Universidade do Minho
Escola de Ciências

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Characterization of Antifungal Activity of a Plant Extract of *Medicago* Genus

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Characterization of Antifungal Activity of a Plant Extract of *Medicago* Genus

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Professor Doutor Rui Pedro Soares de Oliveira

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CHARACTERIZATION OF ANTIFUNGAL ACTIVITY OF A PLANT EXTRACT OF *MEDICAGO* GENUS

ABSTRACT

Medicago L., has been one of the most widely studied legumes for its agricultural importance. These plants contain typical secondary metabolites, such as saponins, which possess several biological properties, namely antifungal activity. Phytopathogenic fungi are pathogens that induce a substantial damage in several economically important crops. Thus, the protection of harvests is essential. The use of chemical fungicides has been banned due to the problems associated with them. Consequently, the search for alternative solutions is increasing, in particularly in the search of natural biological compounds.

Therefore, this work arises with the main goal of evaluated the antifungal activity of a crude *Medicago* extract (ME) in several phytopathogenic fungi. The mycelial growth of *Botrytis cinerea*, *Colletotrichum* species (*C. acutatum*, *C. gloeosporioides*, *C. godetiae* and *C. nymphaea*), *Diplodia corticola* and *Phythophthora cinnamomi*, exposed to ME were measured. The extract exhibited an evident fungitoxic effect against all fungi, showing that the most sensitive was *D. corticola*, with an inhibition rate of 70%. In order to evaluate the phytotoxicity of ME in germination of plants, it was used, under *in vitro* conditions, seedlings of flax (*Linum usitatissimum* L.) plants. ME induced a significant inhibition in the growth of its primary organs. The ME phytotoxic effect in the leaves of strawberry plants, was also evaluated. The results suggested that ME did not cause damage to adult plants. The organism model *Saccharomyces cerevisiae* was also used to understand the toxicity mechanism that ME caused in fungi cells. Through several assays involving the two processes of cell death (apoptosis and necrosis), such as viability tests, assessment of chromatin condensation and nuclear fragmentation, plasma membrane integrity and translocation nucleus-cytosol of Nhp6Ap protein, we concluded that ME may be inducing necrotic death in yeast cells.

Overall, based on results obtained in this work, it was possible to infer that ME has potential as a significant source of natural and biodegradable fungicides for control of pathogen diseases in crop production systems, mainly for application in organic agriculture.

CARACTERIZAÇÃO DA ATIVIDADE ANTIFÚNGICA DE UM EXTRATO DA PLANTA DO GÉNERO *MEDICAGO*

RESUMO

Medicago L., tem sido uma das leguminosas mais estudadas devido à sua importância agrícola. Estas plantas contêm metabolitos secundários específicos, como saponinas, que possuem várias propriedades biológicas, nomeadamente atividade antifúngica. Os fungos fitopatogénicos são agentes patógenos que induzem consideráveis danos em várias culturas economicamente importantes. Assim, a proteção das colheitas é essencial. O uso de fungicidas químicos tem sido banido devido aos problemas associados. Consequentemente, a busca de soluções alternativas está aumentando, particularmente na procura de compostos biológicos naturais.

Portanto, este trabalho surge com o objetivo principal de avaliar a atividade antifúngica de um extrato de *Medicago* (ME) em vários fungos fitopatogénicos. Foi medido o crescimento micelial de *Botrytis cinerea*, espécies de *Colletotrichum* (*C. acutatum*, *C. gloeosporioides*, *C. godetiae* e *C. nymphaea*), *Diplodia corticola* e *Phythophthora cinnamomi*, expostos a ME. O extrato apresentou um evidente efeito fungitóxico contra todos os fungos, mostrando que o mais sensível foi *D. corticola*, com uma taxa de inibição de 70%. Para avaliar a fitotoxicidade de ME na germinação de plantas, em condições *in vitro*, foram utilizadas plântulas de linho (*Linum usitatissimum* L.). ME induziu uma inibição significativa no crescimento dos seus órgãos primários. Foi avaliado o efeito fitotóxico de ME nas folhas de plantas de morango. Os resultados sugeriram que ME não causou danos às plantas adultas. O organismo modelo *Saccharomyces cerevisiae* também foi usado para entender o mecanismo de toxicidade que ME causa em células de fungos. Através de vários ensaios que envolvem os dois processos de morte celular (apoptose e necrose), tais como testes de viabilidade, avaliação da condensação da cromatina e fragmentação nuclear, integridade da membrana plasmática e translocação núcleo-citosol da proteína Nhp6Ap, conclui-se que ME poderá induzir morte necrótica em células de levedura.

Em geral, com base nos resultados obtidos neste trabalho, foi possível inferir que ME apresenta potencial como fonte de fungicidas naturais e biodegradáveis para o controlo de doenças patogénicas que afetam sistemas de produção de culturas, principalmente para aplicação em agricultura orgânica.

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List of Abbreviations and Acronyms

CFU	**C**olony **F**orming **U**nits
dH_2O	**D**eionized **water**
GFP	**G**reen **F**luorescent **P**rotein
HMGB1	**H**igh **M**obility **G**roup **B**ox 1
ME	***M**edicago* **e**xtract
MS	**M**urashige and **S**koog
ns	**n**o **s**ignificant
OD	**O**ptical **D**ensity
PBS	**P**hosphate **B**uffered **S**aline
PCD	**P**rogrammed **C**ell **D**eath
PDA	**P**otato-**D**extrose-**A**gar
PI	**P**ropidium **I**odide
ROS	**R**eactive **O**xygen **S**pecies
rpm	**r**evolutions **p**er **m**inute
S.D.	**S**tandard **D**eviation
YPD	**Y**east-**P**eptone-**D**extrose
YPDA	**Y**east-**P**eptone-**D**extrose-**A**gar

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Chapter I

Introduction

I.1. THE GENUS *MEDICAGO* L.

Medicago L., known as "medics", belongs to the family Fabaceae and includes approximately 87 different species, of which two-thirds are annuals and one-third perennials (Silva et al., 2007; Kicel and Olszewska, 2015), widespread from the Mediterranean to central Asia (Steele et al., 2010). *Medicago* has been one of the most widely studied legumes for its agricultural importance, such as improvement of soil fertility and subsequent crop yield. Medics increase pasture soil organic matter, enhance soil water retention and water availability, and establish a nitrogen-fixing symbiosis with rhizobia improving the soil nitrogen (N) status due to N_2 fixation (Zhu et al., 1998). *Medicago sativa* L. (also called alfalfa or lucerne) is the most known plant of this genus. The estimated world acreage of this plant is 32 million ha, which has a high value as a forage crop and is mainly used as forage for domestic animals (Silva et al., 2007; Steele et al., 2010). This genus also includes *Medicago scutellata* (snail medic) and *Medicago lupulina* (black medic), which are also important forage species, and the legume model species *Medicago truncatula* Gaertn. (Steele et al., 2010). These species are used in medicine, human food, renewable energy production (Rodrigues et al., 2013), green manure, and phytoremediation (Amer et al., 2013), as well as sources of industrial enzymes in biotechnology and phytochemicals (Steele et al., 2010; Barreira et al., 2015), and as a model for genomic studies of Fabaceae (Cannon et al., 2006).

Medicago plants contain typical secondary metabolites such as coumarins, isoflavones, naphthoquinones, alkaloids and saponins. Saponins are the major family of compounds of these plants, characterized by several biological properties (Avato et al., 2006), which are presented in the following sections.

I.1.1. Saponins compounds

Saponins are phenolic compounds present in roots, shoots, seeds, leaves, stems and flowers of *Medicago* plants (Oleszek, 1993). The name saponin is derived from the Latin word "sapo", soap, due to its important physicochemical property and ability to form a colloidal solution in water giving a stable foam when shaken (Wina et al., 2005). Saponins are a structurally class of amphipathic compounds with a lipophilic aglycone that is covalently linked to one (monodesmosidic) or more (di- or tridesmosidic) hydrophilic sugar chains *via* a glycosidic bond. Based on the aglycone structure from which they are derived, saponins are widely classified as steroids, steroidal glycoalkaloids, or triterpenoids (Gholami et al., 2014; Moses et al., 2014). The most abundant monosaccharide units found in these compounds are

xylose, glucose, arabinose, rhamnose and glucuronic acid (Tava and Avato, 2006). The triterpenoid aglycones of saponins are the mostly detected in legumes. Chemical studies demonstrated that these compounds comprise a large group of different sapogenins (Gholami et al., 2014). *Medicago* aglycones consist of a C_{30} (30 carbon atoms) skeleton comprising a pentacyclic structure (Fig. I.1; Sparg et al., 2004). The enzymes involved in the formation of triterpene algycones were already identified, which include the squalene synthase, squalene epoxidase and β-amyrin synthase. The triterpenic pentacyclic nucleus are originated from the cyclization of squalene or oxidosqualene, which involves several steps, catalyzed by the enzyme β-amyrin synthase (Tava et al., 2011). Sapogenins are derived from β-amyrin through various oxidative modifications, and based on the oxidation pattern, two distinct types of aglycones classes. The first class consists of aglycones that are oxidized at the C-28 position, which is regularly accompanied with oxidation at the C-23 position (Fig. I.1). This class includes oleanolic acid, hederagenin, caulophyllogenin, bayogenin, medicagenic acid and zanhic acid (Fig. I.2). The second class exhibits a hydroxy group at the C-24 position (Fig. I.1), and includes the soyasapogenols A, B, and E (Fig. I.2; Gholami et al., 2014). Medicagenic acids are the most biologically active sapogenin found in *Medicago* species (Gestetner et al., 1971; Oleszek et al., 1990; Saniewska et al., 2003).

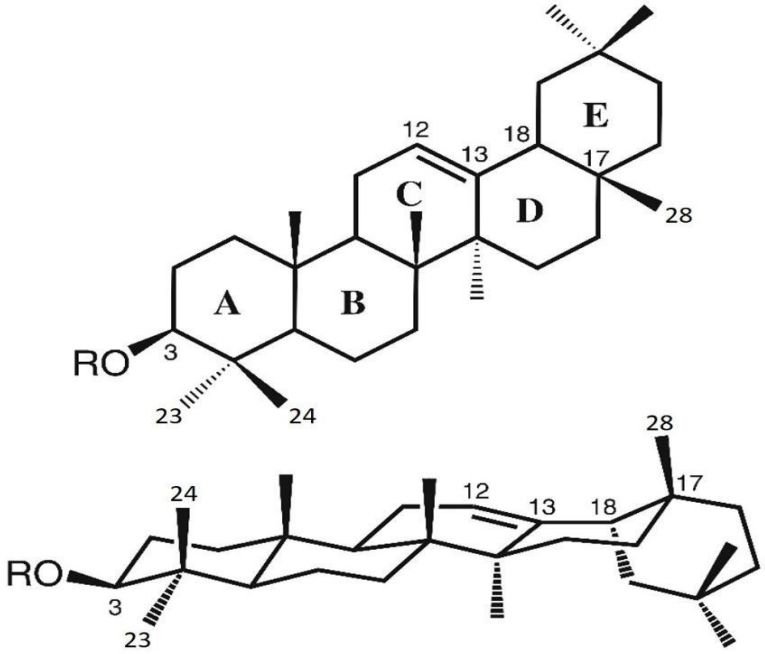


Figure I.1. General chemical structure of *Medicago* aglycones. R=H:β-amyrin (adapted from Tava et al., 2011).

The biological activities of Medicago saponins have been related to their chemical structure. It was shown that monodesmosides saponins (sugar chain linked at C-3 position) are more active than bidesmosides saponins (sugar chain linked at C-3 and C-28 position). The efficacy of these compounds

can be determined by important factors such aglycone, and the nature and position of the sugar in the molecule (Tava and Avato, 2006). Saponins have been reported to possess antimicrobial, insecticidal, fungistatic, nematicidal, allelopathic, hemolytic, antinutritional, antitumoral and cytotoxic properties (Tava and Avato, 2006; Tava et al., 2011; Tava and Pecetti, 2012).

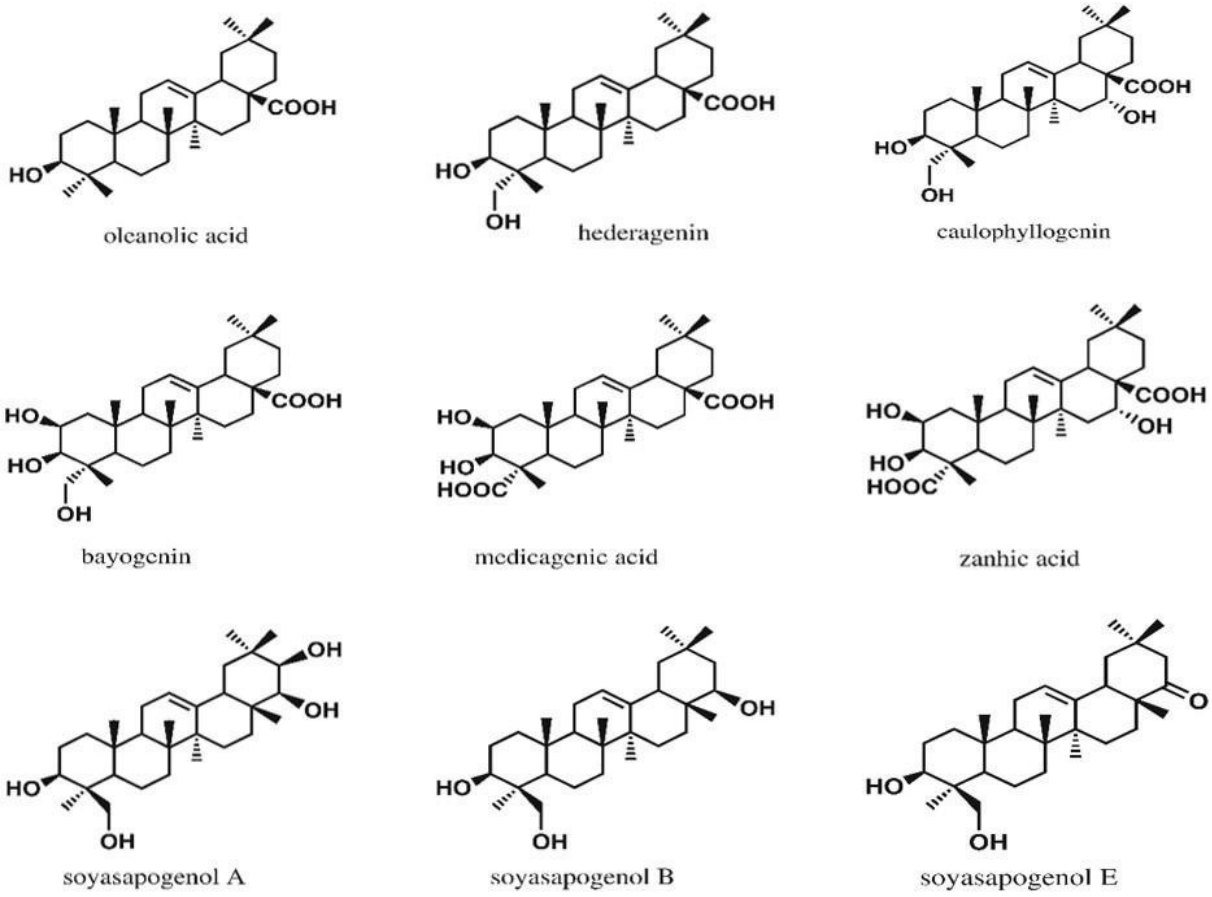


Figure I.2. Aglycones detected in *Medicago* species (adapted from Tava et al., 2011).

I.1.1.1. Antifungal activity of saponins

Saponins are found in many plants, generally in tissues that are most vulnerable to fungal or bacterial attack, or insect predation. Therefore, one of their principal roles is to act in plant defense system (Wina et al., 2005). In response to a pathogen attack, that induce tissue wounds, some plants activate the hydrolysis of saponins derivatives with strong antibiotic activity (Tava and Avato, 2006). The main mechanism suggested to explain this activity is based in their interaction with membrane steroids leading to cell disruption (Howarth et al., 1977).

Medicagenic acid glycosides are highly active exhibiting fungistatic properties, being the major aglycone that contributes to antifungal activity (Nonaka, 1986; Levy et al., 1989). Gestetner et al. (1971),

demonstrated that medicagenic acids, due to special structure features, i.e., two free carboxyl or hydroxyl groups, are essential to fungal growth depression, compared with soyasapogenols, which lack these carboxyl groups, or bear hydroxyl groups at different positions and are devoid of growth-inhibiting activity.

Antifungal activity of *Medicago* plants has been primarily studied with the model fungus *Trichoderma viride*, which appeared particularly sensitive to the presence of saponins in the growth medium (Scardavi and Elliot, 1967; Jurzysta and Nowacki, 1979; Nonaka, 1986; Levy et al., 1989; Oleszek et al., 1990). The activity against this fungus was essentially conditioned by the sugar chain of the saponin moiety (Scardavi and Elliot, 1967), being positively correlated to the ratio of aglycones:sugars (Gestetner et al., 1971).

I.1.1.2. Applications of saponins

Saponins are broadly distributed in many plants and relatively widespread in foodstuffs and herbal preparations. Traditionally, these compounds are used as a natural detergent (Tamura et al., 2012). Over the years, the number of studies on the biosynthesis of saponins has increased, due to their chemical, physical and physiological characteristics. The wide diversity in structures and biological activities of saponins and their biosynthesis intermediates, make these compounds an important starting material for industrial applications, such as for commercial products in pharmaceutical, cosmetic, food and agronomic industry (Gholami et al., 2014; Moses et al., 2014). These compounds can be used as sources of raw materials for the production of steroid hormones and semi synthesis of drugs, and also as food additives and as constituents in photographic emulsions and fire extinguishers (Tava and Avato, 2006; Tamura et al., 2012). Other biological applications include their use as anti-inflammatory, hypocholesterolemic and immune-stimulating as adjuvants in vaccine formulations (Tamura et al., 2012). Plant extracts rich in saponins have been used as ingredients of cosmetic preparations for lipsticks, shampoos and toothpastes (Tava and Avato, 2006). Saponins are also been important bioactive compounds that can be employed in agrochemical industry, such as in nematicidal formulations (Argentieri et al., 2008).

I.2. PHYTOPATHOGENIC FUNGI

Phytopathogenic fungi are pathogens that induce a considerable injury in several economically important crops (Pham et al., 2017). Fungi are the most varied group of plant pathogens accounting for

70-80% of plant diseases (Ray et al., 2017). More than 8.000 fungal species infect seeds, seedlings and mature plants (Ali et al., 2017; Choi et al., 2017). Fungal diseases cause modifications through developmental stages, severe losses and quality problems related to aspect, nutritional value, organoleptic characteristics, and limited shelf life (Ammar et al., 2013). Fungi can survive in an inactive state on living or dead plant tissues until conditions become favorable to their proliferation. Fungal spores can easily spread by wind, water, soil and animals (Ray et al., 2017). Phytopathogenic fungi exhibit a wide range of strategies to infect and colonize plants, resulting in widely diversified pathogenic lifestyles, such as biotroph, hemibiotroph, necrotroph, endophytic and quiescent. Biotrophs have been defined as pathogens that grow and reproduce in living plant tissues, obtaining nutrients through interactions with living plant cells. Hemibiotrophs initially establish a biotrophic relationship with hosts and continue to live when the host cells die. The necrotrophs feed on dead plant cells, killing host tissue before colonizing it (Latijnhouwers et al., 2003). Endophytic fungi live within the host plant cells as symbionts without causing apparent disease. Lastly, quiescent or latent lifestyle is defined for an long period in which the pathogen exists inactive within the host before it changes to an active phase (De Silva et al., 2017).

The phytopathogenic attacks have different levels of specialization that can be observed in plant-fungal interactions. The first level occurs in opportunistic parasites, which enter plants through wounds. The virulence of these fungal species is relatively low and only causes mild disease symptoms. The next level contains pathogens that rely on living plants to grow, and many of the more serious plant pathogens are found in this level. Lastly, the highest level of complexity is reached by obligate pathogens, in which the living host plant is an absolute condition to achieve their complete life cycle (Knogge, 1996). These plant-fungal interactions change their relationship at different stages of their life cycle, depending of environment, physiological maturity, host resistance, and pathogen-associated virulence genes (De Silva et al., 2017). Many phytopathogenic fungi possess mechanisms to actively cross the plant's outer structural barriers, the cuticle and the epidermal cell wall (Geoghegan et al., 2017). For entrance, fungi commonly secrete a cocktail of hydrolytic enzymes, which include cutinases, cellulases, pectinases, and proteases (Knogge, 1996).

Phytopathogenic fungi cause a substantial annual crop loss, estimated up to about 20%. These losses are the major restraints in sustainable agriculture production, especially in intensive cropping systems, representing a continuous challenge to modern agriculture disease management system (Ali et al., 2017). The journal *Molecular Plant Pathology*, in association with fungal pathologists, reviewed the "Top 10" fungal pathogens based on scientific/economic importance. This "Top 10" includes *Botrytis cinerea* in second place and *Colletotrichum* spp. in eighth position (Dean et al., 2012). In addition to

these fungi, *Diplodia corticola* and *Phythophthora cinnamomi* are also considerably relevant, causing substantial damages in important Portuguese crops.

I.2.1. *Botrytis cinerea*

Botrytis cinerea Pers. Fr. is an ascomycete, ubiquitous and destructive pathogen also known as "grey mould fungus" that causes serious pre- and post-harvest diseases in a wide range of hosts, including in at least 200 dicotyledonous plant species around the world (Li et al., 2016). This range includes agronomical important crops and harvested commodities, such as fruit, vegetables, ornamental plants, bulb and cut flowers, which include grape (*Vitis* spp.), tomato (*Lycopersicon esculentum*), strawberry (*Fragaria* x *ananassa*), raspberry (*Rubus* L.), cucumber (*Cucumis sativus*), carnation (*Dianthus caryophyllus*), rose (*Rosa* L.) and oilseed rape (*Brassica napus*) (You et al., 2016; Ayoub et al., 2017). This fungus is considered as a typical necrotrophic, co-opting programmed cell death pathways in the host to achieve infection. Sometimes, this fungus remains quiescent until host physiology changes or environment is conductive. The infestation of this fungus can occur since the seedling stage until product ripening (Dean et al., 2012), and also result in losses during storage and transport of export products (Robles-Kelly et al., 2017). In the infection process, *B. cinerea* use several toxins to facilitate the infection and colonization of plant tissues, through the following steps: penetration of the host surface, killing of host tissue, formation of primary lesion, expansion of lesion, tissue maceration, and sporulation (Choquer et al., 2007). *B. cinerea* is responsible for a very wide range of symptoms that affect different plant organs and tissues (Fig. I.3). The most typical symptoms on leaves and fruits are soft rots accompanied with collapse and water-soaking of parenchyma tissues, followed by a rapid appearance of grey masses of conidia, which are the main produced and dispersed inoculum. Their germination and adhesion on plant surfaces is important for host penetration and colonization (Choquer et al., 2007; Williamson et al., 2007). Due to variety of attack modes, diversity of hosts and survival for long periods as mycelia and/or conidia, *B. cinerea* is difficult to control (Ayoub et al., 2017), remaining a challenge to researchers.

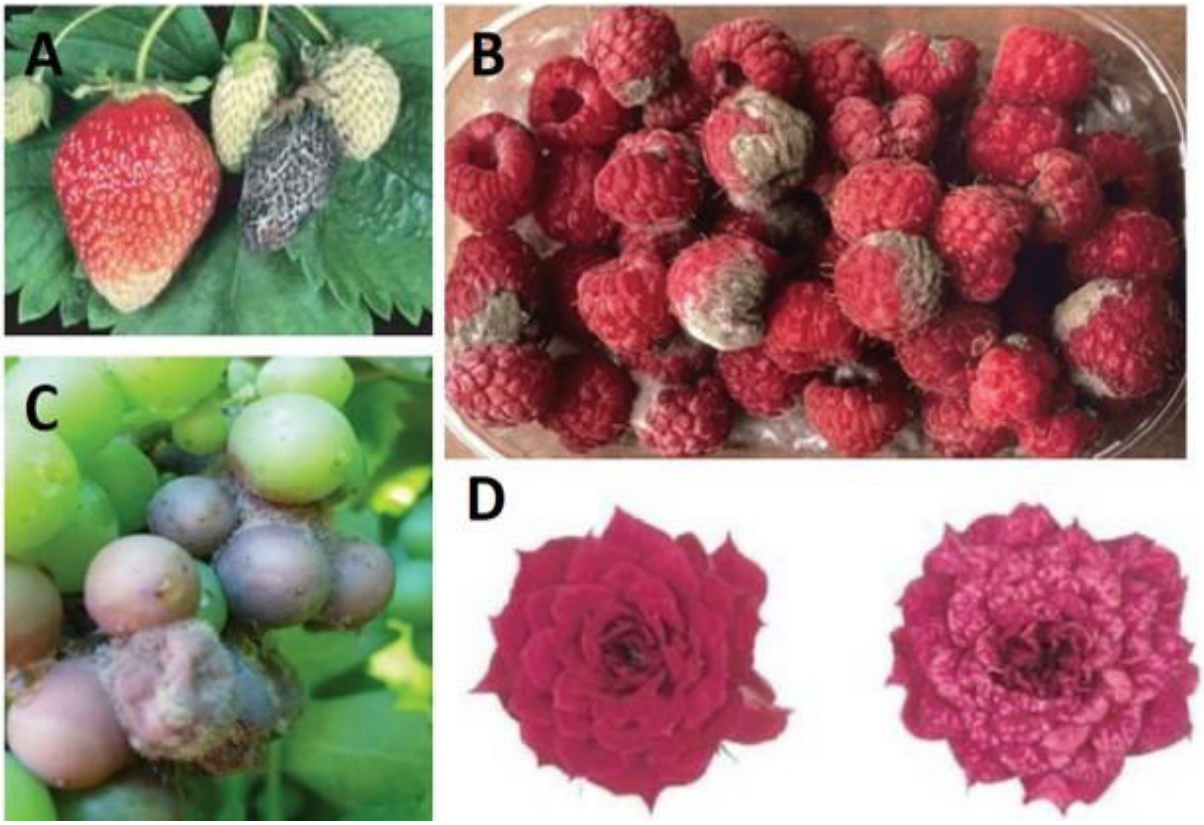


Figure I.1. Symptoms of infection by *Botrytis cinerea* in strawberry **(A)**; raspberry **(B)**; grape **(C)**; and rose **(D)** (Adapted from Williamson et al., 2007; Dean et al., 2012; Agudelo-Romero et al., 2015).

I.2.2. *Colletotrichum* spp.

The genus *Colletotrichum* is one of the most common and important genera of phytopathogenic fungi. Almost every crop grown throughout the world is susceptible to one or more species of this genus (Dean et al., 2012). These species are associated with pre- and post-harvest fruit diseases, and act especially in tropical, subtropical and temperate regions (Bragança et al., 2016). Fruits such as mango (*Mangifera indica*), papaya (*Carica papaya*), banana (*Musa* L.), strawberry (*Fragaria* x *ananassa*), avocado (*Persea Americana*), and citrus (*Citrus* L.) are affected by these fungi (Cannon et al., 1986; Boonruang et al., 2017). Anthracnose disease is the most common symptom associated with *Colletotrichum* infection, that is characterized by a sunken necrotic lesion on leaves, stems, flowers and fruit, on which often orange conidial masses are produced (Bragança et al., 2016). A range of anthracnose disease symptoms caused by this fungus is illustrated in Fig. I.4. However, there are other maladies related to this fungus such as coffee berry disease, red rot sugar cane, brown blotch of cowpea, and crown rot of strawberry and banana (Cannon et al., 1986).

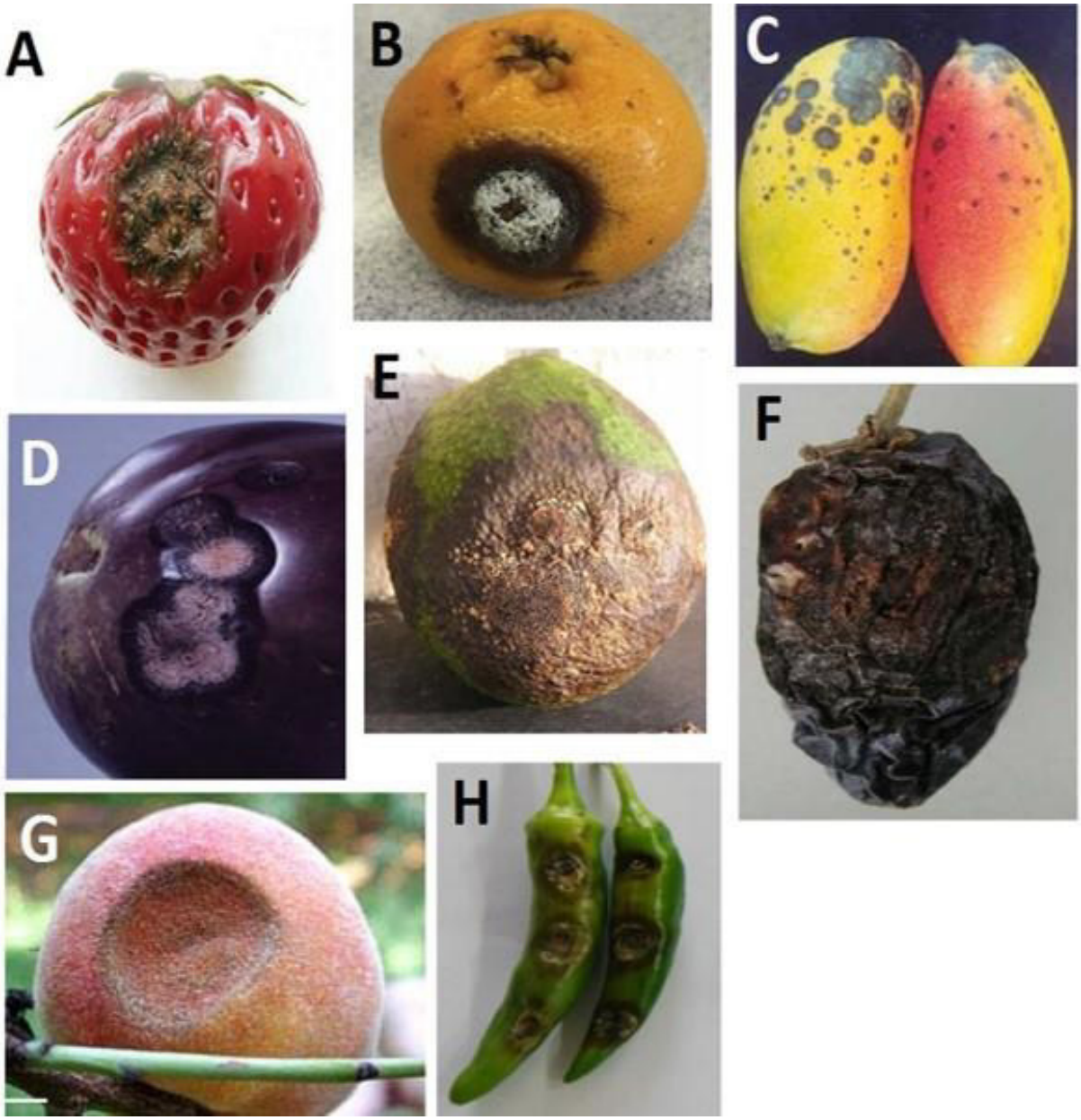


Figure I.1. Anthracnose disease caused by *Colletotrichum* species, *C. nymphaea* in strawberry **(A)**; *C. gloeosporioides* in mandarin citrus **(B)**, mango **(C)**, eggplant **(D)**, and avocado **(E)**; *C. acutatum* in olive **(F)**, peach **(G)** and chili fruit **(H)** (Adapted from Cannon et al., 1986; Schnabel et al., 2006; Nelson, 2008; Gomes et al., 2012; Kamle et al., 2013; Oo and Oh, 2016; De Silva et al., 2017).

Hemibiotrophic is the most common lifestyle of *Colletotrichum* species, however, these also can be necrotrophics, endophytics and quiescent (De Silva et al., 2017). *Colletotrichum* spp., due to unique intracellular hemibiotrophic lifestyle, easy culture and manipulation, has been used as a model pathogen for fundamental, biochemical, physiological and genetic studies (Dean et al., 2012). The initial infection of *Colletotrichum* species usually occurs at the pre-harvest stage when the fungal spores or mycelia infect young fruit through pedicel and peel wounds. Normally, pre-harvest infection remains latent or hidden

until the fruit undergoes ripening, which offers appropriate conditions and nutrients for fungal growth (Boonruang et al., 2017). The fungus reaches a necrotrophic phase, resulting in a significant death of plant cells and the appearance of pathogenic lesions. This delayed onset of symptoms can lead to significant post-harvest losses, through degeneration of apparently healthy products in storage (Cannon et al., 1986). The potential loss of production due to this pathogen is significant. For example, it was reported that this disease can cause post-harvest losses of avocado fruit up to 80% (Boonruang et al., 2017), the bitter rot diseases in apple fruit, can cause up to 50% yield loss (Bragança et al., 2016) and in strawberry the disease causes up to 80% of plant deaths in nurseries and over 50% of yield losses (Xie et al., 2010). *Colletotrichum acutatum* and *C. gloeosporioides* are the most destructive species of *Colletotrichum* in many crops, such as pepper (*Capsicum* spp.; Sun et al., 2015), strawberry (Xie et al., 2010), citrus (Douanla-Meli and Unger, 2017), avocado (Campos-Martínez et al., 2016) and olive (*Olea europaea* L.; Gomes et al., 2012).

I.2.3. *Diplodia corticola*

Diplodia corticola is an endophytic fungus that belongs to the family of Botryosphaeriaceae fungi (Evidente et al., 2006; Fernandes et al., 2014). It is the most widely distributed aggressive pathogen of cork oak (*Quercus suber* L.) trees worldwide, causing serious and negative impacts in their ecosystem, through the limitation of vitality and productivity of these trees (Masi et al., 2016). *Diplodia corticola* has also been implicated in malady of other European oaks, generally known as “oak decline” (Mullerin and Smith, 2015), and in the attack of grapevines (*Vitis* spp.) and eucalypts (*Eucalyptus* L.; Félix et al., 2017). This fungus can affect plants of different ages, inducing symptoms like discoloration or yellowing of leaves, dieback of branches, canker, and vascular necrosis (Fig. I.5; Campanile et al., 2007; Félix et al., 2017). *D. corticola* induces a small collapse and dark brown discoloration of the cortical tissues around the inoculation site (Evidente et al., 2006). The entrance of *D. corticola* is also facilitated by the traditional practice of removing the cork from the oak trunks, causing mild injuries (Mullerin and Smith, 2015). The mechanism of colonization of this fungus is unknown. However, members of the Botryosphaeriaceae family generally enter into plants through wounds, or open stomata for gas exchange. Other members of this family are opportunistic pathogens that often live harmlessly within the host without symptoms, becoming pathogenic when the plant is stressed by environmental factors such as drought, heat, flooding, freezing, transplanting, pruning, herbicide use and soil compaction (Martin and Munck, 2017).

Diplodia corticola is known for its ability to produce phytotoxins, degradative and oxidative enzymes, and cytotoxic proteins, that play a role in the infection mechanism (Félix et al., 2017). *In vitro,* this fungus produces secondary bioactive metabolites with strong activity on host and non-host plants (Mazzeo et al., 2017), which include sapinofuranone B, sphaeropsidines A and C, diplofuranes A and B, diplofuranilones A and B, and diplopyrone (Masi et al., 2016; Félix et al., 2017). Some of these metabolites showed phytotoxic, antifungal and antibacterial activities (Masi et al., 2016). Diplopyrone is a toxic compound to cork oak trees (Mullerin and Smith, 2015) and sphaeropsidin A has been shown to exhibit antimicrobial and anticancer activities (Masi et al., 2016).

As previously mentioned, *D. corticola* is a destructive pathogen of cork oak. This tree is one of the most common forest species in Portugal, which is the world's leading cork producer. In order to avoid severe economic losses, it is important the research for new methods to combat this fungus.

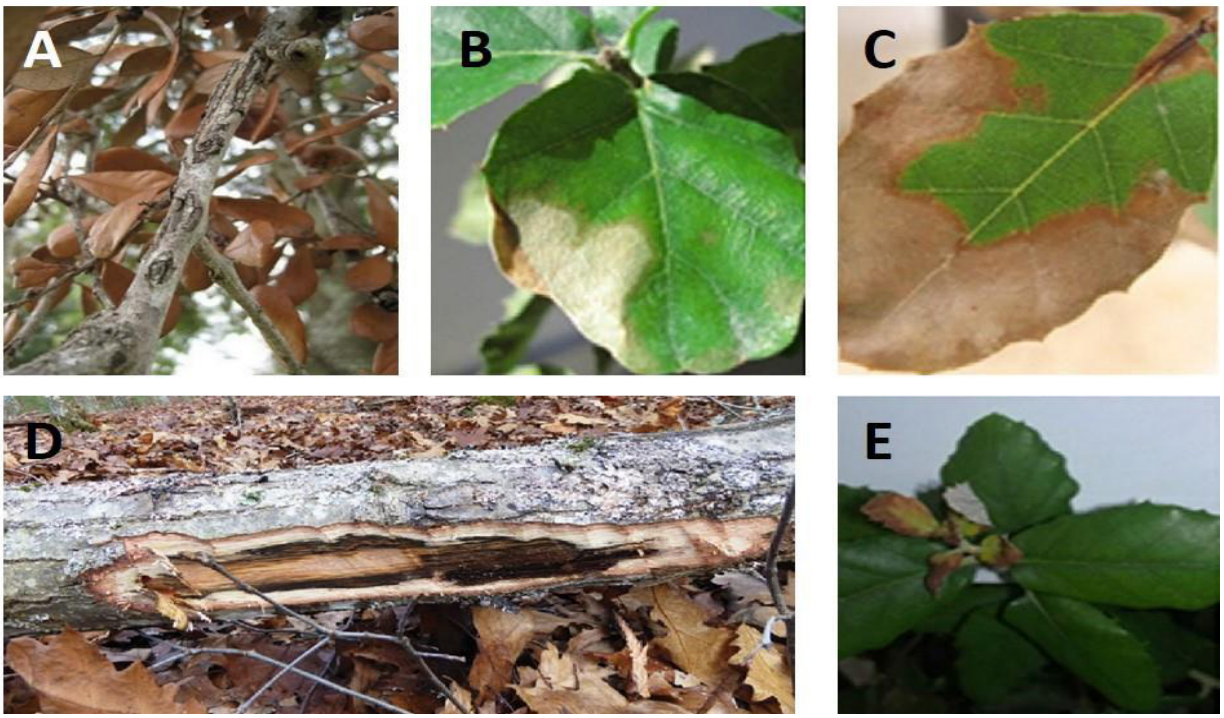


Figure I.1. Oak decline symptoms infected with *Diplodia corticola*. Cankers in live oak branch **(A)**; leaf chlorosis in cork oak **(B, C)**; necrotic lesion on black oak **(D)**, and dieback in cork oak **(E)** (Adapted from Mullerin and Smith, 2015; Félix et al., 2017; Martin and Munck, 2017).

I.2.4. *Phytophthora cinnamomi*

Phytophthora cinnamomi Rands belongs to Oomycetes class and is a highly aggressive filamentous soil-borne fungus, affecting over 3500 plant species, mainly woody plants in mild temperate

and subtropical environments (Migdał et al., 2012; Crone et al., 2013). This fungus is well known for its destructive impact on Mediterranean oak forest ecosystems (Linaldeddu et al., 2014). The holm oak (*Quercus ilex* L.) is the oak specie more susceptible to this fungus (Corcobado et al., 2014a). In addition to this species, *P. cinnamomi* affects several cultures, such as macadamia (*Macadamia* L.; Akinsanmi et al., 2016), eucalypts (*Eucalyptus* L.), pines (*Pinus* L.) and chestnut (*Castanea sativa*; Santos et al., 2015a).

This pathogen is extremely dependent on soil water content, causing massive death of feeder roots, reducing the capacity of hosts to absorb water and nutrients (Coelho et al., 1997). This involves water deficiency symptoms, such as reduction in stomatal conductance and transpiration rate (Sghaier-Hammami et al., 2013). The infection of the roots by *P. cinnamomi* comprises several steps. Initially, the pathogen secretes elicitins into the rhizosphere, facilitating its root penetration. Thus, the host defense genes suffer downregulation, facilitating the pathogen growth and destruction of roots with consequent impairment of water and nutrient uptake. Finally, the leaves suffer chlorosis and wilting, resulting from the changed water and hormonal status of the host (Serrazina et al., 2015). A combination of warming and weather extremes is likely to enhance *P. cinnamomi* dispersal and impact (Corcobado et al., 2014b). In adult trees the symptoms include stem canker, branch dieback, leaf chlorosis and defoliation, thinning of the crown, persistence of immature fruits on the trees after leaf-fall and root rot (Akinsanmi and Drenth, 2013; Corcobado et al., 2014a; Serrazina et al., 2015).

Ink disease is a destructive disease that affect *Castanea sativa* responsible for considerable annual losses of chestnut trees (Branzanti et al., 1999; Santos et al., 2015b). This disease causes root and collar rot of seedlings and adult trees in nurseries, plantations and forests (Vannini and Vettraino, 2001). Portugal is one of the most important producers of chestnut and this disease has been a major threat to chestnut orchards, negatively affecting fruit and timber production (Santos et al., 2015a).

I.3. FUNGICIDES

Fungicides are agents, of synthetic or natural origin, which act to protect plants against fungal diseases by specifically inhibiting or killing the fungus causing the disease (McGrath, 2004). They are also used as a post-harvest treatment to prevent fungi or molds that cause food rot during storage or transport. The fungicides maintaining healthy crops and consistent yields of high quality produce (Regueiro et al., 2015). Fungicides can be protectant or systemic. Protectant fungicides remain on the surface of plants and can damage the plant if absorbed. These fungicides are products suited for

preventive use. Systemic fungicides are absorbed into plants and can move short or long distances from the site of application, possessing preventive and curative activity, thus affecting the pathogen after infection. The mode of action of fungicides is to kill the fungi by damaging their cell membranes, inactivating critical enzymes or proteins, or by interfering with key processes such as energy production or respiration. Other fungicides impact specific metabolic pathways such as the production of sterols or chitin (McGrath, 2004).

Synthetic fungicides have been extensively used to protect crops. Application of these fungicides is cheap and the most effective approaches for the control of plant diseases. These chemical controls are efficient, but have undesirable characteristics such as development of pathogen resistance, environmental pollution, long degradation periods, accumulation in food chain, phytotoxicity to other organisms and public health problems (Joe et al., 2012; Ali et al., 2017; Ayoub et al., 2017). These problems together with the growing interest of consumers for healthier foods has increased the request for fruits containing lower amounts of chemical preservatives (De Oliveira et al., 2017), and the need to find alternative ways to control and protect crops against pathogens. Plants produce a wide range of secondary metabolites that are toxic to pathogens. These compounds occur in healthy plants as a part of their normal growth and development (phytoanticipins), or they are synthesized in response to pathogen attack as part of the defense response (phytoalexins; Ammar et al., 2013). Thus, some of these natural products such as alkaloids, quinones, flavonoids, phenolic acids, saponins, glycosides, tannins and terpenoids, can act as fungicides and might be useful for disease control in agriculture (Sales et al., 2016; Martínez et al., 2017). Botanical fungicides are particularly sought since they are abundant, effective, safe, environment friendly, reduce the dependence on synthetic fungicides, and are not toxic to mammals (Ali et al., 2017; Choi et al., 2017). For the management of phytopathogenic fungi there are some commercial botanical pesticides, such as curcuminoids, cinnamaldehyde and neem (Choi et al., 2017), isolates from turmeric (*Curcuma longa*; Yoon et al., 2013), cinnamon trees (*Cinnamomum*; Yen and Chang, 2008) and neem (*Azadirachta indica*; Singh et al., 1980), respectively.

As previously mentioned in section 1, saponins have been shown to exhibit a wide spectrum of antifungal activity. Saponins obtained from several plants, such as cayenne pepper (*Capsicum frutescens*) (De Lucca et al., 2002), desert date (*Balanites aegyptiaca*), *Quillaja saponaria*, *Yucca schidigera* (Chapagain et al., 2007), and quinoa (*Chenopodium quinoa*; Stuardo and San Martín, 2008) presented fungistatic properties against many phytopathogenic fungi. Emerson and Crandall (1997; Patent No.: 5,639,794), have developed methods and compositions employed saponins of yucca plants (*Y. schidigera* and *Y. valida*) as synergist in combination with natural products, such as aromatic aldehydes, controlling

the colonization and growth of various plant and animal pathogens. De Lucca et al. (2001) patented a fungicidal saponin mentioned as CAY-1 (Patent No.: US 6,310,091 B1). CAY-1 is a fungicidal steroidal saponin isolated from cayenne pepper or others *Capsicum* species (De Lucca et al., 2006). CAY-1, *in vitro*, is significantly lethal against a large variety of fungal organisms associated with diseases in plants, animals and humans, including several pathogenic *Aspergillus* species, *Candida albicans*, *Pneumocystis carinii* (De Lucca et al., 2002), *Colletotrichum* and *Botrytis* species, *Phomopsis obscurans*, *Plasmopara viticola* (De Lucca et al., 2008), among others.

Fungicidal activity of saponins of *Medicago* species such as *M. sativa* (Saniewska et al., 2001; Abbruscato et al., 2014), *M. arborea* (Tava et al., 2005), *M. arabica* (Martyniuk and Bialy, 2008) and *M. hybrida* (Bialy et al., 2006; Saniewska et al., 2006) have also been studied. Due to their significant antifungal activity, saponins of *Medicago* species can be a rich source of natural fungicides.

I.4. *SACCHAROMYCES CEREVISIAE* AS A MODEL ORGANISM

The budding yeast *Saccharomyces cerevisiae* belongs to the Kingdom of Fungi and is a facultative unicellular and anaerobic eukaryotic organism. It is one of the most widely used eukaryotic model organisms (Karathia et al., 2011), because its molecular mechanisms of fundamental cellular processes are very similar to the higher eukaryotes, such as transcription, replication and DNA repair (Grzelak et al., 2006). It has been a very useful model for studies of cellular response to various types of stress (Kwolek-Mirek and Zadrag-Tecza, 2014), and has been used as a model to study aging, regulation of gene expression, signal transduction, cell cycle, metabolism, apoptosis, neurodegenerative disorders, and many other biological processes (Karathia et al., 2011). *Saccharomyces cerevisiae* has the advantage of being easy to handle and cheap cultivation in simple defined media with short generation times and easy accessibility towards molecular and classic genetics (Miloshev et al., 2002; Van Der Klei and Veenhuis, 2006). Moreover, the entire genome sequence of *S. cerevisiae* is available, which enables extensively application of modern system biology approaches (Van Der Klei and Veenhuis, 2006).

Furthermore, *S. cerevisiae* has been used as a model of researches of the inhibitory effect and modes of action of various compounds against phytopathogenic fungi (Polacheck et al., 1991; Simons et al., 2006; Gleeson et al., 2010; Troppens et al., 2013).

I.5. AIMS

Due to the problems caused by chemical fungicides, the search for alternative solutions is increasing, in particularly in the search of natural biological compounds with high antifungal activity. Therefore, this work has a main goal to study the antifungal activity of crude *Medicago* extract (ME) in order to develop a natural and biodegradable product with possible commercial interest. The antifungal activity of saponins obtained from *Medicago* extracts and other plants are well-known, however, the antifungal properties of other bioactive compounds present in crude *Medicago* extracts are poor studied. At industrial level, ME can bring advantages to the use of pure compounds in terms of economy and yield of the production. Thus, to achieve the main goal, the following specific aims have been established:

- Evaluate the antifungal activity of ME in a range of phytopathogenic fungi that affect important crops in Portugal;
- Evaluate the phytotoxic effect of ME on seedlings germination;
- Observe the phytotoxic effect of ME on leaves of adult plants;
- Understand the mechanism of toxicity used by ME in *Saccharomyces cerevisiae* cells.

Chapter II

MATERIALS AND METHODS

II.1. PREPARATION OF *MEDICAGO* EXTRACT (ME)

In this work, two types of extracts (ethanolic and aqueous) were prepared. Aerial parts of *Medicago* plants, collected from an uncultivated land near Albergaria-a-Velha, Aveiro, Portugal, were air-dried in the dark. The plants were crushed with the aid of a blender (Waring Commercial) until it formed a fine powder. For extraction of bioactive compounds, maceration method was used, a process that consists in putting the vegetal material in a cellulose extraction thimble (25 mm (internal diameter) x 100 mm (external length), Whatman) placed in a container with 100 mL of 50% (v/v) ethanol at room temperature and protected from light for 7 days. The ethanolic extract was filtrated with a 0.2 µm syringe filter (VWR) and then stored in darkness at 4 °C. For preparation of aqueous extract, the ethanolic extract was evaporated in a rotary evaporator at 40 °C and 40 rpm (Büchi 461 Water Bath) and freeze-dried (Alpha 2-4 LDplus, Christ). Subsequently, the dried extract was dissolved in sterile deionized water (dH_2O) to 50 mg/mL final concentration. The extract was divided in aliquots and stored in the dark at -20 °C.

II.2. MICROORGANISMS, CULTURE MEDIA AND GROWTH CONDITIONS

The fungi used in this work are represented in Table II.1. The fungi were cultivated in Petri dishes with Potato-Dextrose-Agar medium (PDA, HiMedia Laboratories) in the dark at 25 °C. The yeast strains used were *Saccharomyces cerevisiae* BY4741 (MATa; *his3Δ1*; *leu2Δ0*; *met15Δ0*; *ura3Δ0*) from the strain collection of Euroscarf project, and their mutants *yap1* (BY4741; *YML007w::kanMX4*) provided by Meng-ER (Institut Curie, Paris, France), and BY4741 pUG35- *NHP6A-EGFP*, plasmid provided by Maria João Sousa (University of Minho, Braga, Portugal). The parental strain W303 (MATa; *ade2-1*; *ura3-1*; *leu2-3,112; trp1-1*; *his3-11,15*; *can1*-100), as well as their derivative mutant *yca1* (W303; *yca1::kanMX4*) were provided by Francesc Posas (Universitat Pompeu Fabra, Barcelona, Spain). The strains BY4741, *yap1*, W303 and *yca1* were cultivated on solid YPDA medium [1% yeast extract, 2% peptone; 2% dextrose (Difco); 2% agar (Oxoid)] at 30 °C for 2 days, and then stored at 4 °C. The strain BY4741 pUG35- *NHP6A-EGFP* was cultured on synthetic complete medium (2% glucose; 0.5% ammonium sulphate; 0.7% yeast nitrogen base w/o amino acids; 0.2% dropout mix; 0.01% histidine and tryptophan; 0.02% leucine).

Table II.1. Fungi used in this work and corresponding suppliers.

Fungus	Provided by
Botrytis cinerea	Richard Gonçalves (University of Minho)
Colletotrichum acutatum **isolate PT227**	Pedro Talhinhas (Instituto Superior de Agronomia, University of Lisbon)
Colletotrichum gloeosporioides **isolate 15-025**	Pedro Talhinhas (Instituto Superior de Agronomia, University of Lisbon)
Colletotrichum godetiae **isolate 15-019**	Pedro Talhinhas (Instituto Superior de Agronomia, University of Lisbon)
Colletotrichum nymphaea **isolate CA397**	Pedro Talhinhas (Instituto Superior de Agronomia, University of Lisbon)
Diplodia corticola **isolates CAA008, CAA499 and CAA500**	Ana Cristina Esteves (Centre for Environmental and Marine Studies, University of Aveiro)
Phythophthora cinnamomi **isolate PH107**	Helena Machado (National Institute for Agrarian and Veterinarian Research)

II.3. EVALUATION OF ANTIFUNGAL ACTIVITY ON PHYTOPATHOGENIC FUNGI

To test the effect of ME on the mycelial growth of phytopathogenic fungi, the aqueous extract was incorporated into PDA medium before solidification at 50 °C at different concentrations, 100, 500, 1000 or 2000 µg/mL. Then, preparations were poured into Petri dishes and left to solidify. The negative control consisted of only solvent (dH_2O) in PDA medium. A 5 mm diameter mycelial disc of each fungus was removed from the margins of 12 days old cultures and placed in the middle of the previously prepared Petri dishes. The plates were incubated at 25 °C, protected from light, and the colony diameter of the fungi was measured at 3, 6 and 9 days after incubation. The assay ended when the negative control reached full growth, and the inhibitory activity of each treatment was expressed as the percent growth inhibition compared to the negative control (0% of inhibition), using the following formula (Chapagain et al., 2007):

$$\text{Inhibition } (\%) = \frac{\text{dc} - \text{dt}}{\text{dc}} * 100$$

The terms, dc (negative control) and dt (treated plates) represent the means of 3 replicates of fungal colony diameter (cm).

II.4. Assessment of the phytotoxic effect on flax seedling growth

MS (Murashige and Skoog, 1962) basal medium (Duchefa) was prepared and supplemented with 2% (w/v) sucrose (Fischer Scientific) at pH 5.7 and 0.8% (w/v) agar (VWR, Prolabo). The agar was prepared on a heating plate at 200 °C. The aqueous ME was incorporated in the medium at concentrations of 100, 500, 1000 or 2000 µg/mL. For the negative control, the solvent (dH_2O) was added to the medium as substitute of the extract. The preparations were poured into *in vitro* culture jars and left to solidify.

For this assay, 150 flax seeds were counted (10 seeds per culture jar x 5 concentrations x 3 replicated jars). The seeds were disinfected in 80% (v/v) ethanol for 2 min, followed by bleach (1% active chlorine) for 10 min and then washed 3 to 4 times with dH_2O. After inoculation of the 10 seeds per culture jar, these were maintained in an acclimatized room at 25 °C, under a photoperiodic regime of 16 h and 3 lamps of 36 W with a mean light intensity of 50-95 µmol m^{-2} s^{-1} at 24 °C (± 1 °C). After 15 days of culture, the seedlings were removed from the culture jars, one by one, and the lengths of their primary organs (root, hypocotyl and epicotyl) were measured.

II.5. Observation of the phytotoxic effect on strawberry leaves

The evaluation of ME phytotoxic effect was performed using cultivated garden strawberry, with 4-6 leaves per pot. For this, 3 different concentrations of extract were used: 500, 1000 or 2000 µg/mL. In each pot (4 replicates per treatment), 2 leaves were selected and brushed with 100 µL of respective concentration. The negative control was brushed only with solvent (dH_2O). The leaves were observed every week for 5 weeks, and the extract was brushed again in the third week. The plants were set under uncontrolled environmental conditions.

II.6. EFFECT OF ETHANOLIC ME IN CELLS OF *SACCHAROMYCES CEREVISIAE*

II.6.1. Viability tests

For cells viability assays, a pre-inoculum was prepared from a colony of the desired strain in liquid YPD medium (YPDA without agar), and the culture was incubated at 30 °C and 150 rpm, overnight. The cell proliferation was monitored by reading the optical density at 600 nm (OD_{640}). The OD_{640} was measured and the culture was diluted to 0.1 using fresh YPD medium. Thereafter, the culture was incubated for 4 hours under the same conditions until OD_{640} = 0.4. The culture was divided into 4 aliquots, including the negative control that was prepared only with the cell suspension and the solvent (50% v/v ethanol) and with the different concentrations of extract: 125, 250 or 500 µg/mL. The suspensions were incubated at 30 °C and 150 rpm. At each treatment time (0, 30, 60 and 90 min), the different aliquots were serially diluted in dH_2O to 10^{-4} and then 100 µL of the last dilution of each treatment were spread on YPDA dishes. These were incubated at 30° C for 2 days. After incubation, on each plate, the number of colony forming units (CFUs) was counted. Thus, the percentage of cell viability of the CFUs was calculated, assuming as 100% viability the number of colonies counted in time 0 of each treatment.

II.6.2. Assessment of chromatin condensation and nuclear fragmentation

For this assay, the inoculum of *S. cerevisiae* BY4741 at OD_{640}= 0.4 was divided into 4 aliquots. Each aliquot was centrifuged, washed once with dH_2O, and resuspended in phosphate buffered saline (PBS; 137 mM NaCl; 2.7 mM KCl; 4.3 mM Na_2HPO_4; 1.47 mM KH_2PO_4; pH 7.4). At time 0, the ME was added to 3 aliquots at the concentration of 500 µg/mL,and an equal volume of 50% (v/v) ethanol as added to the fourth aliquot, corresponding to the negative control. The cell cultures were fixed in 70% (v/v) ethanol for 15 min. At different incubation times (0, 30, 60 and 90 min), fluorescent dye Hoechst 33342 (Bio-Rad) was added to the yeast suspension (final concentration of 2 µg/mL). The cells were incubated for 5 min at room temperature, rinsed twice with PBS and resuspended in PBS. Cells were observed using a Leica DM-5000B fluorescence microscope (Leica Microsystems) with appropriate filter for this dye (blue channel) using a 100x oil-immersion objective. Images were acquired with a Leica DCF350FX digital camera and processed with LAS AF Leica Microsystems software.

II.6.3. Assessment of plasma membrane integrity

Integrity of the cell plasma membrane was analyzed by flow cytometry using the dye propidium iodide (PI; Molecular Probes, Eugene, OR). The inoculum of *S. cerevisiae* BY4741 at OD_{640}= 0.4 was diluted to 0.02. The culture was divided into 4 aliquots, centrifuged, washed once in PBS and resuspended in PBS. The ME was added to 3 aliquots at the concentration of 500 µg/mL, and the equal volume of 50% (v/v) ethanol as added to the fourth aliquot, corresponding to the negative control. At different incubation times (0, 30, 60 and 90 min), PI was added to the yeast suspension (final concentration of 2 µg/mL). The cells were incubated for 10 min at room temperature and darkness. The PI-stained cells were measured by flow cytometry in an Epics XLTM (Beckman Coulter) flow cytometer equipped with an argon-ion laser with emission of a 488 nm beam at 15 mW. Red fluorescence, corresponding to FL4 channel, was collected through a 560 nm short-pass dichroic, a 640 nm long-pass and another 670 nm long-pass filter. For each sample, 20.000 events were evaluated, and data were analysed using the FlowJo 7.6 software.

II.6.4. Assessment of the translocation of Nhp6Ap from the nucleus to cytosol

The extract at the concentration of 500 µg/mL was added to a culture of *S. cerevisiae* BY4741 pUG35- *NHP6A-EGFP* at OD_{640}= 0.4. The culture was incubated at 30 °C and 180 rpm. At 0, 30 and 90 min, 20 µL of culture were removed and observed in Leica DM-5000B fluorescence microscope (Leica Microsystems) with appropriate filter for this dye (green channel) using a 100x oil-immersion objective. Images were acquired with a Leica DCF350FX digital camera and processed with LAS AF Leica Microsystems software.

II.7. STATISTICAL ANALYSIS

Experimental data are expressed as mean ± standard deviation (S.D.) calculated from at least three independent experiments. Statistical analysis was performed using one-way ANOVA variance analysis followed by the Turkey's test for multiple comparisons, using the GraphPad Prism 6.

Chapter III

Results and Discussion

III.1. ANTIFUNGAL ACTIVITY OF *MEDICAGO* EXTRACT ON PHYTOPATHOGENIC FUNGI

The efficacy of ME against phytopathogenic fungi mentioned in Chapter II, *in vitro* conditions, was evaluated. The antifungal activity was estimated by measuring the mycelial growth on solid medium using different concentrations of extract (100, 500, 1000 or 2000 µg/mL). The fungi colony diameter was measured after 3, 6 and 9 days of incubation. The percentages of growth inhibition were calculated through comparison of the colony diameter of fungi treated with ME with the negative control (without ME, 0% of inhibition).

III.1.1. Effect of ME on mycelial growth of *Botrytis cinerea*

The antifungal properties of ME were assessed in *B. cinerea*. We observed that all concentrations of ME caused a significant decrease in mycelial diameter of *B. cinerea* in comparison with the negative control (Table 1 Supplementary Material). However, it was not possible to ascertain a direct proportionality between the applied concentration and the mycelial growth. As can be seen in Fig. III.1A, which exhibit the percentage of inhibition at different concentrations at different days (3 and 6), we observed that ME did not induced a dose-dependent effect. The extract was able to reduce growth of *B. cinerea* in nearly 40% after 6 days of incubation to 2000 µg/mL of ME. The lowest inhibition occurred in the 500 µg/mL of ME at day 3 (14.5%), which significantly increased up to 23% on the sixth day. It was also observed that *B. cinerea* morphology underwent changes along the applied concentrations (Fig. III.1B). These alterations were observed for 500, 1000 or 2000 µg/mL of ME, and consisted in a decrease of the mycelium dark color, which possibly could be also indicated of a less dense mycelium or sporulations.

Several studies have been reported the antifungal activity of plant saponins against *B. cinerea* (Verhoeff and Liem, 1975; Saniewska et al., 2001; Cotoras et al., 2004; Saniewska et al., 2006; Ribera et al., 2008; Stuardo and San Martín, 2008; Fischer et al., 2011; Lanzotti et al., 2012). Our results were in concordance with Fischer et al. (2011). Using the same range of concentrations, the authors showed similar growth inhibitions, using saponins obtained from *Quillaja saponaria,* to the obtained in this work. On the other hand, Saniewska et al. (2001), using the same concentrations of saponins *of Medicago sativa*, obtained a considerably lower percentage of growth inhibition than our ME.

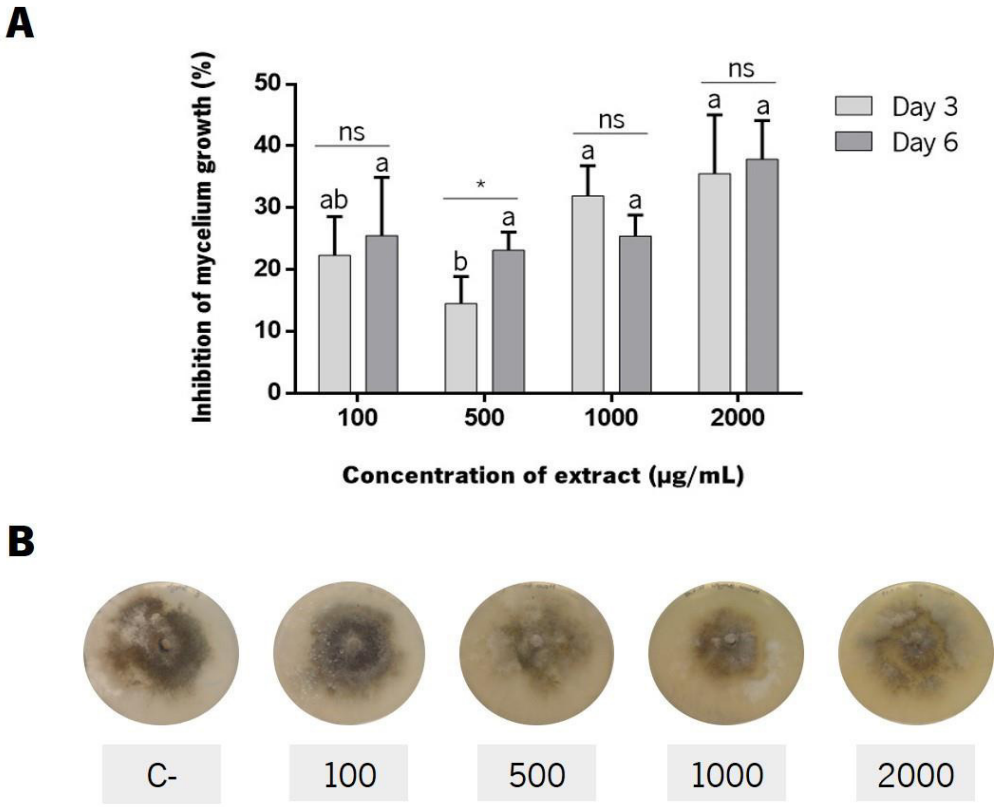


Figure III.1. Effect of ME on mycelial growth of *Botrytis cinerea* on PDA solid medium with incorporation of ME. **(A)** Percentage of growth inhibition determined after 3 and 6 days of incubation at different concentrations of ME, 100, 500, 1000 or 2000 µg/mL. Each bar represents the mean of three independent experiments ± S.D. *P<0.05 indicates significant differences between day 3 and 6 for the same concentration; ns – not significant. The comparison between the different concentrations for the same day are represented by letters. Means followed by the same letter are not significantly different (P<0.05). **(B)** Representative images of ME antifungal activity at different concentrations against *B. cinerea* compared to the negative control (C-) after 6 days of incubation.

III.1.2. Effect of ME on mycelial growth of *Colletotrichum* spp.

Due to the wide variety of *Colletotrichum* species causing serious diseases in economically important crops, antifungal activity of ME was evaluated in 4 different species of this genus, the *C. acutatum*, *C. gloeosporioides*, *C. godetiae* and *C. nymphaea*. The mycelium growth of these species, except *C. gloeosporioides*, was measured up to 9 days of ME incubation. We observed that the antifungal activity was present in all tested fungi. Regarding the growth of *C. acutatum*, ME only showed significant differences for the highest concentration (2000 µg/mL; Table 2 Supplementary Material), exhibiting an inhibition rate of 21.3% (Fig. III.2A). However, a higher inhibition effect (approximately 30%) was seen to 500 µg/mL of ME at day 3, which decreased considerably over the days (Fig. III.2A). In *C.*

gloeosporioides, ME caused significant differences in the growth of all concentrations, inducing a dose-dependent effect (Table 2 Supplementary Material; Fig. III.2B). Thus, ME induced a higher inhibition rate (40.3%) at 2000 µg/mL, and a lower inhibition (13.3%) at 100 µg/mL (Fig. III.2A). We did not detect any significant difference, comparing to negative control, in the mycelial growth of *C. godetiae* (Table 2 Supplementary Material). As can be seen in Fig. III.2A, it is clear that ME did not induce a dose-dependent effect on this fungus. The higher inhibition rate was observed for 2000 µg/mL (26.5%), however, the 500 and 1000 µg/mL of ME showed similar effect on micellium growth. Lastly, we tested ME against *C. nymphaea*. For this fungus, the significant differences were only observed at 1000 and 2000 µg/mL of ME (Table 2 Supplementary Material). As can be seen in Table 2 (Supplementary Material) and Fig. III.2A, a dose-dependent effect of ME was observed to these fungi. Accordantly, a minimum growth inhibition of 12.8% and a maximum inhibition of 34.4% were observed for 100 and 2000 µg/mL, respectively.

Comparing all the ME antifungal activities in *Colletotrichum* species, we found that *C. gloeosporioides* is the most sensitive fungus which was inhibited up to 40%, the other pathogens exhibited growth inhibition rates between 20% and 35%. To *C. gloeosporoides*, *C. godetiae* and *C. nymphaea*, it was observed several differences in mycelial morphology, such as pigmentation alterations, which possibly indicate that fungus physiology was changed.

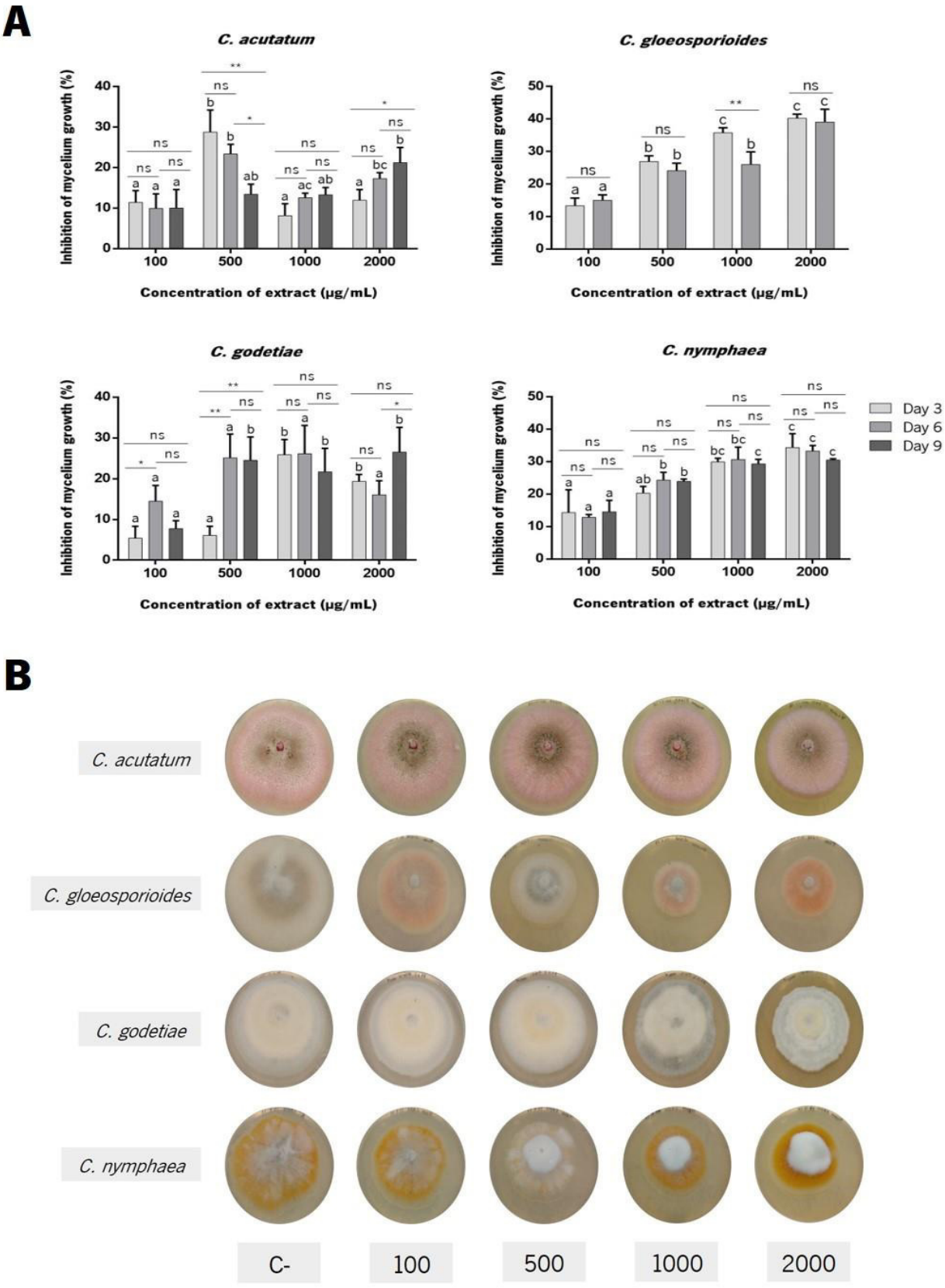


Figure III.2. Effect of ME on mycelial growth of *Colletotrichum* species on PDA solid medium with incorporation of ME. **(A)** Percentage of growth inhibition determined after 3, 6 and sometimes 9 days of incubation at different concentrations of ME,

100, 500, 1000 or 2000 µg/mL. Each bar represents the mean of three independent experiments ± S.D. *P<0.05; **P<0.01 indicates significant differences between the different days for the same concentration; ns – not significant. The comparison between the different concentrations for the same day are represented by letters. Means followed by the same letter are not significantly different (P<0.05). **(B)** Representative images of ME antifungal activity at different concentrations against *Colletotrichum* species compared to the negative control (C-) after 6 (*C. gloeosporioides*) and 9 (*C. acutatum, C. godetiae* and *C. nymphaea*) days of incubation.

III.1.3. Effect of ME on mycelial growth of *Diplodia corticola*

In order to investigate the antifungal activity of ME in different isolates of the same species, 3 isolates of *D. corticola*, CAA008, CAA499 and CAA500 were used. We observed that ME caused a significant decrease in mycelial growth of all isolates at all concentrations (Table 3 Supplementary Material). As can be seen in Fig. III.3B, the growth inhibition caused by ME was quite evident. Through the analysis of Fig. III.3A, it appears that ME caused a dose-dependent effect in CAA008 and CAA500 isolates growth, however few significant differences were observed. On the other hand, ME did not cause a dose-dependent effect in CAA499 growth, however for all concentrations, a significant decreased in the mycelium growth inhibition rate between day 3 and day 6, was observed. CAA008 and CAA500 isolates were shown to be more sensitive to extract than CAA999 isolate, ME was able to inhibit its growth by 69.3%, 68.4% and 36.9, respectively.

We can also conclude that different isolates of the same fungus species may display different susceptibilities to the extract. Our results were in accordance with Nonaka (1986), which studied the effect of *Medicago sativa* saponins on different strains of *Trichoderma viride* fungus. They also found that different strains exhibited variable sensitivity to the same compound. Fischer et al. (2011) observed the same effect of *Quillaja saponaria* saponins in different strains of *B. cinerea*. According to our knowledge, the antifungal activity of saponins in *D. corticola* has never been studied.

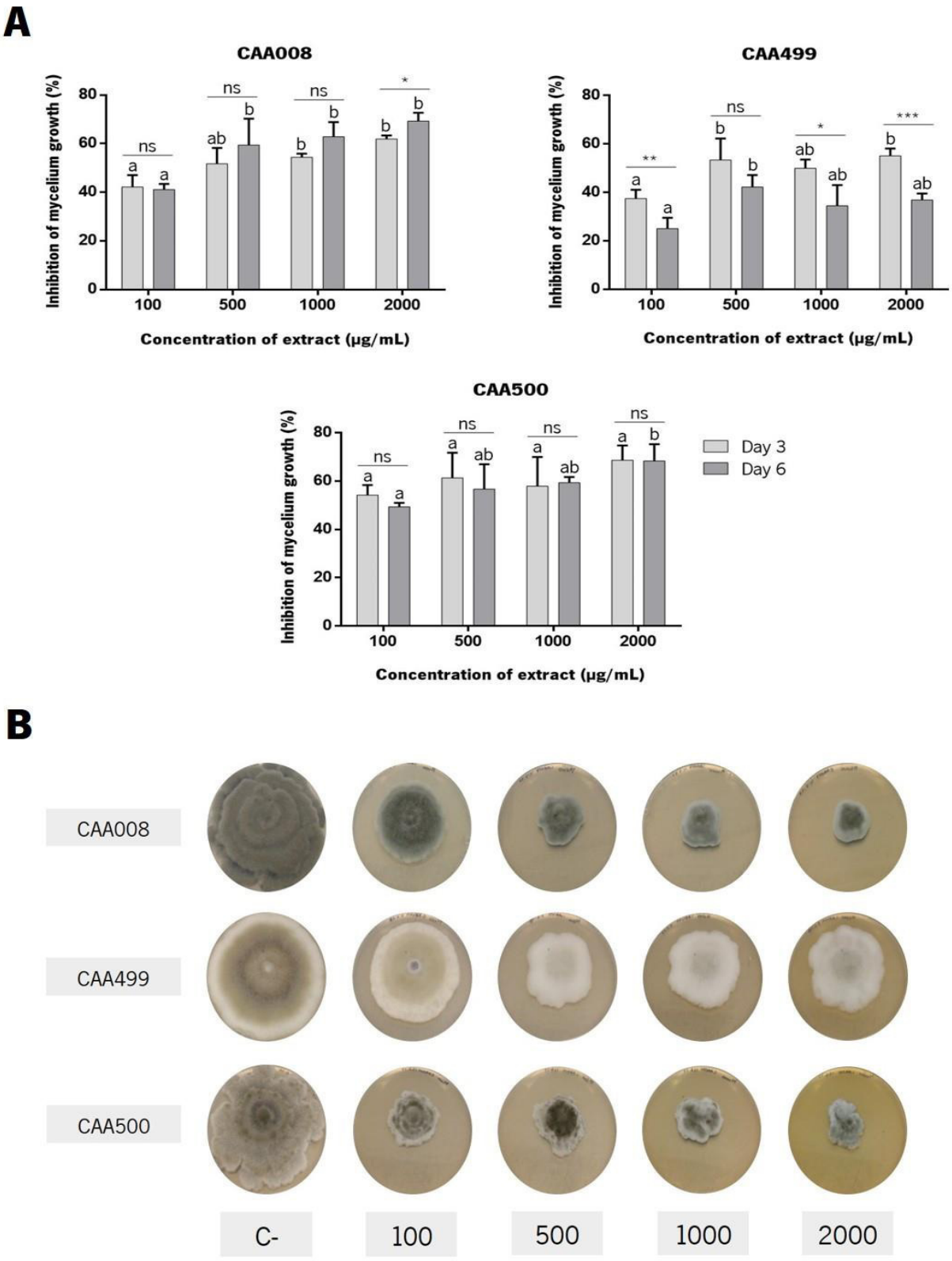


Figure III.3. Effect of ME on mycelial growth of *Diplodia corticola* isolates on PDA solid medium with incorporation of ME. **(A)** Percentage of growth inhibition determined after 3 and 6 days of incubation at different concentrations of ME, 100, 500, 1000 or 2000 µg/mL. Each bar represents the mean of three independent experiments ± S.D. *P<0.05; **P<0.01; ***P<0.001 indicates significant differences between day 3 and 6 for the same concentration; ns – not significant. The

comparison between the different concentrations for the same day are represented by letters. Means followed by the same letter are not significantly different (P<0.05). **(B)** Representative images of ME antifungal activity at different concentrations against *D. corticola* isolates compared to the negative control (C-) after 6 days of incubation.

III.1.4. Effect of ME on mycelial growth of *Phythophthora cinnamomi*

The antifungal activity of ME against *P. cinnamomi* was tested. ME showed no inhibitory effect in the fungus growth (data not shown). Several attempts were performed, and the result was always the same. Even thought, a decrease in mycelium density of *P. cinnamomi* when treated with ME was observed (Fig. III.4). Indeed, the toxicity effect of ME is quite evident, although the mycelium diameter is not affected. These results suggest that the extract induces an effect on the fungus mycelium development. In the future, in order to confirm this hypothesis, antifungal activity can be evaluated through the analysis of mycelium wet weight cultured in liquid medium (Cotoras et al., 2004).



Figure III.4. Representative images of ME antifungal activity at different concentrations, 100, 500, 1000 or 2000 µg/mL, against *Phythophthora cinnamomi*, on PDA solid medium with incorporation of ME, compared to the negative control (C-), after 6 days of incubation (n=7).

Concluding, ME showed an evident fungitoxic effect against all of the investigated fungi. However, variable effects on the growth of those fungi were notice. In most of the tested fungi, the inhibition rates of their mycelial growth were similar among the different concentrations. Curiously, Saniewska et al. (2006) suggested that this effect can be explained due to the fact that even in lower concentrations of saponins, all active targets in mycelium hyphae are already saturated. In the future, higher concentrations of ME should be tested in order to corroborate its effect on mycelial growth. Consistently, Stuardo and San Martín (2008) and Fischer et al. (2011) used high concentrations of *Chenopodium quinoa* and *Quillaja saponaria* saponins up to 7 mg/mL and 30 mg/mL, respectively.

Significant decrease in growth inhibition rate along with time of incubation for the same concentration of ME, was observed in *C. acutatum* at 500 µg/mL, *C. gloeosporioides* at 1000 µg/mL

(Fig. III.2A), and CAA499 isolate (from *D. corticola*) at 100, 1000 and 2000 µg/mL of ME (Fig. III.3A). This suggest that these fungi have the ability to acquire resistance to ME, through a detoxification mechanism. Indeed, several authors have reported that some fungi are able to produce enzymes which specifically detoxify the saponins (Crombie et al., 1986; Osbourn et al., 1994; Bowyer et al., 1995; Wubben et al., 1996; Morrissey and Osbourn, 1999).

The principle of the fungitoxic activity of saponins is based in its interaction with ergosterol, the fungal membrane sterol. Saponins cause changes in membrane permeability and cell death (Ahmed et al., 2012). Some fungi are resistant to the toxic effects of saponins because they have little or no ergosterol in their membranes (Osbourn et al., 1995; Lairini et al., 1996; Ito et al., 2004; Chapagain et al., 2007). ME has shown to induce different inhibitions rates against different fungi. Consequently, this effect might be related to differences in the composition of membrane sterols in the several fungus cells. To corroborate this theory, further assays could be performed in order to evaluate the ergosterol amount in membranes in the tested fungi.

In this work, only the active compounds of the aerial parts of *Medicago* plants were verified. Saniewska et al. (2003, 2005) reported a higher antifungal activity for the saponins from the roots than from the aerial parts of *M. sativa* and *M. arabica*. Consequently, it would be important to test also the antifungal activity of ME obtained from roots and compare them with the results obtained from aerial ones.

III.2. PHYTOTOXICITY EFFECT OF ME ON FLAX SEEDLINGS

To study the phytotoxic effect of ME in germination of plants, flax (*Linum usitatissimum* L.) was used as a model species of dicotyledons plants. This plants can be easily micro-propagated and its seedlings exhibit a simple primary body allowing rigorous measurements of the different organs (Mlynárová et al., 1994), which globally make them good models for phytotoxicity tests.

The lengths of the primary organs of flax seedlings (root, hypocotyl and epicotyl) were measured after 15 days of culture, as shown in Fig. III.5A. The germination of the seedlings in different concentrations of ME (100, 500, 1000 or 2000 µg/mL) was shown in Fig. III.5B. It was possible to notice a quite disparate growth between the seedlings in the same concentration, however, it was also possible to observe that with the increase of ME concentration, the growth of the seedlings decreased (Fig. III.5B). The counts of the number of germinating flax seeds showed that the extract did not significantly influence germination. In all treatments, including the negative control (C-), 93%-97% of seeds germinated. In Fig.

III.5C was represented the mean values of root, hypocotyl and epicotyl lenght of flax seedlings in the different ME concentrations. A dose-dependent inhibitory effect of ME was observed in roots. In this primary organ (Fig. III.5C), a significant decrease in its development for all concentrations was observed. In the highest concentration, 2000 µg/mL, the ME inhibited about 90% of root growth. A minor impact was observed in hypocotyls growing (Fig. III.5C). ME did not induced a significant effect in the hypocotyls lenght, inhibiting only 30% for 500, 1000 and 2000 µg/mL of ME. In the epicotyls length, it was observed a slight stimulus of its growth for the low concentration (100 µg/mL), however, not statistically significant. The extract also caused a drastic inhibition (greater than 90%) of the epicotyls length for the highest concentrations (500, 1000 and 2000 µg/mL). Overall, it was possible to conclude that ME exhibit a phytotoxic effect on the growth of flax seedlings, especially on root and epicotyl development.

Allelopathy occurs when a harmful effect happens from one plant to another (Ashraf et al., 2017). This effect was observed also in our results. It is known that secondary metabolism compounds have natural allelopathic properties that delay the germination, growth or development of many plant species (Sadia et al., 2015). The allelopathic activity of *Medicago* species, mainly *M. sativa*, has been reported by several authors (Oleszek and Jurzysta, 1987; Abdul-Rahman and Habib, 1989; Wyman-Simpson et al., 1991; Oleszek, 1993; Waller et al., 1995; Chung et al., 2000; Li et al., 2005). The saponins are the main secondary metabolites responsible for this activity (Li et al., 2005). Notwithstanding, Oleszek and Jurzysta (1987) concluded that *M. sativa* contains, besides saponins, other water-soluble compounds that may have an inhibitory effect on seedling growth. Since ME used in our experiments was not purified, some compounds present in the extract may be also responsible for the high percentages of inhibition obtained in the flax seedlings. Waller et al. (1995) showed that *M. sativa* saponins affect the germination and seedling growth of wheat (*Triticum aestivum* L.), dandelion (*Taraxacum vulgare* L.), coffeeweed (*Sesbania exaltata* L.) and pigweed (*Amaranthus retroflexus* L.). Curiously, they also observed some growth stimulation in the range of 10-100 µg/mL, and inhibition at higher doses. Our results were similar to the obtained by Waller et al. (1995), since we also observed a slight stimulation in epicotyl growth for a similar concentration. At higher concentrations, ME induced flax seedlings development inhibition. However, the extract did not have an impact in seed germination. The allelopathic compounds may also serve as natural herbicides for weed control in a sustainable manner (Chung et al., 2000). ME revealed to possess high allelopathic properties against flax seedlings, suggesting that can be used as an excellent source of novel herbicides.

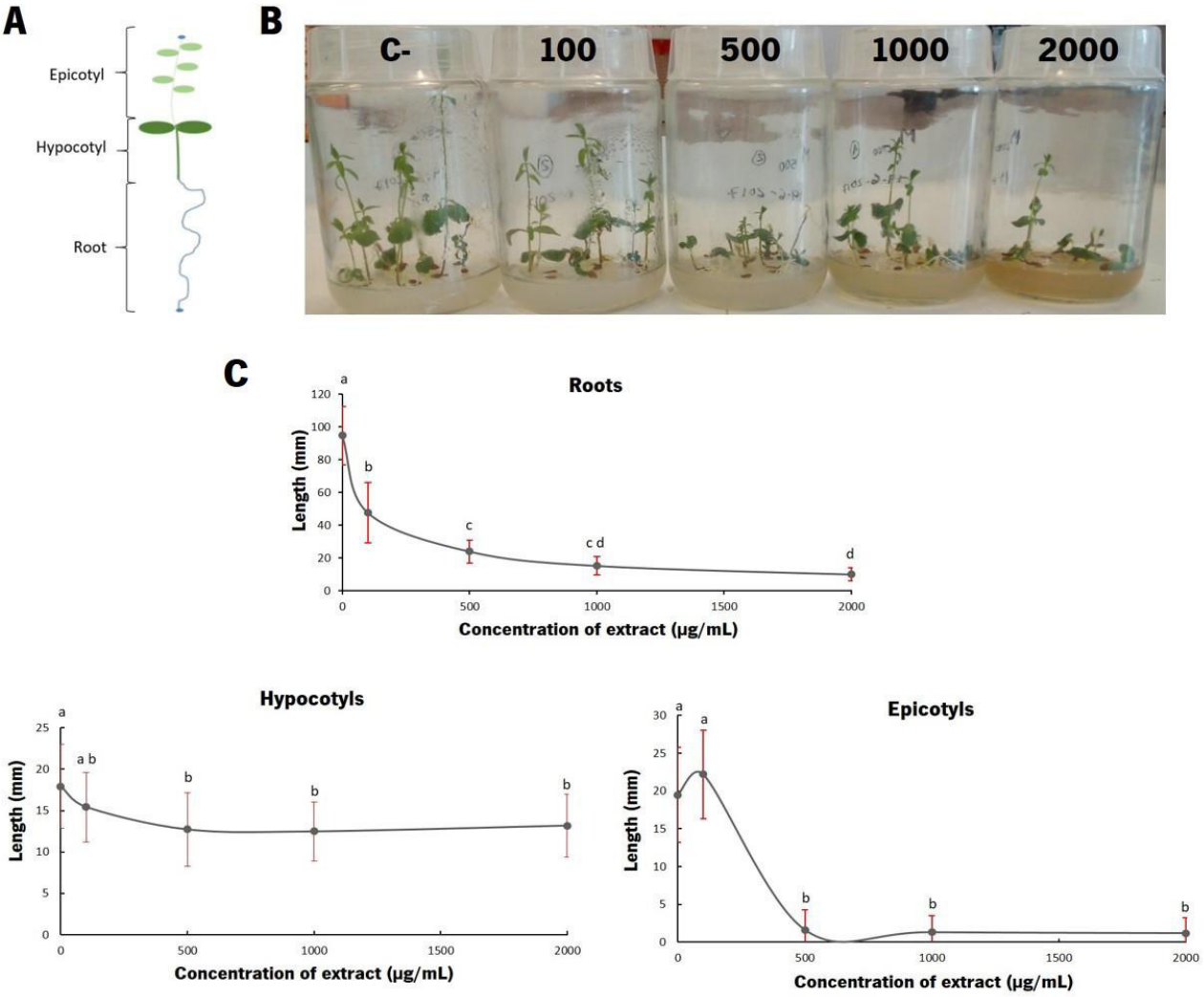


Figure III.5. (A) Illustrative image of the primary organs of a flax seedling (root, hypocotyl and epicotyl). **(B)** Flax seedlings with 15 days in MS medium culture with different ME concentrations, 100, 500, 1000 or 2000 µg/mL. **(C)** Mean values of roots, hypocotyls and epicotyls length (mm) as a function of the ME concentration. Means followed by the same letters are not significantly different (P<0.05).

As previously reported, ME did not significantly affect the hypocotyl development, contrary to described in other primary organs. Hypocotyl is an embryonic organ whose development occurs mostly by cell elongation, while the roots and epicotyls are post-germinative organs that develop by cell elongation and division. Thus, it is reasonable to suppose that ME may interfere in the cell cycle of actively proliferating organs. Waller et al. (1999) hypothesized that a possible mechanism of saponins phytotoxicity may be related to the water absorption at the lipid bilayer interface. It has proposed that at low concentrations saponins promote water transport into the plant root. When saponins are in high concentration, the inhibitory action is manifested due to the formation of high molecular weight matrixes around the rootlet preventing water or nutrients uptake by the plant.

III.3. PHYTOTOXICITY EFFECT OF ME ON STRAWBERRY LEAVES

As previously seen, ME exerts an allelopathic effect in development of flax seedlings. However, the study of ME effect in adult plants, is also very important to address. Thereunto, leaves from cultivated garden strawberry plant were tested with ME. The extract at different concentrations (500, 1000 or 2000 μg/mL) was brushed on 2 leaves per pot and the plants were monitored for 5 weeks. The plants were set under uncontrolled environmental conditions. The results for the first and last day of the assay were represented in Fig. III.6. The brushed leaves are identified with arrows. Comparing the first with the last day, it was possible to observed that ME did not produced significant visual effects on the leaves treated with 500 and 1000 μg/mL of ME. However, in the concentration of 2000 μg/mL of ME, a slight chlorosis (yellowing) was noticed in one of the brushed leaves (represented by red arrow). Nevertheless, the same effect was detected in other non-brushed leaves, as can be seen in representative image of the concentration of 1000 μg/mL (Fig. III.6). Initially, leaves showed chlorosis on the edges that progressed to the center of leaf. Then, chlorosis evolved to necrosis (drying of the edges making them brittle and curved upwards), leading to death of foliar tissue (see in more detail the symptoms in Fig. VI.1 Supplementary Material). These results suggest that the symptoms exhibited in brushed leaf may not be caused by the ME, but due to the exposure of plant to external conditions. Moisture stress, normal aging, cold drafts, lack of light, nutrient deficiency and viral infection are possible causes that may have triggered this type of symptoms. Since our experiments were conducted under uncontrolled environmental conditions, and at autumn-winter period, it is possible that the observed symptoms were induced by cold. In order to infer about this hypothesis, the same experiments should be repeated in the spring-summer period.

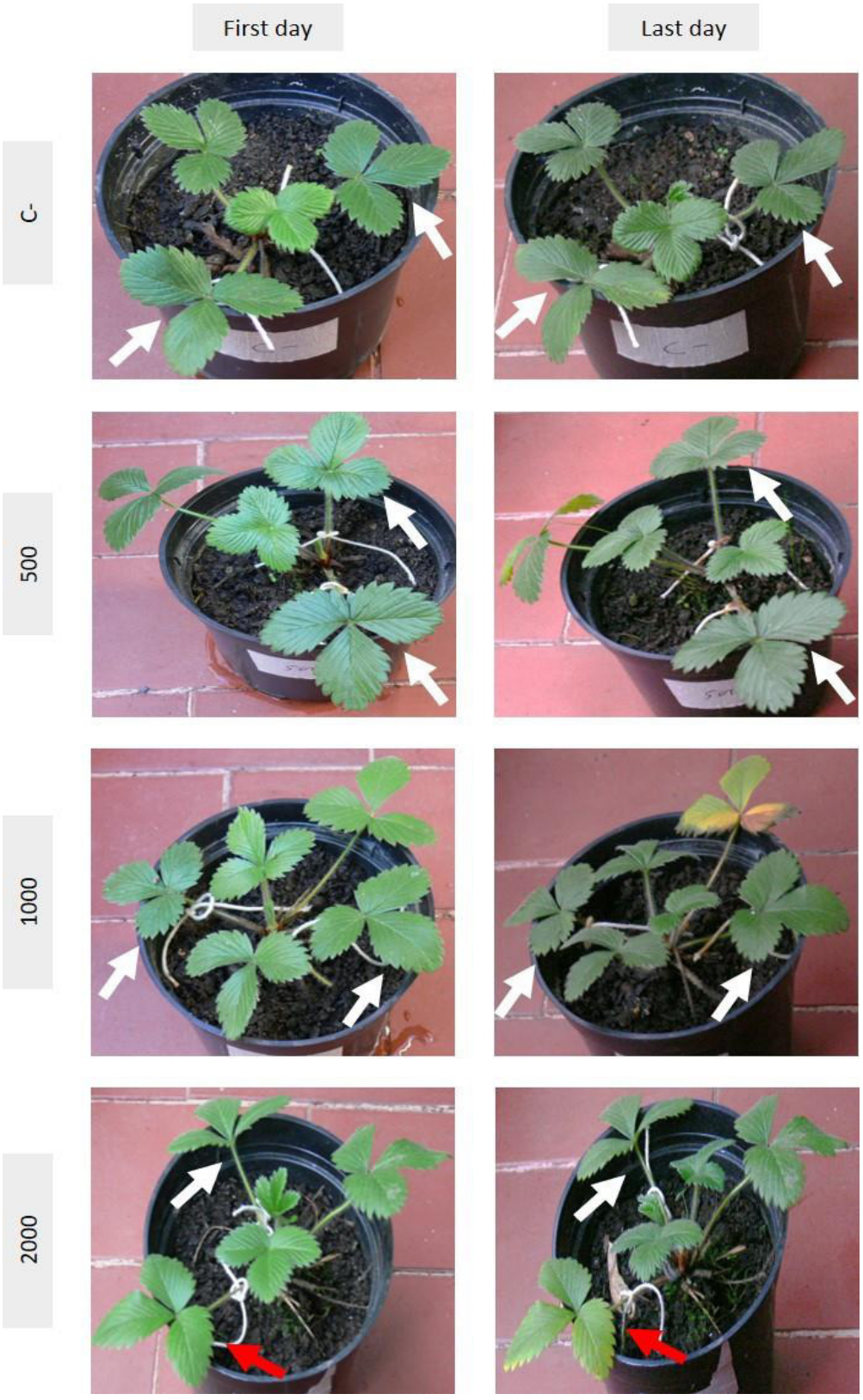


Figure III.6. Representative images of strawberry leaves treated with ME at 500, 1000 or 2000 µg/mL for 5 weeks. Brushed leaves are represented by white arrows and brushed leaves with symptoms are represented by red arrows.

Despite of saponins being known to cause allelopathy in germination and seedling organs development, there are few studies addressing the effect of these compounds on leaves of adult plants. Hoagland et al. (1996) tested the effect of saponins by foliar spray application on 10 weed and crop plants. They did not observe significant effects of phytotoxicity in any of the plants evaluated neither in the shoot fresh weight of plants treated with saponins, suggesting that saponins were either not passing through cuticular membrane barriers, or the concentration of compound used (1.0 mM) was not sufficient to cause phytotoxic effect on foliar tissues. On the other hand, Chapagain and Wiesman (2006) demonstrated, using desert date (*Balanites aegyptiaca*) saponins, that these compounds exhibit a significant ability to penetrate the plant cuticular membrane. Saponins due to their amphipathic structure (lipophilic and hydrophilic portions) function as surface-active agents. They observed that by increasing the concentration of the saponin extracts to 5% (w/v), the penetration rate is inhibited, which might be explained by crystallization of saponin molecules in the oversaturated solution.

In another study, Hoagland (2009) examined the phytotoxic effects of saponins α-tomatine and tomatidine produced by tomato (*Lycopersicon esculentum* L.). Through the extraction and quantification of chlorophyll and by electrolyte leakage bioassays in tissues exposed to these compounds, they observed phytotoxicity effects on several weeds and crop plants, such as hemp sesbania (*Sesbania exaltata*), sicklepod (*Senna obtusifolia* L.), mungbean (*Vigna radiata*), wheat (*Triticum aestivum*), sorghum (*Sorghum bicolor*) and corn (*Zea mays*). These saponins caused several visual phytotoxicity effects, such as chlorosis, browning and necrosis on leaf disks. Therefore, our data were inconclusive, since we do not have enough data to allow us to identify the cause for the observed effects on the strawberry leaves. Further research would be necessary to confirm these hypotheses.

III.4. MECHANISM OF ME-INDUCED TOXICITY IN *SACCHAROMYCES CEREVISIAE* CELLS

Budding yeast (*Saccharomyces cerevisiae*) is an established model organism for research of the modes of action of antifungal compounds (Simons et al., 2006). To better understand the toxicity mechanism caused by ME on eukaryotic cells, several assays involving the two cell death processes (apoptosis and necrosis) were performed. First, we performed viability tests on *S. cerevisiae*. Different strains were used for this assay, including the parental type BY4741 and W303, and their mutant's *yap1* and *yca1*, respectively. The concentrations of extract used were 125, 250 or 500 µg/mL and the

incubation times up to 90 min. The CFUs was counted, and the percentage of cell viability calculated assuming as 100% viability the number of colonies counted in time 0 of each treatment.

The strains BY4741 and *yap1* were used to test whether ME function as an oxidative agent by generating reactive oxygen species (ROS) in *S. cerevisiae* cells. Yap1 is an important transcription factor that is implicated in a cellular pathway that controls the oxidative stress response, up-regulating the expression of several antioxidant genes (Maeta et al., 2004). Deletion of the *YAP1* gene inactivate the ability of the gene product to serve as a positive regulator of transcription, causing significant increased sensitivity to oxidative stress generated by agents such as H_2O_2 (Wu and Moye-Rowley, 1994). As can be seen in the data obtained for parental strain BY4741 (Fig. III.7A) and the *yap1* mutant (Fig. III.7B), the cellular viability decreased with the increase of ME concentration. As expected, in negative control, the CFU increases over time. In BY4741 strain, for 125 µg/mL of ME and 30 min of incubation, the cell viability was reduced to 50% and to 0% for the highest concentrations, 250 and 500 µg/mL of ME. In *yap1* mutant, the lowest concentration, 125 µg/mL of ME, had a slight effect in viability and at 90 min, cell viability decreased about 50%. For the highest concentrations, the total inhibition occurs only at time 90 min.

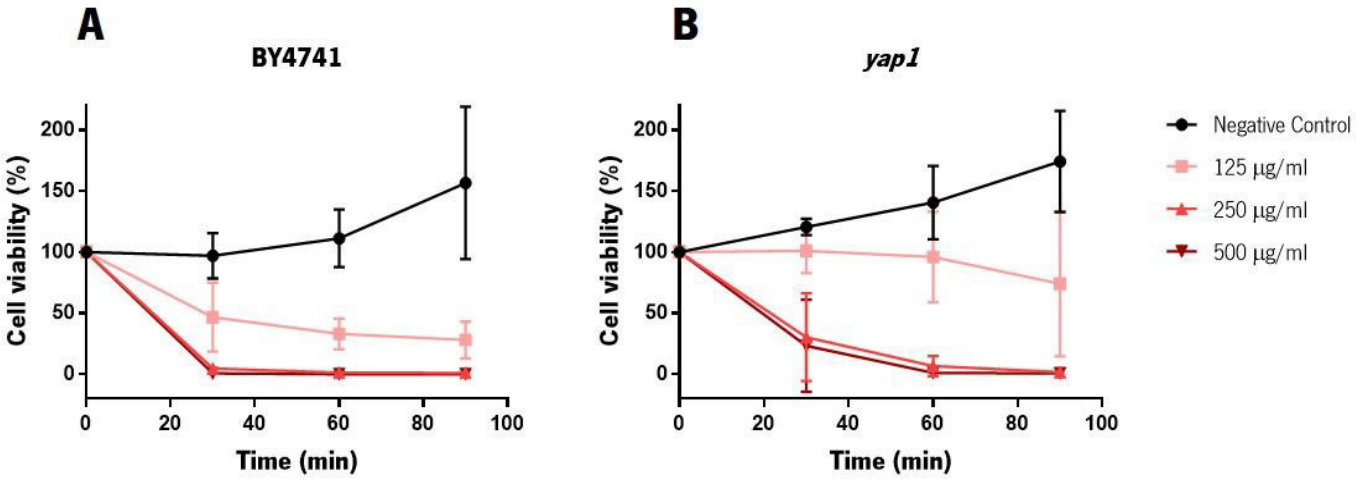


Figure III.7. Percentage of cell viability of *Saccharomyces cerevisiae* parental strain BY4741 **(A)** and their mutant strain *yap1* **(B)** over 90 min of incubation at different concentrations of ME, 125, 250 or 500 µg/mL. Values represent the mean ± S.D. of three independent experiments.

The comparative analysis of the obtained data for these two strains showed that the *yap1* mutant strain is more resistant to ME than its BY4741 parental strain. Disruption of the *YAP1* gene, blocks its participation in defense mechanisms that protect the cells against oxidants, causing hypersensitivity to oxidative stress. We were expecting that the cells of this mutant would be more sensitive to the extract

that the cells of their parental. However, for the three different concentrations used, the cell viability of *yap1* had a less pronounced decrease in viability than BY4741 strain, which suggest that ME does not cause oxidative stress in cells of *S. cerevisiae*.

To test whether ME induces an apoptosis process in cells of *S. cerevisiae*, we used the W303 and *yca1* strains. The *yca1* mutant strain does not possess the *YCA1* gene, that codes the protein metacaspase Yca1 reported as being involved in *S. cerevisiae* programmed cell death (PCD) (Guaragnella et al., 2006). The metacaspase Yca1 is a positive regulator of apoptosis upon stress induction (Wong et al., 2012). The disruption of the *YCA1* gene was shown to increase the cellular resistance to stress by blocking the PCD (Severin et al., 2008). The effect of ME in cell viability of the W303 parental strain was shown in Fig. III.8A. The results obtained, demonstrate that 125 µg/mL of ME is unable to inhibit the cell proliferation. For the higher concentrations, a clear decrease of cell viability was observed in viability up to 30 min of incubation. Thereafter, cells viability continues to decrease, reaching total inhibition at 60 min of 500 µg/mL of ME. For 250 µg/mL of ME, the cell proliferation is null only at 90 min of incubation. The same cell viability pattern was observed in yca1 mutant strain.

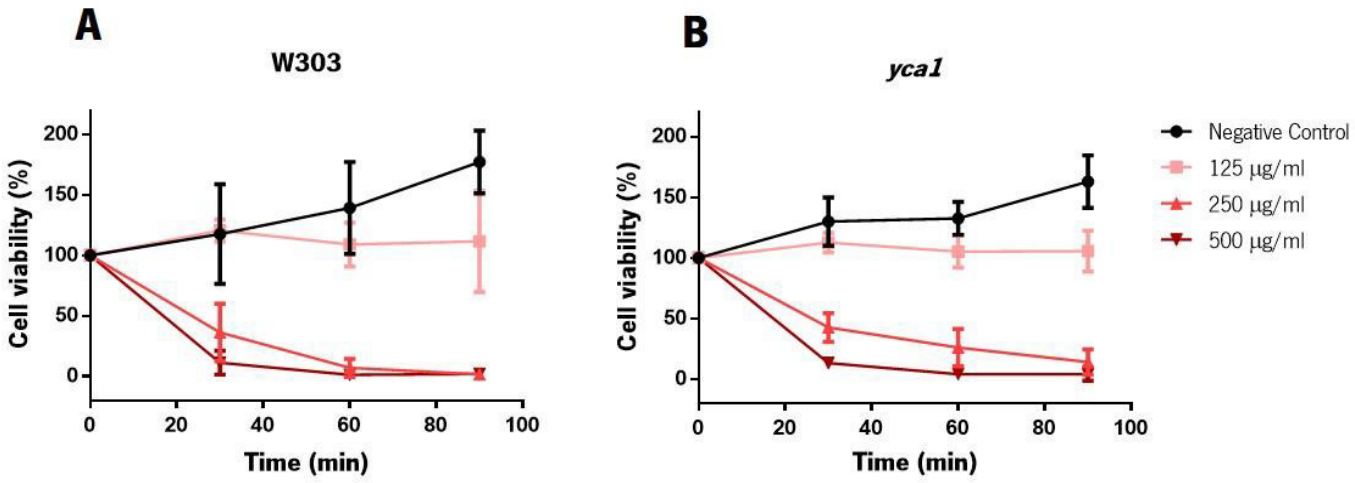


Figure III.8. Percentage of cell viability of *Saccharomyces cerevisiae* parental strain W303 **(A)** and their mutant strain *yca1* **(B)** over 90 min of incubation at different concentrations of ME, 125, 250 or 500 µg/mL. Values represent the mean ± S.D. of three independent experiments.

Comparing the cell viability of the two strains, W303 and *yca1*, no substantial differences between them were found. Since, an increasing number of studies demonstrate the existence of caspase-independent forms of PCD, we cannot discard the hypothesis that ME might induce other forms of PCD in yeast cells.

Apoptosis is a specific mode of cell death characterized by typical morphological and biochemical changes such as chromatin condensation, DNA fragmentation and formation of apoptotic bodies (Belloc et al., 1994), which can be detected by staining with fluorescent dye Hoechst. Thereby, to confirm whether ME induces apoptosis in yeast cells, we monitored, under fluorescence microscope, the nucleus morphology of cells treated with 500 µg/mL of ME, up to 90 min. We did not observe significant differences between the different times of incubation with the negative control in their nuclear morphology, chromatin condensation or nuclear fragmentation over the treatment time (Fig. III.9). Since we also did not observe an involvement of oxidative stress (Fig. III.7), we can conclude that ME does not induce apoptosis in yeast cells.

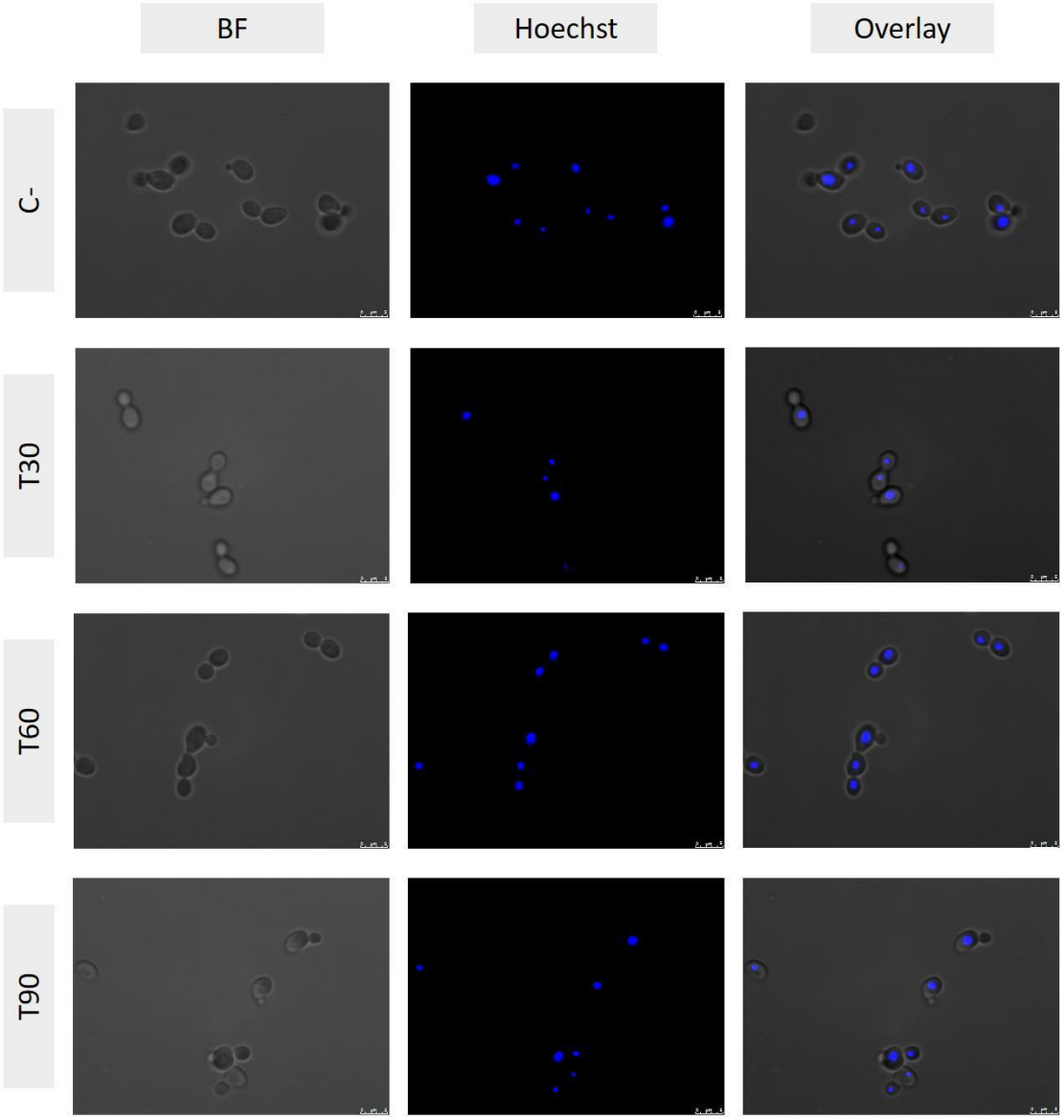


Figure III.9. Representative images of *Saccharomyces cerevisiae* cells cultures at 30, 60 and 90 min of treatment with ME (500 µg/mL) by staining with fluorescent dye Hoechst.

During apoptosis, the integrity of the plasma membrane of cells is preserved and most membrane functions remain unchanged. On the other hand, in cells undergoing necrosis, the process begins through the loss of membrane function and its structural integrity (Bertho et al., 2000). This mode of cell death is characterized by a gain in cell volume (oncosis), swelling of organelles, and subsequent loss of intracellular contents (Kroemer et al., 2009). Propidium iodide is a DNA-specific fluorochrome that stains cells that have lost the integrity of their plasma membrane. In normal situations, the cells are impermeable to PI (Span et al., 2002). Thus, we evaluated the plasma membrane integrity by flow cytometry using PI in *S. cerevisiae* cells exposed to 500 µg/mL of ME. The data were expressed as the percentage of cells with loss of their plasma membrane integrity in comparison with untreated cells (C-). Fig. III.10A displays a representative histogram of cells incubated with ME and negative control. We observed a significant increase of the percentage of cells exhibiting a loss of plasma membrane integrity after 30 (22.6%), 60 (23.6%) and 90 (32.8%) min of treatment with ME, when compared with negative control (Fig. III.10B). Since the loss of plasma membrane integrity is a primary sign of the occurrence of necrosis in cells, the results suggest that ME induces a necrotic process in *S. cerevisiae* cells.

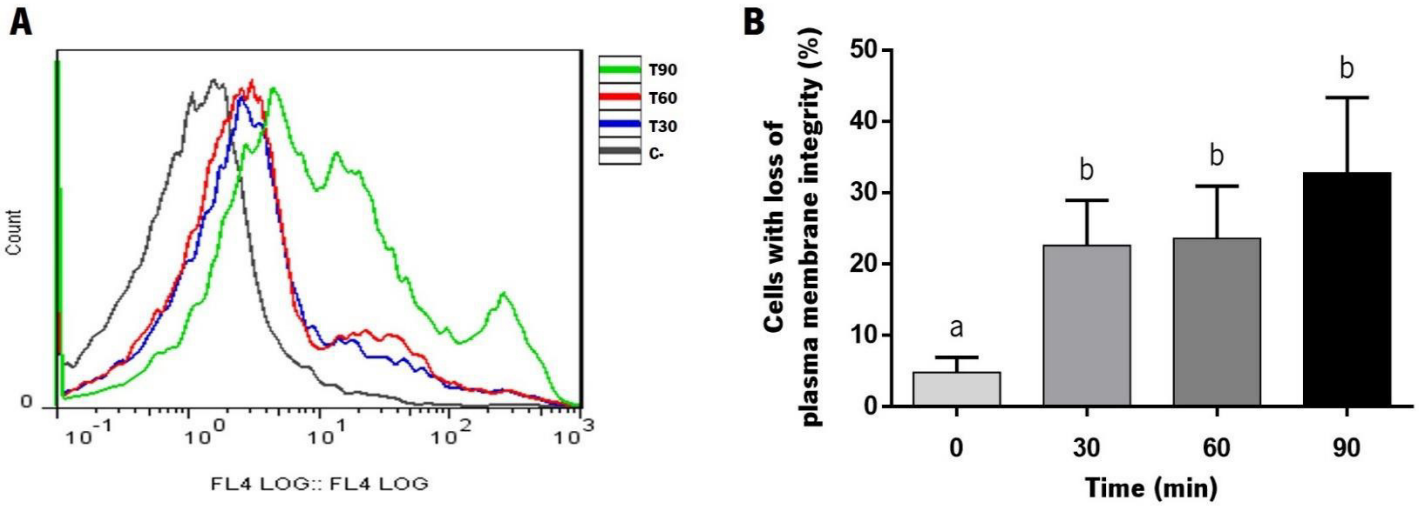


Figure III.10. (A) Representative histogram of the percentage of *Saccharomyces cerevisiae* cells that lost their plasma membrane integrity after 30, 60 and 90 min of incubation treated with 500 µg/mL ME. Untreated cells were used as negative control (C-). **(B)** Percentage of *S. cerevisiae* cells that lost their plasma membrane integrity after 30, 60 and 90 min of incubation treated with 500 µg/mL ME comparing with negative control (time 0). Values represent the mean ± S.D. of three independent experiments. Means followed by the same letters are not significantly different ($P<0.05$).

Necrosis also can be monitored by the nucleus-cytosolic translocation of the human chromatin bound non-histone protein high mobility group box 1 (HMGB1). The nuclear release of Nhp6Ap, the yeast homologue of HMGB1, has also been observed in dying yeast cell and has therefore been considered a

marker of necrosis in this organism (Eisenberg et al., 2009). To confirm whether ME induces a regulated form of necrosis, *S. cerevisiae* BY4741 transformed with an *NHP6A-EGFP* expression plasmid was used. The strain was treated with ME (500 µg/mL) and observed by fluorescence microscopy at 30 and 90 min of incubation. Cells untreated with ME were used as negative control. At time 0 (negative control), the cells exhibited a bright nuclear fluorescence (Fig III.11). After 30 min exposure to ME, a diffuse fluorescence was observed throughout the cell, indicating the translocation of Nhp6Ap from the nucleus to the cytosol, the same result was observed after 90 min. The release of Nhp6A was completed after the first 30 min of treatment, which allows us to conclude that these cells commit into a necrotic regulated death when exposed to ME.

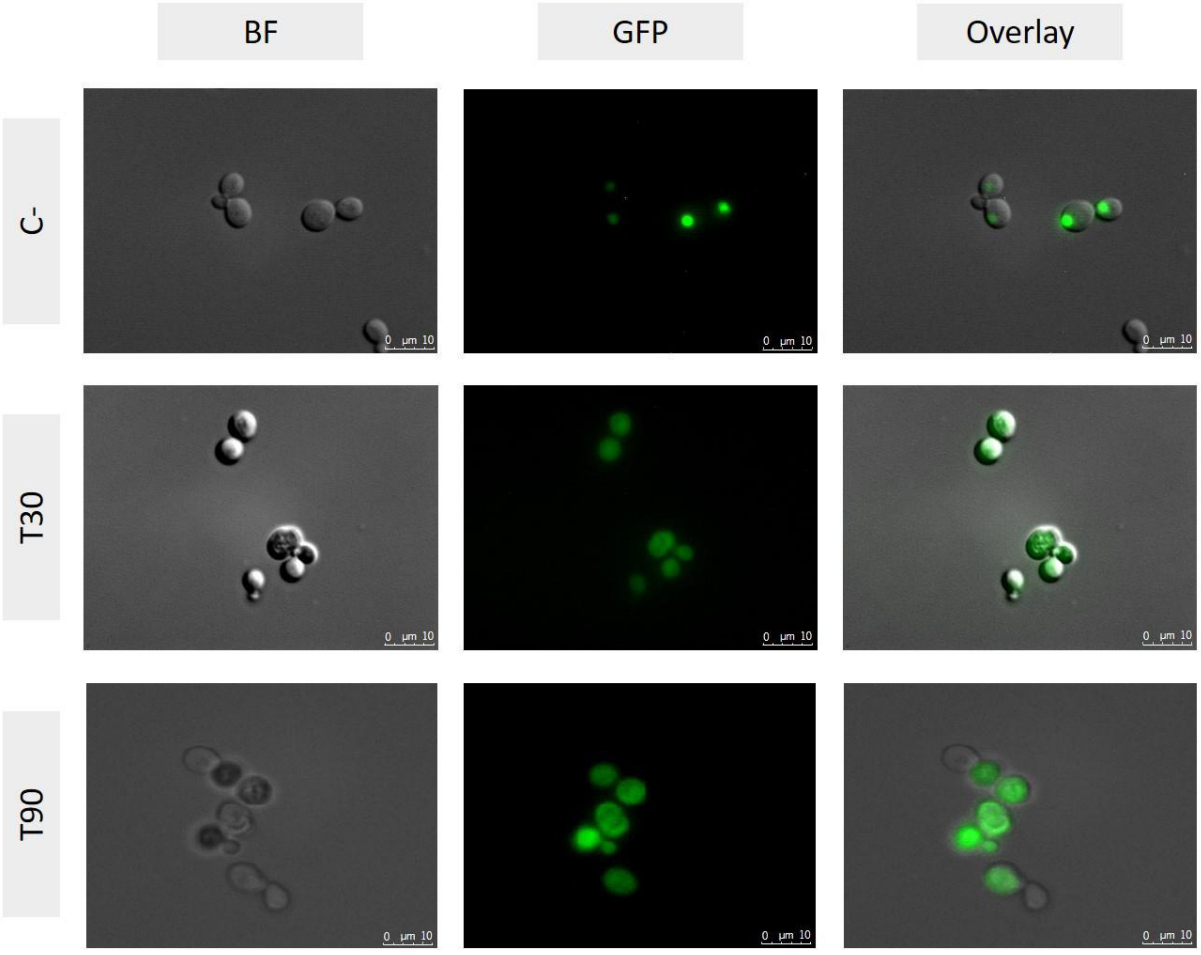


Figure III.11. Representative images of *Saccharomyces cerevisiae* BY4741 *pUG35- NHP6A-EGFP* at 30 and 90 min of treatment with ME (500 µg/mL).

Saccharomyces cerevisiae has been used as a model of studies of the inhibitory effect of various compounds against phytopathogenic fungi (Polacheck et al., 1991; Simons et al., 2006; Gleeson et al., 2010; Troppens et al., 2013). In order to understand the mechanism of toxicity of ME, we performed

several assays related to the two modes of cell death, apoptosis and necrosis. Our results demonstrated that ME induces mainly necrosis in yeast cells.

The mode of action of saponins is not yet fully understood. As discussed in section III.1, these compounds are known to form a complex with fungal membrane sterols, resulting in pore formation and loss of membrane integrity followed by leakage of cell components and cell death (Johnson et al., 1986; Polacheck et al., 1991; Keukens et al., 1995; Armah et al., 1999; Roddick et al., 2001; Sharon et al., 2009; Ahmed et al., 2012). This mode of action has several similarities with the necrotic process, since both cause the loss of plasma membrane integrity and intracellular contents. The antifungal activity of saponins is generally attributed to their membrane-permeabilizing properties (Polacheck et al., 1991; Armah et al., 1999; Ahmed et al., 2012). However, studies on α-tomatine and tomatidine tomato saponins have demonstrated that these compounds may exhibit other modes of action. Simons et al. (2006) have demonstrated in *S. cerevisiae* cells that the activity of tomatidine, unlike α-tomatine, does not involved membrane permeabilization. This compound mimics the effects of sterol biosynthesis inhibitors by targeting the sterol biosynthetic pathway. However, Sandrock and VanEtten (1998) observed that α-tomatine inhibited the growth of *Phytophthora infestans* and *Pythium aphanidermatum* fungi lacking sterols in their membranes, suggesting that α-tomatine can act by a mechanism other than binding to sterols. Ito et al. (2007) have also demonstrated that α-tomatine induces a PCD in phytopathogen *Fusarium oxysporum*. These authors showed that apoptosis was necessary for the antifungal activity of this compound, suggesting a role for mitochondria in the process and that α-tomatine-induced cell death in *F. oxysporum* is ROS- and caspase-dependent. Opposing to these results, we demonstrated that the cell death process induced by ME is a ROS- and caspase-independent pathway (Fig. III.7; III.8). We hypothesize that the apoptotic process does not occur due to the presence of sterols in the membranes of *S. cerevisiae*.

Chapter IV

Final Remarks and Future Perspectives

IV.1. Final Remarks

Nowadays, there is a growing concern about ecological problems caused by synthetic fungicides and their potential lack of safety to human health. Consequently, the search for safe and cost-effective alternatives to chemical fungicides, is needed. Therefore, the fungitoxic properties of biological products has been progressively studied.

In this thesis project, our main goal was to investigate the properties, mainly antifungal, of a crude extract of *Medicago* genus, that will lead in the future to the development of a natural and biodegradable product with possible commercial interest, mainly for application in organic agriculture. Thereunto, we tested the antifungal activity of ME against phytopathogenic fungi that causes substantial damage to many economically important crops. ME was able to inhibit mycelial growth of all tested fungi. Although the results obtained were only from *in vitro* experiments, it has been pointed out that substances which are found to be fungicidal *in vitro*, in almost all cases kill the fungi *in vivo* (Kuhn and Hargreaves, 1987). To assess phytotoxicity of ME, we checked its effect on seedlings growth, using flax plants as a model organism. Its observed that ME caused a decrease in the development of primary organs. We can conclude that ME possesses antifungal and allelopathic activity, these properties may be associated with different compounds that compose the extract, suggesting that ME mechanisms that express their activities, are different. For the application of ME as a fungicide product, it is important that the plant is not affected. Thus, we propose that ME should not be applied to soil, as it would cause impact on early plant growth. Results from the assessment of the phytotoxic activity of ME on cultivated garden strawberry leaves suggests that ME does not cause damage to adult plants.

Saccharomyces cerevisiae is a well-known model to study the inhibitory effect of various compounds against phytopathogenic fungi. Therefore, through several assays involving the two processes of cell death (apoptosis and necrosis), we tried to understand the mechanism of toxicity induced by ME in *S. cerevisiae* cells. We observed, by flow cytometry a loss of plasma membrane integrity, that was an indicative of cells death. We also observed a nucleus-cytosolic translocation of Nhp6Ap protein that is a necrotic marker. From these results, we concluded that ME may be inducing necrotic death in yeast cells.

Overall, through the early data obtained in this work, showed that there is a strong possibility of achieving the desired goal. Based on our results, it is possible to conclude that ME has potential as antifungal agent and could be a significant source of natural fungicides for control of fungal diseases in crop production systems in a sustainable manner, avoiding chemical fungicides applications.

IV.2. Future Perspectives

Given the well-known fact that saponins are the most abundant secondary metabolite compounds present in *Medicago* plants extracts, we assume that ME possesses saponins. Nevertheless, other phytochemicals may be active in ME, which could be influencing the activities, presented here. To determine which compounds are present in the extract, a chemical analysis is required. In this work, it was not possible to do this analysis, however, the next step to follow in this project will be this one.

Since promising results have been obtained in this study, further *in vitro* and *in vivo* assays should be performed in the future. The antifungal activity of ME can be tested with a wider range of phytopathogenic fungi, which produce severe economic losses in agriculture and horticulture. To supplement these assays, these properties may also be observed in fungal spores by microscopy. In this work, we reported that ME was not able to reduce the mycelium expansion of *Phytophthora cinnamomi* fungus, however, was observed a decreased in mycelial proliferation. Thus, these data will clarify whether ME inhibits spore germination and reduces the hyphae development of the exposed fungi.

In order to corroborate the results obtained here, the evaluation of antifungal activity of ME, *in vivo*, is required. Thereunto, ME effect should be tested on fungus-infected plants. These assays will allow us to assess the ME toxicity not only in the fungus, but also in plants.

Although we tried to understand the ME toxicity mechanism using the model organism *S. cerevisiae*, it is fundamental to perform *in vitro* experiments in phytopathogenic fungi cells. The spores can be used for multiple assays to investigate different parameters of toxicity mechanism induced by ME.

Since our main goal is to apply this crude extract at industrial level, elucidation of extract yield from a culture of Medicago plant is essential. Field experiments are required to test different methods of ME application to verify which one is the most effective in fungal control.

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SUPPLEMENTARY MATERIAL

Table 1. Diameter of mycelium of *Botrytis cinerea* treated with ME concentrations of 100, 500, 1000 or 2000 µg/mL at day 6. The values presented are the relative means expressed in centimeters ± S.D. of three independent experiments. **P<0.01; ****P<0.0001 compared with negative control (0).

Days of incubation	Diameter of mycelium (cm) at a *Medicago* extract concentration (µg/mL) of				
	0	100	500	1000	2000
6	8.5 ± 0.0	6.3 ± 0.8 **	6.5 ± 0.3 **	6.4 ± 0.3 **	5.3 ± 0.5 ****

Table 2. Diameter of mycelium of *Colletotrichum* species treated with ME concentrations of 100, 500, 1000 or 2000 µg/mL at day 6 and 9. The values presented are the relative means expressed in centimeters ± S.D. of three independent experiments. *P<0.05; **P<0.01; ***P<0.001; ****P<0.0001 compared with negative control (0); ns – not significant.

Species	Days of incubation	Diameter of mycelium (cm) at a *Medicago* extract concentration (µg/mL) of				
		0	100	500	1000	2000
C. acutatum	9	3.8 ± 0.3	3.4 ± 0.4 ns	3.3 ± 0.3 ns	3.3 ± 0.2 ns	3.0 ± 0.2 *
C. gloeosporioides	6	6.8 ± 0.2	5.8 ± 0.3 **	5.1 ± 0.3 ***	5.0 ± 0.2 ***	4.1 ± 0.3 ****
C. godetiae	9	6.0 ± 1.7	5.6 ± 1.6 ns	4.5 ± 1.1 ns	4.8 ± 1.7 ns	4.5 ± 1.6 ns
C. nymphaea	9	6.1 ± 0.8	5.2 ± 0.6 ns	4.7 ± 0.6 ns	4.3 ± 0.5 *	4.3 ± 0.6 *

Table 3. Diameter of mycelium of *Diplodia corticola* isolates treated with ME concentrations of 100, 500, 1000 or 2000 µg/mL at day 6. The values presented are the relative means expressed in centimeters ± S.D. of three independent experiments. ***P<0.001; ****P<0.0001 compared with negative control (0).

Isolates	Days of incubation	Diameter of mycelium (cm) at a *Medicago* extract concentration (µg/mL) of				
		0	100	500	1000	2000
CAA008	6	8.3 ± 0.4	4.9 ± 0.3 ***	3.4 ± 1.0 ****	3.1 ± 0.5 ****	2.5 ± 0.2 ****
CAA499	6	8.5 ± 0.0	6.4 ± 0.4 ***	4.9 ± 0.4 ****	5.6 ± 0.7 ****	5.4 ± 0.2 ****
CAA500	6	8.0 ± 0.6	4.0 ± 0.3 ****	3.4 ± 0.7 ****	3.2 ± 0.2 ****	2.5 ± 0.7 ****

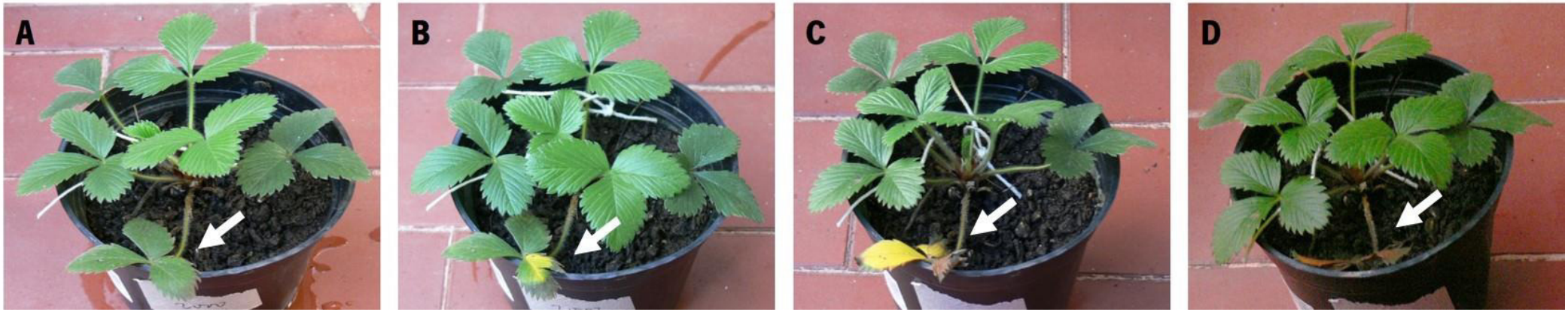


Figure 1. Evolution of symptoms on strawberry leaves. The affected leaf (non-brushed) is represented by the white arrow. **(A)** Leaf without symptoms. **(B)** Appearance of chlorosis (yellowing) followed by necrosis (browning). **(C)** Leaf totally affected, the necrotic parts begin to curve upwards. **(D)** Dead leaf.