Universidade do Minho
Escola de Ciências

Cláudia Sofia de Sá Ferreira

Survey of antifungal activity of plant extracts for the development of natural products for agriculture

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Survey of antifungal activity of plant extracts for the development of natural products for agriculture

Master Thesis

Master's in Molecular Genetics

Work under the supervision of

Professor Doctor Rui Pedro Soares de Oliveira

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Despacho RT - 31 /2019 - Anexo 3

DIREITOS DE AUTOR E CONDIÇÕES DE UTILIZAÇÃO DO TRABALHO POR TERCEIROS

Universidade do Minho, março de 2021

(Cláudia Sofia de Sá Ferreira)

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Despacho RT - 31 /2019 - Anexo 4

STATEMENT OF INTEGRITY

I hereby declare having conducted this academic work with integrity. I confirm that I have not used plagiarism or any form of undue use of information or falsification of results along the process leading to its elaboration.

I further declare that I have fully acknowledged the Code of Ethical Conduct of the University of Minho.

(Cláudia Sofia de Sá Ferreira)

PESQUISA DE ATIVIDADE ANTIFÚNGICA DE EXTRATOS DE PLANTAS PARA O DESENVOLVIMENTO DE ANTIFÚNGICOS NATURAIS PARA A AGRICULTURA

RESUMO

Para controlar as doenças nas plantações provocadas por fungos fitopatogénicos, os agricultores recorrem ao uso de fungicidas sintéticos. Contudo, os fungicidas sintéticos provocam efeitos adversos e potencialmente perigosos na saúde humana e ambiental. Para além disso, também são responsáveis pela seleção de mutações nos patógenos que conferem resistência a este tipo de fungicidas. Uma das alternativas biológicas que tem conquistado o interesse científico é o uso de extratos vegetais obtidos através de plantas. Os extratos vegetais são conhecidos por conterem elevadas quantidades de diversos metabolitos secundários, contribuindo para o potencial antifúngico contra fungos de plantas altamente devastadores.

Este trabalho tem o objetivo de identificar extratos vegetais com atividade antifúngica visando contribuir para práticas agrícolas mais sustentáveis. Foram selecionados seis extratos vegetais, nomeadamente *Carpobrotus edulis*, *Medicago* sp., *Melilotus indicus*, *Plantago major*, *Portulaca oleracea* e *Urtica dioica*. Para determinar a atividade antifúngica, os fungos fitopatogénicos *Botrytis cinerea*, *Colletotrichum acutatum*, *Colletotrichum gloeosporioides*, *Colletotrichum godetiae, Colletotrichum nymphaeae*, *Diplodia corticola* e *Phytophthora cinnamomi* foram expostos aos extratos aquosos. *Diplodia corticola* e *P. cinnamomi* revelaram-se os fungos mais sensíveis ao efeito fungitóxico de *Medicago* sp. e *M. indicus*, respetivamente. A atividade antifúngica do extrato de *Medicago* sp. em condições *ex vivo* também foi avaliada. O extrato vegetal de *Medicago* sp. foi aplicado como tratamento preventivo e o seu efeito foi avaliado em folhas de morangueiro destacadas e artificialmente inoculadas com *Colletotrichum acutatum*. Os resultados mostraram que o extrato foi capaz de eliminar a maioria das colónias de micélio da superfície das folhas.

Concluindo, com base nos resultados obtidos neste projeto, foi possível compreender que os extratos vegetais de *Medicago* sp., *M. indicus*, *P. major* e *U. dioica* podem ser uma fonte interessante de novos fungicidas naturais para desenvolver práticas mais sustentáveis e ecológicas na agricultura.

Palavras-chave: atividade antifúngica; extratos de plantas; fungos patogénicos

SURVEY OF ANTIFUNGAL ACTIVITY OF PLANTS EXTRACTS FOR THE DEVELOPMENT OF NATURAL ANTIFUNGAL PRODUCTS FOR AGRICULTURE

ABSTRACT

To control crops diseases caused by phytopathogenic fungi, farmers use synthetic fungicides. Due to the effects of these synthetic agrochemicals on human and environmental health, development of mutations in pathogens and resistance to various toxins, the continuous use of these approaches is not recommended. Consequently, the search of safer and biodegradable alternatives is increasing, such as the use of plant extracts as natural fungicides. Many plant extracts have gained much popularity and scientific interest for their high content in secondary metabolites, which contribute for the antifungal action against fungi potentially harmful for crops.

This work has the objective of identifying plant extracts with antifungal activity aiming to contribute to sustainable agricultural practices. Six plant extracts, namely *Carpobrotus edulis*, *Medicago* sp., *Melilotus indicus*, *Plantago major*, *Portulaca oleracea* and *Urtica dioica* were selected. To study the antifungal properties, the phytopathogenic fungi *Botrytis cinerea*, *Colletotrichum acutatum*, *Colletotrichum gloeosporioides*, *Colletotrichum godetiae, Colletotrichum nymphaeae*, *Diplodia corticola* and *Phytophthora cinnamomi* were exposed to the cited plant extracts. *Diplodia corticola* and *P. cinnamomi* were the most sensitive fungi to the fungitoxic effect of *Medicago* sp. and *M. indicus*, respectively. The antifungal activity of *Medicago* sp. extract under *ex vivo* conditions it was also evaluated. *Medicago* sp. extract was applied as preventive treatment and its effect was evaluated on detached strawberry leaves, which were artificially inoculated with *Colletotrichum acutatum*. The results showed that the extract was able to eliminate most of the mycelium colonies in the leaves surface.

Concluding, based on the obtained results in the present project, it was possible to understand that *Medicago* sp., *M. indicus*, *P. major* and *U. dioica* extracts could be an interesting source of new natural fungicides to apply in agriculture, and consequently, improve the control of diseases caused by plant fungal pathogens in important crops.

Keywords: antifungal activity; pathogenic fungi; plant extracts.

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LIST OF ABBREVIATIONS AND ACRONYMS

1,8-DHN	1,8-dihydroxynaphthalene
dH_2O	Deionized water
FRAC	Fungicide Resistance Action Committee
MGI	Mycelial Growth Inhibition
MIC	Minimum Inhibitory Concentration
ns	not significant
PDA	Potato Dextrose Agar
ROS	Reactive Oxygen Species
RPM	Revolutions Per Minute
SD	Standard Deviation

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Chapter 1

INTRODUCTION

1.1. PHYTOPATHOGENIC FUNGI

Fungal phytopathogens cause significant damage to several economically important crops (Pham et al., 2017). True fungi and oomycetes are the two major groups of phytopathogenic fungi causing plant lesions, being responsible for approximately 70% - 80% of total plant damages (Raaijmakers et al., 2009; Yang et al., 2019). Fungi are eukaryotic, filamentous, multicellular and heterotrophic organisms that produce a mycelium (Alexopoulos et al., 1996). Oomycetes have identical characteristics but are phylogenetically more identically to brown algae. The main difference between these two groups is the constitution of their cell walls, as true fungi are composed by chitin and oomycetes by cellulose (Raaijmakers et al., 2009). A well-established difference between true fungi and oomycetes is the absence of ergosterol in the cytoplasmatic membrane of the latter (Mendoza and Vilela, 2013). In this project, true fungi and oomycetes will be considered together due to the similarities between their mechanisms of parasitism and the diseases provoked by them.

Fungal spores exhibit resistance to a wide range of agents, such as high levels of ultraviolet and γ-radiation, desiccation, pressure, temperature and toxic chemicals, enabling the survival of spores in extreme conditions (Setlow, 2014). Environmental factors, including wind, water, soil and animals, facilitate the spores propagation to neighboring fields damaging entire harvests (Sephton-Clark and Voelz, 2018). Fungi can remain inactive on living or dead plant tissues, growing on the surface or penetrating them until conditions become advantageous for their proliferation (Ray et al., 2017). Fungal microorganisms have various lifestyles in which they adopt different strategies to interact with host plants, such as biotrophic, necrotrophic and hemibiotrophic (Lo Presti et al., 2015). Biotrophic fungal pathogens contact with the host plant, obtaining nutrients from the living plant tissues (Barnett and Binder, 1973). Necrotrophs establish interactions with the host, expelling a toxic substance that kills the plant tissue in order to extract its nutrients (Barnett and Binder, 1973; Doehlemann et al., 2017). Hemibiotrophic fungal pathogens establish an initial biotrophic phase, switching to a necrotrophic stage later on (Lo Presti et al., 2015; Doehlemann et al., 2017).

Prior estimates, despite outdated, provide a benchmark of crop losses, suggesting that 16% of agricultural production is lost annually due to fungal diseases (Oerke, 2006). Fungal plant pathogens cause several common diseases such as anthracnose, blight, cankers, curls, damping-off, dieback, galls, leaf spots, mildews, root rots, rusts, scab, smuts, and wilts (Ray et al., 2017; Jain et al., 2019). These diseases cause different symptoms on fruits, leaves and stems (Khater et al., 2017). The Journal Molecular Plant Pathology worked together with the fungal pathologists community to create a “Top 10”

of the most damaging fungal phytopathogens based on their scientific/economic relevance, in order to help the scientific community combat plant fungal diseases (Table 1; Dean et al., 2012). In 2014, the fungal pathologists community reunited again to create a "Top 10" of the most plant pathogenic oomycete species also based on their scientific and economic importance (Table 1; Kamoun et al., 2015).

Table 1. Top 10 of true fungi and oomycetes plant pathogens (adapted from Dean et al., 2012; Kamoun et al., 2015).

Rank	Fungal Species	Oomycetes Species
1	*Magnaporthe oryzae*	*Phytophthora infestans*
2	*Botrytis cinerea*	*Hyaloperonospora arabidopsidis* *Phytophthora ramorum*
3	*Puccinia* spp.	-
4	*Fusarium graminearum*	*Phytophthora sojae*
5	*Fusarium oxysporum*	*Phytophthora capsici*
6	*Blumeria graminis*	*Plasmopara viticola*
7	*Mycosphaerella graminicola*	*Phytophthora cinnamomi*
8	*Colletotrichum* spp.	*Phytophthora parasitica* *Pythium ultimum*
9	*Ustilago maydis*	-
10	*Melampsora lini*	*Albugo candida*

The fungi *Botrytis cinerea*, *Colletotrichum* spp. and *Phytophthora cinnamomi* cause several diseases, which affect economically important crops in Portugal. The top 10 of fungal pathogens ranks *B. cinerea* in the second position, *Colletotrichum* spp. in the eighth position and *P. cinnamomi* in the seventh position (Dean et al., 2012; Kamoun et al., 2015). *Diplodia corticola* is also relevant, as it causes substantial damages on Portuguese cork oak forests (Fernandes et al., 2014).

1.1.1. *Botrytis cinerea*

Botrytis cinerea is one of the most destructive necrotrophic pathogens, with the ability to infect more than 200 plant species (Zhang et al., 2018). This species is commonly known as gray mold, named after its most evident symptom of infection, which is the production of abundant aerial hyphae combined with massive sporulation producing gray conidiophores (Pearson and Bailey, 2013). Plant age and fitness, along with humidity and temperature play crucial roles in the extent of disease development. Light intensity and diurnal rhythms are also important factors to determine the development and host infection

(Veloso and van Kan, 2018). The growth of *B. cinerea* depends on favorable conditions, including a humidity higher than 94% and temperatures ranging from 15 °C to 25 °C (Williamson et al., 2007). *Botrytis cinerea* can infect many plant tissues including leaves, stems and flowers of the plant host (Muñoz et al., 2019). This phytopathogen is considered the second most harmful phytopathogen responsible for pre- and post-harvest decay and fruit quality deterioration in greenhouses, open fields and during storage (Dean et al., 2012). *Botrytis cinerea* causes diseases in agronomically important crops, such as cucumber, grapevine, tomato, strawberry, bulb flowers, cut flowers and ornamental plants (Kan, 2005). Infection on the host plant can occur from the seedling to the maturation stage and can survive for a long time as mycelia and/or conidia (Williamson et al., 2007; Zhang et al., 2018).

Over the past decades, *B. cinerea* developed some specific resistance to several synthetic fungicides, including dicarboximides, benzimidazole, phenylpyrrole and hydroxyanilide (Diánez et al., 2002; Myresiotis et al., 2007; Fernández-Ortuño et al., 2012). So, there is a paramount need to exploit other sustainable, cost-effective, and eco-friendly way for controlling diseases associated with *B. cinerea*.

1.1.2. *Colletotrichum* spp.

Colletrotrichum genus belongs to the Glomerellales order and are members of the Glomerellaceae family (Marin-Felix et al., 2017). *Colletotrichum* is a wide-spread fungal genus that causes severe diseases on several plants (including wood and herbaceous plants), with agronomical value worldwide (Cannon et al., 2012). Based on the economic and scientific relevance, this genus was ranked as one of the top ten most damaging fungal pathogens (Dean et al., 2012). Species of the genus *Colletotrichum* damage a large range of plants, such as cereals, fruits, legumes and vegetables, causing yield losses mainly in tropical, subtropical and temperate areas (Bailey and Jeger, 1992; Hunupolagama et al., 2017). Fungal plant pathogens of the genus *Colletotrichum* affect not only the fruit, but also the crown, flowers, leaves, petioles and roots of the host plants (Roy et al., 2018). *Colletotrichum* spp. are known to be responsible for anthracnose, however other diseases including red rot of sugar cane, coffee berry disease, crown rot of strawberry and banana and brown blotch of cowpea, have been reported. Anthracnose causes necrotic and sunken ulcer-like lesion in all parts of the host plant (Agrios, 2005). Three related species of the genus *Colletotrichum*, including *Colletotrichum fragariae*, *Colletotrichum acutatum*, and *Colletotrichum gloeosporioides* can be associated with anthracnose disease (Smith and Black, 1990). *Colletotrichum acutatum* isolates from strawberry produced lesions on wounded and nonwounded pears, peaches, nectarines, and apples (Freeman et al., 2001). *Colletotrichum acutatum*,

C. godetiae and *C. nymphaeae* have also emerged as pathogens associated with olive anthracnose in several Mediterranean countries (Talhinhas et al., 2009; Mosca et al., 2014; Chattaoui et al., 2016). Due to the large number of species of the genus *Colletotrichum* that cause harmful diseases in important crops and their rapid spread in new areas, it is important to understand whether these plant pathogens can be controlled.

1.1.3. *Diplodia corticola*

Diplodia corticola is a fungal plant pathogen belonging to the family Botryosphaeriaceae, that can cause diseases and death on plants (Damm et al., 2007). In addition, Botryosphaeriaceae fungi live harmlessly as endophytes within plants, but may become pathogenic when the plant is stressed by environmental factors including insect damage, water stress, pruning and transplanting (Félix et al., 2017; Martin and Munck, 2017). Among the species of Botryosphaeriaceae isolated from declining oak trees in the Mediterranean region, *D. corticola* seems to be the most widely dispersed and virulent species in oak forests (Alves et al., 2004; Linaldeddu et al., 2009; Tsopelas et al., 2010). This latent phytopathogen is consistently associated with declining of oak trees, and its pathogenicity has been demonstrated not only on Mediterranean but also in USA oak species (Dreaden et al., 2011; Lynch et al., 2013; Linaldeddu et al., 2014). This phytopathogenic fungus attacks different oak, grapevine and eucalypt ecosystems (Úrbez-Torres et al., 2010b; Barradas et al., 2016). Damaged trees can exhibit branch dieback, crown thinning, epicormic shoots, exudates on branches and trunk, root losses, sunken cankers and sudden death symptoms (Linaldeddu et al., 2014). Members of Botryosphaeriaceae family, as *D. corticola*, enter in plants through leaf scars, wounds or open stomata for gas exchange (Martin and Munck, 2017). Cork oak debarking can potentiate *D. corticola* pathogenicity, since cork oak removal represents a stressing event and may cause wounds on these trees (Luque and Girbal, 1989; Costa et al., 2004). In grapevines, most of the Botryosphaeriaceae spores are stuck in trap spores during the rainfall season, which coincides with grapevine pruning, resulting in infections of the exposed xylem (Úrbez-Torres et al., 2010a).

Diplodia corticola is a devastating pathogenic fungus of cork oak, which is one of the most economically and ecologically important forest species in Portugal. As Portugal is the world's leading cork producer, the search for new methods to combat this pathogenic fungus is extremely important to prevent serious economic losses.

1.1.4. *Phytophthora cinnamomi*

The oomycete fungus *P. cinnamomi* is an extremely destructive soil pathogen that affects a wide range of plant hosts, including almost 5000 plant species in more than 70 countries (Jung et al., 2013). *Phytophthora cinnamomi* is the seventh most harmful plant-pathogenic oomycete, based on economic and scientific relevance (Kamoun et al., 2015). The oomycetes class, which includes the genus *Phytophthora* belongs to Oomycota (also called Pseudofungi) phylum (Cavalier-Smith and Chao, 2006; Beakes et al., 2012). *Phytophthora cinnamomi* generates substantial losses in agriculture, forestry and horticulture (perennial fruit, spice and nut crops; Burgess et al., 2017; Lourenço et al., 2019). This oomycete can be a destructive phytopathogen globally in tropical and subtropical agriculture, however it has a larger impact in ecosystems with a Mediterranean type climate (Burgess et al., 2017). Prime examples include the effect of *P. cinnamomi* on chestnuts, particularly American (*Castanea dentata*) and European *(Castanea sativa)*, on Australian eucalyptus and on avocado (*Persea americana*) and macadamia worldwide (Crone et al., 2013; Serrazina et al., 2015; Reeksting et al., 2016; Akinsanmi et al., 2017; Westbrook et al., 2019). Ink disease is one of the most destructive diseases affecting chestnuts, causing specific symptoms such as root and collar rot with dieback of branches, chlorosis, microphylly (i.e. smaller leaves than normal, yellowish foliage and lesions on the central roots and collar; Alistair McCracken, 2013), defoliation, gradual decline and death of susceptible trees (Corredoira et al., 2012). A serious decline of oaks has been reported across the Mediterranean area, affecting mostly the holm (*Quercus ilex* subsp. *Ballota* [Desf.] Samp.), cork (*Quercus suber* L.) and turkey (*Quercus cerris* L.) oaks (Sghaier-Hammami et al., 2013). Symptoms of oak trees decline include leaf discoloration and wilting, defoliation, root rot, trunk exudations and branch dieback (Gallego et al., 1999; Vita et al., 2013).

Phytophthora cinnamomi infects feeder roots and invade woody stems, specially through wounds or natural breaks in the peridermal layer (O'Gara et al., 2015). This pathogenic oomycete can use several mechanisms of dissemination, such as soil spread, water transport, root to root contact and animal and human dispersal (Hill et al., 1994; Ristaino and Gumpertz, 2000; Shearer et al., 2014). The ability of *P. cinnamomi* to cause diseases in a wide range of host plants is uncommon for a plant pathogen and it is a severe threat to worldwide biodiversity and crop production (Hardham, 2005).

1.2. FUNGICIDES

Fungicides are agents, of either natural or synthetic origin, that defend plants from fungal infections by inhibiting or killing the phytopathogenic fungus that causes the infection. The term 'fungicide'

is conventionally used to characterize compounds that control the infections caused by fungi species (Oliver and Hewitt, 2014). There are contact and systemic fungicides. Systemic fungicides are absorbed into plants, while contact fungicides remain on the plants surface. Many contact fungicides are potentially phytotoxic and can damage the plant if absorbed (McGrath, 2004). To avoid losses in agriculture production and protect the crops, the use of agrochemicals including herbicides, insecticides, plant growth regulators and fungicides is required (Oliver and Hewitt, 2014). During the establishment and development of a crop, fungicides are used to control diseases, increase productivity, reduce blemishes and improve the storage life and the quality of the harvested plants. Agents with fungicidal properties are widely used in agriculture to control soil, seed, or air borne fungal pathogens (McGrath, 2004). Half of the world's population relies on agriculture as their main source of income. Therefore, the damage caused by phytopathogens and pests results in substantial economic losses at a global scale (Mamarabadi et al., 2018). The development of synthetical chemical fungicides has been the main method for eradicating or preventing plant diseases (Chen and Dai, 2012).

1.2.1. Synthetic fungicides and mechanisms of action

The introduction of synthetic fungicides (almost 50 years ago) has modernized agricultural production both in quantity and quality by providing fungal diseases control through highly efficient and relatively low toxicity compounds. Synthetic agrochemicals provide optimal growth conditions and protect crops from damage by pests including weeds, insects and phytopathogenic fungi (Hahn, 2014). In agriculture, fungicidal agents are used to protect tubers, fruits and vegetables during storage by being directly applied to ornamental plants, field crops, cereals, trees, and turf grasses. Several substances with several distinct chemical constituents are used as synthetic fungicides (Gupta, 1988). The current classification of fungicides is based on their chemical structures and their mode of action (Table 2; Table 3; Gupta, 2011; Fungicide Resistance Action Committee, 2020).

Table 2. Classification of fungicides based on chemical nature and target site (adapted from Gupta, 2011; Fungicide Resistance Action Committee, 2020).

Chemical class	Examples	Mode of action	Target site
	Benalaxyl	Nucleic acids metabolism	RNA polymerase I

Amides	Metalaxyl		
	Dichlofluanid	Chemicals with multi-site activity	Multi-site contact activity
	Fenhexamid	Sterol biosynthesis in membranes	3-keto reductase, C4- de-methylation
	Flutolanil	Respiration	Complex II: succinate-dehydrogenase
Anilinopyrimidines	Cyprodinil Mepanipyrim Pyrimethanil	Amino acids and protein synthesis	Methionine biosynthesis (proposed)
Benzimidazoles	Benomyl Carbendazim Fuberidazole Thiophanate-methyl	Cytoskeleton and motor protein	ß-tubulin assembly in mitosis
Carbamic acid derivatives	Ferbam Mancozeb Maneb Metiram Thiram Zineb Ziram	Chemicals with multi-site activity	Multi-site contact activity
	Propamocarb	Lipid synthesis or transport / membrane integrity or function	Cell membrane permeability, fatty acids (proposed)
Chloroalkylthiodi-carboximides	Captafol Captan Folpet	Chemicals with multi-site activity	Multi-site contact activity
Conazoles	Azaconazole Bromuconazole Hexaconazole	Sterol biosynthesis in membranes	C14- demethylase in sterol biosynthesis
	Etridiazole	Lipid synthesis or transport / membrane integrity or function	Cell peroxidation (proposed)
Halogenated substituted monocyclic aromatics	Chlorothalonil	Chemicals with multi-site activity	Multi-site contact activity
	Dicloran Tecnazene	Lipid synthesis or transport / membrane integrity or function	Cell peroxidation (proposed)
	Dinocap	Respiration	Uncouplers of oxidative phosphorylation
Morpholines	Dodemorph (liquid) Fenpropimorph (oil) Tridemorph	Sterol biosynthesis in membranes	Δ14-reductase and Δ8→Δ7- isomerase in sterol biosynthesis
Others	Dimethomorph Fludioxonil	Cell wall biosynthesis Signal transduction	Cellulose synthase MAP/HistidineKinase in osmotic signal transduction
	Trifloxystrobin	Respiration	Complex III: cytochrome bc1

Table 3. Classification of anti-oomycetes fungicides based on chemical nature and target site (adapted from Fungicide Resistance Action Committee, 2020).

Chemical class	Examples	Mode of action	Target site
	Metalaxyl		

Amides	Mefenoxam Benalaxyl Kiralaxyl Oxadixyl	Nucleic acids metabolism	RNA polymerase I
Benzamides	Zoxamide	Cytoskeleton and motor protein	ß-tubulin assembly in mitosis
	Fluopicolide Fluopimomide	Cytoskeleton and motor protein	Delocalization of spectrin-like proteins
Carboxylic Acid Amides	Dimethomorph Flumorph Benthiavalicarb Iprovalicarb Mandipropamid	Cell wall biosynthesis	Cellulose synthase
OSBPI (Oxysterol binding protein homologue inhibition)	Oxathiapripolin	Lipid synthesis or transport / membrane integrity or function	Lipid homeostasis and transfer/storage
Phosphonates	Fosetyl-Al	Host plant defense induction	Phosphonates
Thiazole carboxamide	Ethaboxam	Cytoskeleton and motor protein	ß-tubulin assembly in mitosis

According to data provided by Eurostat, in Europe, around 380,000 tonnes of synthetic and inorganic pesticides (average between 2011 and 2018 for 28 European countries) are sold per year. Fungicides and bactericides were the major group of pesticides which recorded the highest sales volumes both in 2011 and 2018 (Eurostat). Due to effects of synthetic fungicides on human and environmental health, mutation in pathogens and resistance to several toxins, the continued use of chemical and synthetic fungicides is not a suitable option to apply in agriculture (Carvalho, 2017).

1.2.1.1. Resistance of synthetic fungicides

Fungicide resistance has been reported in many plant pathogenic fungi worldwide (Al-Balushi et al., 2018; Saito and Xiao, 2018; Weber and Hahn, 2019). Fungal phytopathogenic resistance to fungicides is defined as the combination of three factors: i) presence of naturally occurring resistant individuals, initially at very low frequency, originating from regular mutations, ii) increase in frequency of resistant individuals over time (during the season, from year to year) resulting in resistant sub-populations caused by the selection process of fungicide applications and migration of resistant individuals; and iii) reduction of disease control compared to previous standard treatments (Gisi et al., 2000). The nature of the fungicide and the genetic determinants of resistance influence the rates and patterns of resistance. Fungicide resistance may occur as a result of a single mutation in the target site of action or as a result of multiple gene mutations, conferring a high level of resistance (Lucas et al., 2015). Resistance against antifungal agents includes diverse strategies to combat the action of drugs. There are five major mechanisms of resistance (Figure 1) known to counteract fungicidal effects, including (1) a mutated target

site, which reduces fungicide binding (mechanisms confirmed for benzimidazoles, azoles, quinone outside inhibitors and succinate dehydrogenase inhibitors); (2) overproduction of the fungicide target due to upregulation of the encoding gene; (3) synthesis of an alternative enzyme capable of substituting the target enzyme; (4) metabolic fungicide breakdown due to detoxification by metabolic enzymes; and (5) active efflux or reduced uptake of the fungicide due to the action of ATP-binding cassette transporter or other transporters (Ma and Michailides, 2005; Lucas et al., 2015). Notwithstanding, some other unrecognized mechanisms could also be responsible for the emergence of fungicide resistance (Ma and Michailides, 2005). Lastly, fungicides with different modes of action are needed in disease management to delay fungicide resistance development (McGrath, 2004).

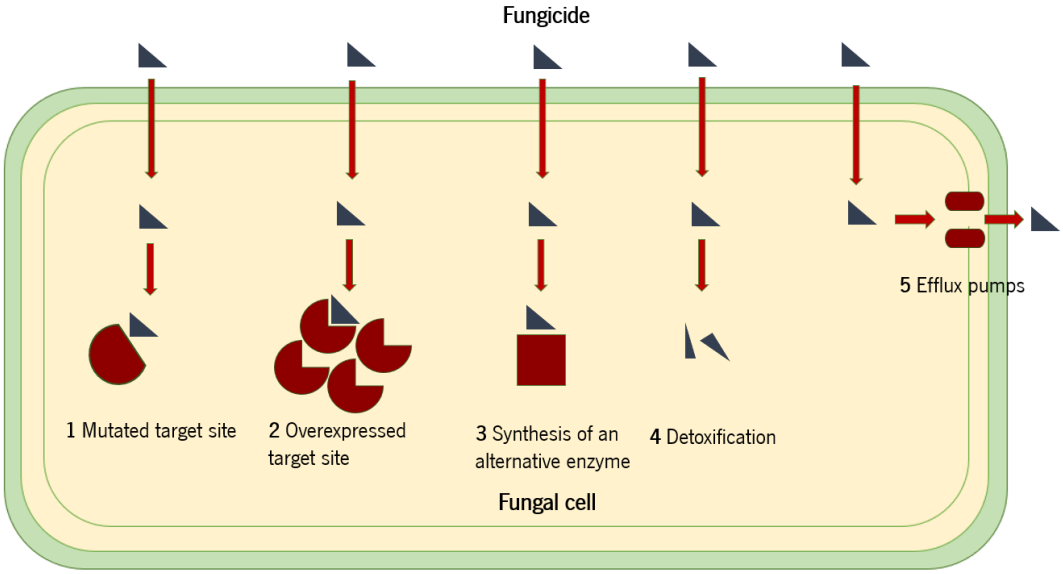

Figure 1. Mechanisms of resistance to fungicides. A mutated target site prevents fungicide binding (1); overexpression of target protein increases concentration of fungicide necessary for inhibition (2); the synthesis of an alternative enzyme inhibit the fungicide to bind (3); degradation of the fungicide by metabolic enzymes (4); and efflux pumps expel fungicides from fungal cell (5) (adapted from Lucas et al. 2015).

1.2.1.2. Environmental impact of synthetic fungicides

Synthetic fungicides affect fungal pathogens and non-target microorganisms, thus substantially impacting the surrounding ecosystems. Some of these non-target microorganisms could help optimize fungicide applications due to their antagonistic activity against different phytopathogens (Walia et al., 2014; Santísima-Trinidad et al., 2018). In addition, fungicides can have unfavorable impacts on non-

target communities, with possible consequences for plant health and productivity (Shi et al., 2019). Organic fungicides incorporate into the soil and their effect may lead to elimination, decrease and/or modification of several soil enzymes, essential for soil fertility (Sukul, 2006). Likewise, fungicide residues, which make their way into surface and ground waters, can cause adverse effects to the structure and functioning of aquatic ecosystems (Wightwick et al., 2010). The presence of fungicides in aquatic habitats cause adverse effects on aquatic invertebrates, including in *Daphnia magna*. The mobility of this small aquatic crustacean is compromised when exposed to certain fungicides, affecting also their reproduction, development and growth at sub-lethal concentrations (Silva et al., 2015; Cui et al., 2017). Penguins and birds accumulate some synthetic fungal agents in the brain and can also transfer some fungicides into eggs from internal tissues, due to their marine diet (Reindl et al., 2013; Falkowska and Reindl, 2015).

Aerial organisms, such as bees, are exposed to fungicides via contaminated food resources like pollen, nectar or water, being, therefore, directly affected by these compounds, which can potentially lead to bee health problems (Bonmatin et al., 2015). Toxicity of fungicides can uncouple and inhibit the respiration of mitochondria in bumblebees (Syromyatnikov et al., 2017). Certain synthetic fungicides produce transcriptional changes *in vitro* that are similar to those seen in brain samples from humans with advanced age, autism, and neurodegeneration, including Alzheimer's disease and Huntington's disease. These chemicals stimulate free radical production and disrupt microtubules in neurons (Pearson et al., 2016).

Synthetic fungicides have the ability to affect soil, water and air quality, with associated risks to humans, flora, and fauna, mainly due to its persistence in soil (Huber et al., 2001). That being said, alternatives for efficient, less toxic crop diseases control are in urgent demand.

1.3. PLANT EXTRACTS

Throughout the years, extensive research efforts have led to the development of several alternatives to synthetic fungicides. One of the several alternatives are the use of plant extracts that have been described to control harvest diseases (in *in vitro* and *in vivo* conditions) and to extend the overall quality and storage life of fresh commodities (Kumari et al., 2015; Han et al., 2017). Plants have secondary biochemical pathways that allow them to synthesize a range of chemicals, often in response to specific environmental stimuli, such as herbivore-induced damage, nutrient deprivation or pathogen attacks (Reymond et al., 2000; Hermsmeier et al., 2001). Plant extracts are considered an appealing alternative to synthetic fungicides, as they provide a wide variety of secondary metabolites, such as

phenolic compounds, flavonoids, xanthones, alkaloids, terpenoids, coumarins and saponins (Arif et al., 2009). These bioactive compounds are generally safer for the environment and human health than synthetic chemicals because of their intrinsic biodegradability, their capacity to interact selectively with the biological targets of interest and their ability to act through multimodal mechanisms of action (Scognamiglio et al., 2019). Nevertheless, there are also some disadvantages associated with using plant extracts, such as the rapid degradation after treatment, the absence of standardized extraction methods for all plants and, generally, their lower efficacy in comparison to synthetic fungicides (Ganesan et al., 2015).

1.3.1. Secondary metabolites

Specific compounds such as secondary metabolites are a large class of naturally occurring molecules that play a crucial role in plant environment interactions and adaptive physiology conferring a selective advantage to plants (Moses et al., 2013). Based on their structure and biosynthetic origin, natural plant products can be classified into different groups: phenols, flavonoids, alkaloids, coumarins, terpenoids, and saponins (Arif et al., 2009). Phenolic compounds are abundant across the plant kingdom, with approximately 10,000 structures identified. Phenolics range from simple low-molecular weight compounds (simple phenylpropanoids, coumarins and benzoic acid derivatives) to more complex structures (flavonoids, tannins and stilbenes) (Kennedy and Wightman, 2011). Flavonoids encompasses about 9000 varieties of compounds and are subdivided into several classes including anthocyanidins, flavones, flavanols, flavanones, flavans, isoflavonoids and biflavonoids (Croft, 1998). These compounds have several important roles in plants such as serving as signaling molecules for plant-microbe symbiosis, protecting cells against ultraviolet light stress and attracting pollinators by influencing flower color (Falcone Ferreyra et al., 2012).

Coumarins comprise a large family of benzopyrone compounds subdivided into four main coumarin sub-types: simple coumarins, furanocoumarins, pyranocoumarins and pyrone-substituted coumarins (Lacy and O'Kennedy, 2004). Coumarins play a momentous role in several pharmacological and medicinal activities, acting as antimicrobials, antioxidants, antiasthmatics, antiviral and anticoagulant (Hwu et al., 2011; Kostova et al., 2011; Bansal et al., 2013; Peng et al., 2013).

Individual alkaloids act as agonists and antagonists to a variety of neurotransmitter systems by direct binding to neuroreceptors and interference with neurotransmitter metabolism, signal transduction, and

ion channel function or by mimicking the structure of endogenous substrates, hormones, neurotransmitter and other ligands (Wink, 2000, 2003).

Terpenes are a diverse group of more than 30,000 lipid-soluble compounds and terpenoids are a modified class of terpenes (Kennedy and Wightman, 2011; Perveen, 2018). Several terpenoids act as repellents, while others function in indirect plant defense by attracting arthropods that prey upon or parasitize herbivores (Schnee et al., 2006; Unsicker et al., 2009; Maffei, 2010).

Saponins are a complex, chemically varied group broadly divided into triterpenoids and sterol saponins, based on the aglycone structure (Moses et al., 2014). These compounds are potent membrane permeabilizing agents associated to several properties, including anti-inflammatory, antifungal, antiyeast, antimicrobial, anticarcinogenic, antiviral, and antioxidant properties (Sparg et al., 2004).

1.3.2. Mechanisms of action of secondary metabolites

Even though the influence of secondary metabolites against phytopathogenic fungi has already been reported, detailed information on their mechanism of action is still very scarce (Orczyk et al., 2020). Various flavonoid compounds have been investigated for their antifungal effect, being a promising, efficient, and cost-effective option for the reduction/inhibition of fungal infections. Flavonoids often inhibit fungal growth due to the following mechanisms: induction of mitochondrial dysfunction; plasma membrane disruption; inhibition of cell formation, cell division, RNA and protein synthesis; and/or efflux mediates pumping system (Aboody and Mickymaray, 2020).

The mechanism of action of coumarins modifies the fungal mitochondrial cell morphology and, consequently, induces apoptosis (Kupidlowska et al., 1994; Razavi, 2011). Thati et al. (2007) found that coumarin derivatives, tested against *Candida albicans*, reduced respiration efficiency due to disruption of cytochrome synthesis in mitochondria, which also contribute to a depletion in ergosterol biosynthesis compromising the integrity of the cell membrane. Guerra et al. (2015) studied the effects of coumarin derivative, 7-hydroxy-6-nitro-2H-1-benzopyran-2-one in culture media with and without exogenous ergosterol, and the results suggested that the coumarin derivative did not act via binding to ergosterol in the plasma membrane (Guerra et al., 2015). They also suggested that this coumarin derivative acts on the fungal cell wall structure corroborating the report of Widodo et al. (2012), who showed that coumarin acts by forming pores in the cell wall, with consequent release of cytoplasmic contents and cell death.

Saponins are able to interact with the fungal ergosterol membrane, not only deforming it but also generating pores in the double lipidic layer, consequently leading to the disturbance of the membrane permeability and inducing cell death (Sparg et al., 2004). This ability to cause perturbation depends on membrane composition and structure of incorporated membrane sterols (Augustin et al., 2011). Steel and Drysdale (1988) found that liposomes prepared with a free 3β-hydroxy sterol (ergosterol, cholesterol, or sitosterol) were more sensitive to disruption by the saponin α-tomatine than the liposome membranes containing sterols lacking a 3β-hydroxy sterol. Some saponins have been shown to influence the properties of different types of membrane proteins, including Ca^{2+} channels and Na^{+}-K^{+} ATPases (Choi et al., 2001; Takechi et al., 2003; Chen et al., 2009).

Accordingly to Fungicide Resistance Action Committee (FRAC) (2020), phenols, sesquiterpenes, triterpenoids and coumarins from *Swinglea glutinosa* extracts affect fungal spores and germ tubes, and induced plant defense. There is no sign of resistance developed to these secondary metabolites.

1.4. GENUS *MEDICAGO*

The plant genus *Medicago* L. belongs to the highly diverse family Fabaceae, and comprises 87 species of both annual and perennial (Small, 2010; Zitouna et al., 2014). Annual medics combine characteristics such as vigorous seedling growth and cold tolerance with high forage quality, prolific seed production, resistance to pests and high morphological diversity (De Haan et al., 1997). This genus includes the widely cultivated crop species *Medicago sativa* L. (alfalfa or lucerne), *Medicago lupulina* L. (black medick) and the legume model *Medicago truncatula* Gaertn. (barrel medic) (Turkington and Cavers, 1979; Small, 2010; Doidy et al., 2019). *Medicago* species are used as medicine, green manure, human food, phytoremediation (developing biotechnology that uses plants, their associated microorganisms, and agricultural techniques for the rehabilitation of environments contaminated by inorganic and organic pollutants), and source of industrial enzymes in biotechnology (Gholami et al., 2014; Panchenko et al., 2017).

Several species of the *Medicago* genus produce a wide range of bioactive compounds, including alkaloids, coumarins, isoflavonoids and a substantially high concentration of saponins (Jurzysta and Waller, 1996; Barnes et al., 2007). In *Medicago* species, saponins are produced in flowers, leaves, roots, seeds and sprouts (Massiot et al., 1991; Kinjo et al., 1994; Bialy et al., 1999; Kapusta et al., 2005). Their plant material content changes as a function of numerous factors, such as plant organs, cutting, year and stage of growth, genotype, and environmental effects (Quazi, 1975; Tava and Pecetti, 1998;

Pecetti et al., 2006). The chemical structure of saponins from several species within the genus has been determined. Generally, they are complex mixtures of high molecular weight triterpene glycosides with medicagenic acid, hederagenin, zanhic acid, bayogenin and soyasapogenols A and B as the main aglycones (Tava and Avato, 2006).

The large biomass produced by *M. sativa*, together with the selection of new saponin-rich cultivars with a defined chemical structure, should make the application of saponins against phytopathogenic fungi in agriculture economically viable (Abbruscato et al., 2014).

1.5. *MELILOTUS INDICUS*

Melilotus Mill. (sweet clover) is one of the most important forage genus of the Leguminosae family, comprising 19 annual or biennual species, mainly distributed in North Africa and Eurasia (Outreach, 1992; Aboel-Atta, 2009). Members of the genus *Melilotus* are known to have high seed yields and can endure extreme environmental conditions, such as high salinity, cold and drought (Rogers et al., 2008; Al Sherif, 2009). In addition, *Melilotus* species have the ability to perform symbiotic fixation with bacteria contributing to high nitrogen rates, which can increase soil fertility and benefit crop rotation (Stickler and Johnson, 1959; Bromfield et al., 2010). The species *Melilotus indicus* (L.) All. (syn. *Melilotus parviflora* Desf.) has been reported to possess anticancer, antioxidant, antibacterial, antifungal, emollient, astringent, strong laxative, and narcotic properties (Abd El-Hafeez et al., 2018; Khan et al., 2018).

Phytochemical analysis has been carried out on *M. indicus*, but data are still limited. This plant contains a large range of phytochemical components such as coumarins, glycosides, flavonoids, tannins, saponins, steroids and terpenoids (Abbas et al., 2013; Ahmed and Al-Refai, 2014; Saleem et al., 2020). Coumarin is one of the most important plant secondary metabolite compound among *Melilotus* species, with *M. indicus* having the highest coumarin content (0.943%) (Nair et al., 2010). The genus *Melilotus* is also referred as wild alfafa and the relationship between *Melilotus* and *Medicago* was already confirmed based on molecular typing (Di et al., 2015). Biological activities of *M. indicus*, including antifungal activity, have not been fully investigated yet. To our knowledge only one report was made regarding antifungal activity of *M. indicus* on soil-borne phytopathogenic fungi (Khan et al., 2018). Due to the phylogenetic similarity, and since antifungal activity of plants from *Medicago* genus has already been extensively studied, we decided to investigate the antifungal activity of this extract against phytopathogenic fungi.

1.6. *PLANTAGO MAJOR*

Plantago major L., commonly known as great plantain, is a perennial plant that belongs to the Plantaginaceae family (Wang et al., 2015). This species prefers light (sandy) and medium (loamy) soils, mainly on roadsides, fields, lawns, and waste places in temperate zones worldwide (Willis, 1980; Stanisavljević et al., 2008). Great plantain is a widespread used medicinal plant with astringent, anaesthetic, analeptic, antiviral, antihistaminic, diuretic and hypotensive properties (Du Dat et al., 1992; Núñez Guillén et al., 1997; Gomez-Flores et al., 2000; Chiang et al., 2002; Nhiem et al., 2011). *Plantago major* extract showed an effectiveness as antifungal and demonstrate a dose-dependent reduction on the total growth, biofilm formation, metabolic activity, and cell surface hydrophobicity of *C. albicans*, which is one of the most prevalent fungal pathogen in humans (Shirley et al., 2017; Zida et al., 2017). This plant also has a high phytoremediation potential by cleaning-up the water and soils contaminated with imidacloprid insecticide (Romeh, 2009). *Plantago major* medicinal properties may be attributed to its biologically active compounds, such as phenolic compounds, flavonoids, iridoid glycosides, terpenoids, benzoic compound, tannins, saponins and sterols (Samuelsen, 2000; Stanisavljević et al., 2008). *Carpobrotus edulis* (L.) N.E.Br. is also a plant known for their wide variety of secondary metabolites such as alkaloids, flavonoids, tannins and saponins (Omoruyi et al., 2012). Alkaloids and terpenoids are phytoconstituents present in ethanolic extract of *Portulaca oleracea* L.. (Durgawale et al., 2018).

To our knowledge, only one report investigated the role of *P. major* extract as antifungal agent against phytopathogenic fungi (Silva et al., 2008).

1.7. *URTICA DIOICA*

Urtica dioica L. is an herbaceous perennial flowering plant, which belongs to the Urticaceae family and it is commonly known as stinging nettle (Kregiel et al., 2018). Nettles grow in tropical and temperate wasteland areas, widespread throughout Europe, North America, North Africa and in parts of Asia (Kregiel et al., 2018; Esposito et al., 2019). The effects of *U. dioica* cultivation on the environment can be potentially favorable for several economic and ecological reasons such as: i) low agronomic inputs, ii) satisfying yields for 10-15 years, iii) soil improvement through overfertilization with nitrogen and phosphate and reduction of soil erosion, iv) biodiversity increases in local flora and fauna, and v) production of new high-quality agricultural raw materials for dyeing, textile and energy sectors (Vogl and Hartl, 2003; Bacci et al., 2011; Di Virgilio et al., 2015). The medicinal properties of *U. dioca* are linked to its antioxidant, antimicrobial, antiulcer, analgesic and hypoglycemic effects (Gülçin et al., 2004; Mavi

et al., 2004; Golalipour and Khori, 2007). Nettle extracts showed *in vitro* antifungal activity by inhibiting mycelium growth of several phytopathogenic fungi (Hadizadeh et al., 2009).

Various factors affect the phytochemical content of *U. dioca* plants, such as the variety, genotype, soil, vegetative stage, climate, harvest time, storage, processing and treatment. All the parts of this plant are a rich source of nutrients and contain a significant number of biologically active compounds, including saponins, flavonoids, tannins, coumarins and sterols (Chaurasia and Wichtl, 1987; Komes et al., 2011; Otles and Yalcin, 2012; Dar et al., 2013; Razika et al., 2017; Kregiel et al., 2018). Due to a varied phytochemical composition, *U. dioica* has a great potential of medicinal applicability and it was already explored against some phytopathogenic fungi (Stephan et al., 2005; Hadizadeh et al., 2009; Andrade Pinto et al., 2010; Torun et al., 2018).

1.8. SCIENTIFIC PROBLEM AND OBJECTIVES

Due to the potentially adverse effects of synthetic fungicides on the environment and human health, mutations in pathogens and resistance to these chemicals, the continued use of chemical and synthetic antifungal agents is not considered a sustainable option (Igbedioh, 1991; Lari et al., 2014). The investigation and development of more eco-friendly antifungal alternatives, such as the search for natural compounds with antifungal activity from plant extracts is increasing. Some advantages of using plant extracts are their biodegradability, their environmentally friendly application and the lower probability of resistance development (as they are a mixture of distinct active compounds with different mechanisms of action) (Okwute, 2012; Tolulope Adenubi et al., 2018). That being said, this work aims to demonstrate that plant extracts have strongly antifungal activity and can be used as natural antifungal agents to control phytopathogenic fungi, and consequently, improve agriculture practices.

To achieve this, the following goals have been established:

In vitro evaluation of antifungal activity of *C. edulis*, *Medicago* sp., *M. indicus*, *P. major*, *P. oleracea*, *U. dioica* and a mixture of extracts (*Medicago* sp., *M. indicus*, *P. major* and *U. dioica*) on a range of phytopathogenic fungi (*B. cinerea*, *C. acutatum*, *C. gloeosporioides*, *C. godetiae*, *C. nymphaeae*, *D. corticola* and *P. cinnamomi*), known to affect important Portuguese crops;

Ex vivo investigation of *Medicago* sp. extract antifungal activity on infected leaves of adult strawberry plants.

Chapter 2

MATERIALS AND METHODS

2.1. ANTIFUNGAL ACTIVITY *IN VITRO*

2.1.1. Biological materials, cultivation conditions and preparation of extracts

Six widely available plant species (Table 4) from distinct families were collected fresh, in different seasons, from uncultivated lands near Póvoa de Varzim, Porto, Portugal. All freshly collected plant materials were air-dried (except *C. edulis* and *P. oleracea*, which were dried at 60 °C and 30 °C, respectively, for two weeks) in the dark, grinded in an analytical mill (IKAA11 Basic) to fine uniform texture and stored in glass jars until use.

Table 4. Plant species used in this work.

Plant	Family	Tissues collected	Season
Carpobrotus edulis (L.) N.E.Br	Aizoaceae	Leaves	Autumn
Medicago L. sp.	Fabaceae	Leaves	Summer
Melilotus indicus (L.) All	Fabaceae	Leaves	Spring
Plantago major L.	Plantaginaceae	Stems and leaves	Autumn
Portulaca oleracea L.	Portulacaceae	Stems and leaves	Autumn
Urtica dioica L.	Urticaceae	Leaves	Spring

Stock aqueous extracts were obtained by soaking 7 g of air-dried and milled plant material in 100 mL of 50% (v/v) ethanol at room temperature and protected from light for 7 days with occasional shaking. Then, the macerated plant suspensions were filtrated with a Whatman filter paper and evaporated using a rotary evaporator at 40 °C and 50 revolutions per min (rpm; Büchi 461 Water Bath). After that, aqueous plant extracts were filtered with a 0.2 µm syringe filter (VWR) in order to obtain a sterile aqueous extract. Finally, the extracts were divided in aliquots and stored in the dark at -20 °C to be used in the future. To determine the aqueous extract concentration, 200 µL were placed in a weighed aluminum foil, incubated at 60 °C and weighed after one day of incubation.

The plant biomass yield was calculated as yield (%) = (WE/WP) × 100, where WE and WP are the weights of the milled plant and the dried plant, respectively.

The fungi used in this work are described in Table 5. Fungal cultures were multiplied in Petri dishes with Potato-Dextrose-Agar medium (PDA, Difco ™) and incubated in the dark at 25 °C.

Table 5. Fungi used in this work and corresponding suppliers.

Fungus	Provided by
Botrytis cinerea	António Teixeira; Hernâni Gerós and Richard Gonçalves (University of Minho)
Colletotrichum acutatum isolate PT227	Pedro Talhinhas (Higher School of Agronomy, University of Lisbon)
Colletotrichum gloeosporioides isolate 15-025	Pedro Talhinhas (Higher School of Agronomy, University of Lisbon)
Colletotrichum godetiae isolate 15-019	Pedro Talhinhas (Higher School of Agronomy, University of Lisbon
Colletotrichum nymphaeae isolate 15-006	Pedro Talhinhas (Higher School of Agronomy, University of Lisbon
Diplodia corticola isolate CAA500	Ana Cristina Esteves (Centre for Environmental and Marine Studies, University of Aveiro)
Phytophthora cinnamomi isolate PH107	Helena Machado (National Institute for Agrarian and Veterinarian Research)

2.1.2. Effect of plant extracts on mycelium growth

The effect of *C. edulis*, *Medicago* sp., *M. indicus, P. major*, *P. oleracea* and *U. dioica* on the mycelial growth of phytopathogenic fungi (*B. cinerea*, *C. acutatum*, *C. gloeosporioides*, *C. godetiae*, *C. nymphaeae*, *D. corticola* and *P. cinnamomi*) was investigated. A mixture of aqueous extracts composed by *Medicago* sp., *M. indicus*, *P. major* and *U. dioica* extracts at 20 mg/mL each were mixed in the same volume proportions and tested. The aqueous extracts were incorporated into PDA medium before solidification at 50 °C at different concentrations: 100, 500, 1000 or 2000 µg/mL. Plates with PDA medium without any extract were used as control. Then, mycelial discs with 8 mm in diameter from the margins of 12 days old fungal cultures were collected using the larger circular part of a sterile 100-1000 µL tip and placed in the center of each Petri dish with a sterile toothpick. Next, all inoculated dishes were incubated at 25 °C, protected from light and the radial mycelial growth was measured at days 3, 6 and 9 after inoculation. Data were expressed as the mean colony diameter along two perpendicular lines on the reverse side of each plate. The assay ended when the negative control reached full growth of the Petri dish total area. Finally, the antifungal activity of each extract was calculated in terms of mycelia growth inhibition (MGI) percentage by using the following formula:

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

, where dc is the fungal colony diameter in negative control and dt is the fungal colony diameter in treated sets.

2.2. ANTIFUNGAL ACTIVITY *EX VIVO*

2.2.1. Leaf samples and optimization of the leaf surface sterilization

Leaves of strawberry (*Fragaria* x *ananassa*; variety Portola) were harvested from field-grown plants (field at the Campus of Gualtar of the University of Minho, in Braga) at the beginning of winter season after 7 months of growing. The strawberry leaves surface was sterilized according to Mirza (2018). Firstly, leaves surfaces were washed with tap water, being dipped for 30 s in 70% ethanol, and for 1 min in 250 mL/L sodium hypochlorite at 0,376 M. Lastly, leaves were washed with sterile deionized water (dH_2O) for 2 min to rinse off the disinfection agents. To define the best conditions for the leaves surface sterilization, 9 more treatments were defined, maintaining all the ethanol and dH_2O conditions and varying the exposure time in sodium hypochlorite (Table 6). Whole strawberry leaves were used in the treatment 1, 8, 9 and 10, and sliced parts of strawberry leaves were used in the remaining treatments (2, 3, 4, 5, 6 and 7). The strawberry leaf from which several parts were obtained was first cut and the resulting sections were subjected to the disinfection process. Sterilized leaves were placed in PDA Petri dishes during 5 days in order to verify whether the surface sterilizations were efficient.

Table 6. Optimization of leaf surface sterilization protocol.

Treatment	Sodium hypochlorite (min)
1	1
2	2
3	3
4	5
5	8
6	10
7	12
8	1’20
9	1’40
10	2

2.2.2. Spore suspension preparation and quantification

Mycelial discs of *C. acutatum* with 8 mm diameter from the margins of 12 days old cultures were collected using the larger circular part of a sterile 100-1000 µL tip, placed in the center of a Petri dish using a toothpick and incubated by 14 days at 25 °C in the dark. In the last day, spores were collected by pouring 15 mL of sterile Milli-Q H_2O over the surface of the mycelia and scraping with a sterile tip in order to disperse spores. The spores were counted under a microscope with a haemocytometer, and the final concentration of 9 x 10^5 spores/mL was adjusted with sterile Milli-Q H_20.

2.2.3. Inoculation and incubation of the leaves

Five transparent Petri dishes were used as inoculation chambers to achieve high humidity during the incubation step. Each Petri dish contained a single leaf treated by brushing both surfaces with *Medicago* sp. extract or/and sterile dH_2O with a sterilized brush (0.5 mL used in each leaf). A preventive treatment was applied in the leaves using two different concentrations (1000 µg/mL and 2000 µg/mL) of *Medicago* sp. extract. *Medicago* sp. extract, at 1000 µg/mL and 2000 µg/mL, was applied on both sides of the corresponding leaves with a brush and dried in a laminar flow chamber. Then, the leaves were inoculated by submerging the adaxial side in the *C. acutatum* suspension for 5 s. Leaves were also subjected to the following controls: i) negative control, which was brushed with sterile dH_2O twice (after the first one has dried); ii) extract control was brushed twice with the maximum concentration applied (2000 µg/mL); and iii) the positive control, which was firstly brushed with sterile dH_2O and then the adaxial side of the leaf were submerged in a suspension of *C. acutatum* spores. The inoculated leaves and controls were transferred into their respective transparent Petri dishes containing one paper filter and two pieces of cotton. Sterile dH_2O was added before the incubation (2 mL to each cotton piece and 1 mL in the paper filter) and in the sixth day of the assay to maintain a moisture environment in the Petri dishes. All the Petri dishes were placed near the window for 12 days so that leaves and fungi become exposed to the circadian rhythm. Strawberry leaves were photographed in the flow chamber on days 3, 6, 9 and 12 of the experiment. In the 12th day, each leaf was evaluated under a stereomicroscope and pictures of the fungal colonies developed on the surface of the leaf were taken. After that, fungal suspensions were prepared by scraping the mycelium colonies present on the strawberry leaves surface with a sterile plastic tip. Then, 3 µL of sterile dH_2O was added to the respective mycelium colonies, resuspended on the leaves surface and transferred to the center of each Petri dish with PDA medium incorporated. Lastly, the Petri dishes were incubated for 12 days at 25 °C in the dark and photographed.

2.3. STATISTICAL ANALYSIS

Data are presented as mean ± standard deviation (SD) of three independent experiments. One-way ANOVA and Kruskal Wallis test were used for multiple comparisons. To identify differences between same day concentrations a letter code was used. For this comparison, mean values followed by the same letters are not significant (ns) (lowercase letters for day 3, capital letters for day 6 and Greek letters for day 9). Comparisons between different days of the same concentration are only represented if they are significant and were represented by the asterisk (*) code. Differences were considered statistically significant if * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$ and **** $p \leq 0.0001$. To observe the antifungal effect of the *Medicago* sp. extract on strawberry leaves, in *ex vivo* conditions, one replicate was performed.

Chapter 3

RESULTS AND DISCUSSION

3.1. PLANT EXTRACTS SELECTION AND RESPECTIVE BIOMASS YIELD

The plants described in Table 7 were selected to test their antifungal activity against a range of phytopathogenic fungi. These plants were chosen because previous studies demonstrated that their were able to inhibit fungal growth (Oh et al., 2000; Motsei et al., 2003; Saniewska et al., 2006; Hadizadeh et al., 2009; Shirley et al., 2017; Khan et al., 2018). For the extraction of secondary metabolites, we selected an ethanol (v/v) 50% solution because the mixture of water and ethanol can extract polar and nonpolar compounds. All the plants biomass yield obtained were above 50%, being *U. dioica* the plant with a maximum biomass yield (96.85%; Table 7).

Table 7. Selected plants, parts of plants used to make the extracts and their biomass yield.

Plant	Parts of plant	Yield (%)
C. edulis	Leaves	52.3%
Medicago sp.	Leaves	57.69%
M. indicus	Leaves	71.31%
P. major	Stems and leaves	61.17%
P. oleracea	Leaves	59.6%
U. dioica	Leaves	96.85%

3.2. ANTIFUNGAL ACTIVITY *IN VITRO* OF PLANT EXTRACTS ON PHYTOPATHOGENIC FUNGI

The inhibitory effects of the four plant extracts and their mixture were studied on the mycelial growth of *B. cinerea*, *C. acutatum*, *C. gloeosporioides*, *C. godetiae*, *C. nymphaeae*, *D. corticola* and *P. cinnamomi*. The antifungal activity was determined by measuring the diameters of fungal growth, in negative control and sample plates, on solid medium incorporated with different concentrations of extracts (100, 500, 1000 or 2000 µg/mL). The mycelial growth was measured on the third, sixth and ninth day after inoculation.

3.2.1. *Medicago* sp. extract

3.2.1.1. Effect of *Medicago* sp. extract on mycelial growth of *Botrytis cinerea*

The antifungal effect of *Medicago* sp. against *B. cinerea*, one of the most harmful phytopathogenic fungi in the world, was evaluated. Mycelium disks were placed in the center of Petri dishes with PDA medium supplemented with different concentrations of the extract. To calculate the percentage of MGI, the diameter of the colonies, as well as the ones of the negative control, were recorded and later compared (Figure 2A; Appendix - Table A1). Although *Medicago* sp. extract was not able to inhibit the mycelial growth of *B. cinerea,* it was possible to observe a decrease in it. Concentration did not affect the degree of growth inhibition as the differences between the treatments were not statistically significant. Although the values were very dispersed and not significantly different, some replicates of the treatments with 100 µg/mL and 500 µg/mL extract showed fairly high percentages of inhibition. In addition, the mycelial density seemed to decrease with increasing concentrations of the extract (Figure 2B), suggesting that less biomass was being produced in the treatments with the extract. These differences were very noticeable at concentrations of 500 µg/mL, 1000 µg/mL, and 2000 µg/mL. Although we tried to weight the fungus mycelium, we faced several complications including the extraction and separation of the mycelium from the agar medium and the weighing of a very low and residual mass. Due to these difficulties the mycelium weight was unsuccessful.

Saniewska et al. (2006) showed that total saponin content from roots of *Medicago hybrida* greatly inhibited the growth of *B. cinerea* at 10 µg/mL on the fourth day of incubation (76.9%). Fischer et al. (2011) studied the effect of saponins from *Quillaja saponaria* plant on this phytopathogenic fungi using a saponin concentration of 1000 µg/mL and 2000 µg/mL, which reduced the mycelial growth between 20% - 60%. There are differences in the percentages of MGI of *B. cinerea* reported by these authors and our study. This may be due to the plant product used in the present work, which is composed by a wide spectrum of bioactive constituents, while the cited authors used more saponin-rich products.

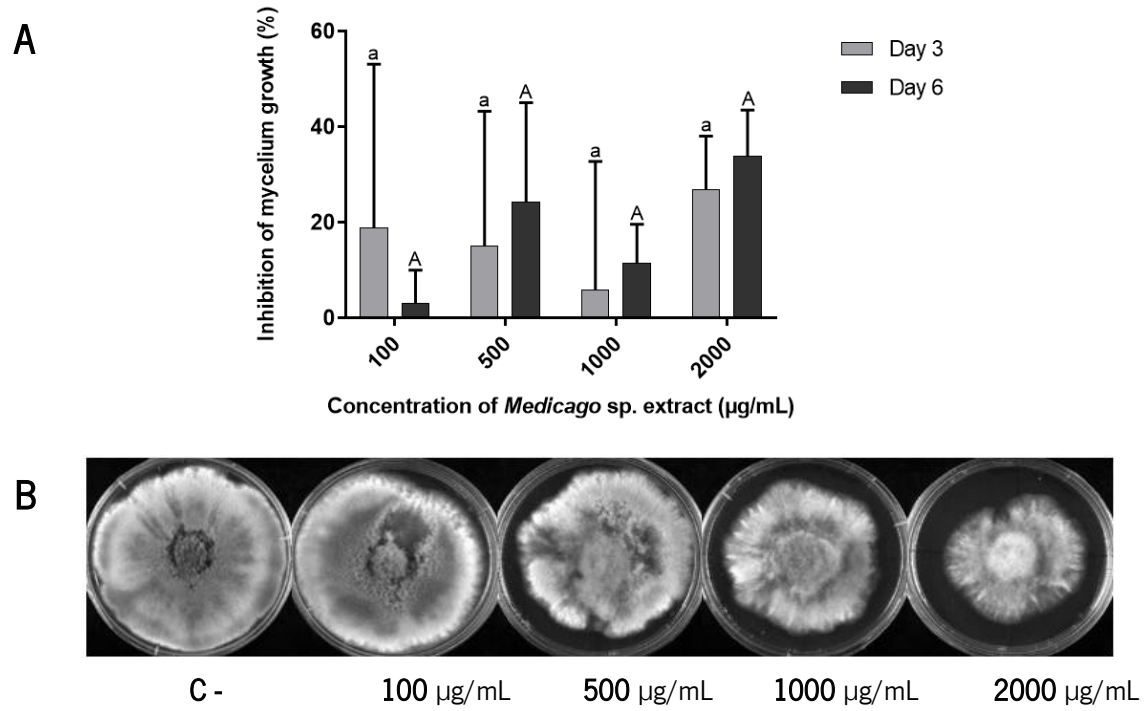

Figure 2. Effect of *Medicago* sp. extract on mycelial growth of *Botrytis cinerea*. The extract was incorporated in PDA medium; a disk of mycelium was placed in the center of the plates and then they were incubated a 25 °C in the dark. (A) The percentage of mycelial growth inhibition determined after 3 and 6 days of incubation at different concentrations of *Medicago* sp. extract, 100, 500, 1000 or 2000 µg/mL. The negative control (C-) was prepared by replacing the extract by the solvent at the highest volume of extract used in the assays. Data are presented as the mean of three independent experiments ± SD. One-way ANOVA and Kruskal Wallis test were used for multiple comparisons. To identify differences between different concentrations of the same day were used the letters code. For this comparison mean values followed by the same letters are not statistically different (lowercase letters for day 3 and capital letters for day 6). Comparisons between different days of the same concentration are only represented if they are significant and were represented by the asterisk code. Differences were considered statistically significant if $*p \leq 0.05$, $** p \leq 0.01$, $*** p \leq 0.001$ and $**** p \leq 0.0001$. (B) Representative images of *Medicago* sp. antifungal activity at different concentrations compared to the negative control (C-) against *Botrytis cinerea* after 6 days of incubation.

3.2.1.2. Effect of *Medicago* sp. extract on mycelial growth of *Colletotrichum* species

Colletotrichum is a wide-spread fungal genus that causes severe diseases in economically important crops. As such, we investigated the antifungal activity of *Medicago* sp. extract in four different species of this genus, including *C. acutatum*, *C. gloeosporioides*, *C. godetiae* and *C. nymphaeae*. The mycelium growth of these species was measured until day 9 of incubation, except for *C. gloeosporioides* (Appendix- Table A2). The *Medicago* sp. extract showed antifungal activity against all species tested (Figure 3). Interestingly, *Medicago* sp. extract caused a dose-dependent effect in all four species. The

percentage of inhibition was statistically different in *C. acutatum* when comparing the higher concentrations (1000 µg/mL and 2000 µg/mL) with the lower concentrations (100 µg/mL and 500 µg/mL) (Figure 3A). In addition, the increasing inhibitions up until the ninth day suggested that the extract has a prolonged effect. In *C. gloeosporioides*, the effect of *Medicago* sp. extract was more notorious in the third day (36.5%) at 2000 µg/mL. *Medicago* sp. greatly inhibited the growth of *C. godetiae* treated with 2000 µg/mL by 42.2% and 39% on days 6 and 9, respectively. Finally, the antifungal activity of *Medicago* sp. extract was evaluated against *C. nymphaeae*. *Colletotrichum nymphaeae* only showed significant differences between 100 µg/mL and 2000 µg/mL concentrations. Accordingly, a maximum inhibition of 29.4% were observed for 2000 µg/mL concentration.

Comparing the four species of the *Colletotrichum* genus tested in this work, we conclude that *C. godetiae* was the most sensitive fungus to *Medicago* sp. extract, showing a percentage of MGI of 42.2%. The remaining fungi, *C. acutatum*, *C. gloeosporioides* and *C. nymphaeae*, exhibited growth inhibition maximum rates between 29.4% - 36.5%. All four species showed changes in mycelial morphology, such as pigmentation, which possibly indicates that the physiology was affected by the extract (Figure 3B).

Pineda et al. (2018) tested the antifungal activity of *Petroselinum crispum* extract against *C. acutatum* at 100, 200, and 400 µg/mL. At 100 µg/mL, essential oil of *P. crispum* exhibited inhibition percentages of *C. acutatum* of almost 60% during the 11 days of the analysis. Methanol extract of *Lantana camara* tested against *C. gloeosporioides* showed a percentage of MGI around 91%. (Dissanayake et al., 2019). In our study, the *Medicago* sp. extract exhibited lower inhibition percentages than the ones reported by these authors. This might be due to differences in the secondary metabolites' compositions and respective amounts.

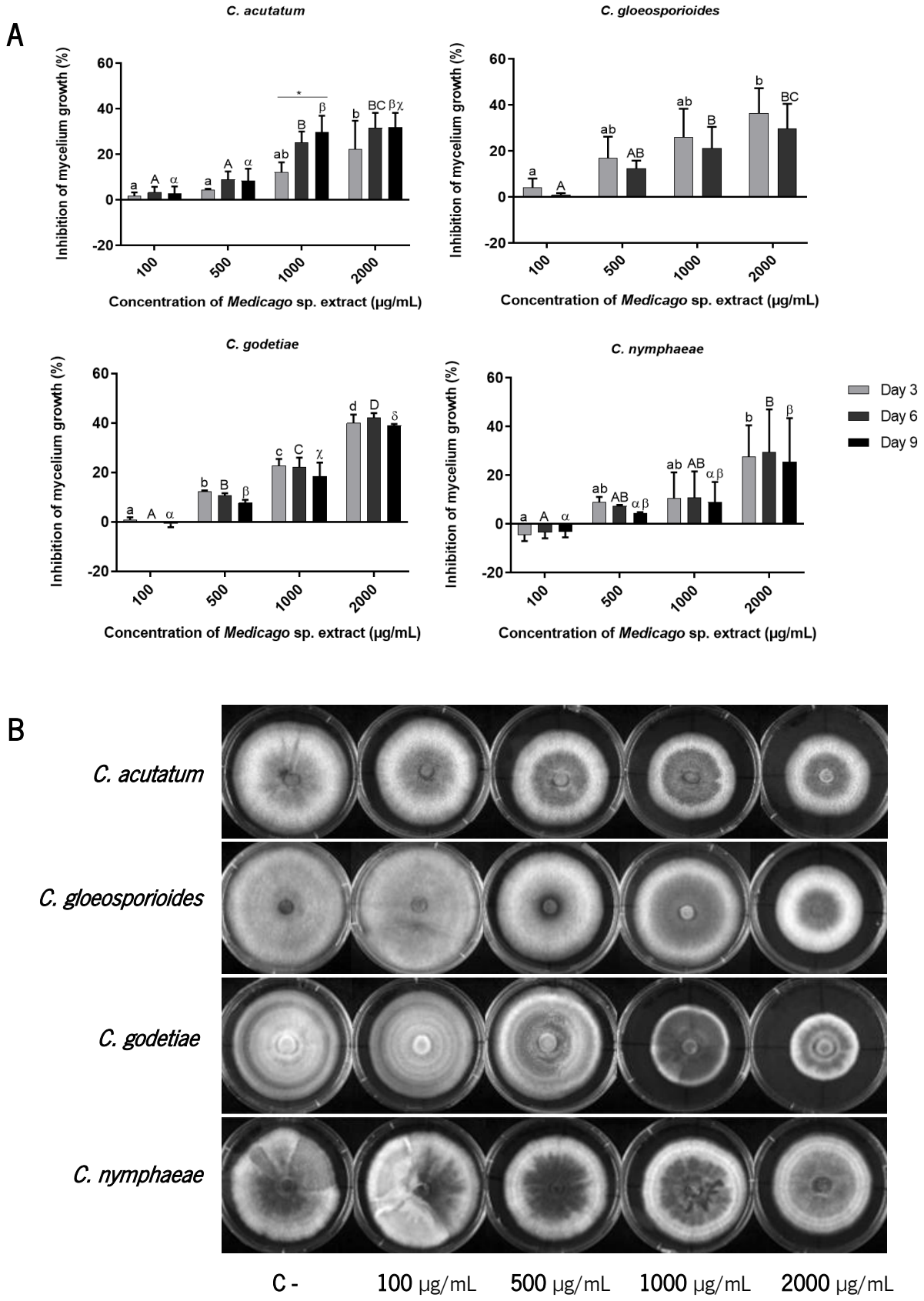

Figure 3. Effect of *Medicago* sp. extract on mycelial growth of *Colletotrichum* species. The extract was incorporated in PDA medium; a disk of mycelium was placed in the center of the plates and then they were incubated a 25 °C in the dark. (A) The

percentage of mycelial growth inhibition determined after 3, 6 and 9 days of incubation at different concentrations of *Medicago* sp. extract, 100, 500, 1000 or 2000 µg/mL. The negative control (C-) was prepared by replacing the extract by the solvent at the highest volume of extract used in the assays. Data are presented as the mean of three independent experiments ± SD. One-way ANOVA and Kruskal Wallis test were used for multiple comparisons. To identify differences between different concentrations of the same day were used the letters code. For this comparison mean values followed by the same letters are not statistically different (lowercase letters for day 3, capital letters for day 6 and Greek letters for day 9). Comparisons between different days of the same concentration are only represented if they are significant and were represented by the asterisk code. Differences were considered statistically significant if $*p \leq 0.05$, $**$ $p \leq 0.01$, $***$ $p \leq 0.001$ and $****$ $p \leq 0.0001$. (B) Representative images of *Medicago* sp. antifungal activity at different concentrations compared to the negative control (C-) against *Colletotrichum* species after 6 days (*Colletotrichum gloeosporioides*) and 9 (*Colletotrichum acutatum*, *Colletotrichum godetiae*, *Colletotrichum nymphaeae*) days of incubation.

3.2.1.3. Effect of *Medicago* sp. extract on mycelial growth of *Diplodia corticola*

Diplodia corticola is regarded as the most virulent fungus involved in cork oak decline. Cork oak is one of the most common forest species in Portugal (ICNF, 2019). So, the antifungal activity of *Medicago* sp. extract was tested against this devastating fungus. *Medicago* sp. extract caused a dose-dependent effect in *D. corticola* growth, however a mycelium reduction was only observed from concentrations 500 µg/mL to 2000 µg/mL (Figure 4A). The antifungal effect of *Medicago* sp. extract on *D. corticola* showed significant differences between 100 µg/mL and 2000 µg/mL concentrations of *Medicago* sp. extract. A maximum inhibition of 60.9% were observed at 2000 µg/mL. To the best of our knowledge, the antifungal activity of *Medicago* extract against *D. corticola* has never been studied.

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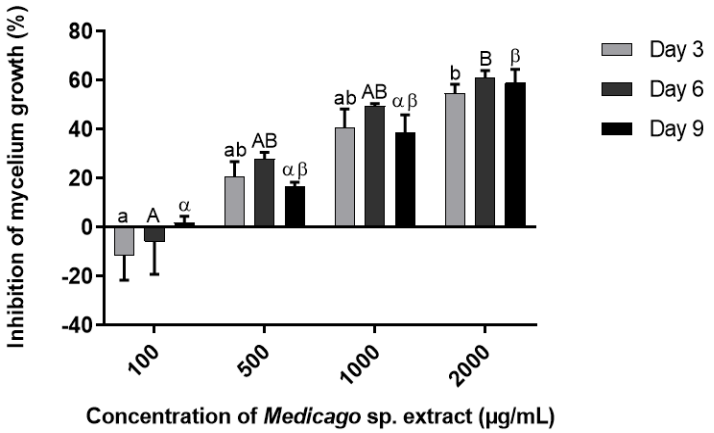

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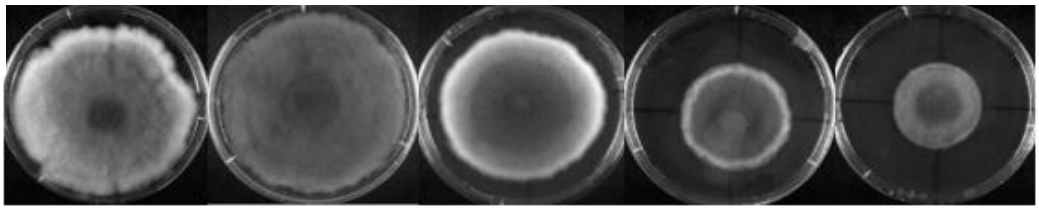

C - 100 µg/mL 500 µg/mL 1000 µg/mL 2000 µg/mL

Figure 4. Effect of *Medicago* sp. extract on mycelial growth of *Diplodia corticola*. The extract was incorporated in PDA medium; a disk of mycelium was placed in the center of the plates and then they were incubated a 25 °C in the dark. (A) The percentage of mycelial growth inhibition determined after 3, 6 and 9 days of incubation at different concentrations of *Medicago* sp. extract, 100, 500, 1000 or 2000 µg/mL. The negative control (C-) was prepared by replacing the extract by the solvent at the highest volume of extract used in the assays. Data are presented as the mean of three independent experiments ± SD. To identify differences between different concentrations of the same day were used the letters code. For this comparison mean values followed by the same letters are not statistically different (lowercase letters for day 3, capital letters for day 6 and Greek letters for day 9). Comparisons between different days of the same concentration are only represented if they are significant and were represented by the asterisk code. Differences were considered statistically significant if $*p \leq 0.05$, $** p \leq 0.01$, $*** p \leq 0.001$ and $**** p \leq 0.0001$. (B) Representative images of *Medicago* sp. antifungal activity at different concentrations compared to the negative control (C-) against *Diplodia corticola* after 9 days of incubation.

3.2.1.4. Effect of *Medicago* sp. extract on mycelial growth of *Phytophthora cinnamomi*

The effect of *Medicago* sp. extract against the oomycete plant pathogen *P. cinnamomi* was also evaluated. As depicted in Fig. 6, *Medicago* sp. extract was able to slightly inhibit the mycelium growth of *P. cinnamomi*. The percentage of MGI increased along the concentrations applied, causing a dose-dependent effect (Figure 5A). The lowest concentration of *Medicago* sp. extract tested, 100 µg/mL, exhibited no effect (0%) on mycelium growth when compared to the negative control. At 2000 µg/mL, *Medicago* sp. extract reduced the radial growth of *P. cinnamomi* by 21.5% on the third day of incubation, however this percentage was not significant when compared to the effect of 100 µg/mL. In the sixth day of incubation, *Medicago* sp. extract showed significant differences between 100 µg/mL and 2000 µg/mL.

Similarly to *B. cinerea* (Figure 2B), *Medicago* sp. extract caused a decrease in the mycelial biomass of *P. cinnamomi* (Figure 5B). This decrease in mycelium density was clearly visible between 500 µg/mL and 2000 µg/mL concentrations. These results suggested that *Medicago* sp. induced an effect on the development of this phytopathogenic fungus mycelium. Since there was less biomass in the presence of the extract, measuring the colonies diameter does not correlate with complete inhibition. Thus, the percentages of inhibition, measured as radial growth, seem to underestimate the inhibition by

the extract. As described for *B. cinerea*, weighing the mycelium was technically difficult to do due to the extraction and separation from the agar medium and its very low total mass resulting in an unsuccessful performed task.

Castillo-Reyes et al. (2015) tested two plants, *Larrea tridentata* and *Flourensia cernua*, against *P. cinnamomi* and obtained minimum inhibitory concentration (MIC) values necessary to inhibit 50% of the fungus of 6.96 mg/L and 8.6 mg/L, respectively. These results show that distinct plant extracts possess antifungal activity against *P. cinnamomi*, however the comparison between results is difficult since they are evaluated in different parameters (MIC_{50} and percentage of MGI). As far as we know, there are no reports studying the antifungal activity of *Medicago* extract against *P. cinnamomi*.

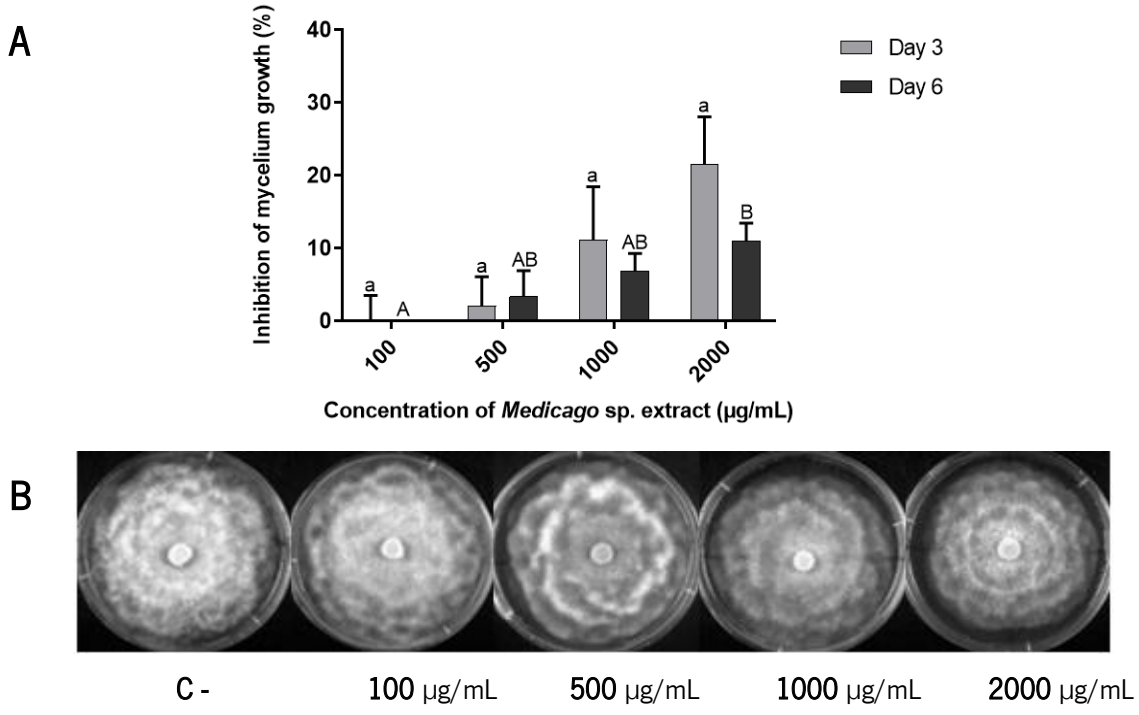

Figure 5. Effect of *Medicago* sp. extract on mycelial growth of *Phytophthora cinnamomi*. The extract was incorporated in PDA medium; a disk of mycelium was placed in the center of the plates and then they were incubated a 25 °C in the dark. (A) The percentage of mycelial growth inhibition determined after 3 and 6 days of incubation at different concentrations of *Medicago* sp. extract, 100, 500, 1000 or 2000 µg/mL. The negative control (C-) was prepared by replacing the extract by the solvent at the highest volume of extract used in the assays. Data are presented as the mean of three independent experiments ± SD. To identify differences between different concentrations of the same day were used the letters code. For this comparison mean values followed by the same letters are not statistically different (lowercase letters for day 3 and capital letters for day 6). Comparisons between different days of the same concentration are only represented if they are significant and were represented by the asterisk code. Differences were considered statistically significant if * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$ and **** $p \leq 0.0001$. (B) Representative images of *Medicago* sp. antifungal activity at different concentrations compared to

the negative control (C) against *Phytophthora cinnamomi* after 6 days of incubation.

Concluding, *Medicago* sp. aqueous extract showed antifungal activity against all phytopathogenic fungi tested. This plant extract not only inhibited the radial mycelial growth but also reduced the mycelial biomass and altered the mycelial morphology over the applied concentrations.

Medicago species are known for their high content in secondary metabolites, mainly in saponins (Tava and Avato, 2006; D'Addabbo et al., 2020). This suggests that antifungal activity of *Medicago* sp. extract revealed against the phytopathogenic fungi tested may be due to the action of saponins. The antifungal activity of plant extracts rich in saponins is probably due to their interference with fungal membrane sterols (mainly ergosterol) by the formation of a saponin/sterol complex, which results in increased membrane permeability and leakage of cell contents or induction of programmed cell death in sensitive fungal cells (Ahmed et al., 2012; Ito et al., 2007). Some fungal species have the capacity to protect themselves from saponin toxicity due to their ability to secrete saponin-detoxifying enzymes, while others have intrinsic resistance due to the specific structure of their cell membrane (Barile et al., 2007; Teshima et al., 2013). The oomycete *P. cinnamomi* was the less sensitive to *Medicago* sp. extract. Barile et al. (2007) reported that *Pythium ultimum*, an oomycete, showed resistance against the effect of the saponin-rich plant *Allium minutiflorum* when compared to true fungi tested. This tolerance of *P. cinnamomi* could be related with the lack of sterols in the membrane of oomycetes. The class of triazole fungicides are inefficient against diseases caused by *Phytophthora* species, since these fungicides target CYP51 enzymes responsible for sterol synthesis, and there is a lack of these enzymes in *Phytophthora* species (Tyler et al., 2006; Gaulin et al., 2010). *Medicago* sp. extract has shown to induce distinct inhibition rates against different fungi. Consequently, this might be related to differences in the composition of the fungal plasma membrane. To corroborate this theory, further assays could be performed to evaluate the ergosterol quantity in membranes on the tested fungi.

3.2.2. *Melilotus indicus* extract

3.2.2.1. Effect of *Melilotus indicus* extract on mycelial growth of *Botrytis cinerea*

The antifungal properties of *M. indicus* were evaluated against *B. cinerea*. *Melilotus indicus* extract caused a slight decrease on fungal growth at all concentrations tested (Figure 6). Unlike the third day, in the sixth day of incubation the mean values of percentage of MGI increased along the applied

concentrations in a robust way, despite they were not significantly different. As observed in the effect of *Medicago* sp. extract on *B. cinerea* (Figure 2A), the values of the three replicates were very dispersed resulting in non-significant differences between the tested concentrations (Figure 6A). However, there were replicas with a percentage of *B. cinerea* growth inhibition around 40%. The effect of *M. indicus* was able to change mycelial density and morphology of *B. cinerea* over the applied concentrations (Figure 6B). These differences were very noticeable at concentrations of 500 µg/mL, 1000 µg/mL, and 2000 µg/mL when compared to the mycelium aspect of the negative control. It was also visible a decrease of mycelium dark color, which could be indicative of a less dense mycelium. Schumacher et al. (2014) reported that dark pigmented sclerotia (compact masses of mycelium with a central core of hyphae with lipid and glycogen reserves) serve as survival structures, which suggests that *M. indicus* was able to reduce the potential for survival and infection of *B. cinerea*.

Combination of three saponins from the bulbs of white onion, *Allium cepa* L., completely inhibited the mycelium growth of *B. cinerea* (Lanzotti et al., 2012). The high percentage of inhibition obtained by the cited authors may be due to the use of isolated and purified saponins. The antifungal activity may vary according to the use of plant extracts and/or purified metabolites against the phytopathogenic fungi. However, the use of plant extracts as natural fungicides are a more cost-effective option. Righini et al. (2019) investigated the antifungal potential of three algae's aqueous extracts, *Anabaena* sp., *Ecklonia* sp. and *Jania* sp. against *B. cinerea*. These algae, that are rich in polysaccharides, influenced fungal growth, ranging from 6% to 9.8% for 2500 µg/mL and 5000 µg/mL, respectively (Righini et al., 2019). These results are lower than those obtained in this work, which could be explained by the differences in chemical composition of these algae and *M. indicus* (mainly composed by coumarins) (Saleem et al., 2020).

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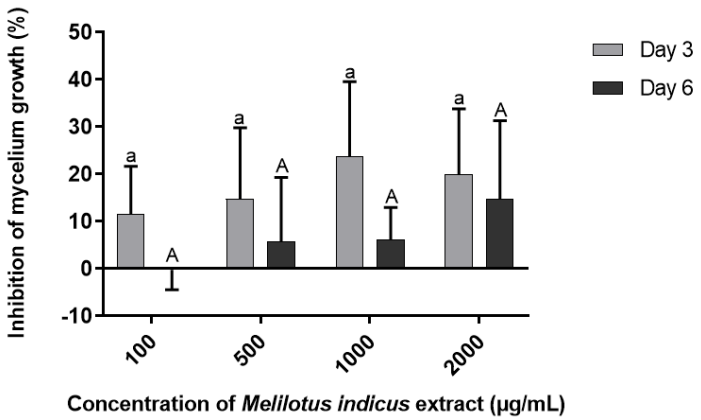

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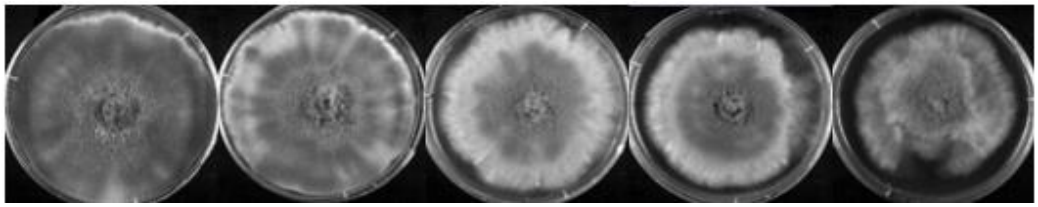

C - 100 µg/mL 500 µg/mL 1000 µg/mL 2000 µg/mL

Figure 6. Effect of *Melilotus indicus* extract on mycelial growth of *Botrytis cinerea*. The extract was incorporated in PDA medium; a disk of mycelium was placed in the center of the plates and then they were incubated a 25 °C in the dark. (A) The percentage of mycelial growth inhibition determined after 3 and 6 days of incubation at different concentrations of *Melilotus indicus* extract, 100, 500, 1000 or 2000 µg/mL. The negative control (C-) was prepared by replacing the extract by the solvent at the highest volume of extract used in the assays. Data are presented as the mean of three independent experiments ± SD. To identify differences between different concentrations of the same day were used the letters code. For this comparison mean values followed by the same letters are not statistically different (lowercase letters for day 3 and capital letters for day 6). Comparisons between different days of the same concentration are only represented if they are significant and were represented by the asterisk code. Differences were considered statistically significant if * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$ and **** $p \leq 0.0001$. (B) Representative images of *Melilotus indicus* antifungal activity at different concentrations compared to the negative control (C-) against *Botrytis cinerea* after 6 days of incubation.

3.2.2.2. Effect of *Melilotus indicus* extract on mycelial growth of *Colletotrichum* species

Antifungal activity of *M. indicus* was investigated in four different species of *Colletotrichum* genus, namely in *C. acutatum*, *C. gloeosporioides*, *C. godetiae* and *C. nymphaeae*. The mycelium growth of these species, except *for C. gloeosporioides*, was measured during 9 days of incubation (Appendix – Table A6). *Melilotus indicus* extract induced a dose-dependent effect in all four species, showing differences in MGI along the applied concentrations (Figure 7A). Regarding the growth of *C. acutatum*, *M. indicus* extract showed significant differences between 100 µg/mL and 2000 µg/mL on day 6 and day 9 of incubation. Thus, this plant extract induced a higher inhibition rate (28.7%) at 2000 µg/mL. *Melilotus indicus* extract was able to significantly reduce the growth of *C. gloeosporioides* by 25.9% at 2000 µg/mL when compared to the percentage of inhibition at 100 µg/mL. This plant extract acted in a similar way in the mycelium growth of *C. godetiae* and in the *C. nymphaeae* since both showed significant differences between 100 µg/mL and 2000 µg/mL on the third, sixth and ninth days of incubation. However, at the highest concentration *M. indicus* extract had a more significant effect on inhibiting the growth of *C. nymphaeae* (24.8%) than *C. godetiae* mycelium (18.9%).

Comparing the antifungal activity of *M. indicus* extract in all *Colletotrichum* species, we found

that *C. godetiae* was the less sensitive fungus, which was inhibited up to 18.9%. The other phytopathogens exhibited growth inhibition rates between 24.8% and 28.7%. It was also observed that the morphology and pigmentation of *C. acutatum*, *C. gloeosporioides*, *C. godetiae* and *C. nymphaeae* underwent changes along the applied concentrations (Figure 7B). In *Colletotrichum* species, melanin is produced by the 1,8-dihydroxynaphthalene (1,8-DHN) pathway (Tsuji et al., 2003). Several studies showed that melanin-based pigmentation is essential for fungal pathogenesis (Tsuji et al., 2003; Langfelder et al., 2003). Our results showed modifications in pigmentation of all *Colletotrichum* species tested, however it is not possible to assure that this extract affected the 1,8-DHN pathway in the four *Colletotrichum* species, since the dark pigment, also called melanin, appear to be darker in *C. godetiae* mycelium at 2000 µg/mL.

Mimosa diplotricha, a plant of the same family (Fabaceae) of *M. indicus* was tested against *C. gloeosporioides* (Deressa et al., 2015). *Mimosa diplotricha* revealed a percentage of radial MGI of 26.6%. Our results are in concordance with the percentage obtained by Deressa and his collaborators, however they used a higher concentration of plant extract. Martinez et al. (2020) tested the antifungal potential of two coumarins, 7-demethylsuberosin and xanthyletin, against *C. gloeosporioides*. Unlike our results, these authors reported that 7-demethylsuberosin reached 43% of mycelial growth inhibition after 8 days of incubation (Martinez et al., 2020). These differences may be explained by the use of two purified coumarins by the cited authors, while in the present work a mixture with other bioactive compounds was used.

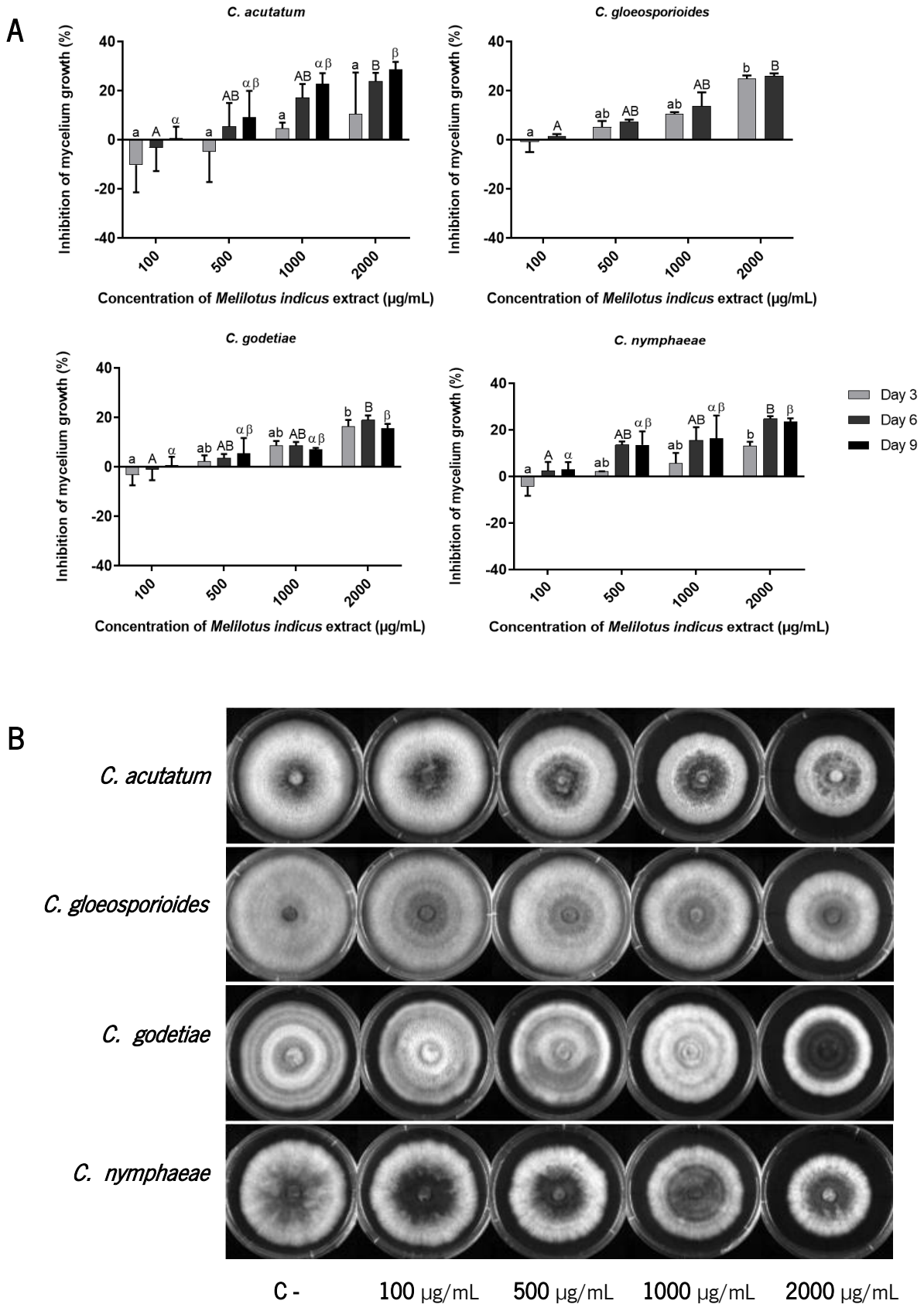

Figure 7. Effect of *Melilotus indicus* extract on mycelial growth of *Colletotrichum* species. The extract was incorporated in PDA medium; a disk of mycelium was placed in the center of the plates and then they were incubated a 25 °C in the dark. (A) The

percentage of mycelial growth inhibition determined after 3, 6 and 9 days of incubation at different concentrations of *Melilotus indicus* extract, 100, 500, 1000 or 2000 µg/mL. The negative control (C-) was prepared by replacing the extract by the solvent at the highest volume of extract used in the assays. Data are presented as the mean of three independent experiments ± SD. To identify differences between different concentrations of the same day were used the letters code. For this comparison mean values followed by the same letters are not statistically different (lowercase letters for day 3, capital letters for day 6 and Greek letters for day 9). Comparisons between different days of the same concentration are only represented if they are significant and were represented by the asterisk code. Differences were considered statistically significant if $*p \leq 0.05$, $** p \leq 0.01$, $*** p \leq 0.001$ and $**** p \leq 0.0001$. (B) Representative images of *Melilotus indicus* antifungal activity at different concentrations compared to the negative control (C-) against *Colletotrichum* species after 6 days (*Colletotrichum gloeosporioides*) and 9 (*Colletotrichum acutatum*, *Colletotrichum godetiae*, *Colletotrichum nymphaeae*) days of incubation.

3.2.2.3. Effect of *Melilotus indicus* extract on mycelial growth of *Diplodia corticola*

The inhibitory effect of *M. indicus* extract was evaluated against *D. corticola*. We observed that *M. indicus* extract caused a decrease in mycelial growth of this plant pathogen at all concentrations tested (Figure 8). This plant extract displayed the highest inhibitory effect (16.7%) at 2000 µg/mL (Figure 8A). At the highest concentration (2000 µg/mL), the mean values of the percentage of MGI were relatively low, but there were replicates with inhibitions greater than 20%. It is worthy to note that *M. indicus* extract revealed significant differences between 100 µg/mL and 2000 µg/mL on the third and sixth days of incubation. As far as we know, there are no studies on the antifungal activity of plan t extracts against *D. corticola*.

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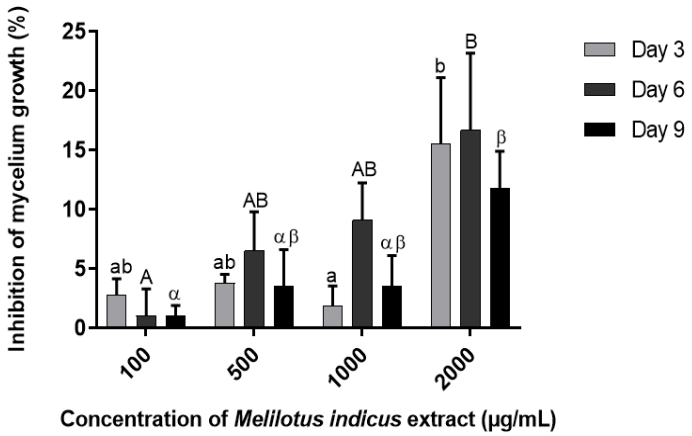

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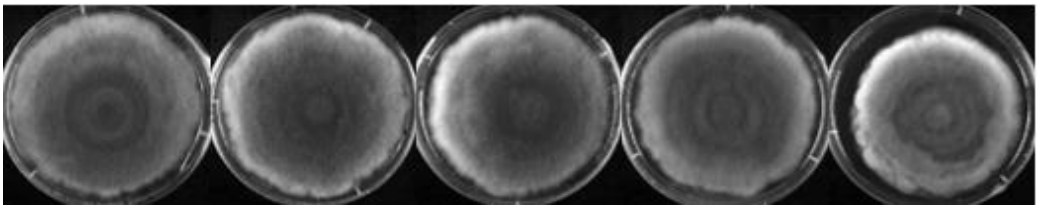


C - 100 µg/mL 500 µg/mL 1000 µg/mL 2000 µg/mL

Figure 8. Effect of *Melilotus indicus* extract on mycelial growth of *Diplodia corticola*. The extract was incorporated in PDA medium; a disk of mycelium was placed in the center of the plates and then they were incubated a 25 °C in the dark. (A) The percentage of mycelial growth inhibition determined after 3, 6 and 9 days of incubation at different concentrations of *Melilotus indicus* extract, 100, 500, 1000 or 2000 µg/mL. The negative control (C-) was prepared by replacing the extract by the solvent at the highest volume of extract used in the assays. Data are presented as the mean of three independent experiments ± SD. To identify differences between different concentrations of the same day were used the letters code. For this comparison mean values followed by the same letters are not statistically different (lowercase letters for day 3, capital letters for day 6 and Greek letters for day 9). Comparisons between different days of the same concentration are only represented if they are significant and were represented by the asterisk code. Differences were considered statistically significant if * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$ and **** $p \leq 0.0001$. (B) Representative images of *Melilotus indicus* antifungal activity at different concentrations compared to the negative control (C-) against *Diplodia corticola* after 9 days of incubation.

3.2.2.4. Effect of *Melilotus indicus* extract on mycelial growth of *Phytophthora cinnamomi*

We also completed the study of *M. indicus* extract by determining MGI on *P. cinnamomi*. Treatments with *M. indicus* extract reduced the mycelial growth on PDA Petri dishes in a concentration-dependent manner, and also showed remarkably significant differences from 100 µg/mL to 2000 µg/mL (Figure 9A). In the two highest applied concentrations, 1000 µg/mL and 2000 µg/mL, the extract proved to be highly effective by reducing *P. cinnamomi* mycelium around 64% and 87.5%, respectively. There was also a notorious decrease in the mycelial biomass of *P. cinnamomi* (Figure 9B). Besides the colony diameter, the reduction in the density of this plant pathogenic oomycete mycelium was clearly visible between 500 µg/mL and 2000 µg/mL. These results suggest that *M. indicus* extract induced an effect on the phytopathogenic fungus mycelium development.

Churngchow and Rattarasarn (2001) revealed that scopoletin, an hydroxycoumarin, displayed antifungal activity by inhibiting the mycelium growth of *Phytophthora* spp.. Despite in this work, we have used a coumarins-rich plant extract instead of purified coumarins, the antifungal potential of *M. indicus* extract was very notorious against the oomycete *P. cinnamomi*. These results suggest that *M. indicus* extract may be an eco-friendly and cost-effective alternative to anti-oomycete synthetic fungicides.

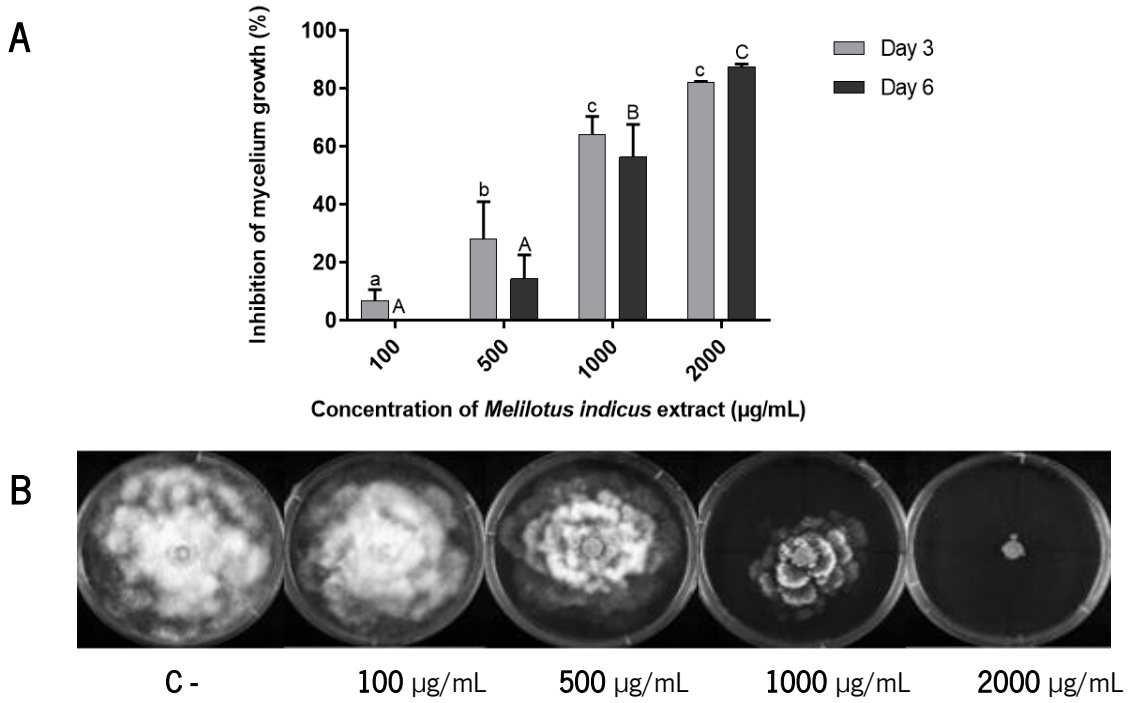


Figure 9. Effect of *Melilotus indicus* extract on mycelial growth of *Phytophthora cinnamomi*. The extract was incorporated in PDA medium; a disk of mycelium was placed in the center of the plates and then they were incubated a 25 °C in the dark. (A) The percentage of mycelial growth inhibition determined after 3 and 6 days of incubation at different concentrations of *Melilotus indicus* extract, 100, 500, 1000 or 2000 µg/mL. The negative control (C-) was prepared by replacing the extract by the solvent at the highest volume of extract used in the assays. Data are presented as the mean of three independent experiments ± SD. To identify differences between different concentrations of the same day were used the letters code. For this comparison mean values followed by the same letters are not statistically different (lowercase letters for day 3 and capital letters for day 6). Comparisons between different days of the same concentration are only represented if they are significant and were represented by the asterisk code. Differences were considered statistically significant if $*p \leq 0.05$, $** p \leq 0.01$, $*** p \leq 0.001$ and $**** p \leq 0.0001$. (B) Representative images of *Melilotus indicus* antifungal activity at different concentrations compared to the negative control (C-) against *Phytophthora cinnamomi* after 6 days of incubation.

Concluding, *M. indicus*, a plant known for their high content of coumarins, showed a considerable fungitoxic effect against all phytopathogenic fungi tested. However, noticeable differences were observed in the MGI of true fungi (*B. cinerea*, *C. acutatum*, *C. gloeosporioides*, *C. godetiae*, *C. nymphaeae* and *D. corticola*) and oomycete (*P. cinnamomi*). In general, the rates of MGI of the tested fungi were similar between the different concentrations, except for *P. cinnamomi*, which revealed an effect dose-dependent.

In *in vitro* assays, the oomycete pathogen *Phytophthora palmivora* showed a higher sensitivity to scopoletin coumarin than the other true fungi tested (Churngchow and Rattarasarn, 2001). Dietrich and

Valio (1973) also showed that a coumarin was significantly more toxic to three oomycetes species than against other fungi tested. A basic coumarin was previously reported to inhibit cellulose biosynthesis in higher plants, which also suggest that the efficacy of coumarins against oomycetes is connected to the structural differences in the cellulose-based cell walls of oomycetes and the chitin-based cell walls of true fungi (Hara et al., 1973).

At the present, the only fungicides available to control infections caused by Oomycota organisms are phosphonate-based compounds (FRAC code 33), phenylamide compounds (FRAC code 4) and four new Oomycota fungicides, such as ethaboxam (FRAC code 22), fluopicolide (FRAC code 43), mandipropamid (FRAC code 40) and oxathiapripolin (FRAC code 49; Fungicide Resistance Action Committee, 2020). The risk of *P. cinnamomi* developing resistance to Oomycota synthetic fungicides is considered high due to their single-site mode of action. This work suggests that *M. indicus* extract could have multiple modes of action, since they can inhibit true fungi and oomycetes. This plant extract may be an excellent and potential sustainable alternative to synthetic anti-oomycetes given its high percentage of MGI of *P. cinnamomi*.

3.2.3. *Plantago major* extract

3.2.3.1. Effect of *Plantago major* extract on mycelial growth of *Botrytis cinerea*

Antifungal activity of the aqueous extract of *P. major* was evaluated against *B. cinerea*. Although this extract slightly inhibited the mycelial growth of *B. cinerea,* there were no statistically significative differences (Figure 10A). Treatments with 500 µg/mL, 1000 µg/mL, and 2000 µg/mL of *P. major* extract exhibited replicas with mycelium growth reduction around 50% - 60%. *Plantago major* extract induced the highest inhibitory effect (12.9%) in *B. cinerea* at 500 µg/mL.

Plantago major extract was able to modify *B. cinerea* mycelial density and morphology over the applied concentrations (Figure 10B). These changes were very evident at 500 µg/mL, 1000 µg/mL, and 2000 µg/mL concentrations when compared to the mycelium aspect of the negative control. It was also noticeable a decrease of mycelium dark color, which could be indicative of a less dense mycelium. As already mentioned, Schumacher et al. (2014) indicated that dark pigmented sclerotia serves as survival structures. The reduction of dark pigmentation suggests that *P. major* extract may reduce the amount of sclerotia, and consequently, the potential for survival and infection of *B. cinerea*.

Bahraminejad et al. (2015) found that *Plantago lanceolata* had no antifungal effect against *B. cinerea*. As far as we know, the antifungal activity of *P. major* extract has never been studied against this phytopathogenic fungi.

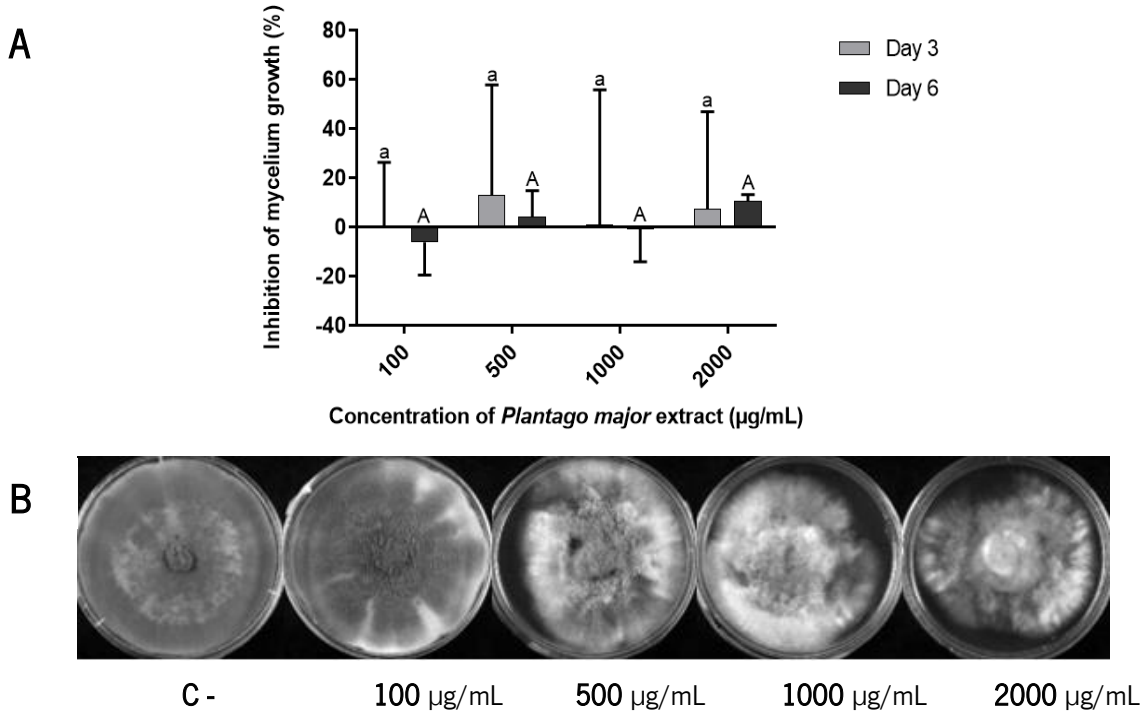


Figure 10. Effect of *Plantago major* extract on mycelial growth of *Botrytis cinerea*. The extract was incorporated in PDA medium; a disk of mycelium was placed in the center of the plates and then they were incubated a 25 °C in the dark. (A) The percentage of mycelial growth inhibition determined after 3 and 6 days of incubation at different concentrations of *Plantago major* extract, 100, 500, 1000 or 2000 µg/mL. The negative control (C-) was prepared by replacing the extract by the solvent at the highest volume of extract used in the assays. Data are presented as the mean of three independent experiments ± SD. To identify differences between different concentrations of the same day were used the letters code. For this comparison mean values followed by the same letters are not statistically different (lowercase letters for day 3 and capital letters for day 6). Comparisons between different days of the same concentration are only represented if they are significant and were represented by the asterisk code. Differences were considered statistically significant if * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$ and **** $p \leq 0.0001$. (B) Representative images of *Plantago major* antifungal activity at different concentrations compared to the negative control (C-) against *Botrytis cinerea* after 6 days of incubation.

3.2.3.2. Effect of *Plantago major* extract on mycelial growth of *Colletotrichum* species

Due to the high variety of *Colletotrichum* species causing serious infections and diseases in economically important crops, the antifungal activity of *P. major* extract was also evaluated against *C.*

acutatum, *C. gloeosporioides*, *C. godetiae* and *C. nymphaeae*. *Plantago major* extract induced a dose dependent effect on the four species of the genus *Colletotrichum* (Figure 11). Regarding the mycelium growth of *C. acutatum*, *C. godetiae* and *C. nymphaeae, P. major* extract caused significant differences between 100 µg/mL and 2000 µg/mL, on day 6 and day 9 (Figure 11A). This plant extract demonstrated statistically significant differences on *C. gloeosporioides* between 100 µg/mL and the two highest concentrations (1000 µg/mL and 2000 µg/mL) in day 3 and day 6. Maximum growth inhibition (25.7%) was obtained against *C. gloeosporioides* with 2000 µg/mL of extract, followed by *C. acutatum* (23.9%) and by *C. godetiae* and *C. nymphaeae* (21.1%).

Comparing the four species of the *Colletotrichum* genus tested in this work, we conclude that the four fungi revealed very similar inhibitions of mycelium growth. *Colletotrichum acutatum*, *C. gloeosporioides*, *C. godetiae* and *C. nymphaeae* demonstrated changes in mycelium pigmentation, which acquires whiter pigmentation along the applied concentrations (Figure 11B). As already mentioned, melanin is produced by the 1,8-DHN pathway in *Colletotrichum* species and are involved in fungal pathogenesis (Tsuji et al., 2003; Langfelder et al., 2003). This suggests that *P. major* extract may affect the 1,8-DHN pathway or some intermediates leading to a loss of pathogenicity of these fungi.

Bazie et al. (2014) found that *P. lanceolata* showed no antifungal activity against *Colletotrichum musae*. Silva et al. (2008) reported that *P. lanceolata* and *P. major* extracts revealed antifungal activity against *C. gloeosporioides.* The results reported by these authors suggest that different species of *Colletotrichum* genus could have different susceptibilities to the same extract (*P. lanceolata*). As in the present work, the cited authors also reported antifungal activity of *Plantago* species against *C. gloeosporioides*.

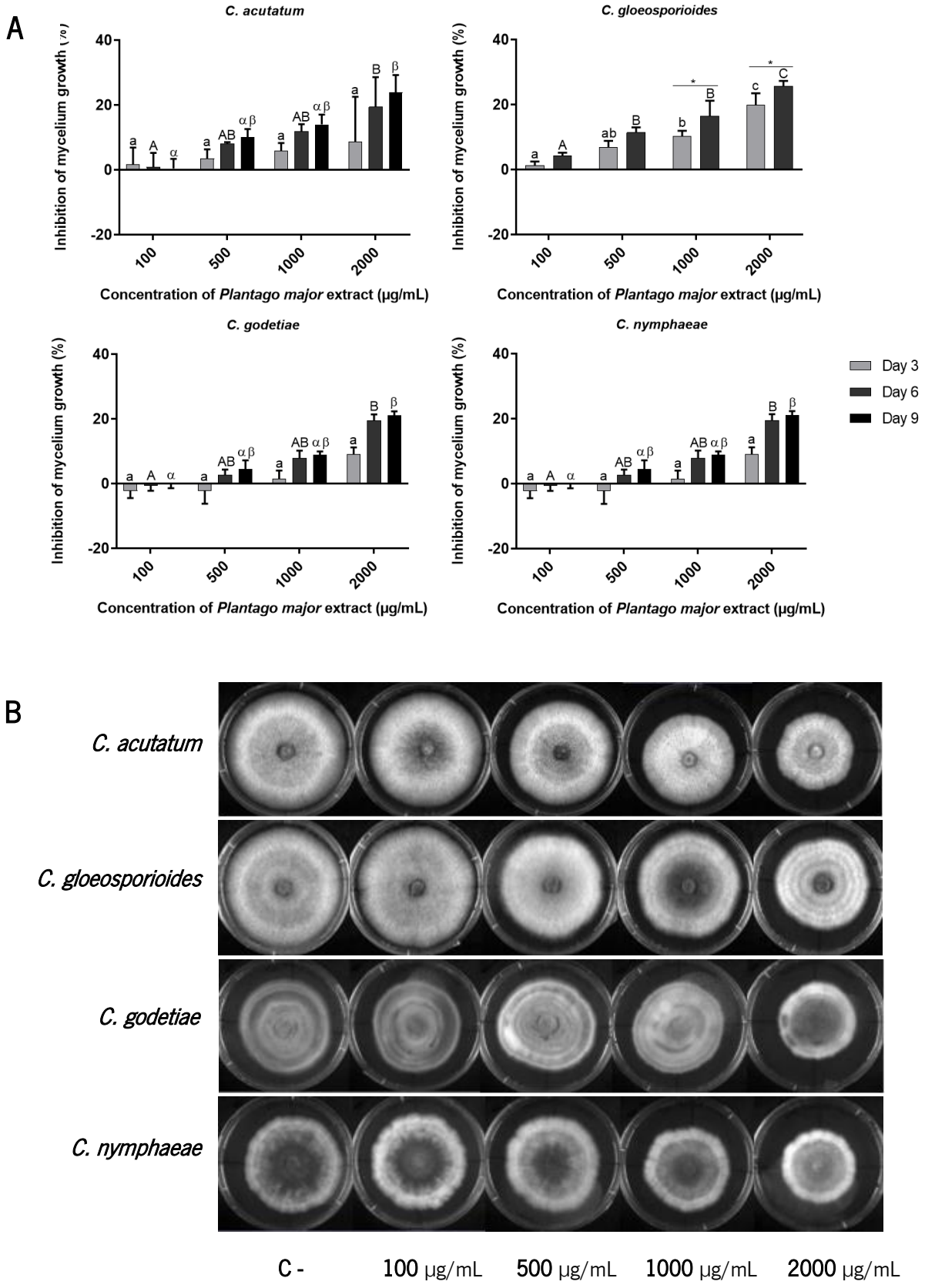


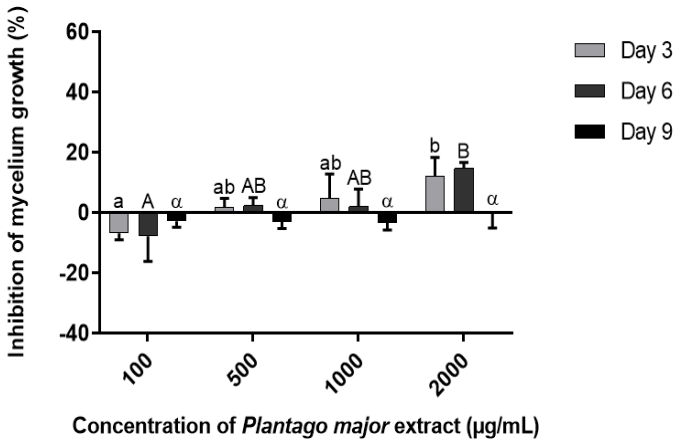
Figure 11 . Effect of *Plantago major* extract on mycelial growth of *Colletotrichum* species. The extract was incorporated in PDA medium; a disk of mycelium was placed in the center of the plates and then they were incubated a 25 °C in the dark. (A) The

percentage of mycelial growth inhibition determined after 3, 6 and 9 days of incubation at different concentrations of *Plantago major* extract, 100, 500, 1000 or 2000 µg/mL. The negative control (C-) was prepared by replacing the extract by the solvent at the highest volume of extract used in the assays. Data are presented as the mean of three independent experiments ± SD. To identify differences between different concentrations of the same day were used the letters code. For this comparison mean values followed by the same letters are not statistically different (lowercase letters for day 3, capital letters for day 6 and Greek letters for day 9). Comparisons between different days of the same concentration are only represented if they are significant and were represented by the asterisk code. Differences were considered statistically significant if $*p \leq 0.05$, $** p \leq 0.01$, $*** p \leq 0.001$ and $**** p \leq 0.0001$. (B) Representative images of *Plantago major* antifungal activity at different concentrations compared to the negative control (C-) against *Colletotrichum* species after 6 days (*Colletotrichum gloeosporioides*) and 9 (*Colletotrichum acutatum*, *Colletotrichum godetiae*, *Colletotrichum nymphaeae*) days of incubation.

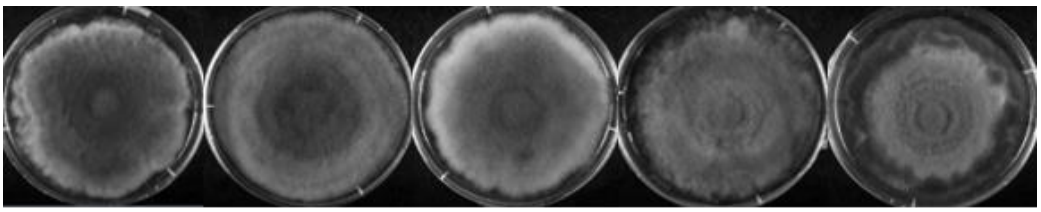
3.2.3.3. Effect of *Plantago major* extract on mycelial growth of *Diplodia corticola*

The antifungal potential of *P. major* extract against *D. corticola* was studied. Mycelial growth of *D. corticola* decreased with the increase of the plant extract concentrations (Figure 12). Until the third and sixth day, the *D. corticola* mycelium grew slowly, but on the ninth day of incubation there was an increase in the mycelium growth resulting in a reduction of MGI. These data suggest an adaptation of *D. corticola* to the action of the extract after 6 days of incubation (Figure 12A). However, there were significant differences between the lowest (100 µg/mL) and the highest (2000 µg/mL) concentrations on the third and sixth days. On sixth day of incubation, *P. major* exerted the highest mycelial growth reduction of 14.6%. Although the effect of inhibiting the growth of the mycelium of this extract is relatively low, *P. major* extract was able to reduce the mycelium density at 2000 µg/mL (Figure 12B). According to our knowledge, the antifungal activity of *P. major* has never been studied against *D. corticola.*

A



B



C - 100 µg/mL 500 µg/mL 1000 µg/mL 2000 µg/mL

Figure 12. Effect of *Plantago major* extract on mycelial growth of *Diplodia corticola*. The extract was incorporated in PDA medium; a disk of mycelium was placed in the center of the plates and then they were incubated a 25 °C in the dark. (A) The percentage of mycelial growth inhibition determined after 3, 6 and 9 days of incubation at different concentrations of *Plantago major* extract, 100, 500, 1000 or 2000 µg/mL. The negative control (C-) was prepared by replacing the extract by the solvent at the highest volume of extract used in the assays. Data are presented as the mean of three independent experiments ± SD. To identify differences between different concentrations of the same day were used the letters code. For this comparison mean values followed by the same letters are not statistically different (lowercase letters for day 3, capital letters for day 6 and Greek letters for day 9). Comparisons between different days of the same concentration are only represented if they are significant and were represented by the asterisk code. Differences were considered statistically significant if $*p \leq 0.05$, $** p \leq 0.01$, $*** p \leq 0.001$ and $**** p \leq 0.0001$. (B) Representative images of *Plantago major* antifungal activity at different concentrations compared to the negative control (C-) against *Diplodia corticola* after 9 days of incubation.

3.2.3.4. Effect of *Plantago major* extract on mycelial growth of *Phytophthora cinnamomi*

The antifungal activity of *P. major* extract was tested against the oomycete *P. cinnamomi*. At 100 µg/mL and 500 µg/mL, *P. major* extract was no capacity to reduce mycelial growth (Figure 13A). The MGI reached a maximum inhibition rate of 32.2% at 2000 µg/mL on the sixth day. There was a significantly difference between 100 µg/mL and 2000 µg/mL of *P. major* extract in day 3 of incubation.

Similarly to the treatments with *Medicago* sp. and *M. indicus*, *P. major* extract also affected the mycelial biomass of *P. cinnamomi* along the applied concentrations (Figure 13B). We also verified that there was no MGI at 1000 µg/mL, however there was a clear decrease in mycelial density. As aforementioned, total inhibition is not only related with mycelium radial inhibition, but also with inhibition of mycelium density These results suggest that *P. major* extract induced an effect on *P. cinnamomi* mycelium development. As in the experiments with other extracts, it was not possible weight the fungus mycelium because was technically difficult remove the mycelium from the agar medium to measure the fungus biomass.

These results suggested that although *P. major* extract had a very slight effect on mycelium radial inhibition, this plant extract was able to highly reduce the biomass of this phytopathogenic fungus. *Plantago cornuti* revealed no inhibition on the mycelium growth of *Phytophthora cambivora* (Nikolova et al., 2017). These results suggest that different *Plantago* species could have distinct effects on *Phytophthora* species, which may be due to the plants extraction methods, and consequently, to their chemical composition.

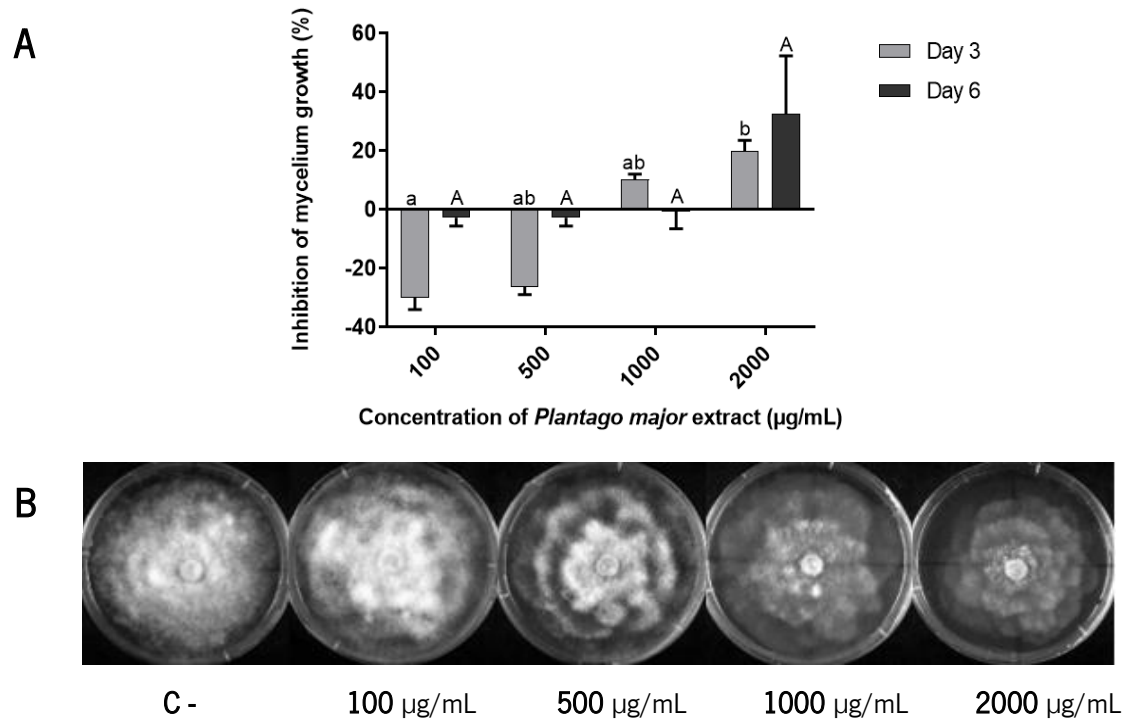


Figure 13. Effect of *Plantago major* extract on mycelial growth of *Phytophthora cinnamomi*. The extract was incorporated in PDA medium; a disk of mycelium was placed in the center of the plates and then they were incubated a 25 °C in the dark. (A) The percentage of mycelial growth inhibition determined after 3 and 6 days of incubation at different concentrations of *Plantago major* extract, 100, 500, 1000 or 2000 µg/mL. The negative control (C-) was prepared by replacing the extract by the solvent at the highest volume of extract used in the assays. Data are presented as the mean of three independent experiments ± SD. To identify differences between different concentrations of the same day were used the letters code. For this comparison mean values followed by the same letters are not statistically different (lowercase letters for day 3 and capital letters for day 6). Comparisons between different days of the same concentration are only represented if they are significant and were represented by the asterisk code. Differences were considered statistically significant if * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$ and **** $p \leq 0.0001$. (B) Representative images of *Plantago major* antifungal activity at different concentrations compared to the negative control (C-) against *Phytophthora cinnamomi* after 6 days of incubation.

Concluding, *P. major* extract revealed relatively low antifungal activity against the plant pathogens,

being *P. cinnamomi* the fungi with the highest mycelium reduction. Lukova et al. (2017) reported that the antifungal activity of *P. major* extract may be due to important groups of bioactive constituents as carbohydrates, phenols, flavonoids, tannins and minor amounts of alkaloids. In addition, these authors also conclude that the chemical content depends on the extraction agent. The moderate concentrations of these secondary metabolites could explain the moderate antifungal activity of *P. major* extract against all phytopathogenic fungi. Nevertheless, it is fundamental a chemical characterization of the *P. major* extract used in this work to obtain robust conclusions.

3.2.4. *Urtica dioica* extract

3.2.4.1. Effect of *Urtica dioica* extract on mycelial growth of *Botrytis cinerea*

The antifungal effect of *U. dioca* extract against *B. cinerea* was tested. The results showed that there were no statistically significant differences between in the effect of *U. dioica* against the pathogen (Figure 14A). The negative controls of this experiment did not present a similar diameter when compared to the *B. cinerea* negative controls obtained with the extracts aforementioned (Appendix Table A1; Table A5;Table A9 and Table A13; Figure 14B). This unexpected lack of reproducibility of growth measurements may be due to the mycelial plugs that contained different amounts of *B. cinerea* spores. This extract only showed a decreased in mycelial growth of *B. cinerea* at 2000 µg/mL, with a maximum growth inhibition rate of 23.5%. In terms of *B. cinerea* biomass, it was possible to observe (Figure 14B) that the treatment with 500 µg/mL had a denser appearance than the negative control. However, the density of the mycelium in the 2000 µg/mL plate was notoriously reduced, indicating an effect of *U. dioca* extract on the mycelium development of this phytopathogenic fungus. These results suggest that although *U. dioca* extract did not exhibit an inhibitory effect on the radial growth of the mycelium, it acted effectively in the reduction of *B. cinerea* density. Torun et al. (2018) found that the boiled water extract of *U. dioica* revealed antifungal activity against *B. cinerea* while other solvents (n-propanol, methanol, ethanol, acetone and ethyl acetate) did not have any effect on this fungus. In the further investigations, the antifungal activity of an *U. dioica* infusion against the phytopathogenic fungi should be tested.

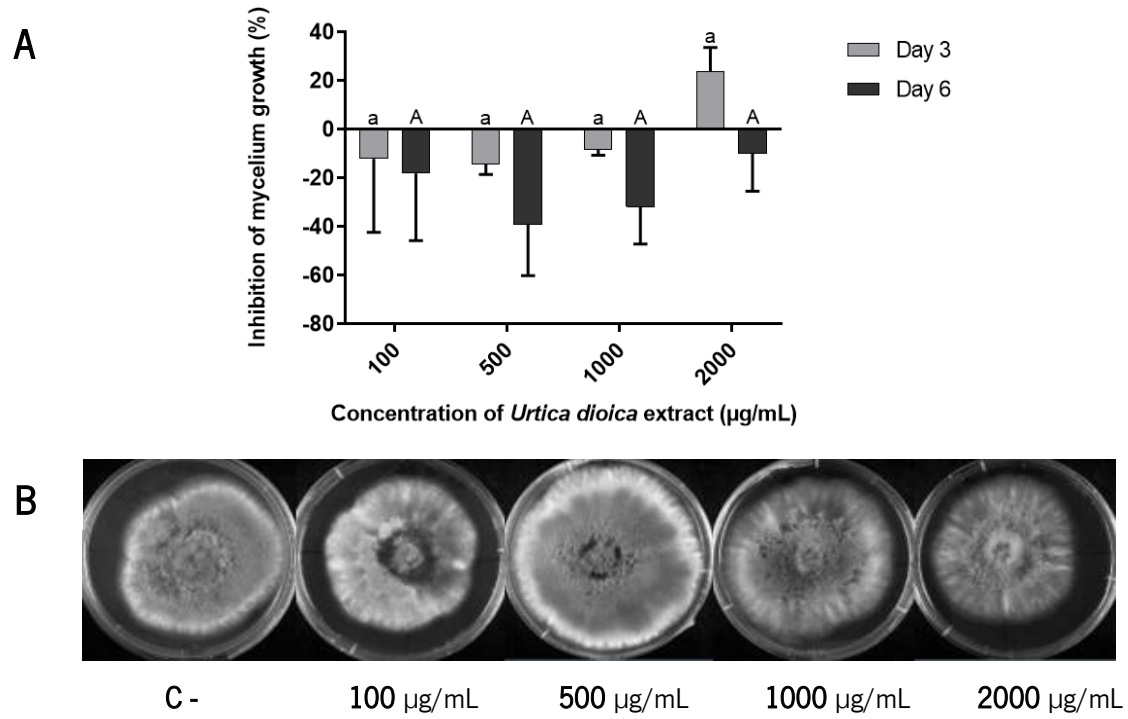


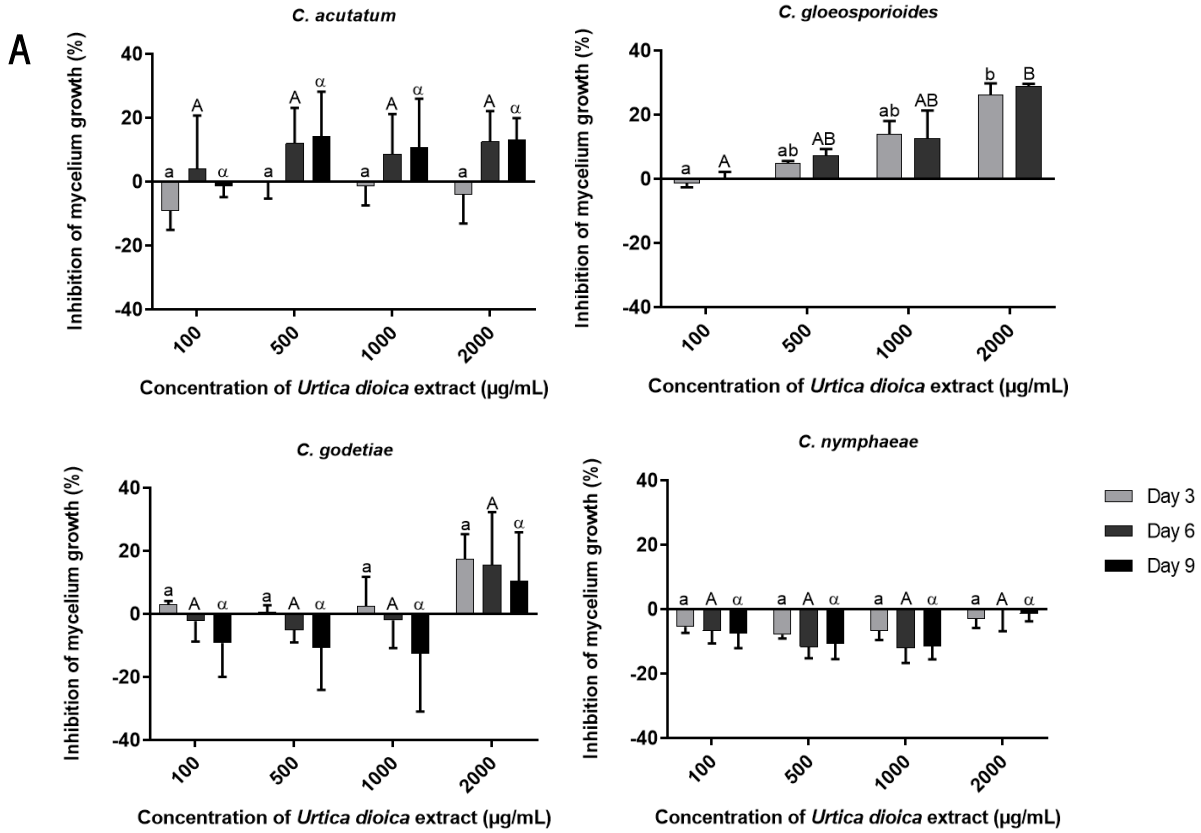
Figure 14. Effect of *Urtica dioica* extract on mycelial growth of *Botrytis cinerea*. The extract was incorporated in PDA medium; a disk of mycelium was placed in the center of the plates and then they were incubated a 25 °C in the dark. (A) The percentage of mycelial growth inhibition determined after 3 and 6 days of incubation at different concentrations of *Urtica dioica* extract, 100, 500, 1000 or 2000 µg/mL. The negative control (C-) was prepared by replacing the extract by the solvent at the highest volume of extract used in the assays. Data are presented as the mean of three independent experiments ± SD. To identify differences between different concentrations of the same day were used the letters code. For this comparison mean values followed by the same letters are not statistically different (lowercase letters for day 3 and capital letters for day 6). Comparisons between different days of the same concentration are only represented if they are significant and were represented by the asterisk code. Differences were considered statistically significant if * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$ and **** $p \leq 0.0001$. (B) Representative images of *Urtica dioica* antifungal activity at different concentrations compared to the negative control (C-) against *Botrytis cinerea* after 6 days of incubation.

3.2.4.2. Effect of *Urtica dioica* extract on mycelial growth of *Colletotrichum* species

Colletotrichum species are one of the most damaging fungal phytopathogens genus (Dean et al., 2012). So, in this work we investigated the antifungal activity of *U. dioica* extract against four species of the genus *Colletotrichum*. We observed that *U. dioica* extract did not cause a dose-dependent effect and there were no significant differences between all applied concentrations in *C. acutatum*, *C. godetiae* and *C. nymphaeae* (Figure 15A). In relation to the growth of *C. acutatum*, this plant extract induced a higher inhibition rate (14.3%) at 500 µg/mL. *Urtica dioica* extract reduced the growth of *C. gloeosporioides*, in

a dose-dependent manner and there were significant differences between 100 µg/mL and 2000 µg/mL. Although there were no inhibitory effect of *U. dioica* extract against *C. godetiae*, it is possible to notice a maximum inhibition rate of 17.5%. This extract also did not show antifungal activity against *C. nymphaeae* as it was not able to inhibit the mycelial growth. Interestingly, in the treatment with 2000 µg/mL of *U. dioica* extract the mean values approached to 0%. There was no evident appearance of morphological alterations in the mycelium aspect of the *Colletotrichum* species along the applied concentrations of *U. dioica* extract (Figure 15B).

Andrade Pinto et al. (2010) studied the antifungal activity of *U. dioca* extract against *Colletotrichum lindemuthianum*. These authors reported that this plant extract did not exhibited antifungal effect on *C. indemuthianum*, which are in concordance with the results obtained in this work for *C. acutatum*, *C. godetiae* and *C. nymphaeae*.



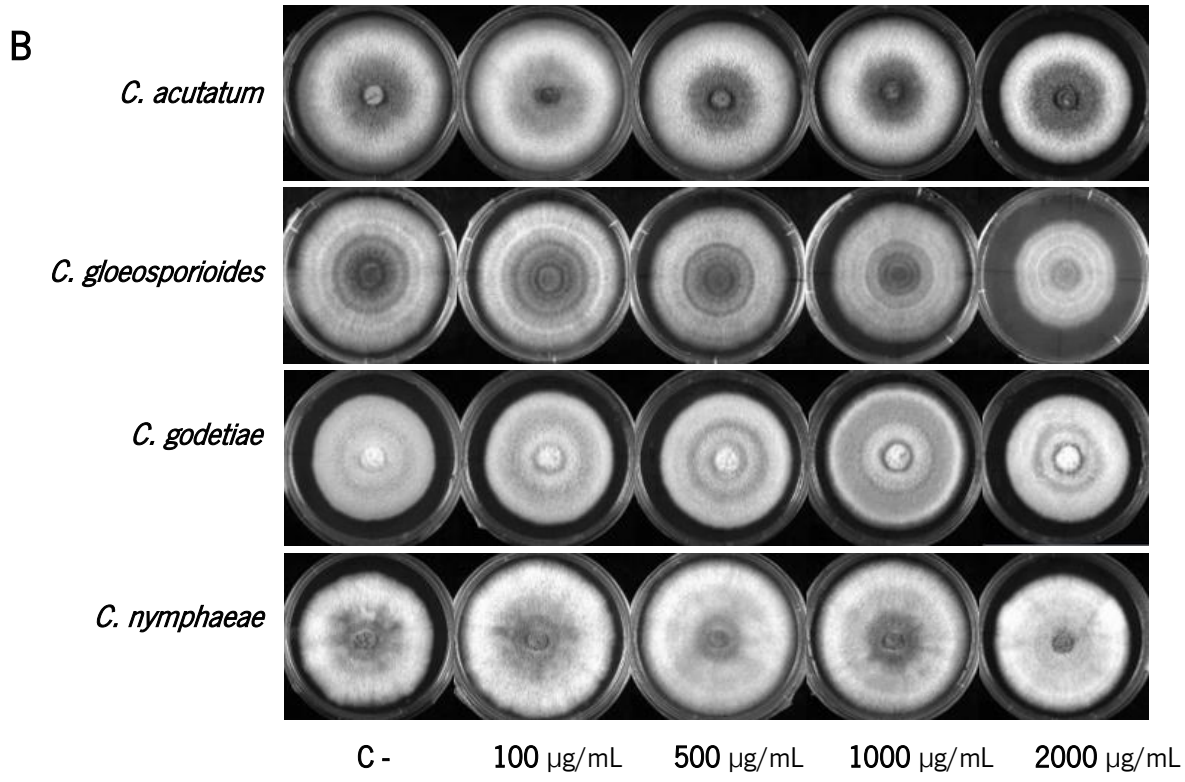


Figure 15. Effect of *Urtica dioca* extract on mycelial growth of *Colletotrichum* species. The extract was incorporated in PDA medium; a disk of mycelium was placed in the center of the plates and then they were incubated a 25 °C in the dark. (A) The percentage of mycelial growth inhibition determined after 3, 6 and 9 days of incubation at different concentrations of *Urtica dioca* extract, 100, 500, 1000 or 2000 µg/mL. The negative control (C-) was prepared by replacing the extract by the solvent at the highest volume of extract used in the assays. Data are presented as the mean of three independent experiments ± SD. To identify differences between different concentrations of the same day were used the letters code. For this comparison mean values followed by the same letters are not statistically different (lowercase letters for day 3, capital letters for day 6 and Greek letters for day 9). Comparisons between different days of the same concentration are only represented if they are significant and were represented by the asterisk code. Differences were considered statistically significant if * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$ and **** $p \leq 0.0001$. (B) Representative images of *Urtica dioca* antifungal activity at different concentrations compared to the negative control (C-) against *Colletotrichum* species after 6 days (*Colletotrichum gloeosporioides*) and 9 (*Colletotrichum acutatum*, *Colletotrichum godetiae*, *Colletotrichum nymphaeae*) days of incubation.

3.2.4.3. Effect of *Urtica dioica* extract on mycelial growth of *Diplodia corticola*

The antifungal potential of *U. dioica* extract against *D. corticola* was tested. Treatments with *U. dioica* extract reduced the mycelial growth of *D. corticola* with statistical significance between 100 µg/mL and 2000 µg/mL (Figure 16A). *Urtica dioica* extract induced the highest inhibitory effect (34.1%) at 2000 µg/mL. It was also possible to verify a decrease in the fungus density, particularly noticeable in the treatments with 1000 µg/mL and 2000 µg/mL of *U. dioica* extract (Figure 16B). According to our

knowledge, there are no studies reporting the antifungal activity of *U. dioica* extract against *D. corticola* or other species of *Diplodia* genus. Thus, this plant extract could be a potential natural fungicide to improve agricultural practices.

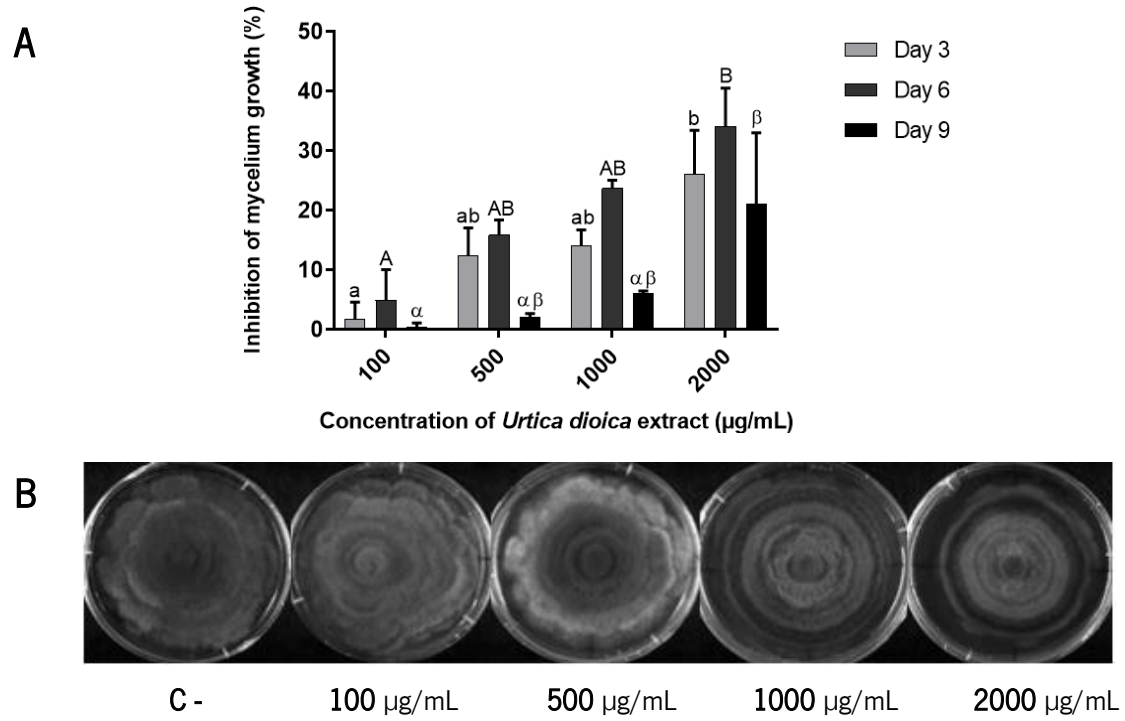


Figure 16. Effect of *Urtica dioica* extract on mycelial growth of *Diplodia corticola*. The extract was incorporated in PDA medium; a disk of mycelium was placed in the center of the plates and then they were incubated a 25 °C in the dark. (A) The percentage of mycelial growth inhibition determined after 3, 6 and 9 days of incubation at different concentrations of *Urtica dioica* extract, 100, 500, 1000 or 2000 µg/mL. The negative control (C-) was prepared by replacing the extract by the solvent at the highest volume of extract used in the assays. Data are presented as the mean of three independent experiments ± SD. To identify differences between different concentrations of the same day were used the letters code. For this comparison mean values followed by the same letters are not statistically different (lowercase letters for day 3, capital letters for day 6 and Greek letters for day 9). Comparisons between different days of the same concentration are only represented if they are significant and were represented by the asterisk code. Differences were considered statistically significant if $*p \leq 0.05$, $** p \leq 0.01$, $*** p \leq 0.001$ and $**** p \leq 0.0001$. (B) Representative images of *Urtica dioica* antifungal activity at different concentrations compared to the negative control (C-) against *Diplodia corticola* after 9 days of incubation.

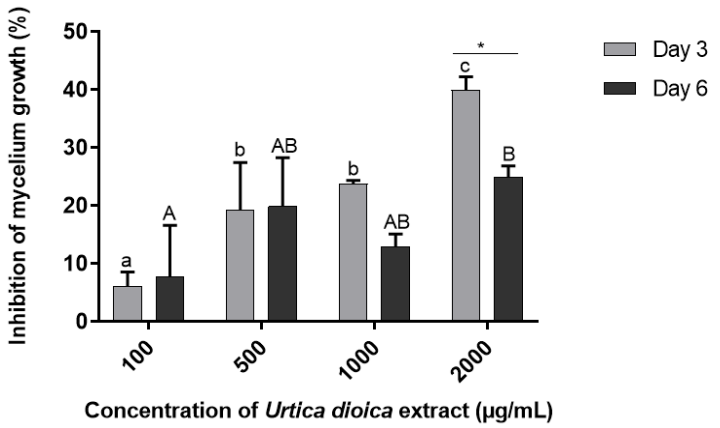
3.2.4.4. Effect of *Urtica dioica* extract on mycelial growth of *Phytophthora cinnamomi*

The antifungal potential of *U. dioica* extract was investigated against *P. cinnamomi*. This plant extract revealed significant differences between 100 µg/mL and the remaining applied concentrations (500, 1000 and 2000 µg/mL) on the third day of incubation (Figure 17A). In day 3 of the assay, the

plant extract at 2000 µg/mL induced the highest mycelial growth reduction (40%), an effect significantly different from (24.9%) the results on the sixth day. This decrease on the percentage of MGI from day 3 to day 6, suggest that *P. cinnamomi* was able to suppress the fungitoxic effect of the extract through a mechanism of detoxification. If this is the case, in field conditions this detoxification might be suppressed with subsequent applications of *U. dioica* extract on the third day after the first extract application. Yang et al. (2018) reported that *Phytophthora cactorum* expressed higher levels of detoxification enzymes and hydrolase activity-associated genes after an exposure to ginsenosides (steroid-like saponins). This supported that the detoxification mechanism plays a crucial role in the rapid adaptability of this pathogen to host plant defense compounds and fungicides.

Urtica dioica extract caused an effect on the mycelium density, similar to those recorded in the mycelia treated with *Medicago* sp., *M. indicus* and *P. major* (Figure 17B; Figure 5B; Figure 9B; Figure 13B). The decrease on the mycelium density is more prominent in the treatments with 1000 µg/mL and 2000 µg/mL of this plant extract. Stephan et al. (2005) found that *U. dioica* extract did not reduced late blight severity on detached potato leaves inoculated with *P. infestans* in a second experiment. Opposite to the *ex vivo* assay performed by the cited authors, in the present work the aqueous extract of *U. dioica* was able to inhibit the mycelial growth of *P. cinnamomi* in *in vitro* conditions.

A



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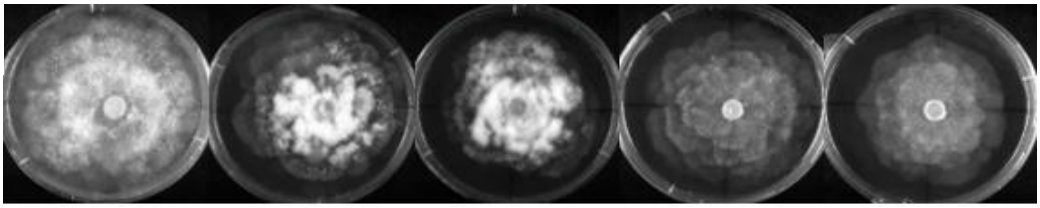


C - 100 µg/mL 500 µg/mL 1000 µg/mL 2000 µg/mL

Figure 17. Effect of *Urtica dioica* extract on mycelial growth of *Phytophthora cinnamomi*. The extract was incorporated in PDA medium; a disk of mycelium was placed in the center of the plates and then they were incubated a 25 °C in the dark. (A) The percentage of mycelial growth inhibition determined after 3 and 6 days of incubation at different concentrations of *Urtica dioica* extract, 100, 500, 1000 or 2000 µg/mL. The negative control (C-) was prepared by replacing the extract by the solvent at the highest volume of extract used in the assays. Data are presented as the mean of three independent experiments ± SD. To identify differences between different concentrations of the same day were used the letters code. For this comparison mean values followed by the same letters are not statistically different (lowercase letters for day 3 and capital letters for day 6). Comparisons between different days of the same concentration are only represented if they are significant and were represented by the asterisk code. Differences were considered statistically significant if $*p \leq 0.05$, $** p \leq 0.01$, $*** p \leq 0.001$ and $**** p \leq 0.0001$. (B) Representative images of *Urtica dioica* antifungal activity at different concentrations compared to the negative control (C-) against *Phytophthora cinnamomi* after 6 days of incubation.

This extract revealed antifungal activity against some plant pathogenic fungi tested in this work. Although *U. dioica* extract had no antifungal effect against *B. cinerea*, *C. acutatum*, *C. godetiae* and *C. nymphaeae*, it just did not reduce the mycelial growth of the later. These results suggest that different species of the same fungi genus may show different susceptibilities to the *U. dioica* extract. This emphasizes the need to investigate the differences in cell wall composition of these fungi and their interaction with the plant compounds of *U. dioica* extract previously analyzed through high performance liquid chromatography and mass spectroscopy techniques.

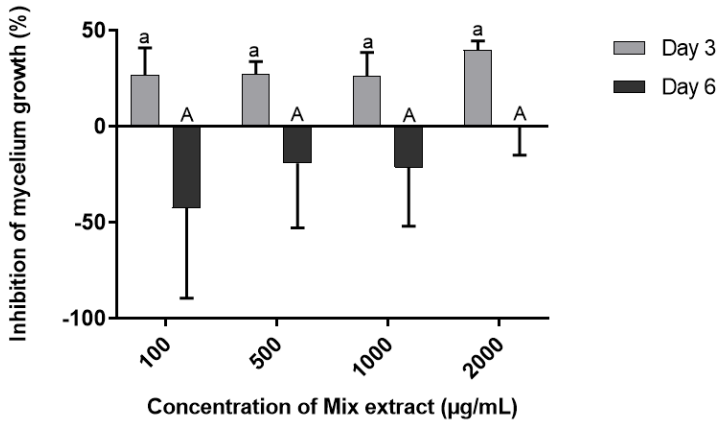
This extract was more efficient in inhibiting the mycelial growth of the oomycete, *P. cinnamomi* (39.9%) than the true fungi. As already explained for the coumarins-rich plant extract, *M. indicus*, the difference in the inhibitory effect of *U. dioica* extract on true fungi and oomycetes could be explained by the presence of coumarins, known for their high effectiveness against oomycetes. Orcic et al. (2014) found three coumarins, esculetin, scopoletin and umbelliferone, in the roots, stems and leaves of *U. dioica* extracts. This suggest that the leaves of *U. dioica* used in this work have a considerable amount of coumarins. In addition, further studies should include roots and stems of *U. dioica* in the extract sample to test the antifungal activity, since Franciškovi ć et al. (2017) reported a higher quantity of scopoletin coumarin in roots than in herb extracts of *U. dioica* plant.

3.2.5. Mix (*Medicago* sp., *Melilotus indicus*, *Plantago major* and *Urtica dioica* extracts)

3.2.5.1. Effect of Mix on mycelial growth of *Botrytis cinerea*

To evaluate the possibility of synergistic action of the extracts that revealed antifungal activity, namely *Medicago* sp., *M. indicus*, *P. major* and *U. dioica*, they were mixed in equal proportions at a concentration of 20 mg/mL each. The antifungal activity of mixture (mix - composed by *Medicago* sp., *M. indicus*, *P. major* and *U. dioica*) against *B. cinerea* was tested. Mycelium disks of the fungus were placed in the center of Petri dishes with PDA medium supplemented with different concentrations of mix. Similarly to the experiment with *U. dioica* extract, the negative controls of *B. cinerea* showed very different mycelium diameters in the three replicates (not shown). Concentration from 100 µg/mL to 1000 µg/mL yielded similar growth reductions (Figure 18A). Although the mix did not show statistically significant differences between concentrations, it was able to induce a maximum mycelium reduction of 39.7% at 2000 µg/mL. As observed with *Medicago* sp. (Figure 2B), *M. indicus* (Figure 6B), *P. major* (Figure 10B) and *U. dioica* (Figure 14B) extracts, the mycelial density was lower with the increasing of mix concentrations (Figure 18B). These differences were very noticeable at concentrations of 500 µg/mL, 1000 µg/mL, and 2000 µg/mL, indicating that inhibition was underestimated, and the total inhibitory effect of mix against this fungus was not only related with the MGI.

A



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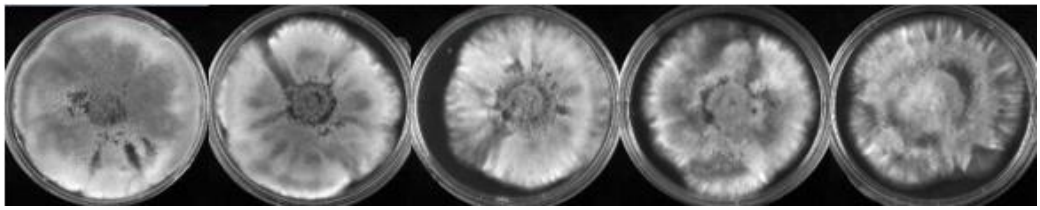

C - 100 µg/mL 500 µg/mL 1000 µg/mL 2000 µg/mL

Figure 18. Effect of mix on mycelial growth of *Botrytis cinerea*. The mix was incorporated in PDA medium; a disk of mycelium was placed in the center of the plates and then they were incubated a 25 °C in the dark. (A) The percentage of mycelial growth inhibition determined after 3 and 6 days of incubation at different mix concentrations, 100, 500, 1000 or 2000 µg/mL. The negative control (C-) was prepared by replacing the extract by the solvent at the highest volume of extract used in the assays. Data are presented as the mean of three independent experiments ± SD To identify differences between different concentrations of the same day were used the letters code. For this comparison mean values followed by the same letters are not statistically different (lowercase letters for day 3 and capital letters for day 6). Comparisons between different days of the same concentration are only represented if they are significant and were represented by the asterisk code. Differences were considered statistically significant if $*p \leq 0.05$, $** p \leq 0.01$, $*** p \leq 0.001$ and $**** p \leq 0.0001$. (B) Representative images of mix antifungal activity at different concentrations compared to the negative control (C-) against *Botrytis cinerea* after 6 days of incubation.

3.2.5.2. Effect of Mix on mycelial growth of *Colletotrichum* species

Colletotrichum species are known for their damages in economically important crops, so the antifungal activity of four species of this genus were evaluated. We observed that mix revealed antifungal activity against the four species tested and statistically significant differences were visible between 100 µg/mL and 2000 µg/mL in all *Colletotrichum* species (Figure 19A). It was possible to ascertain a direct proportionally between the applied concentration and the MGI for *C. acutatum* and *C. godetiae*. The mix was able to reduce the mycelial growth of *C. acutatum* by 23% after 9 days of incubation at 2000 µg/mL. Regarding the growth of *C. gloeosporioides*, this mix induced a maximum inhibition rate of 22.1%. In *C. godetiae*, the higher inhibition rate (23.4%) was observed at 2000 µg/mL. Lastly, mix was tested against *C. nymphaeae*, which revealed the highest inhibition rate (25.3%) of the four *Colletotrichum* species at 2000 µg/mL. Comparing all the mix antifungal activities in *Colletotrichum* species tested in this work, we found that the four species exhibited growth inhibition rates very similar (between 22.1% and 25.3%). The four *Colletotrichum* species exhibited changes in mycelial pigmentation (Figure 19B), which possibly indicates that this extracts combination may affected the fungi physiology. Apparently, it seems that the pigmentation alterations caused by mix are relatively different from the ones obtained with *Medicago* sp. (Figure 3B), *M. indicus* (Figure 7B), *P. major* (Figure 11B) and *U. dioica* (Figure 15B) tested individually. It is possible that the combination of active substances from different extracts could act on several targets, ensuring greater effectiveness and less chance of resistance by fungi. However, more detailed studies of the effect of these extracts, tested individually and mixed, on the morphology are needed.

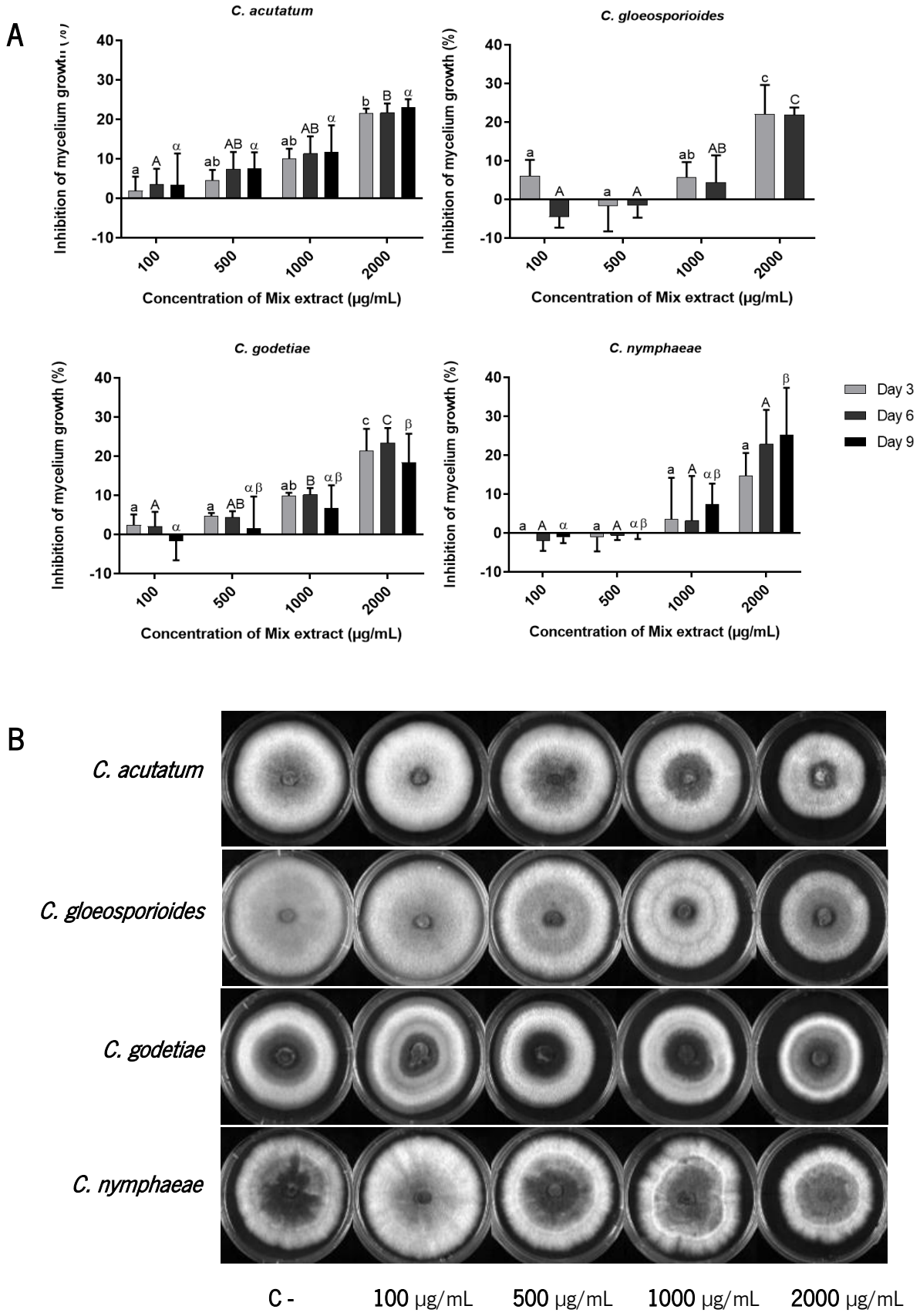

Figure 19. Effect of mix on mycelial growth of *Colletotrichum* species. The mix was incorporated in PDA medium; a disk of mycelium was placed in the center of the plates and then they were incubated a 25 °C in the dark. (A) The percentage of

mycelial growth inhibition determined after 3, 6 and 9 days of incubation at different mix concentrations, 100, 500, 1000 or 2000 µg/mL. The negative control (C-) was prepared by replacing the extract by the solvent at the highest volume of extract used in the assays. Data are presented as the mean of three independent experiments ± SD. To identify differences between different concentrations of the same day were used the letters code. For this comparison mean values followed by the same letters are not statistically different (lowercase letters for day 3, capital letters for day 6 and Greek letters for day 9). Comparisons between different days of the same concentration are only represented if they are significant and were represented by the asterisk code. Differences were considered statistically significant if $*p \leq 0.05$, $** p \leq 0.01$, $*** p \leq 0.001$ and $**** p \leq 0.0001$. (B) Representative images of mix antifungal activity at different concentrations compared to the negative control (C-) against *Colletotrichum* species after 6 days (*Colletotrichum gloeosporioides*) and 9 (*Colletotrichum acutatum*, *Colletotrichum godetiae*, *Colletotrichum nymphaeae*) days of incubation.

3.2.5.3. Effect of Mix on mycelial growth of *Diplodia corticola*

The antifungal effect of mix against *D. corticola* was evaluated. The mix did not show significant differences between the applied concentrations (Figure 20A). In the treatments with mix at 2000 µg/mL, the mycelium growth of *D. corticola* was reduced, exhibiting a maximum inhibition effect of 15.8%. We observed also that the mix caused a decrease in mycelial density at 2000 µg/mL. Although the mix only slightly reduced the mycelium growth, it changed the mycelium density and pigmentation at the highest applied concentration (Figure 20B).

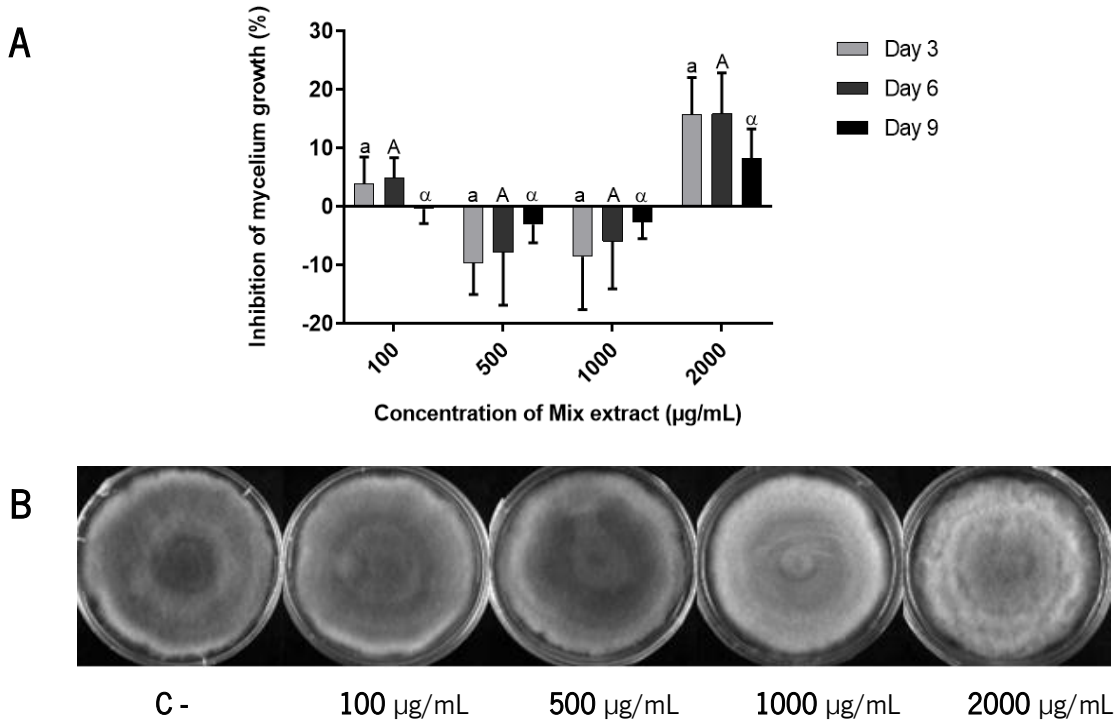

Figure 20. Effect of mix on mycelial growth of *Diplodia corticola*. The mix was incorporated in PDA medium; a disk of mycelium was placed in the center of the plates and then they were incubated a 25 °C in the dark. (A) The percentage of mycelial growth inhibition determined after 3, 6 and 9 days of incubation at different mix concentrations, 100, 500, 1000 or 2000 µg/mL. The negative control (C-) was prepared by replacing the extract by the solvent at the highest volume of extract used in the assays. Data are presented as the mean of three independent experiments ± SD. To identify differences between different concentrations of the same day were used the letters code. For this comparison mean values followed by the same letters are not statistically different (lowercase letters for day 3, capital letters for day 6 and Greek letters for day 9). Comparisons between different days of the same concentration are only represented if they are significant and were represented by the asterisk code. Differences were considered statistically significant if $*p \leq 0.05$, $**$ $p \leq 0.01$, $***$ $p \leq 0.001$ and $****$ $p \leq 0.0001$. (B) Representative images of mix antifungal activity at different concentrations compared to the negative control (C-) against *Diplodia corticola* after 9 days of incubation.

3.2.5.4. Effect of Mix on mycelial growth of *Phytophthora cinnamomi*

The antifungal activity of mix was assessed in *P. cinnamomi*. We observed that all concentrations caused statistically significant differences against *P. cinnamomi* (Figure 21A). The percentage of MGI increased along the concentrations applied causing a dose-dependent effect. At 2000 µg/mL, the mix exhibited a maximum inhibition rate of 72.6% on the third day of incubation. There was a decrease in the growth inhibition rate at all concentrations tested from day 3 to day 6 of incubation, but only at 500 µg/mL and 1000 µg/mL they were statistically significant. These significant decreases suggest that *P. cinnamomi* acquired resistance to the antifungal action of mix through a detoxification mechanism. As already mentioned, *Phytophthora cactorum* can express higher levels of detoxification enzymes and hydrolase activity-associated genes after an exposure to ginsenosides (steroid-like saponins). This information suggests that the saponins content present in this mix could contribute to loss of mycelium inhibitory effect against *P. cinnamomi* along the days. In application in the field, this problem should be avoided by subsequent applications of mix after three days of treatment. The mix was able to reduce considerably the radial diameter of the mycelium, however it appears that the mycelium density remains robust from 100 µg/mL to 2000 µg/mL when compared with the mycelium of the negative control (Figure 21B). This robustness in the mycelium density of *P. cinnamomi* did not happen when the plant extracts that constitute the mix were tested individually (Figure 5B; Figure 9B; Figure 13B; Figure 17B). The lack on the reduction of mycelium density may be due to the wide range of bioactive compounds that can interfere with the action of one another, and consequently, they were not able to potentiate a decrease in the mycelium density. Nevertheless, the mycelium biomass and its density seem to be reduced in the highest concentration (2000 µg/mL) applied.

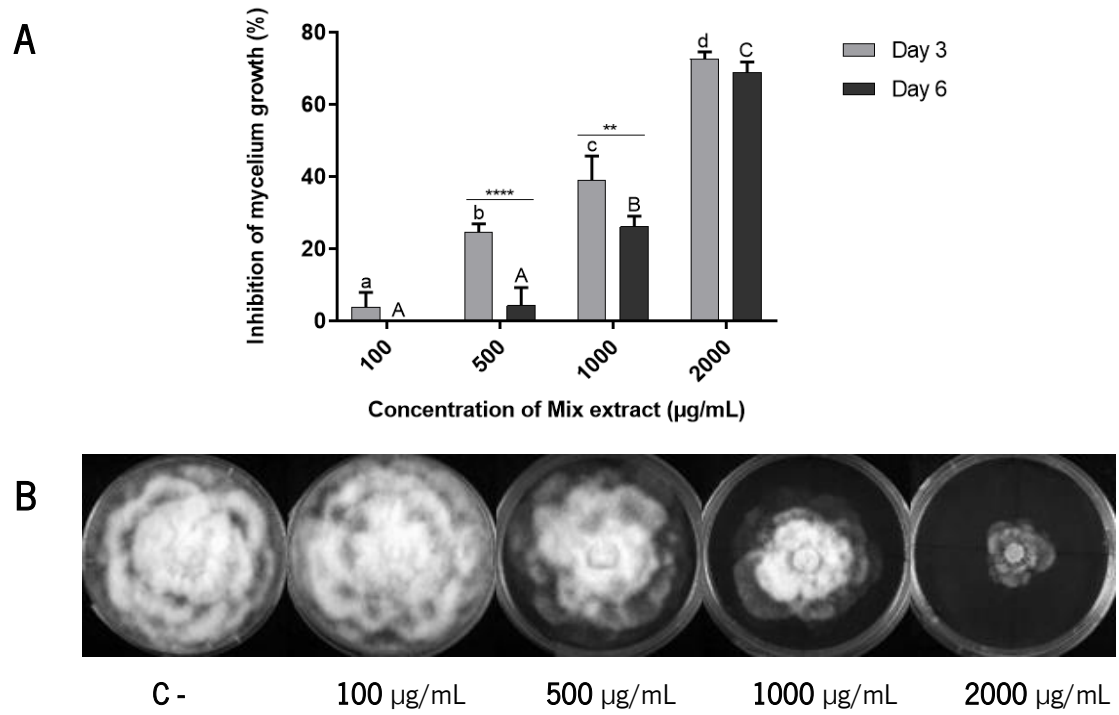

Figure 21. Effect of mix on mycelial growth of *Phytophthora cinnamomi*. The mix was incorporated in PDA medium; a disk of mycelium was placed in the center of the plates and then they were incubated a 25 °C in the dark. (A) The percentage of mycelial growth inhibition determined after 3 and 6 days of incubation at different mix concentrations, 100, 500, 1000 or 2000 µg/mL. The negative control (C-) was prepared by replacing the mixture by the solvent at the highest volume of mix used in the assays. Data are presented as the mean of three independent experiments ± SD. One-way ANOVA and Kruskal Wallis test were used for multiple comparisons. To identify differences between different concentrations of the same day were used the letters code. For this comparison mean values followed by the same letters are not statistically different (lowercase letters for day 3 and capital letters for day 6). Comparisons between different days of the same concentration are only represented if they are significant and were represented by the asterisk code. Differences were considered statistically significant if $*p \leq 0.05$, $** p \leq 0.01$, $*** p \leq 0.001$ and $**** p \leq 0.0001$. (B) Representative images of mix antifungal activity at different concentrations compared to the negative control (C-) against *Phytophthora cinnamomi* after 6 days of incubation.

The present study tested the antifungal activity by using different concentrations of aqueous extracts from six plants, belonging to different plant families, against a range of phytopathogenic fungi. Of the six plant extracts evaluated, four exhibited *in vitro* antifungal activity and a mixture of these four extracts were also tested (Appendix - Table A21). The plant species with the most pronounced antifungal activity were the action of *Medicago* sp. extract against *D. corticola* (60%), *M. indicus* (87.5%) and mix (72.6%) on *P. cinnamomi* mycelium growth. In general, *Medicago* sp. extract was the extract that expressed higher antifungal activity against five (*C. acutatum*, *C. gloeosporioides*, *C. godetiae*, *C. nymphaeae* and *D. corticola*) of the seven tested fungi. According to our knowledge, there are no previous

studies that have reported fungal growth inhibition by *Medicago* species against *D. corticola*. Even without showing statistically significant differences, the mycelium growth of *B. cinerea*, one of most harmful fungus to economically important crops, was moderately reduced by the mix. This also suggests that these extracts have different mechanisms of action, since the mixture had a more pronounced antifungal effect than each extract tested individually against *B. cinerea*. It was possible that chemical constituents from different plant extracts might acted synergistically. In the present work, all the plant extracts tested against *B. cinerea* revealed high SD values. One alternative to avoid the high SD values is to use controlled concentrations of spore suspensions by counting them in a haemocytometer, instead of using a mycelium plug as inoculum of growth experiments.

Antifungal activity of plant extracts may depend on the fungal strain, the chemical composition, and their concentration of secondary metabolites. *Melilotus indicus* and *U. dioica* extracts revealed a very high and moderately high antifungal activity against *P. cinnamomi*, respectively. Antifungal properties of *M. indicus* and *U. dioica* extracts are attributed to the presence of coumarins compounds. The large amount of coumarins (known for their activity against oomycetes) is probably more pronounced in *M. indicus*, which resulted in a higher antifungal effect against the oomycete, *P. cinnamomi*. The chemical composition of *U. dioica* has been studied and coumarins are also present in this plant, however in lower concentrations (Kregiel et al., 2018). These differences in the coumarins concentration could explain the results of this extracts against *P. cinnamomi* since *M. indicus* (87.5%; Figure 9B) displayed a higher percentage of mycelial growth inhibition than *U. dioica* extract (40%; Figure 17B). In addition, when combined with the other two extracts (*Medicago* sp. and *P. major*) into a mixture, the antifungal activity against this oomycete decreased slightly (72.6%; Figure 21B). This antifungal activity decrease could be explained by the compounds of *Medicago* sp. and *P. major* extracts that may blocked the activity of biological compounds of *M. indicus* and *U. dioica* extracts, or due to the dilution of the secondary metabolites in the mix, since each extract was in a lower concentration. In further investigations would be interesting study the synergistic effect of a mix composed by *M. indicus* and *U. dioica*.

In the present study, *C. edulis* and *P. oleracea* did not reveal antifungal properties against *D. corticola* and *P. cinnamomi*. However, there are reports that demonstrate biological activities, including antifungal activity, of these two extracts (Banerjee and Mukherjee, 2002; Omoruyi et al., 2014; Bouftira et al., 2012; Ercisli et al., 2008). *Carpobrotus edulis* and *P. oleracea* have been studied and reported as rich sources of important phytochemicals namely saponins, coumarins, flavonoids, alkaloids and tannins (Castañeda-Loaiza et al., 2020; Okafor and Ezejindu, 2014). However, in the present study these plant extracts did not show antifungal effect, which could be explained by the extraction procedure, and the

harvesting time and environmental conditions (rainfall), which play an important role in determining the phytochemicals and their amount (Oliveira et al., 2009). In further studies, the antifungal activity of *C. edulis* and *P. oleracea* extracts should be investigated with extracts harvested in other seasons in order to conserve the secondary metabolites already characterized in these plants. In the future, we also should perform a phytochemical analysis to discover the chemical constituents of each plant extract that are responsible for the reduction of mycelial growth and its density along the concentrations applied.

3.3. ANTIFUNGAL ACTIVITY *EX VIVO*

3.3.1. Optimization of leaf surface sterilization

Strawberry leaves harvested from the field were sterilized in order to investigate the preventive effect of *Medicago* sp. extract. To eliminate the flora present on the leaves a sterilization protocol was applied, which included 30 s ethanol 70%, then 1 min in 250 mL/L sodium hypochlorite at 0,376 M, and finally 2 min in sterile dH_2O (treatment 1). After the disinfection by these agents, the strawberry leaves were placed on PDA Petri dishes for 5 days to inspect if all microorganisms were removed from the leaves surface. After observing the Petri dishes containing the sterilized leaves for 5 days, it was found that combination of 70% ethanol for 30 s and sodium hypochlorite for 1 min did not eliminate all the microorganisms (Figure 22). These sterilizing agents were maintained in the subsequent treatments to inactivate all the microbes on the surface of leaves by varying the time exposure in the sodium hypochlorite solution. In treatment 2, 3, 4, 5, 6 and 7, we used leaf sections of strawberry in order to save plant resources for the *ex vivo* assay. All leaf sections showed total elimination of the bacterial and fungal content (Figure 22). The complete elimination of the microbial flora may be due to the production of reactive oxygen species (ROS) by the excised parts of the leaf in response to the wounds caused by the excision. The ROS can directly kill plant pathogens by ROS-mediated degradation of their proteins, nucleic acids; deterioration of membranes and by inhibiting germination of spores (Minibayeva et al., 2015). The complete elimination of the microbial flora on the excised parts of the strawberry leaf also suggest that when the leaves are excised the action of the decontamination agents is more efficient. David et al. (1988) demonstrated that sodium hypochlorite efficiently promotes the enzymatic hydrolysis of cell wall cellulose in eucalyptus wood. This suggest that the sodium hypochlorite promotes enzymatic hydrolysis in the sliced tissue and the secondary metabolites are easily oxidized and degraded by the decontamination agents. Although all the sections submerged in the disinfectant agents presented a total

elimination of the microorganisms, they caused spots and black areas in the leaf surface. These lesions were more notorious in extended exposure times in sodium hypochlorite, such as 5, 8, 10 and 12 min. So, in treatment 8, 9 and 10, we decided to investigate sterilization in sodium hypochlorite during 1'20 min, 1'40 min and 2 min. The entire leaves of these treatments did not give acceptable results even by increasing the time of exposure in sodium hypochlorite up to 2 min (Figure 22).

Sodium hypochlorite is one of the most widely used of the chlorine disinfectants due to their inexpensive and fast action to remove dried or fixed organisms and biofilms from surfaces (Merritt et al., 2000; Rutala and Weber, 1997). In the present study, the strawberry leaves surface was not totally sterilized by any treatment applied. So, we proceeded this work by sterilizing the surface of strawberry leaves according to treatment protocol 1. The sterilization verification assay was performed using leaves placed in PDA while the *ex vivo* assay was designed to be performed without any culture medium. Thus, even a partial inactivation of the flora associated with the leaves should not be problematic because it lacked the culture medium to recover viability and proliferate. Although the sterilization protocols allowed the microorganisms proliferation, we thought that this treatment would be more effective in the destruction of normal flora present in the host surface tissue than no leaf disinfection treatment.

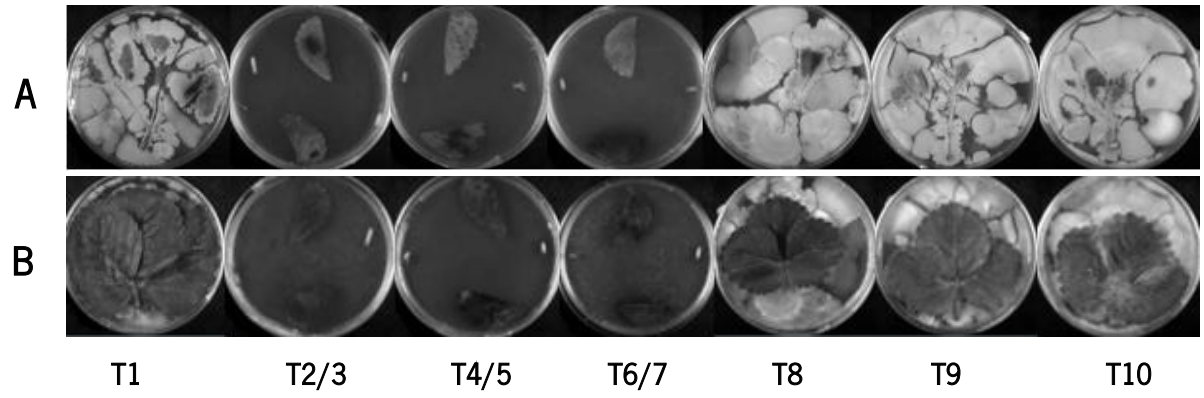

Figure 22. Effect of different surface sterilization treatments of strawberry leaves in PDA Petri dishes. The strawberry leaves were harvested from a cultivation field placed in Campus de Gualtar (University of Minho, Braga), placed in different Petri dishes and incubated at 25 °C in the dark after the sterilization treatment. Back (A) and front (B) of the Petri dishes after 5 days of incubation. T1- leaf exposed to 1 min in sodium hypochlorite; T2 - excised parts of a leaf exposed to 2 min (upside) in sodium hypochlorite; T3 - excised parts of a leaf exposed to 3 min (bottom) in sodium hypochlorite; T4 - excised parts of a leaf exposed to 5 min (upside) in sodium hypochlorite; T5 - excised parts of a leaf exposed to 8 min (bottom) in sodium hypochlorite; T6 - excised parts of a leaf exposed to 10 min (upside) in sodium hypochlorite; T7- excised parts of a leaf exposed to 12 min (bottom) in sodium hypochlorite; T8 - leaf exposed to 1'20 min in sodium hypochlorite; T9 - leaf exposed to 1'40 min in sodium hypochlorite; T10 - leaf exposed to 2 min in sodium hypochlorite.

3.3.2. Effect of *Medicago* sp. extract on strawberry leaves

The hemibiotrophic plant pathogen, *C. acutatum*, is responsible for major losses in strawberry caused by anthracnose disease (Ureña-Padilla et al., 2002). In this work, the *in vitro* antifungal activity of some plant extracts against *C. acutatum* was assessed. Among the tested extracts, *Medicago* sp. extract showed the highest MGI against this fungus. In addition to that, it was very important to investigate the antifungal preventive potential of *Medicago* sp. extract on detached leaves of strawberry plants inoculated with *C. acutatum*. The extract at different concentrations (1000 and 2000 µg/mL) was brushed on both surfaces of the leaves. Then, the adaxial side of the leaves was submerged in a spore's suspension of *C. acutatum*, and each leaf was placed in a Petri dish and monitored for 12 days near the window to mimic the circadian conditions. In the 12th day of the assay, the leaves were evaluated under a stereomicroscope and the observed mycelium colonies present on the leaves surface were transferred into a Petri dish with PDA.

Three- and six-days' post-inoculation, the leaves treated with 1000 and 2000 µg/mL showed a considerable reduction of mycelium colonies present on the surface of strawberry leaves, unlike the positive control (extract treatment was not applied) where pink spots resembling *C. acutatum* mycelium were visible (Figure 23). This suggest that *Medicago* sp. extract possess antifungal properties since it was able to eliminate the mycelium colonies from the leaf's surfaces treated with 1000 µg/mL or 2000 µg/mL concentrations. However, from day 9 until the end of the assay, brownish spots were visible in the treated leaves. The incidence of brown spots was gradually increasing over the days suggesting a progression of the infection caused by *C. acutatum*. Arroyo et al. (2005) found that the infection process of *C. acutatum* in strawberry leaf tissue was characterized by several macroscopic symptoms including brownish spots with 2-8 mm long. According to these authors, the brownish spots on the leaves tissue were caused by *C. acutatum*. In the present work, the detached leaves submersed in the suspension of *C. acutatum* did not present macroscopically visible lesions until 9 days after the inoculation. This symptomless stage in the leaf tissues could also suggest that: i) the extract was effective against the infection caused by *C. acutatum* until the 6th day post-inoculation or ii) the pathogen could remain within the strawberry leaves cell walls without cause any symptom (biotrophic phase), switching to a necrotrophic phase from the 9th to 12th day of the assay.

Apparently, the leaves corresponding to the negative and extract controls remained healthy during the 12 days of the experiment, suggesting that the sterilization protocol was effective and the *Medicago* sp. extract did not have phytotoxic activity.

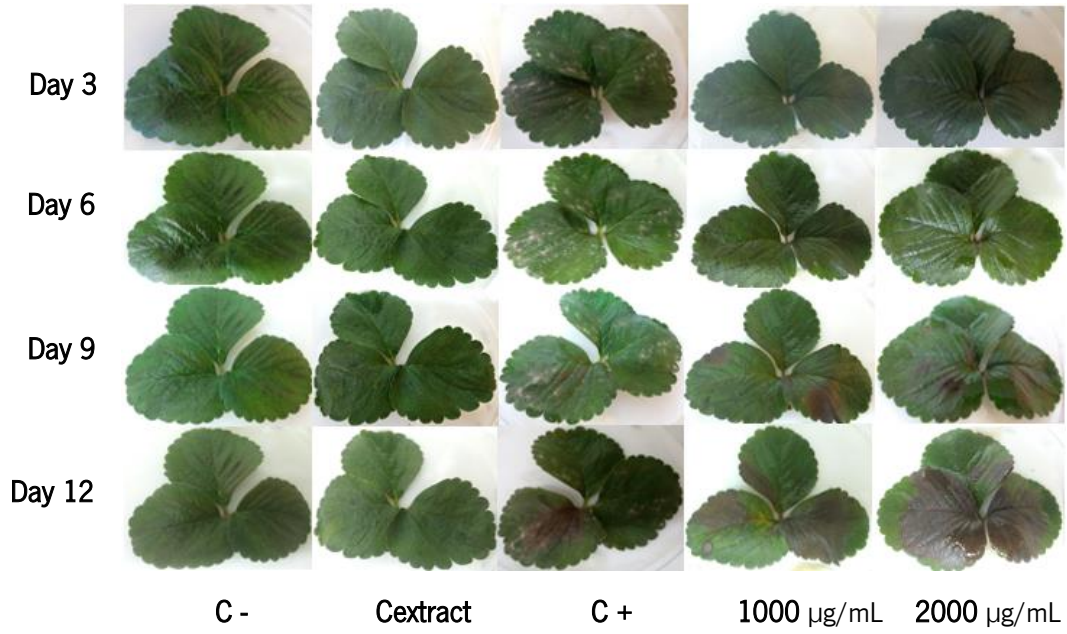

Figure 23. Representative images of antifungal effect of *Medicago* sp. extract on isolated strawberry leaves. The treated leaves were brushed with 1000 µg/mL or 2000 µg/mL of *Medicago* sp. extract. The negative and the positive controls were only brushed with sterile dH_2O; the extract control was brushed with the maximum concentration of the extract used (2000 µg/mL). The adaxial side of the strawberry leaves corresponding to the positive control and those subjected to the treatment with *Medicago* sp. were submerged in a suspension of *C. acutatum*. The Petri dishes containing the leaves were placed near the window to mimic the circadian rhythm and representative images were photographed on the third, sixth, ninth and twelfth day of experiment.

In the 12th day of the assay, we observed the *C. acutatum* infection in strawberry leaves through a stereomicroscope in order to confirm through morphological identification that the infection appears to be caused by *C. acutatum* (Figure 24). As we predicted, the negative and the extract control leaves had no discoloration or visible degraded regions (Figure 24A, 24B). The strawberry leaves, corresponding to the positive control and the treatment with *Medicago* sp. extract at 2000 µg/mL, contained mycelium colonies on the leaves surface (Figure 24C, 24E). In the detached leaf submitted to the treatment with 1000 µg/mL of *Medicago* sp. extract we did not observe mycelium colonies on the surface, however, some rounded structures in the apparent necrotic tissue are visible (Figure 24D).

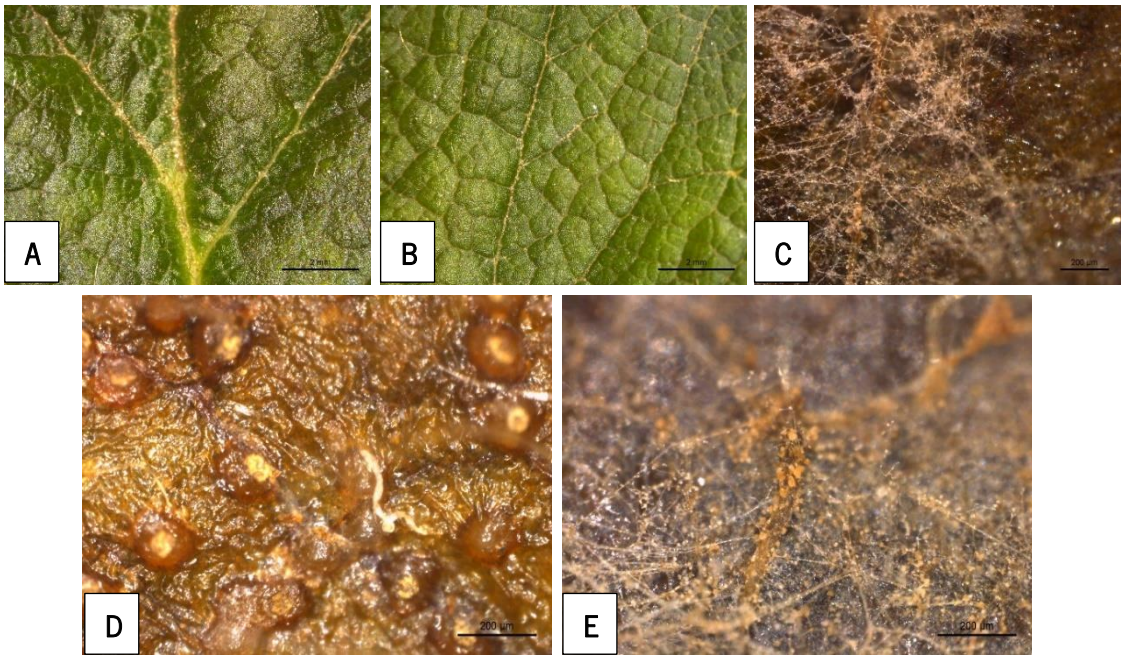

Figure 24. Stereoscope images of antifungal effect of *Medicago* sp. extract on isolated strawberry leaves. The treated leaves were brushed with 1000 µg/mL and 2000 µg/mL of *Medicago* sp. extract. The negative and the positive controls were only brushed with sterile dH_2O; the extract control was brushed with the maximum concentration of the extract used (2000 µg/mL). The adaxial side of the strawberry leaves corresponding to the positive control and those subjected to the treatment with *Medicago* sp. were submerged in a suspension of *C. acutatum*. The Petri dishes containing the leaves were placed near the window to mimic the circadian rhythm for 12 days. (A) negative control - scale bar: 2 mm; (B) extract control - scale bar: 2 mm; (C) positive control - scale bar: 200 µm; (D) treatment with 1000 µg/mL of *Medicago* sp. extract - scale bar: 200 µm; and (E) treatment with 2000 µg/mL of *Medicago* sp. extract - scale bar: 200 µm. Representative images of one performed replica.

To assure that these colonies and the brown spots were caused by *C. acutatum*, fungal suspensions were prepared by scraping the mycelium colonies present on the leaves surface with a sterile plastic tip and resuspending the mycelium in 3 µL of sterile dH_2O. Subsequently, each suspension was placed in the center of each PDA Petri dishes. The Petri dishes of the positive control, 1000 µg/mL and 2000 µg/mL in Figure 25 are the result of suspensions prepared from the leaves of the experiment in Figure 23. After 12 days of incubation at 25 °C in the dark, Petri dishes presented a pink mycelium resembling *C. acutatum* mycelium color, density, and morphology (Figure 25). Although the pink mycelium identical to the *C. acutatum* were obtained it is important to remind that the PDA medium used is optimal for phytopathogenic fungal growth and not the most adequate for the growth of other types of

microorganisms (e.g., bacteria and yeasts). In further investigations, microscopic and molecular analysis should be performed to corroborate these results.

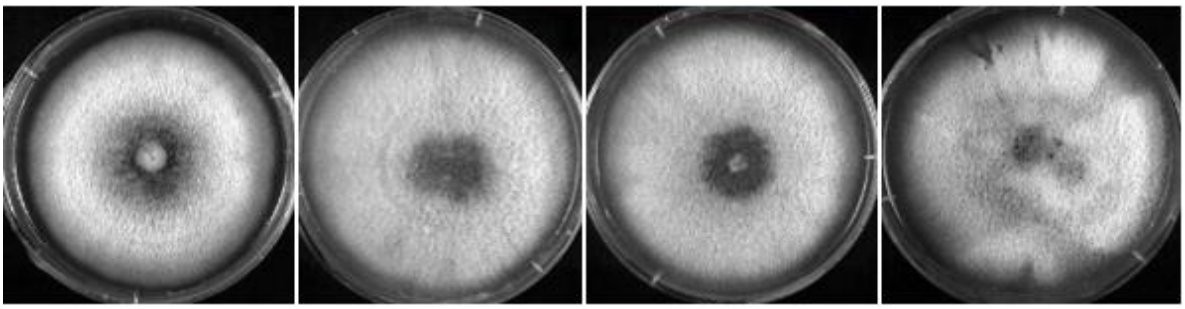

Figure 25. Representative images of *C. acutatum* mycelium removed from strawberry leaves surface on PDA Petri dishes. Fungal suspensions were prepared by scraping the mycelium colonies present on the leaves surface with a sterile plastic tip and resuspending in 3 µL of sterile dH_2O. Each suspension was placed in the center of each PDA Petri dishes and incubated for 12 days at 25 °C in the dark.

In this study, we showed that the preventive treatment with the *Medicago* sp. extract elicited antifungal activity in strawberry leaves against *C. acutatum*. Macroscopic observations revealed that the fungal mycelial colonies were significantly reduced in leaves surface brushed with *Medicago* sp. extract. The effect of the plant extract in reducing *C. acutatum* mycelium in *in vitro* as well as in *ex vivo* experiments, suggested that *Medicago* sp. extract could be an alternative to synthetic fungicides to control anthracnose disease. Although the results of antifungal activity *in vitro* of *Medicago* sp. extract against *C. acutatum* showed that the plant extract inhibited the fungal growth in a dose-dependent manner, this was not possible to conclude in *ex vivo* conditions.

This experiment occurred in the autumn season, which could be a relevant factor for the rapid progression of the brownish spots from the 9th to the 12th day of the assay, since the leaves were old. Parikka and Lemmetty, (2009) found that infection caused by *C. acutatum* in young leaves could be superficial, but in old leaves the fungus easily colonized senescent plant tissues. According to Feil et al. (2003), high temperatures and excess moisture cause rapid decline of the fungus while cold temperatures of winter period can enhance survival because competitive microorganisms are not active. In addition, more research is needed to find a correlation between *C. acutatum* infection and progression in strawberry leaves, the age of foliar tissues and the season of the year when they are harvested from the field.

Chapter 4

FINAL REMARKS AND FUTURE PERSPECTIVES

4.1. FINAL REMARKS

Synthetic fungicides represent a notorious impact in terrestrial, aerial and aquatic ecosystems being a risk to humans and nonhuman biota health. The search of safe, biodegradable, and cost-effective alternatives is needed to control these harmful effects. Therefore, the antifungal properties of biological products such as plant extracts have been looked for and progressively studied.

Accordingly, in this thesis project we proposed to select some plant extracts, namely *C. edulis*, *Medicago* sp., *M. indicus*, *P. major*, *P. oleracea* and *U. dioica*, and study their antifungal activity against a range of phytopathogenic fungi responsible to seriously damaging crops of high economic value. *Medicago* sp., *M. indicus*, *P. major* and *U. dioica* extracts reduced the mycelial growth of all tested fungi, with an exception for *U. dioca* extract, which did not cause a decrease on *C. nymphaeae* mycelium growth. *Medicago* sp. extract revealed considerable percentages of MGI against all fungi tested in *in vitro* experiments. This work allowed us to conclude that the complete inhibition was not only correlated to the measure of colonies diameter, since some all the plant extracts tested individually caused also a decrease in the mycelium density of *B. cinerea*, *D. corticola* and *P. cinnamomi*.

To verify if *Medicago* sp. extract had fungitoxic effect in *ex vivo* conditions, we studied its effect on strawberry leaves infected with *C. acutatum* fungus. This plant extract was able to eliminate most of the fungus colonies present on the leaves surface, where disease symptoms caused by *C. acutatum* were not macroscopically visible until the 9th after the fungus inoculation. That being said, we can conclude that *Medicago* sp. extract possesses antifungal activity in *ex vivo* experiments, however more studies should be performed in order to obtain more robust results. The different results of antifungal activity among these extracts may be associated with different secondary metabolites amounts and composition and their mechanisms of action.

Overall, it is possible to conclude that *Medicago* sp., *M. indicus* have a high potential as antifungal (true fungi) and anti-oomycete agents, respectively. The mixture composed by *Medicago* sp., *M. indicus*, *P. major* and *U. dioica* extracts also revealed a potential natural fungicide, mainly against *P. cinnamomi* (72.6%). These plant extracts could be valuable sources of new natural fungicides for the control and management of fungal disease in agriculture, avoiding synthetic fungicides applications and their harmful effects.

4.2. FUTURE PERSPECTIVES

Several secondary metabolites are described in the literature to be abundant in *C. edulis*, *Medicago* sp., *M. indicus*, *P. major*, *P. oleracea*, *U. dioica* extracts. Therefore, to determine which compounds are present in each plant extract, a phytochemical analysis should be performed. In this work, it was not possible to conduct this analysis, however, it will be the next step to perform in this project. Chemical analysis would also help to create new mixtures with these extracts, based on their diversity and quantity of biological plant metabolites.

Since promising results have been obtained in this study, further *in vitro*, *ex vivo* and *in vivo* assays should be performed. The antifungal activity of these extracts can be tested in a wider range of phytopathogenic fungi and applied in other crops which suffer severe economic losses as strawberry production. In further *ex vivo* assays, a total elimination of the microflora on the leaves surface should be guaranteed to only study the protective effect of the extract applied. Although the infection method used in this work has resulted, in further investigations, controlled concentrations of spore suspensions by counting them in a haemocytometer, should be applied in previously provoked wounds. To supplement these assays, the fungal spores should be observed by microscopy and a DNA extraction should be done to confirm the identity of the fungus used for inoculation. In the future, other types of treatment can be investigated on strawberry leaves, including curative and/or preventive treatments, and the study of different intervals and concentrations of extract applications.

The antifungal activity *in vitro* of some plant extracts should be studied again against *B. cinerea* using another method. The results obtained were very dispersed, but some replicas showed great percentage of MGI. In addition to that, it will be interesting to determine and use a concentration of mycelium spores instead of a mycelium plug in order to avoid dispersed results. In this work, we reported that the extracts were able to reduce the mycelium density and change its pigmentation. In order to corroborate these results, further studies about spore's germination and sporulation should be performed in order to clarify the alterations in the mycelium pigmentation and density of the exposed fungi. The fungal biomass should also be measured through the analysis of mycelium wet weight cultured in liquid medium (Kumawat et al., 2016). The antifungal activity of these extracts should be studied in a wider range of phytopathogenic fungi.

The model organism *Saccharomyces cerevisiae* and their mutant strains should be used in *in vitro* viability assays in order to understand the mechanisms of action of these extracts. It is expected that the strains that do not have the molecular target would be more resistant than the wild-type strain. For

example, for the activity of saponins that bind to ergosterol (Malabed et al., 2017), the strains lacking in the synthesis of ergosterol will presumably be more resistant than the wild-type.

Since one of our goals is to apply these plant extracts at industrial level, it is fundamental in the future grow the plants in the field in a predetermined area, harvest the plants and produce the extract to calculate the yield per cultivation area. In addition, would be also important carry out preventive field trials with different treatment protocols to find out the amount of extract needed to prevent fungal infections. Subsequently, should be studied the feasibility of producing extracts for effective treatments based on obtained results. Lastly, the commercialization of the product, which include a market research, marketing, product design and distribution channels should be tried.

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APPENDIX

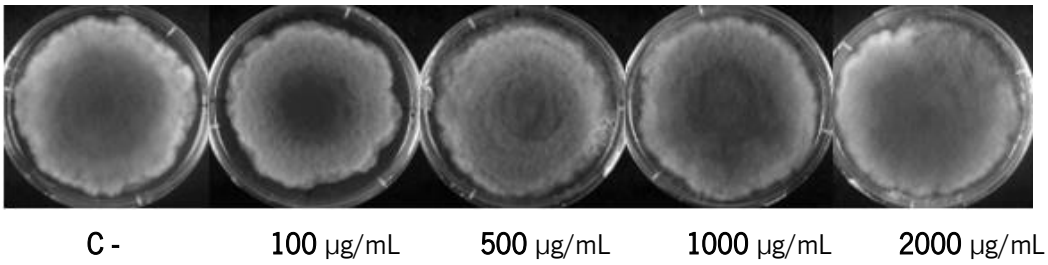

Figure A1. Representative images of *Carpobrotus edulis* antifungal activity at different concentrations compared to the negative control (C-) against *Diplodia corticola* after 9 days of incubation.

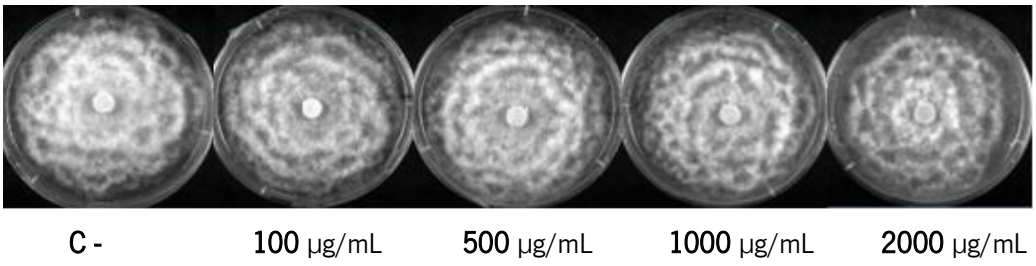

Figure A2. Representative images of *Carpobrotus edulis* antifungal activity at different concentrations compared to the negative control (C-) against *Phytophthora cinnamomi* after 6 days of incubation.

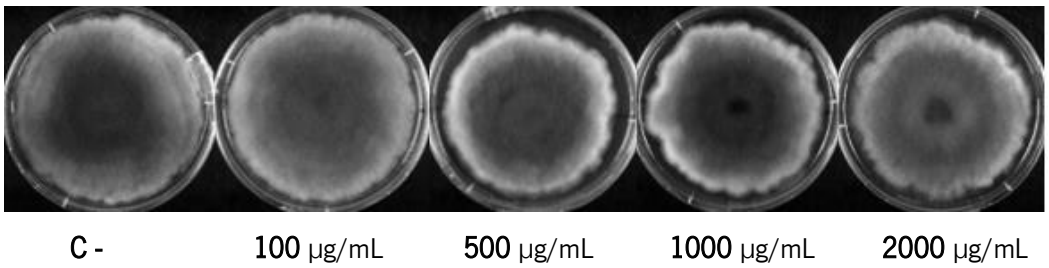

Figure A3. Representative images of *Portulaca oleracea* antifungal activity at different concentrations compared to the negative control (C-) against *Diplodia corticola* after 9 days of incubation.

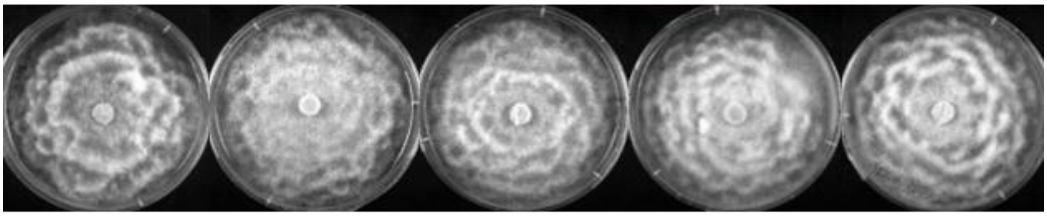

Figure A4. Representative images of *Portulaca oleracea* antifungal activity at different concentrations compared to the negative control (C-) against *Phytophthora cinnamomi* after 6 days of incubation.

Table A1. Diameter of mycelium of *Botrytis cinerea* treated with *Medicago* sp. extract concentrations of 100, 500, 1000 or 2000 µg/mL at day 6 of incubation. The values presented are the relative means expressed in centimeters ± SD of three independent experiments.

Days of incubation	Diameter of mycelium (cm) at a *Medicago* sp. extract concentration (µg/mL) of				
	0	100	500	1000	2000
6	8,3 ± 0,4	8,0 ± 0,3	6,2 ± 1,6	7,3 ± 0,4	5,5 ±1,0

Table A2. Diameter of mycelium of *Colletotrichum* species treated with *Medicago* sp. extract concentrations of 100, 500, 1000 or 2000 µg/mL at day 6 and 9 of incubation. The values presented are the relative means expressed in centimeters ± SD of three independent experiments.

Species	Days of incubation	Diameter of mycelium (cm) at a *Medicago* sp. extract concentration (µg/mL) of				
		0	100	500	1000	2000
C. acutatum	9	7,8 ± 0,1	7,5 ± 0,3	7,1 ± 0,5	5,5 ± 0,5	5,3 ± 0,4
C. gloeosporioides	6	8,4 ± 0,0	8,4 ± 0,1	7,4 ± 0,3	6,7 ± 0,8	6,0 ± 0,9
C. godetiae	9	7,1 ± 0,2	7,2 ± 0,1	6,6 ± 0,1	6,0 ± 0,5	4,4 ± 0,2
C. nymphaeae	9	7,3 ± 0,0	7,5 ± 0,2	7,0 ± 0,1	6,6 ± 0,6	5,4 ± 1,3

Table A3. Diameter of mycelium of *Diplodia corticola* treated with *Medicago* sp. extract concentrations of 100, 500, 1000 or 2000 µg/mL at day 9 of incubation. The values presented are the relative means expressed in centimeters ± SD of three independent experiments.

Days of incubation	Diameter of mycelium (cm) at a *Medicago* sp. extract concentration (µg/mL) of				
	0	100	500	1000	2000
9	8,4 ± 0,1	8,3 ± 0,3	7,0 ± 0,1	5,2 ± 0,6	3,5 ± 0,4

Table A4. Diameter of mycelium of *Phytophthora cinnamomi* treated with *Medicago* sp. extract concentrations of 100, 500, 1000 or 2000 µg/mL at day 6 of incubation. The values presented are the relative means expressed in centimeters ± SD of three independent experiments.

Days of incubation	Diameter of mycelium (cm) at a *Medicago* sp. extract concentration (µg/mL) of				
	0	100	500	1000	2000
6	8,5 ± 0,0	8,5 ± 0,0	8,2 ± 0,3	7,9 ± 0,2	7,6 ± 0,2

Table A5. Diameter of mycelium of *Botrytis cinerea* treated with *Melilotus indicus* extract concentrations of 100, 500, 1000 or 2000 µg/mL at day 6 of incubation. The values presented are the relative means expressed in centimeters ± SD of three independent experiments.

Days of incubation	Diameter of mycelium (cm) at a *M. indicus* extract concentration (µg/mL) of				
	0	100	500	1000	2000
6	8,1 ± 0,5	8,1 ± 0,3	7,6 ± 0,8	7,6 ± 0,1	6,8 ± 0,9

Table A6. Diameter of mycelium of *Colletotrichum* species treated with *Melilotus indicus* extract concentrations of 100, 500, 1000 or 2000 µg/mL at day 6 and 9 of incubation. The values presented are the relative means expressed in centimeters ± SD of three independent experiments.

Species	Days of incubation	Diameter of mycelium (cm) at a *M. indicus* extract concentration (µg/mL) of				
		0	100	500	1000	2000
C. acutatum	9	7,7 ± 0,2	7,6 ± 0,3	6,9 ± 0,7	5,9 ± 0,4	5,5 ± 0,1
C. gloeosporioides	6	8,5 ± 0,0	8,4 ± 0,1	7,9 ± 0,1	7,3 ± 0,5	6,3 ± 0,1
C. godetiae	9	7,1 ± 0,1	7,1 ± 0,3	6,8 ± 0,4	6,6 ± 0,0	6,0 ± 0,2
C. nymphaeae	9	7,3 ± 0,1	7,1 ± 0,3	6,3 ± 0,5	6,1 ± 0,7	5,6 ± 0,2

Table A7. Diameter of mycelium of *Diplodia corticola* treated with *Melilotus indicus* extract concentrations of 100, 500, 1000 or 2000 µg/mL at day 9 of incubation. The values presented are the relative means expressed in centimeters ± SD of three independent experiments.

Days of incubation	Diameter of mycelium (cm) at a *M. indicus* extract concentration (µg/mL) of				
	0	100	500	1000	2000
9	8,5 ± 0,0	8,4 ± 0,1	8,2 ± 0,3	8,2 ± 0,2	7,5 ± 0,3

Table A8. Diameter of mycelium of *Phytophthora cinnamomi* treated with *Melilotus indicus* concentrations of 100, 500, 1000 or 2000 µg/mL at day 6 of incubation. The values presented are the relative means expressed in centimeters ± SD of three independent experiments.

Days of incubation	Diameter of mycelium (cm) at a *M. indicus* extract concentration (µg/mL) of				
	0	100	500	1000	2000
6	8,5 ± 0,0	8,5 ± 0,0	7,3 ± 0,7	3,7 ± 1,0	1,1 ± 0,1

Table A9. Diameter of mycelium of *Botrytis cinerea* treated with *Plantago major* extract concentrations of 100, 500, 1000 or 2000 µg/mL at day 6 of incubation. The values presented are the relative means expressed in centimeters ± SD of three independent experiments.

Days of incubation	Diameter of mycelium (cm) at a *P. major* extract concentration (µg/mL) of				
	0	100	500	1000	2000
6	7, 1 ± 1,4	7,4 ± 1,0	6,7 ± 1,1	7,0 ± 0,9	6,3 ± 0,7

Table A10. Diameter of mycelium of *Colletotrichum* species treated with *Plantago major* extract concentrations of 100, 500, 1000 or 2000 µg/mL at day 6 and 9 of incubation. The values presented are the relative means expressed in centimeters ± SD of three independent experiments.

Species	Days of incubation	Diameter of mycelium (cm) at a *P. major* extract concentration (µg/mL) of				
		0	100	500	1000	2000
C. acutatum	9	8,0 ± 0,1	7,9 ± 0,2	7,2 ± 0,2	6,8 ± 0,2	6,1 ± 0,4
C. gloeosporioides	6	8,4 ± 0,1	8,0 ± 0,1	7,4 ± 0,2	7,0 ± 0,3	6,2 ± 0,1
C. godetiae	9	6,5 ± 0,1	6,5 ± 0,1	6,5 ± 0,2	6,2 ± 0,1	5,4 ± 0,3
C. nymphaeae	9	6,6 ± 0,1	6,7 ± 0,1	6,3 ± 0,1	6,1 ± 0,1	5,2 ± 0,1

Table A11. Diameter of mycelium of *Diplodia corticola* treated with *Plantago major* extract concentrations of 100, 500, 1000 or 2000 µg/mL at day 9 of incubation. The values presented are the relative means expressed in centimeters ± SD of three independent experiments.

Days of incubation	Diameter of mycelium (cm) at a *P. major* extract concentration (µg/mL) of				
	0	100	500	1000	2000
9	8,2 ± 0,2	8,4 ± 0,1	8,5 ± 0,0	8,5 ± 0,0	8,3 ± 0,2

Table A12. Diameter of mycelium of *Phytophthora cinnamomi* treated with *Plantago major* concentrations of 100, 500, 1000 or 2000 µg/mL at day 6 of incubation. The values presented are the relative means expressed in centimeters ± SD of three independent experiments.

Days of incubation	Diameter of mycelium (cm) at a *P. major* extract concentration (µg/mL) of				
	0	100	500	1000	2000
6	8,3 ± 0,2	8,5 ± 0,0	8,5 ± 0,0	8,3 ± 0,2	5,6 ±1,5

Table A13. Diameter of mycelium of *Botrytis cinerea* treated with *Urtica dioica* extract concentrations of 100, 500, 1000 or 2000 µg/mL at day 6 of incubation. The values presented are the relative means expressed in centimeters ± SD of three independent experiments.

Days of incubation	Diameter of mycelium (cm) at a *U. dioica* extract concentration (µg/mL) of				
	0	100	500	1000	2000
6	5,7 ± 0,5	6,6 ± 1,2	7,8 ± 0,7	7,4 ± 0,2	6,2 ± 0,5

Table A14. Diameter of mycelium of *Colletotrichum* species treated with *Urtica dioica* extract concentrations of 100, 500, 1000 or 2000 µg/mL at day 6 and 9 of incubation. The values presented are the relative means expressed in centimeters ± SD of three independent experiments.

Species	Days of incubation	Diameter of mycelium (cm) at a *U. dioica* extract concentration (µg/mL) of				
		0	100	500	1000	2000
C. acutatum	9	7,7 ± 0,1	7,8 ± 0,2	6,6 ± 1,2	6,8 ± 1,2	6,7 ± 0,5
C. gloeosporioides	6	7,7 ± 0,1	7,7 ± 0,1	7,2 ± 0,1	6,7 ± 0,6	5,5 ± 0,1
C. godetiae	9	6,3 ± 0,6	6,8 ± 0,0	6,9 ± 0,2	7,0 ± 0,7	5,6 ± 1,0
C. nymphaeae	9	7,3 ± 0,3	7,8 ± 0,2	8,0 ± 0,0	8,1 ± 0,1	7,4 ± 0,5

Table A15. Diameter of mycelium of *Diplodia corticola* treated with *Urtica dioica* extract concentrations of 100, 500, 1000 or 2000 µg/mL at day 9 of incubation. The values presented are the relative means expressed in centimeters ± SD of three independent experiments.

Days of incubation	Diameter of mycelium (cm) at a *U. dioica* extract concentration (µg/mL) of				
	0	100	500	1000	2000
9	8,5 ± 0,0	8,5 ± 0,0	8,3 ± 0,1	8,0 ± 0,0	6,7 ± 1,0

Table A16. Diameter of mycelium of *Phytophthora cinnamomi* treated with *Urtica dioica* concentrations of 100, 500, 1000 or 2000 µg/mL at day 6 of incubation. The values presented are the relative means expressed in centimeters ± SD of three independent experiments.

Days of incubation	Diameter of mycelium (cm) at a *U. dioica* extract concentration (µg/mL) of				
	0	100	500	1000	2000
6	8,5 ± 0,0	8,3 ± 0,2	6,8 ± 0,7	7,4 ± 0,2	6,4 ± 0,2

Table A17. Diameter of mycelium of *Botrytis cinerea* treated with extract's mix concentrations of 100, 500, 1000 or 2000 µg/mL at day 6 of incubation. The values presented are the relative means expressed in centimeters ± SD of three independent experiments.

Days of incubation	Diameter of mycelium (cm) at an extract's mix concentration (µg/mL) of				
	0	100	500	1000	2000
6	5,3 ± 2,8	6,8 ± 1,3	5,8 ± 1,5	6,0 ± 1,8	5,1 ± 2,1

Table A18. Diameter of mycelium of *Colletotrichum* species treated with extract's mix concentrations of 100, 500, 1000 or 2000 µg/mL at day 6 and 9 of incubation. The values presented are the relative means expressed in centimeters ± SD of three independent experiments.

Species	Days of incubation	Diameter of mycelium (cm) at an extract's mix concentration (µg/mL) of				
		0	100	500	1000	2000
C. acutatum	9	7,7 ± 0,2	7,4 ± 0,5	7,1 ± 0,3	6,8 ± 0,5	5,9 ± 0,3
C. gloeosporioides	6	7,5 ± 0,4	7,9 ± 0,2	7,7 ± 0,3	7,2 ± 0,2	5,9 ± 0,2
C. godetiae	9	7,0 ± 0,5	7,1 ± 0,2	6,8 ± 0,1	6,5 ± 0,1	5,7 ± 0,2
C. nymphaeae	9	7,7 ± 0,1	7,8 ± 0,0	7,7 ± 0,1	7,1 ± 0,3	5,7 ± 0,9

Table A19. Diameter of mycelium of *Diplodia corticola* treated with extract's mix concentrations of 100, 500, 1000 or 2000 µg/mL at day 9 of incubation. The values presented are the relative means expressed in centimeters ± SD of three independent experiments.

Days of incubation	Diameter of mycelium (cm) at an extract's mix concentration (µg/mL) of				
	0	100	500	1000	2000
9	8,3 ± 0,2	8,3 ± 0,2	8,5 ± 0,0	8,5 ± 0,0	7,6 ± 0,6

Table A20. Diameter of mycelium of *Phytophthora cinnamomi* treated with extract's mix concentrations of 100, 500, 1000 or 2000 µg/mL at day 6 of incubation. The values presented are the relative means expressed in centimeters ± SD of three independent experiments.

Days of incubation	Diameter of mycelium (cm) at an extract's mix concentration (µg/mL) of				
	0	100	500	1000	2000
6	8,5 ± 0,0	8,5 ± 0,0	8,1 ± 0,4	6,3 ± 0,3	2,7 ± 0,3

Table A21. Effect of different concentrations, 100 µg/mL, 500 µg/mL, 1000 µg/mL and 2000 µg/mL, of some plant extracts against seven phytopathogenic fungi on day 6 (*B. cinerea*, *C. gloeosporioides* and *P. cinnamomi*) and on day 9 (*C. acutatum*, *C. godetiae*, *C. nymphaeae* and *D. corticola*) of incubation. – absence of antifungal activity (-∞ - 0% of mycelial growth inhibition); + slight antifungal activity (1% - 24.99% of mycelial growth inhibition); ++ moderate antifungal activity (25% - 49.99% of mycelial growth inhibition); +++ moderately high antifungal activity (50% - 74.99% of mycelial growth inhibition); and ++++ very high antifungal activity (75% - 100% of mycelial growth inhibition). * indicate that the plant extract was not studied against the plant pathogen fungus.

		Phytopathogenic fungi						
		B. cinerea	*C. acutatum*	*C. gloeosporioides*	*C. godetiae*	*C. nymphaeae*	*D. corticola*	*P. cinnamomi*
C. edulis	100	*	*	*	*	*	-	-
	500	*	*	*	*	*	-	-
	1000	*	*	*	*	*	-	-
	2000	*	*	*	*	*	-	-
Medicago sp.	100	+	+	+	-	-	+	+
	500	+	+	+	+	+	+	+
	1000	+	+ +	+	+	+	+ +	+
	2000	+ +	+ +	+ +	+ +	+ +	+ + +	+
M. indicus	100	-	+	+	+	+	+	-
	500	+	+	+	+	+	+	+
	1000	+	+	+	+	+	+	+ + +
	2000	+	+ +	+ +	+	+	+	+ + + +
P. major	100	-	-	+	-	-	-	-
	500	+	+	+	+	+	-	-

	1000	-	+	+	+	+	-	-
	2000	+	+	+ +	+	+	-	+ +
P. oleracea	100	*	*	*	*	*	-	-
	500	*	*	*	*	*	-	-
	1000	*	*	*	*	*	-	-
	2000	*	*	*	*	*	-	-
U. dioica	100	-	-	-	-	-	+	+
	500	-	+	+	-	-	+	+
	1000	-	+	+	-	-	+	+
	2000	-	+	++	+	-	+	+
Mix	100	-	+	-	-	-	-	-
	500	-	+	-	+	-	-	+
	1000	-	+	+	+	+	-	+ +
	2000	-	+	+	+	+ +	+	+ + +