Study of the duality plant-fungi

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Resumen Trabajo final de carrera.

Título: Duality plant-fungi. Dualidad planta-hongo.

En el trabajo se ha pretendido estudiar la simbiosis que tiene lugar entre una planta y diversos hongos, la planta a estudiar es el *Pinus pinaster,* y se estudia la relación simbiótica producida entre esta planta y los distintos hongos que podemos encontrar en suelos donde habita dicha planta. Estos hongos son *Suillus bovinus* y *Pisolithus tinctorius.*

La asociación entre hongo y planta se desarrolla en las raíces, formándose unas estructuras denominadas micorrizas, estas asociaciones son fundamentales para la nutrición y desarrollo de la planta.

Con estas asociaciones se consiguen diferentes ventajas para la planta huésped como pueden ser: mejor absorción de agua, mejor absorción de nutrientes, producción de reguladores de crecimiento o protección frente a microrganismos patógenos.

Portugal es un hábitat natural para el *Pinus pinaster*, también conocido como pino marítimo, siendo también muy utilizado para la reforestación, este estudio es importante para proporcionar una base de investigación para una futura reforestación de suelos afectados por incendios, suponiendo que aquellos pinos plantados con simbiosis tengan mejores crecimientos.

Los dos objetivos principales de este trabajo fueron el aislamiento y la identificación de hongos ectomicorrícicos y el desarrollo de un método simple para comprobar la compatibilidad entre en pino marítimo y los hongos ectomicorrícicos.

La conclusión más importante que se obtuvo con la elaboración de este trabajo es que tanto el hongo *Suillus bovinus* como el *Pisolithus tinctorius* son aptos para la formación de micorrizas con el *Pinus pinaster*, así como que la técnica empleada (siembra en vermiculita con turba) es apropiada para la síntesis de las micorrizas.

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Trabajo final de carrera

MEMORIA EXTENDIDA

DUALIDAD PLANTA-HONGO

INTRODUCCIÓN

Este trabajo fue parte de un amplio programa de investigación hecho en la Escuela Superior de Biotecnología, en la Universidade Católica Portuguesa en Oporto, Portugal. Este programa de investigación pretendía hacer un análisis sobre el papel potencial de los hongos ectomicorrícicos en la reforestación, usando el pino marítimo (*Pinus pinaster*). Este trabajo se realizó en la ESB desde Septiembre de 2011 hasta Febrero 2012.

Pino Marítimo (*Pinus pinaster*)

El pino marítimo ocupa una superficie mundial alrededor de las 4,4 millones de hectáreas, de las cuales 4,2 se localizan dentro del área natural de distribución (España, Portugal, Italia, Grecia, Marruecos y Tunisia).

En la península Ibérica la superficie forestal constituida por el *Pinus pinaster* es muy importante, representando el 29% en Portugal, mientras que en España representa el 27%.

El pino marítimo crece hasta 20 o 30 metros de altura y puede alcanzar los 40 metros en condiciones óptimas, crece en suelos frescos y profundos, necesita abundante luz y clima cálido y se localiza en alturas que van desde el nivel del mar hasta los 1000 metros.

El pino marítimo es una especie fundamental para la provisión de la importante industria maderera situada en Galicia y Portugal.

Cuadro 1.Producción de madera de rollo en Portugal y Galicia.

	Total madera (m3)	Pino marítimo (m3)	Proporción de pino marítimo.
Aquitania	10.065.000	9.095.000	90%
Portugal	8.142.000	3.085.000	38%
Galicia	5.196.604	1.913.580	37%
Total	23.403.604	14.093.580	60%

Actualmente, y debido al incremento de los incendios forestales, *Pinus pinaster*, así como otras especies de pinos, están disminuyendo sus áreas de ocupación, especialmente en el sur de la península ibérica, dónde podrían llegar a desaparecer, lo que hace importante el desarrollo de técnicas de reforestación nuevas y eficientes.

Micorrizas

Las micorrizas son el tipo de relación simbiótica más importante entre una planta y un hongo.

Definición de micorriza: Las micorrizas son asociaciones simbióticas esenciales para uno o ambos asociados, entre un hongo (especializado para la vida en suelos y plantas) y una raíz (u otro órgano en contacto con el substrato) de una planta viva, que es fundamentalmente responsable de la transferencia de nutrientes. Las micorrizas se transforman en órganos especializados de la planta que resultan del desarrollo de un contacto íntimo y sincronizado entre planta y hongo.

Las micorrizas son muy importantes en la mayoría de los suelos constituyendo hasta el 70% de la biomasa microbiana.

Por tanto los hongos micorrícicos son organismos simbióticos con una relación mutua con plantas terrestres, y que son capaces de colonizar diversos ecosistemas, se encuentran en el suelo y son muy importantes desde el punto de vista ecológico.

Hay distintos tipos de hongos micorrícicos, clasificados según la interacción con el huésped:

- Hongos Ectomicorrícicos penetran las raíces donde las hifas se sitúan entre las células de la raíz. Normalmente se pueden ver algunos filamentos del hongo en la superficie de las raíces.
- Hongos Endomicorrícicos penetran las células de la raíz, se trata de un grupo muy diverso.

En nuestro caso estudiaremos los hongos ectomicorrícicos ya que son los que forman simbiosis con *Pinus pinaster*.

Estructuras ectomicorrícicas y estados de desarrollo:

Las ectomicorrícas se forman por un crecimiento sincronizado entre las raíces y el hongo compatible, pero sólo ocurre cuando las condiciones del medio son favorables. Este crecimiento se compone de una secuencia de sucesos que tienen lugar en la formación de ectomicorrizas, estos sucesos se pueden resumir como sigue:

1. Sistema de Raíces:
   La mayoría de las raíces que forman micorrizas siguen un patrón de ramificación llamado heterorrizia que consiste en algunas raíces laterales, cortas, que se soportan por una red de raíces largas. Estructuralmente las raíces largas y las cortas son similares, pero la diferencia fundamental es que las raíces cortas crecen más lentamente que las largas.
   El lento crecimiento hace más fácil al hongo ectomicorricico formar la asociación con la planta, ya que esto necesita tiempo.

2. Hifas del suelo:
   Los hongos micorrícicos producen una red de hifas en el suelo, la red consiste en hebras individuales de hifas que constituyen la masa micelial.

3. Contacto con la raíz y proliferación de hifas:
   Las hifas contactan con la raíz corta, la reconocen y se adhieren a sus células epidérmicas, en el ápice de la raíz, donde está el área de crecimiento activo.

4. Raíces micorrícicas:
   Una vez la establecida la asociación las raíces cortas, micorricicas, continúan creciendo en largura y anchura. El tamaño, color, textura y tipo de crecimiento dependen de la planta y el hongo asociados.

5. La red de Harting:
   La red de Harting consiste en una red formada por las hifas éntrelas células huésped, como un laberinto, se sitúa entre las células de la epidermis y las células de la corteza de la raíz, es aquí donde tiene lugar el intercambio de metabolitos entre la planta y el hongo.

6. Reproducción:
   La red de hifas que interconecta hongos ectomicorrícicos en los suelos es también responsable de la reproducción, los cuerpos fructíferos crecen cuando las condiciones del entorno son favorables.

<u>Especificidad ectomicorrícica:</u>
Los estudios de especificidad en ectomicorrizas establecieron que algunos hongos solo fructificaban asociados a algunas especies de plantas, pero también ha sido demostrado que esta especificidad ecológica no implica necesariamente que estos hongos estén restringidos a la formación de ectomicorrizas con estas plantas.
El efecto sobre el crecimiento de la planta depende de la especie de hongo así como de las condiciones ambientales.

<u>Efectos beneficiosos de las ectomicorrizas en la planta huésped:</u>

- Mayor absorción de agua.
- Mayor absorción de nutrientes.
- Producción de reguladores de crecimiento.
- Protección frente a microrganismos patógenos.
- Mayor resistencia a suelos afectados por los efectos negativos de metales pesados.

Efectos beneficiosos de los hongos ectomicorricicos en el suelo:

- Limita la erosión del suelo, ya que las ectomicorrizas ayudan en la retención física de partículas.
- Regeneración del suelo, incrementando la retención de humedad, aire y la descomposición de materia orgánica.
- Incremento de la capacidad productiva de suelos pobres, afectados por desertificación, salinización y erosión.

Producción del inóculo de hongo ectomicorrícico:

Para producir una cantidad suficiente de inóculo y usarla en la práctica una cepa debe crecer fácilmente en un medio puro, sin ser necesariamente complejo, y debe sobrevivir fácilmente a las manipulaciones necesarias para la producción y aplicación del inóculo.

Normalmente los inóculos de hongo ectomicorrícico están compuestos por una biomasa fúngica y un medio de soporte. El medio de soporte debe contener una masa de hongo suficiente y homogénea y protegerla de las malas condiciones que pudieran darse durante la producción y manipulación.

Uno de los métodos más usados para la producción de inóculo micorrízico, consiste en el crecimiento micelial del hongo en un substrato sólido complementado con una solución nutritiva. La experiencia en este campo ha llevado a demostrar que hasta ahora el mejor substrato es una mezcla de vermiculita y turba complementado con el medio modificado Melin-Norkrans (MMN). Esta técnica ha sido adoptada universalmente y se está utilizando con éxito.

OBJETIVOS

Los dos objetivos principales de este trabajo fueron el aislamiento y la identificación de hongos ectomicorrícicos y el desarrollo de un método simple para testar la compatibilidad entre el pino marítimo y los hongos ectomicorrícicos.

MATERIALES Y MÉTODOS

Hongos ectomicorrícicos, aislamiento y mantenimiento en un medio puro:

Los cultivos de hongo, fueron aislados a partir de esporocarpos recogidos en Macedo de Cavaleiros, al norte de Portugal en Octubre y Noviembre de 2011.

El aislamiento en medio puro se realizó a partir de láminas internas del esporocarpo, usando aquellas áreas que no presentaban ninguna fuente posible de contaminación externa, asépticamente (en la cámara de flujo laminar) los fragmentos de esporocarpo eran transferidos a placas Petri, con medio MMN o medio PDA conteniendo estreptomicina.

Estos hongos se mantenían en el medio, y eran revisados frecuentemente para vigilar cualquier contaminación, eliminando aquellas placas contaminadas. Este proceso duró 60 días, manteniendo las placas en condiciones de oscuridad y 25ºC de temperatura.

El aislamiento se realizó a partir de 8 esporocarpos distintos.

Identificación de las especies de hongos:

1. Extracción de ADN:
   El ADN se extraía a partir del hongo aislado.
   Para la extracción de ADN se usó CTAB, método del cloroformo y el isopropanol.
2. Análisis del PCR:
   Esta técnica permite la amplificación de cantidades pequeñas de ADN en millones de veces.
3. Electroforesis en gel de agarosa:
   Una vez tenemos la muestra de ADN se le realiza la electroforesis para determinar si el proceso de amplificación ha resultado con éxito y poder así determinar la secuencia de ADN e identificar el hongo.

Test de compatibilidad y detección de hongos:

Se divide en tres partes, (i) preparación y germinación de semillas, (ii) preparación del inóculo, (iii) inoculación de la planta con el hongo aislado.

- Germinación de las semillas:
  Antes de la germinación las semillas se preparan por lavado y desinfección, usando el siguiente proceso:
  - Lavado durante una hora con agua de grifo.
  - Agitadas durante una hora con agua desionizada y detergente.
  - Lavado con agua de grifo.
  - Agitadas durante una hora con H_2O_2 30%.
  - Lavado con 2 litros de agua desionizada autoclavada.

  Tras esto se disponían en placas de Petri con medio Agar.

  Se dejaban en un cuarto de condiciones controladas de temperatura (20ºC) y luz (12 horas de luz y 12 horas de oscuridad) durante 20 días.

Preparación del inóculo de hongo:

Los hongos se tomaban del inóculo explicado anteriormente y se ponían en placas con MMN o PDA, poniendo en este caso papel de filtro entre el medio y el hongo, para hacer más fácil el paso posterior.

Inoculación Hongo-Planta:

Este proceso consiste en inocular las raíces de las plantas obtenidas con el hongo aislado, la inoculación se producía en un substrato adecuado bajo condiciones asépticas.

Se probaron dos tipos de inoculación:

- En placas de Petri normales con M&S como substrato:

Una semana tras la germinación la planta se transfería a una placa de Petri con medio M&S, se situaban según el sistema "sándwich", primero un papel de filtro, separando las raíces del medio, y sobre las raíces otro papel de filtro. La parte superior de la planta quedaba fuera a través de un agujero. La planta se dejaba una semana en el cuarto de condiciones controladas.

Tras esta semana se producía la inoculación quitando el papel de filtro de encima de las raíces y poniendo en su lugar el papel de filtro con el hongo, cubriendo posteriormente la placa para evitar la luz. Esto se realizó con 10 plantas, 5 con *Pisolithus tinctorius* y 5 con *Suillus bovinus*. Las plantas inoculadas se dejaban en el cuarto de condiciones controladas.

- En placas de Petri cuadradas con distintos tipos de suelo como substrato:

Primero de todo preparamos el substrato; se probaron 4 tipos distintos de substrato:

- 1,2 litros de vermiculita, 400 ml de Turba y 500 ml Agua, autoclavado.
- 1,2 litros de vermiculita, 400 ml de Turba y 500 ml Agua, pasteurizado.
- 1,2 litros de vermiculita, 400 ml de Turba y 500 ml MMN, autoclavado.
- 1,2 litros de vermiculita, 400 ml de Turba y 500 ml MMN, pasteurizado.

Tras la preparación de substrato, la inoculación in vitro tenía lugar en la cámara de flujo laminar para prevenir cualquier contaminación.

Se rellenaban las placas cuadradas de Petri con los distintos substratos, obteniendo 10 placas de cada substrato, después se ponía la planta sobre el substrato dejando la parte superior fuera a través de un agujero y finalmente el hongo se situaba sobre las raíces. Para cada substrato teníamos 5 plantas con *Suillus bovinus* y 5 plantas con *Pisolithus tinctorius*. Las placas eran cubiertas con papel de aluminio para evitar la luz.

Estado de las micorrizas:

El estado de las micorrizas se observaba tras un periodo de 2 meses para determinar la compatibilidad hongo-planta y la influencia de cada substrato.

Identificación de hongos de las micorrizas:

Una vez se detectaba formación de micorrizas, se recogía una muestra de cada planta y se extraía el ADN como se describe anteriormente, para ver si la micorriza formada procedía del hongo inoculado o de alguna contaminación.

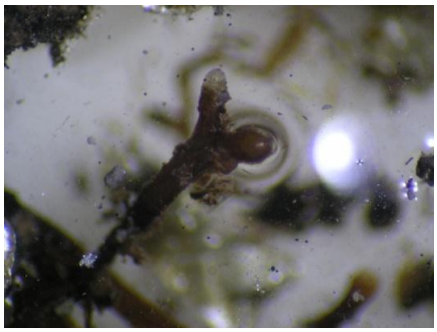
RESULTADOS

1. Germinación de semillas:
   A partir de 80 semillas, germinaron 50, obteniendo un 62,5% de germinación.

2. Plantas micorrícicas obtenidas:
   El siguiente cuadro muestra los resultados:

Medio	Hongo	Nº Placas	Nº Placas con micorrizas	% Formación micorrizas
Vermiculita, turba and agua autoclavada	*Suillus bovinus*	5	1	20%
Vermiculita, turba y agua pasteurizada	*Suillus bovinus*	5	1	20%
Vermiculita, turba y agua autoclavada.	*Pisolithus tinctorius*	5	1	20%
Vermiculita, turba y agua pasteurizada	*Pisolithus tinctorius*	5	2	40%
Vermiculita, turba y MMN autoclavada	*Suillus bovinus*	5	1	20%
Vermiculita, turba y MMN pasteurizada	*Suillus bovinus*	5	2	40%
Vermiculite, peat and MMN autoclaved	*Pisolithus tinctorius*	5	1	20%
Vermiculita, turba y MMN pasteurizada	*Pisolithus tinctorius*	5	1	20%
M&S	*Suillus bovinus*	5	2	40%
M&S	*Pisolithus tinctorius*	5	0	0%

Y aquí podemos ver algunas de las micorrizas obtenidas:



Basándonos en los resultados obtenidos podemos decir que ambas especies de hongos, *Suillus bovinus* y *Pisolithus tinctorius*, pueden formar micorrizas con *Pinus pinaster*, el porcentaje de formación fue alrededor del 20 y 40% en ambos casos. A pesar de que el número de plantas que presentaron micorrizas fue relativamente bajo, es importante decir que algunas placas presentaron contaminación fúngica, y que no se examinaron para prevenir posibles contaminaciones, también en todos los casos las micorrizas detectadas estaban en un estado de desarrollo inicial, indicando la necesidad de extender en el tiempo el experimento para permitir la formación de micorrizas totalmente maduras. Igualmente es posible que, en las placas en las que no se vieron micorrizas, la formación de la estructura estuviera en un momento de desarrollo muy temprano lo que no permitió su identificación.

Relacionado con el medio usado no podemos establecer ninguna conclusión ya que los porcentajes son muy similares.

También hay que recalcar que las plantas en medio M&S estaban muy contaminadas, y no habían crecido lo suficiente para ver micorrizas claras.

Por lo tanto esta técnica no es aconsejable debido a la lenta formación de micorrizas en plantas como el pino marítimo.

Sobre el crecimiento de las plantas con el hongo, todas ellas crecieron de forma similar, independientemente del tipo de hongo o medio en el que estaban.

<u>Resultados de los análisis de ADN a los hongos aislados y de las micorrizas:</u>

Los hongos fueron aislados con éxito, de manera que el proceso pudo continuar.

Para el análisis de ADN se contaba con 8 esporocarpos y 3 micorrizas, después de realizar todas las pruebas pertinentes, obteniendo los siguientes resultados:

Hongo 1 – *Lactarius deliciosus*

Hongo 2 – *Suillus bellinii*

Hongo 3 – *Suillus luteus*

Hongo 5 - *Tricholoma fracticum*

Hongo 6 – *Tricholoma spp*

Hongo 7 - *Tricholoma albobrunneum*

Hongo 11 - *Suillus luteus*

Hongo 12 - *Suillus bovinus*

Micorrizas de Pisolithus – *Pisolithus tinctorius*

Suillus bovinus autoclavada y pasteurizada con contaminantes por tanto no concluyente.

CONCLUSIONES

En relación al aislamiento de hongos, los 8 esporocarpos recogidos, fueron aislados con éxito y su crecimiento posterior fue posible en MMN y PDA.

También, el inóculo de hongo preparado mediante el método utilizado tuvo éxito, facilitando la posterior inoculación de la planta.

A partir de los análisis de ADN obtuvimos 7 especies diferentes de hongos: *Lactarius deliciosus,Suillus bellinii, Suillus luteus ,Tricholoma fracticum,Tricholoma spp,Tricholoma albobrunneum,and Suillus bovinus.*

El resultado de las micorrizas no fue tan bueno como se esperaba, la micorriza de *Pisolithus tinctorius* salió bien, pero las de *Suillus bovinus* tenía contaminantes, por lo que fue no concluyente. La micorriza había que lavarla de contaminantes antes de la extracción de ADN. Sin embargo, debido al tratamiento de calor al que se sometió el substrato, es de esperar que la micorriza perteneciera al hongo inoculado.

En relación con la formación de micorrizas, la conclusión principal es que se demostró que *Suillus bovinus* y *Pisolithus tinctorius* se pueden asociar con *Pinus pinaster* en simbiosis, y que la técnica usada, con turba y vermiculita, es apropiada para la síntesis de micorrizas. Sin embargo, la duración del experimento debe ser ajustada a cada tipo de planta usada.

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INTRODUCTION

This work was part of a broad investigation program made in the Escola Superior de Biotecnologia in the Universidade Católica Portuguesa in Porto, Portugal. This investigation program aimed to make an analysis over the potential role of ectomycorrhizal fungi in the reforestation, using the maritime pine (*Pinus pinaster*). This work has been made in the ESB from September 2011 to February 2012.

MARITIME PINE (Pinus pinaster)

Systematic:

Maritime pine (*Pinus pinaster*) has the following scientific classification: (http://plants.usda.gov)

Kingdom Plantae – Plants

Subkingdom Tracheobionta – Vascular plants

Superdivision Spermatophyta – Seed plants

Division Coniferophyta – Conifers

Class Pinopsida

Order Pinales

Family Pinaceae – Pine family

Genus *Pinus* L.

Species *Pinus pinaster*

Localization:

The world surface area occupied by maritime pine is estimated around 4,4 ha million, in which, 4,2 are located in the natural area of distribution (Spain, Portugal, Italy, Greece, Morocco and Tunisia). The remaining 200,000 ha are located in other areas such as Australia, South Africa, New Zealand, Chile, Argentina and Uruguay (Sanz et al., 2006)

In the Iberian Peninsula, the surface of forest area constitued by *Pinus pinaster* is very important. In Portugal it represents the 29% while in Spain 27%. This is because, both, Spain and Portugal are natural areas of distribution of *Pinus pinaster;* and maritime pine is also used in reforestation programmes. (IFN III (Galicia), IFN 3º Revision (Portugal)) (Anonymous 1989)

Features:

Maritime pine is a tree that grows until 20 or 30 meters high, and it can reach 40 meters in optimal circumstances, it has thick trunk and rough bark.

It grows in fresh and deep soil, principally in loose and sandy soils, and it prefers siliceous soils. It needs abundant light and a warm climate. It lives from sea level to 1000 meters high. (Ginés López. Iberian Peninsula trees and shrubs Guide.)

Economic importance of maritime pine:

Maritime pine is a fundamental specie for the provision of the important wood-processing industry based in Galicia and Portugal. The annual production of industrial roundwood is estimated around 14,1 million

. (Industrial Aplications of *Pinus pinaster* wood. "Technic Cooperation Project for the Development of Industrial Aplication of *Pinus pinaster*". Atlantwood-59)

Chart 1 Round-wood production in Aquitania, Portugal and Galicia.

	Total wood (m3)	Maritime pine (m3)	Proportion of Maritime pine
Aquitania	10.065.000	9.095.000	90%
Portugal	8.142.000	3.085.000	38%
Galicia	5.196.604	1.913.580	37%
Total	23.403.604	14.093.580	60%

Other features that made *Pinus pinaster* important in the forest sector are that it protects against the wind and, due to its radical right and deep rooting it can be used for fixing dunes to allow the recuperation of poor and eroded souls.

The wood, resinous, clear, red or reddish-brown, is durable, heavy and inflexible, used to furniture, poles, formwork, crates, particleboard, joinery, shipbuilding, pulp and fuel. (www.arvoresdeportugal.free.fr)

Provided current situation

Due to the increase of forest fires, *Pinus pinaster* and other pine species currently face a decline in their area of occupation, especially in de south of the Iberian Peninsula, where they could eventually disappear, making important the development of new quick and efficient reforestation techniques. (Ministry of Agriculture, Food and Environment of Spain) (http://www.marm.es/es/biodiversidad/temas/inventarios-nacionales/Pinus_pinaster_tcm7-175331.pdf)

MYCORRHIZAE

Mycorrhizas are the most important type of symbiotic between plant and fungus; there are other kind of associations, such as endophytic or parasitic.

Mycorrhiza, literally means fungus-root, the name comes from the Greek (mycos = fungus, Rhiza = root).

Definition of mycorrhizas: Mycorrhizas are symbiotic associations essential for one or both partners, between a fungus (specialised for life in soils and plants) and a root (or other substrate-contacting organ) of a living plant, that is primarily responsible for nutrient transfer. Mycorrhizas occur in a specialised plant organ where intimate contact results from synchronised plant-fungus development.

So, mycorrhizal fungi are symbiotic microorganisms that have mutual relations with terrestrial plants, successfully colonizing diverse ecosystems (Bonfante, 2003). They are found in the soil and are ecologically very important.

Mycorrhizal fungi are casssified into two different types depending on the interaction with the host:

- Ectomycorrhizal fungi enter the roots where the hyphae pass between root cells. There are usually some fungal filaments on the surface of the finests roots and this can be seen with a naked eye.
- Endomycorrhizal fungi enter the root cells. This is a very diverse group, including the arbuscular mycorrhizal (AM) or vesicular arbuscular mycorrhizal (VAM) fungi. This last group is the most commun in the plant kingdom, so also the most discussed. Only around 150 species of these fungi are known and they can form mycorrhizal associations with 70 % of Angiosperms.

Mycorrhizal fungi infect some cells called the cortex, which are found only on young roots, at the same time pass into the soil, so it forms a bridge to the plant. They provide soil nutrients to the plant and draw energy compounds from the host (St John, 2005)

Mycorrhizal are very important in most soils, and they constitute about 70% of microbial biomass (St John, 2005)

In this case we will study ectomycorrhizal fungi which are the ones forming symbiotic associations with *Pinus pinaster*.

Ectomycorrhizal structures and development stages:

Ectomycorrhizas are formed by a synchronised growth by host roots and compatible fungi, but it only occurs when environmentarl conditions are favorable. That growth is compounded by a sequence of events that takes place in the ectomycorrhizal formation, and those events can be summarized as follows:

1. Root Systems:

Most of the roots that form mycorrhizas follow a branching pattern, this pattern is called heterorhizy consists of some lateral roots, short roots, that are supported by a net of long roots. Structurally short and long roots are similar, but the main difference is that short roots grow slower than long roots do (Wilcox 1964, Kubíková 1967).

The slow growth of shorts roots makes easier to the ectomycorrhizal fungi to form the association with the plant, because it needs time, so, in those species whose roots grow faster is more difficult to form ectomycorrhizas because, that roots develop a periderm that prevents the roots from forming mycorrhizas.

2. Soil hyphae:

Mycorrhizal fungi produce a net of hyphaes in soil. The net consists of individual strands of hyphae that constitute the mycelial mass (Agerer 1991).

3. Root contact and hyphal proliferation:

Hyphae contact the short root, recognise it and adhere onto its epidermic cells, in the apex of the root, which is the actively growing area.

4. Mycorrhizal roots:

Once the association is established, mycorrhizal short roots often continue to grow in length and branching out. The size, colour, texture and branching patterns depend on the association fungi-host plant.

5. The Hartig net:

The Hartig net consists of the net formed by the hyphae between host cells, as a labyrinthine, it is placed between epiderm cells and cortex cells of the

root. It is here where the change of metabolites between fungi and plant takes place.

6. Reproduction:

The hyphal network that interconnects ectomycorrhizal fungi in soils is also responsible for reproduction. Fruit bodies grow when the environmental conditions are favourable.

Ectomycorrhizal specificity:

Specificic studies on ectomycorrizas stablished that some fungi only fructify associated to an species, genus or plant family, but, it has also been demonstrated that this ecologic specificity does not necessarily imply that these fungal species are restricted to the formation ofectomycorrhizae with these plants (Grand, 1968; Molina, 1979a, 1981; Molina and Trappe, 1982a).

So, we can say that the high specificity is not a common characteristic between ectomycorrhizal fungi and plants, so generally vegetal species can associate with a wide range of fungi and conversely.

The effect in the growth of the plant changes depending on the fungi species, and the environmental conditions (Benecke and Gölb, 1974, Mitchell et al., 1984). Also, for the same specific combination fungi-plant, the compatibility changes with the genetic or geographic origin of symbionts (Marx, 1981; Malajczuk et al., 1990). Different fungal strains of the same ectomyicorrizal fungi produce different effects in the plant`s growth (Laiho, 1970; Le Tacon and Bouchard, 1986; Perry et al., 1987).

Beneficial Effects of ectomycorrhizal fungi in the host plant:

- More water absorption: The fungi hyphaes increase the volume of soil explored, so having more area and being easier to transport the water makes the host plant more resistent and safe. (Augé, 2001)
- More nutrients absorption: The best known effect is that ectomycorrhizal plants take up more phosphorus from the soil and grow faster than non-ectomycorrhizal. In addition to phosphorus the hyphae also transport other resources to the host such as ammonium, calcium, sulphur, potassium, zinc, copper and water (Nasim, 2005). This is basically due to the bigger volume covering by the plant with the hiphae, but also due to the fact that fungi are able to promote the

mineralization of organic nutrients and to solubilize minerals by the production of organic acids, making those elements more available to the plants (Högberg et al., 1999; Landerweert et al., 2001; Marchner & Dell, 1994).

- Production of growth regulators: The ectomycorrhizal fungi produce some hormones that affect positively plant development (Brundrett, 1991).
- Protection to antagonistic microorganisms. The mycorrhizosphere has been hypothesized to constitute an environment conducive to microorganisms antagonistic to soilborne pathogen proliferation (Vigo et al., 2000).
- More resistance to soils affected by negative effects of heavy metals due to the ability obtained to immobilize the metals in the roots so they can't pass to other plant's areas.

Beneficial Effects of ectomycorrhizal fungi in the soil:

The beneficial effects of ectomycorrhizal in the soil are so related with their effects in plants because soil and plant are much related too (Bernaza and Acosta, 2006). But we can say that mycorrizae have some effects in the soil that increase so much its potential production and make easier its maintenance.

- Limit the erosione of the soil, because ectomycorrhizas help in the physical retention of soil particles.
- Regeneration of the soil, increasing the posibilities of retention of humidity, air and descomposition of organic matter.
- Increase of the productive capacity in poor soils, afected by the desertification, salinization and erosion due to water and wind.
  (Páez, O.G.Guerrero.2006. Las micorroizas, alternativa ecológica para una agricultura sostenible)

Ectomycorrhizal fungi inocula production:

To produce a sufficient quantity of inocula and use it at practice, an ectomycorrhizal strain has to grow easily in a pure medium, without being

necessary complex medium, and it has to survive easily to the manipulations necessary for production and aplication of inocula (Marx and Kenney, 1982; Le Tacon et al., 1985; Hung and Molina, 1986a).

Frequently, the ectomycorrhizal fungi inocula are compound of a fungal biomass and a carrier material. Inocula will be most effective when production in axenic conditions is achieved and being in an appropriate physiological condition for the mycorrhization start (Joan Pera Alvarez. Ectomycorrhizal fungi selection of *Pinus pinaster* for its aplication in reforestation).

The carrier material has to contain a sufficient and homogeneous fungal mass and to protect it from bad conditions that could happen during the inocula production and manipulation.

The inocula obtaining way has to allow it high scale production with low costs, and it effective aplication in low volumes. Nowadays, most of the inocula kinds only satisfy these requirements in a partial way.

One of most used methods for the mycorrhizal inocula consists of mycelial growth of the fungus on a solid substrate supplemented with nutrient solution which was subsequently incorporated into the substrate or nursery soil (Marx and Kenney, 1982; Harvey, 1991). At first carrier material was formed by cereal seeds, or a mixture of peat, vermiculite and seeds. Good results were obtained (Moser, 1958, 1963; Theodorou, 1967; Takacs, 1961a, b, 1967; Theodorou and Bowen, 1979; Gölb, 1975). But this technique presented contamination problems of saprophytic microorganisms, and it was demonstrated that inocula production in peat and vermiculite substrate (Hacskaylo and Vozzo, 1967), supplemented with liquid culture medium modified Melin-Norkrans (MMN) (Marx and Bryan, 1975), offered better results (Marx, 1980). This technique of inocula production has been adopted universally and has been used with success for the inoculation of forest species all around the world (Marx, 1980; Mexal, 1980; Marx and Kenney, 1982; Cordell et al., 1987a; Le Tacon et al., 1988; Kropp and Langlois, 1990; Marx et al., 1991).

OBJECTIVES

The two main objectives of this work were the isolation and identification of ectomycorrhizal fungi and the development of a simple method to test the compatibility between maritime pine and ectomycorhizal fungi.

MATERIALS AND METHODS

Ectomycorrhizal fungi. isolation and maintenance in pure medium

Pure cultures of fungi used were isolated from sporocarps collected in Macedo de Cavaleiros, norhtern Portugal in October and November of 2011.

The isolation in pure culture was performed from the internal tissues of sporocarp, using those areas that do not present any possible sources of external contamination (Molina and Palmer, 1982). Aseptically (Laminar flow chamber) mycelial sporocarp fragments were transferred to plates of Petri, with MMN medium or PDA medium containing streptomycin.

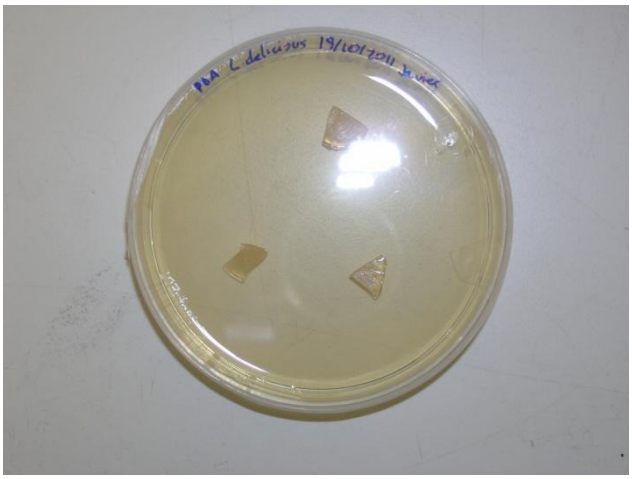
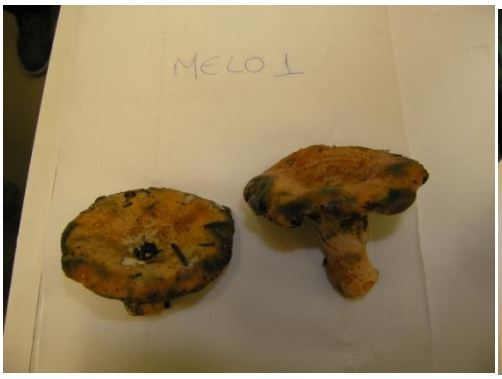


Figure 1. Lactarius deliciosus in MMN

Fungi were maintained in these medium and watched frequently for any contamination. Contaminated plates were immediately discarded or the fungi transferred to another plate. The total process for obtaining pure fungal cultures from isolated sporocarps lasted 60 days. The plates were kept in darkness conditions and 25 ºC of temperature at all times.

The isolated fungi were the following:

- Melo 1:

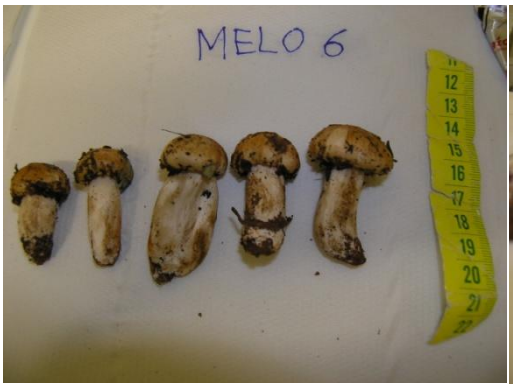


- Melo 2:

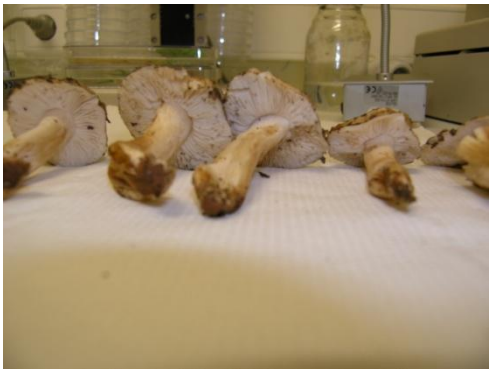
- Melo 3:

- Melo 5:

– Melo 6:



– Melo 7:



– Melo 11:

– Melo 12:

Identification of fungal species:

1. DNA Extraction:

DNA was extracted from the isolated fungi, actively growing on MMN and PDA medium.

For DNA extraction it was used CTAB, chloroform and isopropanol method, using the following protocol (Gardes and Bruns, 1993):

- Cell rupture: A fungi sheet was put it in 300 µl CTAB in an Eppendorf, crushed it with a tip, vortex and placed in the thermo-cycler for 1 hour at 65ºC.
- Protein precipitation: After this 300 µl of chloroform were added (in the chamber of volatiles), the sample vortex and centrifuged for 15 minutes at 15000 r.p.m. The mixture was divided in two phases. The upper phase was taken with a pippete and passed into another eppendorf
- Nucleic acids precipitation: 400 µl of isopropanol were added and the sample kept in the freezer for at least 1 hour at -20 º C. It was then centrifuged for 10 minutes at 15000 rpm.
- Precipitate washing: The eppendorf was reversed to remove the liquid. The DNA was washed with ethanol (70%, 200 µl, washing with the pippete), and we centrifuged machine during 5 minutes at 20000 g, The supernatant was removed with the pippete and the DNA was dried inside the eppendorfs in the oven during 5 minutes.

Finally the DNA was dissolved in 50 µl of ultra-pure water and kept in the freezer for further use.

2. PCR Analysis

This technique allows the amplification of low DNA quantities into hundreds or thousands of times.

First of all we prepare the PCR solution following the next quantities:

For a total of 50 µl:

Buffer: 5 µl

dNTP: 1,25 µl

ITS 1F: 2 µl

ITS4: 2 µl

Taq: 0,5µl

DNA: 2 µl

BSA: 1 µl

Water: 36,25 µl

PCR amplificatiion of the internal transcribed spacer (ITS) region of ribosomal DNA was performed using the primer combination ITS1F (CTTGGTCATTTAGAGGAAGTAA) and ITS4 (TCCTCCGCTTATTGATATGC) on Bio-Rad Termociclador MJ Mini.

Each sample was passed to the Thermo-cycler and ITS programme selected, where following reactions happen:

Denaturation: Separation of two DNA strands by high temperature.

Mating of each short strand of nucleotides (primers) (ITS1F and ITS4) with each separated strands of DNA.

Chain extension: DNA polymerase enzyme extends primers, in the space between both. For the synthesis of complementary sequences of DNA strands, the DNA polymerase uses dNTPs (desoxinucleosides triphosphate).

Buffer allows to maintain the correct pH for the DNA polymerase operation, and BSA prevents from possible inhibitions.

Thermo-cycler process:

5 min – 95ºC; 30 seg – 95ºC; 30 seg – 55ºC; 1 min – 72ºC; 10 min – 72ºC.

3. Agarose gel electrophoresis

After the sample extraction from the thermo-cycler we make an agarose gel electrophoresis to determine if the amplification process was succesful and subsequently determine the DNA sequence and identify the fungi species.
In the electrophoresis PCR samples are placed in an agarose gel, with sybr-safe, which is a specific coloring matter of nucleic acids to make the molecule visible.
During the process nucleic acids go from positive electrode to negative electrode separating molecules by size and shape.

The existence of bands is checked under a blue light.
Once the results are positive DNA purification is done of each sample from its PCR solution, made previously, following the protocol included in the PCR Clean-UP KIT of EzWAY.
The samples obtained were sent to a sequencing company (Stabida) to sequence the DNA.

4. Compatibility tests and fungal screening

Fungal screening was divided in three parts, (i), the seed preparation and germination, (ii) fungal inocula preparation and (iii) plant inoculation with the isolated fungi.

4.1 Seed germination of *Pinus pinaster*.

Before germination the seeds were prepared by washing and disinfecting, using the following process:

- Seed washed for one hour with tap water.
- Seed shaked for 1 hour with deionized water and Tween 20.
- Seed washed with tap water.
- Seed shaked for 1 hour with 30%.
- Seed washed with 2 liters of autoclaved deionized water.

After washing and disinfecting seeds, they were placed in Petri plates (5 or 6 seeds by plate) with agar medium:

- 1% Water Agar; so, 10 gr. Agar for 1 liter water.

Seeds were left in the chamber with controlled conditions of temperature and light: the temperature is 20 ºC, and light recreates the night/day cycle, turning on and off every 12 hours. Seeds stayed under these conditions for around 20 days.

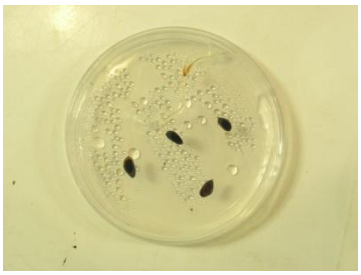


Figure 2 Seeds to germination

4.2 Fungal inocula preparation

For preparing the fungal inocula, fungi were taken from the fungal inócula explained before, and were put in a new petri plate whith MMN or PDA but this time there was a filter paper between the medium and the fungi, so to be easier to take the new fungal inocula preparation.

4.3 Fungi-plant inoculation

The inoculation process consisted in inoculating the roots of plants obtained from seeds with the fungi isolated; the inoculation took place in a suitable substrate, under aseptic conditions.

Two tipes of inoculation were tested:

- In normal Petri plates with M&S as substrate:

One week after germination, the plant was transferred into a Petri plate with M&S medium; they were placed as “sandwich” system, first one filter paper, separating the roots from the medium, and over the roots another filter paper. The plant canopy stayed outside trough a hole. Plants were left in the chamber with controlled conditions during a week.

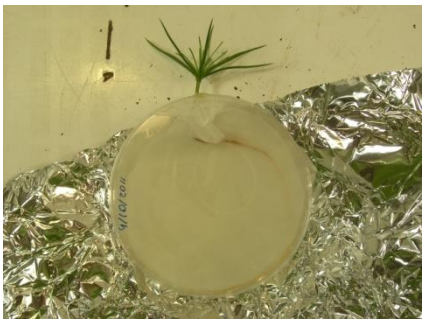


Figure 3 Plant in "sandwich" system

After this week inoculation was made, in the flow laminar chamber, the filter paper over the roots of the plants was removed, and instead, the filter paper containing the fungi inocula preparation was placed over the roots, allowing the contact between fungi and roots. Then, the plate was covered with foil, so the light doesn't reach the fungi and the roots.

The test was performed in 10 plants, 5 with *Pisolhitus tinctorius* and 5 with *Suillus bovinus*.

Inoculated plants were left in the chamber with controlled conditions.

Figure 4 Inoculated plant covered with foil

- In square Petri plates with different kind of soil as substrate:

First of all we prepare the substrate; 4 different kinds of substrate were tested:

- 1,2 liter Vermiculite, 400 ml Peat and 500 ml water. Autoclaved.
- 1,2 liter Vermiculite, 400 ml Peat and 500 ml water. Pasteurized.
- 1,2 liter Vermiculite, 400 ml Peat and 500 ml MMN. Autoclaved.
- 1,2 liter Vermiculite, 400 ml Peat and 500 ml MMN. Pasteurized.

After substrate preparation, an inoculation process in vitro was performed; in the flow laminar chamber to prevent any contaminations.

We fill some square Petri plates with the different substrates, 10 plates were filled of each substrate, after that, a plant was put in the substrate, with a hole so the plant canopy stays outside of the plate, and finally the fungi is placed over the roots. For each substrate we have 5 plants with *Suillus bovinus* and 5 plants with *Pisolithus tinctorius*.

The plates were covered with foil so the light doesn't reach fungi or the roots of plants.

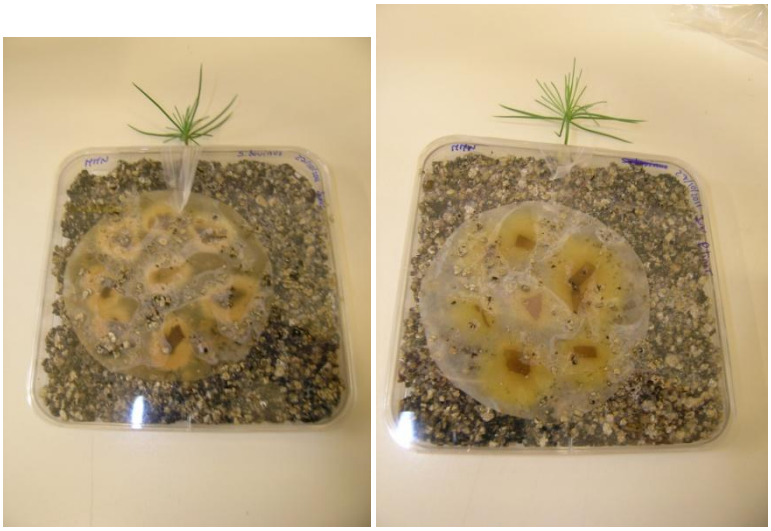


Figure 5. Square Petri plates with plant inoculated with fungi

5. Mycorrhizal status.

Mycorrhizal status was observed after a period of two months so to determine the compatibility fungi-plant and the influence of each type of substrate.

6. Identification of Fungi from Mycorrhizas

Once mycorrhizal formation was detected a sample from each plant was taken and DNA was extracted as previously described, to check if the mycorrhizas formed are from the inoculated fungal or from a contamination. The process is similar to the one used for fungi identification, but in this case RFLP analysis was also performed.

Restriction fragment length polymorphism or RFLP analysis is used to identify a change in the genetic sequence that occurs at a site where a restriction enzyme cuts. Restriction enzymes are proteins isolated from bacteria that recognize specific short sequences of DNA and cut the DNA at those sites. The normal function of these enzymes in bacteria is to protect the organism by attacking foreign DNA, such as viruses.

In this case the enzymes Taq I and Hinf I were used. The RFLP analysis was performed only on successful PCR amplification products (checked by agarose gel electrophoresis).

Hinf Solution for 10 µl for each sample:

- Buffer: 1 µl
- BSA: 0,1 µl
- Enzyme (Hinf): 0,25 µl
- : 5,65 µl
- : 3 µl

Taq Solution for 10 µl for each sample:

- Buffer: 1 µl
- Enzyme: 0,25 µl
- : 5,75 µl
- PCR: 3 µl

  After having each sample the restriction is carried out. In case of Taq I the samples were subjected to 3 hours at 65ºC, and in case of Hinf I, 3 hours at 37 ºC.

  A 3% agarose gel electrophoresis was performed to see the results.

  Later we do the agarose gel electrophoresis to see the results.

RESULTS

1. Seeds Germination

From 80 seeds 50 germinated so we had a 62, 5% of germination rate.

2. Mycorrhizal plants obtained

The following chart shows the results:

Chart 2Mycorrhizal plants obtained

Medium	Fungi	Nº Plates	Nº plates with mycorrhizas	% Formation mycorrhizas
Vermiculite, peat and water autoclaved	*Suillus bovinus*	5	1	20%
Vermiculite, peat and water pasteurized	*Suillus bovinus*	5	1	20%
Vermiculite, peat and water autoclaved	*Pisolithus tinctorius*	5	1	20%
Vermiculite, peat and water pasteurized	*Pisolithus tinctorius*	5	2	40%
Vermiculite, peat and MMN autoclaved	*Suillus bovinus*	5	1	20%
Vermiculite, peat and MMN pasteurized	*Suillus bovinus*	5	2	40%
Vermiculite, peat and MMN autoclaved	*Pisolithus tinctorius*	5	1	20%
Vermiculite, peat and MMN pasteurized	*Pisolithus tinctorius*	5	1	20%
M&S	*Suillus bovinus*	5	2	40%
M&S	*Pisolithus tinctorius*	5	0	0%

The pictures below show some of the mycorrhizas obtained:

Figure 6 Mycorrhiza *Suillus bovinus* in M&S

Figure 7. Mycorrhiza *Suillus bovinus* in Vermiculite, peat and MMN autoclaved

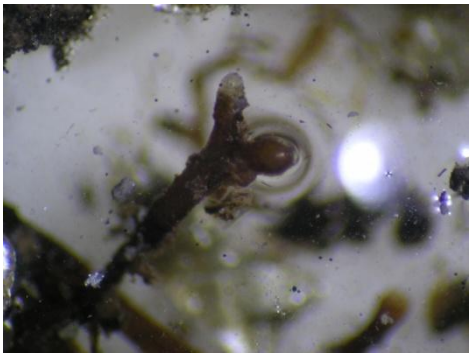


Figure 8 Mycorrhiza *Suillus bovinus* in Vermiculita, peat and water pasteurized

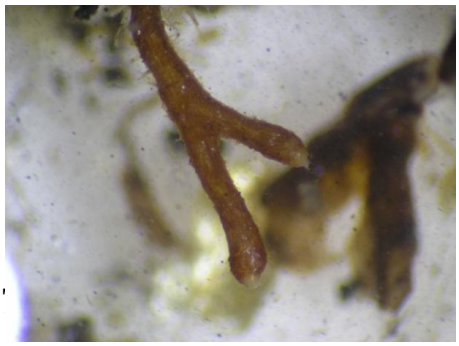


Figure 9. Mycorrhiza *Pisolithus tinctorius* en vermiculite, peat and MMN autoclaved

Based on the results obtained both fungi species, *Suillus bovinus* and *Pisolithus tinctorius*, can form mycorrhizas with *Pinus pinaster*, the percentage of formation was around the 20 and 40% in both cases. Although the number of plants showing mycorrhizas was relatively low, it is important to refer that some plates showing fungal contamination, were not eve opened to prevent the spreading of the contamination. Also, in all cases the mycorrhizas detected were in a preliminary stage of development, indicating the need to extend further the time of the experiment to allow the formation of fully mature mycorrhizas. It is therefore possible, and likely, that in the plates where mycorrhizasl formation was not seen, the structure

formation was in fact in a very early stage of developmen which did not allow it identification.

Related with the medium used we can't get any conclusion because percentages are very similar.

It's important to say that plants in M&S were very contaminated, and they hadn't grown enough to see clear mycorrhizas.

Therefore this technique is not advised for slow forming mycorrhizas plants such as maritime pine.

About the growth of plants with fungi, they all grew similar, regardless of the type of fungi or medium they were in.

3. Isolated fungi results and mycorrhiza results.

Fungi were isolated with success, so the process could continue, as we can see in the figure below:

Figure 10 Isolated fungi.

There were 8 sporocarps and 12 mycorrhizas, and after DNA extraction and PCR analysis we obtained the next sequence:

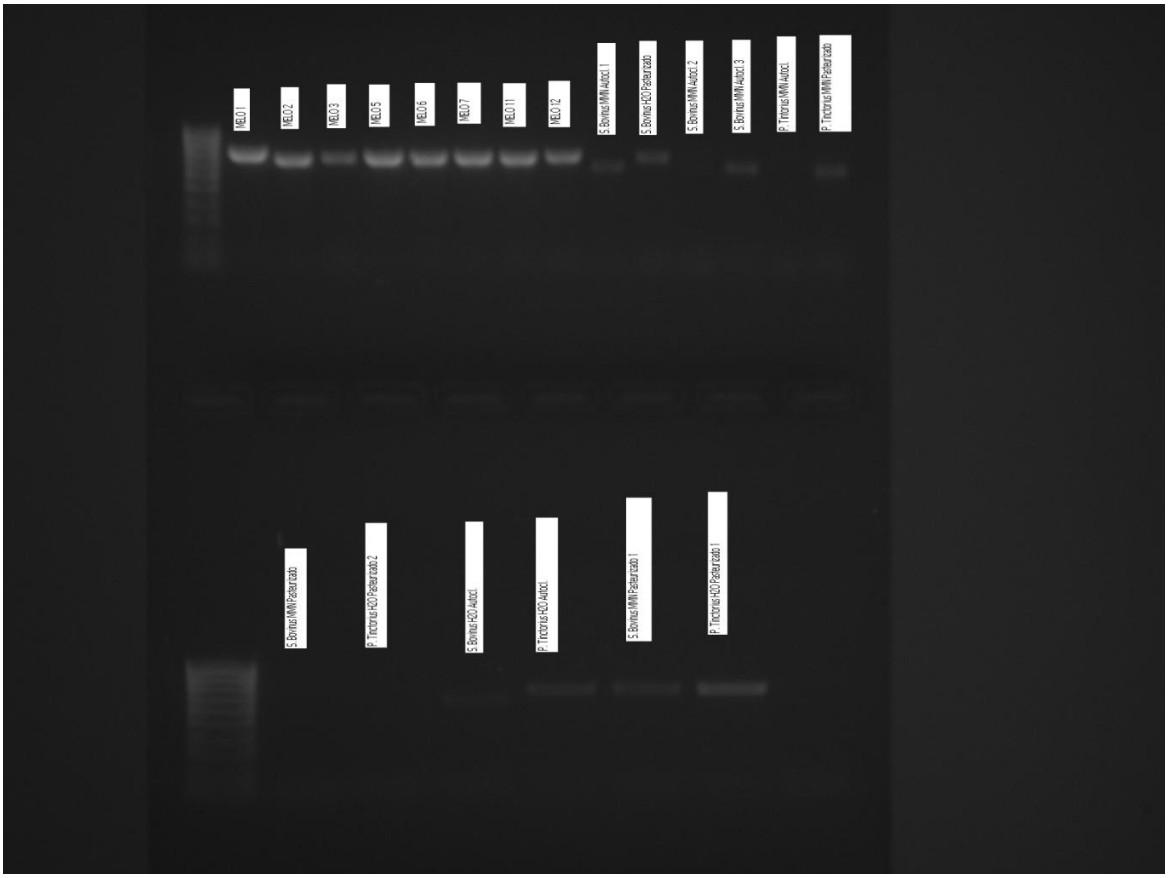


Figure 11. PCR sequence band

The 8 first correspond to sporocarps 1, as seen in all of them we have sequencing. And related to the mycorrhizas, the next 12 bands, we obtained sequencing in 8 of them, we had:

Chart 3. Sequencing bands on mycorrhizas

Medium	Fungi	Samples for analyze	Sequence saw samples
Vermiculite, peat and water autoclaved	*Suillus bovinus*	1	1
Vermiculite, peat and water pasteurized	*Suillus bovinus*	1	1
Vermiculite, peat and water autoclaved	*Pisolithus tinctorius*	1	1
Vermiculite, peat and water pasteurized	*Pisolithus tinctorius*	2	1
Vermiculite, peat and MMN autoclaved	*Suillus bovinus*	3	2
Vermiculite, peat and MMN pasteurized	*Suillus bovinus*	2	1
Vermiculite, peat and MMN autoclaved	*Pisolithus tinctorius*	1	0
Vermiculite, peat and MMN pasteurized	*Pisolithus tinctorius*	1	1

The eight samples that obtained sequencing were made the RFLP analysis:

Figure 12. Sequencing bands obtained after RFLP analysis.

Following the results of the picture, it was decided to make the DNA analysis only in the following 3 samples:

Suillus bovinus in vermiculite, peat and MMN autoclaved.

Pisolithus tinctorius in vermiculite, peat and MMN Pasteurized.

Suillus bovinus in vermiculite, peat and water Pasteurized.

So finally we made the DNA analysis of the 8 sporocarps and 3 mycorrhizas:

Melo 1 – *Lactarius deliciosus*

Melo 2 – *Suillus bellinii*

Melo 3 – *Suillus luteus*

Melo 5 - *Tricholoma fracticum*

Melo 6 – *Tricholoma spp*

Melo 7 - *Tricholoma albobrunneum*

Melo 11 - Suillus luteus

Melo 12 - Suillus bovinus

Pontas: Pisol – *Pisolithus tinctorius*

S. bovinus autoclaved and pasteurized– contaminants – inconclusive

CONCLUSIONS

Related to fungi isolation, the eight sporocarps collected, were isolated with success, and its growth was possible later in MMN and PDA mediums.

Also, the inocula fungi preparation (filter paper) was succesful, making easier the later plant inoculation and it was't any kind of impediment for the fungi growth.

From DNA results we obtained 7 different species of fungi : *Lactarius deliciosus,Suillus bellinii, Suillus luteus ,Tricholoma fracticum,Tricholoma spp,Tricholoma albobrunneum,and Suillus bovinus.*

The results of the mycorrhizas weren't as good as expected, the mycorrhiza of *Pisolithus tinctorius* was right, but mycorrhizas of *Suillus bovinus* had contaminants, so it was inconclusive. The mycorrhiza needed to be cleaned of contaminants prior to DNA extraction. Nevertheless, given the heat treatment that the substrate was subjected to, it is most likely that the mycorrhiza belong to the inoculated fungi.

Related to mycorrhizal formation, the main conclusion is that we proved that *Suillus bovinus* and *Pisolithus tinctorius* can associate with *Pinus pinaster* in symbiosis and the technique using peat and vermiculite used is appropriate to synthesize mycorrhizas; however the duration of the experiment has to be adjusted to each type of plant used.

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