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Tocopherols (vitamin E) production and profile of mycorrhizal fungi before and after *in vitro* elicitation by host plant roots

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List of abbreviations

Akt	Protein kinase B
AM	Arbuscular mycorrhizas
APCs	Cells and antigen-presenting cells
BHT	Butylated hydroxytoluene
CAT	Catalase
CT	Co-culture time
Dw	Dry weight
ECM	Ectomycorrhizas
EMM	Estimated marginal means
F	Flask
Fw	Fresh weight
GPx	Glutathione peroxidase
HPLC	High performance liquid chromatography
HPLC-FL	High performance liquid chromatography coupled to a fluorescence detector
IBA	Indole-3-butyric acid
LDL	low density lipoprotein
MMN	Melin-Norkans medium

MS	Murashige and Skoog medium
MSt	Medium state
Pd	Petri dish
SOD	Superoxide dismutase
VAM	Vesicular-arbuscular mycorrhizas
α-T	α-Tocopherol

Abstract

Mycorrhizal fungi are symbiotic organisms that establish associations with the root system of plants. This special form of living implicates a recognizing process where chemical signals are involved, and oxidative stress mechanisms are most probably activated and overcome. Thus, during this process there is the release of compounds from the secondary metabolism, characterized by their bioactive potential and, therefore, compounds of interest.

Tocopherols, vitamin E vitamers, are some of the molecules released during the oxidative stress process. Actually, vitamin E is the common name for a group of compounds which includes tocopherols and tocotrienols, lipophilic antioxidant compounds with important effects on the body, including aging prevention, immune system strengthening and cancer risk reduction.

Mushrooms have been reported as a source of different antioxidants, among which are tocopherols.

In order to better understand the establishment of the mycorrhizal symbiosis and to promote the production of compounds of interest, namely tocopherols, two species of mycorrhizal mushrooms (*Clitocybe odora* (Bull.) P. Kumm. and *Scleroderma polyrhizum* (J.F. Gmel.) Pers.) and host plants (*Castanea sativa* Mill.) were studied separately and in co-culture. The aim of the present work was: i) evaluate tocopherols composition of mycorrhizal mushrooms from different species/ecosystems; ii) assess tocopherols production of *in vitro* isolated and grown mycelia of the studied species; and iii) analyse tocopherols production of *in vitro* grown mycelia before and after co-culture by host roots, evaluating the effect of host root on the production and profile of tocopherols.

After co-culture of both mushroom species with *Castanea sativa* roots, the tocopherols profile of the three species was determined by high performance liquid chromatography coupled to a fluorescence detector (HPLC-FL). The results obtained showed that the studied species produce rare vitamers of tocopherol, but this mycorrhizal relationship does not cause any oxidative stress for mushrooms or roots, hence the drop in the amount of the different vitamers after the coculture.

Resumo

Os fungos micorrízicos são organismos simbióticos que estabelecem associações com o sistema radicular das plantas. Esta forma especial de vida implica um processo de reconhecimento onde estão envolvidos sinais químicos e os mecanismos de stresse oxidativo são ativados e superados.

A vitamina E é o nome comum para um grupo de compostos que incluem tocoferóis e tocotrienóis, compostos antioxidantes lipofílicos com efeitos importantes no corpo humano, incluindo a prevenção do envelhecimento, fortalecimento do sistema imunológico e redução do risco de cancro. Os cogumelos são referidos como uma fonte de diferentes antioxidantes, entre os quais os tocoferóis.

Os micélios de fungos micorrízicos produzem tocoferóis em condições de cultura *in vitro* e pretendemos: i) avaliar a composição de tocoferóis de fungos micorrízicos de diferentes espécies / ecossistemas; ii) avaliar a produção de tocoferóis de micélios das espécies estudadas isolados e produzidos *in vitro*; e iii) analisar a produção de tocoferóis de micélios cultivados *in vitro* antes e depois da co-cultura com raízes hospedeiras, avaliando o efeito da presença da raiz do hospedeiro na produção e perfil de tocoferóis.

Após a co cultura de micélio com as raízes de *Castanea sativa*, foi realizada uma análise por HPLC para estudar a variação de tocoferóis em diferentes amostras ao longo do tempo. Os resultados obtidos demonstraram que as espécies estudadas produzem vitâmeros raros do tocoferol, mas o estabelecimento destas relações micorrízicas não causa stress oxidativo para fungos ou raízes, daí a queda na quantidade dos diferentes vitâmeros após a co cultura.

1 INTRODUCTION

1.1 Mycorrhizas

Mycorrhizas are symbiotic associations between plants and fungi that colonize the cortex of their roots, being a beneficial association for both partners (Brundrett, 2004).

The term symbiotic was originally used to define both lichens and parasites. Now, many scientists use this term to describe beneficial associations only (Brundrett, 2004). Although the term seems to have been coined several years earlier, in 1977 fungal symbioses have been defined as "all associations where fungi come into contact with living host from which they obtain, in a variety of ways, either metabolites or nutrients" (Cooke, 1977).

This association is triggered by a series of chemical signals emitted by both partners, which implies a recognition process in which oxidative stress mechanisms are overcome. For example, plants release soluble factors, such as strigolactones that activate both the metabolism and branching of the fungal partners (Bonfante & Requena, 2011). Instead, fungi use compounds that activate the signaling transduction pathways that are required for the symbiotic modus of plant cells (Bonfante & Requena, 2011). In fact, these signals are sent by both partners before the actual physical contact, and lead to morphological changes and a complex development of specific structures in both plant and fungus (Adomas Heller et al., 2008). The overexpression and under-expression of stress response or defense genes mark the complex nature of mycorrhizal interactions (Heller et al., 2008).

Mycorrhizas increase plant uptake of water and nutrients, such as nitrogen and essential elements (phosphorus, copper and zinc), particularly when they are in low concentrations or in insoluble forms in the soil. By growing beyond the immediate root zone, the mycelium filaments concentrate the nutrients farther away. Moreover, fungi are suitable for mining minerals from organic and inorganic substrates improving the hydraulic properties of soil (Johnson, 2016; Anabela Martins, 2008). The nutrient flow is a reciprocal process in most mycorrhizal associations (as it is a symbiosis). Therefore, the fungus helps the plant obtaining nutrients and water, while the plant provides the fungus carbon compounds, especially sugars (hexoses), which are absorbed and converted in sugar-derived alcohols, such as mannitol (Anabela Martins, 2008). The fungal mycelium secretes extracellular enzymes that unblock organic complexes (M. S. Carocho, 2011) and the newly released nutrients are then selectively absorbed directly through the cell walls of the mycelium network. In addition to increasing the root absorption surface and obtaining nutrients, another advantage of

mycorrhizal associations is the protection of the plants involved against drought and pathogens (Anabela Martins, 2008; Singh, Kaushal, Kumar, Vimal, & Gupta, 2016). In fact, it has been shown that maintenance and stress resistance of trees improve considerably when plants are inoculated with ectomycorrhizal fungi in nurseries (A Martins, 2010; Anabela Martins, 2008).

Mycorrhizas are classified according to their structure, host families and symbiotic fungal taxa. There are seven types of mycorrhizal associations: i) vesicular-arbuscular mycorrhizas (VAM), widely known as arbuscular mycorrhizas (AM); ii) ectomycorrhizas (ECM); iii) ectendomycorrhizas; iv) ericoid mycorrhizas; v) orchid mycorrhizas; vi) arbutoid mycorrhizas; and vii) monotropoid mycorrhizas. The most common association of all mycorrhizae is the arbuscular mycorrhiza (AM), followed by ectomycorrhizas (ECM) and orchid mycorrhizas (Brundrett, 2004; Shah, 2014).

The main mycorrhizal categories and subcategories are listed and described in **Table 1**.

Table 1: Types and categories of the main mycorrhizal associations (based on Brundrett 2004(Brundrett, 2004)).

Categories	**Subcategories**	**Definition**
Arbuscular mycorrhiza		Mycorrhizal association formed by Glomeromycota fungi in land plants usually with arbuscules and often with vesicles.
Ectomycorrhiza		Associations of higher fungi with land plants with short lateral roots where a hyphal mantle encloses the root and a Hartig net comprising labyrinthine hyphae penetrates between root cells
	Cortical	Hartig net fungal hyphae colonize multiple cortex cell layers of short roots (most associations are in gymnosperms)
	Epidermal	Hartig net fungal hyphae are confined to the epidermal cell layer of short roots (occurs in angiosperms)
	Monotropoid	Exploitative epidermal ECM of myco-heterotrophic plants in the Ericales where individual hyphae penetrate epidermal cells
	Arbutoid	ECM of autotrophic plants in the *Ericaceae* where multiple hyphae penetrate epidermal Hartig net cells

Orchid mycorrhiza	Associations where coils of hyphae (pelotons) penetrate within cells in the plant family *Orchidaceae*

1.1.1 Mycorrhizal fungi

Although mushrooms represent very important biodiversity, part of an autonomous kingdom (Latin fungus = mushroom) (Liu, Hodson, & Hall, 2006), they are among the least exploited resources in the world. However, it is estimated that this kingdom contains 2.2 to 3.8 million species (Hawksworth & Lücking, 2017), including 50,000 fungal species forming mycorrhizal associations (Heijden, Martin, & Sanders, 2015). Thus, there are mycorrhizal fungi associated with plant roots and facilitating the absorption of nutrients, and there are two main different types: Ectomycorrhizae and Endomycorrhizae, which are the most common forms (Stamets, 2000). These soil inhabitants play a very important role in the environment, namely recycling and in particular the biodegradation of organic matter, which is a vital role because they are heterotrophic organisms (Hanson, 2008; Webster & Weber, 2007), as well as their characteristic of establishing reciprocal interactions in the form of a molecular dialogue with plant roots that ultimately leads to symbiosis (Sally & Read, 2008). In plant biology, this symbiosis is important since its establishment ameliorate the nutrient cycle, hence the nutritional status of the host, affects growth, water absorption and protection against root afflictions (Sally & Read, 2008).

The fungi studied in the present work are described below.

1.1.1.1 *Scleroderma polyrhizum* (J.F. Gmel.) Pers.

S. polyrhizum (**Figure 1**), commonly known as star earth ball or dead man's hand, is a well-known edible fungus. However, its consumption is not advisable (Brandenburg, 2010). This species establishes mycorrhizal associations with several trees (e.g., *Caryocar brasiliense* Camb.(Pers, 2000), *Pinus radiata* (Duñabeitia et al., 2018)), being also used in the Traditional Chinese Medicine (Paterson, 2008). The basidiome is globose, regular or irregular and sessile, with a thick peridium from 2 to 10 mm. It is smooth to rough or cracked. It has cottony and fibrous mycelium. The endoperidium is rubescent and has several recurved thick lobes. The gleba is grayish-brown to violaceous-brown, covered by a thin, cottony and white to the dark brown membrane. The taste and odor are similar to rubber. The basidiospores are sub-reticulated, and the basidium is not noticeable but the clamp connections are present. The

species grow lonely or gregarious on soil (mainly sandy-clay soils), being hypogeous when immature and epigeous in the dehiscence (Kuo, 2006).

S. polyrhizum is a common species in Europe (Storey, 1997), North of Africa (B, G, & L, 1993) and United States of America, but rare in Mexico (Pers, 2000). It can also be found in tropical forests (B et al., 1993).

Figure 1(Rodríguez, 2008) **:** Fruiting body of *S. polyrhizum* (J.F. Gmel.) Pers.

The taxonomic classification of *S. polyrhizum* is presented in **Table 2**.

Table 2: Taxonomic classification of *Scleroderma polyrhizum* (J.F. Gmel.) Pers.

Kingdom	Fungi
Division	Basidiomycota
Class	Agaricomycetes
Order	Boletales
Family	Sclerodermataceae
Genus	*Scleroderma*
Species	***S. polyrhizum***
Binomial name	***Scleroderma polyrhizum***

1.1.1.2 *Clitocybe odora* (Bull.) P. Kumm.

C. odora (**Figure 2**), commonly known as aniseed toadstool, is a mycorrhizal edible fungus usually found in leaf litter along the edges of both deciduous and coniferous woodland. Although good edible, this mushroom is most useful as a flavouring, being used after drying (Jordan, 2015). Its cap is convex, then flat with a diameter of 6 to 10 cm. It is green and finally fibrillated. The blades are adnate, very slightly decurrent and moderately crowded. It is sometimes forked, veined in the background, especially on old specimens. Besides, the stalk is cylindrical greenish blue, flaky fibrillose and hairy base slightly thickened. The flesh is dirty white, elastic and not very thick except on the disk. Generally, it has a strong smell of anise and a sweet flavor. The spores are white, non-amyloid, smooth and elliptical (4-5 x 6-7 micrometers). This fungus is solitary, gregarious or clumpy in the woods, especially under hardwoods. It may also occur in conifers woods from July to November. *C. odora* is easy to recognize with its blue-grey tones (Kuo, 2007).

Figure 2(Krisp, 2010) **:** Fruiting body of *C. odora* (Bull.) P. Kumm**.**

The taxonomic classification of *C. odora* is presented in **Table 3**.

Table 3: Taxonomic classification of *C. odora* (Bull.) P. Kumm.

Kingdom	Fungi
Division	Basidiomycota
Class	Agaricomycetes
Order	Agaricales
Family	Tricholomataceae
Genus	*Clitocybe*
Species	***C. odora***
Binomial name	***Clitocybe odora***

1.1.2 *Castanea sativa* Mill.

The chestnut tree can be exploited both for timber as well as fruit. In Portugal chestnuts are mainly cultivated for fruit production, since timber has mostly lost its importance in recent years (INIAV I.P, 2018).

China is the main chestnut producing country with more than 82% of world chestnut production. However, the main cultivated chestnut variety is this country is *Castanea mollissima*. Despite being the world's main producer of chestnuts, China only exports approximately 2% of its annual production. In contrast, Portugal and Spain export almost half of their annual chestnut production, mainly to France, Italy and Brazil. Therefore, this is a very important product in Portugal. The main production area in Portugal of *C. sativa* (European chestnut) is in the Trás-os-Montes region, contributing to 85 % of the total Portuguese production, having a great social and economic importance to the local population, being part of the landscape patrimony (Borges, Gonçalves, Carvalho, Correia, & Silva, 2008).

Although chestnut orchards cover a large area in Portugal (the largest area of *C. sativa* in the European Union) and the Mediterranean climate features have provided unique conditions for the remarkable evolutionary adaptation and divergence of life, the biodiversity of this region has been currently threatened by the habitat loss and degradation, provided by

the pollution levels, drought, non-endemic invasive species spread, overexploitation, etc.(Baptista, Martins, Tavares, & Lino-Neto, 2010; F.S. Reis, 2018). Therefore, given its high importance at different levels (socially and economically), and as the mycorrhization process has several advantages for species survival, taking advantage of the fact that the chestnut tree establishes this type of symbiosis - ectomycorrhizae - with different fungal species, it's *in vitro* mycorrhization has been studied over the last few years. The European chestnut is difficult to propagate by cuttings and show high heterosis of seeds. So, the micropropagation of adult clones emerges as a methodology that aims to overcome this propagation difficulties (Anabela Martins, 2008). Nowadays, there are enterprises that applies modern *in vitro* propagation techniques for the reproduction of plants whose sexual reproduction systems are not efficient or enough to meet market demand, and the chestnut tree is among the plants produced.

With these concepts in mind and considering that the laboratory of Biology and Biotechnology of the School of Agriculture of the Polytechnic Institute of Bragança has been working on the micropropagation of plants of high interest to the region, namely *C. sativa*, these were the studied species and the technique adopted in the present work.

1.1.3 Elicitation

Plant cell cultures are known to be rich sources of valuable pharmaceuticals and biologically active compounds that can replace other therapeutic compounds that are highly requested, but their synthetic production is extremely expensive (Mag, Karwasara, Tomar, & Dixit, 2011). In order to ensure the biosynthesis of desired products in quantities adapted to the commercial needs of exploitation and over-extended periods, elicitation studies have shown promising prospects for increasing yields and reducing production costs (Angelova, Georgiev, & Roos, 2014).

Elicitation has been shown to be an effective approach for inducing and enhancing plant-secreted to defend against pathogen attacks or to respond to some environmental stress (such as salinity, temperature, UV radiation, and oxidative stress) (Angelova et al., 2014). This defense mechanism is triggered by signal molecules called "elicitors" by activating the signal transduction cascade and leading to the activation and expression of genes related to the biosynthesis of secondary metabolites (Gadzovska et al., 2007; Zhao, Davis, & Verpoorte, 2005). Such biotic elicitors are often secreted by the agent and are called exogenous elicitors,

but in some cases, they are endogenous (released by the attacked plant by the action of pathogen enzymes) (Angelova et al., 2014).

1.2 Vitamin E

Vitamin E is an indispensable vitamin for the proper functioning of the human body. It is a natural molecule, usually found in foods from plant origin (*e.g.*, almonds, Pine nuts, hazelnuts, among others), and also of animal origin (*e.g.*, fish, eggs, oysters, etc.) (Revised, 2016). This generic term, vitamin E, is used for a family of chemically related compounds, tocopherols and tocotrienols, consisting of a chromanol head/ring and a prenyl side chain (Herrera & Barbas, 2001).

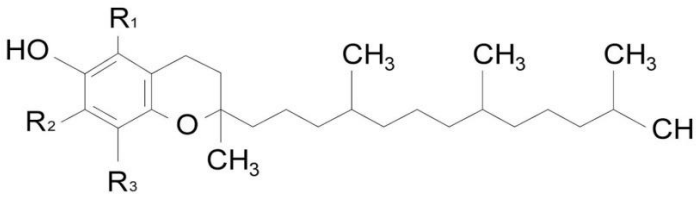
Both structures are analogous, differing between them only in the degree of saturation of their hydrophobic prenyl side chains. Natural vitamins Es are known as α-, β-, γ-, and δ- depending on the methyl or proton groups that are bound to their benzene rings, being α-tocopherol the most abundant form in nature (Engin, 2009).

1.2.1 Different vitamers of vitamin E

Tocols (designation for the eight vitamers collectively) have a 16-carbon phytyl side chain attached to a chromanol ring, the tocopherols side chain being saturated and tocotrienols having three double bonds (Herrera & Barbas, 2001).

1.2.1.1 Tocopherols

The chemical structure of tocopherols and the different isoforms are presented in **Figure 3.**



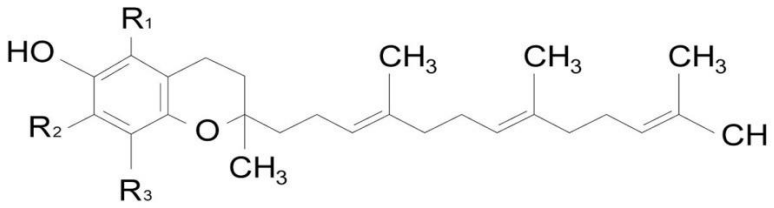
α–tocopherol	$R^1= R^2=R^3= CH_3$
β–tocopherol	$R^1= R^3= CH_3$, $R^2= H$
γ–tocopherol	$R^2=R^3=CH_3$, $R^1= H$

δ–tocopherol	R^3= CH_3, R^1= R^2=H

Figure 3: Chemical structure of the different isoforms of tocopherols.

1.2.1.2 Tocotrienols

Figure 4 present the chemical structure of the different isoforms of tocotrienols.



α–tocotrienol	R^1= R^2=R^3= CH_3
β–tocotrienol	R^1= R^3= CH_3, R^2= H
γ–tocotrienol	R^2=R^3=CH_3, R^1= H
δ–tocotrienol	R^3= CH_3, R^1= R^2=H

Figure 4: Chemical structure of the different isoforms of tocotrienols.

1.2.2 Bioactivity of vitamin E

α-Tocopherol is the most abundant form in nature and has the highest biological activity (Gupta, Suh, State, & Brunswick, 2017). Vitamin E prevents or minimizes the damage caused by free radicals associated with specific diseases, including cancer, arthritis and cataracts, and with aging. This vitamin can prevent the spread of chain reactions induced by free radicals in biological membranes (Ferreira, Barros, & Abreu, 2009). Recent evidence suggests that vitamin E reduces the prevalence of Alzheimer's disease and dementia and has a neuroprotective action against cisplatin peripheral neurotoxicity (McGeer & McGeer, 2001).

Therefore, vitamin E is referred has having mainly antioxidant and anti-inflammatory potential.

1.2.2.1 Antioxidant activity

Antioxidants are vital substances that protect the cells of the body from several "attacks" caused by free radical species which induce oxidative stress in cells leading to molecular damages in cell constituents, such as DNA and proteins (M. Carocho & Ferreira, 2013).The main causes of oxidative stress are smoking, pollution, environmental and chemical toxins (Halliwell, 2000).The oxidative stress is the main cause of many chronic diseases such as diabetes mellitus, cancer, Parkinson's disease and immune dysfunction (Surh, 2003). The prevention of free radical's formation is assured by antioxidants, exogenous (*e.g.*, vitamin C, vitamin E, carotenoids, or phenolic compounds) and endogenous (*e.g.*, superoxide dismutase (SOD), glutathione peroxidase (GPx), catalase (CAT), glutathione reductase, thioredoxin reductase, or glucose-6-phosphate dehydrogenase) (Bouayed & Bohn, 2010), responsible for their neutralization. α-Tocopherol is considered the main lipid-soluble antioxidant in the body (Herrera & Barbas, 2001). Its antioxidant activity is mainly due to its ability to give their phenolic hydrogen to lipid-free radicals (Saini & Keum, 2016). LDLs assume the transport of cholesterol from the liver to the body tissues. The oxidation of LDL is one of the main risks of cardiovascular diseases due to lipid deposition in the arterial wall. The antioxidant activity of tocopherols avoid this risk (Trpkovic et al., 2015). Moreover, the antioxidant activity of α-tocopherol and γ–tocopherol enhance the immune synapse between CD^{4+} T cells and antigen-presenting cells (APCs) and increase interleukin production -2 (IL-2) (Molano & Meydani, 2012).

1.2.2.2 Anti-inflammatory activity

Asthma, rheumatoid arthritis and hepatitis are inflammatory diseases considered to be a major cause of morbidity in human. Chronic inflammation also contributes to the development of cardiovascular diseases (Peter Libby, 2002), neurodegenerative diseases (McGeer & McGeer, 2001) and cancer (Balkwill & Mantovani, 2001; Coussens & Werb, 2002). Tocopherols can act as anti-inflammatories or inhibit the activity of enzymes involved in the biosynthesis of oxidation derivatives (eicosanoids), as well as affecting the transcription of inflammatory genes by modulating the signaling pathways involved in positive regulation of these genes. α-T (α-tocopherol) acts mainly by inhibiting cell signaling. Thus, the inhibition of Protein kinase B (Akt), which plays a key role in several cellular processes, by

tocopherols may be another potential mechanism by which these compounds inhibit the proliferation of inflammatory cells (Constantinou, Papas, & Constantinou, 2008).

Atherosclerosis is a consequence of a complex interaction between inflammation, lipid metabolism, and vascular function (Peter Libby, 2002). In fact, a recent study affirms that supplementation with α-T inhibits the development of atherosclerosis when there is a deficiency of vitamin E (caused by the elimination of tocopherol transfer protein) (Trpkovic et al., 2015).

1.2.3 Fungus as source of vitamin E

Mushrooms are widely consumed because of their exquisite and delicate flavor, as well as for their nutritional and chemical composition (high levels of minerals, protein, fiber and water, and low-fat contents) (Pinto, Barros, Sousa, & Ferreira, n.d.).

Beyond the nutritional characteristics, fungi have also been widely studied for their medicinal properties, particularly because of their richness in bioactive compounds with antioxidant, anti-inflammatory, and anticancer properties, among other bioactivities (Ferreira et al., 2009). Among these molecules polysaccharides and phenolic compounds stand out, but other molecules are also present, namely tocopherols (Pinto et al., n.d.).

It has been shown that the antioxidant activity of fungi is mainly due to the phenolic compounds presented by these natural matrices, followed by tocopherols, ascorbic acid and carotenoids (Barros, Cruz, Baptista, Estevinho, & Ferreira, 2008). Indeed, the presence of vitamin E, namely tocopherols, has been reported in several mushroom species (F.S. Reis, 2018), including the species *C. odora* (Vaz et al., 2011).

Although vitamin E is more abundant in vegetable sources, since these molecules are also associated with plant defense mechanisms, it was decided to study them during the first stages of the mycorrhization process.

2 OBJECTIVES

With the features of the mycorrhization process above mention in mind, two mycorrhizal mushrooms were selected to be isolated and produced by *in vitro* techniques: *C. odora* (Bull.) P. Kumm. and *S. polyrhizum* (J.F. Gmel.) Pers. The mycelia obtained was investigated for its differential capacity to produce tocopherols after isolation *in vitro*. Moreover, these mushroom species were co-cultured *in vitro* with host plant roots, *C. sativa* Mill., in order to evaluate the root potential elicitation on the mushroom's tocopherol profile.

Therefore, the **main objective** of the present work was to evaluate the influence of the roots of *C. sativa* on the tocopherols profile of the fungi *C. odora* and *S. polyrhizum*.

The specific objectives were:

- Isolate and produce by *in vitro* techniques the mycelia of the mushrooms *C. odora* and *S. polyrhizum*;
- Perform the co-culture of the studied mushroom species with the plant *C. sativa*;
- Evaluate the tocopherols profile of both mycelia produced by *in vitro* co-culture, with the host roots.

3 MATERIALS AND METHODS

3.1 Mushroom collection and *in vitro* culture establishment

S. polyrhizum (J.F. Gmel.) Pers. fruiting bodies were harvested in Bragança's outskirts (North eastern Portugal) in 2018. The taxonomic identification of the sporocarps was undertaken at the Polytechnic Institute of Bragança, according to several authors (Guzmán, Cortés-pérez, Guzmán-dávalos, Ramírez-guillén, & Sánchez-jácome, 2013; Ruthes, Smiderle, & Iacomini, 2016).

C. odora (Bull.) P. Kumm. mycelium was obtained from previous cultures maintained in the laboratory of Biology and Biotechnology of the School of Agriculture from the Polytechnic Institute of Bragança, Portugal.

The mycelium isolation **(Figure 5)** was carried out under sterile conditions (laminar flow hood), in Petri dishes (9 cm in diameter) with about 10 mL of solid medium. After isolation, the sub-culture and mycelia mass production was made in solid medium as above and in flasks (250 mL) with 30 mL of liquid medium. The mycelia were maintained in an incubator of the abovementioned laboratory at 25 °C. The medium used was Melin-Norkans medium (MMN) pH 6.3 (NaCl 0.025 g/l; $(NH_4)2HPO_4$ 0.25 g/l; KH_2PO_4 0.50 g/l; $FeCl_3$ 0.005 g/l; $CaCl_2$ 0.050 g/l; $MgSO_4.7H_2O$ 0.15 g/l; thiamine 100 μg/l; malt extract 5 g/l; casamino acids 1 g/l; glucose 10 g/l; and agar 20 g/l (in solid medium)).

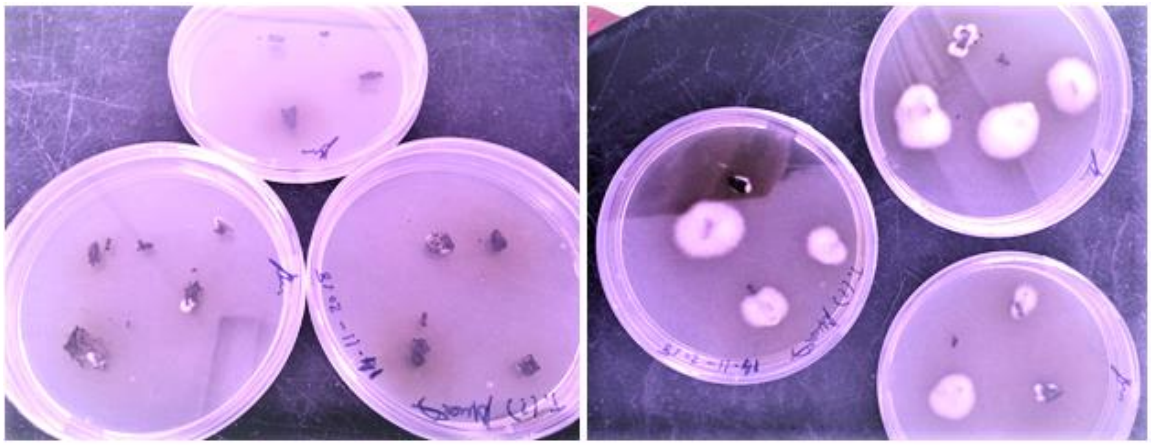


Figure 5: Mycelium of *S. polyrhizum* obtained and maintained by *in vitro* culture.

For the mass production of mycelia, on the solid medium, a thin layer of sterilized cellophane was placed (**Figure 6**), above which the fungi was inoculated, to facilitate the recovery of the mycelium after growth(“A Technique for Mycelial Development of Ectomycorrhizal Fungi on Agar Media,” 2002).

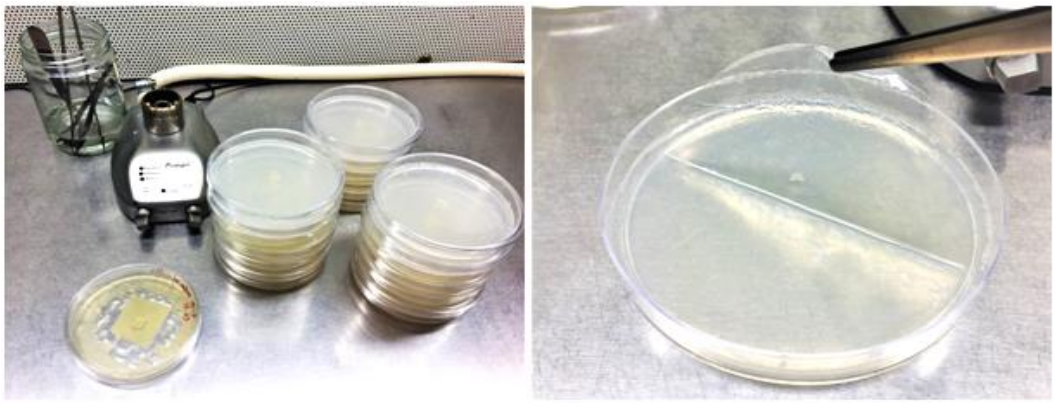


Figure 6: Inoculation of the fungi in solid medium.

After inoculation, it was expected that the surface of the plate was completely covered with mycelium, in order to obtain higher yields for the chemical analyzes (**Figure 7**).

Figure 7: Mass production of fungi in solid medium.

3.2 Chestnut tree establishment *in vitro*

C. sativa plantlets were obtained from previous cultures maintained in the Biology and Biotechnology Laboratory of the School of Agriculture of the Polytechnic Institute of Bragança, Portugal.

Initiation step

The plantlets were sub-cultured in new flasks with a solid culture medium with the purpose of multiplication and elongation of the shoots. The MS (Murashige and Skoog) (Murasnige, 1962) medium pH 5.5 was used (NH_4NO_3 825.0 mg/l; KNO_3 950.0 mg/l; $CaCL_2 2H_2O$ 440.0 mg/l; $MgSO_4.7H_2O$ 370.0 mg/l; $KH_2\ PO_4$ 170.0 mg/l; Fe Na EDTA 80.0 mg/l; myo-inositol100.0 mg/l; KI 0.83 mg/l; H_3BO_3 6.2 mg/l; $MaSO_4.4H_2O$ 22.3 mg/l; $ZnSO_4.7H_2O$ 8.6 mg/l; $Na_2MoO_4.2H_2O$ 0.25 mg/l; $CuSO_4.5H_2O$ 0.025mg/l; $CoCL_2.6H_2O$ 0.025 mg/l; nicotinic acid 0.5 mg/l; pyridoxine HCL 0.5mg/l; tiamine HCL 0.1 mg/l; glycine 20mg/l; sucrose 20.0 g/l; agar8.0 g/l) (Osterc, Fras, Vodenik, & Luthar, 2005).

The explants were maintained in the *in vitro* culture chamber of the above-mentioned laboratory, at 23 °C / 18 °C thermo-period and 16 h / 8h photo-periods.

After about two months of growth, plantlets with 5 cm long or more were obtained (**Figure 8**). The shoots with less than 5 cm were multiplied and sub-cultured in MS medium to continue their proliferation.

Figure 8: Chestnut *in vitro* establishment – propagation step.

Rooting step

The obtained plantlets were removed from the pre-rooting medium by excision of the basal *callus*. In order to increase the exposed surface of the vascular tissue, the basal end of each plantlet was cut at an angle of 30° (Osterc et al., 2005). The shoots were then immersed in a solution of 10 mM indole-3-butyric acid (IBA) (dissolved in KOH and H_2O and autoclaved for 10 minutes) for 10 minutes (**Figure 9**). The shoots were then placed in flasks (3-4 per flask) containing a rooting medium composed of a mixture of MS basal salts at 50% concentration and 3g/l activated charcoal (Osterc et al., 2005), with pH adjusted to 5.5. Containers were placed in the *in vitro* culture chamber of the abovementioned laboratory, at 23 °C / 18 °C thermo-periods and 16 h / 8h photo-periods.

Figure 9: Chestnut *in vitro* establishment – rooting step.

3.3 Plants and fungi co-culture

After rooting (after 3 months), roots were excised and co-cultured with the fungi mycelia for different periods, being used solid and liquid co-culture *in vitro* systems. The induction of the mycorrhizal symbiosis was carried out after the recovery of the formed roots, by placing them in the Petri dishes and flasks containing the inocula of *S. polyrhizum* and *C. odora* well-developed (**Figure 10**). The co-cultures were maintained for 48 hours and 96 hours.

After the established periods, the culture medium, roots and mycelia were recovered separately to assess the parts of the mycorrhizal system richer in the survey compounds.

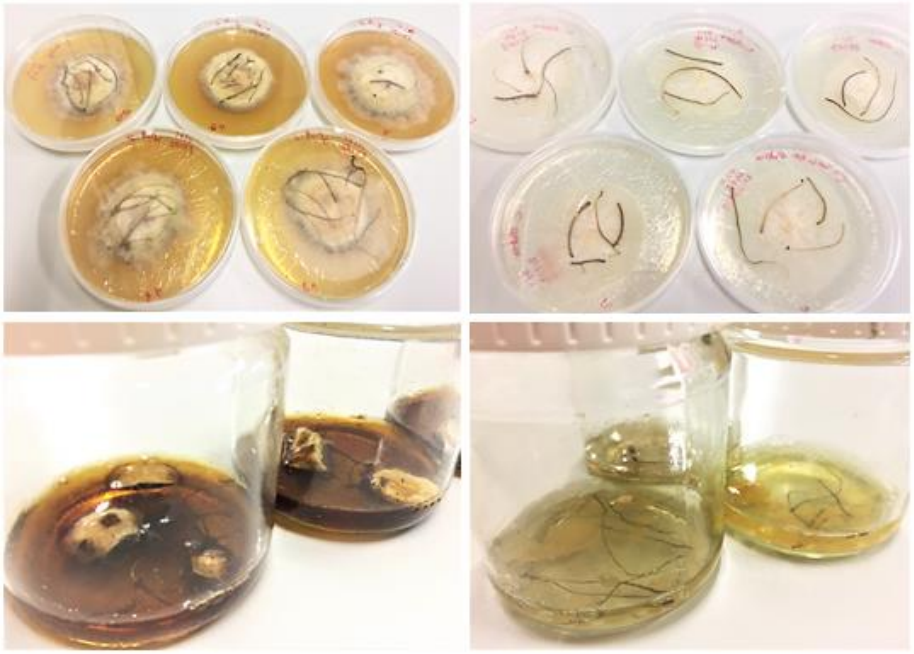


Figure 10: Co-culture of roots and both species of fungi in solid and liquid medium.

For each studied species, Petri dishes and flasks were inoculated with the roots and fungi separately, constituting the control samples.

After the co-culture periods, the mycelia were recovered from the liquid culture medium using a sieve (particle size about 1.5 mm, **Figure 11**) and from the solid medium the recovery was facilitated by using the cellophane (**Figure 12**). Afterwards, the recovered mycelia and roots (**Figure 13**) were weighted in order to obtain the fresh weight (Fw), frozen and lyophilized, matching the dry weight (Dw). The lyophilized samples were then protected from light, until further analysis.

Figure 11: Recovery of mycelium from liquid medium.

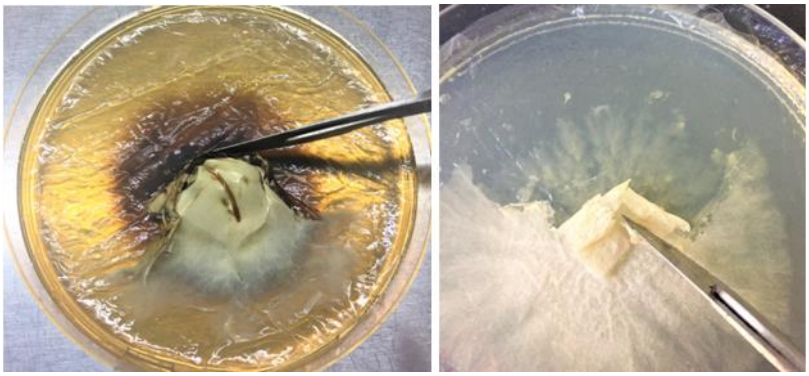


Figure 12: Recovery of mycelium from solid medium.

Figure 13: Roots after co-inoculation.

3.4 Tocopherols analysis

BHT solution (10 mg/mL; 100µL) and tocol solution (2.0 µg/mL; 250 µL for mycelium and 50 µg/mL; 400 µL for plants) were added to the samples (500 mg) prior to the extraction procedure. The samples were homogenized with methanol (4 mL) by vortex mixing (1 min). Subsequently, hexane (4 mL) was added and again vortex mixed for 1 min. After that, saturated NaCl aqueous solution (2 mL) was added, the mixture was homogenized (1 min), centrifuged (5 min, 4000g, 10 ºC) and the clear upper layer was carefully transferred to a vial previously wrapped in an aluminum foil. The sample was re-extracted twice with hexane. The combined extracts were taken to dryness under a nitrogen stream (**Figure 14**), re-dissolved in 1 or 2 mL of *n*-hexane, dehydrated with anhydrous sodium sulphate, filtered through a disposable LC filter disk, transferred into a dark injection vial of 1.5 mL and analyzed by HPLC(Barros et al., 2008). The HPLC equipment (**Figure 15**) consisted of an integrated system with a Smartline pump 1000 (Knauer, Germany), a degasser system Smartline manager 5000, an AS-2057 auto-sampler and a 2500 UV detector at 295 nm (Knauer, Germany) connected in series with a FP-2020 fluorescence detector (Jasco, Japan) programmed for excitation at 290 nm and emission at 330 nm. Data were analyzed using Clarity 2.4 Software (DataApex). The chromatographic separation was achieved with a Polyamide II (250 x 4.6 mm) normal-phase column from YMC Waters (Japan) operating at 30 °C (7971 R Grace Oven). The mobile phase used was a mixture of n-hexane and ethyl acetate (70:30, v/v) at a flow rate of 1 mL/ min, and the injection volume was 20 µL. The compounds were identified by chromatographic comparisons with authentic standards. The quantification was based on the fluorescence signal response, using the internal standard method (Heleno, Barros, João, Martins, & Ferreira, 2009; Filipa S. Reis, Ferreira, Barros, & Martins, 2011).

Figure 14: Drying step under a nitrogen stream.

Figure 15: HPLC-fluorescence system

3.5 Statistical analysis

All data in this thesis are expressed as mean ± standard deviation. Samples were analyzed by a two-way ANOVA with type III sums of squares using the SPSS Software, version 25. This multivariate general linear model treats the two factors, co-culture time (CT) and medium state (MS) as independent, thus allowing the effect of each one to be analyzed independently, providing more insight to their contribution towards the variations in the

sample. If a significant interaction ($p<0.05$) was recorded among the two factors (CT×MS), these were evaluated simultaneously, and some general conclusions and tendencies were extracted from the estimated marginal means (EMM). If there was no significant interaction ($p>0.05$), each factor was evaluated independently using a simple Student's T test (for MSt) or a Tukey's multiple comparison test (for CT) when the means were homoscedastic, and a Tamhane's T2 for non-homoscedastic samples. Homoscedasticity was evaluated using a Levene's test. All analyses were carried out using a significance level of 0.05.

4 RESULTS AND DISCUSSION

Currently, natural antioxidants are among the most investigated compounds by the scientific community. This is largely due to the growing concern about possible detrimental effects of the synthetic alternatives currently used for the prevention / treatment of conditions related to oxidative stress. In fact, the literature shows that fungi are a valuable source of several antioxidants, including phenolic compounds, carotenoids and vitamins (Bernaś & Jaworska, 2016; Molnár, Ősz, Turcsi, & Deli, 2019; Palacios et al., 2011).

In this context, the present study aimed to evaluate the effect of the symbiosis or co-culture between fungi and plant roots in the production of tocopherols by the former.

4.1 Biological material growth

In this work, the *in vitro* culture technique was exploited to produce the species *C. odora* and *S. polyrhizum* and to obtain enough biomass for co-culture with *C. sativa* roots and subsequently analyze their content in tocopherols.

After a few days of inoculation on the MMN medium, it was possible to notice the initial growth in most of the flasks. In five weeks, almost the entire surface of the flasks was covered by the mycelium. In other hand, the mycelia that grew in Petri dishes did not cover the entire surface and stopped growing after about a month and a half.

After one month of inoculation and after the co-culture time, it was possible to collect the biomass and weigh the samples in order to estimate the average growth in each petri dish or flask and to compare the growth of each of the two species in the liquid and solid medium, obtaining the results presented in **Figure 16** and **Figure 17**.

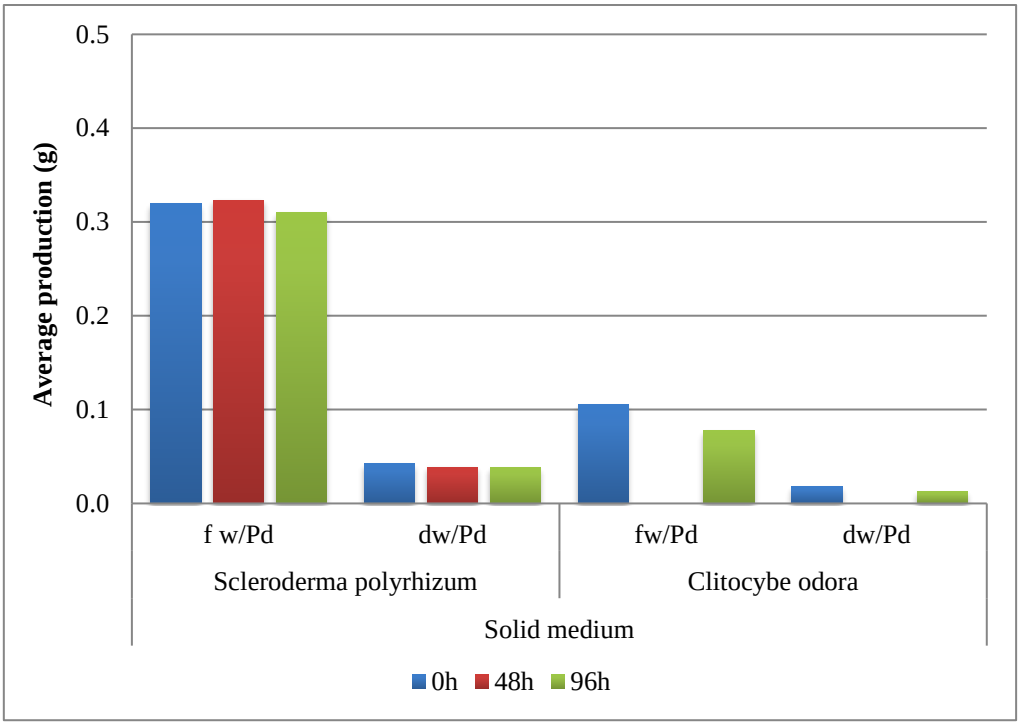


Figure 16: Average mycelium production per Petri dish. Fw/Pd: Fresh weight per number of petri dish, Dw/Pd: Dry weight per number of petri dish.

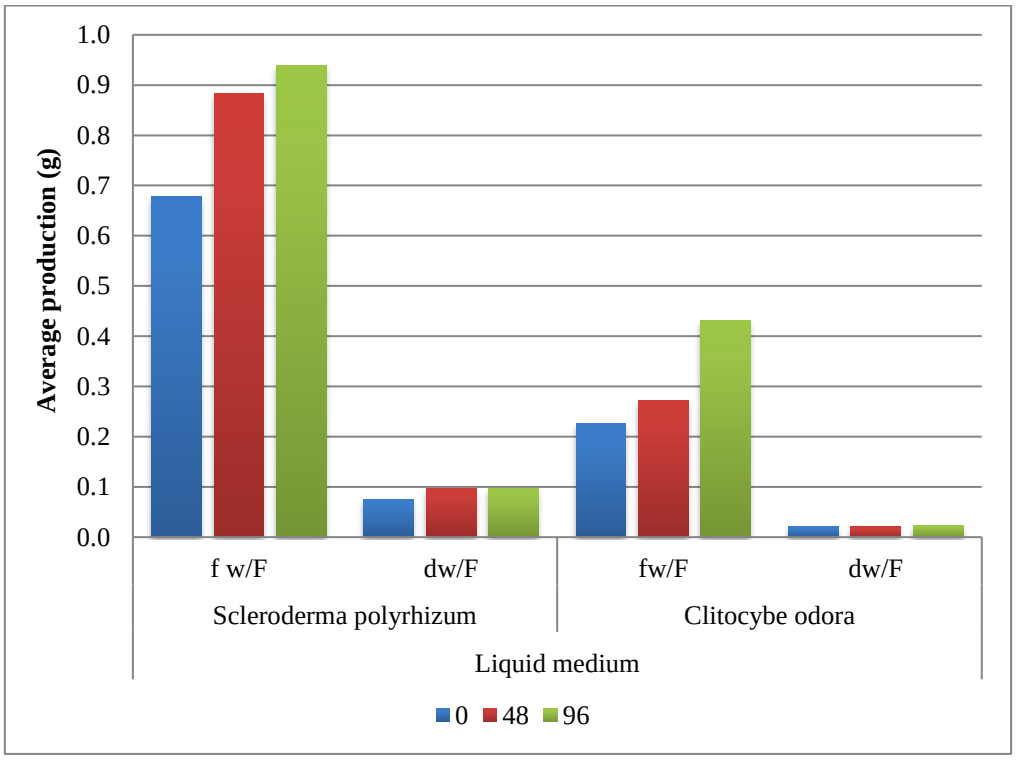


Figure 17: Average mycelium production per flask. Fw/F: Fresh weight per number of flasks, Dw/F: Dry weight per number of flasks.

Through the analysis of the obtained results, it can be verified that the production of *S. polyrhizum* and *C. odora* developed in different ways, depending on the medium (liquid or solid; **Figure 16** and **Figure 17**). For *S. polyrhizum*, the growth was higher in liquid medium compared to solid medium, both in fresh weight basis and dry weight basis. After 96 days, the percentage of growth of *S. polyrhizum* was approximately equal to 0,94g in the solid medium, while in the liquid medium this weight did not exceed 0,32g. On the other hand, for the mycelium production of *C. adora*, the growth differences between the two media was really high. As shown in the **Figure 16**, the production is very low, with a weight around 0,08g, while in liquid medium it reaches up to 0,43g for the samples cocultured 96 hours. With these results, it is possible to conclude that the liquid medium is more conducive to growth of both species, and the biomass production of *S. polyrhizum* is significantly higher than the biomass production of *C. adora.*

The table below (**Table 4**) presents the dry matter ratio of different samples co-cultured in a solid and liquid medium.

Table 4: Dry matter ratio (DW/FW**)** of mycelium and roots (%)

			0h	**48h**	**96h**
S.polyrhizum	Mycelium	Solid medium	13.375	12.005	12.37
		Liquid medium	11.10	10.91	10.26
	Roots	Solid medium	8.406	12.68	11.61
		Liquid medium	8.406	12.60	9.77
C.odora	Mycelium	Solid medium	16.93	_	16.55
		Liquid medium	9.14	7.49	5.483
	Roots	Solid medium	8.406	_	17.12
		Liquid medium	8.406	9.023	10.48

4.2 Tocopherols analysis

As indicated above, all samples, namely the roots of *C. sativa* and the mycelium of the species *C. odora* and *S. polyrhizum,* were analysed before and after co-culture, and the levels of tocopherols present in the samples were determined.

4.2.1 Tocopherols variation in the roots of *C. sativa* plants in co-culture with *C.odora and S. polyrhizum*, in solid and liquid medium

Tocopherols profile of the roots in time zero (T_0) and its variation over time in co-culture **are** shown in **Table 5** and **6** and **Figures 18** and **19**.

Table 5: α-, δ- and total tocopherol variation in the roots of *C. sativa* plants in co-culture with *C. odora*, in solid and liquid medium, expressed as µg/g Dw.

		0 hours	**48 hours**	**96 hours**
Solid medium	α (ug/g)	28.29	-	20.73
	δ (ug/g)	2.46	-	0
	Total (ug/g)	30.76	-	20.73
Liquid medium	α (ug/g)	28.29	11.98	8.54
	δ (ug/g)	2.46	0.28	0
	Total (ug/g)	30.76	12.93	8.54

*The co-culture of 48 hours could not be performed due to contamination complications.

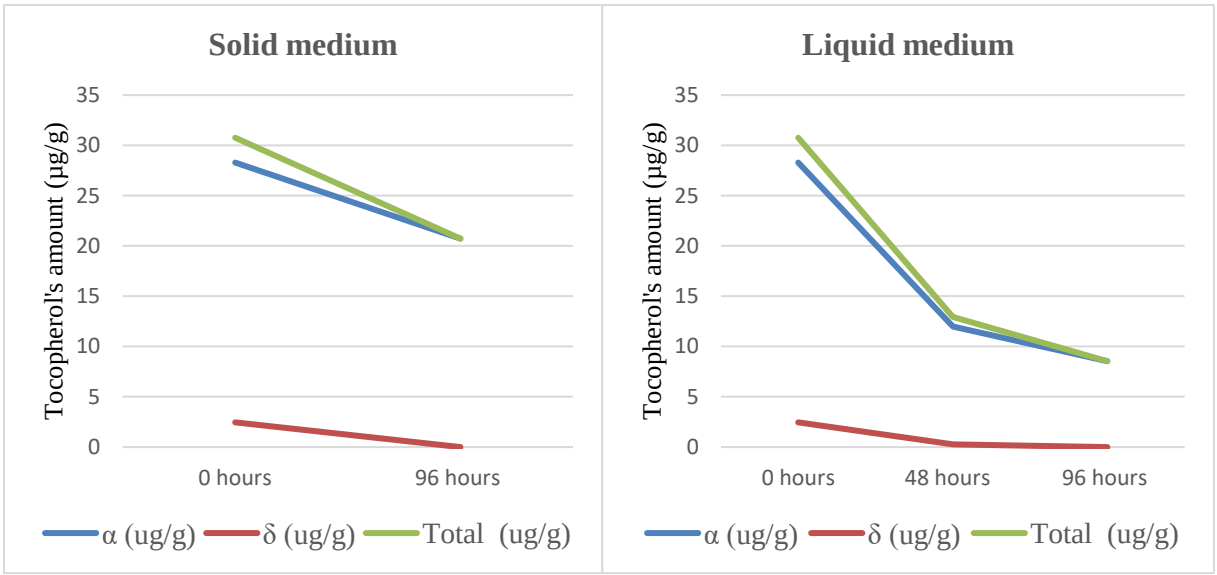


Figure 18: Profile of α-, δ- and total tocopherol in the roots of *C. sativa* plants in co-culture with *C. odora*, in solid and liquid medium, expressed as µg/g Dw.

Table 6: α-, δ- and total tocopherol variation in the roots of *C. sativa* plants in co-culture with *S. polyrhizum*, in solid and liquid medium, expressed as µg/g Dw.

		0 hours	**48 hours**	**96 hours**
Solid medium	α (ug/g)	28.29	23,51	21,38
	δ (ug/g)	2.46	0	0
	Total (ug/g)	30.76	23,51	21,38
Liquid medium	α (ug/g)	28.29	16,73	13,32
	δ (ug/g)	2.46	2,37	0
	Total (ug/g)	30.76	19,11	13,32

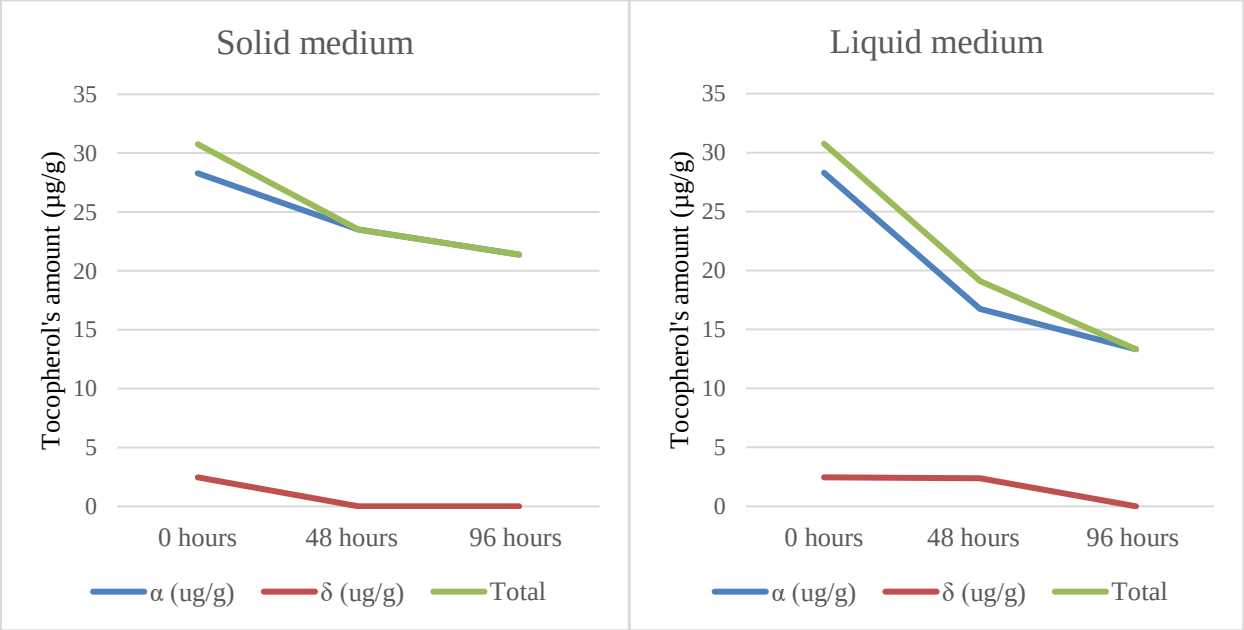


Figure 19: Profile α-, δ-, and total tocopherol in the roots of *C. sativa* plants in co-culture with *S. polyrhizum*, in solid and liquid medium, expressed as µg/g Dw.

First, it has been noticed that, in T_0, the roots had only two vitamers of tocopherols α- and δ-tocopherol, with an amount of α-tocopherol (28.29 µg/g) higher than the amount of δ-tocopherol (2,46 µg/g). The total tocopherols rate found in the roots was 30.76 µg/g, due to the significant contribution of α-tocopherol. As **Figures 18** and **19** show, along the co-culture time, there was a decrease in the α-tocopherol and δ-tocopherol rates, and therefore a total tocopherol decrease. It is clear in the curves that the decrease of tocopherols in the roots co-

cultured with *C. odora* (20.73 µg/g in solid medium and 8.54 µg/g in liquid medium) is more pronounced than in the roots co-cultured with *S. polyrhizum* (21.38 µg/g in solid medium and 13.32 µg/g in liquid medium). It can also be verified that diminution of tocopherols in the roots that were co-cultured in liquid medium is greater than in those co-cultured in a solid medium.

4.2.2 Tocopherols variation in the mycelia of *C. odora* in co-culture with the roots of *C.sativa*, in solid and liquid medium

In *C. odora*'s samples, as shown at **Table 7** and **Figure 20**, the presence of α-tocopherol (0.3 µg/g), γ-tocopherol (2.64 µg/g) and δ-tocopherol (0.48 µg/g) was verified. As abovementioned, the co-culture of 48 hours could not be performed for solid medium due to contamination complications. After co-culture with the roots of *C. sativa*, a significative interaction was detected. In solid medium, for α-tocopherol there is a slight increase of 0.05 ug/g after 96 hours, while the other vitamers were not detected after this time. For the liquid medium, the α-tocopherol levels decreased during the first co-culture period to give a slight increase of 0.04 µg/g during the second period. On the other, δ-tocopherol showed an increase after 48 hours of symbiosis followed by a slight decrease after 96 hours. Regarding the levels of γ-tocopherol, which was the predominant vitamer at the initial time (T_0), it was not detected on both co-culture periods. Total tocopherols decreased in both media.

Table 7: Tocopherols profile of the mycelia of *C. odora* in co-culture with the roots of *C. sativa*, in solid and liquid medium, expressed as µg/g Dw.

		0 hours	**48 hours**	**96 hours**
Solid medium	α (ug/g)	0.3	-	0.35
	γ (ug/g)	2.64	-	0
	δ (ug/g)	0.48	-	0
	Total (ug/g)	3.42	-	0.35
Liquid medium	α (ug/g)	0.65	0.44	048
	γ (ug/g)	3.1	0	0
	δ (ug/g)	2.44	3.96	3.76
	Total (ug/g)	6.19	4.41	4.24

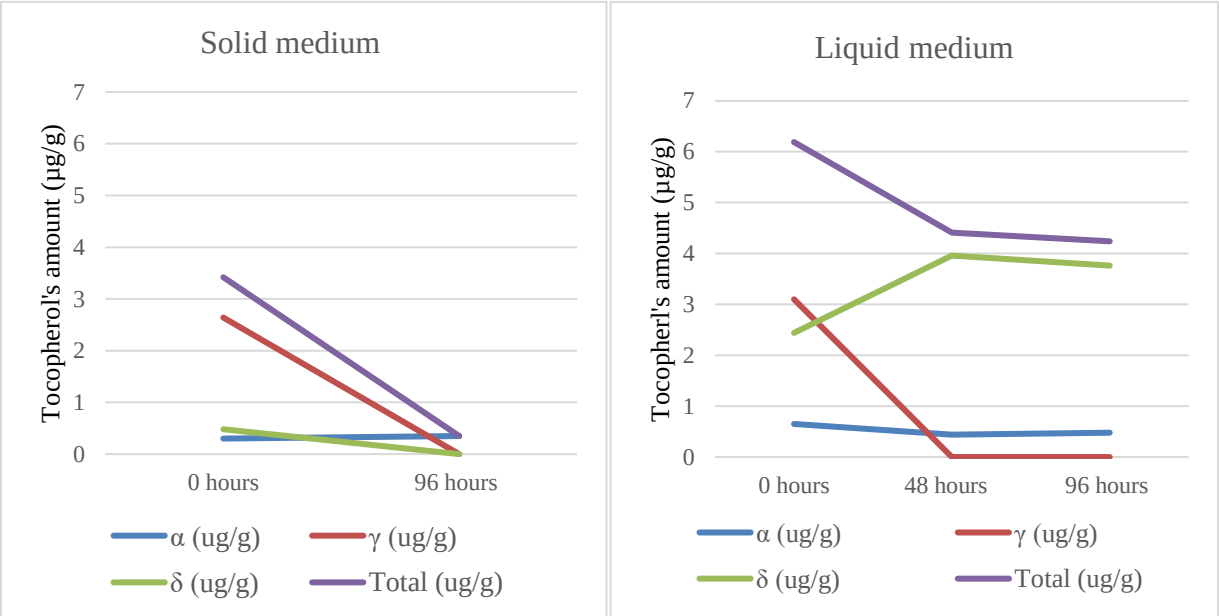


Figure 20: Tocopherols variation in the mycelia of *C. odora* co-cultured with the roots of *C.sativa*, in solid and liquid medium, expressed as µg/g Dw.

4.2.3 Tocopherols variation in the mycelia of *S. polyrhizum* in co-culture with the roots of *C. sativa*, in solid and liquid medium

Table 8 and **Figure 21** shows the presence of two vitamers in *S. polyrhizum* samples, which are α- and δ-tocopherol, with a higher rate of the latter. In this co-culture was also verified a significant interaction, since the levels of α-tocopherol detected on this species co-cultured in solid medium decreased after 48 hours, and then a remarkable increase was observed after 96 hours. Instead, in the co-culture developed in the liquid medium, a gradual decrease was recorded throughout the symbiotic period. Regarding the levels of γ-tocopherol co-cultured in solid medium, it was observed a slight decrease after 48 hours, and a high decrease of 5.36 µg/g after 96 hours. In this co-culture performed in liquid medium, a slight increase in the tocopherols content was observed during the first period, followed by a decrease in the second one (**Figure 21)**.

Table 8: Tocopherols profile of the mycelia of *S.polyrhizum* in co-culture with the roots of *C.sativa*, in solid and liquid medium, expressed as µg/g Dw.

		0 hours	48 hours	96 hours
Solid medium	α (ug/g)	1,27	0,26	0,92
	γ (ug/g)	6,89	6,68	1,32
	Total	8,17	6,95	2,25
Liquid medium	α (ug/g)	0,47	0,16	0,12
	γ (ug/g)	2,29	2,4	1,85
	Total	2,77	2,56	1,97

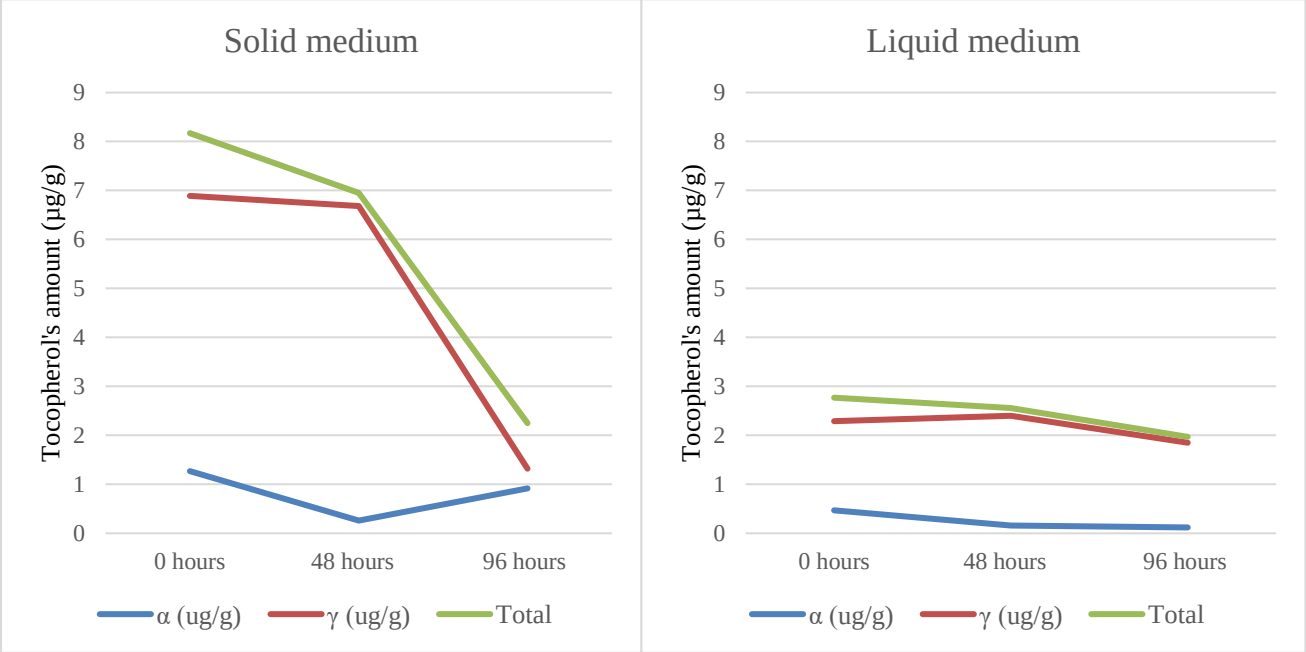


Figure 21: Tocopherols variation in the mycelia of *S. polyrhizum* co-cultured with the roots of *C. sativa*, in solid and liquid medium, expressed as µg/g Dw.

4.3 Statistical analysis

As previously referred, tocopherols, vitamers of vitamin E, are methylated phenols that are synthesized by almost all living organisms, except animals. The role of these liposoluble molecules is an antioxidant one, to reduce oxidation levels in living tissues by halting lipid peroxidation and reacting with singlet oxygen, but also to maintain structural membrane

integrity and aid in intracellular signaling (Munné-Bosch, 2002). During the first stages of symbiosis between plant roots and mushroom hyphae, a great deal of signaling occurs between these two species, and variations in tocopherols and other antioxidant molecules have been reported both for pines (*Pinus pinaster*) but also chestnut trees (*C. sativa*) in association with two mycorrhizal mushrooms, namely *Paxillus involutus* and *Pisolithus arhizus* (M. Carocho & Ferreira, 2013; Carvalho, Ferreira, Barreira, Barros, & Martins, 2013; Filipa S. Reis et al., 2011). In this work, the variations of the different isoforms of tocopherols along 96 hours of symbiosis is reported for the roots of *C. sativa*, with two mushrooms, *C. odora* and *S. polyrhizum*, growing in symbiosis with the chestnut roots, both in liquid and solid mediums. During the 96 hours of contact between the roots and the fungi, two factors influenced the variations in tocopherol content, namely the passage of time and the medium state (solid or liquid) influencing the uptake of nutrients and further altering the symbiotic relationship which in turn influences the production or inhibition of tocopherol isoforms. To better understand the individual influence of these two factors (time and medium state), and to analyze them independently, a 2-way ANOVA was employed, allowing an individualized assessment of each factor. This treatment is used in **Tables 9** to **12**, where **Tables 9** and **10** correspond to the tocopherol isoforms found in the roots of *C. sativa* in co-culture with *C. odora* and *S. polyrhizum*, respectively, while **11** and **12** correspond to the tocopherol isoforms found in the two mushrooms.

Table 9 is divided into two sections, the upper one represents the means of the three co-culture periods (0, 48 and 96 hours), while the lower section displays the means for the two different states (liquid and solid) of the medium used for the co-cultures. The values are represented as means of each co-culture time (CT) including both medium types (MS) in the upper section, and for the lower one, the means of MSt include the three co-culture (CT) time values. This type of representation and interpretation (2-way ANOVA) allows the aforementioned analysis of each factor (time of co-culture and medium state) individually and thus, the standard deviations should not be regarded as the accuracy of an individual analysis as it includes the variation of the non-fixed factor (CT or MSt). If a significant interaction among these two factors is detected (CT×MS $p<0.05$), no multiple comparisons can be carried out, meaning that both factors, ST and MSt, contributed to the variations in tocopherol content, hindering concrete conclusions, although general tendencies can be concluded from the Estimated Marginal Means (EMM) plots. Inversely, if CT×MS is higher than 0.05, the factor is classified individually using either Tukey's or Tamhane T2 tests depending on the homoscedasticity of the distribution for CT, and a Student T test for MSt. Analyzing **Table 9**,

of the four tocopherol isoforms, only two were detected, namely α- and δ-tocopherol. For both, a significative interaction was detected (CT×MS<0.05) and thus no individual classification could be possible, although α-tocopherol showed a tendency to decrease along 48 hours and then increased its quantity. Regarding δ-tocopherol, although a significant interaction was also detected among the co-culture time and medium state, a reduction was detected starting at the 48th hour, with no significant change at the 96th. General conclusions, drawn from the Estimated Marginal Means (EMMS), in **Figure 22** represent the variation of δ-tocopherol over the co-culture period (a), but also compare the two medium states (b). In the a) section a general tendency of reduction in this tocopherol isoform is shown during the 96 hours of co-culture, more evident in the solid medium. The b) section shows that after 48 hours, a higher amount of δ-tocopherol is found in the liquid medium. In terms of the effect of the symbiosis on tocopherol production in the roots of *C. sativa* in co-culture with *C. odora*, it seems that there was a reduction effect in the roots, especially during the first stages. Even though there was a slight increase for α-tocopherol after the 48th hour, the amount never increased to the initial one. This behavior is also verified for the total tocopherols, due to the highest isoform being α-tocopherol. In terms of recovery of these molecules for application in the food or cosmetic industries, it seems that the symbiotic association did not stimulate the production of tocopherols in the roots of the plants. Interestingly, only two of the four isoforms were detected for the roots, the most abundant α- tocopherol and the least abundant but equally potent δ-tocopherol, while β- and γ-tocopherol were not detected during the 96 hours of co-culture.

Table 9: α-, δ- and total tocopherol variation in the roots of *C. sativa* plants in co-culture with *C. odora*, in solid and liquid medium, expressed as µg/g Dw.

		α-tocopherol	δ-tocopherol	Total tocopherols
Co-culture Time (CT)	0 hours	28.3±0.1	2.5±0.1	30.75±0.06
	48 hours	6±6	0.5±0.5	6±7
	96 hours	14±6	0.5±0.5	14±6
p-value (n=10)	Tukey's HSD test	0.458	<0.001	0.007
Medium State (MS)	Liquid	16±9	1±1	17±10
	Solid	16±12	1±1	17±13
p-value (n=15)	Student T-Test	<0.001	<0.001	<0.001
CT×MS (n=30)	*p*-value	<0.001	<0.001	<0.001

For the co-culture time (CT), in each row and within each co-culture period, different letters mean significant statistical differences ($p<0.05$). For the medium state (MS), an asterisk means statistical differences between the two states. The presented standard deviations were calculated from results obtained under different operational conditions. Thus, they should not be regarded as a measure of precision, rather as a range of values.

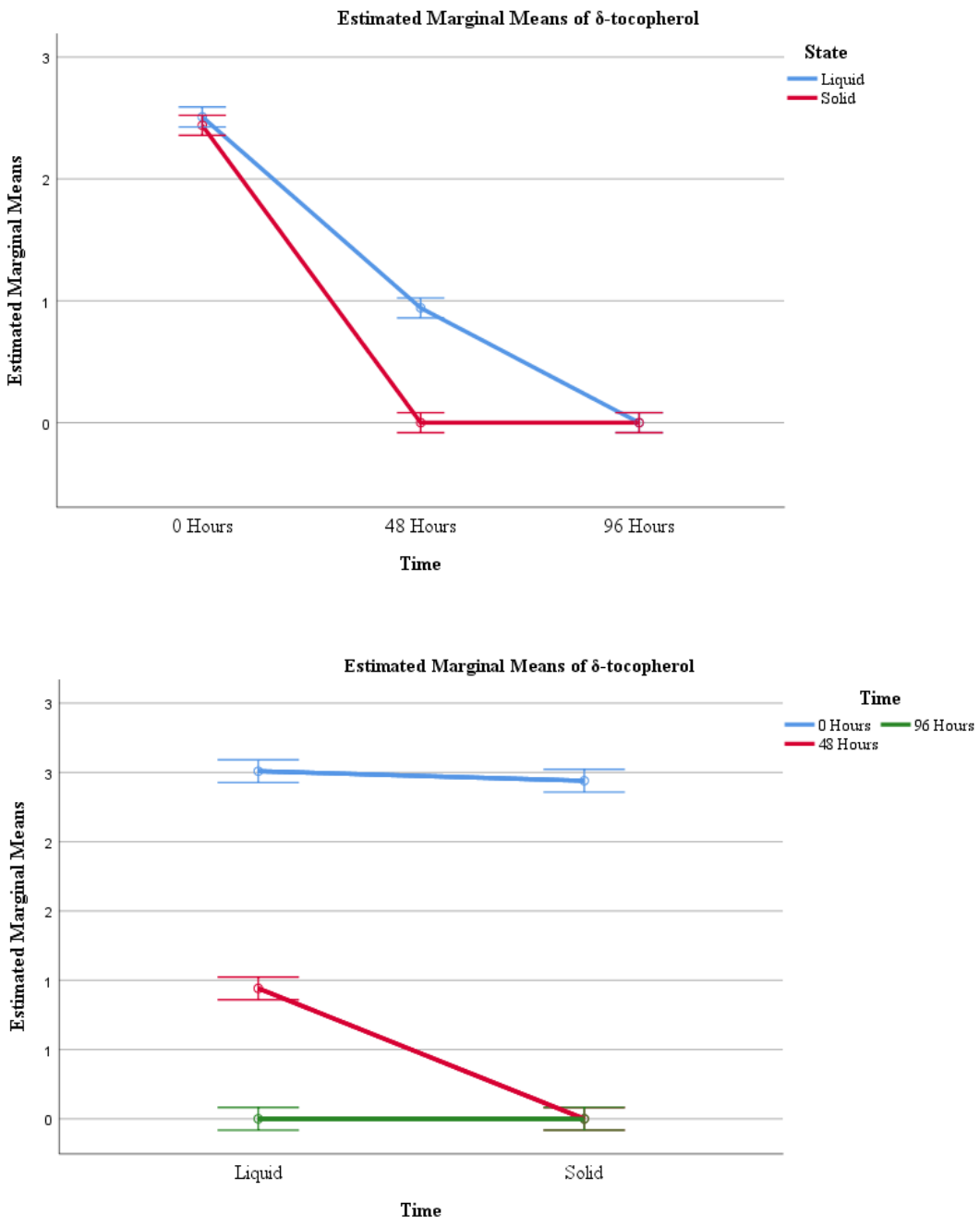


Figure 22: Representation of the EMM plots of δ-tocopherol for the roots of *C. sativa* co-cultured with *C. odora*. a) Representation over the 96 hours. b) Comparison of δ-tocopherol between the two medium states.

Table 10 represents the tocopherol variation in the roots of *C. sativa* in co-culture with *S. polyrhizum* and follows the same layout as **Table 9**. As for the co-culture with *C. odora*, only α- and δ- tocopherol isoforms were detected in the roots of the chestnut plants. Once again, there was a significant interaction between the culture state and the passage of time,

which is normal, explaining that both these factors induced change along the co-culture time, which hindered the extraction of definitive conclusions, and thus, the EMM plots allow for general predispositions, expressed in **Figures 23** and **24**. Once again, a general decrease was observed for both isoforms during the storage time, with α-tocopherol reducing from 28 µg/g to 17, and δ-tocopherol, detected in much lower quantities, as in the roots in co-culture with *C. odora*, decreasing from 2.5 µg/g to virtually zero. In section a) of **Figure 23**, this decrease for α-tocopherol is evident, especially for the liquid co-culture. Section b) further details the amounts of α-tocopherol found in each co-culture, being the highest amount found, as expected at 0 hours, followed by the 48 hours of the co-culture in solid medium. **Figure 24** similar to section a) of **Figure 23** details the reduction of δ-tocopherol, which, for the liquid co-culture during the first 48 hours showed a lower reduction than for the second stage of symbiosis. Once again, the total tocopherols, being the sum of all isoforms showed the same behaviour. Overall, the symbiosis seemed to reduce the amount of α- and δ-tocopherols, being this reduction more pronounced for the α isoform in the liquid co-culture, and inversely, the most drastic reduction for δ-tocopherol was found in solid medium, following the same tendency for this isoform in the roots of *C. sativa* in co-culture with *C. odora*.

Table 10: α-, δ- and total tocopherol variation in the roots of *C. sativa* plants in co-culture with *S. polyrhizum*, in solid and liquid medium, expressed as µg/g Dw.

		α-tocopherol	δ-tocopherol	Total tocopherols
Co-culture Time (CT)	0 hours	28.2±0.2	2.5±0.1	30.75±0.05
	48 hours	20±4	1±1	21±2
	96 hours	17±4	0.1±0.1	17±4
p-value (n=10)	Tukey's HSD test	<0.001	<0.001	<0.01
Medium State (MS)	Liquid	19±6	1±1	21±7
	Solid	24±3	1±1	25±4
p-value (n=15)	Student T-Test	<0.001	<0.001	<0.001
CT×MS (n=30)	*p*-value	<0.001	<0.001	<0.001

For the co-culture time (CT), in each row and within each co-culture period, different letters mean significant statistical differences ($p<0.05$). For the medium state (MS), an asterisk means statistical differences between the two states. The presented standard deviations were calculated from results obtained under different operational conditions. Thus, they should not be regarded as a measure of precision, rather as a range of values.

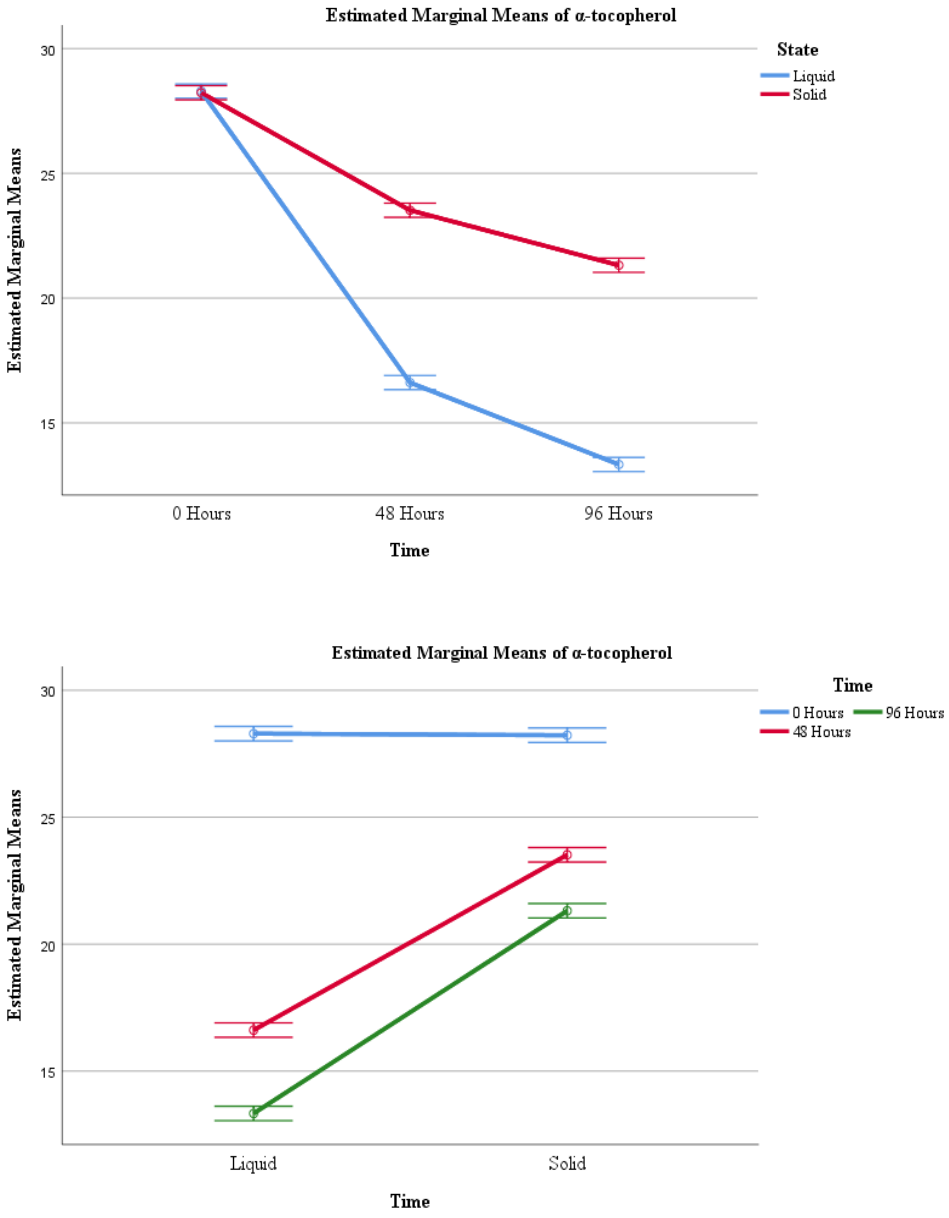


Figure 23: Representation of the EMM plots of α-tocopherol for the roots of *C. sativa* co-cultured with *S. polyrhizum*. a) Representation over the 96 hours. b) Comparison of α-tocopherol between the two medium states.

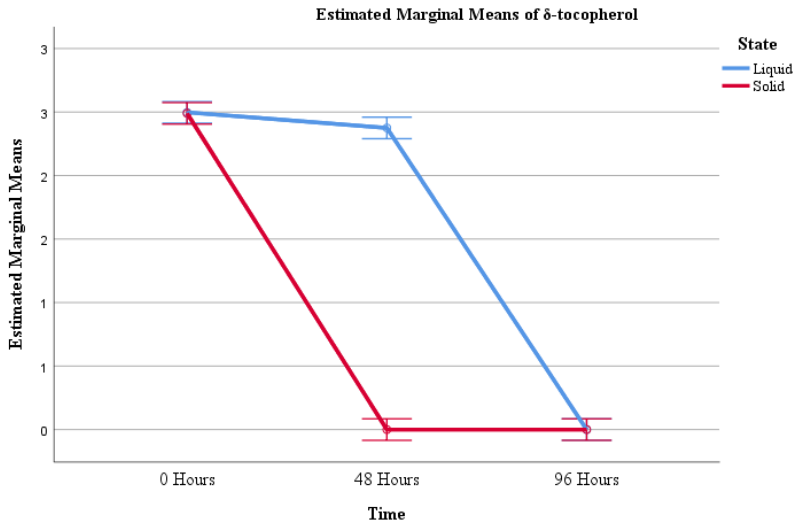


Figure 24: Representation of the EMM plots of δ-tocopherol for the roots of *C. sativa* co-cultured with *S. polyrhizum* over the 96 hours.

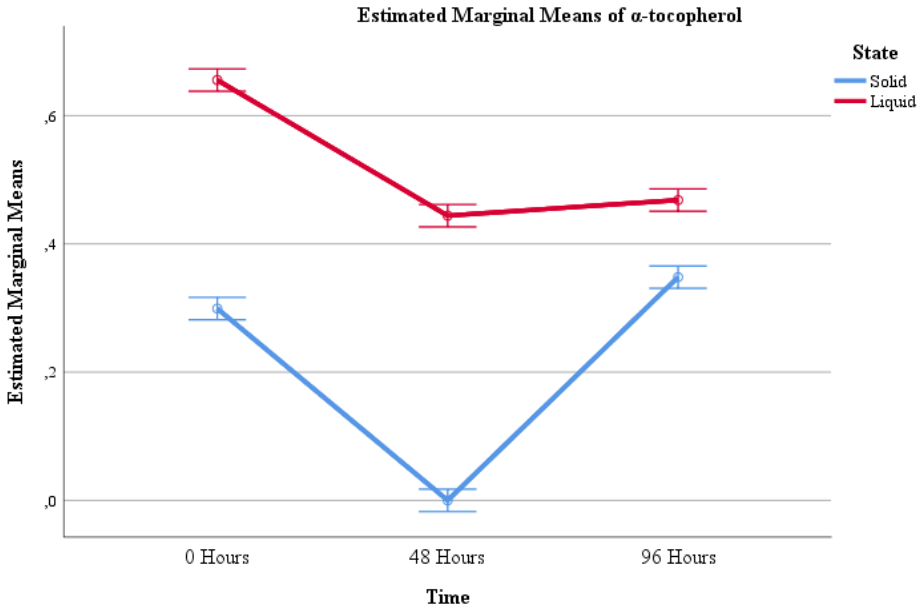
Table 11 and **12**, using the same layout as **9** and **10** also display the variation in tocopherols over the 96 hours in both medium states, but display the tocopherols present in the mycelium of mushrooms of the co-culture, *C. odora* and *S. polyrhizum*, respectively. The mycelia of *C. odora* was the only matrix to display γ-tocopherol, as can be seen in **Table 11**. Once again, significant interaction was detected for all tocopherol isoforms, allowing for general tendencies to be extracted from the EMM plots. Still, it is clear that α-tocopherol showed a decrease from the 0 to the 48th hour, and from there onwards a slight increase, while γ-tocopherol seemed to be completely consumed over the symbiosis, not showing any amounts after the 48th hour. Finally, δ-tocopherol showed an increase in the first period (0 to 48 hours) followed by a decrease. Analyzing the EMM plots to better understand the behavior of the individual isoforms, in **figure 25** section a), the drop from the 0 hour to the 48th is much more pronounced for the solid medium co-culture, as is the increase from the 48th to the 96th hour, while the liquid medium co-culture shows very slight variations. Section b), shows the results over the 96 hours for the γ isoform in which a drop-in quantity is found on the 48th hour and a maintenance of this behavior throughout the remainder of the co-culture. Interestingly, this phenomenon is found for both the solid and liquid co-cultures. Finally, δ-tocopherol, represented in section c) shows a opposite behavior, between the two medium types. For the liquid medium, the EMM plot shows an increase in the first co-culture period and then a very slight decrease, while for the solid medium, a slight decrease is found in the

first stage resulting in the complete consumption of this isoform, and until the 96th hour there is no production of this tocopherol isoform. Once again, the overall amount of tocopherols decreases during the co-culture time, except for δ-tocopherol in the solid co-culture.

Table 11: α-, γ-, δ- and total tocopherol variation in the mycelia of *C. odora* in solid and liquid medium co-cultured with the roots of *C. sativa*, expressed as µg/g Dw.

		α-tocopherol	γ-tocopherol	δ-tocopherol	Total tocopherols
Co-culture Time (ST)	0 hours	0.5±0.2	3±0.3	2±1	5±1
	48 hours	0.2±0.2	0±0	3.96±0.03	4.40±0.01
	96 hours	0.41±0.07	0±0	2±2	2±2
p-value (n=10)	Tukey's HSD test	<0.001	<0.001	<0.001	<0.01
Medium State (MS)	Liquid	0.5±0.1	1±1	3.4±0.7	4.920.9±2
	Solid	0.2±0.2	1±1	2±2	2±2
p-value (n=15)	Student T-Test	<0.001	<0.001	<0.001	<0.001
ST×MS (n=30)	*p*-value	<0.001	<0.001	<0.001	<0.001

For the storage time (CT), in each row and within each storage period, different letters mean significant statistical differences ($p<0.05$). For the medium state (MS), an asterisk means statistical differences between the two states. The presented standard deviations were calculated from results obtained under different operational conditions. Thus, they should not be regarded as a measure of precision, rather as a range of values.


Estimated Marginal Means of α-tocopherol
State
Solid
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Estimated Marginal Means
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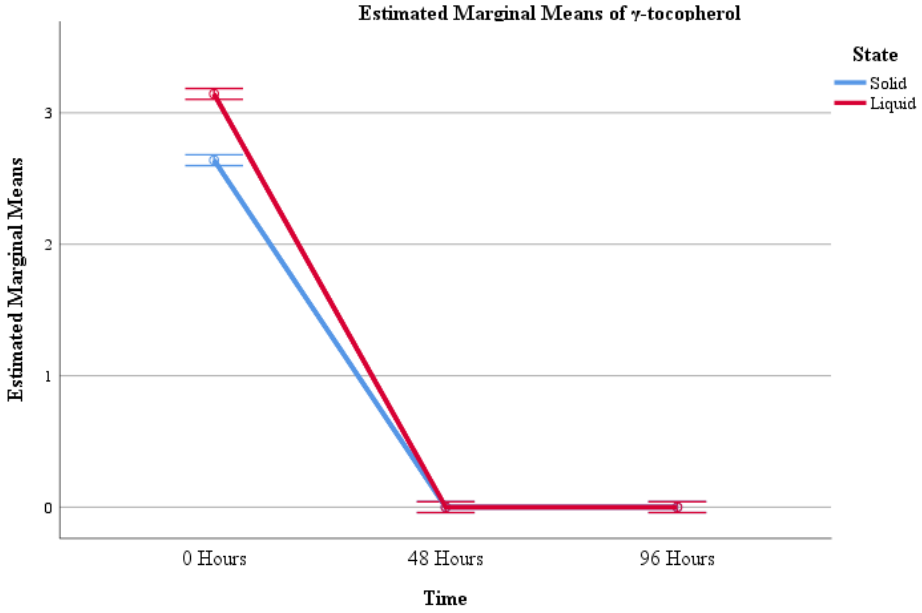
0 Hours
48 Hours
96 Hours
Time


Estimated Marginal Means of γ-tocopherol
State
Solid
Liquid
Estimated Marginal Means
3
2
1
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0 Hours
48 Hours
96 Hours
Time

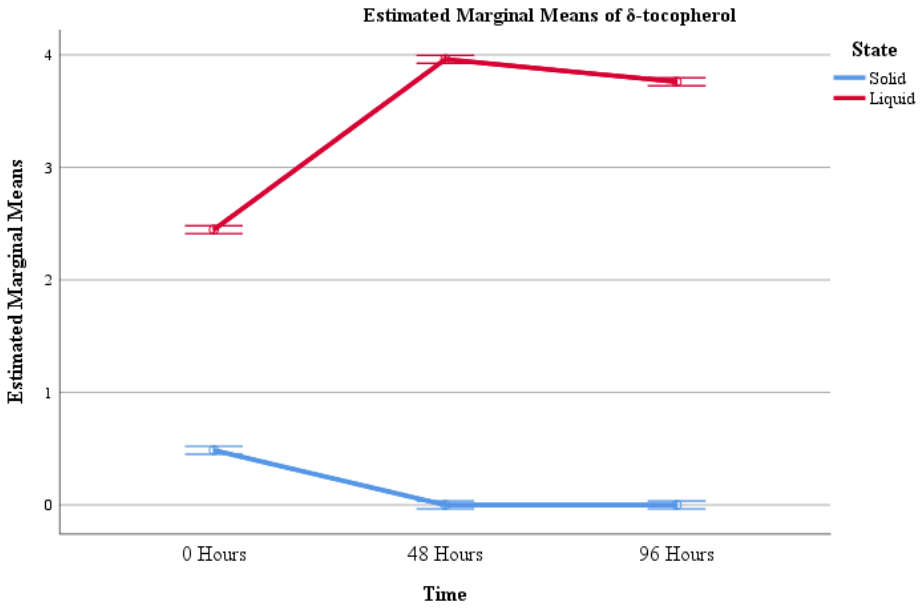

Figure 25: Representation of the EMM plots of α-tocopherol (a), γ-tocopherol (b) and δ-tocopherol (c) for the mycelia of *C. odora* co-cultured with the roots of *C. sativa* over 96 hours.

Table 12, pertaining to the tocopherols found in the mycelia of *S. polyrhizum*, shows the two isoforms found, namely α- and δ-tocopherol, being the most abundant the latter isoform. Once again, a significative interaction was detected for the co-culture time and the state of the medium, allowing for tendencies to be drawn from the EMM in **Figure 26**. **Section a)** of **Figure 26** shows the behavior of α-tocopherol during the 96 hours of co-culture, and it is clear that this isoform in the mycelia of *S. polyrhizum* showed a sharp decrease in the first 48 hours, and a small recovery from the 48th to the 96th hour, while the amounts in the mycelia in the liquid co-culture started at a lower quantity and gradually decreased over the whole culture period. **Section b)** of **Figure 26** shows the behaviour of total tocopherols (the EMM plots of δ-tocopherol were not suitable for general conclusions, although the total tocopherols represent in a certain manner the same behavior as the δ isoform), and it is clear that the liquid co-culture showed higher amounts at time 0, which gradually decreased until the 48 hours and then sharply decreased in the second phase. The liquid co-culture showed an overall lower quantity of total tocopherols which was also gradually reduced over time, although in a slighter manner. Once again, the overall reduction of tocopherols was detected in the mycelia of *S. polyrhizum*, in line with what was detected in the mycelia of *C.odora* and the roots of *C. sativa*, aside from one exception, δ-tocopherol in the mycelia of *C. odora* in the liquid co-culture for the first 48 hours. In conclusion, the fact that there was a reduction of

the detected isoforms of tocopherols could prove that either they were dispatched to quench oxidative behavior of free radicals towards the cells involved in the symbiotic development, or they could be consumed in signaling cascades during the symbiotic recognition, and thus not being produced in the first steps of the mycorrhization. In a similar work, Carvalho et al. (2013)(Carvalho et al., 2013) studied the mycorrhization of *C.sativa* and two other mushrooms, *Paxillus involutos* and *Pisolithus arhizus* during a co-culture of 72 hours. Interestingly, all isoforms of tocopherols were detected both in the roots, stems and mycelium of the mushrooms and also in the medium where the mycorrhizae had formed, except β-tocopherol which was not detected in the medium or mycelium of *Paxillus involutus*. The mycelia showed higher amounts of tocopherols, dominated by γ-tocopherol, which was hardly detected in the mushrooms of the present study. Still, in line with the discoveries of the present study, the one by Carvalho et al. (2013)(Carvalho et al., 2013) also concluded that the overall amount of tocopherols reduced along the co-culture time, confirming that ectomycorrhizal fungi do not stimulate the production of tocopherols in the roots of the chestnut plants, at least in the first hours of symbiosis. Further studies are required, in order to verify the variation of the tocopherol isoforms further than the initial 96 hours, to completely comprehend the variation of these incredibly important molecules in mycorrhizae.

Table 12: α-, γ-, δ- and total tocopherol variation in the mycelia of *S. polyrhizum* in solid and liquid medium co-cultured with the roots of *C. sativa*, expressed as μg/g Dw.

		α-tocopherol	γ-tocopherol	Total tocopherols
Co-culture Time (ST)	0 hours	0.9±0.4	4±2	5±3
	48 hours	0.21±0.05	4±2	5±2
	96 hours	0.5±0.4	1.6±0.3	2.1±0.2
p-value (n=10)	Tukey's HSD test	<0.001	<0.001	<0.01
Medium State (MS)	Liquid	0.2±0.1	2.2±0.2	2.4±0.4
	Solid	0.8±0.4	5±3	6±3
p-value (n=15)	Student T-Test	<0.001	<0.001	<0.001
ST×MS (n=30)	*p*-value	<0.001	<0.001	<0.001

For the storage time (CT), in each row and within each storage period, different letters mean significant statistical differences ($p<0.05$). For the medium state (MS), an asterisk means statistical differences between the two states. The presented standard deviations were calculated from results obtained under different operational conditions. Thus, they should not be regarded as a measure of precision, rather as a range of values.

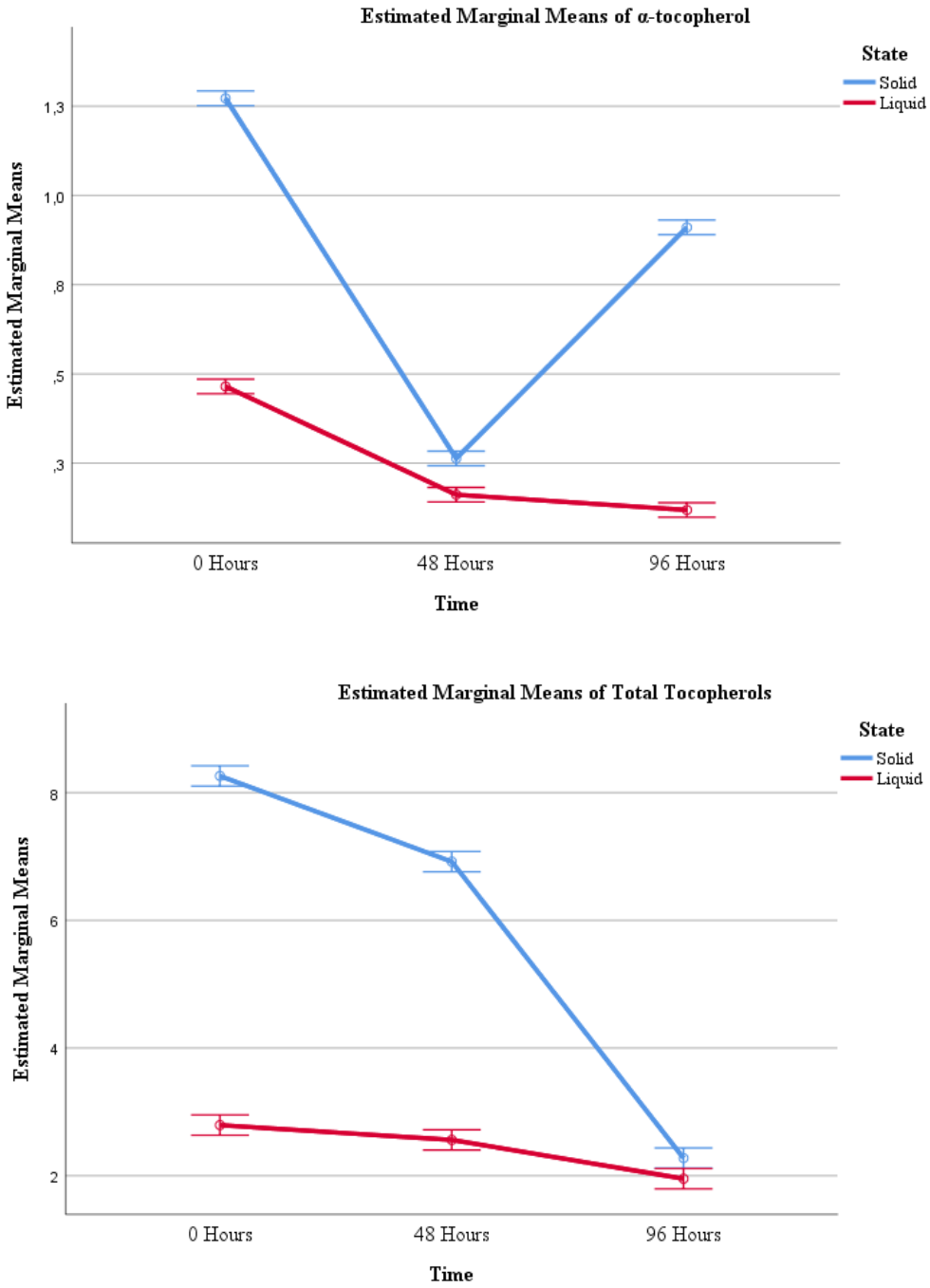

Figure 26: Representation of the EMM plots of α-tocopherol (a) and total tocopherols (b) for the mycelia of *S. polyrhizum* co-cultured with the roots of *C. sativa* over 96 hours.

5 CONCLUSION AND FUTURE PERSPECTIVES

Previous studies showed that vitamin E composition of mycorrhizal fungi *in vivo* and *in vitro*, and mycelium samples from *in vitro* cultures were very promising for the production of total tocopherols compared to the fruiting bodies.

The present study aimed to analyze and evaluate the production of antioxidant compounds (tocopherols) in samples of two species of ectomycorrhizal fungi, *C. odora* and *S. polyrhizum*, isolated and in symbiosis with the roots of *C. sativa* in order to see if this initial symbiosis phases constitute a condition of oxidative stress that will trigger the defense of fungi hence their elicitation to produce tocopherol.

By analyzing the vitamin E composition of the mycorrhizal fungi studied, significant quantities of tocopherol have been found of which several vitamers are rare (gamma and omega), but during the time of co-culture with the roots, these rates have decreased and some were gone after 96 hours.

In conclusion, mycorrhization process is not a stress condition for either fungi or roots since it did not increase the production of tocopherols and even the stress caused by *in vitro* culture was inhibited.

For future perspectives, to take advantage of the ability of mushrooms in the production of several vitamers which have a very important therapeutic role, a line of work and analyzes that must be done to found the stressful conditions and the system that triggers the elicitation of fungi and increase the production of tocopherols. This system can be biotic or abiotic (light, temperature, nutrient...).

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