Evaluation of *Trichoderma* and Mycorrhiza inoculation strategies in the nursery against Grapevine Trunk Diseases by the use of molecular detection tools

Catarina da Cunha Maia Leal
Engenharia de Viticultura e Enologia
Departamento de Geociências, Ambiente e Ordenamento do Território
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Orientadores:
Felipe Gaínza Cortés, Investigador, Viña Concha y Toro
Cecília Rego, Professora Auxiliar, Instituto Superior de Agronomia

Coorientador:
Anabela Carneiro, Professora Auxiliar convidada, FCUP

Todas as correções determinadas pelo júri, e só essas, foram efetuadas.

O Presidente do Júri,

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Abstract

Nowadays, Grapevine trunk diseases (GTD's) are considered the most difficult challenge in viticulture all over the world. GTD's are very injurious for the sustainability of the winemaking industry because they can affect grapevines of all ages, the fungi involved in this diseases have an endophytic phase, and there is a lack of efficient chemical control methods. As consequence of this emerging problem, several studies were led worldwide, both to in-depth the knowledge on the GTD's and to find eco-friendly solutions able to control this diseases. *Trichoderma* and arbuscular Mycorrhiza fungi (AMF) showed a lot of potential in decreasing the incidence of GTD's in vineyards as well as increasing the vine ability to survive in stress conditions.

The present work tested the effect of different *Trichoderma* strains (Tifi®: *Trichoderma artroviride*, Mamull®: *Trichoderma gamsii*, Sherwood strain: *Trichoderma virens*, Trichoderma spp. 1 and 2: *Trichoderma* spp.) against the main GTDs-associated fungi (*Phaeomoniella Chlamydospora*, *Inocutis* spp., *Eutypa lata*, *Diplodia seriata* and *Neofusicoccum parvum*) *in vitro*. On another essay, grapevine nursery plants were inoculated with commercial products based on AMF mixes (AEGIS-Gel®: *Rhizophagus irregularis*, *Glomus mosseae*; Oiko-Rhiza®: *Glomus brasillanum, Glomus clarum, Glomus deserticota, Rhizophagus irregularis, Glomus mosseae, Glomus monosporum, Gigaspora margarita*) to evaluate the colonization and arbuscules development capacity. Besides this experiments, was created a molecular detection system for *Trichoderma* and AMF detection based on PCR techniques.

As results, the molecular detection system set up on Concha y Toro laboratory, is able to identify *Trichoderma* and AMF on root samples, which can be used as a pre-diagnosis to evaluate the efficiency of the inoculations. Tifi® was the most effective treatment against all GTDs fungi growth, and, unexpectedly, the native "Sherwood" strain showed some potential on inhibition of GTDs fungi growth. In the case of AMF potential evaluation, both plants inoculated with AEGIS-Gel® and Oiko-Rhiza® showed significant differences in Frequency (F%), Colonization intensity (M%) and Arbuscles abundance (A%) compared with the control (non-inoculated plants). Plants inoculated with Oiko-Rhiza® showed significant higher nitrogen and arginine contents when compared with AEGIS-Gel®-inoculated and control plants. Together, these results show the potential use of this beneficial microorganisms (namely *Trichoderma* and AMF) in order to

strengthen and improve the performance of Viña Concha y Toro grapevines against GTDs.

Key-words: Grapevine trunk diseases, *Trichoderma*, Arbuscular Mycorrhiza fungi, molecular detection, ITS region.

Resumo

Hoje em dia, as Doenças do lenho da videira são consideradas o maior desafio na viticultura em todo o mundo. As doenças do lenho são bastante prejudiciais para a sustentabilidade da indústria vinícola, pois podem afetar videiras de todas as idades, causando o declínio e possível morte das plantas. Os fungos envolvidos nestas doenças apresentam uma fase endofitita e não existem ainda métodos eficientes de controlo químico. Como consequência deste problema emergente, vários estudos foram desenvolvidos em todo o mundo, tanto para aprofundar o conhecimento sobre as doenças do lenho, quanto para encontrar soluções ecologicamente responsáveis, capazes de controlar este tipo de doenças. *Trichoderma* e Micorrizas arbusculares mostraram um grande potencial na diminuição da incidência de doenças do lenho em vinhas, bem como no aumento da capacidade das plantas sobreviverem em condições de *stress*.

O presente trabalho testou o efeito de diferentes espécies de *Trichoderma* (Tifi®: *Trichoderma artroviride*, Mamull®: *Trichoderma gamsii*, Sherwood strain: *Trichoderma virens*, Trichoderma spp. 1 and 2: *Trichoderma* spp.) contra os principais fungos associados a doenças do lenho (*Phaeomoniella Chlamydospora*, *Inocutis* spp., *Eutypa lata*, *Diplodia seriata* and *Neofusicoccum parvum*) *in vitro*. Num outro ensaio, videiras de viveiro foram inoculadas com produtos comerciais à base de misturas de Micorrizas arbusculares (AEGIS-Gel®: *Rhizophagus irregularis*, *Glomus mosseae*; Oiko-Rhiza®: *Glomus brasillanum, Glomus clarum, Glomus deserticota, Rhizophagus irregularis, Glomus mosseae, Glomus monosporum, Gigaspora margarita*) com o objetivo de avaliar a capacidade de colonização e de desenvolvimento de arbúsculos. À parte destes ensaios, foi desenvolvido um sistema de deteção molecular de *Trichoderma* e Micorriza arbuscular baseado em técnicas de PCR.

Como resultados, o sistema de deteção molecular desenvolvido no laboratório da empresa Concha y Toro, foi capaz de identificar *Trichoderma* e Micorrizas arbusculares em amostras de raízes, que pode ser utilizado como um pré-diagnóstico para avaliar a eficiência das inoculações. Tifi® foi o tratamento mais eficaz contra o desenvolvimento de todos os fungos associados a doenças do lenho, e, inesperadamente, a “Sherwood” *strain* demonstrou algum potencial na inibição do crescimento de fungos associados a doenças do lenho. No caso da avaliação do potencial de Micorrizas arbusculares, ambas as plantas inoculadas com AEGIS-Gel® e Oiko-Rhiza® apresentaram diferenças na

frequência (F%), intensidade de colonização (M%) e intensidade arbuscular (A%) quando comparadas com o controlo (plantas não inoculadas). As plantas inoculadas com Oiko-Rhiza® apresentaram um teor em azoto e arginina significativamente superior quando comparadas com plantas inoculadas com AEGIS-Gel® e plantas controlo. Juntos, estes resultados demonstram o potencial do uso de microrganismos benéficos (nomeadamente *Trichoderma* e Micorriza) para fortalecer e melhorar a performance das vinhas da Concha y Toro contra doenças do lenho da videira.

Palavras-chave: Doenças do lenho da videira, *Trichoderma,* Micorriza arbuscular, deteção, região ITS.

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Abbreviations list

AM - Arbuscular Mycorrhiza

AMF – Arbuscular Mycorrhizal fungi

ANOVA – Analysis of variance

BDA – Black dead arm

bp – Base pairs

cfu – Colony forming unit

CMD – Cornmeal dextrose agar

DNA – Deoxyribonucleic acid

D. *seriata – Diplodia seriata*

E. *lata – Eutypa lata*

F. *mediterranea – Famitiporia mediterranea*

GLSD – Grapevine leaf strip disease

GTD's – Grapevine trunk diseases

H. *rufa – Hypocrea rufa*

ITS – Internal transcribed spacer

LSU – Large subunit

N. *parvum – Neofusicoccum Parvum*

P. *chlamydospora – Phaeomoniella chlamydospora*

PCR – Polymerase chain reaction

PDA – Potato dextrose agar

Pm. *minimum – Phaeoacremonium minimum*

q-PCR – Quantitative polymerase chain reaction

rDNA – Ribosomal deoxyribonucleic acid

RNA – Ribonucleic acid

rRNA – Ribosomal ribonucleic acid

SSU – Small subunit

T. *artroviride – Trichoderma artroviride*

T. *gamsii – Trichoderma gamsii*

T. *ganense – Trichoderma ganense*

T. *harzianum – Trichoderma Harzianum*

T. *saturnisporum – Trichoderma saturnisporum*

T. *viride – Trichoderma viride*

VCT – Viña Conha y Toro

1. Introduction

1.1 General Introduction

Grapevine trunk diseases (GTDs) are a group of diseases caused by several fungal pathogens that live and colonize the wood of the perennial organs of vines (Bertsch *et al.,* 2013). Nowadays, trunk diseases are considered the most difficult challenge in viticulture all over the world and are rapidly concerning all wine producing countries. GTDs are very injurious for the sustainability of the winemaking industry because the pathogens responsible for these diseases colonize the wood of the plant causing its death on shorter or longer term. This type of diseases can affect not just adult vines (more than 7-10 years) restricting the productivity (Fig.1) and decreasing the longevity of the plants (Fig. 1) (ex: Esca, Eutypa and Botryosphaeria dieback), but also young vines (less than 7-10 years) and most important, this diseases can affect mother-plants and rootstocks/canes in the nursery (Halleen *et al.,* 2003). So, bearing in mind the world economic cost for replacement of dead grapevines is around 1.5 billion dollars per year (Hofstetter *et al.,* 2012), that replaced grapevines could probably be affected with trunk diseases and may need to be replaced again in a few years creating a vicious cycle.

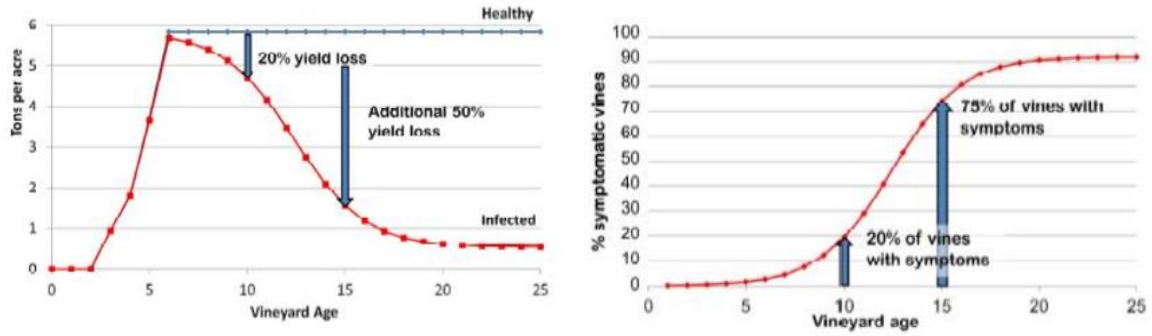

Figure 1 - Percentage of symptomatic vines and yield loss of vineyards affected by trunk diseases along 25 years.

In this global context, Chile, an important wine producer, has 209kha of planted vineyards, a number that has been growing for the past four years, making Chile the ninth country with more vine surface area (OIV, 2017). However, it is estimated that 22% of this vineyards are affected with trunk diseases (Díaz *et al.,* 2011). In Chile, as

worldwide, there are three most relevant GTDs, Esca complex, Eutipiosis and *Botryosphaeria* dieback which affect significantly the productivity and longevity of the vines. Unfortunately, these diseases are spread very easily from vine to vine, vineyard to vineyard and nowadays from country to country. Although there is a 2 year quarantine period for every plant material that is imported into Chile, trunk diseases fungi have an endophytic phase (the pathogen is inside the plant, however is harmless), so the plants may only show foliar symptoms three, or more years after infection. Therefore, this quarantine period is not enough to guarantee and prevent the entrance of GTDs into the country.

For that reason and knowing that this type of diseases has no effective treatment, it is extremely important to detect the GTDs early at the mother blocks, protect the vines from these fungi diseases and strengthen them so that they can withstand better in hostile environments.

1.2 Aims of the project

The work presented on this master thesis is an integral part of a large project aimed at obtaining virus/GTDs-free grapevine plants and strengthening them at nursery stage with different beneficial microorganisms to cope with the productive vineyard conditions, financed by public/private grants from the Chilean state agency "Corporación de Fomento" (CORFO).This project aims to strengthen nursery grapevines with beneficial microorganisms such as, *Trichoderma* spp. and Mycorrhiza, in order to reduce the incidence of trunk diseases in Concha y Toro grapevines.

The presented work has one general objective: evaluate the effectiveness of different strains of *Trichoderma* and the best strategy to use *Trichoderma* and Mycorrhiza against trunk diseases fungi in nursery grapevines. To achieve this general purpose, four specific objectives were set: evaluate the virulence of commercial products and native species of *Trichoderma* spp. against wood fungi *in vitro*; evaluate the efficiency of internal transcribed spacer (ITS) primers on commercial products and native species of Arbuscular Mychorrizal fungi; evaluate and design primers for different species of *Trichoderma* spp.; evaluate the efficiency of different inoculation techniques of *Trichoderma* spp. and arbuscular Mycorrhiza (AM) fungi in nursery vines.

2. State of art

2.1 World and Chile Viticulture

Grapevine was one of the first fruit species to be domesticated and nowadays it´s the world's most economically important fruit crop (Keller, 2010).

Currently, grapes are cultivated in six of the seven continents, transversely most of the different climates, from oceanic, and transition temperate, to continental, mediterranean, tropical and even arid climates (Schultz and Stoll, 2010). The most favorable areas for grape production are positioned between 30° and 50° of latitude in the Northern Hemisphere and between 30° and 40° of latitude in the Southern Hemisphere, corresponding to the areas with more of a temperate climate (Reisch *et al.,* 2012).

The grape production is so important worldwide that the global production reached 7.5 million of hectares in 2016 (OIV, 2017). The majority of the produced grapes are used in the winemaking industry, which leads to a production of approximately 267 million of hectoliters in 2016 (OIV, 2017).

Chile is an important wine producer with a vineyard area of 214 thousands of hectares, a number that it's been growing in the past few years (OIV, 2017). In 2016, Chile occupied the 9th place in the countries with the larger vineyard areas, and it´s the 8th largest world wine producer, with 10.1 million of hectolitres produced in 2016 (OIV, 2017).

Though the grapevine production is economically and culturally important in Chile, as much as in the other countries, there are different types of factors that can reduce the production, the quality of the products and the longevity of the plants. Grapevine trunk diseases, are considered the most difficult challenge in viticulture all over the world and are concerning all wine producing countries, due to their method of action and lack of effective treatment.

2.2 Grapevine Trunk diseases

2.2.1 Esca Complex

2.2.1.1 Introduction

The studies on Esca carried out since the 1980's have led to a deep revision of this trunk disease, specially regarding the pathogens involved and the symptom expression. The more recent one revealed Esca as a "complex of diseases" characterized by different diseases and different syndromes according to the stage of the vine life, the type of wood and foliar symptoms, and/or to the pathogens infecting and acting into the vine (Fig. 2).

The old denomination of "Esca" as a wood white decay causing "apoplexy" (sudden wilting of the vine) and typical foliar symptoms is replaced by five different diseases/syndromes (dark wood streaking, Petri disease, grapevine leaf stripe disease (GLSD), white rot, and Esca proper) grouped under the name of "Esca complex." These syndromes differ based on the age of the symptomatic vines and the pathogens involved (Mondello *et al.,* 2018).

In white decay and Esca proper, basidiomycetes are involved, while in all the others, vascular ascomycetes as *P. chlamydospora*, *Phaeoacremonium* spp., are the common factor, and more recently, in Petri disease, *Cadophora* spp.. The dark wood streaking is a wood symptom proving the presence of the pathogens in vines from the nursery, while the Petri disease is typical of very young vines, a decline developing in new plantations. The typical wood symptom in both is the vascular discoloration. The Petri disease could also show foliar symptoms (chlorosis) and decline, up to death of the vines. The GLSD syndrome is characterized by the typical tiger-stripe pattern on symptomatic leaves and is often associated with a partial or complete apoplexy in affected plants. It can be found in both young and adult vineyards, in vines showing only vascular pathogens infections or also in those showing wood decay. A longitudinal orange-brown stripe under the bark can usually be noticed after removing the bark. The white decay is determined by basidiomycetes fungi (in Europe, *Fomitiporia mediterranea*), which determine only symptoms on the infected wood. Finally, the name Esca proper was retained to refer to the original name (Esca as decayed wood used as tinder), typical of adult vineyards, and characterized by the contemporary presence of the main vascular pathogens plus the basidiomycetes species and the development of foliar symptoms (Gubler *et al.,* 2015; Mondello *et al.,* 2018; Surico, 2009).

Figure 2 - Different diseases and different syndromes of Esca Complex (Mondello *et al.,* 2018).

The fungi that cause the complex of diseases hibernate in the infected wood during the winter, and it´s dissemination is essentially through the wind rain. They penetrate the wounds, in particularly, pruned wounds, release toxins and cause cells to die from distance making them dark but consistent. Then the fungus penetrates the dead cells digesting them and the tissues acquire a spongy and colourless aspect, responsible by sending new toxins to the following tissues and therefore advancing in the plant. The area that is not affected by fungi allows the plant to remain functional, however, the disease progressively worsens and destroys all plant tissues causing the plant to die (Martin and Cobos, 2007).

Symptoms of GLSD may manifest in a slow evolution, where the margins of the leaves show necrosis, with spots in the center that end up causing a stain on the whole leaf (Fig. 3). These symptoms appear on the basal leaves first, evolving through the entire shoot. The bunches show a disruption and possible necrosis as well (Fig. 3). If the disease has a very rapid evolution (apoplexy), part of the vine can dry in a few days or even hours. This form of the disease is rare and manifests itself in the hottest time of the year, after a plentiful rain in which the vines appear healthy but don't budburst the following year. The leaves and bunches die due to lack of nutrients, caused by the failure of the vessels. The shoots can present a dark or a whitish spongy spot, depending on the disease stage.

Figure 3 - Symptoms of grapevine leaf stripe disease in leaves and bunches.

2.2.1.2 Control methods

Application of sodium arsenite was formerly the best curative treatment, however, it was banned since the year 2000 due to its toxicity (carcinogen), eliminating the possibility of having an effective chemical treatment against this disease.

Thus, it became mandatory to use cultural and biological means of struggle to reduce the spread of the disease (Rolshausen *et al.,* 2010).

The first caution to have is adopting training systems that create smaller pruning wounds when installing a new vineyard. Avoid big pruning wounds and burn the affected wood are other ways to avoid the spread of this disease. Protect the punning wounds with products like bitumen or ointment can also prevent the infection (Rolshausen *et al.,* 2010).

The use of fungi that are antagonistic to Esca fungi, such as *Trichoderma*, can be used for biological control, but studies are necessary to understand the function and potential of these organisms against Esca complex (Rolshausen *et al.,* 2010). Recently, Mondello *et al.*, (2018) did a review of fifteen years of trials with chemicals and biocontrol agents and concluded that the biological control agents are effective against GTDs and can be a good solution for this diseases.

2.2.2 Botriosphaeriae dieback

2.2.2.1 Introduction

Botryosphaeriae dieback known for a very long time as “slow stroke”, is produced by fungi species from Botryosphaeriaceae family (Díaz and Latorre, 2013) (Fig. 6). To date, several studies have allowed the identification of at least 21 different species in the

Botryosphaeriaceae occurring in grapevines worldwide (Urbez-Torres, 2011). Other fungi like *Lasiodiplodia theobromae*, *N. parvum* and *Botryosphaeria dothidea* can be associated with this disease as well.

The fungus overwinters on the bark of affected vines and start to producing spores during the spring. Spores are spread through the wind and rain drops to other vines. When in contact with the tissues (pruning wounds, cuts, or natural cracks), they can germinate if the temperatures are above 5ºC (Hillis *et al.,* 2017). The fungi, gradually invade the vascular tissues of the trunk and thread their way to the roots. This results in the formations of cankers, wood necrosis and dieback of the shoots (Pitt *et al.,* 2012).

The most characteristic symptoms of this disease is the whitening and dead of some branches, while others still healthy. The cankers are not normally detected until the branch is dead. If the top part of the branch is removed, the wood on the inside is brown or reddish-brown instead of white. In some cases, it´s possible to see the cankers on the branches (Pitt *et al.*, 2012) (Fig. 4).

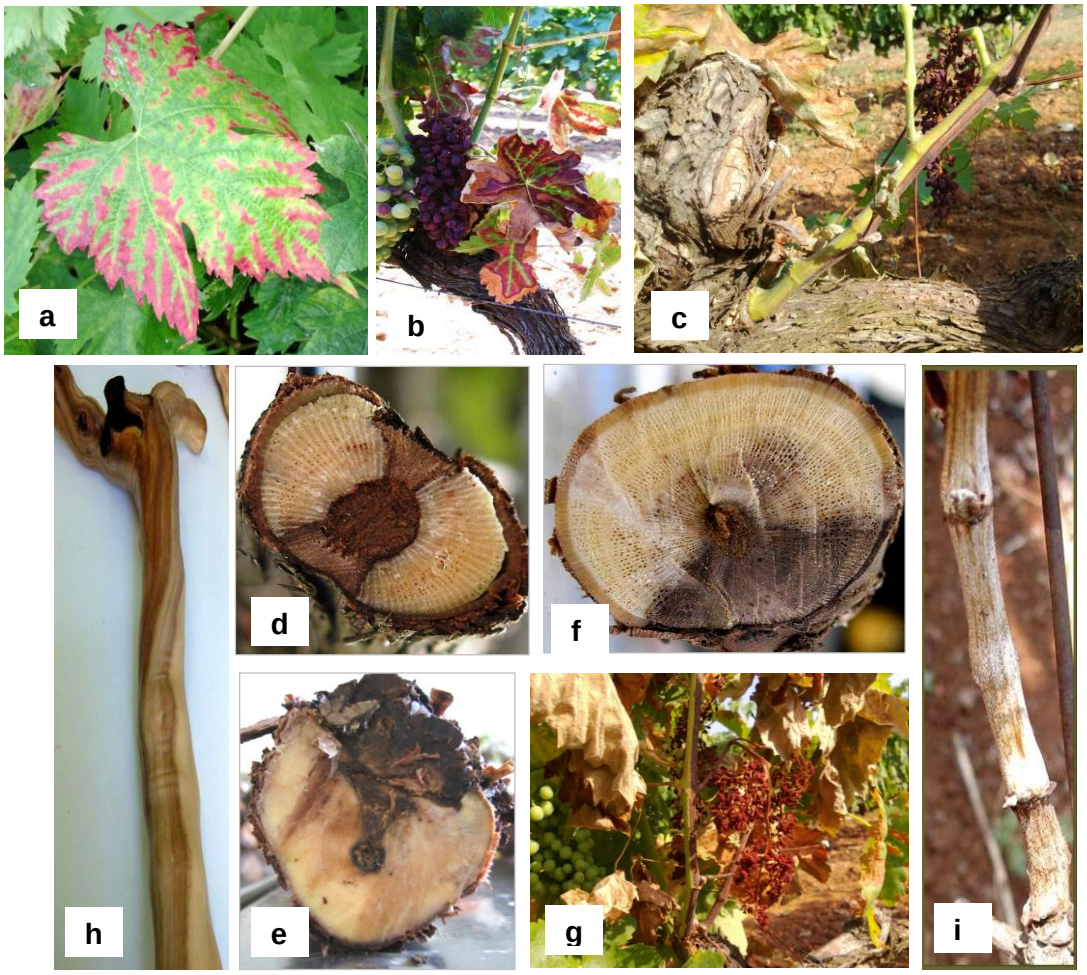

Figure 4 – Different symptoms associated with Botryosphareria dieback in leaves (a, b), stems (c), internal wood tissues (d,e.f), bunches (g) and canes (h, i).

The first symptoms of Botryosphaeria dieback (Fig. 4, a), often confused with black dead arm (BDA) by *D. mutila*, and synonym of Botryosphaeria canker, are visible from budburst in spring and consist of dead spurs/buds and in a stunted or delayed growth (Fig. 5). It can also appear in a severe form similar to Esca apoplexy. Foliar symptoms, when present, can be similar to chlorosis or to Esca. Botryosphaeriaceous fungi could determine also bunch rot on grapes (Fig. 4, g). The wood of Botryosphaeria-infected plants shows the presence of brown necrotic sectors, typically arc-shaped and sectorial, wedge shaped necrosis (Fig. 4, d, e, f).

Figure 5 - Symptoms of Botriosphaeria dieback: dead spurs/buds and in a stunted or delayed growth.

Botryosphaeriaceae species	
Sexual stage	Asexual stage
Botryosphaeriaceae species and authority	Anamorph - Teleomorph connection[a]
Botryosphaeria dothidea (Moug. ex Fr.) Ces. & De Not.	*Fusicoccum aesculi*
Diplodia corticola A.J.L. Phillips, A. Alves & J. Luque	*"Botryosphaeria" corticola*
Diplodia mutila (Fr.) Mont.	*"Botryosphaeria" stevensii*
Diplodia seriata De Not.	*"Botryosphaeria" obtusa*
Dothiorella iberica A.J.L. Phillips, J. Luque & A. Alves	*"Botryosphaeria" iberica*
Dothiorella americana J.R. Úrbez-Torres, F. Peduto & W.D. Gubler	Unknown
Guignardia bidwellii (Ellis) Viala & Ravaz	Unknown
Lasiodiplodia crassispora T.I. Burgess & Barber	Unknown
Lasiodiplodia missouriana J.R. Úrbez-Torres, F. Peduto & W.D. Gubler	Unknown
Lasiodiplodia theobromae (Pat.) Griff. & Maubl.	*"Botryosphaeria" rhodina*
Lasiodiplodia viticola J.R. Úrbez-Torres, F. Peduto & W.D. Gubler	Unknown
Neofusicoccum australe (Slippers, Crous & M.J. Wingf.) Crous, Slippers & A.J.L. Phillips	*"Botryosphaeria" australe*
Neofusicoccum luteum (Pennycook & Samuels) Crous, Slippers & A.J.L. Phillips	*"Botryosphaeria" lutea*
Neofusicoccum macroclavatum (T.I. Burgess, Barber & Hardy) T.I. Burgess, Barber & Hardy	Unknown
Neofusicoccum mediterraneum Crous, M.J. Wingf. & A.J.L. Phillips	Unknown
Neofusicoccum parvum (Pennycook & Samuels) Crous, Slippers & A.J.L. Phillips	*"Botryosphaeria" parva*
Neofusicoccum ribis (Slippers, Crous & M.J. Wingf.) Crous, Slippers & A.J.L. Phillips	Unknown
Neofusicoccum viticlavatum (Van Niekerk & Crous) Crous, Slippers & A.J.L. Phillips	Unknown
Neofusicoccum vitifusiforme (Van Niekerk & Crous) Crous, Slippers & A.J.L. Phillips	Unknown
Phaeobotryosphaeria porosa (Van Niekerk & Crous) Crous & A.J.L. Phillips	Unknown
Spencermartinsia viticola (A.J.L. Phillips & J. Luque) A.J.L. Phillips, A. Alves & Crous	*Dothiorella viticola*

Figure 6 – List of *Botryosphaeriaceae* species, responsible for the sexual and asexual stage of Botryosphareria dieback.

2.2.2.2 Control methods

Like in the Esca complex there are not a lot options to treat this disease except for cultural methods such as prune bellow the visible canker or replace the vine if the disease is in an advanced stage, avoid large pruning wounds and avoid pruning before rainy days, treat the pruning wounds with sealants or paints, grow less susceptive varieties, do supplemental irrigation and destroy all affected wood from the vineyard (Úrbez-Torres *et al.,* 2006). Recently, (Gramaje *et al.,* 2018) did a review concerning management of grapevine trunk diseases and conclude that management of GTDs must be integrated and holistic, with an interdisciplinary approach.

2.2.3 *Eutypa* dieback

2.2.3.1 Introduction

Eutypa dieback is caused by different species of *Eutypa*, *Eutypella*, *Cryptovalsa*, *Cryptosphaeria* and *Diatrype*. *Eutypa dieback* was known in France and some other countries as one of the most destructive diseases of woody tissues in grapevines. Since it has a slow development until the appearance of the symptoms, many winegrowers do not give it enough attention. In the absence of control measures and above all prevention, the symptoms can lead the dead of the plant and the spread of the disease to the whole vineyard. This disease was not officially reported in Chile.

The ascospores of the fungus initially infest and colonize the tissues of the xylem through open wounds propagating to the phloem of the trunk and arms of the vines. After an incubation period of 3 years or more, a canker is formed around the wound and the main symptoms appear in the young tissues leading to wilt, necroses on the leaf edges and dead of the inflorescences and branches (Moller and Kasimatis, 1981; Moller and Lehoczky, 1980).

In temperate regions, perithecias of *E. lata* reach maturity in the spring, and ascospores are spread by the raindrops. In regions where temperatures are close to zero throughout the winter, the spread of the ascospores is higher in the end of the spring and early summer.

The infection begin when the ascospores get in contact with new open wounds and the susceptibility of the wounds decrease significantly two weeks after pruning and after four weeks the wounds are completely healed (Persoon, 1974) The ascospores take approximately 12 hours to germinate at a temperatures between 20 and 25ºC. Germination occurs within the xylem near the surface of the wound, and the mycelium develops slowly within the vessels and after some time in the trunk.

The disease, as previously mentioned, advances very slowly in the vine, showing no symptoms in the first or second year after the infection. After the third or fourth year, a canker is usually seen and the symptoms on the leaves appear. The economic impact of the disease doesn't occur until the plant reaches maturity, causing more money losses (Persoon, 1974).

The new branches during the first months of growth show deformations and discoloration. The young leaves are smaller than normal, with bent and colorless exterior, torn edges and the possibility of necrotic spots. The affected shoots have an irregular bunch with larger and smaller berries. The symptoms are mostly visible in the spring, once the affected shoots become more visible (Fig. 7). Symptoms in the foliage become more extensive with each passing year until they reach the entire arm and there is a significantly decrease in yield (DeScenzo *et al.,* 1999).

Figure 7- Symptoms of *Eutypa dieback*.

2.2.3.2 Control methods

When the disease is spread in a large part of the vines in a vineyard, it becomes extremely difficult to control it only with dissemination control measures. However, if just few plants are affected, the elimination of the affected pruning wound can reduce the inoculum of the disease in the vineyard, reducing its pressure. Unfortunately, pruning provides many open wounds, and therefore many points of infection, so this must be done at a time when the spread of diseases is reduced. When pruning is performed early in winter there is a higher incidence of the disease, so later pruning is recommended (Chapuis *et al.,* 1998).

2.3 Solution for a sustainable eco-friendly vineyard

In theory, if we choose healthy plants from the nursery without any type of diseases and propagate them, we should be able to create a new nursery free of diseases. And if we took those plants to a new vineyard we should be able to create a healthy vineyard with no diseases. However this is practically impossible once the air and soil are contaminated with fungi responsible for trunk diseases (Díaz *et al.*, 2013).

Therefore, since it´s impossible to grow vineyards completely free from wood diseases, in order to reduce its incidence, we need to protect the vines from trunk diseases and strengthen them so that they can withstand better in hostile environments.

So, in this project we are going to use two beneficial microorganisms that protect and strength vines, *Trichoderma* and Mycorrhiza.

2.4 Studied microorganisms

2.4.1 Trichoderma

Trichoderma is a genus of asexual fungi primarily isolated from soils, roots or leaves of plants which are present in every type of soils (tropical and temperate) (Fig. 8) (Howell, 2002).

This type of fungi presents high genetic diversity, and can be used to produce various products of commercial and ecological interest. *Trichoderma* are extracellular proteins producers and are well-known for their ability to produce enzymes that degrade cellulose and chitin although they produce other important types of enzymes (Kubicek and Harman, 1998). There are strains capable of producing different antibiotic-capable compounds. These fungi can compete with other microorganisms, for compounds, like, key compounds, released by the seeds that stimulate the germination of pathogenic fungi of plants in the soil, besides competing with these microorganisms for space and nutrients (Bolar *et al.,* 2001). So, therefore, *Trichoderma* are bio fungicides that can be used to eliminate trunk diseases fungi in grapevines.

In addition to this ability of directly attacking or inhibiting the growth of pathogens, recent studies indicate that *Trichoderma* induces systemic and localized resistance against these agents (Bolar *et al.,* 2001).

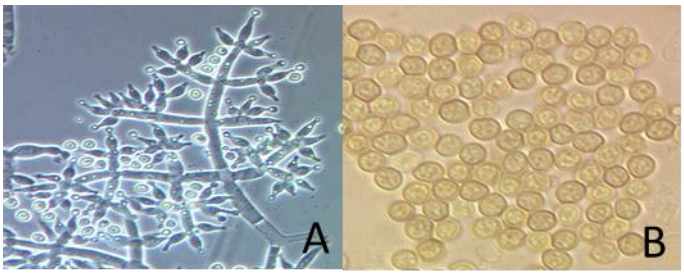

Figure 8 - *Trichoderma harzianum* hyphae (a) and *Trichoderma aggressivum* conidiospores (b).

2.4.1.1 Historical review

The first description of the gender *Trichoderma* was back in 1974 by Persoon who described four different species in which only *Trichoderma viride* was remained unchanged (Persoon, 1974). Then, in 1865, Tuslane brothers established the connection between *T. viride* and *Hypocrea rufa*, the teleomorph equivalent of *Trichoderma*, using as base the stomatic hyphae format and comparing with the *Trichoderma* conidiophore (Tulasne, 1930).

During the following years some discovers were made however, the different species assigned to the genus *Trichoderma*/Hyocrea were very difficult to distinguish morphologically. In 1927, Abbott was able to classify four different groups, but only three

were added to the classification (Abbott, 1926). In 1939, Bisby observed species of *Hypocrea rufa* and *Hypocrea gelatinosa* and was not able to distinguish between them, assuming that *H. gelatinosa* was only a state of growing or an immature condition of *H. rufa.* Also, in 1956, Dingley characterized several species of *Hypocrea*, relating them with its anamorph, *T. viride (Dingley, 1957; Samuels et al., 2006).*

However, only in 1969 the development of a concept for identification was initiated by Rifai that used sexual characteristics to distinguish between *Trichoderma* species, dividing them in nine groups (morphologically similar species in each group) (Rifai, 1969).

Thereafter, several new species of *Trichoderma* were reported, and by 2006, the genus already presented more than 100 defined species (Druzhinina *et al.,* 2006).

2.4.1.2 Morphology

Trichoderma spp. cultures, usually grow fast between 25-30 °C, however, they are thermo sensitives at 35°C. Initially, the colonies are transparent in CMD medium (cornmeal dextrose agar) or white in PDA medium (Potato dextrose agar). The mycelium don´t show up on CMD medium, however, the conidia create aggregates loose or compact, usually green or yellow (Gams *et al.,* 1998).

Conidia, ovoid and hyaline, are formed from conidiogenic cells. These are at the end of pyramid-like branched structures known such as conidiophores (Grondona *et al.*, 1997).

The shape of conidia varies from globose to ellipsoidal, ovoid or cylindrical short with the basal part more or less conical and truncated. The range of the size of the conidia in *Trichoderma* is not large, however, some species can always be differentiated by small size differences. The surface of the conidia appears smooth in the majority of the species in microscopic observation, nevertheless, may also have rough appearance as in *T. viride*, and may have a wing shape or bubble projection as in *T. saturnisporum* and *T. ganense*. The conidia pigments can range from colourless to dark green, or less frequent gray or brown (Gams *et al.,* 1998).

Chlamydospores are common in a large number of species, although uniform, globular or ellipsoidal, terminal or intercalary, smooth-walled, yellowish or greenish forms with a

diameter of 6-15 µm in most species can be present in a uniform manner (Gams *et al.,* 1998).

2.4.1.3 Mycoparasitism

Trichoderma are capable of parasitizing other fungi. The events that give rise to mycoparasitism are very complex. *Trichoderma* strains detect a fungus and develop towards it (Chet *et al.,* 1981). The sense of location of *Trichoderma* functions, at least partially, due to the expression of enzymes that degrade the cell wall. Different species may follow different types of induction, but the target fungus always produces a small amount of exochitinases. This enzyme catalyzes the release of oligomers from cell wall cells by the target fungus, which, in turn, induces the production of fungitoxic endocitinases by *Trichoderma* (Brunner *et al.,* 2003), which attack the target fungus even before contact (Viterbo *et al.,* 2002; Zeilinger *et al.*, 1999). When *Trichoderma* comes in contact with the target fungus, *Trichoderma* move around it forming an appressoria (specialized cell typical of many fungal plant pathogens that is used to infect the host). Once in contact with the fungus, *Trichoderma* produces several enzymes that degrade cell walls (Kubicek and Harman, 1998) and probably different types of antibiotics (Schirmböck *et al.,* 1994). The combined activities of these products lead to a parasitism in the target fungus, destroying theirs cell walls and, finally, killing it. This mechanism of mycoparasitism is very important since it explains how *Trichoderma* directly attack plant pathogens.

2.4.1.4 Induced resistance in plants

Induced resistance in plants has not been much studied in the past, perhaps because researches were more focused on the direct effects that *Trichoderma* have on plant diseases.

Resistance induction systems in plants by *Trichoderma* are very complex; however we can describe three possible ways of inducing resistance. Two of them involve the direct production of proteins related to the pathogen. The production of these proteins is usually caused by wounds or necrosis that the pathogen (ex: insects) origin in the plant. In the

case of insects the plant produces salicylic acid as a signal molecule and in the case of herbivores the plant produces jasmonic acid as the signal molecule (Hammerschmidt *et al.*, 2001). The pathway induced by jasmonic acid and salicylic acid, cause a cascade of reactions that induce the systemic response in the plant.

The third type of induced resistance has been described as being induced by a nonpathogenic agent. It is similar to the previous types as it results in a systemic plant response. However, it functions differently, since the pathogen-related proteins are not induced by the colonization of pathogens in the roots, but in their absence. Nevertheless, in the presence of the pathogen the response is more intense than in its absence (Bostock *et al.*, 2001).

2.4.1.5 Increase of the roots and the plant growth

Trichoderma and other beneficial microorganisms that colonize the roots are capable of increasing the growth of the plant. Intuitively, this idea may seem counterproductive since these organisms induce resistance in plants, and this process spends a lot of energy on the plant. However, many resistance-inducing fungi also increase the growth of roots and stalks (Lindsey and Baker, 1967; Lumsden and Vaughn, 1993; Pozo *et al.,* 2002). *Trichoderma* increases root growth and, consequently, plant productivity. In many cases, the responses are the result of direct effects on the plant, reducing the activity of microorganisms that destroy the roots, and inactivating toxic compounds present in the soil. *Trichoderma* increases the plant nutrient uptake and nitrogen use, and helps to solubilize soil nutrients (Pozo *et al.,* 2002). The way these processes occur at the molecular level is still unknown, however, it is known that there are differences in the responses of plants depending on their species.

2.4.2 Mycorrhiza

Mycorrhiza are symbiotic associations, essential for one or both partners, between a fungus (specializes in soil and plant development) and a root (or another part of the plant capable of symbiosis) responsible for the transfer of nutrients (Brundrett, 2004).

The fungal hyphae associate with the roots of the plant making the plant more able to absorb water and minerals from the soil, making it more resistant to adverse conditions (Fig 9.). In return, the plant provides the fungus with amino acids and carbohydrates essential for its growth.

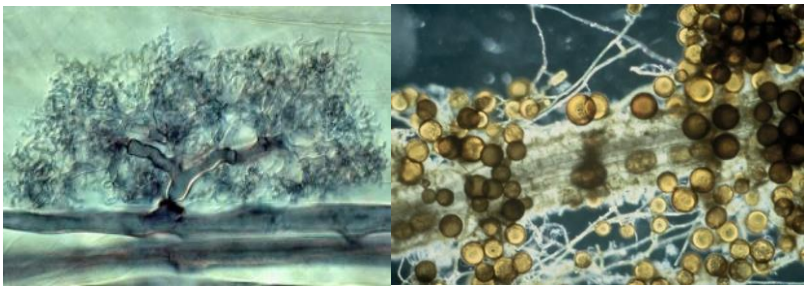

Figure 9 - Mycorrhizal mutual association with plant roots.

2.4.2.1 Historical review and classification

It is believed that arbuscular Mycorrhiza (AM) has been described for the first time in 1842 by Nägeli, 1842, however, his drawings were only remotely similar to arbuscular Mycorrhiza. Only, Frank, 1885, came up with the name “Mycorrhiza” to an association between tree roots and ectoMycorrhizal fungi for the first time.

Nowadays, at least 80% of terrestrial plant families form AM symbiosis (Newman and Reddell, 1987; Wang and Qiu, 2006). Arbuscular Mycorrhizal fungi belong to the Glomeromycota, which was originally divided into four orders (*Paraglomerales, Glomerales, Diversisporales* and *Archaeosporales*) with around 150 to 200 species (Schübler *et al.,* 2001). In 2010, Schübler and Walker made a reformulation of the species within these orders.

On the basis of morphological and anatomical features, Mycorrhiza are divided into three types: ectoMycorrhiza, endoMycorrhiza and ectendoMycorrhiza. EndoMycorrhiza are the most common Mycorrhiza and the first to evolve. They belong to *Glomeromycota* family and are associated with about 80% of plant species, including vines. EndoMycorrhiza are characterized as the name indicates by the development of the fungus inside the tissues of the host (plant). In this group the arbuscular Mycorrhiza are included in which the fungus develops inside and outside the cells of the cortex, creating shrubs that are highly branched structures (Brundrett, 2004).

The AM symbiosis usually starts with a spore present in the soil. When humidity is enough, the spore germinate originating a short germination mycelium, and travels through the soil, in order to find a plant host. This process is possible through a chemical gradient coming from the plant roots, a combination of signals (Bonfante and Genre, 2010). In case the spore is not able to find a host, its hyphae re-enter a dormant stage until the next opportunity. In the instance that an adequate plant host is in the vicinity of the germinating spore, the AM fungus releases its own cocktail of chemical signals that work as recognition signal. In case the plant recognizes the AM, it releases root exudates molecules that induce hyphae growth and branching. The hyphae of the AM fungus make their way to the inner layer of plant cells in the growing root tip, or area of active exchange, and the hyphal terminus will enter in a plant cell space with the help of specialized cells called apressoria. The cytoplasm of the cell moves over to allow the fungus to introduce the arbuscule, that is the structure that permits the fungus and plant cells to have direct membrane exchange of nutrients and photosynthates, or sugars (Fig. 10). As the plant develops, the AM (particulary the arbuscules) moves along with the growing root tip (Bonfante and Genre, 2010). The extracellular phase consist in a mycelium that will extend in the soil to capture water and nutrients, releasing spores that will guarantee the reproduction of the AM (Bonfante and Genre, 2010) (Fig. 10).

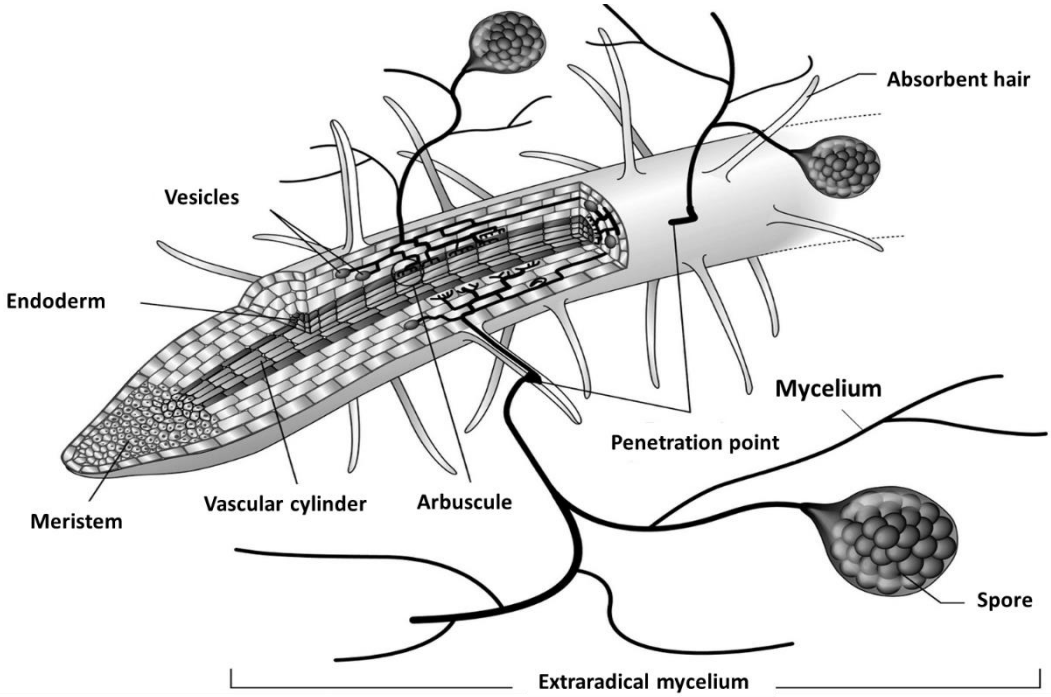

Figure 10 - Scheme of an arbuscular Mycorrhiza (AM), showing the structures present on the soil (extracellular mycelium and spores), and the structures inside the plant root (arbuscules and vesicles). (Adapted from https://www.agrireseau.net, accessed on 17/02/2019)

As previously mentioned, arbuscular Mycorrhiza are able to form a symbiosis with the roots of plants such as vines, simplifying the absorption of water and minerals by the plant. As such, these associations can offer innumerous advantages to the plants, and although they don´t protect the plant from diseases like *Trichoderma*, Mycorrhiza make them more capable of living in stressful environments, like water stress, that is very important nowadays due to the climatic changes. Besides that, Mycorrhizas present numerous other advantages for vines, which will be discuss hereafter (Fig. 11).

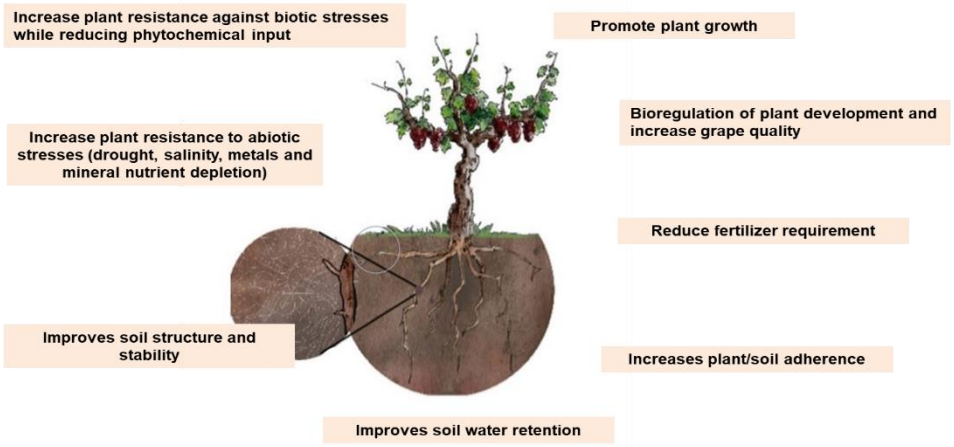

Figure 11 - Benefits of arbuscular Mycorrhizal symbiosis for grapevines (Adapted from Trouvelot *et al.*, 2015).

2.4.2.2 Growth rate and nutrition

Nutrition plays an essential role in the functioning of the vine and the quality of the wine. Fertilization is very often the used technique to ensure that plants have all the nutrients necessary for development in the soil since most soils are poor in nutrients, however it´s

a very complex process since it depends not only on the characteristics of the soil but also the type of plant.

Arbuscular Mycorrhiza (AM) increase the absorption capacity of phosphorus, nitrogen, and other nutrients, and reduce drastically the need for fertilizer use in the soil.

Phosphorus deficiency occurs mostly in acid soils that are promising to its fixation, but also in areas with high rainfall (Menge, 1982). The main source of phosphorus for plants is soluble phosphorus ($HPO4^{2-}$), which results from the destruction of organic forms transformed by soil microorganisms. This form of phosphorus has a very low concentration in soils due to its slow diffusion. In roots with AM, the mycelium forms an extensive network between the depletion zones of the roots, decreasing the diffusion distance of phosphorus in the soil (Harrison, 1999).

Although soil nitrogen input is often reduced to minimize vegetative growth of the plant and improve grape quality, nitrogen extraction from the soil is crucial for grapevine metabolism (Wermelinger *et al.,* 1991). The role of AM in extraction nitrogen from the soil is not fully studied, however, is estimated that AM's can capture $NO3^-$ and $NH4^+$, as well as organic nitrogen from the soil (Gachomo *et al.,* 2009). AM are also able to capture organic nitrogen by decomposing organic materials (Hodge and Fitter, 2010), and in vines, Mycorrhizal nitrogen input may also increase plant biomass (Karagiannidis *et al.*, 2007).

2.4.2.3 Increased tolerance of vines to abiotic stresses

Abiotic stresses can be responsible for large losses in vineyard productivity. Drought, salinity or heavy metals can cause serious problems on vines. In very dry conditions, the yield of the plants is very low and the perenniality of the plant can disappear. However, in dry regions, the AMF mycelium and/or shrubs development, increase significantly the area of root absorption and decrease the effect of the dryness in the plant (Schreiner *et al.,* 2007).

Soil salinity is related to the content of dissolved salts in soils and can be a limiting factor for viticulture productivity, especially in arid and semi-arid regions (Belew *et al.,* 2010). When too high, salinity inhibits the growth of the plants and affects the quality of the berries (Walker *et al.,* 2002). It has been scientifically proven that AM occurs in soils with

high salinities (García and Mendoza, 2007) and decrease the salt content in the soil, consequently reducing plant stress and raising the input of nutrients such as phosphorus, iron, nitrogen, zinc and cooper. The increase of phosphorus in the plant is probably the best way for the plant to reduce stress caused by salinity, but there are other less studied pathways (Giri and Mukerji, 2004).

Copper-based fungicides are the most used in vineyards for the treatment of diseases such as downy mildew, increasing the concentration of this compound in soils to toxic levels (Giri and Mukerji, 2004). To persist in toxic soils, AM´s developed a series of strategies that avoid the damage caused by metals such as copper (Ferrol *et al.,* 2009). Although the cellular mechanisms behind this adaptation are still poorly understood, AM are already widely used for viticulture in soils with high concentrations of copper.

2.4.2.4 Protection against biotic stress

Most vines suffer from diseases such as mildew and powdery mildew, which cause significant losses in production and require the use of many fungicidal chemicals. However, these fungicides are not completely effective against trunk diseases.

Plants can develop Mycorrhizal-induced resistance that promotes systemic protection against various types of pathogens (Cameron *et al.,* 2013). These reactions are similar to those previously reported in this thesis for *Trichoderma.*

2.4.2.5 Increasing of soil stability

The majority of the vineyards are planted on terraces with a high slope increasing the risks of soil erosion. The symbiosis between Mycorrhiza and vines results in a complex network around the soil (Cavagnaro *et al.,* 2005). This network creates a link between roots and soil, increasing soil stability and improving soil structure. Moreover, the production of “sticky” hydrophobic substances by glomalin-rich Mycorrhiza also contribute to soil stability and water retention (Bedini *et al.*, 2009).

2.5 Molecular markers as a tool to identify fungal species

Once grapevine trunk diseases partakes no effective treatment, early detection is crucial in order to avoid the destruction caused by this diseases. Also, during a biocontrol research work, it is important to follow the success of microorganisms inoculation. Advances in molecular technology have a great potential for the rapid detection and identification of fungi for several purposes. Numerous targets within the fungal genome have been evaluated, focusing on using sequences within the ribosomal DNA (rDNA) gene complex. This section of the genome includes the 18S, 5-8S and 28S genes which have a relatively conserved nucleotide sequence among fungi. It also includes the variable DNA sequence areas of the intervening internal transcribed spacer (ITS) (White *et al.,* 1990).

2.5.1 ITS gene

According to White *et al* 1990, DNA sequences present on ribosomal RNA provide ways to analyze the phylogenetic relationships on all taxonomic levels. As they are present in all organisms and because they have multiple copies on the genome, the ribosom DNA sequences are adequate to study phylogenetic relations between organisms. These regions are the most important in fungi, since they are separated by intergenic spacer sequences that can change the position and direction of the transcription process according to the fungus (Bruns *et al.,* 1992; Hillis and Dixon, 1991).

The ribosomal DNA region is divided on three different genes. The first one, is the small unity (SSU) localized on 17-18S position, has a slow evolutionary development, being useful on studies that evaluate the kinship degree between organisms. The second region, the large one (LSU), is located on 25-28S and has variable regions on the first 900 base pairs, so this area is the most used in phylogenetic studies (White, 1990) (Fig. 12).

The third region is the ITS region and it´s located between 18S and 28S regions, and by being an highly variable conserved region in all fungi, allows the user to distinguish between fungi species. Besides, the ITS region is small and easy to amplify, being widely used in the identification of fungus species. ITS region is divided in two parts, ITS-1

located between 18S and 5.8S, and ITS-2 between 5.8S and 28S (Atkins and Clark, 2004; Schlötterer *et al.,* 1994; White, 1990).

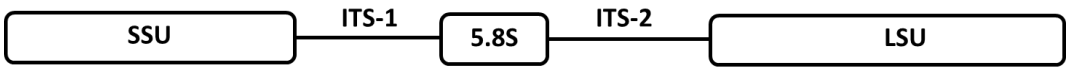

Figure 12 - Schematic representation from the ribosomal DNA region (Adapted from Hagn *et al.,* 2007).

On *Trichoderma*, the ITS region has a significant role on molecular characterization, since each one of the *Trichoderma* species already have sequences of this gene on Genbank (Druzhinina *et al.,* 2006). Once the ITS region is one of the most studied DNA region and it´s very specific, it has been the most used to identify the gender, however, when it comes to the species, some care must be taken, since in very similar species, may not exist differences on the ITS region (Druzhinina *et al.,* 2006).

3. Material and Methods

3.1 *In vitro* assay

3.1.1 Source of *Trichoderma* and pathogenic fungi

For this experiment, two *Trichoderma* commercial products were used: Tifi® from "Agrotecnologias Naturales" prepared with 1.5% p/p of *T. atroviride* Strain MUCL 45632 and Mamull® from "Bio Insumos Nativa SpA" prepared with *Bionectria ochroleuca* strain *Mitique*, *T. gamsii* strain *Volqui* and *Hypocrea virens* strain *Ñire*, all at the concentration of 0.33% p/p (1 x 10^7 cfug). These commercial products were diluted in 1/10 parts of sterilized water and put in a PDA medium plate during five to seven days to grow.

Additionally, three native strains were used in this lab essay: one of *T. virens "*Sherwood" strain isolated from the Sclerophyll forest in the Maule region of Chile (available on the Phytopathology laboratory bank on the University of Talca, Chile) and two *Trichoderma* spp. strains isolated from organic vineyards of Concha y Toro Holding, located at O´Higgings region, Chile.

The trunk diseases fungi tested were *P. chlamydospora, Inocutis* spp, *E. lata, D. seriata* and *N. parvum* taken from the Phytopathology laboratory bank on the University of Talca, Chile.

Before the beginning of the experiment (the time depends on the growth rate of the fungus) new cultures of all fungi were made by, taking a 5 mm diameter mycelial disc from the stored culture and placed in the centre of Petri dishes containing potato dextrose agar.

3.1.2 Potato dextrose agar medium preparation

Ten grams of mashed potatoes, 10 g of dextrose (Difco™, Detroit, MI) and 15g of agar (Bacto™ Agar BD, Difco™, Detroit, MI) were dissolved in 500ml of distilled water in an Erlenmeyer flask (500ml) and 50µl of lactic acid were added to the mixture. The flasks were sealed with the cork not completely closed and aluminium paper covering the top, and autoclaved (Huxley HL 340 autoclave) at 121°C for 15min. The autoclaved media were allowed to cool and thereafter 25ml of the autoclaved medium was added to each Petri dish inside the laminar flow chamber (sterilized environment).

3.1.3 Paired cultures assay

3.1.3.1 Paired cultures assay "13 days assay"

This *in vitro* experiment was performed with one *Trichoderma* native strain or commercial product against one trunk disease fungi in plate. All *Trichoderma* spp. were tested against all trunk disease fungi and each treatment consisted of three replications. The inoculation was carried out under sterilized conditions. A mycelial disc of *Trichoderma* spp. and the pathogenic fungus (5mm of diameter) were taken from the active growing area of each colony with a core sampler and placed in opposite sides of the plate, at approximately 0.5 cm distance from the periphery of the dishplate (Fig. 13). The dishes were incubated at 20-25°C with 16h of light exposure and 8h of darkness and photographed every day during thirteen days.

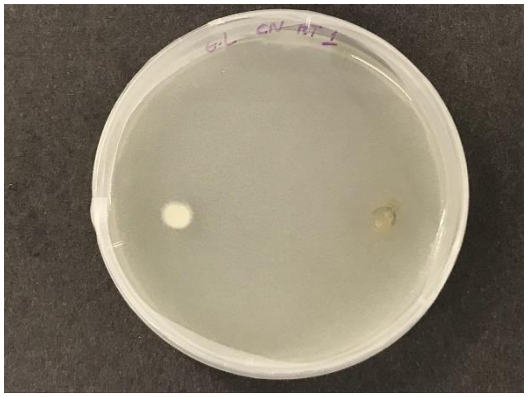

Figure 13 – Paired culture assay “13 days” on the first day of the assay.

3.1.3.2 Paired cultures assay “20 days assay”

This assay was generally similar to the above described 13 days assay, except that in the “20 days assay”, the wood fungi were placed in the dish five days before the *Trichoderma* spp. (Fig. 14).

The dishes were stored at 20-25°C with 16h of light exposure and 8h of darkness and photographed every day during twenty days.

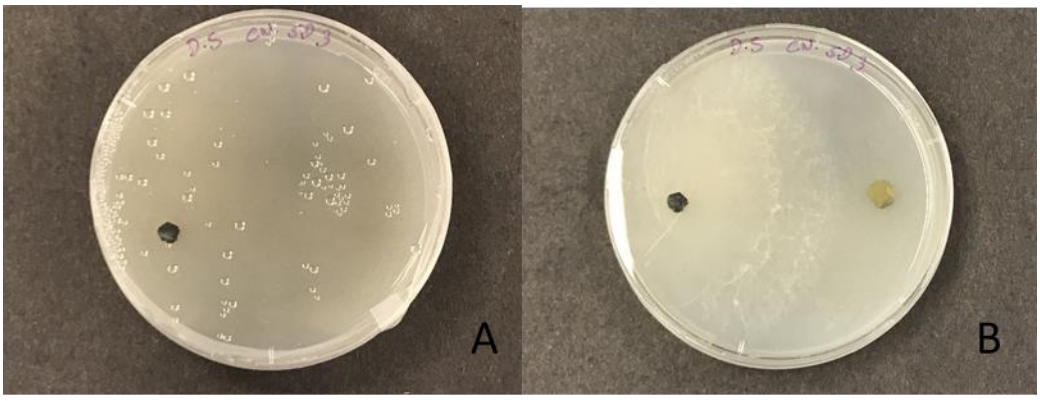

Figure 14 - (A) Paired cultures “20 days assay” on the first day of the experiment with a mycelial disc of wood fungi and (B) paired cultures “20 days assay” on the fifth day of the assay with a mycelial disc of wood fungi” and *Trichoderma* spp.

3.1.3.3 Paired cultures “triple assay”

The triple assay was performed like the “13 days assay”, however, 15cm diameter dishes were used and two wood fungi and one *Trichoderma* were placed in each dish, against

each other (Fig. 15). In this experiment, the *P. chlamydospora* and *Inocutis* spp., were not tested due to their slow growth rate. The dishes were stored at 20-25°C with 16h of light exposure and 8h of darkness and photographed every day during thirteen days.

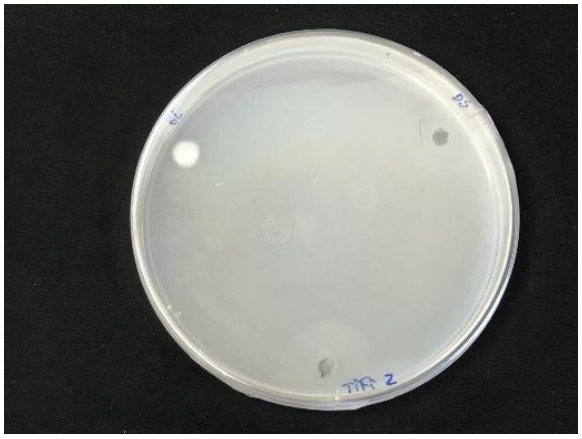

Figure 15 - Paired cultures “triple assay” on the first day of the experiment.

3.1.4 Statistical analysis

During the experiment, paired cultures assays were photographed and pictures were analyzed using ImageJ software (Image Processing and Analysis in Java), in order to define the area occupied by each fungus on the dish. This process was daily repeated for all the different assays.

The statistical analysis was performed using one-way ANOVA and mean comparisons were made using Turkey’s test with a significance level of 5%.

3.2 *Trichoderma* and Mycorrhiza molecular detection

3.2.1 Biological Material

In this experiment were used two Mycorrhiza commercial products, two *Trichoderma* commercial products, roots of plants inoculated with these commercial products and roots without inoculation.

The Mycorrhiza commercial products were AEGIS-GEL® from ITALPOLLINA, prepared with *Glomus intraradices* and *Glomus mosseae* at the concentration of 50 spores/cc and

Oiko-Rhiza-E from OIKOS CHILE LTDA, prepared with *Glomus brasillanum, Glomus clarum, Glomus deserticota, Glomus intraradices, Glomus mossae, Glomus monosporum* and *Gigaspora margarita* at the concentration of 500 spores/cc.

The *Trichoderma* commercial products were, Tifi® from "Agrotecnologias Naturales" prepared with 1.5% p/p of *Trichoderma atroviride* (Strain MUCL 45632), and Mamull® from "Bio Insumos Nativa SpA" prepared with *Bionectria ochroleuca Mitique* strain, *Trichoderma gamsii Volqui* strain and *Hypocrea virens Ñire* strain, all at the concentration of 0.33% p/p (1 x 107 cfu/g).

All the plant materials (inoculated roots with Mycorrhiza and *Trichoderma* commercial products and inoculation and no inoculated roots) were taken from the Concha y Toro nursery. Three plants were randomly collected in order to create a composite sample for each treatment. Without removing the plant from the nursery field, the lateral roots (the thinnest roots are more likely to be mycorrhized than the principal roots) were removed and put in a properly identified bag (approximately 15gr per sample). The samples were washed with tap water and then twice dried in paper. After, the smallest roots were selected, grinded in a mortar and then frozen using liquid nitrogen. Each 3-5gr sample was stored in a falcon tube at -80°C until being used.

In order to establish the best DNA extraction protocol and the best PCR conditions to extract and amplify the Mycorrhiza commercial products, a sample with higher concentrations of Mycorrhiza was used, in this case, grass. The samples were collected from the Concha y Toro field, washed with tap water and then twice dried in paper. After that, the samples were grinded in a mortar, frozen using liquid nitrogen, and stored at -80°C until being used.

3.2.2 DNA extraction

DNA extraction was performed using a protocol based on the DNeasy® PowerSoil® Kit from Qiagen®. Some modifications were made to the original manufacturer protocol. Firstly, a root sample of 0.1g was heated at 60°C for 20 min with the cell lyses solution in an orbital shaker incubator and the manufacturer protocol was followed from this point on. DNA quantification was performed using the Tecan Infinite® 200 PRO NanoQuant (photometer) and the Invitrogen Qubit 4 Fluorometer, using the provider procedures.

3.2.3 Primer Design

In order to ensure the success of the *Trichoderma* inoculation, molecular detection was required to detect the presence of the commercial product strains on the plant roots. For that propose, specific primers were designed for each strain targeting the ITS region of the genome. This is a highly variable conserved region in all fungi, which allows the user to distinguish between species. Besides, ITS sequences are small, easy to amplify and there is currently a large number of sequences described and available on GenBank®. A hundred ITS sequences of a specific species were saved from the GenBank® in a word file. Ten sequences at a time were aligned on ClustalW2 to find a consensual sequence (part of the sequence that repeats in every aligned sequences) and the ten consensual sequences, therefore obtained were again aligned on ClustalW2 in order to find the last consensual sequence. After running this consensual sequence on Blast® to determine the similarity between it and the biological sequences from each the species evaluated, the sequences were introduced in two different softwares, Primer3Plus and Geneious that created the required primer sequences to amplify our consensus sequence. In the last step, the final primers sequences (forward and reverse) were sent to Integrated DNA Technologies, Inc, to synthesize.

In this experiment, we created sets of primers for *T. gamsii, T. atroviride* and *T. harzianum* (two sets of primers) to be tested (sequences are on attachment 1 supplementary table 2). Besides the designed primers, another set of primers from Hagn *et al.,* 2007 (UT primers) was tested in this experiment.

On the case of Mycorrhiza, no primers were newly designed. The primers tested were from Krüger *et al.,* 2009. This mixed primer set, amplifies an arbuscular Mycorrhiza rRNA fragment of approximately 1500 bp that covers part of the SSU region, part of the LSU region and the entire ITS region, combining a mix of forward primers and a mix of reverse primers (Krüger *et al.,* 2009), in order to be able to amplify DNA from all *Glomeromycota* species (Fig. 16) (sequences are on attachment I supplementary table 1).

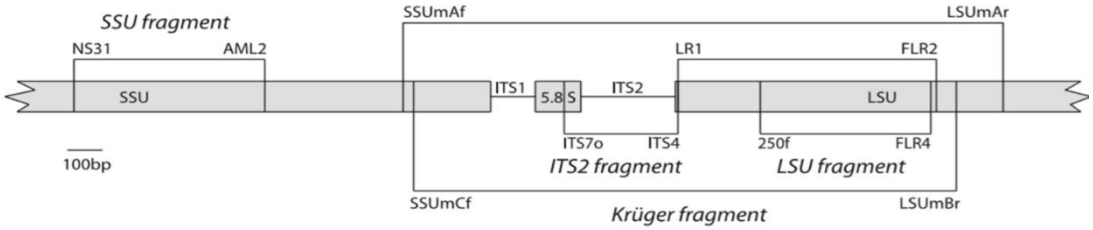

Figure 16 - Target nuclear ribosomal DNA regions and primers locations (Adapted from Kohout *et al.,* 2014).

Also, a set of primers specific for Glomus sp. was tested from Alkan *et al.*, 2006 (sequences are on attachment I supplementary table 1).

3.2.4 Mycorrhiza and *Trichoderma* detection

To detect the presence of *Trichoderma* and Mycorrhiza in root samples molecular detection approach was performed, in which the Polymerase chain reaction (PCR) (Mullis and Faloona, 1987) technique was applied. For this reaction the GoTaq (R)® Green Mater Mix from Promega was used as described in the instruction's manual. The PCR consisted in a first step of denaturation (2 min, 95°C), followed by 30 to 35 cycles of denaturation (15 seconds, 95°C), annealing (15 seconds, primers annealing temperature) and extension (1 minute for 1kb amplified, 72°C) followed by a final extension step (5 minutes, 72°C). The standard 10 µL and 25 µL reaction mixes used on the lab and its components are indicated on table 1, however there were some exceptions indicated on the attachment I table 4. The primers used on this experiment are specified on the attachments. An Applied Biosystems, Veriti 96 Well thermal cycler was used and programmed as specified before, depending on the primers used on the reaction (specific conditions of annealing are described on the attachments). Before any reaction, all the samples were diluted to 10ng/µl in order to dilute any PCR inhibitors that could be present on the sample.

Table 1. PCR reaction components using GoTaq (R)® for 10 and 25µl of reaction per sample.

Reagents	Volume for 10µl of reaction per sample	Volume for 25µl of reaction per sample	Final concentration
Sterile water	2.6 µl	9.5 µl	-
Forward primer	0.2 µl	0.5 µl	10 pMol
Reverse primer	0.2 µl	0.5 µl	10 pMol
GoTaq (R)®	5 µl	12.5 µl	1 X
DNA sample	2 µl	2 µl	50-100 ng

Results of the PCR reactions were observed by electrophoresis (Ritchie *et al.,* 1976) on agarose gel at 1.5% (m/v) from Bacto™ Agar BD on TAE 1X buffer (diluted from VWR Amresco Life Science TAE 50X). GeneRuler DNA Ladder Mix (Thermo Scientific) was used as molecular weight marker and TAE 1X was used as the electrophoresis buffer. The electrophoretic separation was performed between 80-110 V, during 40-50 minutes. The fluorescence obtained with the use of GelRed on the agarose gel preparation, allowed the DNA visualization on a UV transilluminator. The images were shot with a Canon Rebel T3 EOS 1100D and analysed with Vision Works LS image acquisition and analysis, UVP V7.1 RC 3.54.

3.2.5 Sequence Analysis

In order to identify the species present in our set of samples, the PCR products and/or the gel fragments were purified using Purelink™ Quick gel Extraction and PCR Purification combo kit and then, sent to Macrogen, Inc (Korea) to sequenciation on both ways (forward and reverse). To refer that the first samples were sequenced based on the gel fragments, but since the results were not satisfactory, the second group of samples were sequenced based on the gel fragments and the PCR products to obtain more accurate results.

The reverse sequences resulting from sequenciation were run on GenScript to create a reverse complementary sequence. This sequence was posteriorly aligned with the forward sequence from the sequenciation on Clustal Omega. The consensus sequence was then run on Blast® to identify the specie.

3.3 Grapevine nursery assay

3.3.1 Grapevine nursery process

In general, the grapevine propagation process is quite similar among all the areas in the world. Chile is not an exception and Concha y Toro, in particular, follow the generic grapevine nursery process.

The grafting process in Chile starts in May, when cuttings from rootstock and scion mother plants are collected. These cuttings are stored at 5-6°C and high relative humidity (90%) (Cold treatment). In August/September, the cuttings are submersed in hydration tanks for about four days (hydration). After the hydration, buds are manually removed and rootstocks and scion cuttings are mechanically grafted with an omega-cut grafting machine (grafting) and after, the grafted area is sealed in a melted wax formulation. Grafted plants are stored at 26-27°C and 80-85% of relative humidity for about 20 days to promote the callus formation (stratification). After this time, plants with a good callus, buds and root formation are selected (budburst and selection) and transplanted to the nursery fields for one growing season in order to grow roots and buds. Following this year, plants are uprooted and stored at 5-6°C until being distributed and planted in vineyards.

3.3.2 Treatments and applications

This experiment was performed on Concha y Toro´s nursery during the 2017/2018 season and partakes three phases: the cleaning process, the *Trichoderma* inoculation and the Mycorrhiza inoculation. Each one of these phases includes three different treatments. The assay started with 4050 plants, 3 replicas for each 27 different treatment combinations, and each replica with 50 plants.

3.3.2.1 Cleaning process

In this assay, it were used three different cleaning processes, VCT process (implemented in Concha y Toro´s nursery) (L0), neutral anolyte from Aquannolyte® (L1) and hot water treatment (HWT) (L2), applied in different phases of the vine nursery process. The scheme of the vine nursery process used in Concha y Toro with the treatments is detailed on attachment II, supplementary table 5.

The VCT process included five steps. The first step occurred right before the cold treatment with a 10min immersion of the cuttings in a solution with two contact fungicides with preventive action, Rovral® 50% (6g/l) (BASF Crop Protection) and Pomarsol® Forte 80% (Bayer Crop Protection) (10g/l). The second step was done during hydration and consisted in an 8 hours treatment with a strong anti-infectious agent (chlorine dioxide >5%) 1ml/l. The third step was performed right before the grafting with a 6 hours

treatment with two systemic fungicides, Tecsa® Clor 50% (PROTECSA®) (1g/l) and Polyben® 50 WP *(Anasac* Chile S.A.). The fourth step occurred at the plant selection after the budburst, and involved the use of 10g of copper sulphate per bin as a fungicide. The final step happened right before the packaging and consisted in a 10min imersion of the plants in a solution with two contact fungicides with preventive action, Rovral® 50% (6g/l) and Pomarsol® Forte 80% (10g/l) (Tab. 2).

Table 2. List of commercial products used on the VCT cleaning process.

Commercial Product	Active ingredient	Manufacturer	Concentration
Rovral® 50%	Iprodione	BASF Crop Protection	6g/l
Pormasol Forte® 80%	Tetramethylthiuram disulphide (Thiuram)	BASF Crop Protection	10g/l
Tesca®Clor 50%	Chloryde Dioxide	PROTECSA®	1g/l
Polyben® 50WP	Benomyl	Anasac Chile S.A.	1 g/l

The neutral anolyte (disinfecting and sterilizing natural product, commercialized as electrolyzed water) treatment had five different steps. The first two steps, one on before the cold treatment and one on the hydration treatment, consisted in the immersion of the cuttings in a 25% neutral anolyte (Aquannolite®) solution for 6h. The third and four steps, right before the grafting and right before the stratification done with the pulverization of the plant material with a neutral anolyte (Aquannolite®) at 25%. The last step was right before the packaging and consisted in the immersion of the plant material in a 25% neutral anolyte (Aquannolite®) solution for 6h.

The hot water treatment had two steps. The first step happened right before the cold treatment and consisted in the immersion of the cuttings in a 54°C water solution for 5 minutes. The second step occurs on the hydration and consists in the immersion of the plant material in a 50°C water solution for 30 minutes.

3.3.2.2 Trichoderma treatment

In this experiment were used three different treatments, Mamull® ($1x10^7$cfu/g) (T1), Tifi® ($2x10^8$ cfu/g) (T2) and a water treatment (control) (T0), applied before and after the

grafting by an approximately 5 second aspersion in a 2% water solution. Mamull® is prepared with *Bionectria ochroleuca* strain *Mitique*, *Trichoderma gamsii* strain *Volqui* and *Hypocrea virens* strain *Ñire*, all at the concentration of 0.33% p/p (1 x 10^7 cfu/g) (Bio Insumos Nativa SpA) and Tifi® is prepared with 1.5% p/p of *Trichoderma atroviride* strain MUCL 45632 from Agrotecnologias Naturales.

3.3.2.3 Mycorrhiza treatment

In this experiment were used three different Mycorrhiza treatments, AEGIS-GEL® (2.5 cc/plant) (M1), OIKO-RHIZA® (5 g/plant) (M2) and a control (without treatment) (M0), applied by an approximately 5 second immersion before the plantation in the nursery. The products were prepared according to the manufacturer instructions.

3.3.3 Performed analysis

Several analysis were performed in this experiment in different stages of the grapevine nursery process.

3.3.3.1 Trunk diseases fungi and Trichoderma presence

The detection of trunk diseases fungi and *Trichoderma* strains on the plant roots was achieved using molecular detection tools such as PCR and q-PCR, before starting the assay (on the scions and mother plants), right after the grafting, and after removing the plants from the nursery field.

3.3.3.2 Budburst percentage

After the stratification, the percentage of budburst was determined using the following formula:

$$\%Budburst = \frac{number\ of\ plants\ with\ a\ successfull\ budburst}{number\ of\ grafted\ plants}\ x\ 100$$

3.3.3.3 Mycorrhizal analysis

Three months after the plants were transplanted to the nursery field, and when they were uprooted nine random root samples from each treatment were removed from the plants. This roots were dyed using a protocol based on Vierheilig *et al.*, 1998 and Pitet *et al.,* 2009. After, the samples were observed at a optical microscope in order to quantify the Mycorrhizal colonization.

The Mycorrhizal colonization estimation was perfomed according to Trouvelot *et al.,* 1986. This method involves observing the root fragments and grade them from 0 to 5 based on the Mycorrhizal colonization (Fig. 17). This classification allows a rapid estimation of the level of Mycorrhizal colonization in each fragment and the abundance of arbuscles. The values are placed on an Excel file to calculate the paramethers of estimation.

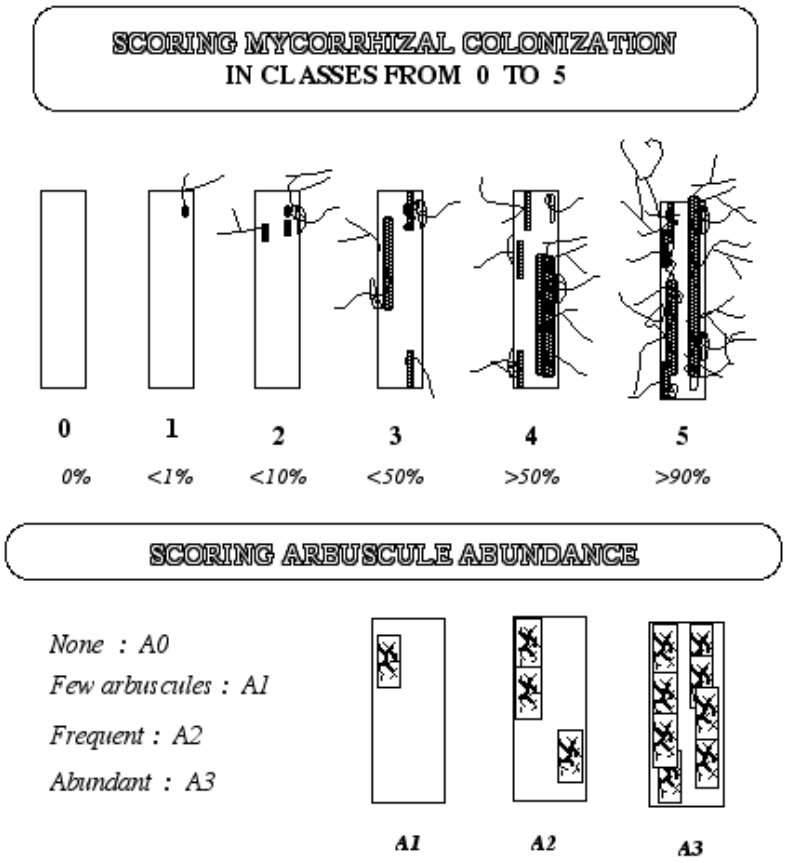

Figure 17 - Attributed value to the root fragments in function of its Mycorrhizal colonization and attributed value of the arbuscules abundance to calculate the estimation parameters of colonization and Mycorrhizal activity (Trouvelot *et al.*, 2015)

- Mycorrhiza frequency (F%) on the root system:

$$F\% = \frac{\text{number of fragments with Mycorrhiza}}{\text{Total number of fragments}} x100$$

- Mycorrhizal colonization intensity (M%) on the root system:

$$M\% = \frac{95n5 + 70n4 + 30n3 + 5n2 + n1}{\text{Total number of fragments}}$$

n5 = number of fragments categorized as 5
n4 = number of fragments categorized as 4
n3 = number of fragments categorized as 3
n2 = number of fragments categorized as 2
n1 = number of fragments categorized as 1

- Mycorrhizal colonization intensity (m%) on the root fragments:

$$\mathrm{m\%} = \mathrm{M}\frac{\text{Total number of fragments}}{\text{Number of fragments with Mycorrhiza}}$$

- Arbuscules abundance in the mycorrhized parts of the root fragments:

$$a\% = \frac{100mA3 + 50mA2 + 10mA1}{100}$$

Where:

$$mA3 = \frac{95n5A3 + 70n4A3 + 30n3A3 + 5n2A3 + n1A3}{Number\ of\ fragments\ with\ Mycorrhiza}\ x\frac{100}{m\%}$$

$$mA2 = \frac{95n5A2 + 70n4A2 + 30n3A2 + 5n2A2 + n1A2}{Number\ of\ fragments\ with\ Mycorrhiza}\ x\frac{100}{m\%}$$

$$mA1 = \frac{95n5A1 + 70n4A1 + 30n3A1 + 5n2A1 + n1A1}{Number\ of\ fragments\ with\ Mycorrhiza}\ x\frac{100}{m\%}$$

- Arbuscules abundance on the root system:

$$A\% = a\%\ x\frac{M\%}{100}$$

The parameters F%, M%, and A% were used to achieve results. The remaining parameters, m% and a%, were only calculated to achieve A%.

3.3.3.4 Chemical Nutrient analysis

After the plants were uprooted from the nursery field, the roots were removed from three random plants from each treatment. Dry weight as determined and subsequently, the nitrogen content, macro and micronutrients levels were evaluated.

The dry weight was determined by weighting the plant roots before and after a night in the greenhouse at 80°C, the nitrogen content was determined using the Kjedahl method (Bradstreet, 1954), and the macro and micronutrients level were determined using the ICP OES method (Larrea-Marín *et al.,* 2010).

3.3.4 Statistical analysis

All the data from these assays were analysed using a Multi-factor ANOVA (Statgraphics 18®). Means comparisons were made using the Turkey's test with a significance level of 5%.

4. Results

The purpose of this project was to evaluate different biological treatments using different *Trichoderma* spp. and Arbuscular Mycorrhizal Fungi (AMF) strains and their efficiency on nursery plants. For that three different type of assays were performed. The first one was a dish assay prepared in order to understand which *Trichoderma* strain is the most effective against pathogen fungi *in vitro*. The second was to create and test primers to detect *Trichoderma* spp. and AMF strains present on the nursery plants using PCR-based methods. For the last one, was performed a field experiment testing the effect of different *Trichoderma* spp. and AMF treatments on nursery grapevine plants.

4.1 *In vitro* Assay

In this experiment the real conditions of the field were recreated in order to test which *Trichoderma* strains are the most effective against pathogen fungi, so they can after be tested on the field.

In these experiments, two commercial products made from *Trichoderma* spp. species (Tifi® and Mamull®) and one native species of *Trichoderma* spp. ("Sherwood" strain) were tested against five different trunk diseases fungi in order to evaluate their virulence.

The trunk diseases fungi tested in this experiment were *P. chlamydospora, Inocutis spp, E. lata, D. seriata* and *N. Parvum*.

4.1.1 Paired cultures assay

4.1.1.1 Paired cultures assay "13 days assay"

P. chlamydospora and *Inocutis* spp. presented a very slow mycelial growth rate being easily inhibited by all *Trichoderma* treatments. Even though, Tifi® presented significant larger areas of mycelial grown on the dish than the other two treatments during the first days, at the end of the assay, all *Trichoderma* were effective in inhibiting these trunk diseases fungi under study (Fig. 18).

Regarding *E. lata*, the commercial products Mamull® and Tifi® show to be effective in controlling. On the contrary, were effective against, however the “Sherwood” strain was only capable of stop its growth. The “Sherwood” strain grew significantly less than the commercial products, and though Tifi® grew significantly more than Mamull® on the first seven days of the assay, at the end there were no significant differences between both treatments (Fig. 19).

Concerning *D. seriata*, all treatment but the “Sherwood” strain of *Trichoderma* were effective, Tifi® eliminated *D. seriata* on day 8, while Mamull® was only able to do it on day 11. Tifi® presented significant larger mycelial growing areas than Mamull® and “Sherwood” strain, Mamull® and the “Sherwood” strain presented no significant different values during the assay (Fig. 20).

Finnaly, Tifi® was the only effective treatment against *N. parvum.* Both, Mamull® and the “Sherwood” strain were able to stop the mycelial growth of *N. parvum*, however, both grew significantly less than Tifi® in the Petri dish. There were no significant differences between the Mamull® and “Sherwood” strain treatments (Fig. 21).

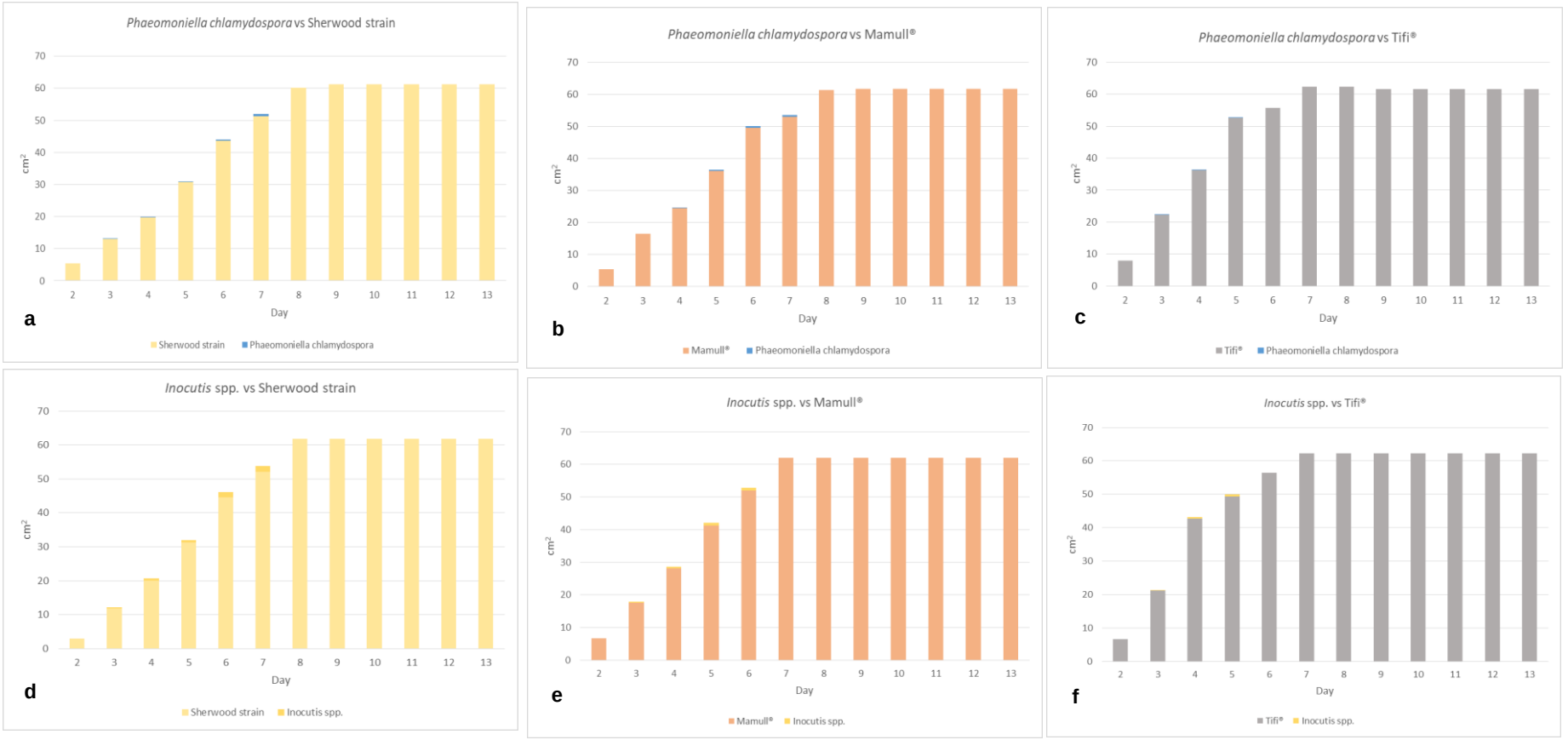

Figure 18 – Results obtained in paired cultures assay "13 days" with *Phaeomoniella chlamydospora*, *Inocutis* spp. and *Trichoderma* strains ("Sherwood"strain, *Trichoderma virens* isolated from the Sclerophyl forest in the Maule region of Chile, Tifi®: *Trichoderma atroviride* commercial product; Mamull®: *Bionectria ochroleuca, Trichoderma gamsii* and *Hypocrea virens* commercial product): a) *Phaeomoniella chlamydospora versus Trichoderma* "Sherwood" strain; b) *Phaeomoniella chlamydospora versus* Mamull; c) *Phaeomoniella chlamydospora versus* Tifi®; d) *Inocutis* spp. *versus Trichoderma* "Sherwood" strain; e) *Inocutis* spp. *versus* Mamull®; f) *Inocutis* spp. *versus* Tifi®.

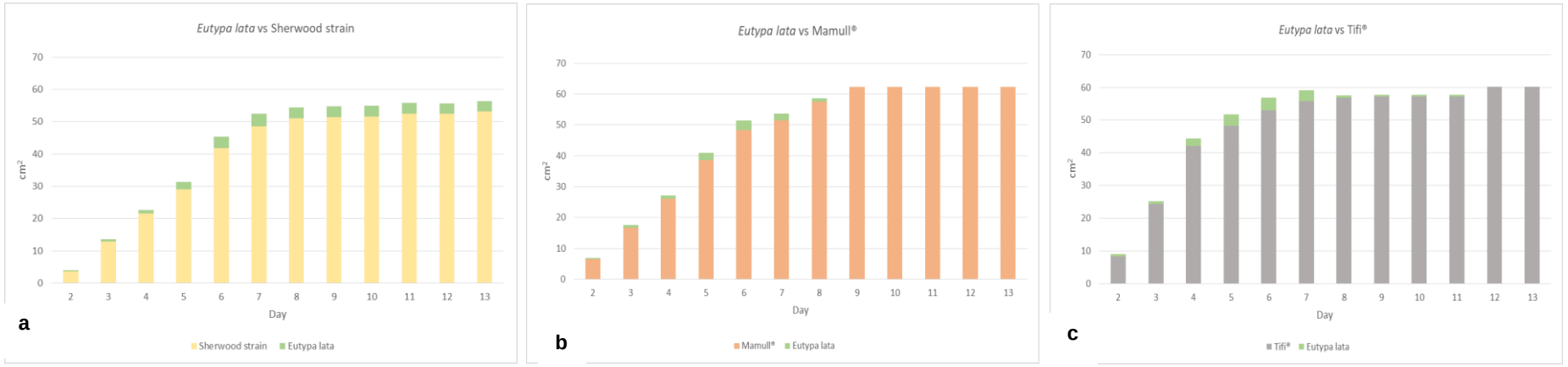

Figure 19 – Results obtained in paired cultures assay "13 days" with *Eutypa lata* and *Trichoderma* strains (*Trichoderma virens* "Sherwood"strain isolated from the Sclerophyl forest in the Maule region of Chile, Tifi®: *Trichoderma atroviride* commercial product; Mamull®: *Bionectria ochroleuca, Trichoderma gamsii* and *Hypocrea virens* commercial product): a) *Eutypa lata versus Trichoderma* "Sherwood" strain"; b) *Eutypa lata versus* Mamull®; c) *Eutypa lata versus* Tifi®.

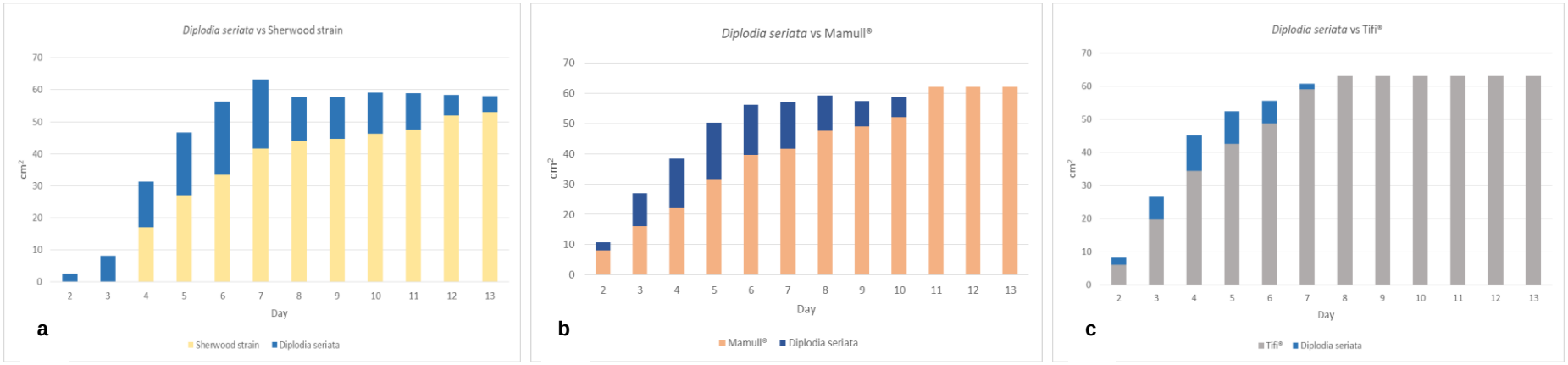

Figure 20 – Results obtained in paired cultures assay “13 days” with *Dipolodia seriata* and *Trichoderma* strains (“Sherwood”strain, *Trichoderma virens* isolated from the Sclerophyl forest in the Maule region of Chile, Tifi®: *Trichoderma atroviride* commercial product; Mamull®: *Bionectria ochroleuca, Trichoderma gamsii* and *Hypocrea virens* commercial product): a) *Dipolodia seriata versus Trichoderma* “Sherwood” strain”; b) *Dipolodia seriata versus* Mamull®; c) *Dipolodia seriata versus* Tifi®

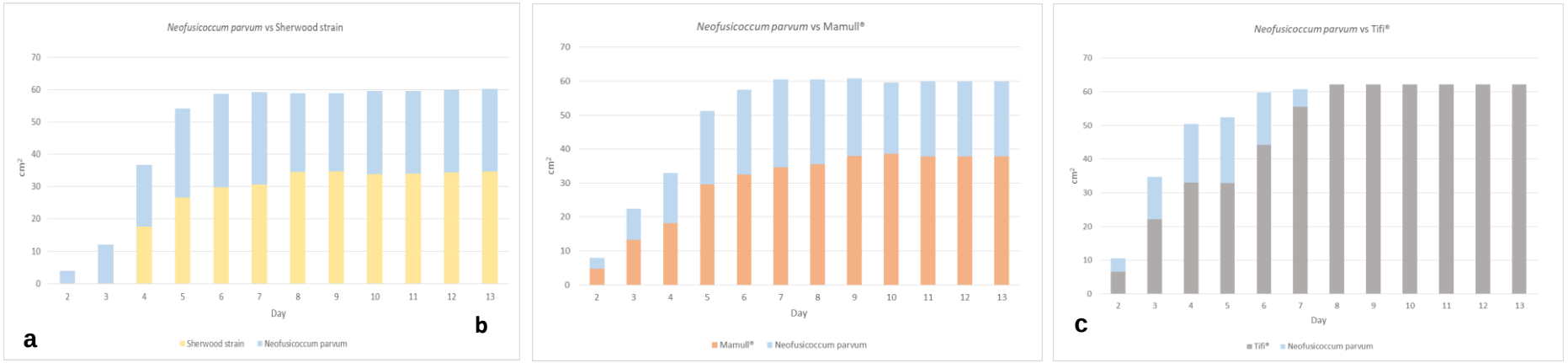

Figure 21 – Results obtained in paired cultures assay “13 days” with *Neofusicoccum parvum* and *Trichoderma* strains (“Sherwood”strain, *Trichoderma virens* isolated from the Sclerophyl forest in the Maule region of Chile, Tifi®: *Trichoderma atroviride* commercial product; Mamull®: *Bionectria ochroleuca, Trichoderma gamsii* and *Hypocrea virens* commercial product): a) *Neofusicoccum parvum versus Trichoderma* “Sherwood” strain”; b) *Neofusicoccum parvum versus* Mamull®; c *Neofusicoccum parvum versus* Tifi®.

4.1.1.2 Paired cultures assay "20 days assay"

In order to emulate the field conditions of trunk diseases, the *Trichoderma* was introduced five days later. This allowed evaluate the *Trichoderma* capacity to control the pathogen fungi when they're well established.

Results with *P. chlamydospora* and *Inocutis* spp. showed that, even with five days of advance, they didn´t grew significantly which revealed that all *Trichoderma* treatments were equally effective (Fig. 22).

In the previously essay, when placing in the dish at the same time of trunk disease fungus, Mamull® was slightly more effective against *E. lata* than Tifi®, however, when *E. lata*, is placed five days before to develop on the media dish, the only effective *Trichoderma* treatment is Tifi®. It showed significant higher values of mycelial grow than previously essay. There are no significant differences between the Mamull® and the "Sherwood" strain treatments (Fig. 23).

The "Sherwood" strain was not effective against *D. seriata* when it was placed on medium dish at the time as trunk disease fungi but, if the pathogen was placed five days after, the "Sherwood" strain appears to be effective in inhibiting it. The Tifi® treatment was the first treatment able to inhibit *D. seriata* on the 13th day, followed by Mamull® on the 14th day. "Sherwood" strain was the last treatment able to inhibit *D. seriata* on 15th day; however, there are no significant differences between *Trichoderma* treatments (Fig. 24).

As in the previous assay, Tifi® was the only treatment effective against *N. parvum*, occupying significant larger areas with mycelial growth since day 13th day (Fig. 25).

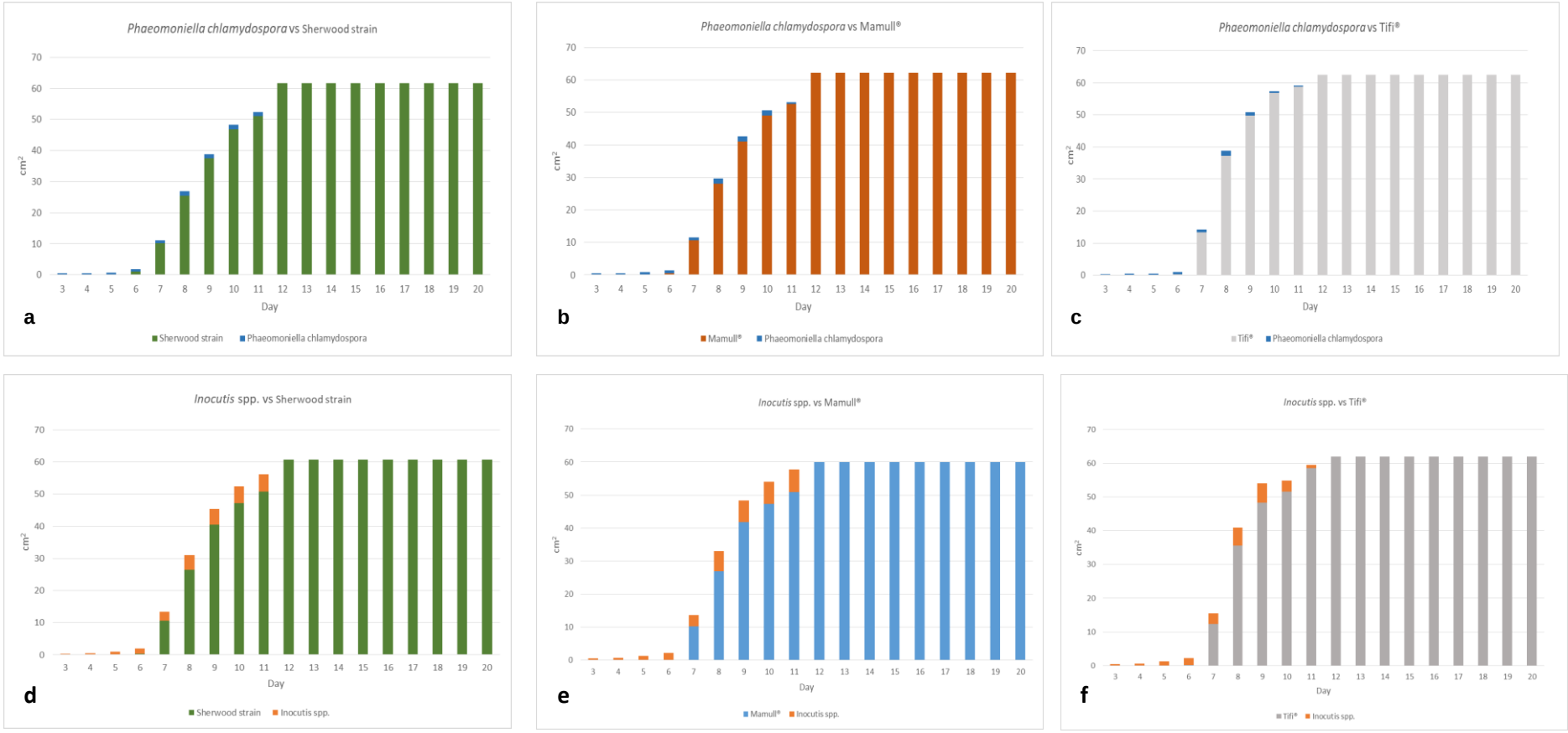

Figure 22 - Results obtained in paired cultures assay “20 days” with *Phaeomoniella chlamydospora*, *Inocutis* spp. and *Trichoderma* strains (*Trichoderma virens* “Sherwood”strain isolated from the Sclerophyl forest in the Maule region of Chile, Tifi®: *Trichoderma atroviride* commercial product; Mamull®: *Bionectria ochroleuca, Trichoderma gamsii* and *Hypocrea virens* commercial product): a) *Phaeomoniella chlamydospora versus Trichoderma* “Sherwood” strain”; b) *Phaeomoniella chlamydospora versus* Mamull®; c) *Phaeomoniella chlamydospora versus* Tifi® d) *Inocutis* spp. *versus Trichoderma* “Sherwood” strain; e) *Inocutis* spp. *versus* Mamull®; f) *Inocutis* spp. *versus* Tifi®

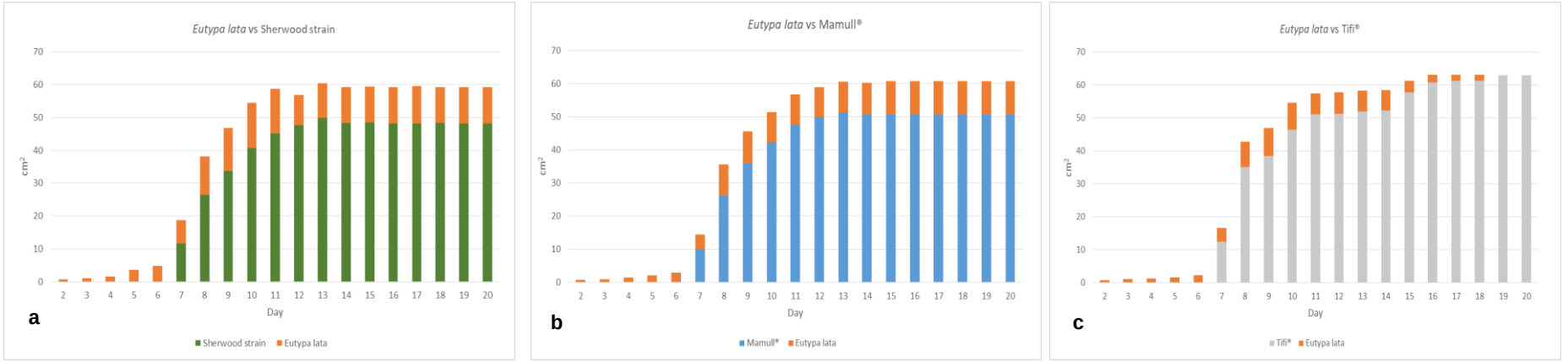

Figure 23 - Results obtained in paired cultures assay “20 days” with *Eutypa lata* and *Trichoderma* strains (*Trichoderma virens* “Sherwood”strain isolated from the Sclerophyl forest in the Maule region of Chile, Tifi®: *Trichoderma atroviride* commercial product; Mamull®:*Bionectria ochroleuca, Trichoderma gamsii* and *Hypocrea virens* commercial product): a) *Eutypa lata versus Trichoderma* “Sherwood” strain”; b) *Eutypa lata versus* Mamull®; c) *Eutypa lata versus* Tifi®.

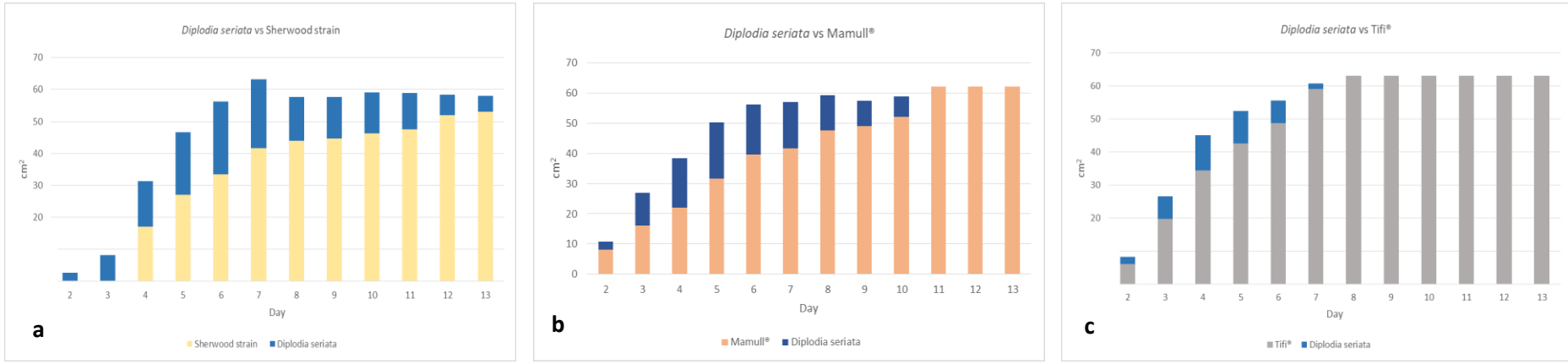

Figure 24 - Results obtained in paired cultures assay “20 days” with *Dipolodia seriata* and *Trichoderma* strains (“Sherwood”strain, *Trichoderma virens* isolated from the Sclerophyl forest in the Maule region of Chile, Tifi®: *Trichoderma atroviride* commercial product; Mamull®: *Bionectria ochroleuca, Trichoderma gamsii* and *Hypocrea virens* commercial product): a) *Dipolodia seriata versus Trichoderma* “Sherwood” strain”; b) *Dipolodia seriata versus* Mamull®; c) *Dipolodia seriata versus* Tifi®.

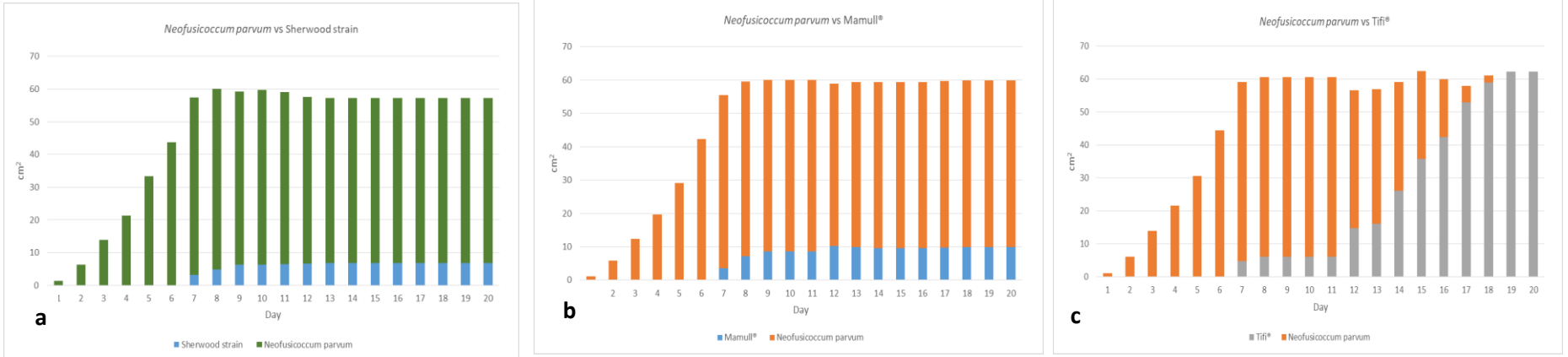

Figure 25 - Results obtained in paired cultures assay “20 days” with *Neofusicoccum parvum* and *Trichoderma* strains (“Sherwood”strain, *Trichoderma virens* isolated from the Sclerophyl forest in the Maule region of Chile, Tifi®: *Trichoderma atroviride* commercial product; Mamull®: *Bionectria ochroleuca, Trichoderma gamsii* and *Hypocrea virens* commercial product): a) *Neofusicoccum parvum versus Trichoderma* “Sherwood” strain”; b) *Neofusicoccum parvum versus* Mamull®; c *Neofusicoccum parvum versus* Tifi®.

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4.1.1.3 Paired cultures triple assay

The objective of this assay was to simulate the field conditions where *Trichoderma* treatments have to inhibit simultaneously more than one pathogenic fungus. In order to emulate the fungal complexes, two trunk diseases fungi were placed *versus* one *Trichoderma* treatment on a 15cm PDA dish and stored at 25°C during 13 days. *D. seriata*, *E. lata* and *N. parvum* were selected due to their fast growth rate and Mamull®, Tifi®, the "Sherwood" strain the two *Trichoderma* spp. strains were used.

Against *D.seriata* and *N.Parvum,* the "Sherwood" strain and Mamull® have showed significant smaller mycelial grow than Tifi®, however were significant better treatments than the *Trichoderma* spp 1 and 2. These last two treatments were the worst treatments against this combination, showing significant lower occupied areas than the other *Trichoderma* treatments and the *N. parvum* on the dish (Fig. 26).

Against the combination *E.lata*/*D.seriata*, the "Sherwood" strain, Mamull® and Tifi® were effective against *Diplodia*, however, there was no effective treatment against *E. lata*. The *Trichoderma* spp 1 and 2 were not effective inhibiting any of the fungi (Fig. 27).

There was no effective *Trichoderma* treatment against *E. lata* and *N. parvum* combined, since there was no treatment able to inhibit any of the fungi from the dish. Tifi® was the closest treatment to eliminate *D. seriata* and *N. parvum* from the dish growing significant more than all the other treatments since the 9th day. Tifi® *versus E. lata* showed no significant differences on its growth than other treatments, however *N. parvum* grew significant less with Tifi® than with the other *Trichoderma* treatments (Fig. 28).

During the entire assay, there were no significant differences between the "Sherwood" strain, Mamull® and Tifi®, which showed significant higher values of occupied area than *Trichoderma* spp 1 and 2, which showed no significant differences between each other.

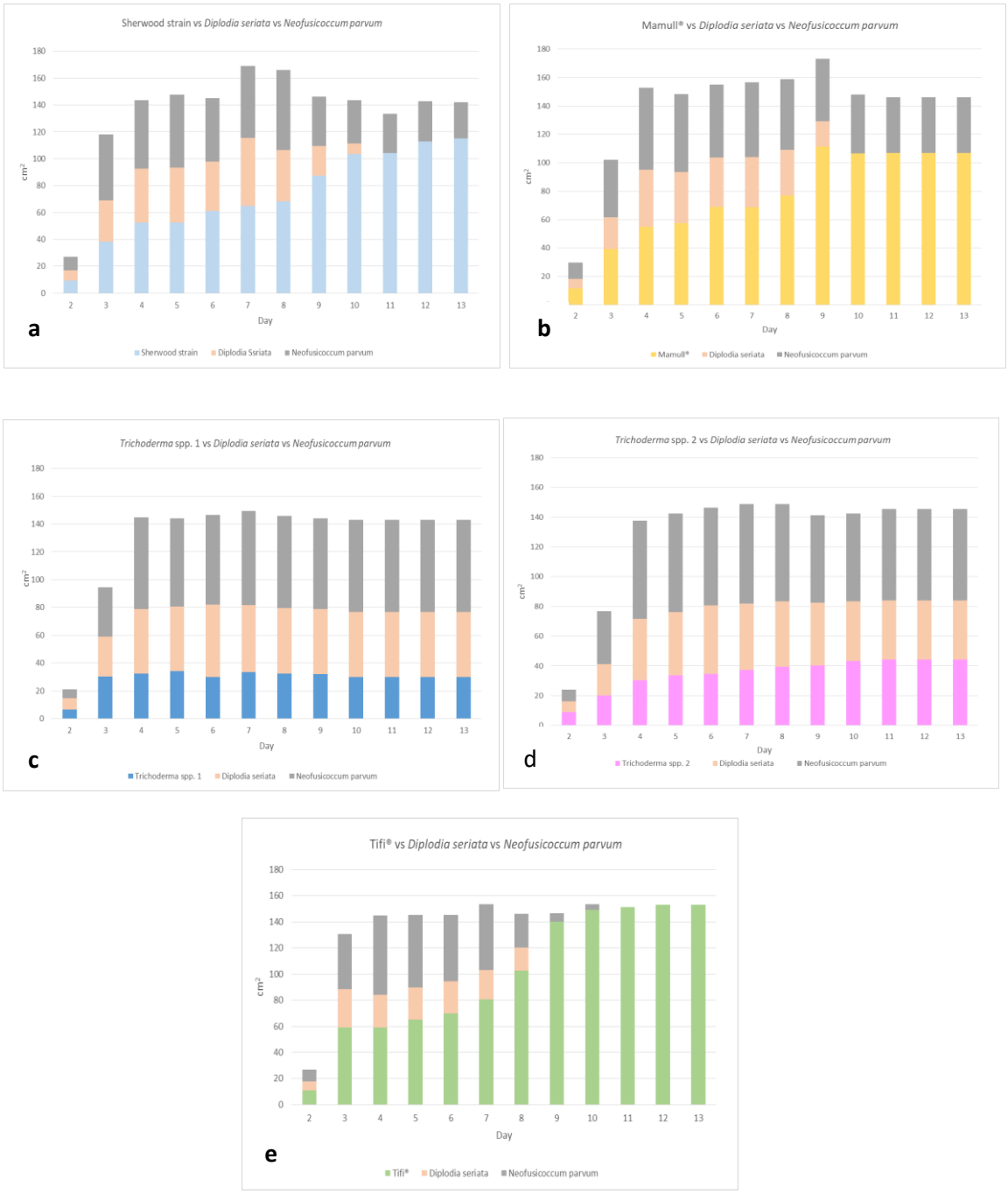

Figure 26 - Results obtained in paired cultures triple assay between *Diplodia seriata*, *Neofusicoccum parvum* and *Trichoderma* strains: a) *Diplodia seriata* and *Neofusicoccum parvum versus Trichoderma* "Sherwood" strain; b) *Diplodia seriata* and *Neofusicoccum parvum versus* Mamull®; c) *Diplodia seriata* and *Neofusicoccum parvum versus Trichoderma* spp1; d) *Diplodia seriata* and *Neofusicoccum parvum versus Trichoderma* spp2. e) *Diplodia seriata* and *Neofusicoccum parvum versus* Tifi®.

Figure 27 – Results obtained in paired cultures triple assay between *Eutypa lata, Diplodia seriata* and *Trichoderma* strains: a) *Eutypa lata, Diplodia seriata versus Trichoderma* "Sherwood" strain; b) *Eutypa lata, Diplodia seriata versus* Mamull®; c) *Eutypa lata, Diplodia seriata versus Trichoderma* spp1; d) *Eutypa lata, Diplodia seriata versus Trichoderma* spp2; e) *Eutypa lata, Diplodia seriata versus* Tifi®.

Figure 28 - Results obtained in paired cultures triple assay between *Eutypa lata*, *Neofusicoccum parvum* and *Trichoderma* strains: a) *Eutypa lata* and *Neofusicoccum parvum versus Trichoderma* "Sherwood" strain; b) *Eutypa lata* and *Neofusicoccum parvum versus* Mamull®; c) *Eutypa lata* and *Neofusicoccum parvum versus Trichoderma* spp1; d) *Eutypa lata* and *Neofusicoccum parvum versus Trichoderma* spp2.; e) *Eutypa lata* and *Neofusicoccum parvum versus* Tifi®.

4.2 AM fungi ITS primers test on commercial products

In pursuance of knowing the efficiency of the Mycorrhiza inoculation, molecular tools can be used to detect these microorganisms on the nursery grapevine plants in an early stage of their life. For that purpose, the efficiency of Mycorrhiza ITS primers was tested so they can after be used on the field experiment.

In order to establish the best DNA extraction protocol and the best PCR conditions to extract and amplify the Mycorrhiza commercial products, a sample with higher concentrations of Mycorrhiza was used, in this case, grass.

Results obtained with several different DNA extraction protocols from grass samples in order to attain a good quality DNA sample to use on the PCR reactions (DNeasy Plant Mini Kit®, Cetyltrimethylammonium bromide), revealed that DNeasy PowerSoil kit® was the protocol that allows the obtention of better samples (Tab. 3).

Table 3. Quality DNA parameters obtain from grass samples using TECAN infinity® 200 (absorbance at 260 and 280 nanometers, concentration and the ratio) and Qubit® (concentration).

Sample I.D	Abs 260nm	Abs 280nm	Conc (ng/µl)	Ratio	Quibit quant. (ng/µl)
Grass 1	0.11365	0.0601	113.65	1.89	96.6
Grass 2	0.12895	0.0689	128.95	1.87	119.0

Grass samples were submitted to a PCR using general fungi ITS primers (ITS 4 and 5) in order to detect fungal DNA (sequences are on attachment I supplementary table 3).

Results of electrophoretic separation of the PCR products of grass samples (grass 1 and grass2) and a negative control amplified with ITS 4 and 5 primers revealed bands corresponding to what was expected (500 bp) (Fig. 29).

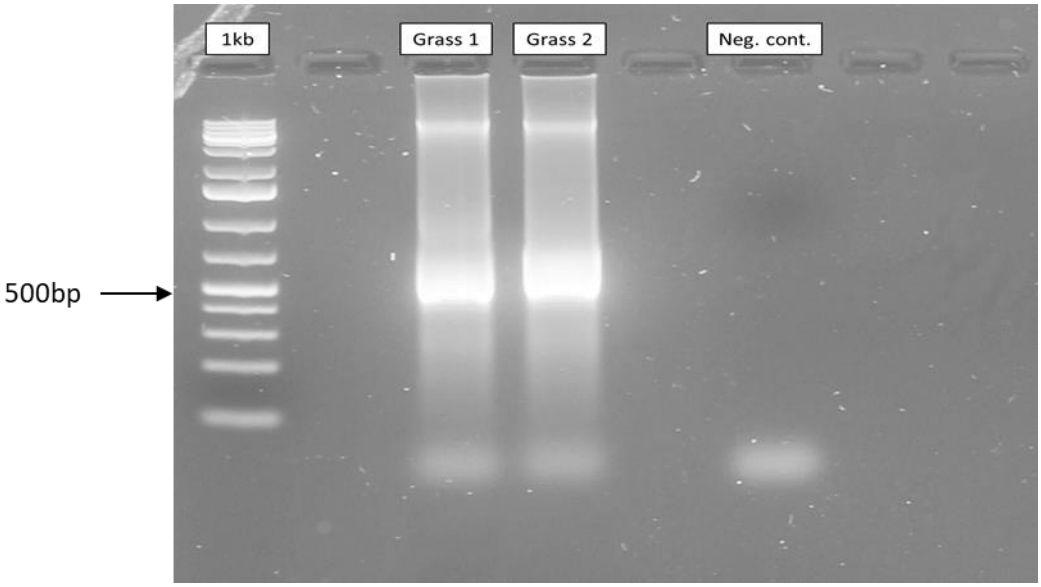

Figure 29 - Electrophoretic separation of the PCR products of grass samples (grass 1 and grass 2) and a negative control amplified with ITS 4 and 5 primers.

After that, the samples were amplified using Mycorrhiza specific ITS primers (Krüger *et al.,* 2009), that combine a mix of forward primers and a mix of reverse primers, in order to be able to amplify DNA from all *Glomeromycota* species. In order to set up the better conditions to amplify Mycorrhizal DNA from the commercial products, a temperature gradient for these primers was performed using the grass samples. The bands with 1500 bp were expected, however an unspecific amplification (600 bp) was obtained (Fig. 30).

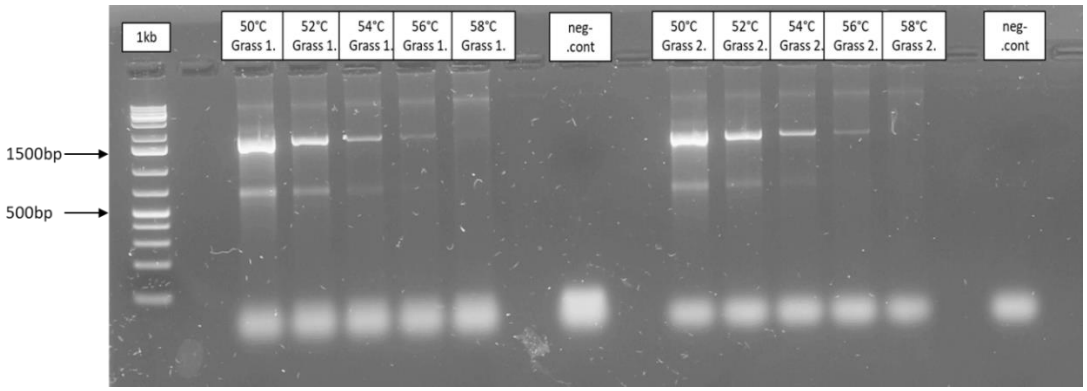

Figure 30 - Electrophoretic separation of the PCR products of grass samples (grass 1 and grass 2). Each sample was amplified using the Krüger primers at 50°C, 52°C, 54°C, 56°C and 58°C. A negative control was used to each sample amplified at 50°C.

Several protocols were followed in order to extract DNA directly from the commercial products, however, none of the attempts provide an Oiko-Rhiza® sample with enough

DNA concentration to use on PCR technique. Therefore, the AEGIS-gel® commercial product and roots inoculated with AEGIS-gel® and Oiko-Rhiza® DNA samples were extracted after several attempts, using DNeasy PowerSoil kit (Tab. 4).

Table 4. Quality DNA parameters obtain from AEGIS-gel commercial product and roots inoculated with AEGIS-gel® and Oiko-Rhiza® using TECAN infinity® 200 (absorbance at 260 and 280 nanometers, concentration and the ratio) and Qubit® (concentration).

Sample I.D	Abs 260 nm	Abs 280 nm	Conc. ng/µl	ratio	Qubit quant. (ng/µl)
AEGIS-gel C.P.	0.04115	0.0227	41.15	1.81	-
Oiko-Rhiza roots	0.04127	0.0239	41.27	1.73	32.5
AEGIS-gel roots	0.07090	0.0394	70.9	1.80	61.2

After the successful DNA extractions and amplification of the samples, using general fungi ITS primers (ITS 1 and 4, and ITS 1 and 5), electrophoresis revealed all the predictable bands (Fig. 31).

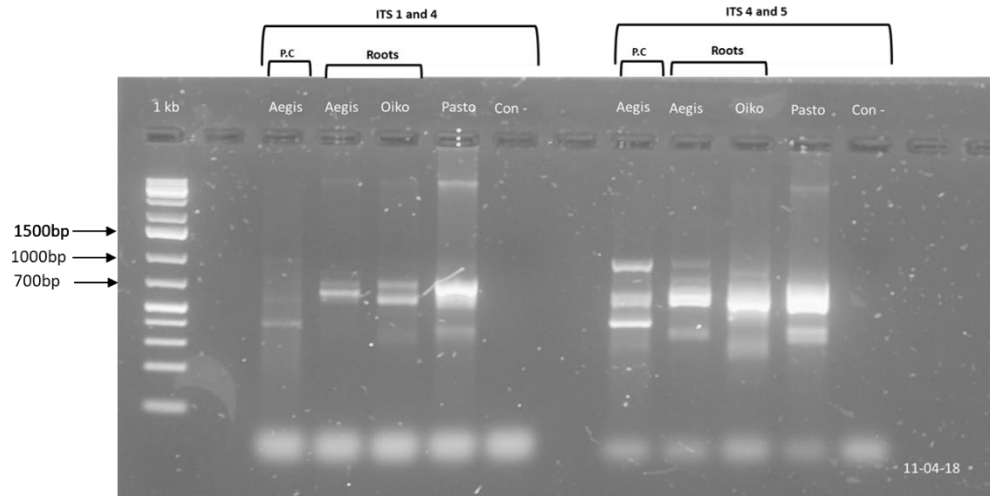

Figure 31 - Electrophoretic separation of the PCR products amplified with ITS 1 and 4, and ITS 4and 5 primers. The samples were AEGIS-gel® commercial product and roots inoculated with AEGIS-gel® and Oiko-Rhiza®. Each set of primers had a negative control.

After, the samples were tested with the Krüger primers making a temperature gradient. The samples were again tested using 10µl of PCR mix and 25µl of PCR mix, using the temperature of 50°C. Since no results were obtained on this amplifications, the 10µl mix samples were re-amplified (Fig. 32).

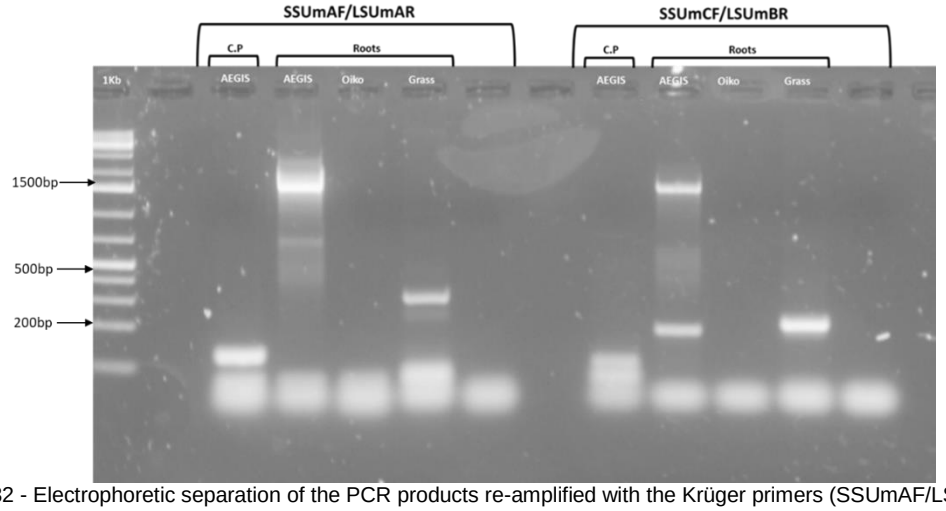

Figure 32 - Electrophoretic separation of the PCR products re-amplified with the Krüger primers (SSUmAF/LSUmAR and SSUmCF/LSUmBR). The samples were AEGIS-gel® commercial product and roots inoculated with AEGIS-gel® and Oiko-Rhiza®. Each set of primers had a negative control.

This process was repeated (test the samples with Krüger primers and re-amplify the results) (Fig. 33).

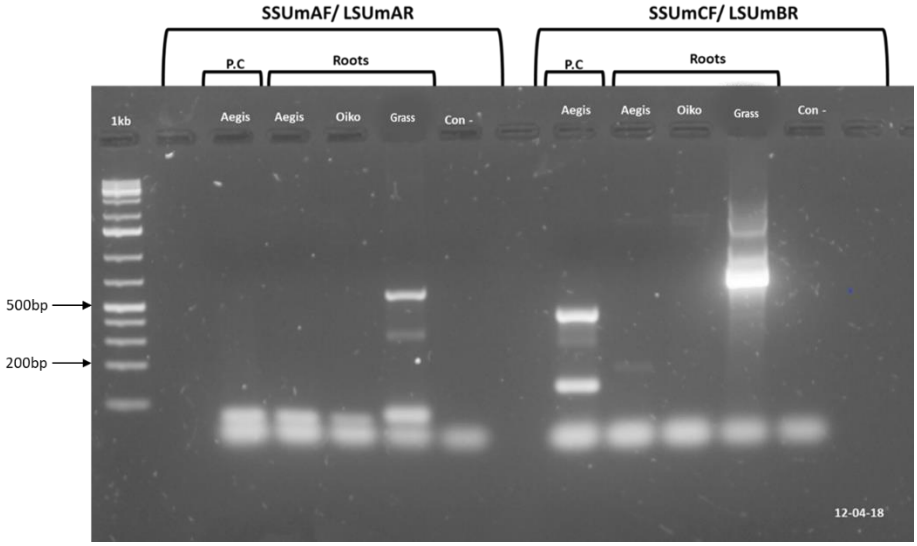

Figure 33 - Electrophoretic separation of the PCR products re-amplified with the Krüger primers (SSUmAF/LSUmAR and SSUmCF/LSUmBR). The samples were AEGIS-gel® commercial product and roots inoculated with AEGIS-gel® and Oiko-Rhiza®. Each set of primers had a negative control.

The expected size of the bands obtained would be 1000 bp, however, all the visible bands were purified and sent to sequencing. On some of the results was possible to

identify *Glomus* spp., however most of the results were inconclusive due to unspecific amplification.

Since the results were not conclusive, DNA was extracted from samples of roots inoculated and non-inoculated with Mychorriza using DNeasy PowerSoil kit®, however, the samples did not amplify with any of the constitutive primers (Actin, NS, or ITS). Therefore, the samples of roots inoculated with AEGIS-gel® were used to test another set of primers from (Alkan *et al.,* 2006), with three different concentrations. This primers amplify a sequence of 101 bp, that targets the ITS1 region and the 18S rRNA gene (SSU region), and are specific for *Glomus intraradices* present on AEGIS-gel®. For all concentrations, the predictable size band (100 bp) was found since these primers were designed to qPCR (Fig. 34).

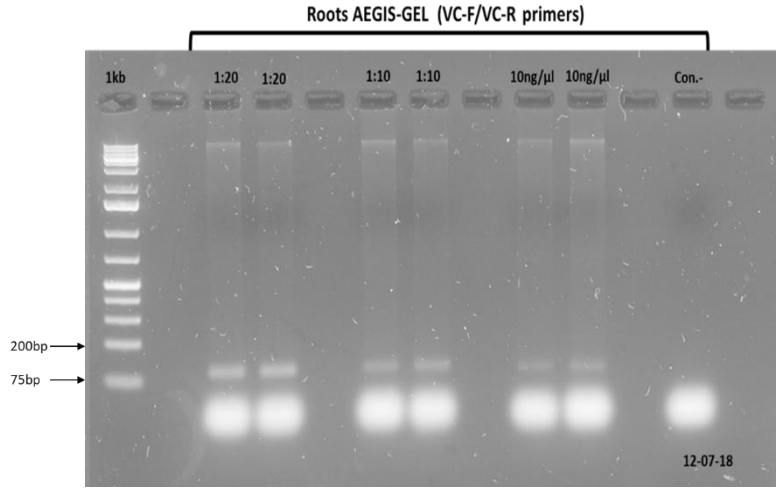

Figure 34 - Electrophoretic separation of the PCR products amplified with the VC primers (VC-F and VC-R). The samples were two samples of roots inoculated with AEGIS-gel® diluted to three different concentrations (1:20, 1:10 and 10ng/µl) and a negative control.

The PCR product and all the bands from the gel were purified and sent to sequencing. The results were ran on BLAST (NCBI) and was identified the genus *Glomus* spp., however the specie was not conclusive.

4.3 Design and evaluation of primers for different species of *Trichoderma*

Since there are few *Trichoderma* primers that differentiate between strains, some primers were designed for specific *Trichoderma* strains present on the commercial products used on the other assays. Therefore, it is possible to detect this *Trichoderma* on the nursery grapevine plants in an early stage of their development, and monitoring the success of inoculations.

In order to design and evaluate ITS primers for different species of *Trichoderma,* DNA was extracted from Tifi® and Mamull® (commercial products) using DNeasy Plant Mini Kit® from Qiagen® (Tab. 5).

Table 5 - Quality DNA parameters obtain from Tifi® and Mamull® commercial products using TECAN infinity® 200 (absorbance at 260 and 280 nanometers, concentration and the ratio) and Qubit® (concentration).

Sample I.D	Abs 260nm	Abs 280nm	Conc ng/µl	Ratio
Tifi	0.00403	0.00244	4.03	1.65
Mamull	0.01223	0.00604	12.23	2.02

Tifi® and Mamull® commercial products were after amplified using general fungi ITS primers (ITS 4 and 5). The electrophoretic separation of the PCR products amplified with ITS4 and ITS5 primers revealed the expectable size, (bands around 500 bp (Fig. 35). Amplification products were purified and sent to sequencing, and it was possible to identify *Trichoderma* spp.

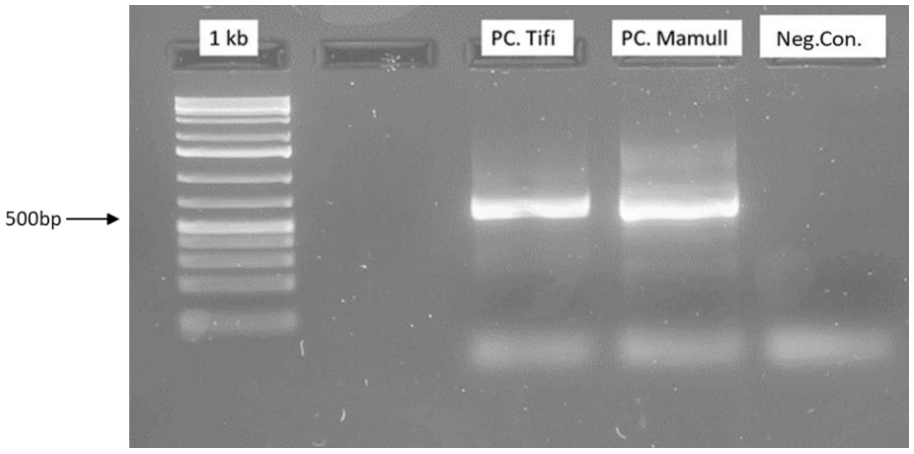

Figure 35 - Electrophoretic separation of the PCR products amplified with ITS 4and 5 primers. The samples were Tifi® and Mamull® commercial products and a negative control.

Therefore, specific primers were designed for *Trichoderma gamsii*, *Trichoderma artroviride* and *Trichoderma harzianum* (2 sets of primers were designed) that amplify a 250 bp sequence from the ITS region. This particular *Trichoderma* strains can be found on the used commercial products (Tifi® and Mamull®). It was also used a set of primers from Hagn *et al.*, 2007, that amplify a 540 bp sequence that targets the ITS region, part of the SSU region (18S) and part of the LSU region (28S) called UT.

To test the efficiency of these primers, DNA was extracted from Mamull® and Tifi® directly, and roots inoculated with these same products. The annealing temperature for each primer was determined performing a temperature gradient on a sample from Mamull®. With all the primers, it is possible to obtain one band always with the same size (around 200-250 bp) (Fig. 36).

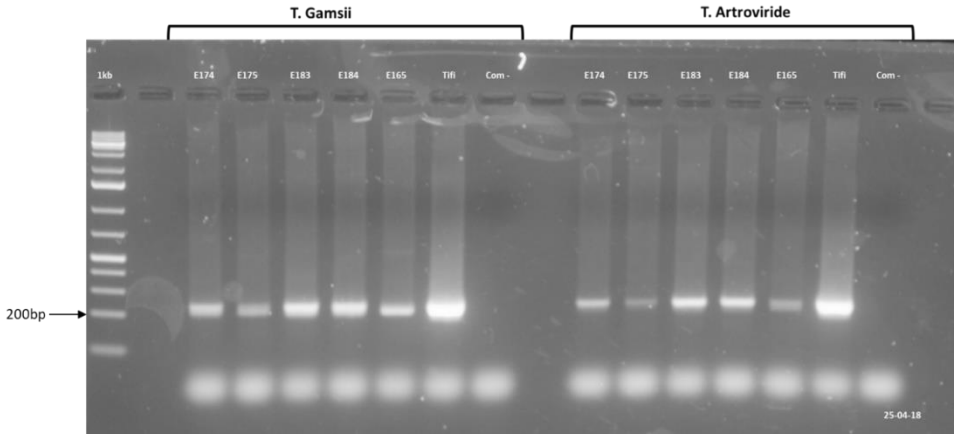

Figure 36 - Electrophoretic separation of the PCR products amplified with the designed primers for *Trichoderma* strains (*T. gamsii, T. artroviride, T. harzianum*1 *and T. harzianum*2). The sample was Mamull® commercial product and was tested at 50°C, 52°C, 54°C, 56°C, 58°C and 60°C for each primers set. A negative control was used for each set of primers.

The PCR reaction was repeated with the same sample and the correct annealing temperatures for each primer, the bands were purified and sent to sequencing (Data not showed).

Along with this, the PCR was repeated with samples from Tifi®, roots inoculated with *Trichoderma* and roots without inoculation. Once again, one expected band always with

the same size (approximately 200 bp) was obtained with the primers designed for this experiment, and one band with 500 bp when the UT primers were used which also was predictable according to the author (Fig. 37). The bands were purified from the gel and sent to sequencing.

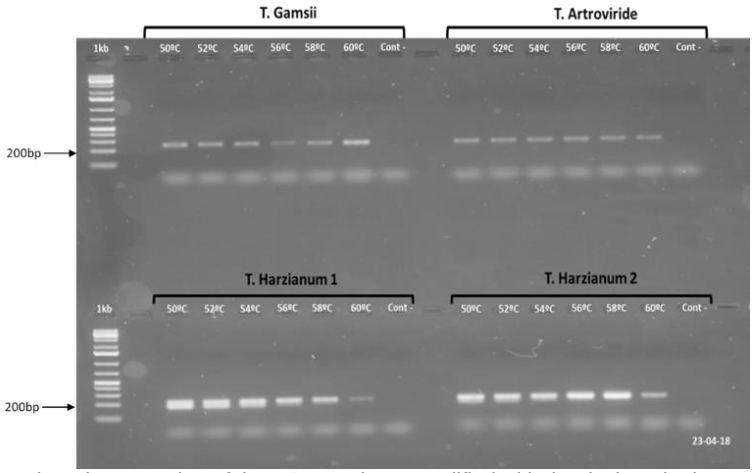

Figure 37 - Electrophoretic separation of the PCR products amplified with the designed primers for *Trichoderma* strains (*T. gamsii, T. artroviride, T. harzianum*1 *and T. harzianum*2) and the UT primers. The samples were roots inoculated with *Trichoderma* commercial products (E174, E175 and E165) and samples without *Trichoderma* inoculation (E183 and E184). A negative control was used for each set of primers.

The results were analysed on BLAST (NCBI), and the genus *Trichoderma* spp. was identified, however the specie identification didn´t show conclusive results.

Hereafter, DNA was extracted from dishes inoculated with Tifi®, Mamull® and a native *Trichoderma virens* "Sherwood" strain (Tab. 6), and tested with the same primers.

Table 6. Quality DNA parameters obtain from dishes inoculated with *Trichoderma* "Sherwood" strain, Mamull® and Tifi® using TECAN infinity® 200 (absorbance at 260 and 280 nanometers, concentration and the ratio) and Qubit® (concentration).

Sample I.D	Abs 260nm	Abs 280nm	Conc. ng/µl	Ratio	Qubit conc. ng/µl
"Sherwood"	0.0908	0.0547	90.8	1.66	42.8
Mamull	0.1886	0.1396	188.6	1.35	83.9
Tifi	0.0263	0.0139	26.3	1.89,	11.5

The bands number and size were all according to what was expected (Fig. 38). The PCR product and the bands were purified and sent to sequencing. Once again, the genus *Trichodema* spp. was identify on every sample, however the specie was not conclusive.

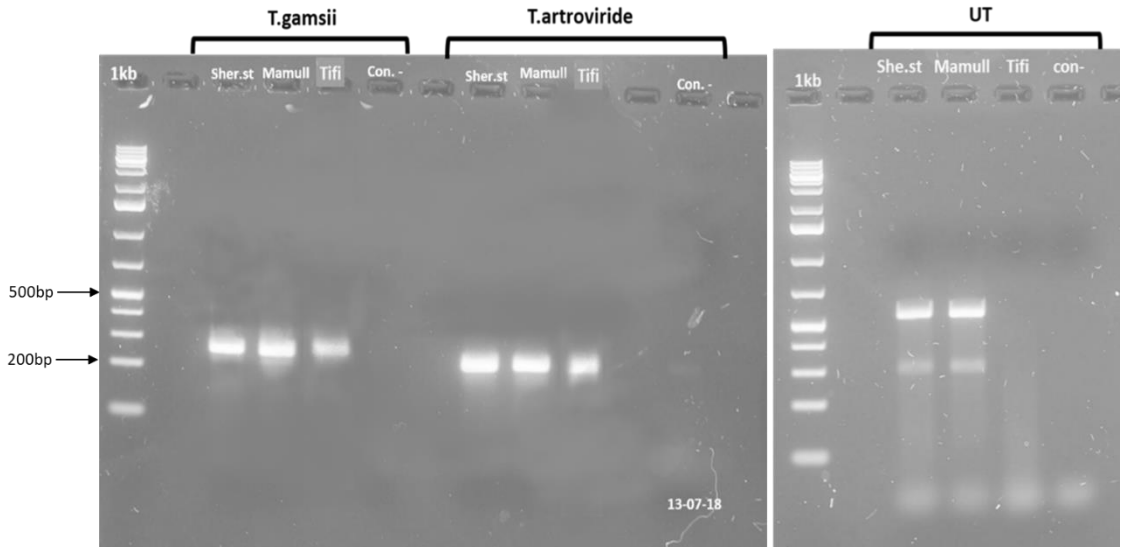

Figure 38 - Electrophoretic separation of the PCR products amplified with *T. gamsii, T. artroviride* (designed primers) and UT primers. The samples were DNA from dishes inoculated with Mamull®, Tifi® and "Sherwood" strain. A negative control was used for each set of primers.

4.4 *In vivo* assay

With the goal of understanding the effects of *Trichoderma* and Mycorrhiza inoculation on grapevine nursery plants along with the nursery cleaning process, a field assay was performed on Concha y Toro nursery during the 2017/2018 season. Three different factors were tested: the cleaning process, the *Trichoderma* inoculation and the Mycorrhiza inoculation. Each factor presented three different levels or treatments. The cleaning processes was the VCT process (a chemical procedure already implemented in the company) (L0), the neutral anolyte cleaning (L1), or the hot water treatment (L2), the *Trichoderma* inoculation was performed with Mamull® (T1), Tifi® (T2), or water (control) (T0) and the Mycorrhiza inoculation could be done with AEGIS-gel® (M1), Oiko-Rhiza® (M2) or no treatment (control) (M0). Each possible treatment (for example LOTOMO) had three replicas made with 50 plants each.

Several analysis were performed in this experiment in different stages of the grapevine nursery process. Before the assay started, after grafting and at harvest (11 months later), plants were tested for the presence of trunk diseases fungi using a destructive protocol (it is necessary to destroy the plants to do the determinations). However, due to an unexpected behaviour during the grafting and sproud stage of the nursery (too many plants died or not qualify with the quality standards), there was not enough plants to have a significant number of replicates (only one plant was analysed according to these parameters, which was not conclusive for the entire experimental units).

4.4.1 Budburst percentage

After stratification, the percentage of budburst was determined as described on the methods. No interaction was observed between the three factors, and only the cleaning process affected the budburst percentage. Therefore, the neutral anolyte cleaning technique presented significant lower budburst percentages than the VCT process (Fig. 39).

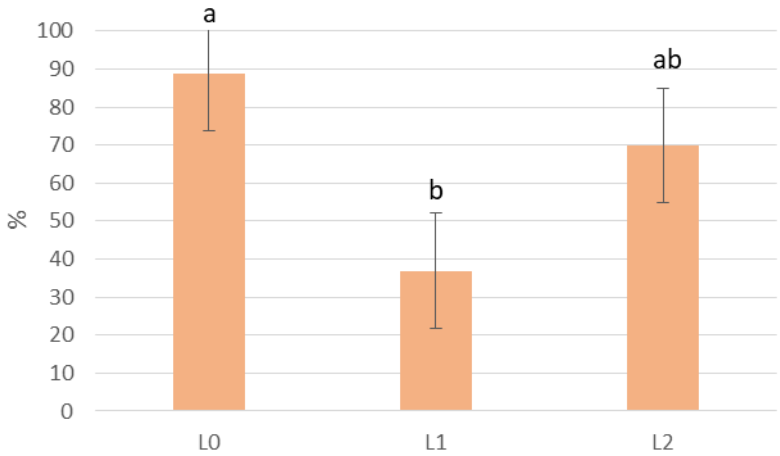

Figure 39 - Graphic representation of the budburst percentage according to the cleaning method: VCT treatment (L0), neutral anolyte treatment (L1) and Hot water treatment (L2).

4.4.2 Mycorrhiza analysis

Three months after the plants were transplanted to the field, and at the moment they were harvest from the field, nine random plants from each treatment were collected and tested for Mycorrhiza frequency on the root system (F%), Mycorrhiza colonization at the root system (M%) and arbuscules abundance on the root system (A%).

4.4.2.1 Mycorrhiza frequency on the root system

Three months after the plants were transplanted the analysis showed a difference on the frequency of Mycorrhiza on the root system (F%), in which, when the chemical cleaning process (L0) was used, the treatment using Oiko-Rhiza (M2), presents significant higher frequencies than the control (MO) (Fig. 40). However, after 11 months of the transplantation, no significant differences were found on the Mycorrhiza frequency on the root system.

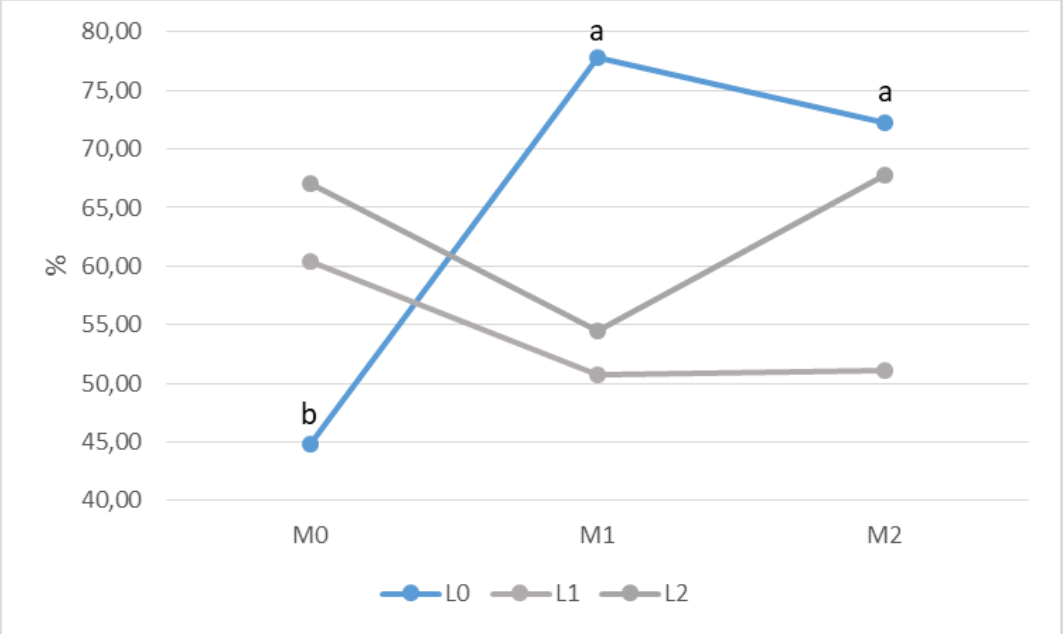

Figure 40 - Graphic representation of the interaction between the Mycorrhiza treatment (No treatment (M0), AEGIS-Gel® (M1) and Oiko-Rhiza® (M2)) and the cleaning process (VCT treatment (L0), neutral anolyte treatment (L1) and Hot water treatment (L2)) on the frequency of Mycorrhiza on the root system three months after the transplantation.

4.4.2.2 Mycorrhiza intensity on the root system

Three months after the plantation, the intensity of Mycorrhization on the root system showed significant differences on the cleaning method, in which the VCT method (L0), has significant more intensity of Mycorrhization than the neutral anolyte (L1) (Fig. 41).

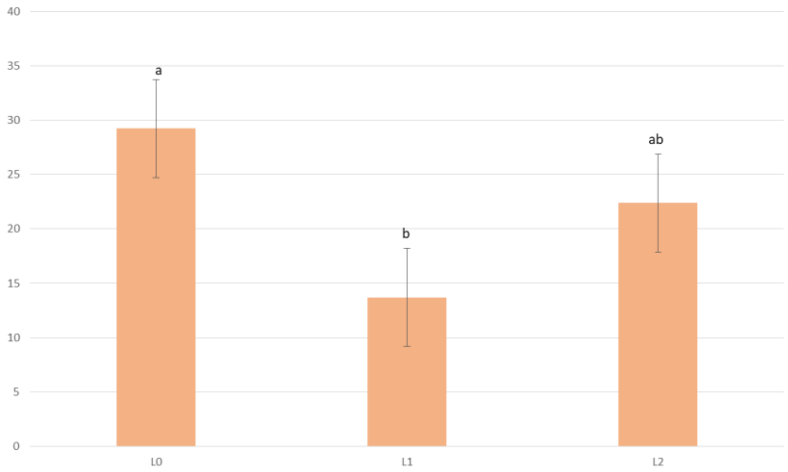

Figure 41 - Graphic representation of the Mycorrhiza intensity on the root system 3 months after the transplantation according to the cleaning method: VCT treatment (L0), neutral anolyte treatment (L1) and hot water treatment (L2).

However, 11 months after the plantation, the cleaning process didn't seem to have an impact on the Mycorrhization intensity. In fact, there is an interaction between the *Trichoderma* and the Mycorrhiza treatment, in which, when Oiko-Rhiza® (M2) is used, the treatment using Tifi®, presents higher Mycorrhiza intensity than when Mamull® is used. In general, after 11 months there was a slightly increase on the Mycorrhiza intensity on the root system (Fig. 42).

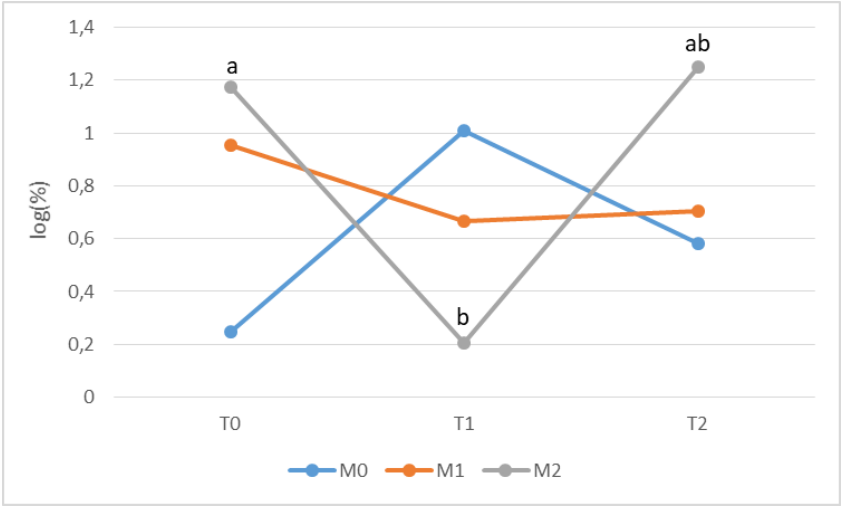

Figure 42 - Graphic representation of the interaction between the Mycorrhiza treatment (No treatment (M0), AEGIS-Gel® (M1) and Oiko-Rhiza® (M2)) and the *Trichoderma* treatment (Water treatment (T0), Mamull® (M1) and Tifi® (T2)) on the intensity of Mycorrhiza on the root system 11 months after the transplantation.

4.4.2.3 Arbuscular intensity on the root system

Concerning the arbuscular abundance on the root system, three months after the transplantation, there was an interaction between the Mycorrhiza treatment and the cleaning process. When used some Mycorrhiza treatment (M1 or M2), the plants with the VCT cleaning process (L0) had more arbuscules on the roots than the neutral aquanolyte (L1) (Fig. 43).

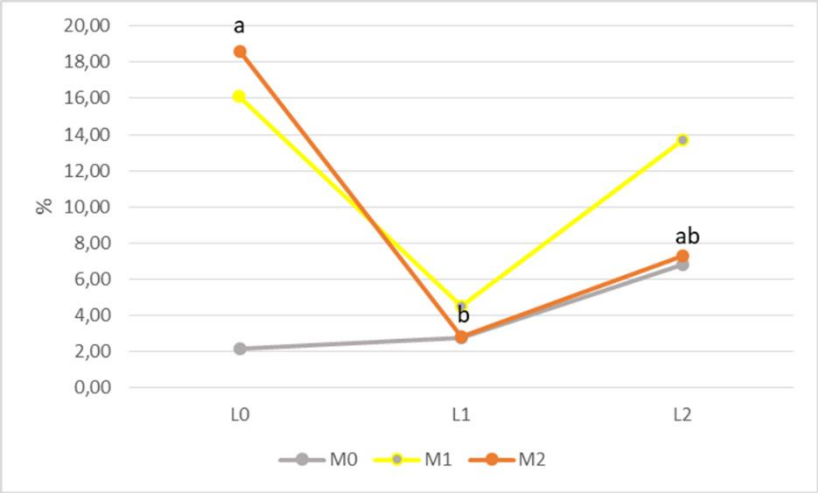

Figure 43 - Graphic representation of the interaction between the Mycorrhiza treatment (No treatment (M0), AEGIS-Gel® (M1) and Oiko-Rhiza® (M2)) and the cleaning process (VCT treatment (L0), neutral anolyte treatment (L1) and hot water treatment (L2)) on the Arbuscular intensity on the root system three months after the transplantation.

Eleven months after the transplantation, the results showed an increasing of the arbuscular abundance on the root system percentage compared to the results after three months, and the treatments using Mycorrhiza (M1 and M2) had higher percentages of arbuscular Mycorrhiza than the control (M0), however there were no significant differences between the treatment combinations.

4.4.3 Chemical analysis

After harvesting the plants from the nursery field, the roots were removed from three random plants from each treatment to be analysed for the arginine and nitrogen content, other macro and micronutrients (calcium, potassium, magnesium, sodium, phosphorus, silica, boron, iron, copper, zinc, and manganese) levels and dry weight.

4.4.3.1 Macro and micro nutrients

The Mycorrhiza treatment was the only factor influencing the nitrogen and the arginine content, being that, the plants with Oiko-Rhiza® (M2), had higher nitrogen content than the control plants (M0) (Fig. 44). The rest of macro and micro nutrients didn´t show significant differences.

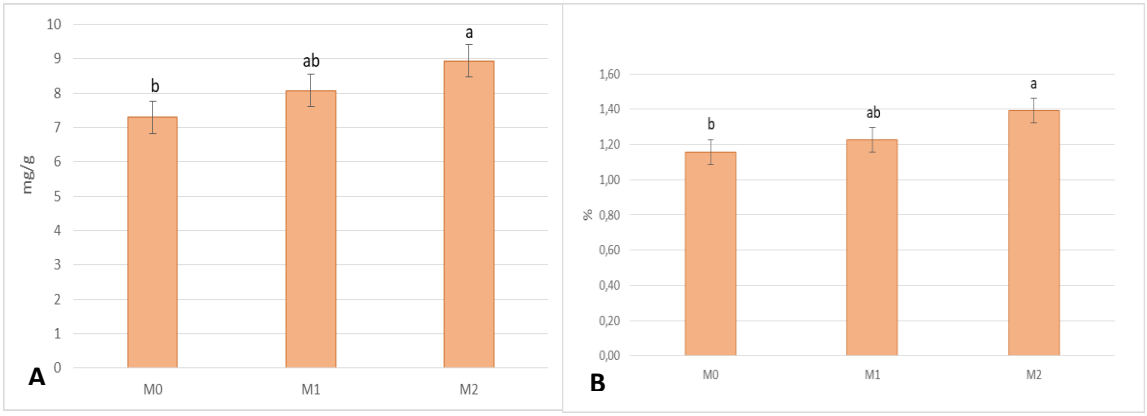

Figure 44 - Graphic representation of the nitrogen content (A) and the arginine content (B) according to the Mycorrhiza treatment: No treatment (M0), AEGIS-Gel® (M1) and Oiko-Rhiza® (M2).

4.4.3.2 Root dry weight

Regarding roots dry weight, all factors affect the results, which means that each treatment behaves differently according to the combinations its insert on figure 45.

When using the combinations, neutral anolyte and Oiko-Rhiza®, HWT and no Mycorrhiza, and Hot water Treatment (HWT) and Oiko-Rhiza®, we had significant higher dry weight than when no *Trichoderma* treatment was used.

When combining HWT with Tifi®, the values for dry weight were higher with AEGIS-Gel®, however when using neutral anolyte without *Trichoderma* treatment, Oiko-Rhiza® was the best Mycorrhiza treatment. If using neutral anolyte with Tifi®, the treatment without Mycorrhiza was the one with higher roots dry weight. When using no Mycorrhiza combined with Tifi® or Oiko-Rhiza® without *Trichoderma* the neutral anolyte treatment presented better results for roots dry weight. The results obtained for dry weight are very ambiguous which suggest the necessity of more data from another seasons.

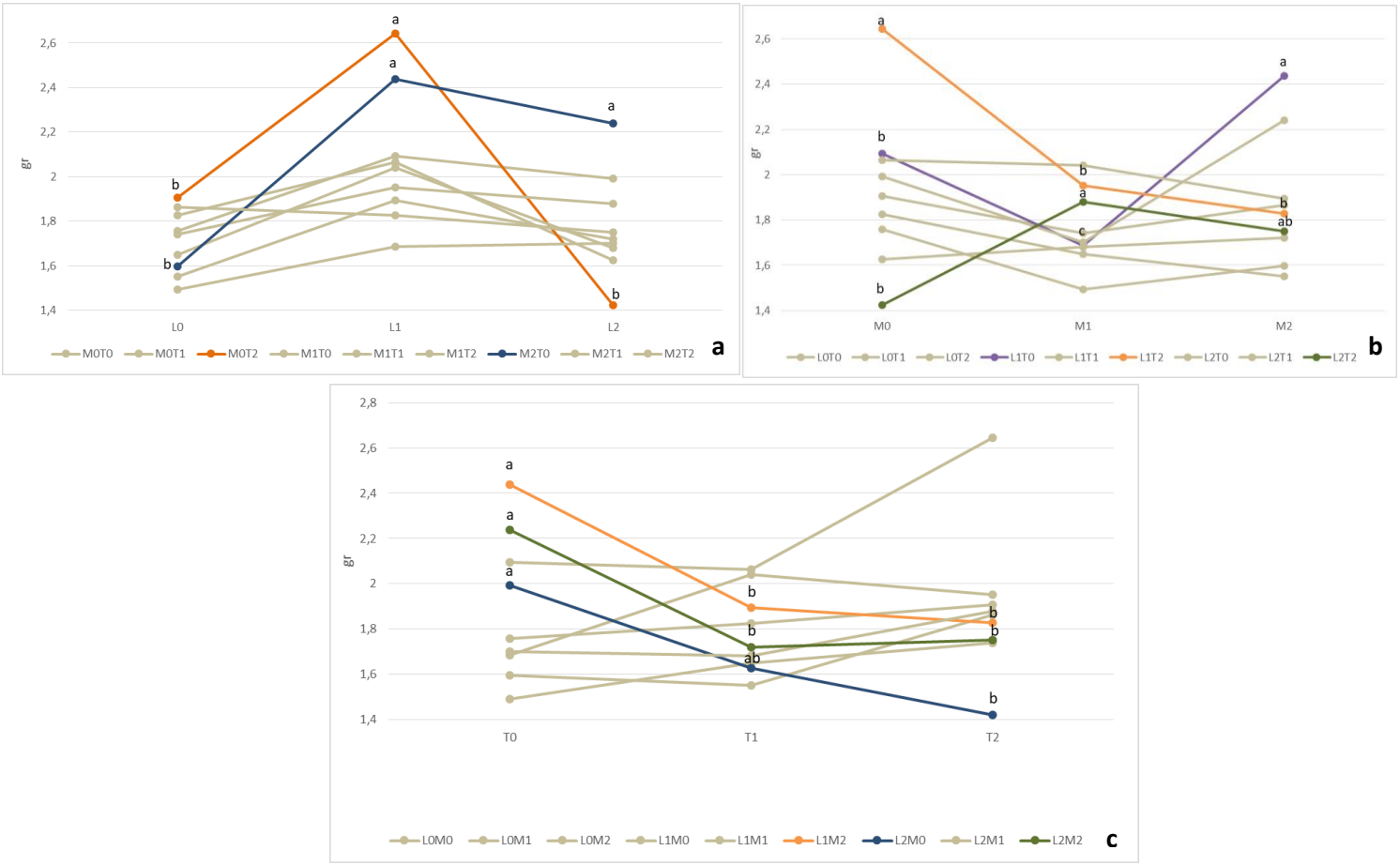

Figure 45 - Statistical analysis between the interactions of the three treatments evaluated: a) according to the cleaning process; b) according to the Mycorrhiza treatment and c) according to the *Trichoderma* treatment.

Despite these preliminary results and bearing in mind the assay is going to continue along the next years, the results indicate that the best cleaning process seems to be the VCT treatment, and the best Mycorrhiza treatment seems to be the Oiko-Rhiza®. About the *Trichoderma* treatment it is not possible to select a treatment with clear better results

than the others based on this assay so far, indicating the necessity of continuing the assay during the next seasons.

5. Discussion

Grapevine trunk diseases can affect vines of all ages, from old vines with more than 7 years, to young vines, rootstock/canes and mother-plants. Moreover, the fungi that cause these diseases have an endophytic phase of many years, in which the disease is present on the plants, however there are no visible symptoms. This, along with the fact that there are no effective treatments, makes GTDs the principal challenge in viticulture all over the world.

Since GTDs infect every vineyard and there is no effective cure for them, prevention practices and beneficial microorganisms like *Trichoderma* and Mycorrhiza can be the solution to control these diseases. Early diagnosis allows the viticulturist to prevent the spores dispersion in the field, and the inoculation of Mycorrhiza and *Trichoderma* strains protects the vines from GTDs and strength them so they can withstand better in hostile environments.

Despite the fact that *Trichoderma* and Mycorrhiza are already been used on vineyards all over the world, it is important to understand how they interact, which strains are better to use on each case, which is the best inoculation technique, and how to detect the presence of trunk diseases fungi and beneficial microorganisms. Therefore, this project was divided on four different assays that intend to answer these questions.

5.1 *In vitro* assay

In this assay *Trichoderma* strains were tested against trunk diseases fungi on PDA Petri dishes to test *Trichoderma* virulence and pathogens aggressiveness.

When tested against the *Trichoderma* strains, *P. chlamydospora* and *Inocutis* spp. were easily overgrowth and inhibited by them. When putting both the pathogen and the *Trichoderma* at the same time, the growth rate of *P. chlamydospora* and *Inocutis* spp.

was too slow when compared to the growth rate of the *Trichoderma* strains that easily overgrow the pathogens (Fig. 18). Even when putting the pathogens five days before the *Trichoderma* strains, the growth of the pathogens was not significant, and the three *Trichoderma* treatments (native "Sherwood" strain, Mamull® and Tifi®) were able to overgrow both pathogens very fast (Fig. 22). According to Begoude *et al.,* 2007, *Trichoderma* can grow until 20mm per day at 25°C, while *P. chlamydospora* grows only approximately 1 to 1.5mm (Whiting *et al.,* 2001) and *Inocutis* spp. around 3mm per day (Begoude *et al.,* 2006), which proves that even with five days of advantage the *Trichoderma* growth rate is significantly higher making it easy to inhibit the grow of the pathogens. Within an industrial perspective, focused on the impact of *Trichoderma* inoculations on grapevine plants, these results provide evidence of the advantages of an early *Trichoderma* inoculation. When applied at an early stage of the grapevine development, *Trichoderma* species are known to prevent trunk diseases infections and symptoms, along with growth stimulation (Di Marco and Osti, 2007; Di Marco *et al.,* 2004; Fourie and Halleen, 2006; Kotze, 2008). This finding, together with the results obtained in this assay, reinforce the importance of applying *Trichoderma* at an early stage of plant development, in order to let the *Trichoderma* establish largely in the plants before they are planted on a soil infected with GTDs spores. Being that, pathogens with slower growth rate such as *P. chlamydospora* and *Inocutis* spp., are not able to develop and cause symptoms on the plants. Being that, the next possible assay to test the virulence of this two pathogen fungi consisted of putting the pathogen a long time before the *Trichoderma* in order to let it develop until occupies at least half of the dish. For this reason, these two fungi were not used on the triple assay.

E. lata is a fungus responsible for one of the most aggressive grapevine trunk disease in the world nowadays, *Eutypa* dieback. When testing *E. lata* against *Trichoderma* native species and commercial products the results were different between the 13 and the 20 days assay. When using *Eutypa* and the *Trichoderma* at the same time, the commercial products Mamull® (*Bionectria ochroleuca, Trichoderma Gamsii* and *Hypocrea virens*) and Tifi® (*Trichoderma atroviride*) are able to overgrowth *E.* lata and kill it (Fig. 19). However the *Trichoderma virens* native "Sherwood" strain was only able to inhibit the growth of Eutypa. Kotze *et al.* (2011) tested *T. harzianum* and *T. artroviride* against *E. lata* using similar conditions, and all the *Trichoderma* strains were able to overgrowth Eutypa excepting for two indiscriminate *Trichoderma* spp strains. However, when allowing *E. lata* develop for five days, only Tifi® was able to overgrowth the Eutypa

inoculum, while Mamull® and the native strain were only able to inhibit its growth (Fig. 23). This probably explains why *Eutypa dieback* is so aggressive, because once is establish, it is hard to successfully eliminate it. Once again, it is important to highlight the necessity of a *Trichoderma* inoculation on an early stage of grapevine stage, in order to avoid *E. lata* infections later on infected soils.

When it comes to *D. seriata* the results are different. If testing both *D. seriata* and the *Trichoderma* strains at the same time, the two commercial products were able to overgrow the pathogen and inhibited it completely, but the native strain was only able to stop and decrease its growth (Fig. 20), however, in 13 days was not able to completely overgrow the pathogen (Fig. 24). On the other side, when growing *D. seriata* on the dish for 5 days, all the *Trichoderma* strains were able to eliminate the pathogen. This may occur because when the pathogen is already installed, the native strain of *Trichoderma* develop more virulently since its growth became more effective on controlling the pathogen, nevertheless, this is a supposition and cannot be proved. However, when introducing the "Sherwood" strain after five days of *D. seriata*, despite the fact that the native strain eliminate the pathogen, the pathogen was able to stop the growth of the *Trichoderma* strain, which means that, even after *D. seriata* was dead the "Sherwood" strain was not able to overgrow the pathogen and occupy the entire dish as happened in every other case. It´s been hypothesized that fungi associated with grapevine trunk diseases produce a variety of compounds, such as biosynthesized phytotoxic metabolites, that affect not only the host but a large range of organisms (Bénard-Gellon *et al.,* 2015; Cobos *et al.,* 2010). This can be the explanation for this phenomenon, however, despite the importance of *D. seriata* as a phytopathogen, little is known about the toxicity and the modes of action of this compounds (Bénard-Gellon *et al.,* 2015; Cobos *et al.,* 2010).

Countless studies have been proving that even though *D. seriata and N. parvum* are two of the most aggressive grapevine trunk diseases fungi*, N. parvum* is the most aggressive one (Bellée *et al.*, 2017; Massonnet *et al.,* 2017; Urbez-Torres, 2011; Úrbez-Torres *et al.,* 2009). In the current study, the results obtained corroborate those affirmations. As expected, *N. parvum* was the most aggressive GTD fungi in this study since, when growing at the same time, the pathogen and the *Trichoderma*, although the native strain and Mamull® were able to stop the growth of *N. parvum*, only Tifi® was able to overgrow the pathogen and inhibit it (Fig. 21). When placed against Mamull® and the native strain,

the pathogen and the *Trichoderma* created an inhibition zone that none of the fungi was able to pass. When putting *N. parvum* in the Petri dish for five days before the *Trichoderma*, Mamull® and the native strain were barely able to develop creating an inhibition zone that stoped the pathogen from overgrow them, but not being able to grow as much as the pathogen. Once again, Tifi® was the only *Trichoderma* capable of overgrow and inhibit completely the pathogen.

Although the results from this assays were very positive, showing that there are *Trichoderma* commercial products that are effective against GTDs by themselves, the reality is that in the field these fungi usually coexists, meaning that *Trichoderma* treatments need to be effective against more than one fungus at a time. Therefore, we performed an assay where we tested one *Trichoderma* against two trunk diseases fungi on the same Petri dish. For this assay *P. chlamydospora* and *Inocutis* spp. were not used due to their slow growth, and two new native strains of *Trichoderma* were included.

When testing *Trichoderma* against *D. seriata and N. parvum*, the results were consistent with the results from the previous assays. The "Sherwood" strain and Mamull® were able to eliminate *D. seriata* but not *N. parvum*, proving that *N. parvum* is more aggressive than *D. seriata (Bellée et al., 2017; Massonnet et al., 2017; Urbez-Torres, 2011; Úrbez-Torres et al., 2009)*. Tifi®, once again was the only *Trichoderma* treatment able to overgrow and inhibit both pathogens together from the Petri dish (Fig. 26).

When together, the combination *E. lata* and *N. parvum* was the most aggressive, since no *Trichoderma* treatment was able to inhibit any pathogen during the thirteen days of the assay, however, Tifi® decreased the growth of both pathogens (Fig. 28). This assay could be extended in time in order to evaluate the *Trichoderma* capacity to cope N. *parvum*, since its growth seemed to decrease from the day 13 of the assay. The reason for this aggressiveness of this combination it is not known since the biological life cycles, basic molecular aspects and the mechanisms involved on the pathogenicity of this fungal species is not very studied yet (Bénard-Gellon *et al.,* 2015; Cobos *et al.*, 2010).

When testing *E. lata* and *D. seriata* together, the "Sherwood" strain, Mamull® and Tifi® were all able to overgrow and eliminate *D. seriata*, however, none of them were able to eliminate *E. lata*. In all cases, the growth of *E. lata* was much reduced, however an inhibition zone was formed and both the *Trichoderma* and *E. lata* kept the same area coexisting in the dish (Fig. 27).

The two *Trichoderma* spp. native strains had a very small growth on every pathogen combination, and were not able to overgrow and eliminate any pathogen, always creating inhibition zones and coexisting with both pathogens. This probably happened due to the fact that these strains were isolated from plants that have symptoms of GTDs, showing that the *Trichoderma* strains on these plants are not very effective against these diseases. Therefore, on future assays would be interesting to isolate *Trichoderma* from healthy plants surrounded by plants with trunk diseases symptoms in order to isolate native strains with potential to overgrow pathogens and being possibly used in the control of GTDs.

In conclusion, Tifi® was the most effective *Trichoderma* commercial product, which is supported by the fact that Tifi® recently obtained a Chile certification as a successful biological controller for GTDs on grapevines. It is important to highlight the efficiency of Tifi® against *E. lata*, one of the most aggressive pathogens on grapevine vineyards nowadays. Mamull® is a commercial product with strains isolated from vines exposed to lower temperatures that the ones used on this assay. Therefore, the fact that Mamull® didn´t present results as good as Tifi® may be because the temperatures used on this assay were not favourable to its development, showing the importance of repeating this assays with lower temperatures. Despite the fact that the “Sherwood” native strain was not as effective as the commercial products it showed some potential on inhibiting pathogens growth. *E. lata* and *N. parvum* were in general the most aggressive pathogens, improving their aggressiveness when together in a complex.

5.2 AMF ITS primers test on commercial products

Arbuscular Mycorrhizal fungi (AMF) can bring a large range of benefits to the modern viticulture such as increase grapevine nutrition and growth through a larger access to soil nutrients and also by raising phosphorus, nitrogen, and other elements intake (Biricolti *et al.*, 1997; Nikolaou *et al.,* 2002); upsurge the tolerance to abiotic stress such as soil salinity, water stress, and heavy metal toxicity (Belew *et al.*, 2010; Kohler *et al.,* 2008; Komárek *et al.*, 2010); protect grapevines against biotic stress (not well studied yet) (Cameron *et al.*, 2013; Jung *et al.,* 2012); increase soil stability and improve grape quality and production (Bedini *et al.,* 2009; Wilson *et al*., 2009). Therefore, in this study

we first, developed and applied a system for early detection of AMF and tested the effects of AMF inoculation on grapevine nursery plants.

AMF have been traditionally identified by the morphology of their spores, or, in their absence, the intraradical structures (Merryweather and Fitter, 1998). Spore production is highly dependent on physiological parameters of the AMF and on environmental conditions, making this way of identification not very accurate (Bever *et al.,* 1996). So, the identification techniques that might solve this problem involve molecular detection.

Molecular identification techniques have the potential to revolutionize AMF ecology because they offer the opportunity to identify AMF in any given root sample without the need for spores. Besides this techniques, there is also the potential of identifying hyphae in the soil, but the necessary techniques to this identification have not been yet satisfactorily developed (Bever *et al.,* 1996).

Most of the problems with molecular identification techniques, such as the DNA quantity needed, were solved by introduction of polymerase chain reaction (PCR). PCR amplification is now one mandatory step on any molecular technique. With PCR it is possible to amplify the genome or part of it directly from a spore, a root sample or a soil sample (Reddy *et al.,* 2005).

Despite the fact that PCR technique is widely used and very accurate, AMF take extra complications when it comes to molecular detection, some of them detected on this study. The major constraint is their non-cultivability due to their obligate symbiotic nature (Reddy *et al.,* 2005). This fact makes it very complicated to extract DNA samples, since the commercial, products tested on this study had a very small amount of AMF spores in their constitution, and it was not possible to grow them on any medium, resulting on samples with very low DNA concentrations.

Besides that, AMF mycelium is deeply embedded within the roots (Van Tuinen *et al.*, 1998) making it really hard to extract DNA from the roots, and also, the root samples are often composed by a lot of other organisms whose DNA can also be extracted with commercial kits used to extract AMF DNA. For that reason, in this assay, several DNA extraction protocols were used to extract AMF DNA from root samples and commercial products directly, and most of the samples had not enough quality or quantity, or despite presenting good quantity and quality of DNA did not amplified using constitutive or specific primers. In our case the best protocol was the DNeasy PowerSoil kit, that

although provide good quality samples, when it comes to commercial products, not always worked using AMF specific primers. Therefore, in a first phase of this assay, grass samples (highly colonized by AMF) were tested using several DNA extraction protocols, and after, different sets of primers in order to set up the best protocol to AMF molecular detection.

Another issue to the AMF molecular detection is the primer selection. It is very difficult to design primers for all glomalean fungi excluding other fungi and plants, otherwise, numerous different species will be co-detected (Van Tuinen *et al.,* 1998). The solution for this problem can be designing primers for specific groups of AMF which is also very difficult since the rDNA sequences are often identical within species (Buckler *et al.,* 1997). Also, one root can be colonized by more than one AMF which makes hard to distinguish between species.

Therefore, we decided to use the primers designed for Krüger *et al.* (2009) instead of design new primers for Arbuscular Mycorrhiza. Krüger (2009) tried to include the LSU region of the AMF DNA, since the studies that used only the SSU or the ITS region couldn´t amplify all species of arbuscular Mycorrhiza. In this study, they prepared a mix of primers to create two sets of primers that included LSU, SSU, and ITS region of AMF DNA (Fig. 16). According to Krüger, these primers have a relatively good efficiency on root and spores samples, an excellent specificity (discrimination against non-targeted fungi and plant DNA), and work for all *Glomeromycota*.

When these primers were tested on grass samples, commercial products of Mycorrhiza and roots inoculated with the same commercial products, the grass samples were the only ones that we enabled the amplification of these primers. Being that, is was decided to reamplify the PCR product using the same primers. On the two reamplifications performed, the only samples able to amplify the expected bands (1500-1800bp) were the grass samples and roots inoculated with AEGIS-Gel®. The remaining samples amplified smaller sized bands, that when ran on BLAST, came back non-conclusive due to the DNA damage or low DNA concentrations on the PCR products. On the grass samples and AEGIS-Gel®, it was able to identify *Glomus spp*., however the species identification was not conclusive. Besides that, these samples presented other smaller sized bands (200 bp, 500 bp and 600bp) that belonged to plant DNA or non-targeted fungi. This may happened since the results were obtained with a reamplification using the PCR product as a new target. When repeating the same PCR using the PCR product

as a new target, sometimes the border of the amplicons degrade or the amplicon fragmentize, so, that could originate some unspecific amplification patterns.

After this experiment, it was decided to try specific primers to *Glomus intraradices* designed by Alkan *et al.* (2006), in order to avoid the errors of not so specific primers. This set of primers was designed to be used on q-PCR, however on this assay they were applied with the purpose to be used in a near future to set up a q-PCR detection system. With these primers it were only tested roots inoculated with AEGIS-Gel® because there was not enough quantity of the samples, used on the last assay. The set-up of these primers was easier than the one of Krüger primers and the DNA amplified on the first amplification. The bands are small (100bp), because the primers were designed for q-PCR. The results of the sequencing allowed us to identify *Glomus* spp.*,* however the species identification was not conclusive.

As conclusion, this study was not able to identify Mycorrhizal DNA until the species, only until the gender. This is probably a result of bad DNA samples and not enough DNA concentration, which is not very plausible since the constitutive amplifications worked showing the presence of DNA, or not enough quality, due to contaminations of DNA from plant or non-targeted fungi extracted along with the targeted DNA, which seems more plausible. Besides that, the sequenciation of the samples amplified with the Krüger primers was made using the purified gel, which proved afterwards not being as effective as the sequenciation performed on PCR product. Being that, the next step seems to be improving the DNA extraction protocol in order to obtain cleaner and better samples, and probably try new sets of primers and “nested PCR procedures”.

5.3 Design and evaluation of primers for different species of *Trichoderma*

Unlike what happened with Mycorrhiza, the molecular detection system to identify *Trichoderma* was easier to set up, once *Trichoderma* is easily grown *in vitro* and easy to extract. The DNA extraction of commercial products (Mamull® and Tifi®) and roots inoculated with Mamull® and Tifi® resulted on samples with enough DNA concentration and quality.

In the case of *Trichoderma* detection, it was decided to design new primers targeting the ITS region of *Trichoderma*. Four different set of primers were designed, one specific for species of *Trichoderma* present on Tifi® and Mamull® *(T. gamsii, T. artroviride, T. harzianum1 and T. harzianum 2*). The fragment amplified by these primes is approximately 200bp long.

Each result of the PCR reactions with the primers designed on plants inoculated and non-inoculated with *Trichoderma* showed one clear band with around 200bp as expected. After sending the purified PCR products for sequencing, the results showed that all amplified sequences could be assigned to *Trichoderma* species. Also, these primers specificity to *Trichoderma* DNA was good, since they did not amplified non-targeted fungi or plant DNA. However, despite the fact of designing the primers with DNA sequences from specific strains, the results were not conclusive to the species.

When tested with pure cultures of the *T. artroviride* "Sherwood" strain, Tifi® and Mamull®, the results of the designed primers were the same, one clear band at approximately 200bp that represented only *Trichoderma* DNA, however, the species identification was not conclusive.

Being that, the designed primers proved to be specific to *Trichoderma* spp., not amplifying non-targeted fungi or plants and targeting the ITS region of the *Trichoderma* DNA. The fact that the ITS sequences from different *Trichoderma* species are very similar (Lieckfeldt *et al.,* 1998; Singh *et al.,* 2014) it is the reason which why it is not possible to identify the species with conventional PCR.

Besides creating this four sets of primers, it was tested one set of primers from Hagn *et al.* (2007). This primers amplify sequences with approximately 540bp that target the ITS 1, ITS 2 and 5.8rRNA regions of *Trichoderma* DNA.

When tested with samples from roots of plants inoculated and non-inoculated with Tifi® and Mamull® the UT primers from Hagn *et al.*(2007) (primers that amplify the ITS region, part of the SSU and part of the LSU region of *Trichoderma* spp.) amplified one clear band on every sample around 500bp as expected. When sent to sequencing all the sequences belonged to *Trichoderma* species as expected. When tested with pure cultures of *T. artroviride* and Mamull® one extra band appeared at approximately 300bp that showed no results when send to sequenciation, so probably represented some type of

contamination. When tested with a pure culture of Tifi®, the UT primers did not amplify DNA, probably due to a DNA degradation on the sample.

In conclusion, as many other studies in the past (Meincke *et al.,* 2010; Zhihui *et al.,* 2008) including Hagn *et al.* (2007) it was decided to test on this assay, several primers that are being developed in order to improve the molecular detection and identification of *Trichoderma*, however, just like what happened to the primers designed during this work, this studies were not able to identify any specific strain of *Trichoderma*.

Until today, there is not enough information of the *Trichoderma* and Mycorrhiza DNA that enables to identify to the species using viable methods. There are some methods of species identification such as next generation sequencing (NGS) and metagenomics approaches, however, in an industrial perspective, these methods are too expensive and not very practical to implement in a company, due to the time some of them require, and the necessary equipment to perform them. Being that, and considering that this project was develop to be applied in a company, the importance is not identify the species, but to have an implemented control system for the nursery process. Therefore, the primers designed and set up on this project are being used to identify the presence of *Trichoderma* and Mycorrhiza along with the tinction process (already set up to Mycorrhiza and currently being develop to *Trichoderma*). Other technique being develop at the Concha y Toro laboratories is the q-PCR technique that compares the number of DNA fragments copy of Mycorrhiza and *Trichoderma* under inoculated plants, being an indirect procedure to discriminate between inoculated and non-inoculated plants.

5.4 *In vivo* assay

However the *in vitro* assays are extremely important to select the best inoculation strains, it is also important to know how *Trichoderma* and Mycorrhiza inoculations affect the plant physiology and morphology. For that an assay was performed on Concha y Toro nursery during the 2017/2018 season, to test the effects of *Trichoderma* and Mycorrhiza inoculation combined with the cleaning process on nursery grapevines. The assay tested three different factors: the cleaning process, the *Trichoderma* inoculation and the Mycorrhiza inoculation. Each factor presented three different levels or treatments. The cleaning processes was the VCT process (already chemical procedure implemented in

the company) (L0), the neutral anolyte cleaning (L1), or the HWT (L2), the *Trichoderma* inoculation was performed with Mamull® (T1), Tifi® (T2), or water (control) (T0) and the Mycorrhiza inoculation were done with AEGIS-Gel® (M1), Oiko-Rhiza® (M2) or no treatment (control) (M0). Each possible treatment (for example LOTOMO) had three replicates made with 50 plants each.

Several analyses were performed in this experiment in different stages of the grapevine nursery process. Since the assay is very complex (three levels with three factors), it is hard to correlate the results and avoid external effects to influence the results, has happened with the presence of trunk diseases fungi, supposed evaluated before the assay, after the grafting and 11 months after, at harvest. Probably associated with the plant status in mother block before the harvest, too many plants were lost during the grafting process or not qualified with the nursery quality standards making it impossible to determine the presence of fungi during the assay.

5.4.1 Budburst percentage

As expected the budburst was only affected by the cleaning process used on the plants since the *Trichoderma* and Mycorrhiza treatments were not done at that point.

The cleaning process using a neutral anolyte (Aquannolyte) showed significant lower budburst percentages than the VCT treatment. The neutral anolyte solutions contain a mixture of oxidizing substances made of molecules of carbon, oxygen, nitrogen and phosphorus at a neutral ph. These substances such as HOCL, CL^-, CLO, H_2O_2, pronounced microbiocidal effectiveness against bacteria, viruses and fungi (Prilutskiĭ *et al.,* 1996). In addition to the fact that H_2O_2 can function as a signaling molecule in plants (Desikan *et al.,* 2004; Foyer and Noctor, 2005), and that the increasing of H_2O_2 on the grapevine buds can inhibit the catalase activity inducing budburst (Pérez and Lira, 2005; Perez and Rubio, 2006), it is also well-known the oxidative damages caused by increases in H_2O_2 levels in plant tissues (Prilutskiĭ *et al.,* 1996). Although the concentrations of Aquanolyte used on this assay were the recommended by the supplier, this product lacks studies of its effects on plants, since has only been tested to clean surfaces (counters, floors, etc.). Being that, the effects of this particular product on plants, particularly in the budburst is not well-known and the fact that, there is a slightly

difference between the concentration of H_2O_2 that is beneficial for the budburst and the concentration that causes oxidative stress on the plant tissues, may be plausible explanations for the lower budburst percentages using this cleaning process.

5.4.2 Mycorrhiza analysis

Three different analysis were performed (Mycorrhiza frequency on the root system (F%), Mycorrhiza colonization at the root system (M%), and arbuscules abundance on the root system (A%)) on two different times of the assay (3 months and 11 months after the plantation in nursery field). The specific results for each parameter are complex and sometimes inconclusive as discussed further on. However, if we simplify the results analysing the Mycorrhiza factor by itself, it´s evident that the arbuscules abundance on the root system increases on eleven months which indicates that the Mycorrhiza are functional, and although the Mycorrhiza frequency on the root system decrease, the intensity of the Mycorrhization increased during the assay, proving the success of the Mycorrhization process.

Also, it is clear that the cleaning process used on the nursery affected the Mycorrhization process after 3 months of the transplantation, however after 11 months this effect wasn't seen anymore.

However, due to the complexity of this assay, there are some external factors that can affect the Mycorrhization that should be discuss in future studies.

5.4.2.1 Mycorrhiza frequency on the root system

After three months of the plantation, both, the Mycorrhiza treatment and the cleaning process affected the Mycorrhiza frequency on the root system. Being that, when there is a Mycorrhiza treatment (M1 or M2) the VCT cleaning process was the most effective one on Mycorrhiza frequency. As mentioned before, the Aquannolyte product used on this assay has not been tested on plant material, and in lightly high concentrations may cause oxidative damages on the plant tissues (Prilutskiĭ *et al*., 1996). Also, despite the fact that the hot water treatment is being used to disinfect plant material since the nineteen century, is proved to delay root and bud development in certain varieties and rootstocks

(Waite and Morton, 2007). As mentioned before, AM fungi colonize their hosts not only by contact but also as a result of complex and poorly understood signalling mechanisms (Bonfante and Genre, 2008). Being that, the fact that the neutral anolyte and the HWT cleaning treatment affect the plant tissues weakening the plant, may block the release of signal substances by the plant hindering the Mycorrhiza to connect with it, explaining why the Mycorrhiza frequency is lower when these treatments are used.

Eleven months after the transplantation, the frequency of Mycorrhiza on the root system decreased slightly, however there were no significant differences between treatments. There are no certainties about the reasons that led to this slightly decrease, however it may be due the phenology of the Mycorrhiza under the nursery level, in which there might have a decrease on the frequency but an increase on intensity of Mycorrhization and an increase of the Mycorrhizal activity, as can be seen on the next section.

5.4.2.2 Mycorrhiza intensity on the root system

After three months of the plantation, the cleaning process was the factor that affects the intensity of Mycorrhiza on the root system. The results corroborate with the results from the Mycorrhiza frequency, in which the VCT process showed significant better results. This probably is explained, as mentioned before, by the damage that HWT and the neutral anolyte can cause to the plant preventing it from releasing the signal substances and consequently hindering Mycorrhization.

At the moment of harvest (11 months after the transplantation) the cleaning process no longer affected the Mycorrhiza intensity, however the Mycorrhiza and the *Trichoderma* treatments did. In general, the treatment with AEGIS-Gel® was the most advantageous one, since worked very satisfactorily with any *Trichoderma* treatment. The treatment with Oiko-Rhiza® presented the worst results when combined with Mamull®, and the treatment without Mycorrhiza or *Trichoderma* didn´t work either very well. The interactions between Mycorrhiza and *Trichoderma* are very poorly studied and can probably be highly affected by external factors such as temperature, soil type, biotic and abiotic stresses, which indicates the need of more studies on this subject.

5.4.2.3 Arbuscular intensity on the root system

It is important to notice the relevance of this parameter once the arbuscules are the structures that penetrate the root cells and allow the Mycorrhiza to associate with the plant (Gianinazzi-Pearson *et al.,* 1996). Therefore, the presence of these structures allows to realize if the Mycorrhiza is functional or not more than just its presence.

As happened with other Mycorrhiza parameters, 3 months after the transplantation, the cleaning process affected the arbuscular intensity, such as the Mycorrhiza treatment, and as in the other parameters, the VCT process was the one that presented better results when combined with a Mycorrhiza inoculation (M1 or M2), probably for the reasons explained before. When it comes to differences between Mycorrhiza treatments, there were no significant differences since both products worked similarly with every cleaning process.

Eleven months after the transplantation there was a substantial increase on the arbuscular intensity on the root system on every treatment, however there were no significant differences between treatments. The substantial increase on the arbuscular percentage showed that the Mycorrhiza present on the plants was active and in consequence functional. Despite the fact that no significant differences were found, there is a tendency that shows higher values of arbuscular intensity on inoculated plants when compared to the control. Moreover, the soil used on this assay was not tested for nitrogen and other nutrients content. Therefore, if the soil is not deficient on any nutrient, the Mycorrhiza are not able to reach their maximum potential since the plant is not under any stress, which may lead them to show no differences when compared to the treatment. As described below, the nitrogen content is higher on plants inoculated with Mycorrhiza, however, there were no differences on the rest of macro and micronutrients, which corroborates the thought that maybe the soil has no nutrient deficiency, inhibiting the Mycorrhiza to achieve its maximum potential.

5.4.3 Chemical analysis

5.4.3.1 Macro and micro nutrients

After the harvest the plant roots were analysed to the arginine and nitrogen content, other macro and micro nutrients (calcium, potassium, magnesium, sodium, phosphorus, silica, boron, iron, copper, zinc, and manganese) and dry weight.

The behaviour of arginine and nitrogen content was similar, in which the Mycorrhiza treatment was the factor that influenced. The plants treated with Oiko-Rhiza® (M2), had significant higher nitrogen and arginine content that the control plants (M0). The plants treated with AEGIS-Gel® (M1) had higher nitrogen and arginine content than the control plants and less than the Oiko-Rhiza® plants however none of these differences were significant. When associated with plant roots, AMF created a mutualistic interaction based on biotrophic nutrient exchanges between AMF and the plant. The host plant supplies the Mycorrhiza with carbon, and the Mycorrhiza enhances the ability of the plant to get water and nutrients from the soil (Smith and Read, 2008). Along with other advantages, AMF are known to enhance the plant nitrogen, phosphorus and other nutrients uptake, which explains why the inoculated plants present higher amounts of nitrogen (Smith and Read, 2008). Arginine is known for having an important role on the nitrogen intake, namely on plants with Mycorrhiza symbiosis (Abuzinadah *et al.,* 1986; Frungillo *et al.,* 2014; Jin *et al.,* 2005; Smith and Read, 2008), which explains why it present the same behaviour as the nitrogen content. However, when it comes to the other micro and macro nutrients, there were no significant differences among the Mycorrhiza treatments. One possible explanation for this results can be the fact that the analysis were performed only on roots. Although the nutrients uptake happens essentially on the plant roots, these nutrients have the ability to move inside the plant to where they are most needed (Kaitaniemi and Honkanen, 1996). Therefore, the extra concentration of macro and micronutrients provided by the AMF association may be translocated to other parts of the plant such as shoots or leaves, and not being detected on the roots. Another possible explanation, as pointed before, may be the fact that the soil had no deficiency of any of these nutrients, and the ideal conditions did not allow the Mycorrhiza to reach its maximum potential showing no differences when compared with the control.

5.4.3.2 Root Dry weight

The results show that every factor affects dry weight (Cleaning process, *Trichoderma* and Mycorrhiza). This means that the values for root dry weight vary according to the combination of treatments, and the need to analyse individually every different combination. Although the analysis of each possibility is very complicated, it is important to understand why this happened. The root dry weight (root biomass) consists on the root weight without the water and it is used to understand the plant growth and development. However, the root growth is affected by all types of external and internal factors such as biotic stresses (salinity, temperature, drought, etc) and biotic stresses (parasites, plant self-regulation). Being that, it is explainable that the results for dry weight are very complex, since there are several other factors that were not take in account.

In conclusion regarding the nursery assay, the VCT seemed to be the most effective cleaning process along *Trichoderma* and Mycorrhiza inoculations. Both Mycorrhiza treatments showed positive results although Oiko-Rhiza® was the one with the best results. However, in an industrial context, the costs to implement Oiko-Rhiza® are significantly higher than the costs to implement AEGIS-Gel®, so this is probably the better solution to the company. Based only on this assay, it is not possible to identify the best *Trichoderma* treatment, because it is necessary, in the case of *Trichoderma*, to evaluate the plants under a controlled infection with different GTDs to understand if the treated plants present a better response against this diseases than the control plants, and are able to control the GTDs spread of the diseases into the plant.

6. Conclusion

The principal aim of this project was to evaluate the effectiveness of different strains of *Trichoderma* and the best strategy to use *Trichoderma* and Mycorrhiza against trunk diseases fungi in nursery grapevines. The first part of this objective was achieved by performing an *in vitro* assay that tested five *Trichoderma* native strains and commercial products against different trunk diseases fungi. In this assay the commercial product Tifi® was the most effective treatment against all trunk diseases fungi with special emphasis

to *E. lata* in which Tifi® was the only effective treatment after *Eutypa* was established. The commercial product Mamull® improved its effectiveness at lower temperatures showing worst results than Tifi® on this assay. The native "Sherwood" strain presented some potential on pathogen growth inhibition, being however less effective than Mamull® and Tifi®. The *Trichoderma* spp. 1 and 2 native strains were not effective against any trunk disease fungi because were isolated from symptomatic plants of organic vineyards.

For establishing the best strategy to use *Trichoderma* and Mycorrhiza against trunk diseases fungi in nursery grapevines two different works were performed. In one, it were designed and tested *Trichoderma* and Mycorrhiza primers in order to follow the *Trichoderma* and Mycorrhiza development after the inoculation. On both cases, the primers were only able to identify the genus *Trichoderma* spp. and *Glomus* spp. respectively. Although this was not the desired result, these primers were able to detect the presence of *Trichoderma* and Mycorrhiza and can be used as a pre-diagnosis. Therefore, it is necessary to differentiate between the inoculated and non inoculates plants to understand if the inoculation was successful. For that there are two different techniques being develop at Concha y Toro now, the q-PCR technique that allows distinguishing between the DNA concentration of an inoculated plant and a control plant (not inoculated). The other technique, to be used along with the q-PCR that is the most viable one at the industry environment is the tinction method that allows one to see on a microscope the roots with Mycorrhiza (which is already being used) (Pitet *et al.,* 2009; Vierheilig *et al.,* 1998) and *Trichoderma* (which is being developed).

The other assay performed to establish the best strategy to use *Trichoderma* and Mycorrhiza against trunk diseases fungi in nursery grapevines was the *in vivo* nursery assay. This experiment is quite complex and is intended to continue on the next seasons in order to collect more data and adjust the design. Consequently, the results obtained are preliminary and sometimes not completely clear. However, so far it was possible to clearly identify the VCT process as the most effective cleaning process. Both Mycorrhiza products showed good results even though the Oiko-Rhiza® was slightly more effective. Yet, since this project is being developed in an industry context, the AEGIS-Gel® seems to be a better solution due to the price difference when compared to Oiko-Rhiza®. When it comes to the *Trichoderma* treatment there were no conclusive results on this experiment yet, however based on the *in vitro* treatment, Tifi® would be the best product if it was necessary to choose one now.

7. Future perspectives

Although these experiments were successful on testing the best products and techniques to prevent trunk diseases fungi on nursery grapevines, on an industrial perspective, it is extremely important to continue these kind of experiments in order to obtain robust results. Repeating the *in vitro* assay testing different temperatures and different *Trichoderma* strains (using strains isolated from sane plants surrounded by symptomatic plants) can be significant to develop better *Trichoderma* products. The q-PCR technique to monitor the success of *Trichoderma* and Mycorrhiza inoculations must be implemented along with the tinction process to *Trichoderma*. Lastly, the *in vivo* experiment must be extended for the next seasons to collect more data and in order to simplify this complex design, withdraw the cleaning process in which the results of this experiment were obtained.

In a scientific perspective, it is imperative to study more accurately the interactions between *Trichoderma* and Mycorrhiza, and the plant, and more important the factors that can affect these interactions that allow the identification of new markers as signal responses of the pathogenic or beneficial interaction between plant/pathogen/beneficial microorganisms. It is important to complement the use of *Trichoderma* and Mycorrhiza with another beneficial microorganism like endophytic bacteria's that might allow the winegrowers to "close" the windows for the entrance of pathogens in a more sustainable point of view.

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9. Attachments

I. List of primers used on this study

Supplementary table1. List of primers used on Mycorrhiza detection.

Primer I.D.	Primer mix	Sequence (5'-3')	Annealing temp.	Amplification product
SSUmAf	SSUmAf1	TGGGTAATCTTTTGAAACTTYA	50°C	~1500bp
	SSUmAf2	TGGGTAATCTTRTGAAACTTCA		
LSUmAr	LSUmAr1	GCTCACACTCAAATCTATCAAA	50°C	
	LSUmAr2	GCTCTAACTCAATTCTATCGAT		
	LSUmAr3	TGCTCTTACTCAAATCTATCAAA		
	LSUmAr4	GCTCTTACTCAAACCTATCGA		
SSUmCf	SSUmCf1	TCGCTCTTCAACGAGGAATC	50°C	~1500bp
	SSUmCf2	TATTGTTCTTCAACGAGGAATC		
	SSUmCf3	TATTGCTCTTNAACGAGGAATC		
LSUmBr	LSUmBr1	DAACACTCGCATATATGTTAGA	50°C	
	LSUmBr2	AACACTCGCACACATGTTAGA		
	LSUmBr3	AACACTCGCATACATGTTAGA		
	LSUmBr4	AAACACTCGCACATATGTTAGA		
	LSUmBr5	AACACTCGCATATATGCTAGA		
VC-F	-	GAGACCATGATCAGAGGTCAGGT	56°C	101bp
VC-R	-	GGTCATTTAGAGGAAGTAAAAGTCGTAAC	56°C	

Supplementary table 2. List of primers used on *Trichoderma* detection.

Primer I.D.	Sequence (5'-3')	Annealing temp.	Amplification product
T.gamsii-F	GGAGGGACCAACCAAACTCT	55°C	~200bp
T.gamsii-R	TGAAATGACGCTCGGACAGG		
T.artroviride-F	CGGAGGGACCAACCAAACTC	55°C	~200bp
T.artroviride-R	TGAAATGACGCTCGGAGAGG		
T.Harzianum1-F	ACTCCCAAACCCAATGTGAA	54°C	~200bp
T.Harzianum1-R	CGATGCCAGAACCAAGAGAT		

T.Harzianum2-F	AACTCCCAAACCCAATFTGA	56°C	~200bp
T.Harzianum2-R	CGATGCCAGAACCAAGAGAT		
uTf	AACGTTACCAAACTGTTG	54°C	~540bp
uTr	AAGTTCAGCGGGTATTCCT		

Supplementary table3. List of primers used on constitutive PCR reactions.

Reagents	**Volume for a standard PCR reaction (primers ITS, T.gamsii, T.artroviride, T.Harzianum1, T.Harzianum 2 and UT)**	**Volume for a PCR reaction with Krüger primers**	**Volume for a PCR reaction with VC primers**
Sterile water	2.6 µl	2 µl	2.2 µl
Forward primer	0.2 µl	0.5 µl	0.4 µl
Reverse primer	0.2 µl	0.5 µl	0.4 µl
GoTaq (R)®	5 µl	5 µl	5 µl
DNA sample	2 µl	2 µl	2 µl

Supplementary table 4. PCR reaction components using GoTaq (R)® for 10µl of reaction per sample for different sets of primers.

Primer I.D.	**Sequence (5´-3´)**	**Annealing temp.**	**Amplification product**
ITS1	TCCGTAGGTGAACCTGCGG	56°C	~500bp
ITS4	TCCTCCGCTTATTGATATGC	56°C	~500bp
ITS5	GGAAGTAAAAGTCGTAACAAGG	56°C	~500bp

II. General nursery process, cleaning processes, Mycorrhiza and *Trichoderma* treatments.

Supplementary table 5. Concha y Toro nursery process, Mycorrhiza and *Trichoderma* applications and cleaning process detailed. The cleaning processes are VCT process (L0), neutral anolyte (L1) and thermotherapy (L2). The Trichoderma treatments are Mamull® (T1), Tifi® (T2) and water (control) (T0). The Mycorrhiza treatments are AEGIS-Gel® (M1), Oiko-Rhiza® (M2).

		Acquisition of material	Pre-cold	cold	hydration	Grafting	Stratification	Budburst and selection	Plantation (nursery)	harvest	Packaging
Cleaning process	L0		10min Rovral © 50% (6g/l) + POMARSOL © FORTE 80% (10g/l)		8h Chlorine dioxide>5% (1ml/l)	6h Tesca© 50% (1g/l) POLYBEN (50WP)		10g cuper/bins			10min Rovral © 50% (6g/l) + POMARSOL © FORTE 80% (10g/l)
	L1		6h Aquannolyte © 25%		6h Aquannolyte © 25%	Pulverization Aquannolyte © 6%	Pulverization Aquannolyte © 6%				6h Aquannolyte © 25%
	L2		5min 54°C		30min 50°C						
Trichoderma treatment	T0					Aspersion with water	Aspersion with water				
	T1					Aspersion with Mamull ($1x10^7$ufc/g)	Aspersion with Mamull ($1x10^7$ufc/g)				
	T2					Aspersion with Tifi ($2x10^8$ufc/g)	Aspersion with Tifi ($2x10^8$ufc/g)				
Mycorrhiza treatment	M0								No treatment		
	M1								Submersion in AEGIS-GEL (2.5 cc/planta)		
	M2								Submersion in OIKO-RHIZA (5g/planta		