Universität für Bodenkultur Wien

Influence of NP-fertilization and water stress on the fungal root colonisation of *Quercus coccifera* L. subsp*. calliprinos*

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Declaration

I here declare to the Institute of Forest Ecology (IFE), University of Natural Resources and Life Sciences, BOKU, Vienna that this is the original grounding research work done by independently and all other sources of text passages, ideas and thoughts are accordingly acknowledged. So, this written research work has not been presented to any other educational institutions for prizes.

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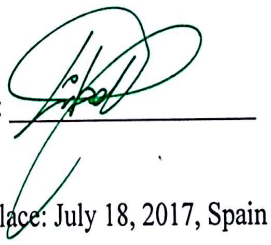
Signature: 

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Abstract

Desertification, extreme climatic events, scarcity of P, and N deposition are only a few of anthropogenic problems at we face nowadays, but can be the most important for the continuity of life on Earth. These problems are projected to worsen the next decades and will become more extremes in fragile regions as the Mediterranean biome. Little is known about root endophytic populations in these environments but there is strong evidence that they can play a key role to cushion the extreme events and enhance the ecosystems resilience. In this work, we attempt to deepen in the role of endophytic and mycorrhizal fungi of a Mediterranean specie, Quercus coccifera subsp. calliprinos. 72 oak seedlings of 1.5-year-old seedlings were grown for 485 days in a multi-factorial experiment greenhouse in Sde Boqer, Israel. The plants were exposed to moisture and dry conditions by one hand, and different N:P ratios by other. After, we calibrated a staining method to finally do a qualitative and quantitative analysis under microscope. We found presence of Ectomycorrhizae, Arbuscular mycorrhiza and Dark Septate Endophytes and we did a quantification of the last. We observed a positive correlation between DSE colonization and N:P ratios, a peaks of colonization in 9 and 20 ratios, and higher colonization in moisture conditions.

Key words: dark Septate Endophytes; DSE; fungal symbiont; Quercus coccifera; Quercus calliprinos; mycorrhiza; mediterranean endophytes

Motivation

By one hand, the curiosity of a part of the ecology and biology completely unknown for me, motivated this work, and by other, to believe that this little studied field, the role of microorganisms living-in-plants, still have a lot for discover, and also will be key to understand the future conditions and scenarios that we face in the next years on Earth, how demonstrate each time more evidences of the specific and ecosystemic role of these microorganisms. Also, to improve the capabilities and skills through the work-study-learning process.

Other reasons that motivate this study were:

- Very little information about the mycorrhizal-endophytic status of Quercus
- Very little information about mycorrhizal-endophytic microorganisms in Mediterranean Environments
- Increase of evidences of the important specific and ecosystemic role of this microorganisms in past-present and future focus

1. Introduction

The deforestation and desertification are increasing, the desserts advancing, the resources are running out at an uncontrolled rate, and the element cycles are deeply modified until point that currently, we need 1.5 planets to provide the current resource needs (Steffen et al., 2011). Such is the footprint that these impacts are leaving in this new Earth epoch that this period is called by some authors as "*Anthropocene*" (Crutzen, 2006; Steffen et al., 2011).

One of the most important modified cycles is the phosphorous cycle. This element, is protagonist in some essential processes such as at cell level, where it is found on the genetic material in the chromosome, surrounding the DNA and RNA molecules. Also, have a fundamental role in the cell energy-cycle in form of adenosine triphosphate molecule (ATP) and it is present in the external part of the cell membranes, permitting only the partial entrance in the adequate quantity of some elements trough sodium-potassium pump (Hernández and Munné-Bosch, 2015). Phosphorus with N and K, are the three main nutrients to plant nutrition and tissue concentrations (Marschner, 2011). In the photosynthetic organisms, the phosphorous joined with other elements like carbon and siliceous, and combined with the photosynthetic products, its essential to build their tissues and form resistant structures.

In the antiquity, the societies maintained the natural rhythms living according to nature, where, all the organic matter was recycled. Today, we are losing phosphorous at an exponential rate, and paradoxically, in the "developed countries" we have all the surface waters contaminated by the phosphorus fertilizers, which are transported to the oceans and sedimenting in the ocean bottom. We are not conscious that due to the nature of geologic cycle and the lack of atmospheric phase, this element will be not substitutable nor recoverable at human time scale (Ashley et al., 2011).

Phosphorous peak, the point in that phosphorus will start to decrease is predicted to arrive 2030-2040. This event is directly related with the increase in the demand of a growing world population, to fertilize crops and feed animals for meat production. (Cordell et al., 2009). To face these challenges, the understanding of relations between living beings like the fungal microorganisms living in plant roots are thought to be key to overcome the current and future scenarios and improve the plant resistance and ecosystems resilience in the actual and future

complex context of climate change and resources scarcity (Porras-Alfaro and Bayman, 2011; Giauque and Hawkes, 2013). The potential of some microorganisms to enhance the plants phosphorus status benefits need to be much more studied. Some of them like mycorrhizae are well studied (Bothe, 2010), while another's have been neglected.

Symbiosis

The interrelation between organisms is one of the three vital functions inherent to all types of life. Organisms are evolving and developing mechanisms and associations to overcome the evolutionary barriers, adapting to biotic and abiotic changes over the large planetary environments (Solomon and Berg., 2013; Richardson et al., 2014). Of these relations, close relationships between two or more organisms pertaining to different species are common in all ecosystems, going from mutualism to parasitism with a great diversity of behaviours. Coined by Anton de Bary (1879) first used the word Symbiosis, (from the Greek "living together") to discribe relations that can be observed ranging from phylogenetically close related species to the most distantly related organisms like eukaryotic and bacterial organisms (Dimijan, 2000).

Symbiosis is so important that some organisms cannot live without this symbiosis, in this case is coined "obligated symbiosis" (Rayner et al.,1915; Bignell, 2000; Douglas et al., 2011). Even the most essential and basic life pillars, like the eukaryotic cell, comes from an endosymbiotic process, in which a bacterium was enclosed within a more complex cell, which gave rise to the present eukaryotic cell (Mereschkowsky, 1905, 1910; Margulis, 1970).

Mycorrhiza

In the long evolutionary path, plants were early symbiotically associated with a great diversity of organisms, which allowed them to overcome giant evolutionary barriers like the colonization of the land environments from the sea and ocean, as evidenced by 440-410 Million-Year-Old fossils found (Smith and Read 2010; Redecker *et al*., 2000). These fossils show an ancestral type of symbiosis between a group of fungi called "Arbuscular Fungi" and the roots of the protracheophyte *Aglaophyton major* pertaining to plant kingdom (Wang et. al, 2006). These organisms belong to three phyla, *Glomeromycota*, *Ascomycota* and *Basidiomycota* (Solomon and Berg., 2013). Such relations, early coined by Frank as *Mycorrhizae* literally means "root"

and “fungi” (Frank, 1885) appears in the 92% of the plant families (Wang et. al, 2006). Its considered that some plants currently without this symbiotic-mutualistic relation had this relation in the past (Smith and Read, 2010). Estimations suggest that around 50000 fungal species form mycorrhizal associations with 250000 plant species (Marcel et al., 2014).

Then “mycorrhiza”, define a functional structure between two type of organisms, pertaining to plant (root) and fungi kingdoms, in which two members have benefits, although also some negative effects have been recorded (Marcel et al., 2014).

Mycorrhizal relations are keys to understand the global and local cycling of several elements, fundamentally carbon and phosphorous. The mycorrhiza exchange of nutrients with the host, providing them phosphorus in exchange for carbon. This effect is possible mainly due to the extension of the radicular system by the fungi mycelium, which allows to root explore more soil volume and access to fine pores which the normal roots can’t explore (Smith and Read, 2010; van der Heijden et al., 2014). This effect has many benefits to the plant, in different levels, since the nutritional state is enhanced.

The mycorrhizal symbiosis is dominated by two main types of relations, Arbuscular Mycorrhizae (AM) and Ectomicorrhyzae (EM). Although both differ in many aspects, they are characterized by form on intra-cellular and extra-cellular structures as interphase respectivly for substances exchange (Smith and Read, 2010).

Many of the fungi having AM relations belong to the *Glomeromycota* phyla (Smith and Read, 2010). This group of symbiotic fungi are characterized by forming two important components, the fungal structures within or between the cells of the root (interfaces) and the external mycelium in the soil (Smith and Read, 2010). Usually the structures are arbuscules (Figure 1b) within the cortical-cells, specifically between the cell wall and cell membrane, and storage vesicles (Figure 1a) located between the cells. The arbuscules and vesicles are connected to the mycelium, which in turn extends towards the rhizosphere, and outer environments. In the soil the external mycelia is growing and absorbing substances, and inside the plants, the exange of nutrients is done through the interfaces (arbuscules). In some cases, AM have been recorded having different interface structures, but it need much more research, since there are a lot of factors influencing these changes (Smith and Smith, 1990).

Ectomycorrhizal (EM) relations belong mainly to *Ascomycota* and *Basidiomycota* phyla. EM is characterized mainly by form three types of structures: a hyphal network between the epidermal and cortical cells, never intracellular, coined as "Hartig net" (Figure 1d), a sheath or mantle covering the root (Figure 1c) which develop characteristic branching patterns (useful to identify them), and the external mycelium through which the fungi take up nutrients from the soil (Smith and Read, 2010).

Currently, EM relations are likely the most studied in the field of symbiotic relations in plants. Such ecological benefits are mainly enhanced water and nutrient uptake which in turn generate and increase plant strength and fruit size, reduction of irrigation requirements and defence against pathogens and herbivorous (Smith and Read, 2010). At the same time, other close groups such as endophytes in woody angiosperms and gymnosperms have been neglected, because they are harder to study (Moricca and Ragazzi, 2007), and due to that until recently, no strong benefits nor harmful properties had been detected associated to endophytes (Johnson et al., 2013; Card et al., 2014; Saikkonen et al., 2016).

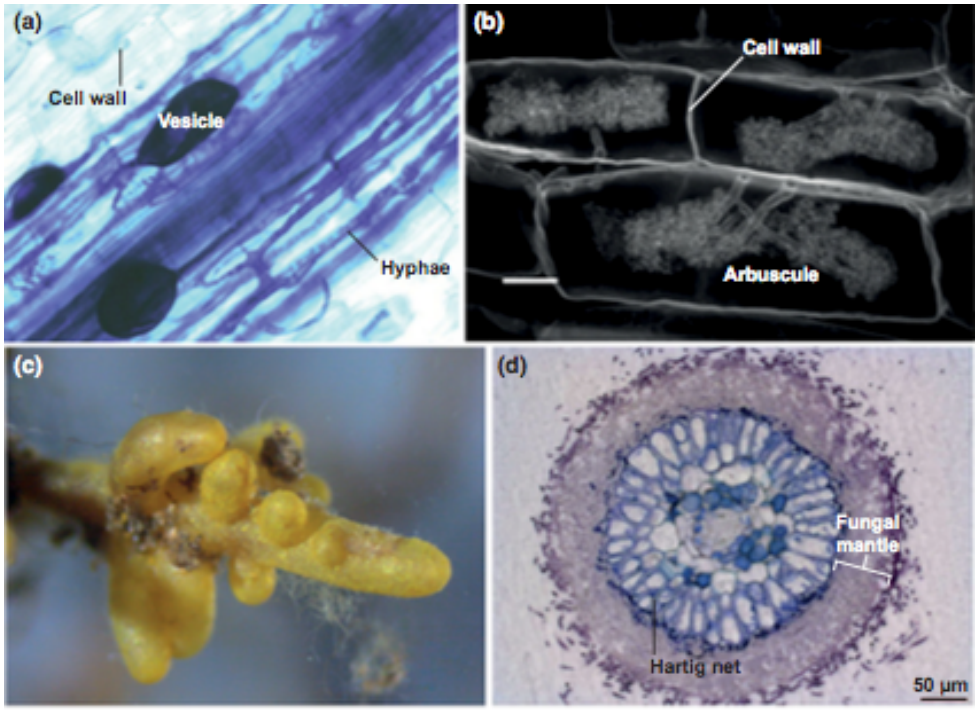


Figure 1. Typical structures of Arbuscular Mycorrhizae (a,b) and Ectomycorrhizae (c,d). Marcel et al., 2014

Endophytes

One of this neglected groups are the "Endophytes", microorganisms mainly pertaining to fungi and bacteria but also insects, nematodes, protozoa which live in the inner part of plant tissues, that are developing a part or all their life cycle, either in leaves, stems, trunk or roots without cause apparent symptoms (Chanway, 1998; Strobel, 2003; Sieber and Grünig, 2006; Müller and Döring, 2009).

Among the endophytes, the group of fungal microorganisms may be hyperdiverse (Petrini, 1991; Strobel and Daisy 2003; Huang et al., 2007) and have a global distribution. They have been reported from a range of natural ecosystems and form associations with a high variety of members in the plant kingdom from vascular plants to marine algae and mosses (Moricca and Ragazzi, 2008).

In addition, the same variability of behaviours is observed when we examine the general behaviours of the global symbiotic relations, this was coined like "the continuum" (Johnson et al., 1997). These interactions, goes from mutualism to antagonism, conditioned by factors such as environmental conditions, plant age, allelochemistry, or mode of transmission, what have been coined as "Mutualism-Parasitism Continuum" (Jhonson et al., 1997). For example, the anamorphic coelomycete *Discula Quercina* (West), an endophyte usually occurring in Oak native forests, changed its behaviour from innocuous to phytopathogenic due to external factors, supporting the hypothesis that in some cases, environment stress and senescence in plants turn the nature of the endophyte from mutualist or innocuous to pathogenic (Moricca and Ragazzi, 2008). This is a fundamental field to explore for the next decades.

The vast majority of fungal endophytes research have been focused in grasses (Schardl, 2004; Moricca and Ragazzi, 2008), but has practically been forgotten in woody species in Mediterranean environments (Moricca and Ragazzi, 2008). The few studies which have studied endophytes have often focused on the economical perspective, like the diseases in crop plants (Ragazzi et al., 1999; Kim et al., 2007). Its predicted that due to technical difficulties, this research is very limited and. When more plants are examined and more technics and technology developes, much more entophyte species will be discovered (Moricca and Ragazzi, 2008).

Another interesting point of view is the pharmacological perspective, these microorganisms are being investigated from this angle since it was discovered that bioactive agents, novel metabolites produced and stored in the plant tissues, can help to cure diseases through compounds with anticancer, antimicrobial and antiviral properties (Strobel et al. 2003; Selim et al., 2012). For example Taxol, a diterpenoid used for treatment of ovarian and breast cancers, is currently really expensive, what makes it non-available to most of the people. Now was isolated from fungal endophytes of *Taxus* trees, what open new good expectations to obtain this substance easily, and thus become it cheaper for people (Stierle et al. 1993).

Dark Septate Endophytes

Dark Septate Endophytes are conidial or sterile septate fungal endophytes which have melanised structures such as intercellular and intracellular hyphae and microsclerotia in plant roots and have known or likely affinities within ascomycetes (Jumpponen and Trappe, 1998). It has also been suggested that hyaline septate hyphae may be associated with DSE colonisation (Haselwandter and Read 1982; Newsham 1999; Yu et al. 2001). Although these organisms have been known since the early twentieth century, there are few studies, due to difficulties like the absence of asexual phase in many species (Fraccaro and Carrenho, 2017).

The relation between these organisms and plants, have been recorded in more than 600 plant species, in which they may improve nutrient uptake and maintain adequate water relationships for plant survival overall in arid environments (Barrow and Osuna 2002; Barrow 2003). Unusally they are not host specific but can colonize several different plant species (Mandyam and Jumpponen., 2005; 2011). They are characterised by have four types of structures, not always presents simultaneously, dark septate hyphae, hyaline septate hyphae with and without a lipid reserve and microsclerotia (Figure 2) (Jumponen and Trappe, 1998).

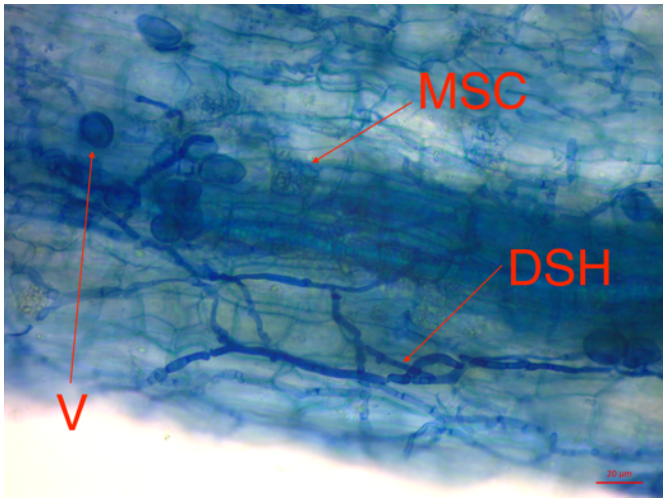


Figure 2. Three typical structures of DSE; vesicles (V), microsclerotia (MSC) and dark septate hyphae (DSE)

In the Northern Hemisphere in temperate, boreal and subarctic plants are frequently dominated by dark septate endophytes (Sieber 2002; Addy et al. 2005; Grünig et al. 2008). DSE in different environments as Alpine, Antarctic and Temperate forests, where demonstrated to play an important and still unknown role, due to its functions either by its mutualism or pathogenic, and having observed benefits like increased growth, enhanced nutrition from inorganic and readily sources, or from organic and recalcitrant sources, protection against herbivorous animals, insects and other microbial organisms, benefits very similar in some cases to mycorrhizal organisms (Mandyam and Jumpponen., 2005).

About the effects recorded, Haselwandter and Read (1982) isolated and reinoculated DSE of *Carex* species from European Alps and, when inoculated in the Carex species in a controlled experiment they found several benefits for the host, such as increase in the P content of shoots and increase of dry weight of the roots. Also, Jumponen et al (1988) examined the effects of the endophyte *Phialocephala fortinii* in *Pinus contorta* from a glacier forefront soil and it was observed a significant increase in P absorbed in inoculated plants than in non-inoculated. Newsham (1999) studied the effect of the DSE root fungus in seedlings of the annual grass *Vulpia ciliate ssp. ambigua* and it was founded a significant increase on root length and root N content, shoot root and total phosphorous content confirming that this is a mutualism case.

Their importance is growing due to recent discoveries regarding benefits to plants. For example facilitation by DSE increases the accessibility to organic nitrogen, phosphorus and sulphur sources (Mandyam and Jumpponen, 2005). They are appearing as a new resource to face the

current and future challenges, "*with potential to apply them in managing sustainable agroecosystems by promoting environmentally friendly practices"* (Spagnoletti et al., 2016).

Quercus

The genus *Quercus* is an important clade in the northern hemisphere, in terms of species diversity, ecological dominance and economic value. Oaks are widely distributed through all these ecosystems, appearing from temperate deciduous forest, temperate and subtropical evergreen forest, subtropical and tropical savannah, subtropical woodland, oak-pine forest, oak-'pinion'-juniper woodlands, various kinds of 'cloud forest', tropical premontane forest, tropical montane forest, matorral (summer rain chaparral), and a variety of Mediterranean climate vegetation, including chaparral (French: maqui), oak woodland, and evergreen oak forest (Nixon, 2006).

In Mediterranean environment, *Quercus coccifera* is the dominant *Quercus*, which appears forming large shrub extensions around the Mediterranean Sea.
This experiment was realized above *Quercus coccifera L. var. calliprinos (Webb) Boiss* or *Palestinian oak* species in a greenhouse in Israel. This country pertains to Paleartic global Ecoregion which contains the Mediterranean maqui or chaparral biome (Figure 3). Specifically, the habitats of Palestinian oak are situated in *Eastern Mediterranean schlerophyllous-broadleaf forests* (Figure 4).

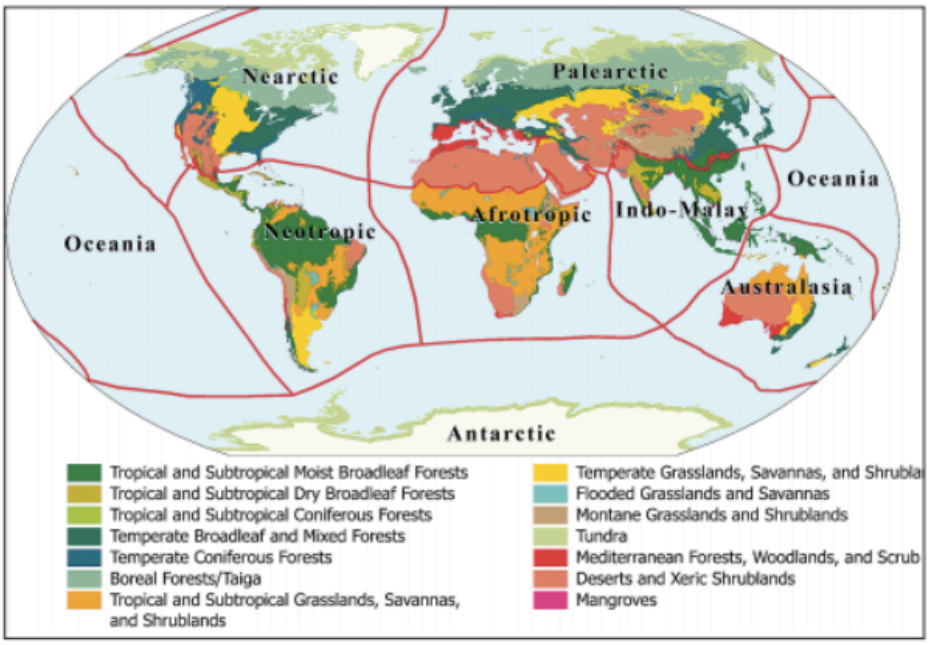


Figure 3. Map of Ecoregions. Olson et al, 2001

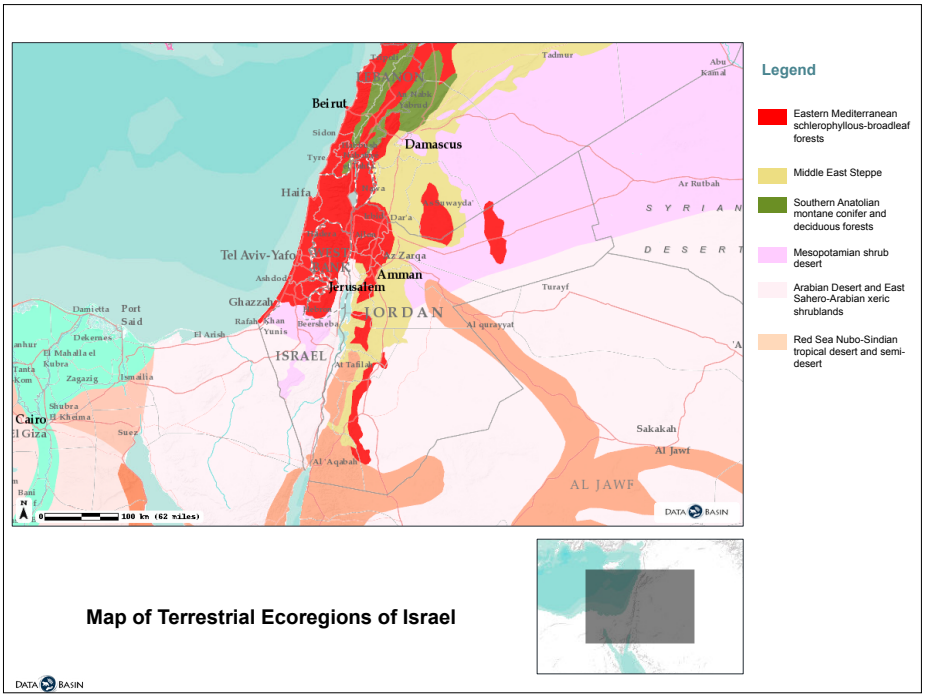


Figure 4. ***Ecoregions of Israel map.*** *Modified of Olson et al., 2001*

Aims

This study arises from the necessary to carry out more research in the field of symbiotic root microorganisms in environments like Mediterranean or Chaparral biome, where there is a big lack of those (Moricca and Ragazzi 2007). These microorganisms are thought to have key functions at specific and ecosystemic level, such as increase of drought resistance for the host, and P unlocking and assimilation. This knowledge is fundamental nowadays, in a context of Climate Change, and for projected future conditions, in which its expected more droughts, extreme events, and a scarcity of phosphorous due the human intervention in this cycle (Ashley et al., 2011).

Also, is necessary to do more research from the perspective of global pathogenic events occurring in forests, like the "*declining oak*" phenomena, in which exist evidences of some endophytes could be implicated (Thomas et al., 2002).

Among oak forests in Japan, the *Quercus* genus shared many ectomycorrhizal and also endophytes with subordinate plant species. In the order Helotiales there are several functional groups of fungi, among them ectomycorrhizal, saprophytic and endophytes, that benefit their plant hosts by mineralizing nitrogen. Helotiales endophytes can be major participants in the belowground plant fungal association (Toju et al., 2013).

Considering the global diversity and distribution of endophytes and *Quercus* genus, in which have been recorded to host a wide variety of EM associations (Harley and Smith, 1983; Smith and Read 2008), endophytic associations (Moricca and Ragazzi 2007; Collado et al., 1999), here is hypothesised that it will host also Dark Septate Endophytes.

This thesis attempts to deepen in the ecology of DSE, analysing root samples of the Mediterranean specie *Quercus coccifera subsp. Calliprinos* (Webb) Boiss. coming from a multi-factorial greenhouse experiment in Israel, in which the plants were exposed to moisture and dry conditions by one hand, and different N:P ratios by another.

The method used will be a modified of standard protocols to staining, and then submit the samples under stereo and optic microscope to do a quantitative and qualitative analysis with specialized microscope imaging software, to obtain data and argument and discuss the possible implications and consequences of this results.

This thesis is expected to contribute to generate useful information in this fields, and at the same time provide in the process the learning to the author.

General objectives

- Develop a functional method to quantify Ectomycorrhiza (EM), Endomycorrhiza (AM) and Endpohytes colonization in *Quercus coccifera* L. *var*. *calliprinos* (Webb) *Boiss*. roots

- Quantify the Ectomycorrhiza (EM), Endomicorrhiza (AM) and Endophytes infection in *Quercus coccifera L. var*. *calliprinos (Webb) Boiss*. roots

- Reveal the effects of different N and P fertilization ratios and different water irrigation treatments on root colonization

Hypothesis

- We expect high fungal infection in lower irrigation treatment

- We expect high fungal infection in high N/P ratio

2. Materials and Methods

This bachelor thesis is a complementary study from another experiment of greater scope realized in an Agrarian Experimental Station Israel.

2.1 *Quercus coccifera* L. *subsp. calliprinos* (Webb) *Boiss.*

The studied species, pertains at *Fagaceae* family and *Quercus* genus. The first species name proposed was *Quercus calliprinos* Webb., but after a comparison with different species databanks (GBIF, 2016; ThePlantList, 2010; CatalogueofLife, 2017; Kew Royal Botanic Garden, 2017) it was observed that the specie its treated as a *Quercus coccifera L.* where *Quercus calliprinos* Webb. it's a synonym. In other sites, there are still discussion, or *Quercus calliprinos* Webb. is treated as a variety or subspecies from the other (Vigo, 2006; oaknames, 2007; flowersinisrael, 2005).

It's interesting to note, that there are evident morphologic differences between occidental and oriental *Quercus*, how for example, tree and acorns size, or the growth habit which are bigger (almost 20 m front a 4-5 m) in the oriental *Quercus* (Paffeti et al., 2001; Vigo, 2006).
Due to this considerable morphologic difference, we decide to add the subespecie name, to specify in the oriental Mediterranean *Quercus*.

This specie is present in the "Mediterranean" or "Chaparral" biome, which is within the largest biogeographic realm, the "Palearctic". It occurs in a well-marked strip around the Mediterranean Sea, only broken in its north half in most of the Italian coast and in its south half in the northeast zone of the African coast, specifically Tunisia, Libya and Egypt. In Figure 5 is evident that *Q. coccifera* and *Q. calliprinos* is treated as the same due to the species distribution. In Israel, occurs from central to north costal plane environments (Figure 6a), and pollen evidences has been showed the higher ancient dominance prior to human activity (Liphschitz et al., 1990).

Always occupying Israel maquis ecosystem, this woody-perennial phanerophyte (Raunkiaer, 1934) dominate the *Q. calliprinos – P. palaestina* (Liphschitz and Biger, 1990) association.

This shrub-tree, reaches 20 m but usually, it appears forming shrubs or small trees, which are the most typical structure of this ecosystems (Figure 6b). It leaves has dentate or serrate edges, spinescents, serrate, and entire (wildflowers, 2017). The evolutionary adaptations have given to this resistant plant a very strong and lignified stem, with a cereous layer containing chitin protection in its leaves. This plant has a sclerophyllous nature, characteristic in the Mediterranean maquis species, which permits to be adapted at these hard environments, usually with a strong drought period in the summer season, hence, occurring the driest epoch and the rain scarcity together. This lignification and hardness of it tissues it's a very efficient form to alleviate the excessive water loss, and permits an optimum storage of water, necessary in these environments.

This specie has unisexual flowers, grouped in catkins which transform in an acorn which appears from March-May and it have not soil specificity (González, 2006).

From the historic-cultural aspect, this tree is widely known by the citation in several ancient religious texts such as Bible. Near the region of Hebron there is one thought to be over 850 years old (wildflowers, 2017).

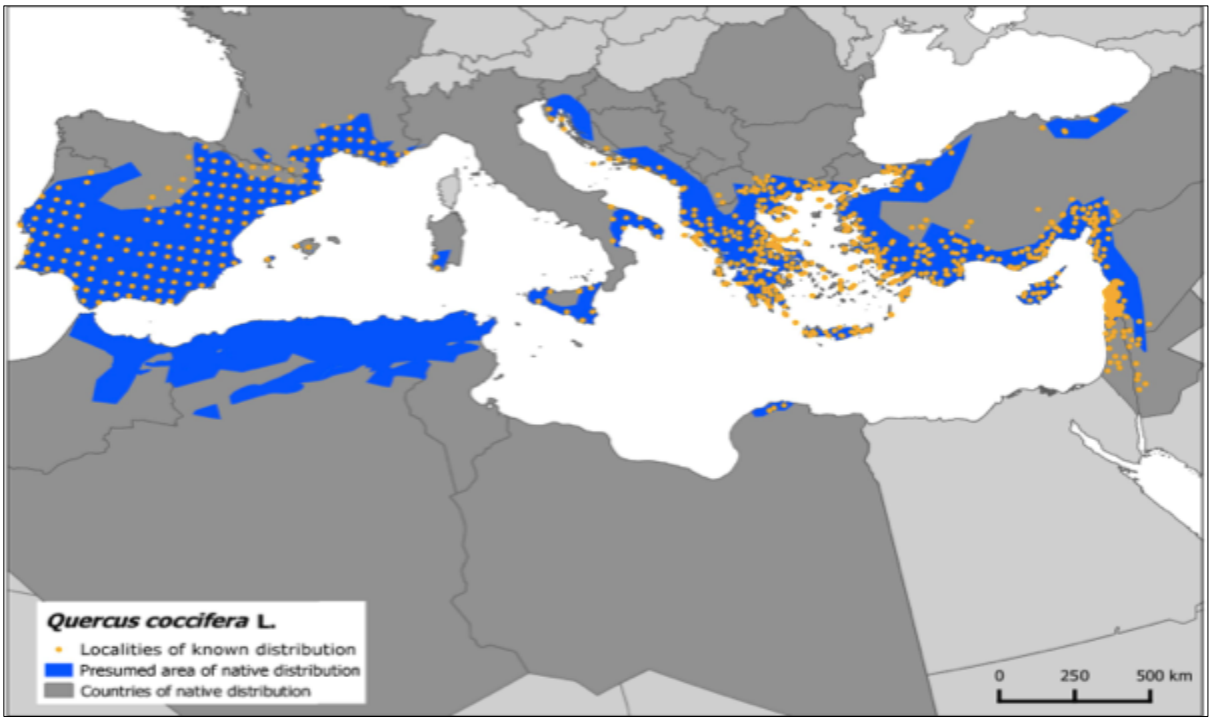


Figure 5. Global distribution of Quercus coccifera L. Wazen et al., 2015

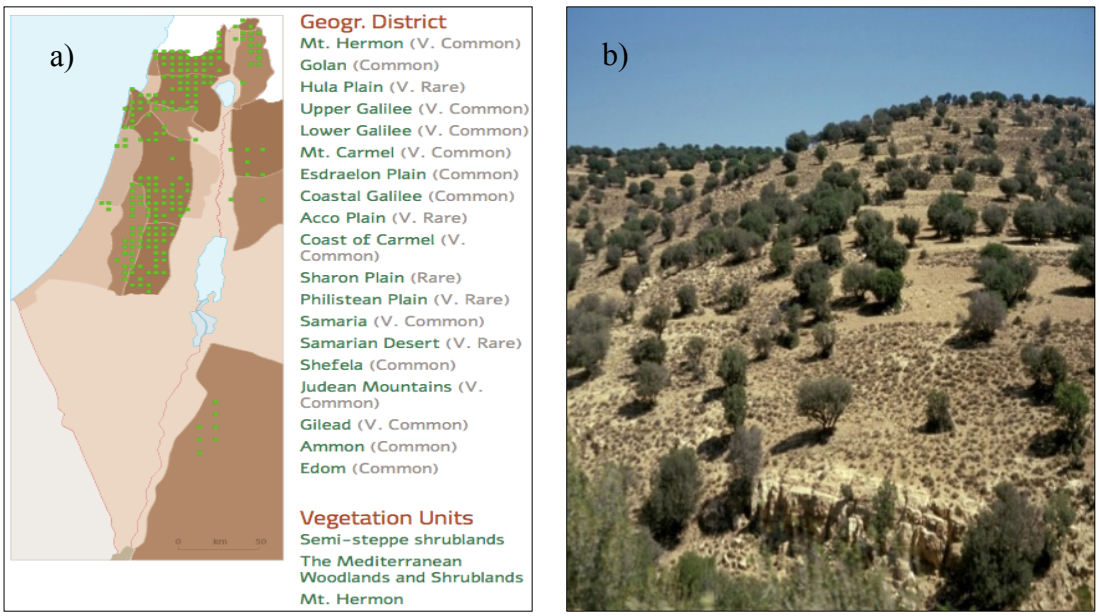


Figure 6. Distribution of Quercus coccifera var. calliprinos in Israel (a) & typical communities of the last (b) flora.org

2.2 *Experimental setup*

For the experiment, 72 oak seedlings (*Quercus coccifera* subsp. *calliprinos*) of 1.5-year-old oak seedlings were grown for 485 days in a greenhouse in Sde Boqer, Israel. Trees were planted in 18 liter white buckets, filled with sand. Mycorrhiza infection was performed by preparing a soil solution based on Rendzina soil from one of the field sites (southern Judean Mountains). The soil was wet sieved, and 200 ml of the solution were given to each the plant. This procedure was performed twice during the experiment. The greenhouse experiment comprised two irrigation treatments and six fertilization treatments with different Nitrogen and Phosphorus levels and ratios (Table 1). Fertilization treatments were designed to establish an N to P gradient ranging from low N to P ratio (0.94) to high N to P ratio (43).

In addition, two different irrigation treatments were established. Saplings of the dry treatment were irrigated with an amount of 333 ml per day during winter, and 400 ml per day during summer days, and saplings of the moist treatment with 666 ml H_2O per day during summer days, and 800 ml per day during winter days respectively. Fertilization was performed according to the schedule in Table 1. Electronic conductivity of all fertilizer solutions was similar to avoid differences in salinity stress. Micronutrients, were added three times during the experiment by means of 100ml 0.5M Hoagland solution. Mycorrhiza infection was

performed by preparing a soil solution based on Rendzina soil from one of our field sites (southern Judean Mountains).

Table 1. Different nutrient compounds and concentrations applied to Quercus coccifera var calliprinos seedlings

Fertilizer	P	N	N to P ratio	KH_2PO_4 (mg)	KNO_3 (mg)	K_2SO_4 (mg)	$NaNO_3$ (mg)	10.5%NH_4NO_3 (ml)	$MgSO_4$*7 H_2O (mg)
F1	2	1.88	0.94	8.79	8.8	14	3.36	2.92	9.90
F2	2	4.14	2.07	8.79	19.38	0	7.2	7.2	9.2
F3	0.5	2.03	4.07	2.20	12	19.2	1.6	2.92	9.90
F4	0.5	4.25	8.5	2.20	24.27	1.2	3.8	7.2	9.2
F5	0.1	2.04	20.45	0.44	12.8	20.4	1	2.92	9.90
F6	0.1	4.26	42.62	0.44	25.57	3.2	2.76	7.2	9.2

Each fertilizer and irrigation treatment has 6 replications, which comes up to a total amount of 6*2*3*2=72 samples.

Trees were harvested in February / March 2016 after an experimental period of 485 days. The trees were cut at their base and were divided into their compartments fine roots (Rf), coarse roots (Rc), shoots (Sh), and leaves (Le). Roots were washed. One representative fine root strand was scanned for recording root area, root diameter, root length and root volume. Total root area and total root length was calculated based on the representative subsamples.

A second representative fresh root strand was prepared to determine anatomy. A third representative fresh root strand was stored in 7% Glycerin solution and frozen at -20 degrees for mycorrhizae analysis. Then those samples of the third strand were sent to BOKU, to realize the mycorrhizal and endophytic analysis.

2.3 Mycorrhizal and Endophytic colonization

72 samples + 38 extra-samples were received. The first step was the classification of the samples by priority groups, wet - dry conditions, type of fertilizer irrigation, and extra-samples, and subsequently stored in a freezer at -18ºC.

2.3.1 Ectomycorrhizal assessment

Several samples of extra-samples group were randomly chosen and defreeze, and after that, submitted at visual analysis under Stereo-microscope. The first observation was based in the grid intersection method drawn on petri dish (Figure 7), (Brundrett ett al., 1996).

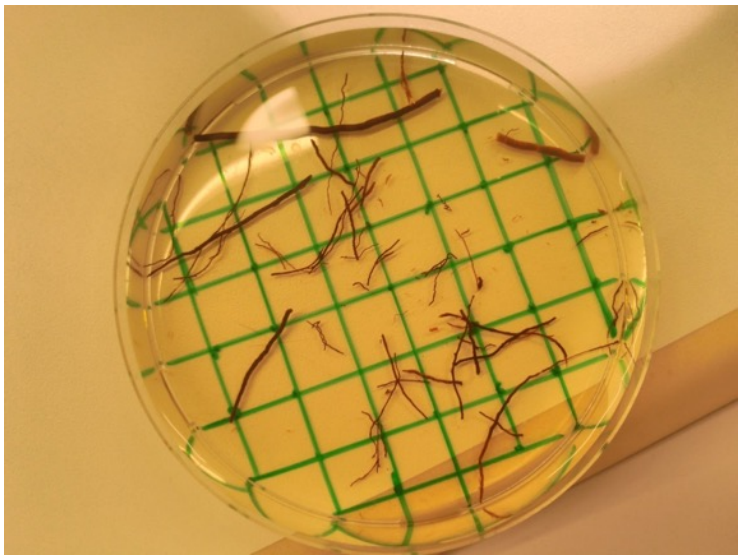


Figure 7. Petri dish intersection method. Liber Domínguez Espí

Some ways to identify the type of ectomycorrhizal fungi were consulted, Agerer Colour Atlas of Ectomycorrhizae (1987), and the ectomycorrhizal determination web page deemy (Agerer and Rambold, 2004) in order to take a contact and to know some resources, methodology and authors.

2.3.2 Arbuscular Mycorrhizae and Endophytes assessment

The microscope (Carl Zeiss Primo Star HAL/LED Full-Kohler, Trieb R, SF20, Foto) was prepared to do the observations, adapting a digital cam (ZEISS AxioCam ERc5s) through an adapter lens (Kamera/Adapter P95-C 1/2 0,5x fur Primo), installed in the photo-tube. This lens reduced the scale 0.5 times and, consequently, after it was necessary re-calculate the scale with the program used Zen 2 Blue Edition (Jena 2014 by Carl Zeiss Microscopy GmbH Version) to visualize and take photos.

The methods to assess the Endomycorrhizal fungi and fungal Endophytes root infection are the same, because these microorganisms are very similar in terms of biologic structures and chemical compounds. The base methods were modifications of Philips and Hayman (1970), Brundrett et al., (1984) and Vierheilig et al (1998).

This method is fundamentally based by one hand on a previous elimination of the root pigmented compounds located in the cell (Clearing and Bleaching processes), such as lignified walls of the of the epidermis and hypodermis and phenolic materials, which impede a clear vision through the root, and by other hand a selective staining (Staining process) which permits stablish selective bonds between the ink and the fungi structures, after the clearing with an acidified water to remove the ink rest (Vierheilig et al., 1998). The final aim of this method is to preserve the root and at the same time to have a good observation of the inner tissues.

After a carefully search it was observed that the vast majority of information regarding these methods are focused in the grass species, against a big lack of information regarding wood species (Bevege, 1968; Graham et al., 1991;). None specific information regarding the studied specie was found. Due to this, the first step was the calibration and adjust of the method. This method is different for each plant due to the specific characteristics which confers high or less dark pigmentation like lignin and suberin by one hand, and the intrinsic aggressively of the treatment by the other (Vierheilig et al., 2005). Usually, when more age and woody is the specie, more lignified and difficult to observe, hence, a stronger treatment it's necessary.
Otherwise these compounds would impede the clear vision and its consequence it would get a non-representative result.

In this case, the evergreen specie studied, *Quercus coccifera var. calliprinos*, showed a strong pigmentation (Fig 8f), hence, an accuracy calibration was necessary.

I. Clearing process

This process is based fundamentally in the debilitation and break of the cellular wall, trough increase temperature and pressure, warming the roots in KOH. The method was as follow:

- Defreeze the root samples (Figure 8a) which was 1,5h approx.
- 7 autoclavable glasses filled with 70 ml of 10% KOH dissolution
- Then 0.4 g of each sample were weigh out, and introduced in the glasses (Figure 8b and c). Sometimes, the roots were too big o disintegrated from the begin, in that case these roots were de-estimated. (With this quantity of sample, it was got approximately 10-15 pieces or subsamples of which at the end of the process were chosen 3 to realize the counting)
- Introduce in the first autoclave process (Figure 8d and e) at 121ºC and 10 min (alternative to lengthy boiling method) and rest overnight

The next table (2) shows the different protocols (1-7) tested in the clearing and bleaching steps. First, different concentrations (1 and 3%) of alkaline treatment (KOH) and then bleeching with (H_2O_2). Variations in the number of autoclave processes (1, 2, and 3) including overnight (6 and 7).

Table 2. Protocols assessed for the clearing and bleaching steps and results

Modified protocols	Clearing treatment	Alkaline treatment	Result
1	10% KOH + **1** Aut (15 min 121)	10 min **1**% H_2O_2	Dark
2	10% KOH + **1** Aut (15 min 121)	10 min **3**% H_2O_2	Dark
3	10% KOH + **2** Aut ((1x10 min 121) + (1x10 min 121))	10 min 3% H_2O_2	Dark
4	10% KOH + **2** Aut ((1x15 min 121) + (1x10 min 121))	30 min 3% H_2O_2	Dark
5	10% KOH + **3** Aut ((1x15 min 121) + (2x10 min 121))	30 min 3% H_2O_2	Destructed
5	10% KOH + 3 Aut ((3x10 min 121) +	30 min 3% H_2O_2	Destructed
6	10% KOH + **2** Aut ((1x15 min 121) + (1x10 min 121))*	30 min 3% H_2O_2	Dark
7	**10% KOH + 2 Aut ((1x10 min 121) + (1x10 min 121))***	**45-55 min 3% H_2O_2**	**Clear**

*Overnight between first and second autoclave processes; Aut: Autoclave treatment

- The next day, the samples were checked, always was observed a solution with a "coffee" appearance pigments (Figure 8f) soaked three times under the tap water, and the KOH renovated. Then, a second treatment followed in Autoclave with the same program that the day before (121ºC and 10 min)

- After one and half hour (more time than the process duration), the autoclave was opened and the flasks extracted (Figure 8g)

- After 5 minutes cooling, soaked under tap water two times (Figure 8h)

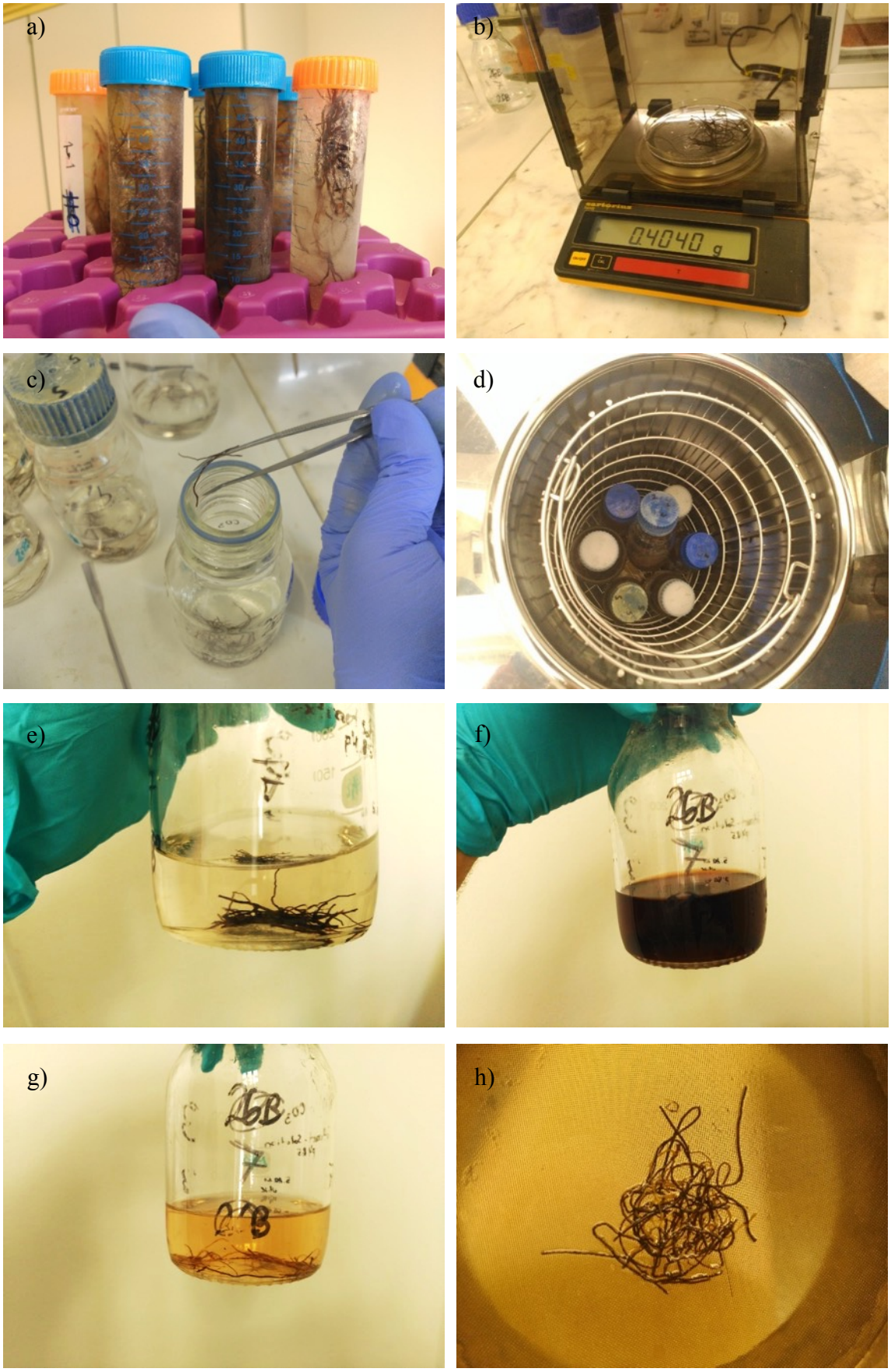


Figure 8. Clearing process. Defrosting, weighting and soaking samples in KOH (a-c), autoclave process and results (d-h)

II. Bleaching process

The bleaching is the partially empty and bleached of the cell content in an alkaline medium, due to the oxidation by the Hydrogen Peroxide (H_2O_2), and its function is to remove the resting pigments that remain from the previous step (Figure 9d). This step is not necessary in most of the grass and other herbaceous species, but due to the schlerophyllous nature of this specie it was necessary to do so.

The process was as follow:

- After clearing treatment and a testing (Figure 9a), a dissolution of 3% H_2O_2 was prepared and after 70 ml were filled in 100 ml glass beakers (Figure 9b, c) then the subsamples were introduced inside during 45 to 50 minutes

Figure 9. Bleaching process. Testing roots (a;)pigments coming out (a, b and c), remaining H_2O_2 solution with pigments (d)

III. Staining and Mounting processes

After to get information about several methods for the staining step, it was chosen an ink-vinegar method, due to several advantages since usually the used compounds for the staining step have hazard and dangerous properties, for example lactic acid or phenol; acidic glycerol; trypan blue; cotton blue or different inks. Even some of them have been recently labelled like carcinogenic. Another advantages are, cheaper, easily to obtain, and tested equally effective than the others (Vierheilig et al., 1998 and 2005).

The procedure was as follow:

- Extraction the roots from the H_2O_2 and rinse two times with a sieve under tap water
- Introduce the subsamples in petri dishes and choose the non-destructed
- Cut in 1.5-2cm pieces (Figure 10a)
- Fill in Eppendorf's tubes of 2 ml capacity with 1 ml of ink solution (5% Pelikan Blue Ink + 5% Acetic acid) (Figure 10b)
- Introduce two root pieces of roots in each Eppendorf close them (different root subsamples in different Eppendorfs)
- Introduce them in the heater and heat 20 minutes at 90º (Figure 10c)
- Extract them, and wait 2 minutes to cooler (Figure 10d)

The next table (3) shows the different protocols assayed for the boiling root pieces with ink step, which were 2, 10, 15, 20, 30 minutes and the results.

Table 3. Conditions assessed for the boiling root step with ink and previous acidification step

Protocols	Alkaline treatment	Heating (min)	Acidify (min)	Result
1	45-55 min 3% H2O2	2	5	No stained
2	45-55 min 3% H2O2	10	10	Stained and clear
3	45-55 min 3% H2O2	15	15	Stained and clear
4	45-55 min 3% H2O2	20	20	Stained and clear
5	45-55 min 3% H2O2	30	25	Destructed

- Open the Eppendorf tubes, and introduce the subsamples in slightly acidified water 20 minutes (Figure 10e)

- Then mount 5 pieces in marked slides, using PVLG (Polyvinyl alcohol-lactic acid-glycerol) (Figure 10f-h)

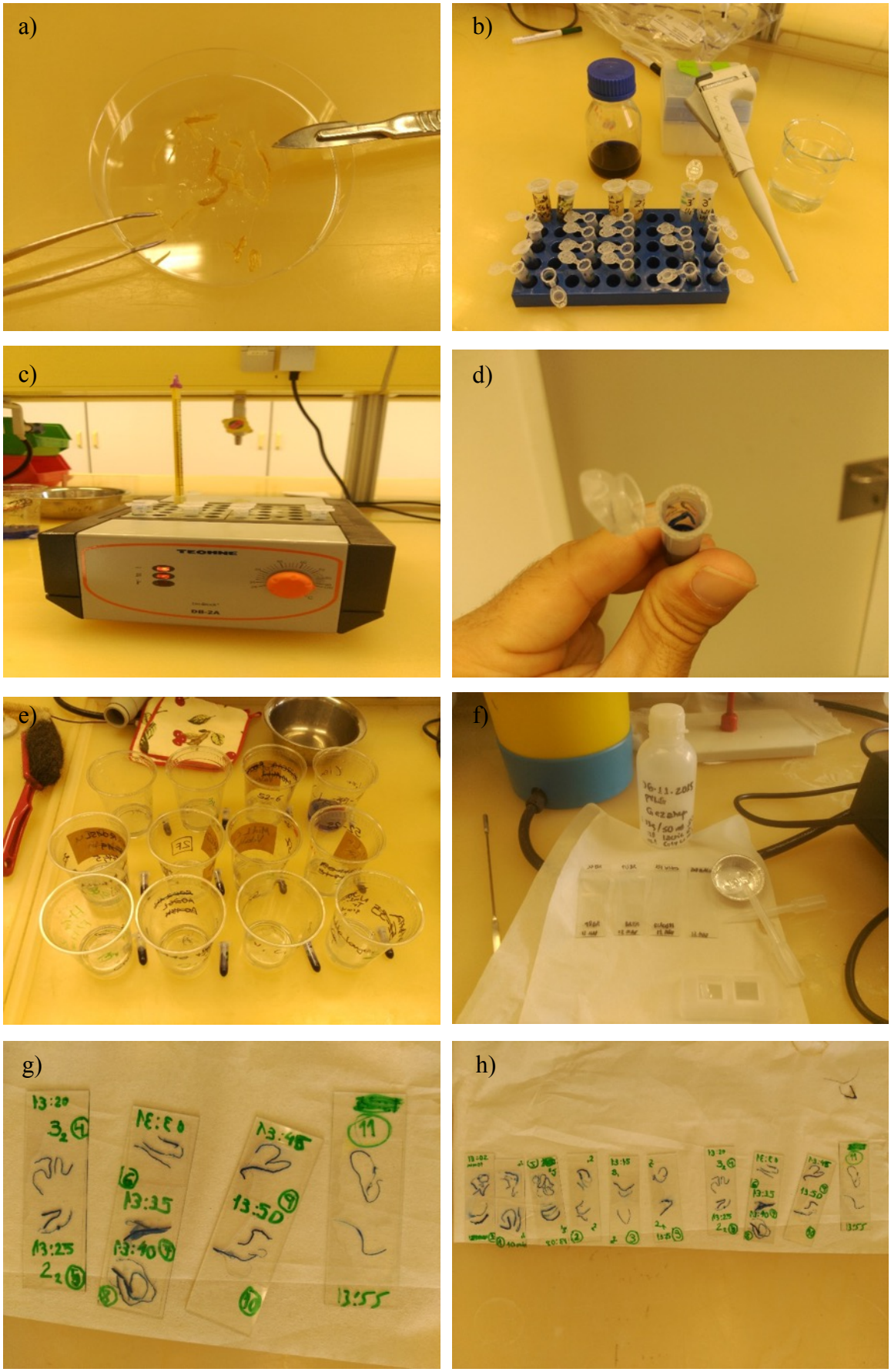
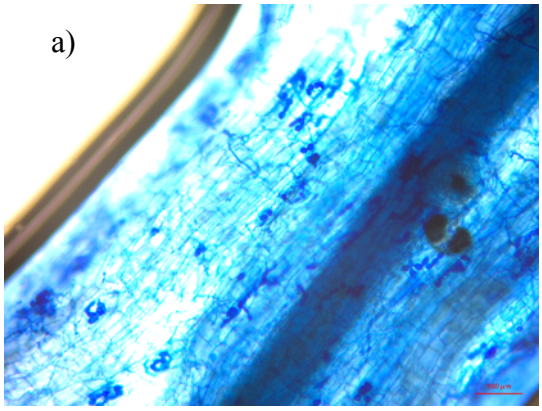


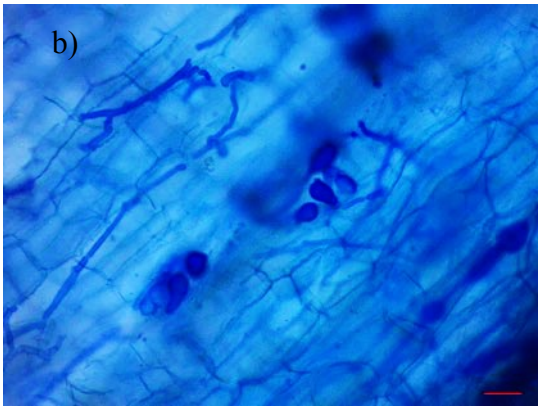
Figure 10. Staining process. Cutting and introducing in Eppendorf tubes cleared and bleached root subsamples (a and b), boiling with ink (c), soaking with acidify water (e) and mounting them in slides (f - h)

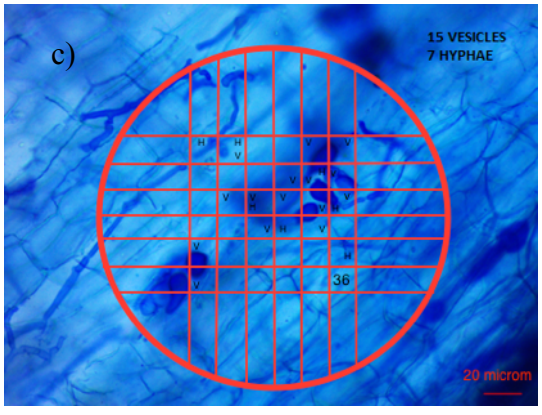
IV. Quantification

This is a simple method developed with the aim of record all the fungal structures inside the root tissues. It consists in quantify all the structures, in this case, vesicles (spherical structures) (Figures 11a, b) and hyphae (long structures) (Figures 11a, b) observing through a grid-length eyepiece which was inserted in the ocular lens. The observed surface, contained a grid of 6 x 6 squares (Figure 11c), in which hyphae and vesicle separately either complete or a part into each square were recorded like a one unitary square. For this record were used two manual counters.

Of the five stained pieces by sample, the three best conserved regarding the cortex were chosen. This choice was done with visual observations criteria, which were developed after so many repetitions. Then, in each of the 3 subsamples 10 observation points were determined and quantified, trying to have careful doing the same angle in the microscope stage control. The Figure 11d shows an example of the data-record table, where appears the sample number, the subsample number, and the number of observation and quantification of both vesicles (V) and hyphae (H) in each subsample.







d).

SAMPLE N	17					
REPLICATE	1		2		3	
OBSERV	V	H	V	H	V	H
1	34	4	18	9	0	0
2	27	7	2	17	0	0
3	27	6	0	21	0	0
4	12	6	24	8	0	0
5	19	9	14	21	0	0
6	46	11	8	22	0	0
7	26	12	5	9	0	0
8	12	21	0	0	0	0
9	36	13	12	11	0	0
10	15	18	16	19	0	0

Figure 11. Quantification method. Fungal endophyte structures in a stained subsample root piece (a, b) representation of quantifying method in grid lent (c) and data collection table (d). Magnifications of 10 and 40x respectively. Bar representing 100 µm (a) and 20 µm (b, c)

V. Obtained parameters

- **Vesicles Infection (VI):**

$$\frac{Total\ n\ of\ Vesicles\ Counted\ into\ the\ grid\ squares}{36\ total\ squares} * 100$$

- **Hyphae Infection (HI):**

$$\frac{Total\ n\ of\ Hypahe\ Counted\ into\ the\ grid\ squares}{36\ total\ squares} * 100$$

- **Vesicles Infection + Hyphae infection (VI+HI) :**

$$\left(\frac{\boldsymbol{Total\ n\ of\ Vesicles\ Counted\ into\ the\ grid\ squares}}{\boldsymbol{36\ total\ squares}} * \mathbf{100}\right) + \left(\frac{\boldsymbol{Total\ n\ of\ Hypahe\ Counted\ into\ the\ grid\ squares}}{\boldsymbol{36\ total\ squares}} * \mathbf{100}\right)$$

3. Results

It was only possible to quantify one fungi group, the Dark Septate Endophytes, although evidences of EM and structures like-AM were found. Of 72 samples, 4 were not assessed (loosed), in which 2 were from moisture and 2 from dry conditions. Of the remaining 68, in 16 were not possible to quantify the colonization due to degradation of the tissue (mostly) or by the excessive dark pigmentation (Figure 12). In this 16 samples, 8 were from moisture and 8 from dry conditions (Tables 4 and 5). The remaining 52 samples (26 of dry conditions and 26 of moisture) were assessed with the quantify method commented above (Table 4).

Table 4. Summary of total samples. Non-checked samples, presence/absence structures confirmed and complete quantification in moisture and dry conditions.

	Moisture	**Dry**	**Total**
Loosed	2	2	4
Only Presence Assessed (Destructed samples)	8	8	16
Quantify (only DSE)	26	26	52

About the relation of destructed samples and ratios, the results were: two destructed samples in moisture conditions in the ratio 1, one destructed sample in moisture conditions and two in dry conditions in ratio 2; none in ratio 4; none in moisture conditions and one in dry conditions in ratio 9; three in moisture conditions and one in dry conditions in ratio 20; two in moisture conditions and four in dry conditions in ratio 43 (Table 5).

This is a total of two destructed samples in ratio 1; three in ratio 2; zero in ratio 4; one in ratio 9; four in ratio 20 and six in ratio 43 (Table 5).

Table 5. Destructed samples in respect to N:P ratio. Absolute values

N:P Ratio	Moisture abs	Dry abs	Total abs
1	2	0	2
2	1	2	3
4	0	0	0
9	0	1	1
20	3	1	4
43	2	4	6

In the next graph, the same results but in relative values:

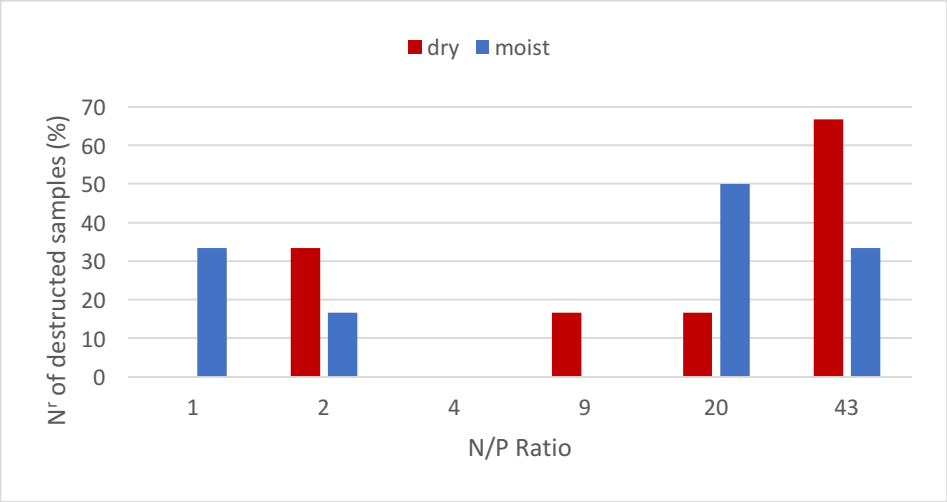


Figure 12. Destroyed samples in moisture (blue) and dry (red) conditions with respect to N/P ratios. Relative values

3.1 Method Calibration

The testing of the method always showed too dark tissues with one autoclave treatment (Fig 14a). With more of two autoclave treatments the tissues always showed high degradation (Figure 14c). Testing the bleaching step, it was observed that excessive time or concentration with H_2O_2 resulted in excessive emptied of the cell contents (Figure 13d).

The optimum method, finally was as follow:

- one autoclave the first day (121ºC and 10 min) followed of overnight
- the next day in the morning a second autoclave process previous change of KOH (121ºC and 10 min)
- soak two times with tap water and following 50 minutes bleaching with 60 ml of 10% H_2O_2
- 20 minutes heater-staining in 1ml Eppendorf's tubes
- 20 minutes more resting with slightly acidified water with a few drops of household vinegar, in this case 5ml of Acetic Acid/1000ml water miliq

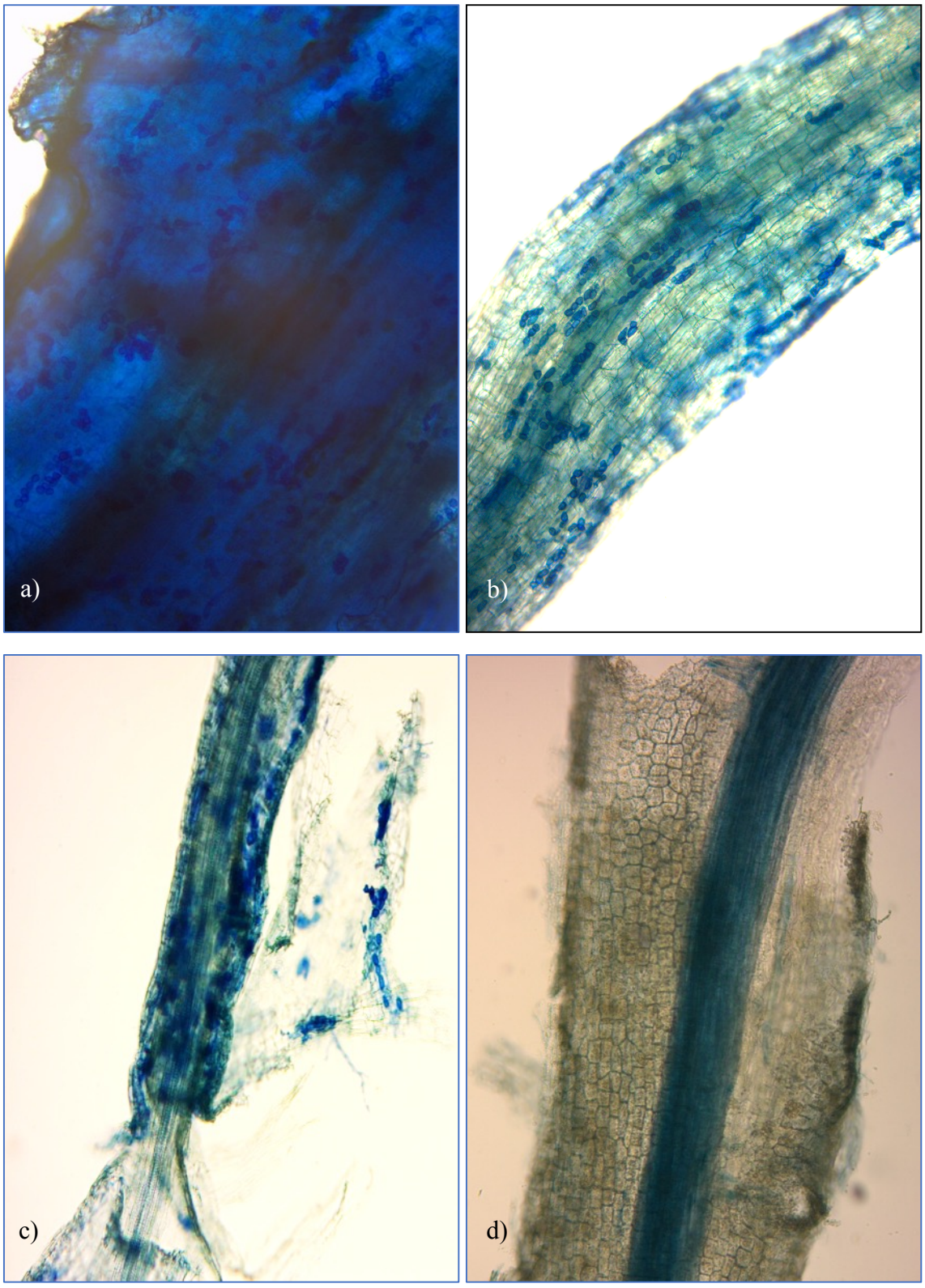


Figure 13. Results of a root piece of Quercus coccifera subsp. calliprinos submit to different autoclave treatments. 1 (a), 2 (b), 3 (c) and excessive bleaching 4 (d)

3.2 Ectomycorrhizal colonization

The samples showed a partial disintegration and detached of the epidermal tissue. The roots didn't have branched structures (Figure 15a, b, c), but it was observed a lot of scars and smalls fragments on the bottom, and in the storage tubes (Figure 15 a-d). Some were identified as characteristic ectomycorrhizal structures of the sheath (Figure 15h), thickened tips (Figure 15e-g) and fragmented branching patterns (Figure 15b, d).

Finally, due to the excessive fragmentation of the root (Figure 15a, b, c, d), a complete ectomycorrhizal analysis was reject.

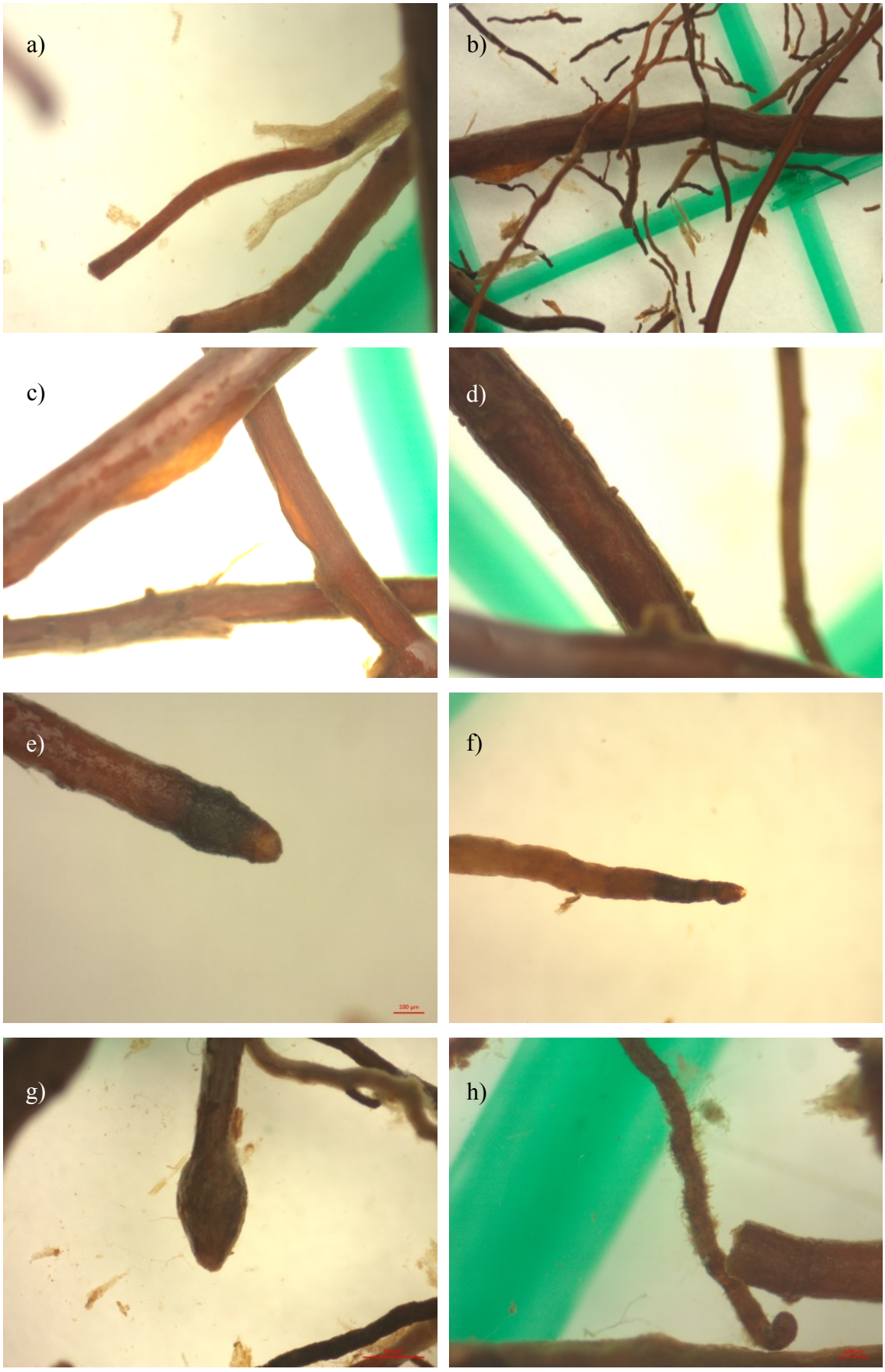


Figure 14. Evidences of Ectomicorrhizal colonization (b, e-h) in Quercus coccifera subsp. calliprinos roots. Bars: 1mm

3.3 Arbuscular Mycorrhiza colonization

Only a minimal amount of formations-like-arbuscules were observed. These structures were observed in most of cases in several steps before the staining process, when the subsamples were progressively observed (Fig 15a, b) to check the effectivity of the process. After the complete treatment, usually these presumed arbuscular structures disappeared (Figure 15c, d), what did impossible the quantification.

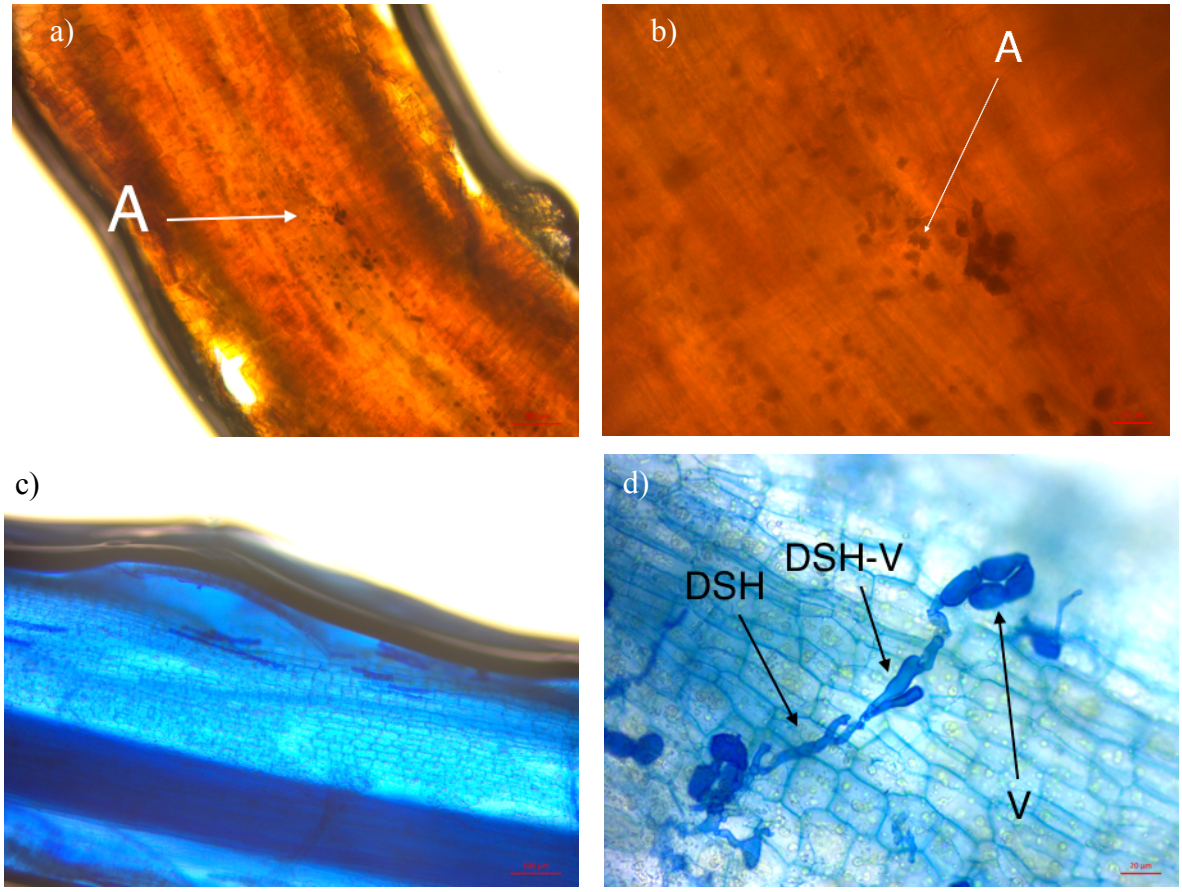


Figure 15. Arbuscular structures in root piece in several steps of the staining process. Clearing process (a,b) after staining process (c,d) (DSE appeared) A=Supposed Arbuscule; DSH=Dark Septate Hyphae; V=Vesicles

3.4 Dark Septate Endophytes colonization

This type of microorganisms was the predominant in terms of colonization. Before (Figure 16a) and after staining (Figure 16b) it showed a good observation quality (Figure 16a-h), hence, it was proceeded to do a quantification. The observed structures which confirmed this type of colonization were "Dark Septate Hyphae" (Inter-Intracellular structures) (Figure 16b, c, d), "Vesicles" (Intracellular structures) (Figure 16a, b, c, e, f), "Microsclerotia" (Intracellular structures) (Figure 16g, h), and the inter-connexions points among them (Figure 16b, c). In addition, figures that show the intermedium stem between the Dark Septate Hyphae and Vesicles were found (Figure 15d).

The Microsclerotia (Figure 16g, h), aggregations of irregularly lobed hyphae (Stoyke, G., & Currah., 1991) were the less frequent structure. Always appeared colonizing the intracellular space and containing a connection with the hyphae network (Figure 16g, h). Sometimes, structures forming Microsclerotia appeared to be at an intermediate stage, between vesicle and hyphae, containing apparently intermediate structures between the two (Figure 15d and 16g).

Dark Septate Hyphae appeared mainly colonizing the root in a longitudinal pattern (Figure 16c), both inter and intracellularly, as Jumpponen and Trappe (1998) described (Figure 16b, f). Also, the hyphae appeared projecting its structures towards the perpendicular to the root axis (Figure 16b, c). The hyphae were always septate (Figure 16b-f) and usually connecting both types of structures, Vesicles and Microsclerotia (Figure 16h). The width of the hyphae was around 6μm although no data were recorded (Figure 16b, c, d). The Vesicles, easily identifiable by the characteristic rounded form, also appeared in the intracellular spaces, and connected to the hyphae network (Figure 16b, c, e). It was observed a curious like-philamented nucleus into the vesicles (Figure 16e).

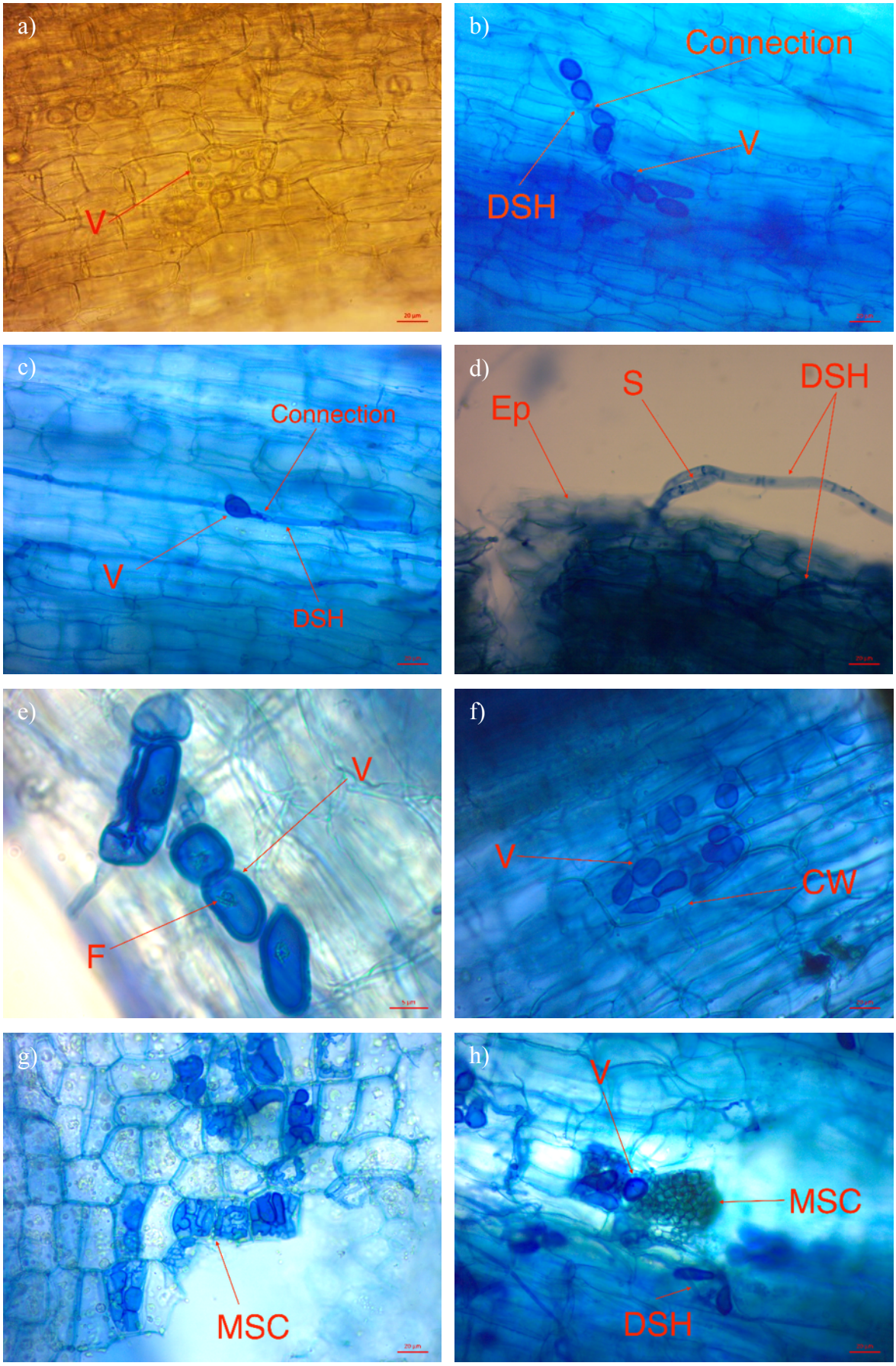


Figure 16. Pre-and post-staining (a, b) and diversity of structures found. Dark Septate Hiphae (b, c, d); Vesicles (a, b, c, e, f); Microsclerotia (g, h). V: Vesicles; DSH: Dark Septate Hiphae; Ep: Epidermis; F: Filaments; CW: Cell-Wall; S: Septa (d)

Of the 68 samples finally examined (including the 16 with only presence/not), 34 pertained to dry conditions and 34 to moisture conditions. Of dry conditions, in 24 samples were recorded endophytic structures (hyphae + vesicles) and 31 in moisture conditions. This is in relative terms, 91 % in moisture conditions, and 70% in dry conditions (Figure 12), which is a significant difference, higher in moisture conditions.

This is a total of 55 of 68 samples with presence of endophyte structures, which is 80.9 % of the total of examined samples.

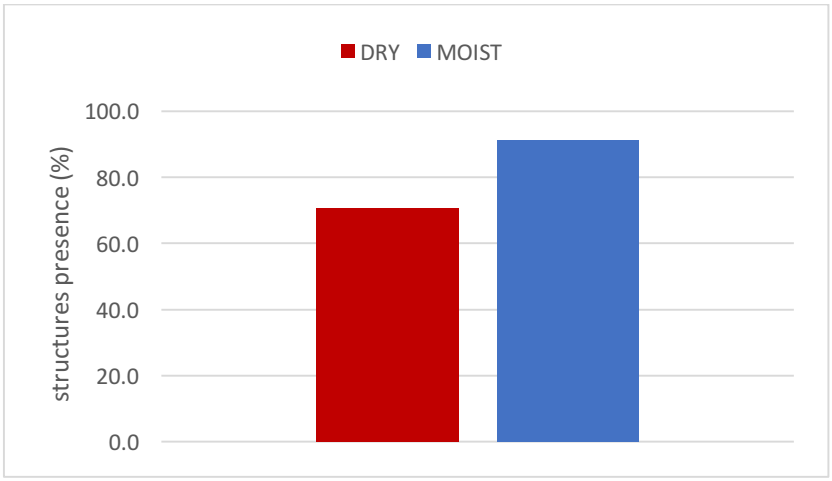


Figure 17. DSE total relative presence of root fungal structures in dry (red) and moisture (blue) conditions

We assessed the colonization regarding to six N:P ratios applied to the plants, ranging from 0.94 to 42.62, and represented by 1, 2, 4, 9, 20 and 43 (Table 6). The colonization was assessed trough the predominant two types of structures, vesicles (V) and Dark Septate hyphae (DSH). For DSH and dry conditions, the lowest value of colonization was 1.1% in the N:P ratio 1, in turn 23,1% for moisture in the ratio 2. The peaks were 37.8% in the ratio 9 in dry and 62,2% in ratio 20 in moisture conditions (Table 6).

For vesicles and dry conditions, the lowest value of colonization was 0,7 % in the N:P ratio 2, in turn 21,4% for moisture in the ratio 2. The peaks were 42.6% in the ratio 43 in dry and 40.4% in ratio 1 in moisture conditions (Table 6).

The maximum colonization peak is 101.7% (Figure 20) (About 200%) in the ratio 20 and moisture, and 4.4% (about 200) in dry and ratio 1.

Table 6. Ratios of P, N and N:P applied to the seedlings

N to P ratio	Represented
0.94	1
2.07	2
4.07	4
8.5	8
20.45	20
42.62	43

The colonization results in relative values are represented in the next Table (**7**) and the Figures 18 (DSH), 19 (V) and 20 (DSH+V).

*Table 7. Mean relative colonization of root samples in different N:P ratios and **dry (D) and moisture (M)** conditions.*

	Vesicles (%)		Hyphae (%)		V+H (%)	
Ratio	**D**	**M**	**D**	**M**	**D**	**M**
1	2.6	40.4	1.1	54.1	4.4	94.5
2	0.7	21.4	7.9	23.1	8.6	44.5
4	8.5	28.7	15.7	38.2	24.2	66.8
9	23.4	32.6	37.8	42.8	61.2	75.5
20	7.6	39.4	10.3	62.2	18.0	101.7
43	42.6	25.5	16.1	46.4	58.8	71.9

In the case of DSH colonization (Figure 18), it can be observed a non-normal distribution. In dry conditions, there is a positive correlation from the ratio 1 to 9, but after there are a big decrease, from 37 to 10 % to arrive at the last ratio (43) with 16% of colonization. In turn, in moisture conditions, there is a positive correlation among the ratio 2 to 20. The values start with 54%, and then a decrease occurs arriving to 23 in the second ratio. From 2 to 20 ratios the values increased and in the last decrease until 46.

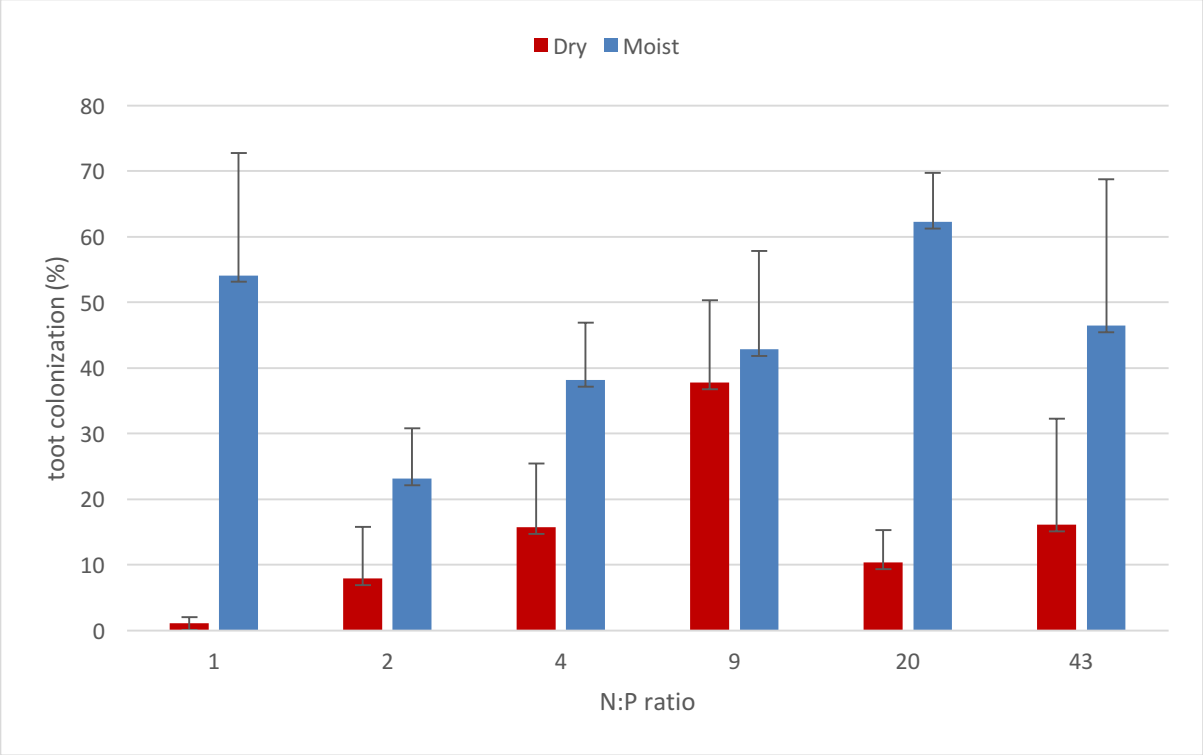


Figure 18. Relative Dark Septate Hyphae colonization regarding N:P ratios. P<0.05

In the case of V colonization (Figure 19), also it can be observed a non-normal distribution. In dry conditions, the values start with 2.6% of colonization (ratio 1). After there is a decrease, arriving to 0,7 % (the lowest value of the experiment) and until the ratio 9 there is an increase, having a value of 23.4%. Then the colonization decrease until 7,6% in the ratio 20 for finally increase another time until arrive to 42.6%.

In turn, in moisture conditions, the values start with 40.4%, and then a decrease occurs arriving to 21.4% in the ratio 2, from this until the 20 the values increased until arrive to 39.4%, but in the last there are a decrease until 25.5%.

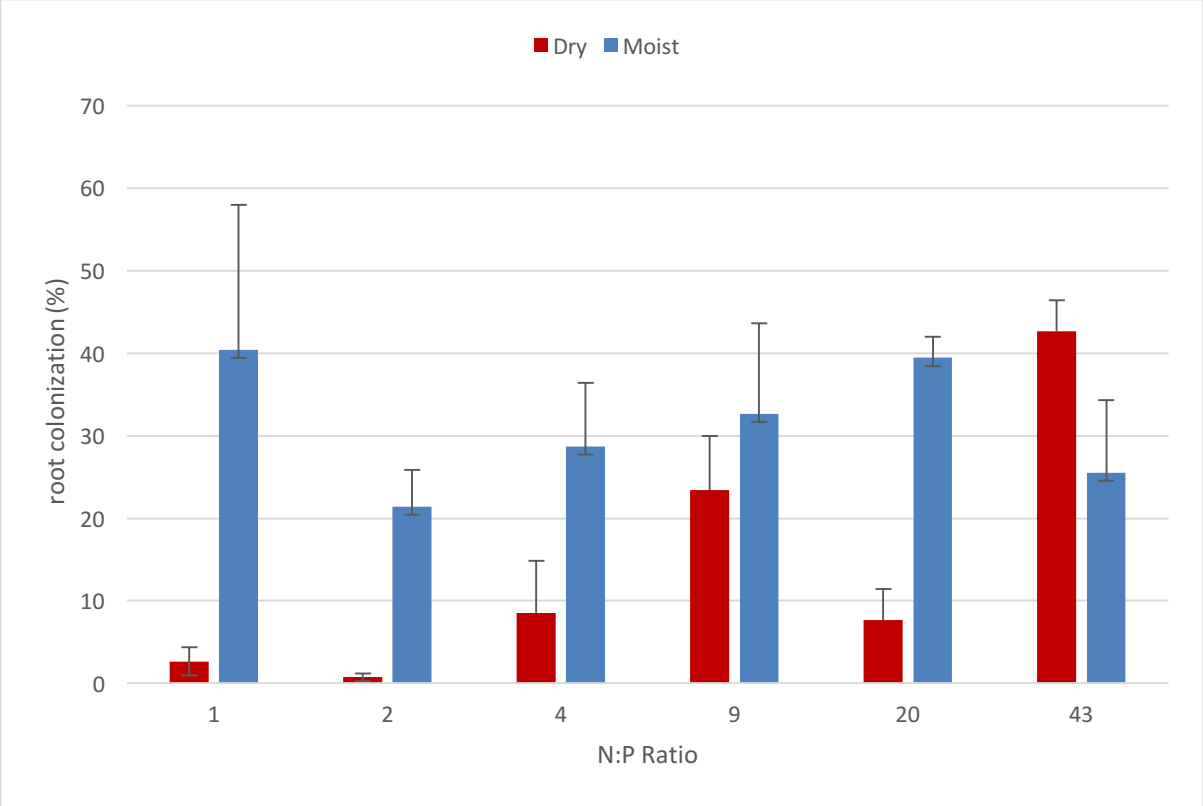


Figure 19. Relative Vesicles colonization regarding N:P ratios. P<0.05

The next graph (Figure 20) shows the result of sum of the two previous ones, but due to the similarity of both, the tendency in this one continues being very similar. The relative data are above 200%. For the dry conditions the colonization increase from the first ratio with 4,4% of colonization until the ratio 9 with 61,2%, and then decrease in the ratio 20 to finally increase in the ratio 43, until 58,7% of colonization.

For the moisture conditions the values starts with 94%, and then a decrease occurs arriving to 44% in the second ratio. From 2 to 20 ratios the values increased, but in the last decrease until 72%.

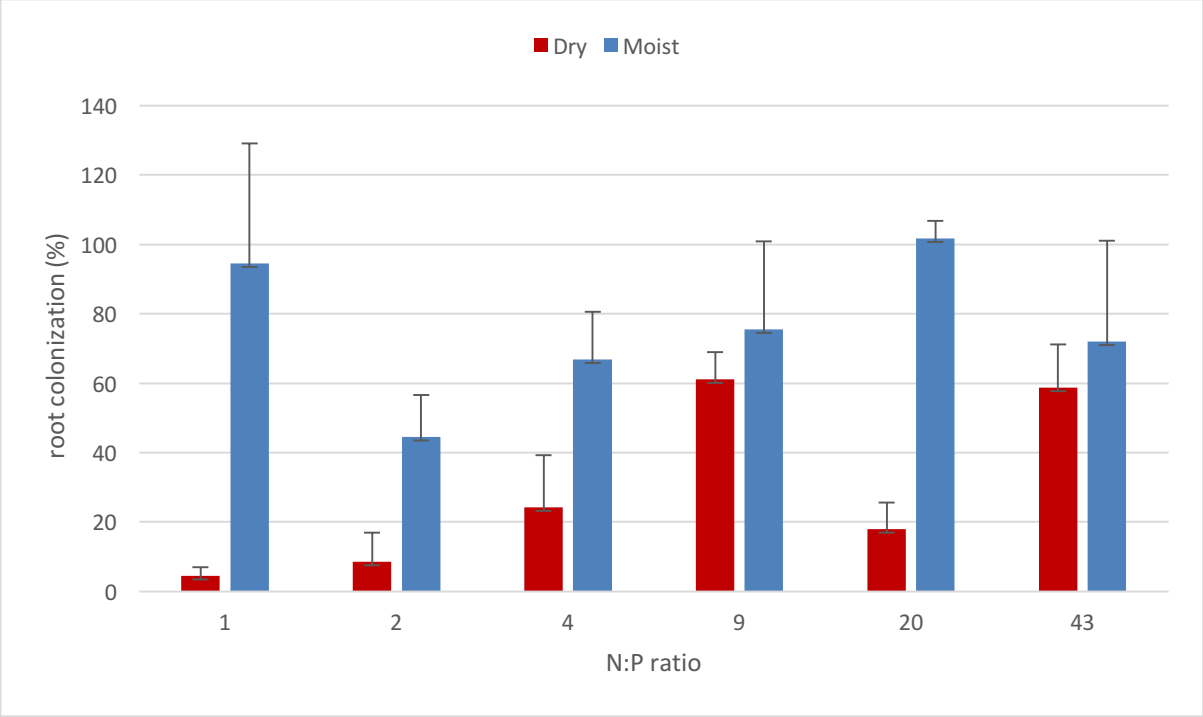


Figure 20. Relative Vesicles plus Hyphae colonization regarding N :P ratios. P<0.05

4. Discussion

First, its necessary to point out that several samples were destructed in the process. It was predicted that this would happen when the samples were examined under microscope for the first time. It was evidenced by the presence of gaps and partially break of the root epidermis in most of the samples. After a carefully check, we detected that a mistake had been realized in the greenhouse experiment of Israel. This error, it was done after to harvest the seedlings and prepare the samples, when they were storage in 7 % of glycerine, instead of 50% how several authors recommend (Brundrett et al., 1996; Vierheilig, 2005). This early mistake derived in a completely freezing of the samples once in the freeze, what caused the partially disintegration of the external tissues of the roots (Figure 14 a, b, c, d). Consequently, an increase of the roots sensitivity during the staining process occurred. For this reason, the results of this thesis are reduced and should be token carefully.

It is interesting to note that, the peak of destructed samples was in in the higher N:P ratio (43) in dry conditions, and the second higher N:P ratio (20) for the moisture conditions (Figure 12).

- Could be related with the large amount of nitrogen?

Some studies such as that of Li et al., (2014) observed a decrease of tissue thickness in some species caused by high N concentrations or the high sensitivity in salinity stress environments (in this case could be represented by dry conditions), which might increase the root sensitivity to some N compounds (Bernstein and Kafkafi, 2002). It's impossible to know the reason with only our data, but this evidences could be consistent since the maximum of destructed samples here, occurred in dry conditions and the higher N:P ratio (Figure 12).

4.1 Method calibration

The develop of the method was based on to prevent the tissue and fungal structures destruction by one hand and to have enough potential to remove the strong pigmentation on the other. The highest difficulty was de variety of responses in one equal treatment, which likely was caused by the previous bad state of the samples, and the sclerophyllous nature of the plant (Vierheilig et al., 1998; 2005).

It was observed that the quality of the method was substantially improved when after the first autoclave process the samples were resting in KOH overnight. Then the second day on the morning, was done the second autoclave process.

Another interesting observation was an improve of the quality of the observation after the storage of some samples in 50% glycerine, 50% Miliq water, after the clearing and bleaching process. This improvement was discovered by chance when by lack of time the samples where storage to continue with the process the next day. After, this advice was found in the literature (Vierheilig, 2005).

4.2 Ectomycorrhizal colonization

Due to the dark colour and scarcity of branched structures, we hypothesized with the genus *Tomentella* (Jakuks et al., 2005), pertaining to *Thelephoreaceae* family and *Basidiomycota* division. This genus is present in Eurasia and specifically is also abundant in Mediterranean environments, having been recorded in several *Quercus* species, like *Q. cerris* (Jakuks et al., 2005) and *Q. ilex* (Clavería and de Miguel., 2005). This last specie is very close to the studied stpecie, *Quercus coccifera subsp. calliprinos*, occurring together in many ecosystems.

To find quality results will be necessary to do more accuracy morphotyping analysis with a previous correct storage protocol, and use other methods such us DNA sequencing, phylotyping (molecular characterization) or culture methods, which give more precise results, often identifying genus and specie levels (Suz et al., 2008).

4.3 Arbuscular Mycorrhiza colonization

The presumed arbuscular structures were very few and without a hyphae network connecting them (Figure 21a, c) which are one of the defining characteristics to corroborate the presence of this fungal microorganisms. This fact is likely also related with the previous degradation state of root tissues.

Due to the lack of this hyphae network connecting the arbuscules, and so scarce presence, the quantification of arbuscular mycorrhiza was reject.

These structures were observed in most of cases in several steps before the staining process, when the subsamples were progressively checked (Figure 15 a-d) to observe the effectiveness of the process. This found probably indicate that these structures don't resisted the aggressiveness of the treatment, by other hand, Dark Septate Fungi were found in a good state of conservation how it will be discussed in the next point.

If this presence of structure-like-arbuscles would be confirmed like a AM, this result would be consistent with several reports which evidenced a trend of co-occurrence between AM and DSE in terrestrial plants (Ruotsalainen *et al*. 2002; Trowbridge & Jumpponen (2004); Fuchs & Haselwandter 2004; Beauchamp *et al*. 2005; Cázares *et al*. 2005; Lingfei *et al*. 2005; Pietikainen *et al*. 2005; Muthukumar *et al*. 2006; Menoyo *et al*. 2007; Postma *et al*. 2007;). Wagg et al. 2008 confirmed that when there is coexistence, the fungi tend to occupy different niches within the same root, and Trowbridge & Jumpponen (2004) confirmed that there is not antagonistic relationship between them. When appeared, arbuscules always occurred in the inner part of the cortex surrounding the endodermic tissue (Figure 21a, c), while the DSE appeared mainly in the outer part of the cortex near the epidermis (Figure 21b, d) and within it. Therefore, both microorganisms trended to occupy different places. According to the commented authors, this co-colonization could have occurred here.

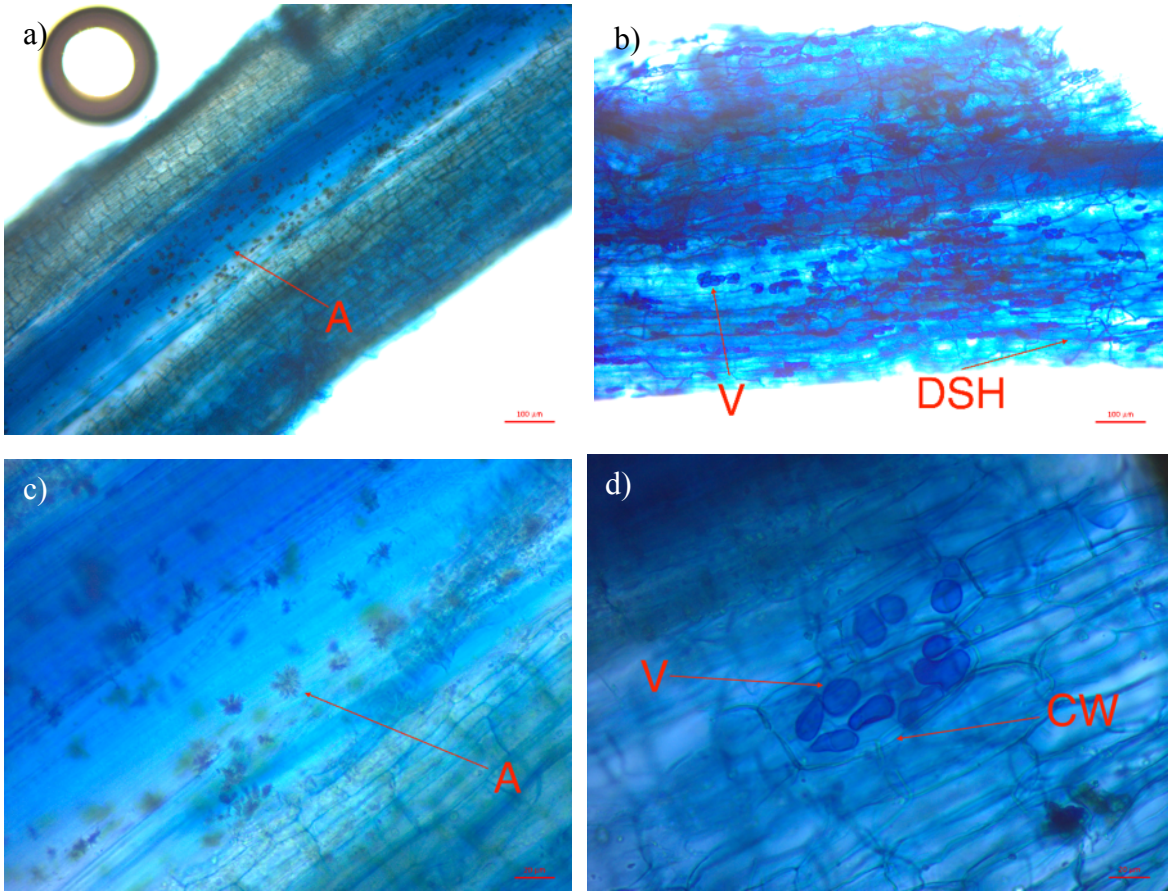


Figure 21. Comparison among places occupied by AM structures (a, c) and DSE structures (b, d). V= Vesicles; CW=Cell Wall; AÇ=Suposedly arbuscules; DSH=Dark Septate Hypahe. Bars a, b= 100µm and c, d= 20µm

According to Heper (1983) the application of high amounts of nitrate would increase the AM colonization, which in his case was of *Glomus mosseae*. He also found that, when high concentrations of phosphorous were found in roots, the colonization was inhibited, in turn, when there was low concentration the colonization appeared to be related with the N content of the host tissue. When low N treatments, poor colonization, with very few arbuscules and intracellular hyphae.

Jhonson et al. (2003) found that, in grasslands species, mycorrhizal response to N enrichment is mediated by ambient soil fertility and that N enrichment decreases allocation to AM structures at sites with ample soil P, but it increases allocation to mycorrhizae at sites with P-deficient soils.

Requena et al (1996) studied some indigenous woody plants in a desertified semiarid ecosystem in the southeast of Spain, were most of the plants examined were recorded heavily AM indicating a trend of this mycorrhizae type with stressed, degraded and dry (230 mm of

annual mean precipitation) environments. Also, they observed a seasonality variation in the colonization rate, which was higher in the end of summer than in winter.

The results of Sylvia and Neal (1990) showed the importance of N and P nutrients in colonization, specifically, when plants were deficient in N, colonization don't was supressed by increase of P, but when N was sufficient, P increase supressed the colonization.

Following these trends, in case of being able to quantify this structures-like-AM, we should be obtained more AM colonization with high N:P ratios and dry conditions.

Jumponen et al. (1998) studied *Pinus contorta* grown on glacier forefront soil inoculated with the dark-septate fungus *Phialocephala fortinii,* and they found a functional intracellular structure possibly analogous to arbuscules or the hyphal coils of ericoid mycorrhizae. Since the plants with the inoculation of this DSE improved their P absorption, they suggested that this structures analogue to arbuscules formed by *P. fortinii* could be doing the exchange of this nutrients. In this study, they don't have photos of this structures, but maybe our apparent arbuscules (Figure 22c) could be also structures formed by our DSE. It is proposed for another study, to determine the connexion of this structures previous non-destructive staining process.

4.4 Dark Septate Endophytes colonization

This study confirms that *Quercus coccifera* subsp. *calliprinos* host Dark Septate Endophytes, thus providing useful and new information and being congruent with the currently known global and ubiquity occurrence of this group of fungal microorganisms in all ecosystems (Jumpponen and Trappe, 1998; Mandyam and Jumpponen, 2005; Sieber and Grünig, 2006). These fungal microorganisms appeared in most of the samples, even when the plants were exposed to a wide range of treatments with several N:P ratios, and dry/moisture conditions. At the same time, this found confirms a strong adaptation to several environmental conditions by DSE into a stressing ecosystem, the Mediterranean. In addition, their structures survived when the others AM and EM were highly degraded, which shows a great capacity of survival by this group of fungal microorganisms. The last was an unexpected found.

Regarding the destruction of the mycorrhizal structures:

- why this group was more resistant than the others to the storage conditions and the subsequent aggressive staining process?

Probably the response to this question is closely related with the presence of melanin, a pigment that this group of fungi contains, mainly in their hyphae and microsclerotia (Jumpponen and Trappe, 1998). Melanin is present and is synthetized in all biological kingdoms (Nosanchuk and Casadevall, 2003) and is thought to play a significant role in fungal cell wall mechanical strength (Nosanchuk et al., 2015). Also confer it scavenging of free radical, thermo-tolerance, metal ion sequestration, cell development and protection to radiation damage (Cordero and Casadevall, 2017). Melanin is frequently present in organisms which live in adverse environments (Bell and Wheeler, 1986; Jumpponen et al., 1998; Newsham et al. 2009). They protect DSE from both drought and extreme temperatures allowing them to have a global distribution how suggest several works such as Cázares (1992) and Jumponen et al. (1998) when isolated several DSE from an alpine glacier foreland in USA.

We speculate that dark colour in DSE can mean high concentrations of melanin's in their tissues, easily observable in the Figures 21 and 22, and that soft colour in the presumed

arbuscules (Figure 21a, c) can be due to the lack of melanin. High concentrations in DSE could be the reason by DSE were better preserved.

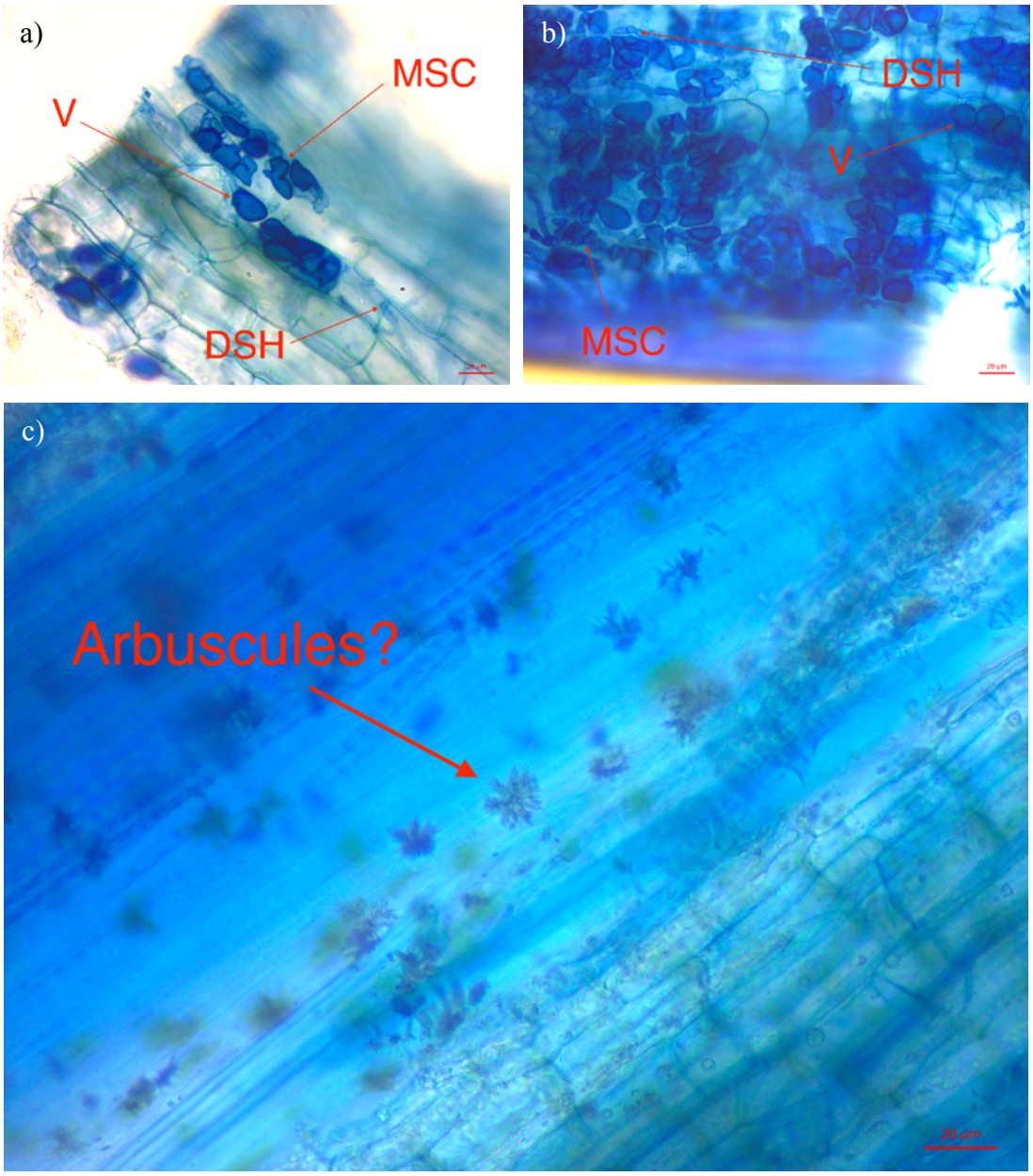


Figure 22. Different colours can be due to differences in melanin concentration among DSE (a, b) and (apparent) arbuscules (c)

In our experiment, plants had only 3 years when were harvested in February/March which is a non-stress epoch in terms of temperature and water irrigation (Table 1). Roots showed in decreasing order, hyphae, vesicles, and microsclerotia (without quantification by lower quantities), which can be consistent with evidences found by Marins and Carrenho (2007),

when affirm that "*hyaline hyphae are common during periods of intense plant metabolism, while dark hyphae and microsclerotia appear mainly during later stages of colonization or in periods of stress*".

- Regarding the colonization in dry and moisture conditions:

We found a 70.6% of samples colonized (with presence) in dry conditions, and 91.2% in moisture conditions. This result does not support our hypothesis of "*We expect more fungal colonization in dry conditions*" which was based on the evidences of most studies consulted, where is usually demonstrate higher presence and colonization of DSE when more stressed, extreme and dry is the environment (Jumpponen and Trappe 1998; Kovács and Szigetvári 2002; Newsham et al. 2009; Knapp et al. 2012, 2015; Muniania, 2016). Although some works have found the opposite effect, such as Herrera et al (2011), assessed some endophytes groups (DSE included) in a semiarid grassland, where they recorded more endophytes in moisture conditions. They suggested that it could be due to a common but complicated strategy of water translocation, similar to that proposed and shown in the arbuscular mycorrhizae.
Similarly, Lingfei et al (2005) also found a positive correlation between humidity, sunlight hours and DSE colonization in a grassland site of southwest China, and a significant seasonality variations trough time in one year which was different in different host plants.

It's necessary to do more studies, to understand these environmental changing hydric preferences by DSE. For next studies, here its proposed to take samples in a seasonal periodicity, because in different epoch and rains, or in chamber experimentation, the parameters can be related much more easily, than only samples token in one specific point in time.

- Regarding the relation among N:P ratios and colonization:

In the ratios 20 and 43 there was a high samples destruction (4/12 and 6/12) (Table 5) during the staining process, what have the consequence of less observation points recorded. This means that the result is less close to the reality (lower quality) than the others.

In the central ratios (2-9) (Figure 20) there is a progressive increase of the colonization, that is when more N:P ratio more colonization, but in the extremes 1, 43 and 20 there are different trends, very hard to explain. This central trends are consistent with studies in that Mycorrhizae are positive correlated with N:P ratios such as Hepper (1983). They also found that high levels of phosphorous significantly inhibited the colonization, but if they increased also N levels at the same time, this inhibition was not produced. In addition, it was affirmed that N:P ratio affect directly at the concentration of both elements in root tissue, and this factor is the most important governing the mycorrhizal infection.

The peaks of high levels for DSE colonization are the ratio 9 in dry and 20 in moisture conditions. The lowers are 1 for dry and 2 for moisture. This indicate that DSE develops in large extent in high proportions of N regarding P. We could discuss this for example if we would have data of P absorbed /biomass increased/decreased, focussing in this higher or lower peaks. For example, if we observe significant data in the decrease of biomass coinciding with the ratios 9 dry and moisture 20 we could affirm that the DSE might be behaving as a pathogenic, since the point of view of resources consumption (nutrients), maybe into the mutualism-parasitism continuum proposed by Johnson (1997). The same could be used at inverse.

The vesicles and hyphae showed the same variation of colonization regarding the N:P ratios, between dry-dry (Figure 18 and 19) and moisture-moisture (Figure 18 and 19). Only variate in dry condition the ratios 1 and 2 which are increasing in the Hyphae and decreasing in Vesicles.

In mycorrhizas, there are evidences that plant nutritional state influentiate the exudates of the roots, mainly aminoacids and sugars, and exudates in turn are suggested to influentiate the colonization. For example, Ratnayake, Leonard and Menge (1978) found less sugar and amino acids in exudate from phosphorus- deficient plants and they suggested that this induced higher level of infection in the roots, while Bowen (1969) found that amounts of exudate from pine roots was much less under nitrogen-deficient conditions and that its amino acid composition was also altered. Here is proposed that this mechanism could be also related with the DSE and this is an interesting experiment to add to this.

About biomass and DSE in a review of Newsham (2011) after inoculating DSE in plants, he found only positive effects, regarding shoot and root biomass, shoot nitrogen (N) and phosphorus contents, with increases of 26–103% for plants inoculated, relative to uninoculated controls. Inoculation increased total, shoot and root biomass by 52–138% when plants had not been supplied with additional inorganic N, or when all or the majority was supplied in organic form. Inoculation with the DSE *Phialocephala fortinii* was found increasing shoot and root biomass, shoot P concentration and shoot N content by 44–116%, relative to inoculated controls. The analyses here suggest that DSE enhance plant performance under controlled conditions, particularly when all, or the majority, of N is available in organic form.

Li et al., (2012) studying a grass-endophyte association, found that benefits depended strongly of N and P availability. When N or P was limited, infected plants accumulate significantly more aboveground biomass and total biomass than non-inoculated plants. When both were limited, the benefits decreased, according also with Ravel et al 97 and Malinowski et al 98. When they supplied N and P at the same time, they didn't find advantages in infected plants respect non-infected, and they suggest that could be by a scarcity of nutrients in the medium.

Also Haselwandter and Read (1982) and Ruotsalainen et al. (2002) suggest that DSE are more abundant in habitats poor in P.

Results obtained by Jumpponen and Trappe (1998) led Jumpponen (1999) to the conclusion that growth enhancement by the DSE may be due to improved phosphorous and nitrogen uptake (Jumpponen et al. 1998).

Haselwandter, (1987) affirms that there is some indication that DSE fungi aid alpine plants in uptake of phosphorus and nitrogen (Mullen et al., 1998).

Another study found about DSE and N:P relations is the Getachew (2016) master thesis. He investigated colonization in teff plants Ethiopia recording hyphae colonization, and he found levels around 5% of colonization in ratios of 2 N:P (40 kg/ha of N and 20 kg of P), which is near our results in hyphae colonization (7,9%), but only in dry conditions. He also found no significant differences between control and this fertilise treatment and found a positive correlation among AM and DSE.

To get faithful results, we should across our colonization data with the results of several physiological parameters obtained in the "mother" (previous) experiment which was conducted in Göttingen (Dirks et al., non-published). Thus, we could give sense to our experiment and try to find a possible relation in the behaviour and role of DSE through parameters of plant physiology.

Due the problems with the 20 and 43 N:P ratios commented above, we speculate that we could have obtained higher colonization than we have.

Mandyam Jumpponen 2005 studied in pure culture, a fungus which showed to be able to hydrolyse P sources unavailable to the plant, like rock and tricalcium phosphates. It also improved seedling nutrition by supplying P from these sources. The improved P nutrition had as a consequence a typical mycorrhizal response, increased shoot and root biomass. The plants colonised by *A. ustus*, with their access to immobile P sources, were more efficient in P use than were uninoculated plants.

Assuming this increase, for example in 10% more, maybe it could be possible to relate the photosynthetic P use efficiency with the colonization, since it would have apparently, a positive correlation in both cases.

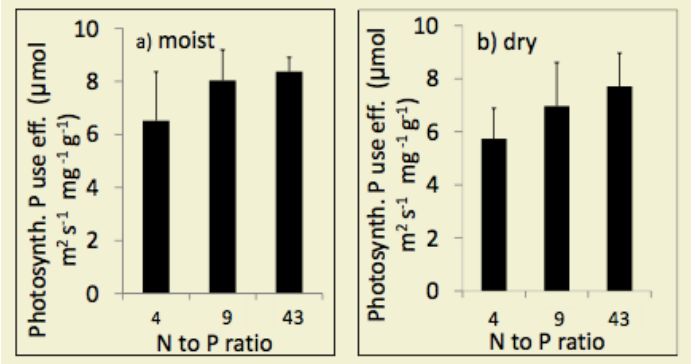


Figure 23. Photosynthetic P use efficiency of Q. coccifera subsp. calliprinos regarding N:P ratio. Dirks et al. (Non published)

Regarding biomass P use efficiency, we have similar trend in moisture conditions and colonization and if we increase another time the ratio 43, we could have an inverse proportional relation with the dry conditions.

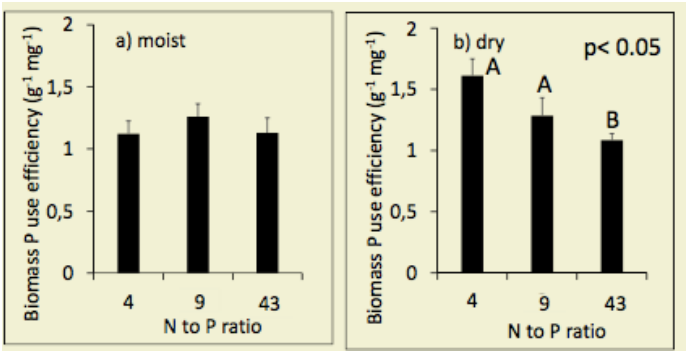


Figure 24. Biomass P use efficiency of Q. coccifera subsp. calliprinos regarding N:P ratios. Dirks et al. (Non published)

Maintaining the last two ratios increased in a 10% more (colonization) could have an inverse proportional relation among biomass and colonization. Also in the central ratios 2 to 20 in dry conditions, having the peak of colonization in the ratio 9 and being 8 the peak in in biomass.

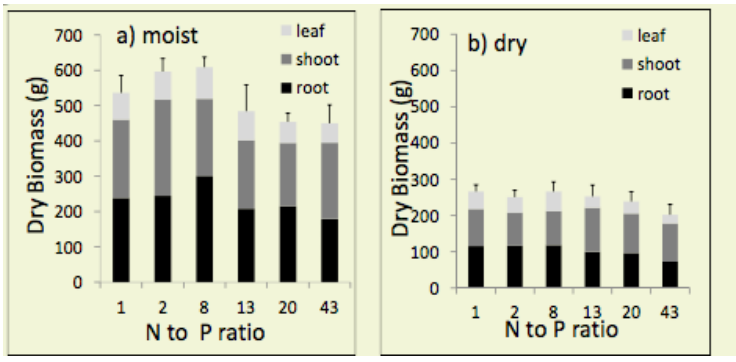


Figure 25. Biomass of Q. coccifera subsp. calliprinos regarding N:P ratios. Dirks et al. (Non published)

Also, there are similar results regarding moisture conditions between root to shoot ratio and colonization, only different in ratios 4 in root-shoot ratio and 9 in colonization which are increasing regarding the ratio before and decreasing in colonization. No similarities to dry conditions.

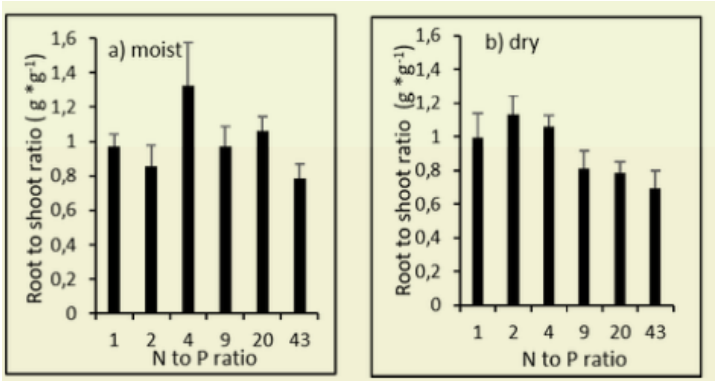


Figure 26. Root to Shoot ratio of Q. coccifera subsp. calliprinos regarding N:P ratios.Dirks et al. (Non published)

In a thesis of Lukešová (2013) she affirms that found not transport of N from the DSE to the plant and the endophyte acted as a mild parasite under experimental conditions but on the other hand it was able to enhance P uptake. In this case, there are non-apparent similarities with the $H^{33}PO_4$ uptake and our data.

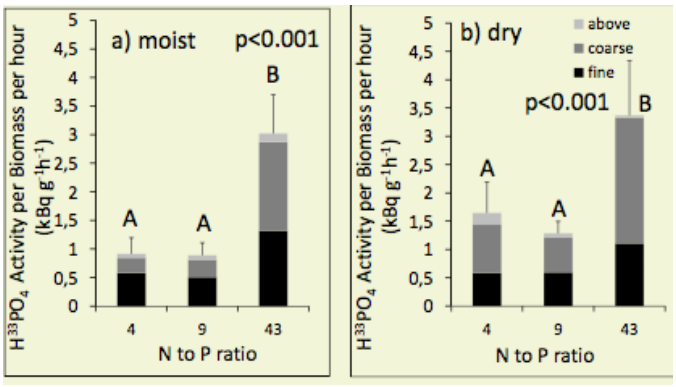


Figure27. $H^{33}PO_4$ uptake efficiency of Q. coccifera subsp. calliprinos regarding N:P ratios. Dirks et al. (Non published)

There is a negative correlation among colonization and $H^{33}PO_4$ transport efficiency (assuming an increase of colonization in the last ratio). It could mean that DSE could be negatively influencing this transport, because we have high colonization in the 43 N:P ratio, and this transport efficiency have the minimum values in the same ratio. It means that DSE could be behaving like pathogenic among the continuum in high ratios, that is in high proportions of N in comparison with P.

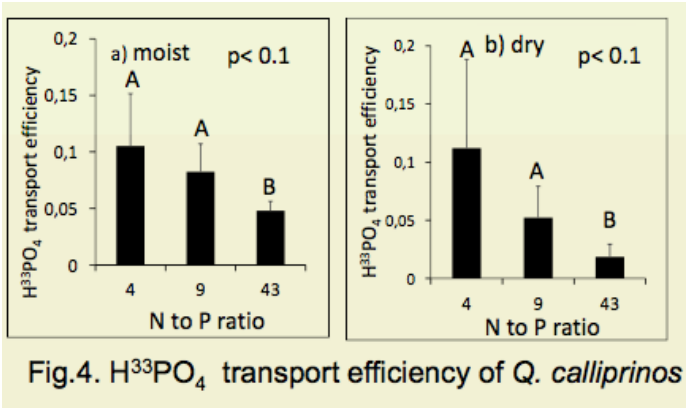


Figure 28. $H^{33}PO_4$ transport efficiency of Q. coccifera subsp. calliprinos regarding N:P ratios. Dirks et al. (Non published)

We assumed the scenario that we could have obtained more colonization in the last N:P ratio (43) to adjust some of this results obtained in the bigger experiment. Also would be possible a decrease in the last ratios, by saturation N levels. To know these results, its necessary to do this experiment in well conditions, with quality samples and with enough number of repetitions.

5. Conclusion

This study confirms that *Quercus coccifera* subsp. *calliprinos* potentially host Dark Septate Endophytes, EM and presumed AM. Thus here is provided useful and new information and congruent with the currently known global and ubiquity occurrence of this groups of fungal microorganisms in all ecosystems. On the other hand, these fungal microorganisms appeared in most of the samples, even when the plants were exposed to a wide range of treatments with several N:P ratios, and dry/moisture conditions. At the same time, our findings confirms a strong adaptation to several environmental conditions by DSE into a stressing ecosystem, the Mediterranean. In addition, their structures survived when the others like presumed AM and EM were highly degraded, which shows a great capacity of survival by this group of fungal microorganisms. The last was an unexpected found.

Also, we can affirm that the increased N:P ratio affects directly proportional to the DSE colonization between N:P ratios of 2 and 9 and we speculate that this relation could be also occur in ratios of 20 and 43.

It means that in future conditions of climate change, N deposition and P scarcity in terrestrial ecosystems we can have more colonization of this microorganisms in this species? It can be possible, but there are too much variables and not enough data, we can only to speculate with this opened question.
Visual analyses methods are still necessary and useful, but is also necessary join them to new genetic molecular methods to have each time more quality and real information regarding taxonomic classification and ecology of this groups of microorganisms.

While a lot of studies exist about EM and AM, many aspects of DSE functions, role and ecology remains practically unknown. The relations among this groups and the ecosystems will be key to understand the plant-climate responses and the elements cycles in the currently context.

6. Bibliographic References

Addy, H. D., Piercey, M. M., & Currah, R. S. (2005). Microfungal endophytes in roots. *Canadian Journal of Botany*, *83*(1), 1-13.

Agerer, R. 1987–2002. Colour atlas of ectomycorrhizae. *Einhorn-Verlag, Schwäbisch Gmünd, Germany*.

Agerer, R., and Rambold, G. (2004). DEEMY—an information system for characterization and determination of ectomycorrhizae. *München, Germany*.

Ashley, K., Cordell, D., & Mavinic, D. (2011). A brief history of phosphorus: from the philosopher's stone to nutrient recovery and reuse. *Chemosphere*, *84*(6), 737-746.

Beauchamp, V. B., Stromberg, J. C., & Stutz, J. C. (2005). Interactions between Tamarix ramosissima (saltcedar), Populus fremontii (cottonwood), and mycorrhizal fungi: effects on seedling growth and plant species coexistence. *Plant and Soil*, *275*(1), 221-231.

Bell, A. A., & Wheeler, M. H. (1986). Biosynthesis and functions of fungal melanins. *Annual review of phytopathology*, *24*(1), 411-451.

Bent, E., & Chanway, C. P. (1998). The growth-promoting effects of a bacterial endophyte on lodgepole pine are partially inhibited by the presence of other rhizobacteria. *Canadian Journal of Microbiology*, *44*(10), 980-988.

Bernstein, N., & Kafkafi, U. (2002). Root growth under salinity stress. *Plant roots: The hidden half*, *3*, 787-805.

Bevege, D. I. (1968). A rapid technique for clearing tannins and staining intact roots for detection of mycorrhizas caused by Endogone spp., and some records of infection in Australasian plants. *Transactions of the British Mycological Society*, *51*(5), 808IN16-810.

Bignell, D. E., & Eggleton, P. (2000). Termites in ecosystems. In *Termites: evolution, sociality, symbioses, ecology* (pp. 363-387). Springer Netherlands.

Bothe, H., Turnau, K., & Regvar, M. (2010). The potential role of arbuscular mycorrhizal fungi in protecting endangered plants and habitats. *Mycorrhiza*, *20*(7), 445-457.

Bowen, G. D. (1969). Nutrient status effects on loss of amides and amino acids from pine roots. *Plant and Soil*, *30*(1), 139-142.

Brundrett, M. C., Piche, Y., & Peterson, R. L. (1984). A new method for observing the morphology of vesicular–arbuscular mycorrhizae. *Canadian Journal of Botany*, *62*(10), 2128-2134.

Brundrett, M., Bougher, N., Dell, B., & Grove, T. (1996). Working With Mycorrhizas in Forestry and Agriculture.

Card, S. D., Faville, M. J., Simpson, W. R., Johnson, R. D., Voisey, C. R., de Bonth, A. C., & Hume, D. E. (2014). Mutualistic fungal endophytes in the Triticeae–survey and description. *FEMS microbiology ecology*, *88*(1), 94-106.

Catalogue of Life (2017). Species 2000 & ITIS Catalogue of Life, 30th June 2017. Digital resource at http://www.catalogueoflife.org/col/details/species/id/24df09191a9df5316bd5b4b3917f57b3/synonym/0d45d9e6bb4f7ef21b23fb973a7e2224 Species 2000: Naturalis, Leiden, the Netherlands. ISSN 2405-8858.

Cazares, E. (1992). *Mycorrhizal fungi and their relationship to plant succession in subalpine habitats* (Doctoral dissertation).

Cázares, E., Trappe, J. M., & Jumpponen, A. (2005). Mycorrhiza-plant colonization patterns on a subalpine glacier forefront as a model system of primary succession. *Mycorrhiza*, *15*(6), 405-416.

Clavería, V., & De Miguel, A. M. (2005). Análisis de la comunidad ectomicorrícica de un carrascal de Navarra (España). *Bulletin de la Société d'histoire naturelle de Toulouse*, *141*, 97-101.

Collado, J., Platas, G., González, I., & Peláez, F. (1999). Geographical and seasonal influences on the distribution of fungal endophytes in Quercus ilex. *The New Phytologist*, *144*(3), 525-532.

Cordell, D., Drangert, J. O., & White, S. (2009). The story of phosphorus: global food security and food for thought. *Global environmental change*, *19*(2), 292-305.

Cordero, R. J., & Casadevall, A. (2017). Functions of fungal melanin beyond virulence. *Fungal Biology Reviews*.

De Bary, A. (1879). *Die erscheinung der symbiose*. Verlag von Karl J. Trübner.

Dimijian, G. G. (2000). Evolving together: the biology of symbiosis, part 1. *Proceedings (Baylor University. Medical Center)*, *13*(3), 217.

Dirks et al. (Non published) Phosphorus uptake and Phosphorus use efficiency of Palestine oak (*Quercus calliprinos*) under future drier climate: a three-factorial climate chamber experiment

Douglas, A. E. (2011). Lessons from studying insect symbioses. *Cell host & microbe*, *10*(4), 359-367.

Flowers in Israel (2005) Martha Modzelevich
http://www.flowersinisrael.com/Quercuscalliprinos_page.htm (June 2017)

Frank, B. (1885). Ueber die auf Wurzelsymbiose beruhende Ernahrung gewisser Baume durch unterirdische Pilze. *Ber. dt. Bot. Ges.(3)*, 128-45.

Fuchs, B., & Haselwandter, K. (2004). Red list plants: colonization by arbuscular mycorrhizal fungi and dark septate endophytes. *Mycorrhiza*, *14*(4), 277-281.

Getachew (2016): The Interaction between Fertilization and Arbuscular Mycorrhizal Inoculation on Growth of Eragrostis tef in Amhara Region, Ethiopia. *Master Thesis BOKU*

Giauque, H., & Hawkes, C. V. (2013). Climate affects symbiotic fungal endophyte diversity and performance. *American journal of botany*, *100*(7), 1435-1444.

Global Biodiversity Information Facility (2016). GBIF Backbone Taxonomy. GBIF Secretariat. Checklist Dataset http://www.gbif.org/species/2879177 (June 2017)

González, G. L. (2006). *Los árboles y arbustos de la Península Ibérica e Islas Baleares: especies silvestres y las principales cultivadas* (Vol. 1). Mundi-Prensa Libros.

Graham, J. H., Eissenstat, D. M., & Drouillard, D. L. (1991). On the relationship between a plant's mycorrhizal dependency and rate of vesicular-arbuscular mycorrhizal colonization. *Functional Ecology*, 773-779.

Grünig, C. R., Queloz, V., Sieber, T. N., & Holdenrieder, O. (2008). Dark septate endophytes (DSE) of the Phialocephala fortinii sl–Acephala applanata species complex in tree roots: classification, population biology, and ecology. *Botany*, *86*(12), 1355-1369.

Harley, J. L., & Smith, S. E. (1983). *Mycorrhizal symbiosis*. Academic Press Inc

Haselwandter, K., & Read, D. J. (1982). The significance of a root-fungus association in two Carex species of high-alpine plant communities. *Oecologia*, *53*(3), 352-354.

Haselwandter, K., Bonn, G., & Read, D. J. (1987). Degradation and utilization of lignin by mycorrhizal fungi. *Mycorrhizae in the next decade, practical applications and research priorities*, 31.

Hepper, C. M. (1983). The effect of nitrate and phosphate on the vesicular-arbuscular mycorrhizal infection of lettuce. *New Phytologist*, *93*(3), 389-399.

Hernández, I., & Munné-Bosch, S. (2015). Linking phosphorus availability with photo-oxidative stress in plants. *Journal of experimental botany*, *66*(10), 2889-2900.

Herrera, M. A., Salamanca, C. P., & Barea, J. M. (1993). Inoculation of woody legumes with selected arbuscular mycorrhizal fungi and rhizobia to recover desertified Mediterranean ecosystems. *Applied and Environmental Microbiology*, *59*(1), 129-133.

Herrera, J., Poudel, R., Nebel, K. A., & Collins, S. L. (2011). Precipitation increases the abundance of some groups of root-associated fungal endophytes in a semiarid grassland. *Ecosphere*, *2*(4), 1-14.

Huang, W. Y., Cai, Y. Z., Hyde, K. D., Corke, H., & Sun, M. (2007). Endophytic fungi from Nerium oleander L (Apocynaceae): main constituents and antioxidant activity. *World Journal of Microbiology and Biotechnology*, *23*(9), 1253-1263.

Jakucs, E., Kovács, G. M., Agerer, R., Romsics, C., & Erős-Honti, Z. (2005). Morphological-anatomical characterization and molecular identification of Tomentella stuposa ectomycorrhizae and related anatomotypes. *Mycorrhiza*, *15*(4), 247-258.

Johnson, N. C., Graham, J. H., & Smith, F. A. (1997). Functioning of mycorrhizal associations along the mutualism–parasitism continuum. *The New Phytologist*, *135*(4), 575-585.

Johnson, N. C., Rowland, D. L., Corkidi, L., Egerton-Warburton, L. M., & Allen, E. B. (2003). Nitrogen enrichment alters mycorrhizal allocation at five mesic to semiarid grasslands. *Ecology*, *84*(7), 1895-1908.

Johnson, L. J., de Bonth, A. C., Briggs, L. R., Caradus, J. R., Finch, S. C., Fleetwood, D. J., ... & Tapper, B. A. (2013). The exploitation of epichloe endophytes for agricultural benefit. *Fungal Diversity*, *60*(1), 171-188.

Jumpponen, A. M., & Trappe, J. M. (1996). Population structure of Phialocephala fortinii on a receding glacier forefront. In *Mycorrhiza in integrated systems–from genes to plant*

development. *Proceedings of Fourth European Symposium on Mycorrhiza*. *Commission of the European Union, Luxemburg*(pp. 128-130).

Jumpponen, A., Mattson, K. G., & Trappe, J. M. (1998). Mycorrhizal functioning of Phialocephala fortinii with Pinus contorta on glacier forefront soil: interactions with soil nitrogen and organic matter. *Mycorrhiza*, *7*(5), 261-265.

Jumpponen, A. R. I., & Trappe, J. M. (1998). Dark septate endophytes: a review of facultative biotrophic root-colonizing fungi. *The New Phytologist*, *140*(2), 295-310.

Jumpponen, A. R. I. (1999). Spatial distribution of discrete RAPD phenotypes of a root endophytic fungus, Phialocephala fortinii, at a primary successional site on a glacier forefront. *The New Phytologist*, *141*(2), 333-344.

Knapp, D. G., Pintye, A., & Kovács, G. M. (2012). The dark side is not fastidious–dark septate endophytic fungi of native and invasive plants of semiarid sandy areas. *PLOS one*, *7*(2), e32570.

Knapp, D. G., Kovács, G. M., Zajta, E., Groenewald, J. Z., & Crous, P. W. (2015). Dark septate endophytic pleosporalean genera from semiarid areas. *Persoonia: Molecular Phylogeny and Evolution of Fungi*, *35*, 87.

KovAcs, G. M., & Szigetvari, C. (2002). Mycorrhizae and other root-associated fungal structures of the plants of a sandy grassland on the Great Hungarian Plain. *PHYTON-HORN-*, *42*(2), 211-224.

Li, X., Ren, A., Han, R., Yin, L., Wei, M., & Gao, Y. (2012). Endophyte-mediated effects on the growth and physiology of Achnatherum sibiricum are conditional on both N and P availability. *PloS one*, *7*(11), e48010.

Li, Q., Mao, H., Dong, X., Zuo, Z., & Zhou, J. (2014). The effects of nitrogen on micro-structure of tomato leaf. In *2014 Montreal, Quebec Canada July 13–July 16, 2014* (p. 1). American Society of Agricultural and Biological Engineers.

Lingfei, L., Anna, Y., & Zhiwei, Z. (2005). Seasonality of arbuscular mycorrhizal symbiosis and dark septate endophytes in a grassland site in southwest China. *FEMS Microbiology Ecology*, *54*(3), 367-373.

Liphschitz, N., & Biger, G. (1990). Ancient dominance of the Quercus calliprinos-Pistacia palaestina association in mediterranean Israel. *Journal of Vegetation Science*, *1*(1), 67-70.
Lukešová, T. The role of DSE (Dark Septate Endophytes) in plant communities in forest ecosystem.

Malinowski, D. P., Alloush, G. A., & Belesky, D. P. (1998). Evidence for chemical changes on the root surface of tall fescue in response to infection with the fungal endophyte Neotyphodium coenophialum. *Plant and Soil*, *205*(1), 1-12.

Mandyam, K., & Jumpponen, A. (2005). Seeking the elusive function of the root-colonising dark septate endophytic fungi. *Studies in Mycology*, *53*, 173-189.

Mandyam, K., Fox, C., & Jumpponen, A. (2012). Septate endophyte colonization and host responses of grasses and forbs native to a tallgrass prairie. *Mycorrhiza*, *22*(2), 109-119.

Margulis, L. (1970). *Origin of eukaryotic cells: Evidence and research implications for a theory of the origin and evolution of microbial, plant and animal cells on the precambrian Earth*. Yale University Press.

Marins, J. F. D., & Carrenho, R. (2017). Arbuscular mycorrhizal fungi and dark septate fungi in plants associated with aquatic environments. *Acta Botanica Brasilica*, *31*(2), 295-308.

Marschner, H. (2011). *Marschner's mineral nutrition of higher plants*. Academic press.

Mereschkowsky, C. (1905). *Über natur und ursprung der chromatophoren im pflanzenreiche*.

Mereschkowsky, C. (1910). Theorie der zwei Plasmaarten als Grundlage der Symbiogenesis, einer neuen Lehre von der Entstehung der Organismen. *Biol. Centralbl*, *30*, 278-288.

Moricca, S., & Ragazzi, A. (2008). Fungal endophytes in Mediterranean oak forests: a lesson from Discula quercina. *Phytopathology*, *98*(4), 380-386.

Mullen, R. B., Schmidt, S. K., & Jaeger III, C. H. (1998). Nitrogen uptake during snowmelt by the snow buttercup, Ranunculus adoneus. *Arctic and Alpine Research*, 121-125.

Müller, P., & Döring, M. (2009). Isothermal DNA amplification facilitates the identification of a broad spectrum of bacteria, fungi and protozoa in Eleutherococcus sp. plant tissue cultures. *Plant Cell, Tissue and Organ Culture*, *98*(1), 35-45.

Muniania, C. N. (2016). *Seasonal variation and potential roles of dark septate fungi in an arid grassland* (Doctoral dissertation, Western Illinois University).

Muthukumar, T., Senthilkumar, M., Rajangam, M., & Udaiyan, K. (2006). Arbuscular mycorrhizal morphology and dark septate fungal associations in medicinal and aromatic plants of Western Ghats, Southern India. *Mycorrhiza*, *17*(1), 11-24.

Newsham, K. K. (1999). Phialophora graminicola, a dark septate fungus, is a beneficial associate of the grass Vulpia ciliata ssp. ambigua. *New Phytologist*, *144*(3), 517-524.

Newsham, K. K., Upson, R., & Read, D. J. (2009). Mycorrhizas and dark septate root endophytes in polar regions. *Fungal Ecology*, *2*(1), 10-20.

Newsham, K. K. (2011). A meta-analysis of plant responses to dark septate root endophytes. *New Phytologist*, *190*(3), 783-793.

Nixon, K. (2006). Global and neotropical distribution and diversity of oak (genus *Quercus*) and oak forests. *Ecology and conservation of neotropical montane oak forests*, 3-13.

Nosanchuk, J. D., Stark, R. E., & Casadevall, A. (2015). Fungal melanin: what do we know about structure?. *Frontiers in microbiology*, *6*.

Nosanchuk, J. D., & Casadevall, A. (2003). The contribution of melanin to microbial pathogenesis. *Cellular microbiology*, *5*(4), 203-223.

Olson, D. M., Dinerstein, E., Wikramanayake, E. D., Burgess, N. D., Powell, G. V., Underwood, E. C., ... & Loucks, C. J. (2001). Terrestrial Ecoregions of the World: A New Map of Life on Earth: A new global map of terrestrial ecoregions provides an innovative tool for conserving biodiversity. *BioScience*, *51*(11), 933-938.

Paffetti, D., Vettori, C., & Giannini, R. (2001). Relict populations of Quercus calliprinos Webb on Sardinia island identified by chloroplast DNA sequences. Forest Genetics, 8(1), 1-12.

Petrini, O. (1991). Fungal endophytes of tree leaves. In *Microbial ecology of leaves* (pp. 179-197). Springer, New York, NY.

Phillips, J. M., & Hayman, D. S. (1970). Improved procedures for clearing roots and staining parasitic and vesicular-arbuscular mycorrhizal fungi for rapid assessment of infection. *Transactions of the British mycological Society*, *55*(1), 158IN16-161IN18.

Pietikäinen, J., Pettersson, M., & Bååth, E. (2005). Comparison of temperature effects on soil respiration and bacterial and fungal growth rates. *FEMS Microbiology Ecology*, *52*(1), 49-58.

Porras-Alfaro, A., & Bayman, P. (2011). Hidden fungi, emergent properties: endophytes and microbiomes. *Annual review of phytopathology*, *49*.

Postma, J. W., Olsson, P. A., & Falkengren-Grerup, U. (2007). Root colonisation by arbuscular mycorrhizal, fine endophytic and dark septate fungi across a pH gradient in acid beech forests. *Soil Biology and Biochemistry*, *39*(2), 400-408.

Ratnayake, M., Leonard, R. T., & Menge, J. A. (1978). Root exudation in relation to supply of phosphorus and its possible relevance to mycorrhizal formation. *New Phytologist*, *81*(3), 543-552.

Raunkiaer, C. (1934). The life forms of plants and statistical plant geography; being the collected papers of C. Raunkiaer. The life forms of plants and statistical plant geography; being the collected papers of C. Raunkiaer.

Ravel, C., Courty, C., Coudret, A., & Charmet, G. (1997). Beneficial effects of Neotyphodium lolii on the growth and the water status in perennial ryegrass cultivated under nitrogen deficiency or drought stress. *Agronomie*, *17*(3), 173-181.

Rayner, M. C. (1915). Obligate symbiosis in Calluna vulgaris. *Annals of Botany*, *29*(113), 97-133.

Redecker, D., Kodner, R., & Graham, L. E. (2000). Glomalean fungi from the Ordovician. *Science*, *289*(5486), 1920-1921.

Requena, N., Jeffries, P., & Barea, J. M. (1996). Assessment of natural mycorrhizal potential in a desertified semiarid ecosystem. *Applied and Environmental Microbiology*, *62*(3), 842-847.

Richardson, J. L., Urban, M. C., Bolnick, D. I., & Skelly, D. K. (2014). Microgeographic adaptation and the spatial scale of evolution. *Trends in Ecology & Evolution*, *29*(3), 165-176.

Royal Botanic Gardens Kew. (2017) Seed Information Database (SID). Version 7.1. Available from: http://apps.kew.org/wcsp/namedetail.do?name_id=172209 (June 2017)

Ruotsalainen, A., Väre, H., & Vestberg, M. (2002). Seasonality of root fungal colonization in low-alpine herbs. *Mycorrhiza*, *12*(1), 29-36.

Ruttenberg, K. C. (2003). The global phosphorus cycle. *Treatise on geochemistry*, 8, 682.

Saikkonen, K., Young, C. A., Helander, M., & Schardl, C. L. (2016). Endophytic Epichloë species and their grass hosts: from evolution to applications. *Plant molecular biology*, *90*(6), 665-675.

Scervino, J. M., Gottlieb, A., Silvani, V. A., Pérgola, M., Fernández, L., & Godeas, A. M. (2009). Exudates of dark septate endophyte (DSE) modulate the development of the arbuscular mycorrhizal fungus (AMF) Gigaspora rosea. *Soil Biology and Biochemistry*, *41*(8), 1753-1756.

Schardl, C. L., Leuchtmann, A., & Spiering, M. J. (2004). Symbioses of grasses with seedborne fungal endophytes. *Annu. Rev. Plant Biol.*, *55*, 315-340.

Schulz, B. (2006). Mutualistic interactions with fungal root endophytes. *Microbial Root Endophytes*, 261-279.

Selim KA, El-Beih AA, Abdel-Rahman TM, El-Diwany AI (2012) Biology of endophytic fungi. CREAM 2:31–82.

Sieber, T. N., & Grünig, C. R. (2002). Fungal root endophytes. *Plant roots: the hidden half. Marcel Dekker, New York*, 887-917.

Sieber, T., & Grünig, C. (2006). Biodiversity of fungal root-endophyte communities and populations, in particular of the dark septate endophyte Phialocephala fortinii sl. *Microbial root endophytes*, 107-132.

Sylvia, D. M., & Neal, L. H. (1990). Nitrogen affects the phosphorus response of VA mycorrhiza. *New Phytologist*, *115*(2), 303-310.

Smith, S. E., & Smith, F. A. (1990). Structure and function of the interfaces in biotrophic symbioses as they relate to nutrient transport. *New Phytologist*, *114*(1), 1-38.

Smith, S. E., & Read, D. J. (2010). *Mycorrhizal symbiosis*. Academic press.

Solomon, E., & Berg, L. R. (2013). Biología, Cengage Learning. *SA, México*.

Spagnoletti, F. N., Tobar, N. E., Di Pardo, A. F., Chiocchio, V. M., & Lavado, R. S. (2017). Dark septate endophytes present different potential to solubilize calcium, iron and aluminum phosphates. *Applied Soil Ecology*, *111*, 25-32.

Steffen, W., Persson, Å., Deutsch, L., Zalasiewicz, J., Williams, M., Richardson, K., ... & Molina, M. (2011). The Anthropocene: From global change to planetary stewardship. *AMBIO: A Journal of the Human Environment*, *40*(7), 739-761.

Stierle, A., Strobel, G., & Stierle, D. (1993). Taxol and taxane production by Taxomyces andreanae, an endophytic fungus of Pacific yew. *SCIENCE-NEW YORK THEN WASHINGTON-*, *260*, 214-214.

Strobel, G. A. (2003). Endophytes as sources of bioactive products. *Microbes and infection*, *5*(6), 535-544.

Stoyke, G., & Currah, R. S. (1991). Endophytic fungi from the mycorrhizae of alpine ericoid plants. *Canadian Journal of Botany*, *69*(2), 347-352.

Strobel, G., & Daisy, B. (2003). Bioprospecting for microbial endophytes and their natural products. *Microbiology and molecular biology reviews*, *67*(4), 491-502.

Suz, L. M., Martín, M. P., Oliach, D., Fischer, C. R., & Colinas, C. (2008). Mycelial abundance and other factors related to truffle productivity in Tuber melanosporum–Quercus ilex orchards. *FEMS microbiology letters*, *285*(1), 72-78.

The Plant List (2010). Version 1. Published on the Internet; http://www.theplantlist.org/tpl1.1/record/kew-171917 (June 2017).

Thomas, F. M., Blank, R., & Hartmann, G. (2002). Abiotic and biotic factors and their interactions as causes of oak decline in Central Europe. *Forest Pathology*, *32*(4-5), 277-307.

Toju, H., Sato, H., Yamamoto, S., Kadowaki, K., Tanabe, A. S., Yazawa, S., ... & Agata, K. (2013). How are plant and fungal communities linked to each other in belowground ecosystems? A massively parallel pyrosequencing analysis of the association specificity of root-associated fungi and their host plants. *Ecology and evolution*, *3*(9), 3112-3124.

Trehane, P. (2007 onwards), The Oak Names Checklist. Published on the internet http://www.oaknames.org/search/fullname.asp?id=2517 (June 2017)

Trowbridge, J., & Jumpponen, A. (2004). Fungal colonization of shrub willow roots at the forefront of a receding glacier. *Mycorrhiza*, *14*(5), 283-293.

Vierheilig, H., Coughlan, A. P., Wyss, U., & Piché, Y. (1998). Ink and vinegar, a simple staining technique for arbuscular-mycorrhizal fungi. *Applied and environmental microbiology*, *64*(12), 5004-5007.

Vierheilig, H., Schweiger, P., & Brundrett, M. (2005). An overview of methods for the detection and observation of arbuscular mycorrhizal fungi in roots. *Physiologia Plantarum*, 125(4), 393-404.

Vigo, J. B. (2006). *Les comunitats vegetals. Descripció i classificació* Barcelona, España: Universitat de Barcelona: 978-84-475-2891-2

Wagg, C., Pautler, M., Massicotte, H. B., & Peterson, R. L. (2008). The co-occurrence of ectomycorrhizal, arbuscular mycorrhizal, and dark septate fungi in seedlings of four members of the Pinaceae. *Mycorrhiza*, *18*(2), 103-110.

Wang, B., & Qiu, Y. L. (2006). Phylogenetic distribution and evolution of mycorrhizas in land plants. *Mycorrhiza*, *16*(5), 299-363.

Wazen, N., & Fady, B. (2016). Geographic distribution of 24 major tree species in the Mediterranean and their genetic resources.

Wildflowers of Israel (2017) http://www.wildflowers.co.il/english/plant.asp?ID=659 (June 2017)

Yu, T., Nassuth, A., & Peterson, R. L. (2001). Characterization of the interaction between the dark septate fungus Phialocephala fortinii and Asparagus officinalis roots. *Canadian Journal of Microbiology*, *47*(8), 741-753.