pinea* L.) is an economically important forest tree in the Mediterranean region and has been the target of breeding and selection through micropropagation mainly for its ecological and ornamental aspects. A crucial step in micropropagation is adventitious rooting of microshoots, which often is highly inefficient in most conifer species including stone pine. Hence, we conducted *in vitro* co-culture of *Pinus pinea* microshoots with the ectomycorrhizal fungus *Pisolithus arhizus* (isolated from natural stands) in order to promote adventitious root growth and plant survival during acclimatization. Significant differences were found in the number of branches, in the number of roots plus branches, in total length of roots, in total length of roots plus branches, in average root length and in the length of the longest root in inoculated plants during *in vitro* rooting compared with non-inoculated plants. The roots of inoculated plants also grew better in vermiculite and during acclimatization in a mixed substrate compared with roots of control plants resulting in the development of vigorous root system. Overall, mycorrhizal inoculation increased the survival rate of the regenerated pine.

Key words: co-culture, *in vitro* mycorrhization, micropropagation, root system.

INTRODUCTION

Stone pine (*Pinus pinea* L.) is an important species widely distributed in the Mediterranean region (Capuana and Giannini 1995). The trees have been exploited for their edible seeds (pine nuts) since prehistoric times and currently, in addition to being cultivated for the seeds, it is also a widespread horticultural tree (Nergiz and Donmez 2004). *P. pinea* has been successfully introduced into North Africa as well as Argentina and South Africa. In other places e.g. California, Scotland, and Southern England it is usually confined to parks and gardens (Fady et al. 2004). In Portugal, there is large-scale production of clonal stone pine genotypes using grafting and *in vitro* organogenesis from mature embryo cotyledons (Alpuim 2000, Carneiro 2002, Zavattieri et al. 2009, Ragonezi et al. 2010a). However, the number of rooted microshoots obtained through organogenesis is low, because similarly to other conifers, stone pine rooting is difficult and genotype dependent (Cuesta et al. 2006, Ragonezi et al. 2010b). Another *in vitro* method of micropropagation is somatic embryogenesis, which in a few pine species is relatively efficient but has limited use in stone pine due to difficulties in somatic embryo maturation and conversion to plants (Carneros et al. 2009).

P. pinea, like other conifers, forms ectomycorrhizas (ECM), which have beneficial impact on plant growth in natural environments (Read 1991) and forest ecosystems (Grove and Le Tacon 1993). Central to the success of these symbioses is the exchange of nutrients between symbionts (Smith and Read 1997) and the extended function of the root system (Smith

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and Read 1997, Read and Perez-Moreno 2003, van der Heijden et al. 2003). This relationship with fungi grants conifers an ecological advantage to withstand the harsh living conditions in sandy soils and coastal areas. Many Basidiomycetes fungi and some Ascomycetes fungi are characterized primarily by the presence of a mantle (sheath) and a Hartig net consisting of modified fungal hyphae that develop among root cells (Smith and Read 1997). This relates not only to the extent of root colonization but also to the development of hyphae in the soil (Colpaert et al. 1992, Thomson et al. 1994). Generally, gymnosperms have a Hartig net that develops around epidermal and cortical cells (Peterson and Massicotte 2004).

Pisolithus arhizus (Scop.) Rauschert is a cosmopolitan fungus in warm temperate regions of the world and forms ECM associations with a wide range of tree species, both angiosperms and gymnosperms (Marx 1977, Chambers and Cairney 1999). Moreover, *P. arhizus* is well adapted to diverse environmental conditions, is relatively easy to grow *in vitro* and has the ability to recognize and become associated with host plants (Béguiristain and Lapeyrie 1997, Reis et al. 2011).

Several studies have shown the potential of using ECM fungi in conifer propagation (Gay et al. 1990, Niemi et al. 2000, 2004, 2005). Inoculation of specific fungi can enhance root formation and or subsequent root branching of cuttings in vivo and in seedlings (Karabaghli et al. 1998, Niemi et al. 2000, Normand et al. 1996). Rincón et al. (1999) identified at least eight genera of fungi that formed ECMs in control inoculation of *P. pinea* seedlings in the nursery (*Amanita*, *Hebeloma*, *Laccaria*, *Lactarius*, *Pisolithus*, *Rhizopogon*, *Scleroderma*, and *Suillus*), but the numbers of mycorrhizal short roots varied among isolates. Fungal inoculations can increase the plant ability to overcome the stress related with nursery and growth after transplantation (García et al. 2011, Fini et al. 2011). It has been previously shown that ECM-derived fungal isolates promoted sustained root growth of *in vitro* propagated stone pine microshoots, but in that study most of the fungal-isolates that induced renewed root growth were not identified (Oliveira et al. 2003).

The aims of the present study were first, to isolate from a pure stand of *P. pinea* the ectomychorrizal fungus, identify it and obtain pure culture of the mycelia and second, to determine whether *in vitro* inoculation of stone pine micropropagated plants with *P. arhizus* would promote the adventitious root growth and thereby improve acclimatization by decreasing the loss of plants during weaning.

MATERIALS AND METHODS

Collection of P. arhizus fruiting bodies from a Pinus pinea stand

Fruiting bodies of *P. arhizus* were collected from a pure *P. pinea* stand (N 38° 25'; W 7° 56') in January 2010. Identification was done on-site at the collection time and later confirmed in laboratory. Morphological identification was based on keys, monographs and field guides (Pegler et al. 1995, Calonge 1998, Gerhardt et al. 2000). Specimens were stored at 4ºC before sterilization and isolation procedures.

Mycelia isolation and fungal cultures

For the asepsis, the fruiting bodies were cut into large pieces, placed in running water for 10 min and then in 70% ethanol (Sigma-Aldrich®, Sintra, Portugal) for 2 min. Next, the pieces were rinsed with sterile distilled water in a laminar flow unit, placed in 20% (v/v) sodium hypochlorite 10% (v/v) (commercial bleach with 5% free chlorine) for 10 min and rinsed four times in sterile water. The large pieces were then cut in small pieces (50 mm^3) for growth or were stored at -20ºC. For growth, the pieces of fruiting bodies were placed in Petri dishes with a medium. The cultures were kept at 25ºC in dark and subcultured at weekly intervals. Three different media were initially tested for *P. arhizus* isolation and culture; BAF (biotin-aneurin-folic acid agar; Oort 1981), Hagen (Modess 1941) and MMN (modified Melin-Norkrans, Marx 1969). The media were autoclaved for 20 min at 121ºC and the pH was adjusted to 4.5-5.0 for Hagen, 5.7-6.2 for MMN and 5.8-6.3 for BAF. Hundred mg ml^{-1} Rifampicin (Sigma-Aldrich®, Sintra, Portugal) was added to each medium after autoclaving. After 14 days in culture, Hagen medium was chosen for mycelium maintenance and growth because it was cost-effective and suitable for making mycelium plugs for the co-cultures.

Identification of the fungal isolates

DNA was extracted from fresh mycelia using NucleoSpin® Plant Kit for extraction of genomic DNA (Macherey-Nagel, Cascais, Portugal). The quality and quantity of the obtained DNA was evaluated by agarose gel. The region containing partial portions of the small subunit (18S), both internal transcribed spacers (ITS) and the 5.8S of the rDNA repeat unit was amplified using the oligonucleotides primers ITS 5 (5'-GGAAGTAAAAGTCGTAACAAGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') (Gardes and Bruns 1993). PCR reactions were carried out on a MyCycler Thermal Cycler (BIO-RAD, Amadora, Portugal) and consisted of initial denaturing at 95ºC for 3 min followed by 30 cycles at 92ºC each 30 s, 55ºC for 30 s, and 72ºC for 1 min. The reaction was completed by a 10 min extension at 72ºC. PCR products were analyzed by agarose gel (1%) electrophoresis, purified with the NucleoSpin Extract II Kit (Macherey-Nagel, Cascais, Portugal) and sequenced by capillary electrophoresis using the ABI PRISM 3730 xl sequencer with the Kit BDT v1.1 (Applied Biosystems, Porto, Portugal). The

sequences of the ITS region were aligned with those of related fungal strains retrieved from the GenBank databases for the homology analysis.

Plant material

Mature seeds of *P. pinea* derived from cones of open pollinated select trees (adapted for pinion production) were obtained in March 2009. The pine stand is located in Mata de Valverde farm, National Forestry Station, Alcácer do Sal. Seeds were stored in a cold chamber at 4ºC until used. Seeds were used to obtain cotyledon explants. The seed coat was cracked with a nut cracker and discarded. The megagametophytes were surface disinfested by immersion in 70% (v/v) ethanol (Sigma-Aldrich®, Sintra, Portugal) for 2 min followed by three rinses in sterile bi-distilled water. They were then immersed in sodium hypochlorite 10% (v/v) (commercial bleach with 5% free chlorine) for 25 min followed by four rinses in sterile water. All of the following steps were carried out under aseptic conditions. An embryo was excised from the megagametophyte by making a longitudinal incision with a scalpel and by gently pulling the edges of the cleft with two forceps. Finally, the cotyledons were excised from the embryo axes with a cut at their bases. Plants obtained from all the cotyledons of a seed were designated as one clone.

Microshoot induction from embryo cotyledons

The cotyledons were cultured separately in a Petri dish with semi-solid Woody Plant Medium (WPM) (Lloyd and McCown 1980) supplemented with 22.2 µM 6-benzylaminopurine (BAP), 20 g l^{-1} sucrose, 7 g l^{-1} agar (Sigma-Aldrich®, Sintra, Portugal) and with pH adjusted to 5.8 before autoclaving. The culture chamber conditions during the shoot induction stage were 25º/19ºC (day/night), 16 h photoperiod provided by cool-white fluorescent light at 80 µmol m^{-2} s^{-1}. After a month, the explants with shoot buds were transferred to fresh WPM medium with 2 g l^{-1} of activated charcoal (Sigma-Aldrich®, Sintra, Portugal) to promote shoot elongation.

Rooting of microshoots

Elongated microshoots, ~2 cm long, were transferred to rooting medium based on WPM with half concentration of the macronutrients, 7 g l^{-1} agar and different carbon sources (see below), and adjusted to pH 5.8 before autoclaving. For root induction, the medium was supplemented with 10.7 µM α-naphthalene acetic acid (NAA) and 0.12M glucose (WPMRI). The cultures were kept for two weeks in a growth chamber at a constant temperature of 19ºC. The first week the cultures were in darkness and the second week under a 16 h photoperiod. Root expression was obtained after transferring the microshoots to WPM consisting of half concentration of the macronutrients, without plant growth regulators and with 0.058 M glucose (WPMRE) and incubated in a growth chamber at 16-h photoperiod provided by cool-white fluorescent lamps at 80 µmol m^{-2} s^{-1} and 25º/19ºC day/night. Roots appeared after 3 to 6 weeks.

Co-culture in vitro

After the rooting induction and expression stages, 51 rooted plants were transferred to 100 ml Erlenmeyer flasks containing double layer WPM. The bottom medium layer was without sucrose (45 ml in the flask) and the top medium layer was with 1.5% sucrose (5 ml in the flask) to allow fungal growth as previously described by Oliveira et al. (2003) (Fig. 1A). Twenty six plants were inoculated with *P. arhizus* and the other 25 (not inoculated) served as controls. Before fungal inoculation, care was taken to ensure that both groups of plants were uniform with respect to the number and length of roots. The inoculation was done with fresh mycelia from the cultures grown in the dark at room temperature. Five mm agar plugs with the fungal "lawn" were cut out with a cork borer and placed in the vicinity of a plantlet. All plantlets remained in the double layer medium for 4 weeks at 16 h photoperiod provided by cool-white fluorescent lamps at 80 µmol m^{-2} s^{-1} and 25º/19ºC day/night. At the beginning of the mycorrhization experiment the initial position and length of roots of all plants in both groups (control and inoculated) were marked on the bottom glass of the flask with permanent marker pen. At weekly intervals, new colour marks were made to trace root growth inside the flask. The total growth of a plant root was the sum of all incremental measurements made at weekly intervals with a ruler put on the glass bottom of the flask (Fig. 1B). After four weeks, plants were taken out of the culture medium and before transplanting to sterile vermiculite the total number of roots and branches per plant were counted and the primary roots and root branches length were measured.

The verification of the accuracy of root measurements in vitro

A parallel experiment was done with the aim to verify the accuracy of the indirect measurements (IM) of the length of primary roots and root branches through the glass bottom of a flask. These measurements were made on 20 plants using the same procedure as described above during *in vitro* culture (through the bottom glass of a flask). At the end of this experiment plants were taken out and their roots were measured again (direct measure, DM). Data obtained with both methods were statistically compared.

Acclimatization

After the co-culture, the plants including controls went through acclimatization with the aim to follow the growth of the root system. The acclimatization was

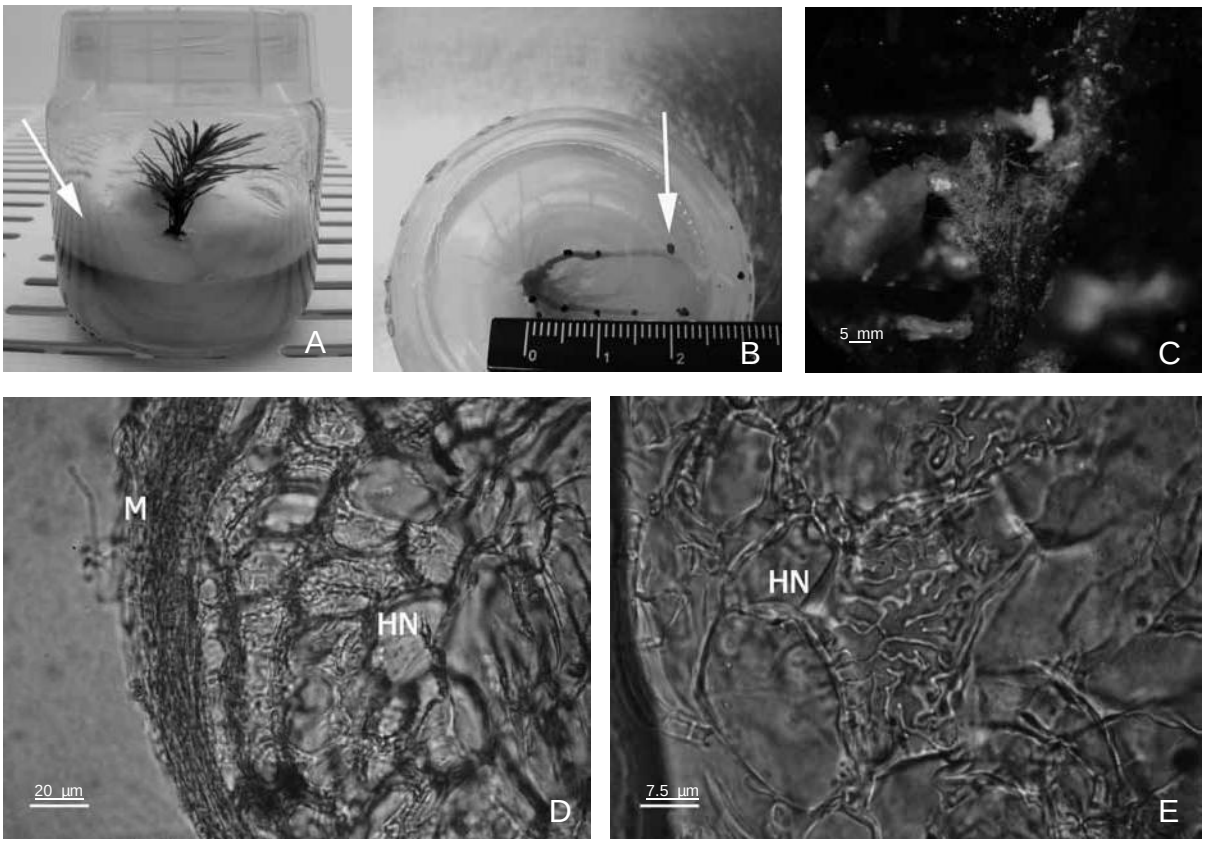

Fig. 1. *In vitro* co-culture of *Pinus pinea* and the mycelium of *Pisolithus arhizus*. A) in a double layer WPM medium. Arrow indicates place of inoculation with the mycelium, B) Bottom of a culture vessel showing colour mark (arrow) made to measure the weekly root growth with a ruler, C) Details of a dichotomous ectomycorrhizal structure covered with yellowish-brown mycelium collected from a plant 30 days after inoculation, D and E) Cryostat transversal root section colonized by *P. arhizus* showing the mantle (M) developed at the root apex of a short root and internal Hartig-net hyphae (HN) (200 ×) (Scale bar 20 µm), D). Details of the transversal section showing well-differentiated Hartig-net (HN) in cortical cells (2000 ×) (Scale bar 7.5 µm) (E).

done in two stages: 1) in sterile vermiculite and 2) in sterile mixed substrate (vermiculite : perlite : peat, 2 : 1 : 1 - Europerlita Española S. A.). The acclimatization lasted 10 weeks in a growth chamber at 25/19ºC day/night and 16 h photoperiod (270 mmol $s^{-1}m^{-2}$), and at the relative humidity of 80%. Plants were watered as required alternating sterile water and liquid WPM (without glucose).

Vermiculite

Plants were planted into plastic cups (~15 cm high and 5 cm in diameter) with sterile vermiculite. Number and length of roots and root branches were recorded at the time of transfer from *in vitro* to vermiculite and also after 2-week growth at the moment of transplanting to sterile mixed substrate in larger vessels (~20cm high and 4 cm in diameter).

Mixed substrate

After 2-week growth in mixed substrate, plants were carefully removed, the root system of each plant measured as described above (first measurement) and plants were replanted. Measurements were repeated after 4 more weeks at the end of the experiment (second measurement). The lengths of the primary roots and root branches were recorded.

Anatomical and histological studies

Mycorrhized root samples from the mixed substrates were collected at the end of the experiment (6 weeks) and identified for histological and anatomical studies. Roots were fixed in 4% glutaraldehyde diluted in 0.1 M HEPES buffer (N-2 Hydroxyenthyl piperazine-N´-2-ethane sulfonic acid), pH 6.8 and stored in a refrigerator for 24 h. Afterwards roots were washed two times in 1N PBS (phosphate buffer saline) with 4% sucrose for 15 min and finally washed in PBS with 15% sucrose. After washing, roots were placed in PBS with 15% sucrose and 7.5% of microbiology gelatin (Merck®, Lisboa, Portugal) for 1 to 2 h at 37°C.

Other Petri dishes with the same gelatin base were prepared and after 1 h when the solution solidified, roots

were placed on the surface of the gelatin and covered with 1 cm layer of molten gelatin solution. Petri dishes were stored in a refrigerator for 1 h; blocks (1 cm × 1 cm × 1 cm) of gelatin with roots were cut out, frozen and stored at -80ºC. Longitudinal and transverse sections, approximately 5-10 µm thick, were cut at -33ºC using a cryostat (Cryosect, Seaward Ltd, London), transferred to glass slides and stained with a common fountain pen ink. Sections were observed under an Olympus microscope at the magnification of 1125 ×.

Statistical analyses

When plants are inside flasks, counting roots and root branches can be easily done without risk of errors. The same cannot be done for root lengths, length of the longest root, and the total root length. Therefore, indirect measurement (IM) made through the glass bottom of flasks were compared with direct measurements (DM) of the same plants at the time of transplantation to vermiculite. This was done by fitting a straight line forced through the origin (DM = *a* IM) and examining the 95% confidence interval of regression coefficient *a*.

Numbers of roots and branches and the lengths of plants, which were randomly assigned to control and treatment groups at the time of inoculation with the fungus were compared by two-tailed exact or approximate Student's *t* tests after checking for homocedasticity using the two-tailed *F* distribution.

The experiment was a 2 × 4 factorial design with inoculation (inoculated and non-inoculated) and stage (*in vitro*, vermiculite, first and second period of growth in mixed substrate) as main effects. In order to maximize the independency of effects of inoculation at each stage, growth was expressed for each variable as the difference between the measured values at the end and at the beginning of each stage. Therefore, growth could have negative, zero, or positive values. For example, negative values in number of roots could result from root loss when plants were uprooted at the end of a given stage, zero values when there was no change in root number, positive values when there was an increase in root number between the beginning and the end of a given stage.

Main effects and interactions were analysed by factorial ANOVA. Prior to that homocedasticity was investigated using the two-tailed F distribution and whenever heterocedasticity was found data was transformed using the Box-Cox transformation (Box and Cox 1964, Rohlf 1992). Because of the significance of interactions between the main factors (see Results and discussion) the effects of inoculation were investigated separately for *in vitro*, vermiculite and mixed substrate stages by Student's t tests. Comparisons were one-tailed and prior to t tests, homocedasticity was investigated using the two-tailed *F* distribution. Whenever heterocedasticity was found comparisons of means were done using the approximate Student's *t* distribution. A comparison-wise significance level of 0.05 was used throughout. Data are expressed as means ± standard errors.

RESULTS AND DISCUSSION

Pisolithus arhizus

Representative voucher specimen of the fruiting body collected in the pine stand was deposited at Évora University Herbarium (UEVH-FUNGI 2001610). In Hagen medium the mycelia were yellowish-ochraceous with paler margin. The subsequent ITS rDNA sequencing, with 644 bp, showed 99-100% homology with *P. arhizus* sequences found in NCBI (National Center for Biotechnology Information), confirming the success of our isolation and culture procedures. *P. arhizus* sequence was deposited in GenBank (NCBI identification number: HQ896485).

Root growth in vitro

Straight line equation forced through the origin could always be fitted to describe the relationship between direct measurements (DM) and indirect measurements (IM). For the length of the longest root DM = 0.957 IM ($p < 10^{-4}$, $R^2 = 0.981$; 95% confidence interval for the regression coefficient 0.890 - 1.024) and for total root length DM = 0.990 IM ($p < 10^{-4}$, $R^2 = 0.982$; 95% confidence interval for the regression coefficient 0.923 - 1.056). Thus no significant differences occurred in pairwise comparisons between DM and IM of root length. The IM allowed the determination of root lengths, which was used as reference at the beginning of the experiment and could be used, if desired, to follow root growth during co-culture. To our knowledge, this was the first time that the indirect method of root length determination during *in vitro* culture was addressed and effectively used.

There were no significant differences between the plants chosen for inoculation with the fungus and control plants before the onset of the experiment in the number of roots and in the total length of roots. Conversely, significant differences were found between the two groups of plants before the onset of the experiment in the number of branches, in the number of roots plus branches, in the length of the longest root and in the average root length calculated as the ratio of total length of roots / number of roots (Table 1). Throughout the experiment, plant mortality was observed only in control plants, in which three plants died during the growth in vermiculite.

Interaction between the main factors inoculation and stage was not significant for the change of number of branches ($p = 0.320$), change of number of roots plus branches ($p = 0.224$) and change of average length of roots ($p = 0.359$), and for these variables neither was the factor inoculation. Conversely, significant interac-

Table 1. Means ± standard errors of variables in plants assigned to control and to inoculation at the onset of the experiment.

Variable	Assigned to control	Assigned to inoculation	p
Number of roots	2.6 ± 0.3	1.8 ± 0.2	0.055
Number of branches	0.8 ± 0.3	0.0 ± 0.0	0.044
Number of roots plus branches	3.4 ± 0.4	1.8 ± 0.2	0.003
Total length of roots (mm)	29.5 ± 3.3	36.3 ± 5.5	0.301
Length of the longest root (mm)	11.5 ± 0.9	18.0 ± 1.9	0.007
Average root length (mm)	11.8 ± 1.5	22.7 ± 4.1	0.024

Differences were investigated by exact or approximate Student's *t* tests and at the probability level 0.05. Sample size was n = 13.

tion between main factors was found in the change of number of roots (p = 0.016), change of total length of roots (p = 0.014), change of total length of roots plus branches (p = 0.002) and change of length of the longest root (p = 0.049). Therefore, the effects of inoculation were separately analysed for each growth stage using Student's *t* tests (Table 2).

Inoculated plants had significantly higher changes in the number of root branches and in the number of roots plus branches, during *in vitro* growth compared with control plants. In addition, the number of root branches in inoculated plants but not of roots, significantly increased during the co-culture, while in control plants no change occurred in the number of roots and in the number of roots plus branches. It is well known that *Pisolithus* spp. secret hypahorine, indole-3-acetic acid (IAA) and other growth regulators that trigger morphological changes in the root system (Martin et al. 2001, Niemi et al. 2000). The involvement of *P. arhizus* in promoting root branching to form second and third-order laterals in both conifer and angiosperm hosts were previously reported by Chambers and Carney (1999). The results of our study on *in vitro* root growth confirmed what was determined *in situ* by other researchers.

Root growth in vermiculite

During growth in vermiculite differences were found in the change of length of the longest root, in the change of total root length, in the change of total length of roots plus branches and in the change of average length of roots. No significant differences were observed in the change of number of roots or of branches, but like during *in vitro* growth, if differences occurred, mean changes in inoculated plants were significantly higher than mean changes in control plants. The magnitude of changes in inoculated plants during vermiculite growth was between 8 fold (change in total length of roots) and 15 fold (change in length of the longest root) greater than in control plants. Also, on average the change in control plants was up to 10% of the corresponding values and in inoculated plants it was up to 73%.

These results are largely in agreement with the findings of Ostonen et al. (2009) on the effects of different ECM on the anatomotype of the root system in Alnus spp. They observed that different functional parameters of short ECM roots, e.g., specific root area (SRA), specific root length (SRL), and root tissue density (RTD) were modified depending on the

Table 2. Means ± standard errors of variables with significant differences between control and inoculated plants.

Growth stage	Variable	Control	Inoculated	*p*
In vitro	Change in number of branches	0.0 ± 0.0	1.4 ± 0.6	0.018
	Change in number of roots plus branches	0.0 ± 0.0	1.4 ± 0.6	0.018
Vermiculite	Change in total length of roots (mm)	3.8 ± 3.1	29.2 ± 7.2	0.003
	Change in total length of roots plus branches (mm)	2.8 ± 4.2	32.3 ± 7.1	0.002
	Change in average root length (mm)	0.9 ± 1.2	9.7 ± 3.4	0.014
	Change in length of the longest root (mm)	1.0 ± 2.0	14.7 ± 3.7	0.002
Mixed substrate, 2nd measurement	Change in number of roots	-1.0 ± 0.3	-0.1 ± 0.1	0.001
	Change in number of roots plus branches	-1.0 ± 0.3	-0.1 ± 0.1	0.001
	Change in total length of roots (mm)	-4.2 ± 8.8	17.4 ± 7.6	0.038

Differences were investigated separately for each stage by exact or approximate Student's *t* tests and at the probability level 0.05. Sample size in control was n = 10 except *in vitro* where n = 13; in inoculated plants always n = 13.

colonizing fungal species. There are other examples of root anatomotype changes (Reithmeier 2011), however most of the research was done in nurseries and in the natural environment.

Root growth in mixed substrate

No significant differences were found during the first 15 days in mixed substrate between inoculated and control plants suggesting that the two groups grew at the same rate. These results indicated that the substrate, nutritionally richer than vermiculite was sufficient for the growth of control plants or that the effect of the fungus was less evident compared with the nutritionally poor conditions used during the first acclimatization stage. But this lack of effects was temporary because during the second period of acclimatization in mixed substrate significant differences were found in the change of number of roots, change of number of roots plus branches and change in total root length. These changes were significantly higher in inoculated plants than those in control plants. Reduction in the number of roots and, as a consequence, in total root length, likely resulted from the second uprooting of plants from mixed substrate which was necessary to count and measure the roots. Overall it suggests that inoculated plants were more robust and able to better withstand the stress imposed by uprooting than control plants.

These results demonstrated the dynamic interaction of the substrate organic matter with ectomycorrhizal colonization and root growth. Rosling (2003) extensively analysed the relationship between mycelium growth and the activity of ectomycorrhizal fungi in different soil substrates and its potential to modify their microenvironment and subsequent root colonization. Rincón et al. (2005) have also demonstrated an interaction between the substrates and the optimal fungal growth and root colonization. Although, our study focused only on stone pine root growth and not on the fungal mycelia behaviour in the employed substrates, the results of others might suggest that similar interaction occurred in the present work, which to our knowledge is the first attempt to investigate root development during such a short period of time.

Mycorrhiza anatomical and histological studies

Plants were very useful for the identification of target structures and for the observation of the symbiosis with minimal disturbance to the rest of the root architecture. Samples of the dichotomous roots from the plants grown in mixed substrate and collected after acclimatization were used for the anatomical and histological observations (Fig. 1C). According to our previous experience, the symbiotic structures were highly variable in their complexity, but the transverse sections of the ectomycorrhizal roots showed a well developed mantle and Hartig net (Fig. 1D and E).

Co-culture of *P. pinea* plants with *P. arhizus* effectively helped to overcome one of the most common problems associated with the *in vitro* rooting: the inhibition of adventitious root growth under the culture conditions and the survival of the plants *ex vitrum*. Even in nutritionally poor vermiculite used during the early stage of acclimatization, none of the inoculated plantlets died and we observed a vast mycorrhizal symbiosis establishment. Moreover, fewer roots were lost during transplantation, which likely resulted from morphological modifications of the mycorrhized roots such as the presence of the hyphae around the roots and the internal Hartig net which increased root thickness and contributed to a more robust root system.

The adventitious rooting of *P. pinea* was initially induced *in vitro* by NAA and at the time of inoculation there were no significant differences in the number of roots per plantlet. Therefore, it may be surmised that the significant differences in root growth observed during *in vitro* co-culture stage was not caused by the external auxin supply necessary for the root induction stage. Also, the fact that the change in number and length of roots and branches was significantly higher in the inoculated plants suggests the importance of the fungal presence. Both increased root branching and length were beneficial for acclimatization of stone pine plantlets.

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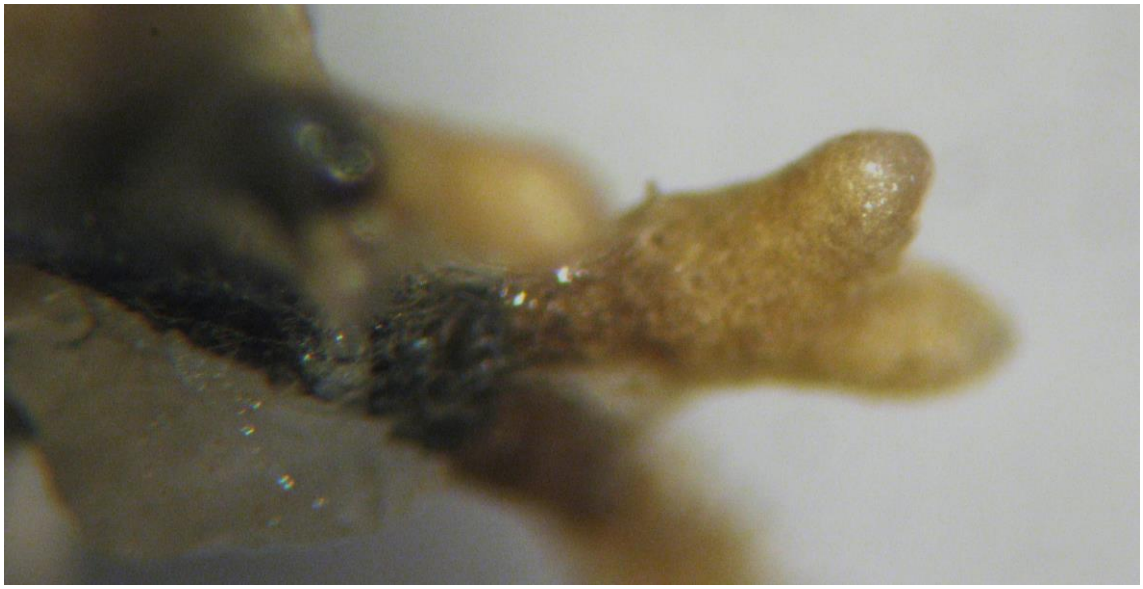

Chapter 7

O-coumaric Acid Ester, a Potential Signaling Molecule Detected During Early *in vitro* Co-culture Between *Pinus pinea* L. Plantlets and the Ectomycorrhizal Fungus *Pisolithus arhizus* (Scop.) Rauschert

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_O_-coumaric acid ester, a potential signaling molecule detected during early _in vitro_ co-culture between _Pinus pinea_ L. plantlets and the ectomycorrhizal fungus _Pisolithus arhizus_ (Scop.) Rauschert

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Abstract

In ectomycorrhizal symbiosis, plant roots and fungi function together as a unit. Previously, we showed improvement in various root parameters during *in vitro* co-culture of *Pinus pinea* and *Pisolithus arhizus* before physical contact occurred, as well as increased survival rate of the inoculated plantlets during acclimatization. To better understand the positive effects on root growth parameters before the establishment of physical contact between these partners, the biochemical compounds released to the liquid phase of the co-culture medium were analysed. It is known that biochemical signals lead to the development of complex structures in both the plant and the fungus that constitute an ectomycorrhiza. The results of HPLC-UV and LC-DAD-MS analysis of the liquid phase medium samples that were collected from 1 to 10 days of *in vitro* co-culture are presented. *O*-coumaric acid ester, a phenolic compound, was identified in root exudates of stone pine from the second day and its presence was detected for up to 10 days of co-culture. This result contributes to the understanding of the role of phenolic compounds in pine/ ectomycorrhiza symbiosis establishment and also explains some of our previous results.

Keywords Adventitious roots, ectomycorrhiza, phenolics, stone pine, symbiosis

Introduction

Ectomycorrhizae (ECM) are symbiotic structures between plant roots and fungi. In the ectomycorrhizal symbiosis, the host (plant roots) and the mycobiont (ectomycorrhizal fungus) function collectively as an entity. The development of ECM in plants frequently allows them to get established in habitats that neither symbiont could occupy independently (Nehls et al. 2000).

ECM development involves a series of complex processes that occur simultaneously in symbionts. Extramatrical hyphae, the mantle and the intraradicular hyphal network are active metabolic bodies that provide essential nutrients (e.g. nitrogen, phosphate) to the host plant and carbohydrates for the fungal partner making this a mutualistic association (Allen 1991; Varma and Hock 1994; Smith and Read 1997; Martin et al. 2001).

Successful colonization of roots by mycorrhizal fungi and further development of the ectomycorrhizal structure, results from a coordinated series of events mediated by biochemical signals (Seddas et al. 2009). During ECM establishment the molecular dialog initiate developments that lead to physical steps in the association once the detection or attraction of the partner occur before physical contact (Harrison 2005).The fungus must face the host defense mechanisms and be able to initiate the mutual nutrient transfer across the root-fungus interface (Reis et al. 2011). This is achieved by an intense cell activity before and after physical contact between partners. In a recent review by Bonfante and Genre (2010) the identification of several novel nutrient transporters has revealed some cellular processes that underlie symbiosis, but the biochemical signals prior to physical contact and their functions still need to be elucidated, especially for ectomycorrhizal fungi.

Martin et al. (2001) suggested that rhizospheric signals including auxins, flavonoids, alkaloids, cytokinins, and other metabolites produced by both partners could act in a synergistic or in antagonistic way. More recently plant phenolic compounds such as *p*-coumaric acid, coumarin, naringenin and other flavonoids were also cited as potential candidates of signals during mycorrhizal formation (Lynn and Chang 1990; Mandal et al. 2010; Amalesh et al. 2011; Plett and Martin 2012; Hassan and Mathesius 2012). Phenolic compounds are ubiquitous in plants and participate in several important functions which enable them to adapt to changing biotic and abiotic environments (Boudet 2007).

Several studies have shown the benefits of using ECM fungi (*Amanita*, *Hebeloma*, *Laccaria*, *Lactarius*, *Pisolithus*, *Rhizopogon*, *Scleroderma*, and *Suillus*) in conifer micropropagation (Grange et al. 1997; Wallander 2000; Rai 2001; Wu et al. 2003; Taylor et al. 2004; Niemi et al. 2004; Adriaensen et al. 2006). Among many advantages the mycorrhized plants (either with arbuscular mycorrhizal (AM) or ECM fungi) were more efficient in water and nutrient absorption through an increased area of soil colonization, had increased pathogen resistance and increased transplantation survival compared with non mycorrhized plants. In addition some ECM and ericoid fungi could breakdown phenolic compounds present in the soil that might interfere with nutrient uptake (Allen et al. 1989; Brundrett 1991; Grandmaison et al. 1993; Newsham et al. 1995; Little and Maun 1996; Bending and Read 1997; Cordier et al. 1998; Bratek et al. 2002).

Recently, we demonstrated that *in vitro* co-culture of *Pinus pinea* plantlets with *Pisolithus arhizus* helped to overcome the cessation of adventitious root growth and resulted in a root system that was better adapted to post transplantation stress. None of the inoculated plantlets died in spite of using exclusively sterile vermiculite in the early phase of acclimatization during which a vast mycorrhizal symbiosis was established. Moreover, fewer roots were lost during transplantation which was facilitated by the morphological modifications of the mycorrhized roots such as the presence of the hyphae around the roots and the internal Hartig net, which increased root thickness and contributed to a more robust root system (Ragonezi et al. 2012).

In this study the objective was to characterize the chemical nature of the mediators and the period of *in vitro* co-culture during which the signaling between *P. arhizus* and roots of *P. pinea* occurred. We present biochemical results of high-performance liquid chromatography (HPLC-UV) and a liquid chromatography - diode array detector - mass spectrometry (LC-DAD-MS) analysis of the metabolites released into the liquid phase of the double layer medium during the first days of plant/fungus co-culture.

Material and methods

Reagents

Acetic acid (glacial) 100% anhydrous for analysis EMSURE® ACS, ISO, Reag. Ph Eur contained the SVHC above 0.1% (VWR® Carnaxide, Portugal) was stored at room temperature in the dark. Methanol gradient grade for liquid chromatography LiChrosolv® Reag. Ph Eur contained no SVHC above 0.1% was obtained from VWR® and stored as

above. All other chemical reagents used for preparation of solutions were reagent grade from Merck® (Lisbon, Portugal).

Plant material and micropropagation of P. pinea

Mature seeds of *P. pinea* were obtained in March 2009 from selected 'plus' trees (Alcácer do Sal region, Portugal) and were stored in a cold chamber at 4ºC until used. For the description of shoot organogenesis see Oliveira et al. (2003). For rooting, elongated microshoots, ±2 cm long, were transferred to a rooting medium based on Woody Plant Medium (WPM) (Lloyd and McCown 1981) with half concentration of the macronutrients, 0.65% agar (Difco Bacto-Agar®) and different carbon sources (see below), and adjusted to pH 5.8 before autoclaving. For root induction, the medium was supplemented with 10.7µM naphthalene acetic acid (NAA) and 0.12M glucose (WPMRI). The cultures were kept for two weeks in a growth chamber at a constant temperature of 19 ºC. The first week the cultures were in darkness and the second week under a 16 h photoperiod. Root expression was obtained after transferring the microshoots to WPM consisting of half concentration of the macronutrients, without plant growth regulators and with 0.058 M glucose (WPMRE) and incubated in a growth chamber provided with 16 h photoperiod by cool-white fluorescent lamps at 80 µmol m^{-2} s^{-1} and 25º/19ºC day/night temperatures. Roots appeared after 3–6 weeks.

Fungi purification and identification

Collection of fruiting bodies of *Pisolithus arhizus* (Scop.) Rauschert and molecular identification of pure isolate was described by Ragonezi et al. (2012). *P. arhizus* sequence was deposited in the GenBank (NCBI identification number: HQ896485).

Co-Culture Technique

Preparation of the Double-phase Medium

After the root induction and expression phases the plantlets were transferred to 6.6 x 5.9 cm glass culture vessels (100ml, Sigma-Aldrich®, Sintra, Portugal) containing Double-phase Medium. All medium components: perlite (Europerl®), WPM with 6g/l gellan gum (Phytagel™, Sigma-Aldrich®) (WPMS) and WPM without gelling agent and carbohydrate (WPML) were first sterilized. Subsequently, the Double-phase Medium (approved patent Nº 105239 of the National Institute of Industrial Property, INPI) was prepared in a horizontal laminar flow chamber as follows: 1) 15 ml of molten WPMS was poured into each vessel together with 0.9 g of perlite. After the medium cooled down and solidified the perlite remained at the top and together formed the "solid phase" medium layer which was then flipped over inside the

vessel with a sterile metal spatula, 2) 30 ml of WPML were poured into the culture vessel forcing the “solid phase” medium to float on its surface due to the perlite. In consequence, the upper part of the “solid phase” medium had no physical contact with the liquid medium (Fig. 1).

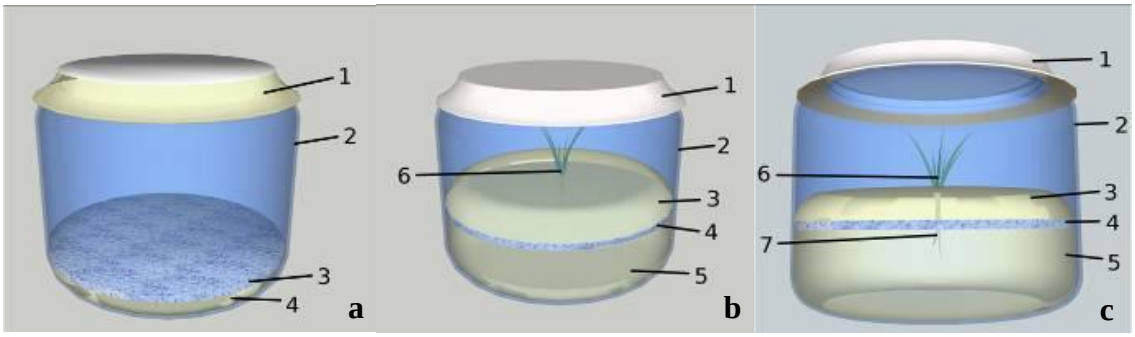


Fig. 1 - Illustration of double-phase medium preparation and plant culture; a, 1 – cover; 2 – flask; 3 and 4 - WPMS medium with perlite at the top. b and c, 3 and 4 – WPMS medium with perlite facing down, 5 - WPML, 6 - plantlet; 7 – root (Patent approved by INPI Nº 105239).

***Co-culture* of *P. pinea plants* with *P. arhizus* mycelia**

First, *P. pinea* plantlets were placed in the double-phase medium (Fig. 2). The roots were introduced into the liquid phase of the double-phase medium by making an opening in the upper semi-solid medium using heat sterilized perforating tool. The surface of the semi-solid medium was then inoculated with fresh mycelia of *P. arhizus* and the co-culture vessels were capped and transferred to the culture chamber with a 16h photoperiod, 24ºC/19ºC day/night, respectively. The cultures were kept under cool white lamps (Philips Master LD36W/840) with photosynthetic photon flux density of 90 µmol m^{-2} s^{-1}. The development of plants and mycelia was monitored daily.

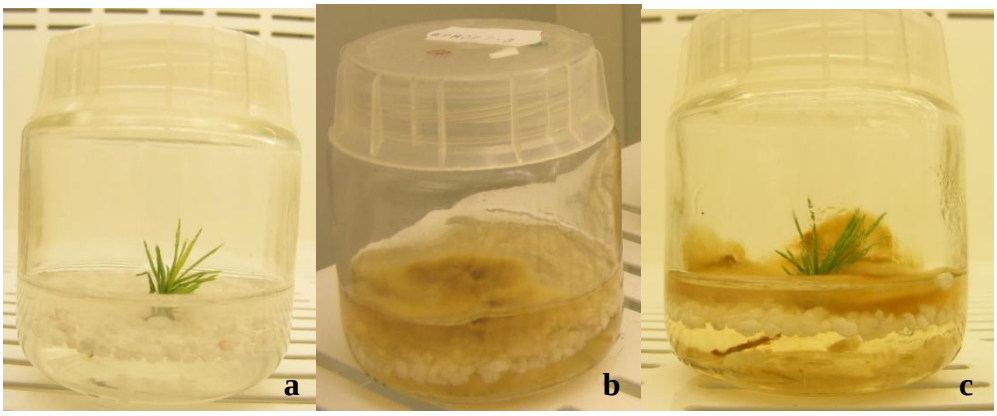


Fig. 2 - Negative controls: pine plantlet without fungus (a) and fungal mycelium without pine plantlet (b). *In vitro* co-culture of *Pinus pinea* and *Pisolithus arhizus* mycelia in double-phase medium (c).

Negative controls

Identical double-phase medium and culture conditions were used for two negative controls; pine cultures without fungal inoculation and mycelium culture without pine plants.

Biochemical analysis

Collection of liquid medium samples

The vessels containing the co-cultures (plantlets and fungus) and those containing only pine plants or *P. arhizus* (negative controls) were transferred to the horizontal laminar flow unit in order to collect samples of the liquid phase medium for the analysis. Using a sterile spatula, the edge of the solid phase was slightly lifted and the vessel was tilted to allow the liquid medium flow into a 50 mL centrifuge tube (Falcon™). The tubes were filled up to half their volume (about 25 mL each). The sampling was done on day 1, 2, 3, 5 and 10 of culture. The tubes with liquid medium samples were immediately stored at -20ºC.

Preparation of samples

Target substances **-** The preparation of standard solutions (SS) of target substances (IAA, Rutin and IBA) for chromatographic analysis was done as follows: 1 mg of the tested substance was dissolved in 10 mL of methanol, corresponding to a final concentration of 100 mg dm^{-3}. The solutions were then diluted 1:10 (also in methanol) to provide work solutions (WS) for injections.

Samples **-** Samples of liquid phase medium collected at different time intervals (1^{st}, 2^{nd}, 3^{rd}, 5^{th} and 10^{th} day) were freeze-dried in a Christ® Alpha 1-4 Freeze dryer (Biotech International, Germany) and subsequently lyophilized. Each lyophilized sample was resuspended in 3 mL of miliQ water:methanol (40:60 v/v) solution. The liquid was filtered through a nylon syringe filter (0.45µm, VWR®), into HPLC-UV/LC-DAD-MS vial (VWR®) and frozen at -20ºC. For injection into HPLC-UV the samples were thawed on ice in the dark.

Doped substances **-** Sample doping was performed using SS of the target substances. SS was diluted in the liquid phase of the sample: 1 mL of SS and 9 mL of sample. The mixture was filtered through a nylon syringe filter (0.45µm) into HPLC-UV/LC-DAD-MS vial and frozen at -20ºC. For injection into HPLC-UV the samples were thawed on ice in the dark.

Analysis of samples by HPLC-UV

All samples were analyzed in an HPLC-UV system ELITE LaChrom VWR HITACHI equipped with a VWR HITACHI L-2100 pump, a Rheodyne injector and a VWR HITACHI L-2400 UV detector. The reversed phase analytical column was a Supersher 100 Merck RP-18 (250 x

4.6 mm, 5µm) with a pre column LiChrospher 100 RP-18. The data acquisition and automatic processing were performed using an EzChom Elite Software.

The separation was achieved in isocratic mode, and the mobile phase was composed of 60% methanol, 1% acetic acid and 39% miliQ water, for 45 minutes, at a flow rate of 0.7 mL min^{-1}. All analyses were performed at room temperature, the injection volume was 20 µL and the chromatographic profile was recorded at 210 nm.

Analysis of samples by LC-DAD-MS

LC-DAD-MS (Liquid chromatography - Diode array detector - Mass spectrometry) analyses were carried out in a LCQ Advantage Thermo Finnigan mass spectrometer equipped with an electrospray ionization source and using an ion trap mass analyzer. The conditions of analysis were: capillary temperature 300ºC; source voltage 4.5 kV, source current 100 µA, and capillary voltage -45 V in negative ion mode. The mass spectrometer equipment was coupled to an HPLC system with autosampler (Surveyor Thermo Finnigan) and diode array detector (DAD). The analytical column was a reversed phase Zorbax Eclipse XDB (C_{18}, particle size 3.0 µm, 150 mm x 2.1 mm). The chromatographic separation was performed with mobile phase at a flow rate of 0.2 mL min^{-1}, by injecting 20 µL of each sample and the elution program was similar to the one used in the HPLC-UV analysis. The DAD detector was scanned from 200 to 500 nm and the chromatographic profile was recorded at 210 nm.

Results

HPLC-UV analysis

In order to identify potential signalling compounds, samples of the liquid phase medium collected after the 1^{st}, 2^{nd}, 3^{rd}, 5^{th} and 10^{th} day of *P. pinea* and *P. arhizus* co-culture were analysed by HPLC-UV. With the exception of the 1^{st} day sample, in all other samples a clear peak was recognized. For example, the chromatograms of the 2^{nd} day samples showed a peak at retention time (RT) 11.50 min (Fig. 3A), but there was no corresponding peak in the negative control (plant culture without fungus) (Fig. 3B).

To determine the identity of the unknown compound (UC), standard solutions of target substances, which have been previously identified in ectomycorrhizal symbiosis such as IAA, Rutin and IBA were analyzed with the same method and at the same conditions as the samples. When the 2^{nd} day sample was doped with these target compounds the RT of UC did not correspond to any RT of the target substances (Fig. 4 A, B and C).

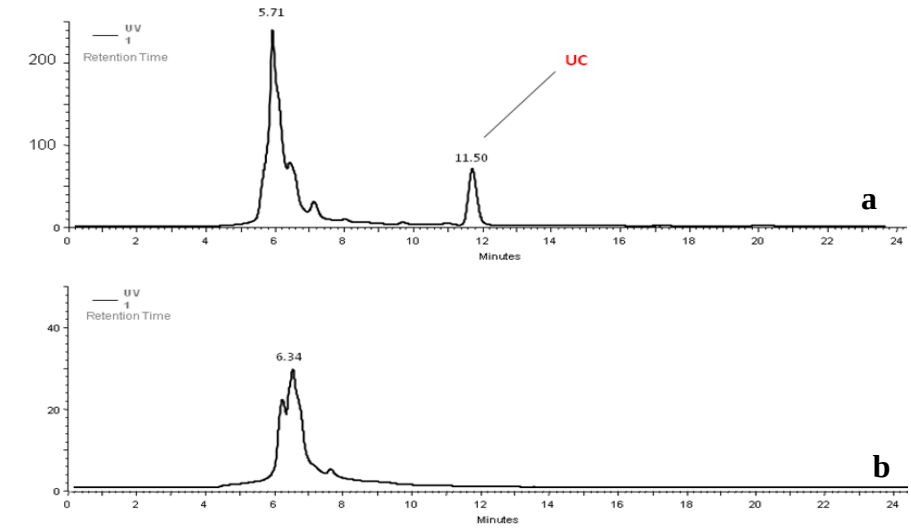


Fig. 3 - HPLC-UV chromatograms of the medium sample collected from co-culture of *P. pinea* plantlet and *P. arhizus* on the 2nd day (a) and *P. pinea* microshoot without fungal inoculation collected on the 2nd day (b). Peak at RT 11.50 min corresponds to the unknown compound (UC).

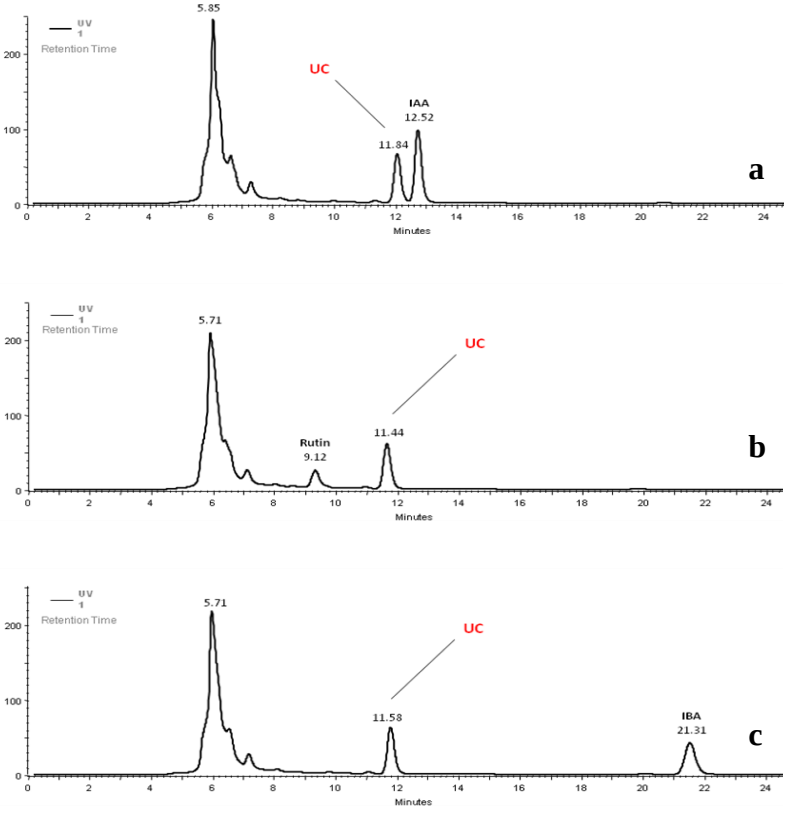
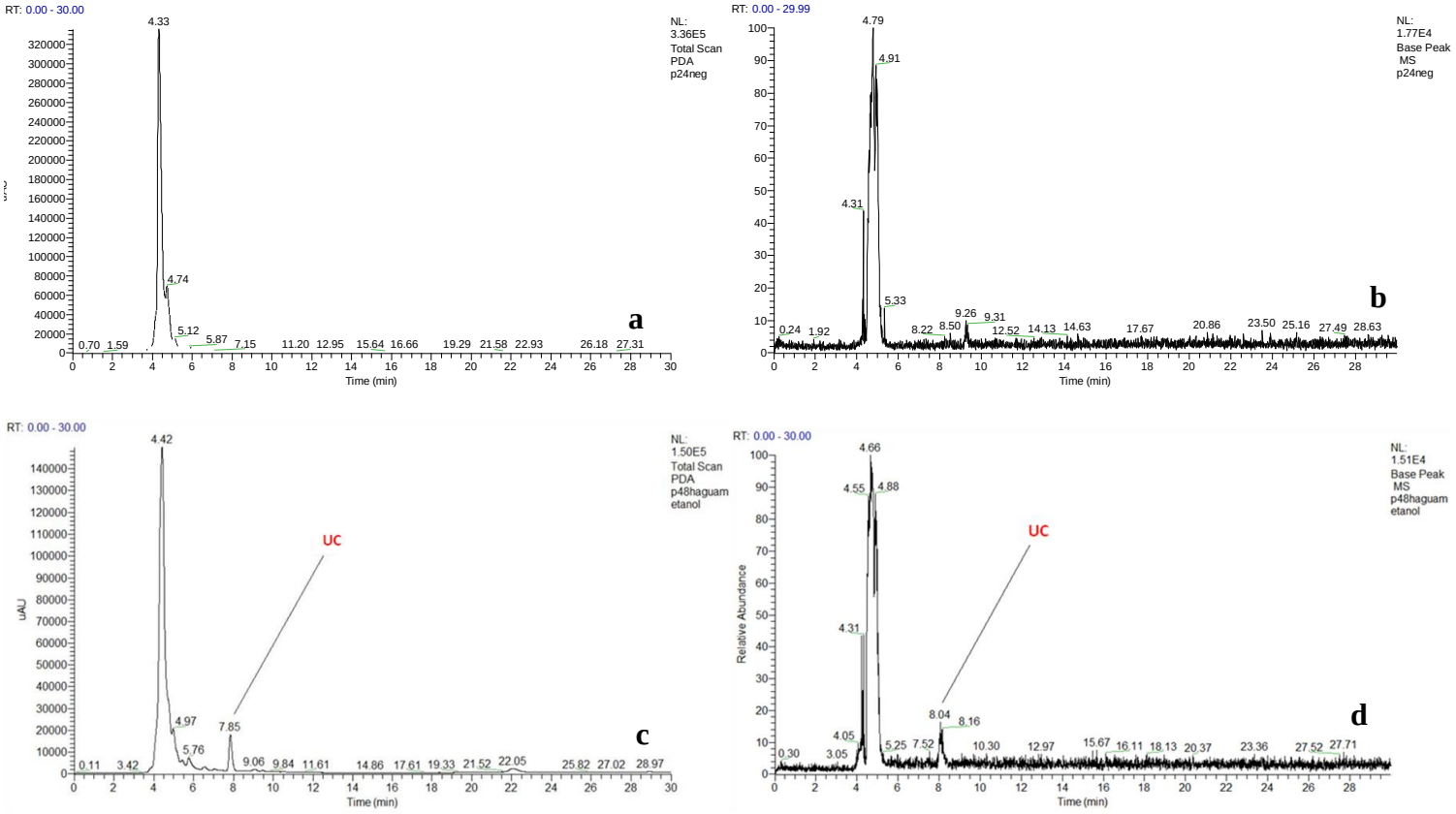


Fig. 4 - HPLC-UV chromatograms of samples from the co-culture of *P. pinea* plantlet and *P. arhizus* collected on the 2nd day and doped with IAA (a), Rutin (b) and IBA (c). Peaks at RT 11.84, 11.44 and 11.58 min correspond to the unknown compound (UC).

LC-DAD-MS analysis

Samples of the liquid phase medium collected after the 1st, 2nd, 3rd, 5th and 10th day of co-culture were also examined by LC-DAD-MS. The results were consistent with those obtained by HPLC-UV. Only in the 1st day medium sample from plant/fungus co-culture the peak for the UC did not appear. In all other co-culture samples the presence of the metabolite was confirmed. When peak areas were compared among the samples, there was a slight trend toward increased amounts with longer co-culture time.

The chromatograms of all collected samples revealed several peaks which likely corresponded to other compounds present in the culture medium (Fig. 5). The 2nd day sample was used as reference for the identification of the UC by LC-DAD-MS. Fig. 5c shows the DAD total scan and Fig. 5d the total ion current chromatograms with RT of 7.85 and 8.04 min, respectively. The UV spectrum of this compound, obtained on-line by the DAD detector, showed two wavelength maxima at 222 and 276 nm (Fig. 6a). The full MS spectra showed an ion signal at m/z 231 with abundance of 100% corresponding to the molecular [M-H] parent ion, and at m/z 145, the peak corresponding to benzoyl fragment characteristic of benzoic acids due to the loss of OH or OR groups (Fig. 6b).



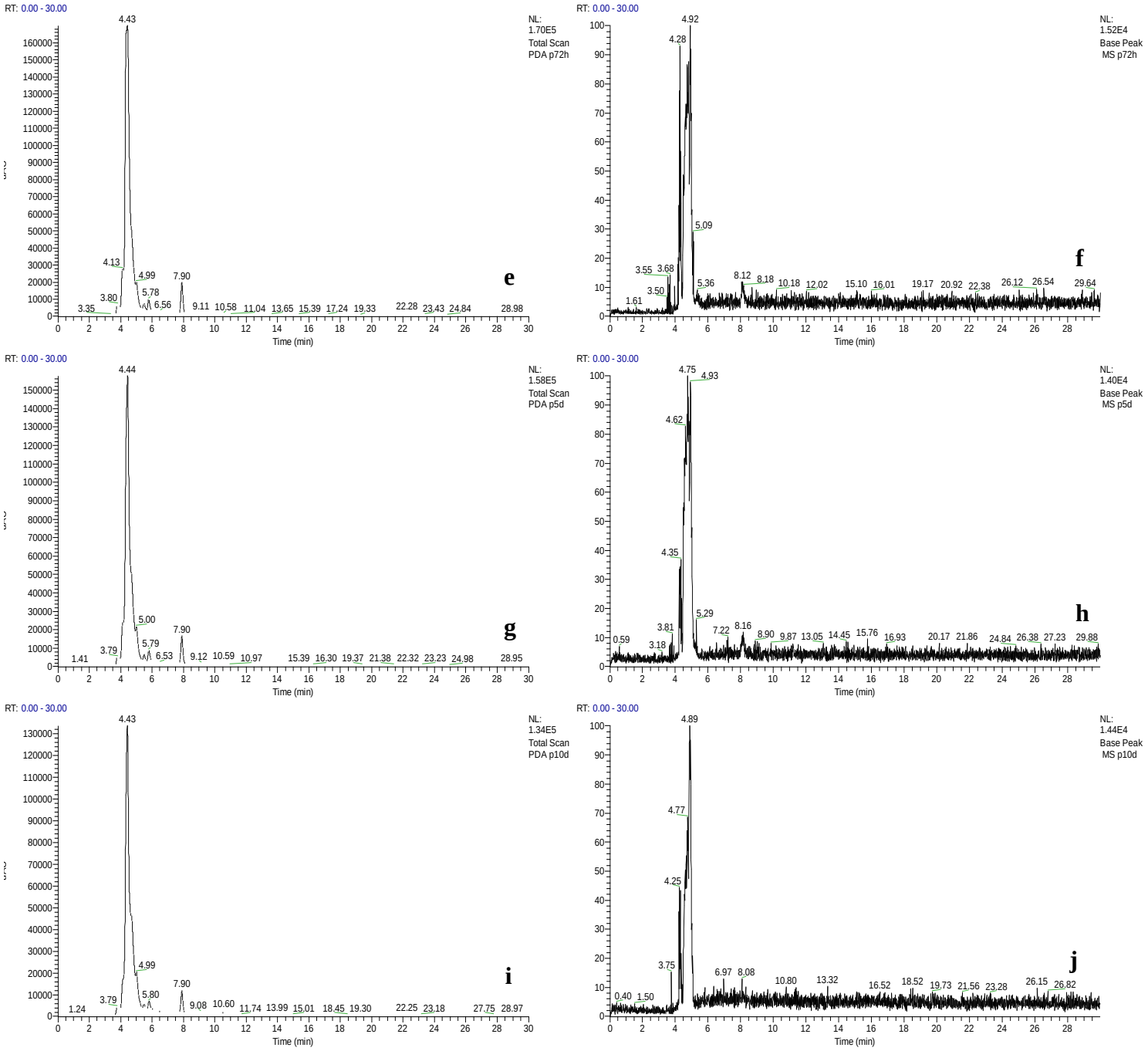


Fig. 5 - DAD total scan (a, c, e, g, i) and total ion current (b, d, f, h, j) chromatograms of the *P. pinea/P. arhizus* co-culture medium samples collected at different time intervals. Letters a and b correspond to the 1st day, c and d to the 2nd day, e and f to the 3rd day, g and h to the 5th day, and I and j to the 10th day.

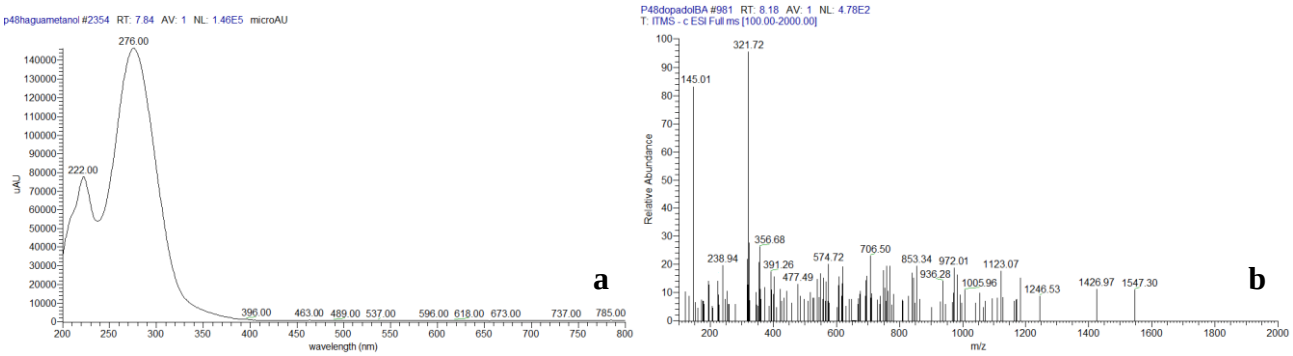


Fig. 6 - UV (a) and mass spectra (b) of the unknown compound (UC) in the *P. pinea/P. arhizus* co-culture sample collected on the 2nd day.

The UC was identified as an ester of *o*-coumaric acid (also known as 2-Coumaric acid; trans-2-Hydroxycinnamic acid; (2E)-3-(2-hydroxyphenyl) acrylic acid; O-hydroxycinnamic acid; 2-Propenoic acid, 3-(2-hydroxyphenyl)-(E)-) and *o*-hydroxyphenylacetic acid). The chemical structure of this compound and its identification was based on the fragmentation pattern and UV spectra that were similar to those reported by Atoui et al. (2005) (Fig. 7).

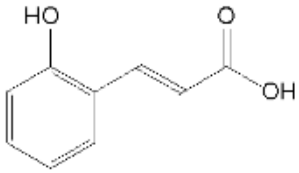
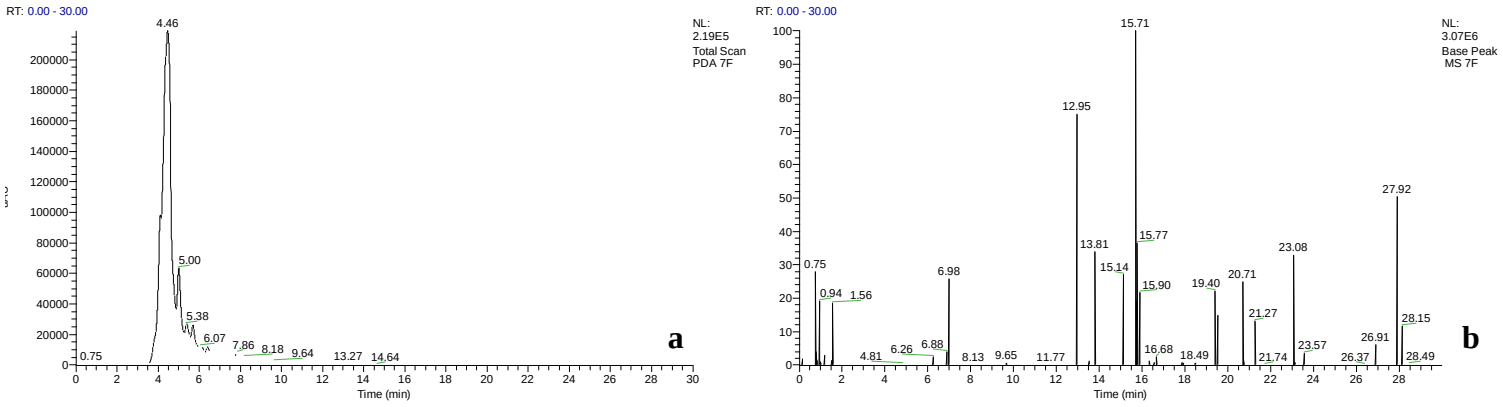


Fig. 7 - Chemical structure of *o*-coumaric acid.

LC-DAD-MS analysis also confirmed the results obtained by HPLC-UV in negative controls (*P. arhizus* mycelium and *P. pinea* microshoots cultured separately) in which no ester of *o*-coumaric acid was identified (Fig. 8).



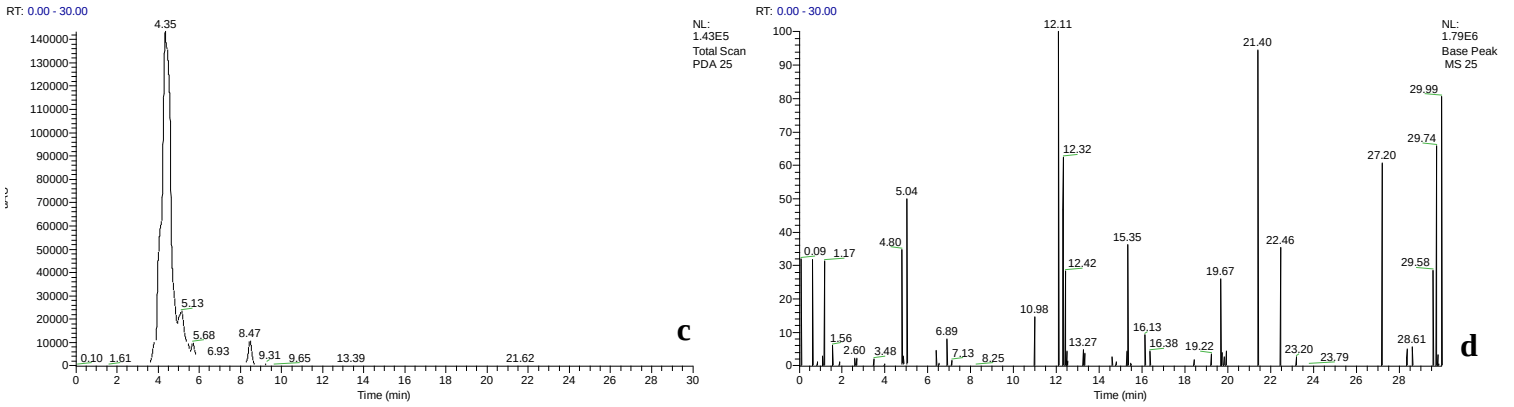


Fig. 8 - DAD total scan (a, c) and total ion current (b, d) chromatograms of liquid medium phase samples of the two negative controls. Letters a and b corresponds to the culture of *P. arhizus* mycelia, c and d to the culture of *P. pinea* plantlets.

Discussion

Signalling compounds in the ECM symbiosis are less known than those in the arbuscular associations. In AM symbioses flavonones, flavones and isoflavones are capable of stimulating spore germination, hyphal elongation and hyphal branching (Siqueira et al. 1991b; Graham 1991; Tsai and Phillip 1991; Harrison and Dixon 1993; Scervino et al. 2005a). In the case of legumerhizobia symbiosis other phenolic acids like hydroxybenzoic and hydroxycinnamic acids that are derived from the general phenylpropanoid pathway act as signalling molecules in the initiation of the symbioses, and as agents in the plant defense (Mandal et al. 2010). In ECM associations indole auxins, phenolic compounds and flavonoids were implicated as signaling molecules. It is well known that *Pisolithus* spp. secret hypahorine, IAA and other growth regulators that trigger morphological changes in the root system of host plants (Niemi et al. 2000; Martin et al. 2001). The involvement of *P. arhizus* in promoting branching to form second and third-order lateral roots in both conifer and angiosperm hosts were previously reported by Chambers and Carney (1999). In earlier study we demonstrated that the presence of *P. arhizus* during *in vitro* co-culture with *P. pinea* plantlets enhanced the adventitious root growth, before the physical contact occurred, and later after mycorrhization improved the survival rate of plants during acclimatization (Ragonezi et al. 2012).

In the present study, we identified a putative signalling compound that was present in the liquid phase of the double phase medium in the co-cultured *P. pinea* plantlets *with P. arhizus.* LC-DAD-MS analysis identified the substance as an ester of *o*-coumaric acid, which is a phenolic acid produced by plants and other organisms as secondary metabolite and a common chemical constituent in the plant kingdom (Abdul-Raman and Habib 1989; Vega et al. 2008; Ngoc et al. 2009; Sellami et al. 2009; Canuto et al. 2012). Among many effects

phenolic acids exert allelopathy and are implicated in the development of ecological interactions with the adjoining plant and with some rhizospheric organisms (Kefeli et al. 2003). For example, *o*-coumaric acid abundantly produced by *Euapatorium adenophorum* (a notorious weed worldwide) had a selective allelopathic effect that was toxic for *Arabidopsis thaliana* seed germination and plant growth but not for its own seed germination (Zheng et al. 2012). This result suggests that *o*-coumaric acid might render *E. adenophorum* a competitive advantage over neighbouring plants during its invasion and establishment. Whether *P. pinea* *o*-coumaric acid has an allelopathic effect requires further elucidation.

Our results demonstrated that *o*-coumaric acid ester was produced in the first two days of the co-culture and that its concentration tended to increase during the first 10 days. No *o*-coumaric acid ester was identified in the negative controls suggesting that pine roots might have secreted this compound in response to the presence of fungal mycelium. It is therefore plausible that *o*-coumaric acid ester played a signalling role in establishing the relationship with ECM associations before the physical contact.

Metabolites such as butanoic acid, cinnamic acid, *o*- and *p*-coumaric acid, vanillic acid or p-hydroxybenzamide occurred in the rhizosphere of *Arabidopsis* when challenged with the Gram-negative bacterial pathogen *Pseudomonas syringae* pv. tomato (Bais et al. 2005). The authors reported that bacteria could effectively modify the antimicrobial plant response, because the strains which were partly resistant to these compounds were able to block the exudation of antimicrobials using a mechanism based on the type III secretory system (Bais et al. 2005).

In this study *P. arhizus* mycelium grew continuously in co-cultures and this could be attributed to a similar ability possessed by *Pseudomonas* strains to modify and/or use *o*-coumaric acid for its benefit. Similar results were obtained by Zeng and Mallik, (2006) who studied the detoxifying effect of ECM fungi (*Laccaria laccata*, *L. bicolour* and *Paxilus involutus)* of black spruce (*Picea mariana*) phenolic compounds produced by the understory plant *Kalmia angustifolia.* Ferulic acid and *o*-coumaric acid were degraded within 10 days and the degraded amount depended on the fungus species. The authors concluded that certain ECM fungi not only offered protection to the host plants against phenolic allelochemicals released from neighbouring plants, but could also use them as carbon source. This is one of the mechanisms that ECM fungi use to control species interactions in higher plants by changing the rhizosphere chemistry. In our study on day 10 of co-culture the compound was either not degraded yet or the degradation by the fungus (or utilization for growth) was substituted by the plant secreting new *o*-coumaric acid ester. In another study

by Münzenberger et al. (2003) detoxification of ferulic acid by ECM fungi *Laccaria amethystina* and *Lactarius deterrimus* grown in liquid culture showed different detoxification pattern. Both studies confirmed the ability of the symbiont to degrade and detoxify phenolic compounds and that this ability was not only species-specific, but also specific to different strains of the same fungus species.

To our knowledge, this is the first time that an *o*-coumaric acid derivative was identified in the mutualistic interaction between pine species and *P. arhizus* and that this compound possibly participated in the initial cross-talk between the partners and also that *P. arhizus* could live and grow in the presence of *o*-coumaric acid ester.

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Chapter 8

Patent - System and Method for *in vitro* Culture of Plants for Analysis of Metabolites Released by the Root System Nº 105239

Patent approved by the National Institute of Industrial Property Portugal (INPI)

Castro MR, Ragonezi C, Oliveira P, Zavattieri, A. Patent INPI Nº 105239

Patent - System and method for *in vitro* culture of plants for analysis of metabolites released by the root system Nº 105239

Abstract

This invention is about the system development and *in vitro* culture method made up by two phases, solid and a liquid, the first to the plant support and the second to allow the analysis of the surrounding medium of the roots. This system is designed to allow an easy extraction of the metabolic compounds released/disseminated in the liquid phase during the root growth, in order to facilitate further processing and biochemical characterization. Considering that a proper root growth is a key factor for further strengthening and establishment of the plants in the field, the determination of the biochemical substances responsible for a better plant performance may lead to his artificial synthesis and implementation as an additive for organic fertilizers. Since is a closed system with known variables, this method could be applied in tests with pesticides, herbicides and other similar products.

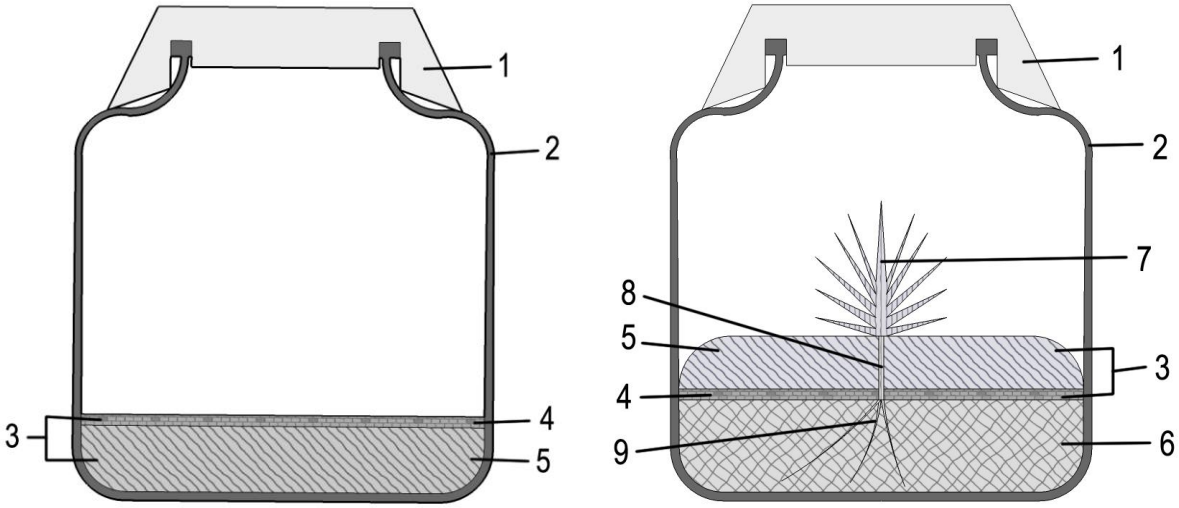

Fig. 1 - A - Illustrative scheme of the final appearance of the solid phase system preparation in a culture flask of *in vitro* plants. B: Schematic illustration of the final aspect of the method with inclusion of rooted plants. Figures numbers legend: 1 - cover; 2 - flask; 3 - solid phase; 4 - floating substrate; 5 - gelled culture medium; 6 - liquid phase; 7 - shoot; 8 - stem; 9 – roots

Claims (based on Figure 1)

1. Culture system for *in vitro* plants analysis of metabolites released by the root system characterized to contain a nutritive liquid medium (6) which supports a floating inert substrate (4) and a solid nutrient medium (5).

2. System according to claim 1 characterized by containing partners of biotization on the gelified surface (5).

3. Method of preparing the *in vitro* culture system described in claims nºs 1 and 2, characterized by the following sequence of steps:

a. Preparation of nutrient media use in the solid phase (3) and in the liquid (6);

b. Addition of the gelling agent to the nutrient medium from the solid phase, dissolving by heat, and distribution for the *in vitro* plant culture flasks already containing the floating substrate, before they reach the gelification temperature;

c. Solid phase inversion to guide the floating layer (4) under the gelling medium (5);

d. Pour the nutritive liquid phase in the already prepared flasks with the solid phase with the floating layer facing downward, resulting in the liquid phase (6);

e. Perforation of the solid phase (3) for insertion of the root system (9) and the stem (8) of the plant;

f. Plant culture, optionally incorporating other organisms on the gelling medium of the solid phase;

g. Extraction of the liquid phase (6) for further analysis.

4. Method according to claim 3, characterized by recycling of the solid phase (3) of the same plant (7, 8 and 9) by introducing a new liquid phase (6) for studies and analyzes with temporal component.

5. Method according to claim 3, characterized by recycling of the solid phase (3), by introducing a new liquid phase (6) and a new plant (7, 8 and 9).

Chapter 9
Final considerations and Future perspectives

Since each of the research issues addressed by this thesis and the respective outcomes have already been extensively reported in the different publications, only a summary of the main findings and a brief critical review are provided here. For more detailed information, the reader is referred to specific chapters.

The vegetative propagation of most conifer species through the rooting of cuttings is often limited by poor rooting ability. Micropropagation is also plagued by difficulties during the rooting phases of microshoots. An exhaustive review of conifer rooting was carried out and the findings published (Adventitious rooting of conifers: influence of physical and chemical factors, Chapter 2) to amass the available information on this specific topic. Based on the information compiled, various physical and chemical factors were adjusted in order to improve the rate and quality of *in vitro* rooting of stone pine, which were hitherto extremely low. An improvement in microshoot rooting of up to 70% in most clones was achieved; these results far surpassed the rooting success obtained by other researchers. A protocol was finally defined for a combination of suitable auxin and carbon source concentration in half strength WPM macronutrients; light source and light intensity were also adjusted during root induction and expression phases. These results were presented at an international meeting (see Annexes) and published (Influence of light quality and intensity on adventitious root formation in microshoots of *Pinus pinea* L., Chapter 3).

Although great advances have been made in rooting percentages, the roots obtained stopped growing which prevented the acclimatization of the pine plants in substrate. A new strategy was developed based on the co-culture of *P. pinea* and different ectomycorrhizal fungi**.** The reasons for this new approach were based on previous findings showing positive results in root system growth in the presence of various fungi tested during the expression phase of stone pine rooted microshoots (see Oliveira et al. 2003). This association produced positive results, since it effectively helped to overcome one of the most common problems associated with *in vitro* rooting: the cessation of adventitious root growth under the culture conditions, as well as the improvement of various rooting parameters. Additionally, it helped to mitigate the stressful transition from the *in vitro* to *ex vitro* phase since the association was extremely beneficial for the acclimatization of stone pine plantlets (*Pisolithus arhizus* (Scop.) Rauschert improves growth of adventitious roots and acclimatization on *in vitro* regenerated plantlets of *Pinus pinea* L., Chapter 6).

As explored above as part of this thesis, in mycorrhizae symbiosis, plant roots and fungi function together as a unit. In some cases, adventitious roots regenerated by microshoots can develop mycorrhizal-like structures (dichotomous) without fungal presence which closely resembles inoculated roots. The results, presented in Mycorrhiza-like structures in rooted microshoots of *Pinus pinea* L (Chapter 4) demonstrated the appearance of mycorrhiza-like structures in both rooted microshoots

and axenic embryo root cultures of *P. pinea*. Many environmental or cultural conditions influenced the capacity of this species to produce mycorrhizal-like structures; also the use of PGRs can induce similar root morphologies in the absence of fungi. In this regard, it is also important to emphasize that the use of histological and anatomical techniques was essential for differentiating mycorrhized from non-mycorrhized roots.

The identification of ectomycorrhizal collecting fungi species in pine stands was carried out in the field by means of morphological characterization and then the isolates confirmed by means of molecular techniques. Using a suitable molecular method, in this case the M13-PCR technique, it was possible to separate the different groups that can be found in pine stands. In addition, sequence analysis of ITS regions was employed to identify and confirm the identity of fungi during mycorrhization stages (if the species used at the beginning of trials is the same in the final stage). These results are presented in the article entitled Molecular approach to characterize ectomycorrhizae fungi from Mediterranean pine stands in Portugal (Chapter 5).

The nature of the signals released by the ECM symbionts, and how these signals behave within the partners, and whether these molecules may in the future be used in axenic cultures to mimic fungi in order to improve the rooting system and acclimatization, provide a stimulus for the chemical analysis of the signalling compounds present in ECM/pine plant co-cultures. In the analysis of the co-culture medium, the identified signaling compound *o*-coumaric acid ester appeared in the first hours of indirect contact between *P. pinea* and *P. arhizus* mycelium. The biochemical signalling compound found in this study also contributes towards an understanding of the role of phenolic compounds for pine/ECM symbiosis establishment and could also explain our previous results which showed an increase in various root parameters during co-culture before physical contact (*O*-coumaric acid ester, a potential signaling molecule detected during early *in vitro* co-culture between *Pinus pinea* L. plantlets and the ectomycorrhizal fungus *Pisolithus arhizus* (Scop.) Rauschert, Chapter 7).

The co-culture between pine plants and *P. arhizus* mycelium was developed in a culture medium that had two layers of semi-solid medium. During the first attempts to identify the signalling compounds it became evident that the use of gelling agents such as gellan gum (Phytagel) or agar (Agar-Agar) posed an obstacle to the extraction of the target compounds from the media, especially at low concentrations. For this reason, a novel system composed of semi-solid-liquid culture media was developed that allowed the analysis of the metabolites released by the roots (approved Portuguese Patent - System and method for *in vitro* culture of plants for analysis of metabolites released by

the root system, Chapter 8). In order to determine the chemical composition of the medium surrounding the roots, several studies were required to ascertain whether a compound could be detected in a minute concentration. The exclusive use of liquid culture medium without support for the plant was a non-viable solution because the plant tended to sink. Using a filter paper platform to support the plant seemed to provide the best way of dealing with this issue in the short term; however, after full imbibition the plantlets showed hydric stress and produced morphological abnormalities due to hyperhydricity. In our trials, we replaced the filter paper with a different support platform which maintained the plantlet above surface of the liquid medium, the roots in the medium and simultaneously eased the root metabolic exudates extraction. In this way, such difficulties were overcome.

As a final point, it should be emphasized that during this thesis a large number of different techniques had to be learned and subsequently applied for the successful accomplishment of tasks. Complexities presented in the thesis range from micropropagation protocols to histological, molecular and analytical techniques, and the process of mycorrhization *in vitro*. The living organisms presented here belong to different kingdoms with completely different characteristics and were able to interact in a positive way when conditions were ideal. A large number of papers have explored mycorrhization *ex vitro* or *in vitro* mycorrhization in petri dishes using pine seeds. For this paper, we had to acquire a great deal of knowledge in the field and experiment with innovatory techniques. Lastly, we are pleased to report that all the thesis aims were achieved on time.

Future perspectives

Further studies should focus on increasing our knowledge of the symbiotic association between plant species and microbial inoculants in biotization procedures. In the field of signalling, additional work is required to elucidate how the interaction starts because the beginning of the association is crucial and it determines the success of the relationship. It is also important to experiment with different types of ECM fungi in associations with the purpose of determining the most efficient ones for the restoration of root growth, acclimatization and transfer to soil.

The findings of this thesis should be interpreted in accordance with the explicit characteristics of each species and the specificity of both partners is a crucial feature for the establishment of association.

This thesis has provided new and relevant information about the symbiotic association between *Pinus pinea* and *Pisolithus arhizus*, which may also be applied to different types of conifer/ECM associations or any other plant species or microbial inoculants, as long as the association promotes positive effects.

Finally, it should be stressed that this thesis opens the way for new avenues of research to be explored. For instance, ectomycorrhiza-like structures need further investigation as regards the genes involved and the stressful situations that activate them. In the case of signalling mediators, each phase of the effective establishment of symbiosis between *P. arhizus,* as well as other ECM fungi associated with stone pine could be chemically analyzed. In addition, it would be interesting to carry out metabolic tests by adding the *o*-coumaric acid in to the plants growth medium. Furthermore, complementary research should be carried out on the survival of *in vitro* inoculated fungi in the nursery and under field conditions after outplanting. This could be done by using different techniques to study the competence of the ECM fungi introduced artificially versus the naturally-occurring fungi.

Other publications (poster/oral presentations and published sequences)

Other vegetative propagation techniques including rooted cuttings, micropropagation by organogenesis, etc.

S2–6

Micropropagation of recalcitrant pine (*Pinus pinea* L.) – An overview of the effects of ectomycorrhizal inoculation

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Stone pine *(Pinus pinea* L.) is an economically important forest species in some regions of Iberian Peninsula. Portugal and Spain have nearly 500,000 ha of stone pine stands, representing 85% of worldwide distribution. The main use of this species is for the production of seeds (pinion) for food industry. In addition to its enormous profitability as a producer of seeds, it has beneficial impact on soil protection, dunes fixation and is a pioneer species particularly for cork and holm oaks degraded ecosystems. Stone pine plantations are today a major source of income for forestry holdings. Investments have targeted breeding, reforestation, forest management and harvesting. The maternal inheritance of desirable characteristics such as cone weight, number of seeds per cone and seed length is considerably high in this species thus encouraging the selection of seeds from "plus" trees. The selected trees have been propagated by grafting and micropropagation. However, grafting generates high variability due to scion-rootstock interaction that varies production levels. The production of clonal plants from selected seeds by micropropagation techniques has advanced very slowly due to the recalcitrance of this species in tissue culture and particularly to adventitious rooting of microshoots. Due to the tremendous importance of developing a reproducible tissue culture method for clonal propagation, a study has been carried out for over a decade to enhance rooting and acclimation. During this period of time, continuous increments in the multiplication rate and rooting frequency were achieved by introducing variations in culture media composition and conditions. Auxins, carbohydrates, light quality and duration, temperature at different concentrations and levels as well as compounds such as coumarin; salicylic acid, polyamines, etc. were tested for induction and expression phases of adventitious rooting. Despite these efforts, microshoots regenerated through organogenesis from mature embryo cotyledons failed to root or to have sustained root growth. At this point, an *in vitro* co-culture technique of stone pine microshoots with ectomycorrhizal-fungi was introduced to overcome the adventitious root growth cessation *in vitro* and improve root development during acclimation phase. An overview of the results showing the positive effect of fungal inoculation in promoting root growth *in vitro* and on plantlet survival during acclimation will be presented. Preliminary results of biochemical signals between *Pinus pinea/Pisolithus arhizus* during early steps of *in vitro* culture detected by liquid chromatography-mass spectrometry that might be responsible for the positive effect on root growth will be also presented.

Keywords: acclimation, co-culture, ectomycorrhiza, *in vitro* adventitious rooting, micropropagation, stone pine.

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OP47

Biotization of the mediterranean stone pine (*Pinus pinea* L.)

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In vitro mycorrhization of micropropagated plants can be used to resume the growth of the root system and to improve the acclimation phase by enhancing root functionality and improving the mineral and water status of the plants. To obtain these benefits an extensive characterization and identification of field ectomycorrhizas (ECM) that are associated with the target plant species is crucial. Not all fungi can promote *in vitro* rooting or other beneficial effects, hence it is necessary to test in co-culture each fungus–host plant–clone combination. To select effective clone–fungus interaction, the signals released by mycorrhizal symbionts, and how they influence the behavior of the partners have to be studied. In this context, we present the results of our four year-work on *in vitro* mycorrhization of *Pinus pinea* L. The effectiveness of inoculation of rooted plantlets, their survival rate as well as morpho-histological and physiological characterization are described in comparison with non inoculated plants during acclimation phase.

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MYCORRHIZA-LIKE STRUCTURES DURING *IN VITRO* CULTURE OF STONE PINE (*PINUS PINEA* L.). A MATTER OF STRESS?

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Keywords: fungus-plant interactions, mycorrhizal systems, genotype, micropropagation, *Pinus pinea*, osmotic potential

Pinus pinea is one of the most important species grown in the Iberian Peninsula and it is Portugal's largest edible seeds producer. The induction and improvement of *in vitro* rhizogenesis of microshoots of *Pinus pinea* L. was developed in our laboratory using *in vitro* co-culture system of pine micro-shoots with ECM fungi. Unexpectedly, extensive dichotomous and coralloid branching of lateral roots occurred during *in vitro* rooting at the expression phase in our control plants. On the other hand, non inoculated plants that remained in the culture medium for longer than a month, in increasingly dry medium, developed more numerous mycorrhizal-like structures. This would suggest a correlation between osmotic and/or nutritional stress and the abundance of these mimicing structures. Results of changes in the osmotic potential of the culture medium (water content) and their influence on the number of dichotomous branching as well as the genotype dependence on the production of such structures will be presented. Analysis of dichotomous and coralloid roots (derived from *in vitro* cocultures) with and without fungus inoculation, were analyzed during the acclimation phase through histological observation. The cryostat sections revealed anatomical differences, both internal and external. The dichotomous branching of short lateral roots and the formation of coralloid organs are diagnostic of ectomycorrhizas in many pine species, but the micorrhyzae-like structures found in the control plants show a striking similarity to those of ectomycorrhizas. This phenomenon has been observed previously in other pine species and might be indicative of the long coevolution of these two kingdoms for millions of years. Therefore, it is possible that in the past mycorrhiza-like structures might have been erroneously assumed as plant-fungi associations.

THE USE OF ECTOMYCORRHIZAL FUNGI TO RESTORE ROOT GROWTH DURING *IN VITRO* ROOTING AND MINIMIZE LOSSES DURING THE ACCLIMATION OF STONE PINE (*PINUS PINEA* L.)

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Keywords: fungus-plant interactions, *in vitro* mycorrhization, *Pisolithus arhizus,* rooting, acclimation

The ICAAM Institute aims to study Mediterranean forest ecosystems in all aspects. Our Plant Breeding and Biotechnology Laboratory of ICAAM has always been involved in biotechnology of Mediterranean woody species, and has developed various *in vitro* techniques for vegetative propagation of *Quercus* and *Pinus* (Ragonezi *et al.* 2010, Zavattieri *et al.* 2009). The power of clonal propagation for the improvement of these woody species is indisputable. However, despite the fact that a lot of improvement in micropropagation has been made, we have always faced problems in the rooting phase (lack or reduced root growth), acclimation (water stress, loss of plants) and transfer to the field (low adaptability, low plant establishment). In this context, a few years ago we found an adequate "natural solution" for the *in vitro* mycorrhization. Different ECM fungi from pure and mixed stands of *Pinus pinea* were tested for their ability to enhance root formation and root sustainability, acclimation performance and survival of plants. Results of growth and physiological parameters during the *in vitro* and *ex vitro* acclimation phases of microplants inoculated with *Pisolithus arhizus* growing in different substrates and different conditions during *ex vitro* development and colonization will be presented.

Literature

Ragonezi, C., Castro, M.R., Klimaszewska, K., Lima, M., Zavattieri, M.A. 2010: Influence of light quality and intensity on adventitious root formation in microshoots of *Pinus pinea* L. *Acta Hort.,* (ISHS) 865:287- 291.

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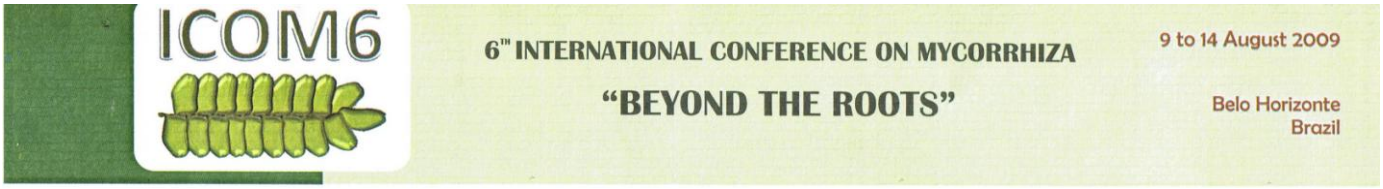

P8.2 – Misleading Mycorrhiza?

Author: Carla Ragonezi

Co-authors: Castro, M.R.; Santos-Silva,C.; Lima, M.; Klimaszewska, K; Vaz, M; Zavattieri, Mª.A. Pg. 82

Pinus pinea L. is an important Mediterranean forest species, mostly due to its edible seeds. Those seeds constitute a relevant resource for the Portuguese economy. To enhance *in vitro* rhizogenesis of *P. pinea* microshoots a co-culture system with ectomycorrhiza-derived fungi was developed. Plant and fungi were grown in double-layer WPM medium (with ½ the macronutrients and 0.2% of sucrose). Acclimation was made in pots containing mixed substrates, or in peat rhizotrons. Furthermore, axenic root cultures were prepared with roots excised from microshoots previously induced with auxin treatment. Observations were made monthly and root samples were taken from co-culture system and axenic cultures for further histological analysis. Structures similar to pine ectomycorrhiza (fine dichotomous and coralloid branching) occurred not only in co-cultures but in uninoculated controls, continuing to develop in the acclimation phase even in the absence of any symbiosis, and also in the axenic root cultures. The latter observation shows that axenic cultures may mimic the signaling from ectomycorrhizal fungi that is believed to induce the characteristic branching. This can be exploited in order to understand how plants perceive their mutualistic partners. Other anatomical and histological differences were found between mycorrhiza-like and fungal-induced structures.

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Analysis and mastering of root growth signalling by ectomycorrhizal fungi in *Pinus pinea* L. microshoot cultures

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Stone pine (*Pinus pinea* L.) is one of the most characteristic conifers in the Mediterranean basin. This species is of great economic importance because of its edible seeds (pine nuts). In order to ensure that Portugal remains one of the main world producers of pine nuts it is necessary to propagate superior individuals characterized by high seed yield and quality. Attempts have been made to develop a tissue culture method for clonal propagation using cotyledon explants (Oliveira et al., 2003) but poor rooting of microshoots and genotype dependence limited the use of this technique. Subsequently, our research effort was focused on rooting improvement by changing the medium carbon source and physical conditions (light and temperature) during rooting induction. In parallel experiments we also demonstrated that microshoot coculture with ectomycorrhizal fungi could rescue halted root growth and also help with successful acclimatization and outplanting. Of a random sample of 12 fungi derived from *P. pinea* ectomycorrhizas, at least six had consistently the same effect. Thus, our objectives are to ameliorate mass propagation of selected pine genotypes using ectomycorrhizal fungi for *in vitro* rooting, and to characterize biochemically the interactions between fungi and microshoots. Two hypotheses are being tested: 1) that the fungi promote rooting with diffusible mediators; 2) once these mediators are identified, that this action can be reproduced in axenic cultures. Results of the first year co-cultures, as well as anatomical and morphological characterization of the root systems derived from co-cultures and their comparison with axenic cultures will be presented.

Mycorrhiza-like structures in rooted microshoots of *Pinus pinea* L. Latest developments of a new insight.

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Pinus pinea L. (stone pine) is one of the major plantation species in Iberian Peninsula, being Portugal the largest edible seed producer in the world. The induction and improvement of in vitro rhizogenesis of microshoots of *Pinus pinea* was developed in our laboratory using a co-culture system with ECM fungi. In the acclimation phase in mixed substrates, or in rhizotrons, anatomical and morphological studies were done to observe the evolution of the root system in microshoots from the co-culture system vs. control plants. Surprisingly, extensive dichotomous and coralloid branching of lateral roots was seen to occur spontaneously in inoculated, as well in control and, moreover, similar branching occurred in liquid culture of excised seedling roots without the presence of ECM fungi, both with, and without hormone induction. The striking similarity of these organs with pine ectomycorrhizas prompted their anatomical analysis since dichotomous branching of short lateral roots and the formation of coralloid organs are diagnostic of ectomycorrhizas. This fact was observed before in other pines and proves the long co-evolution of these two kingdoms for millions of years. Therefore, natural structures may be erroneously assumed as plant-fungi associations. Plant and fungal symbiont where cultured in double-layer WPM medium with half the macronutrients and 1% of sucrose. Using rhizotrons and histological cuts it was possible to identify between natural (or chemical induced) and fungal-induced structures. Observations were made every month and showed the formation of small mycelia in several inoculated clones and appearing of Micorrhyzae-like structures, both dichotomous and coralloid in symbiotic and control plants. Further analysis showed anatomical differences between the fungal induced and control plants formations. Root samples were taken to perform the histological sections, both fresh hand-sectioned or fixed. After histological cutting, other anatomical differences were found, at a much smaller scale, both internal and external. However, the presence of Hartig net was not confirmed. These results suggested that the development of ECM-like structures might have occurred spontaneously. This new approach pursues the understanding of the physiological mechanisms related with the production of these interesting structures, as a survival mechanism for pine trees and as a launching pad for fungi colonization of the root systems.

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Achieving *in vitro* rooting in recalcitrant pine

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Stone pine (*Pinus pinea* L.) is one of the most characteristic species in the Mediterranean basin, being Portugal one of the main world producers. Its economic importance is based on its edible seeds. Stone pine planting stocks are largely derived from seeds collected from natural stands, and the conventional method of propagation is by planting nursery-grown seedlings. However, with this propagation procedure, the quality of the resulting material is unknown. Modern techniques for clonal propagation include grafting and cuttings of desirable genotypes. Such methods are labour-intensive, and therefore not ideal for large-scale multiplication of elite trees. Due to the tremendous importance of developing a reproducible tissue culture method for clonal propagation, an intensive study has been carried out in our group for over a decade to overcome the recalcitrance of this species to root *in vitro*. During this period of time, slow but continuous increments of the rooting percentage of this species was achieved. Studies were carried out introducing variations in media composition. Auxins and carbohydrates were tested at different concentrations, light, and temperature were used at different levels and many other different compounds (coumarine; salicylic acid, polyamines, etc) were also tested, for both the induction and expression phases of the rhizogenic process. Changes in the methods of applying auxins were also studied (quick-deep vs. pulse treatment). Before 2008, the highest rooting percentage obtained was 41,37% (Zavattieri et al., 2007). As a result of successive observations that most of the clones rooted approximately ten days after their transference to the expression medium (response to hormone treatment), but others took approximately one month, a new experiment was conducted to evaluate the effect of media dilution in promoting root induction and development. The only change introduced in the above protocol consisted in a reduction of macronutrients in the basal media. This simple modification of the previous established protocol significantly increased the total rooting percentage in all clones tested. The rooting percentages per clone ranged from 34% to 90%. Thus, in 140 microshoots of *Pinus pinea*, 88 rooted, giving an overall percentage of 62,85% which represents an increase of 20% comparatively with media with full strength macronutrients.

Key words: Pinus pinea, Stone pine, rhizogenesis, adventitious root formation.

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NCBI Sequences

Ragonezi, C, Klimaszewska K, Santos-Silva C, Martins MR, Caldeira AT, Zavattieri A 2011 *Pisolithus* sp. P1001 18S ribosomal RNA gene, partial sequence, internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence, and 28S ribosomal RNA gene, partial sequence. GenBank: HQ896485.1 National Center for Biotechnology Information, GENBANK, USA

Pisolithus sp. P1001 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence

GenBank: HQ896485.1

LOCUS HQ896485 645 bp DNA linear PLN 25-JUL-2011
ACCESSION HQ896485
VERSION HQ896485.1 GI:340561862
ORGANISM *Pisolithus* sp. P1001
Eukaryota; Fungi; Dikarya; Basidiomycota; Agaricomycotina; Agaricomycetes; Agaricomycetidae; Boletales; Sclerodermatineae; Pisolithaceae; Pisolithus.
AUTHORS Ragonezi,C., Klimaszewska,K., Santos-Silva,C., Martins,M.R., Caldeira,A.T. and Zavattieri,A.
TITLE Direct Submission
JOURNAL Submitted (10-JAN-2011) Chemistry Department, University of Evora, Rua Romao Ramalho, 59, Evora 7000 671, Portugal
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Ragonezi C, Caldeira AT, Martins MR, Santos-Silva C, Klimaszewska K, Louro R, Ganhao E, Zavattieri A 2012 *Lactarius deliciosus* isolate UEZB1 internal transcribed spacer 1, partial sequence, 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence, and 28S ribosomal RNA gene, partial sequence. GenBank: JQ066791.1 National Center for Biotechnology Information, GENBANK, USA

Lactarius deliciosus isolate UEZB1 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence

GenBank: JQ066791.1

LOCUS JQ066791 439 bp DNA linear PLN 20-MAR-2012
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ORGANISM *Lactarius deliciosus*
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Agaricomycetes; Russulales; Russulaceae; Lactarius.
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