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Evaluation of biocidal potential of some plant species from the Mediterranean wild flora

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To Dalila
always by my side

Acknowledgments

1 Table of contents

List of tables

List of figures

Summary

The use of environmentally friendly methods for pest control is gaining an increasing attention in agricultural practice. By one side, the consciousness of the environmental and health risks linked to the widespread use of synthetically-derived pesticides are increasing worldwide. The high persistence of chemicals in the environment and in the food chain, and the development of highly resistant pest populations, including weeds, insects, bacteria, and fungi, are among the most concerning adverse effects of their uncontrolled use. Second, there are some special cropping systems, such as those for organic production, where the use of chemicals is discouraged, when not banned.

In this general frame, an increased number of farmers is seeking alternative technical choices for pest management.

The main objective of this work was to contribute to the development of sustainable methods for crop protection, giving value to the native resources of the Mediterranean area through a detailed study on the biocidal activity of secondary metabolites produced by some selected wild plants, with a view to their possible practical applications.

Three Mediterranean wild species, namely *Artemisia arborescens*, *Dittrichia viscosa* and *Rhus coriaria* were selected with this purpose, due to their already acknowledged biological activity and the availability of plant biomass. Those plant species have been investigated and tested against weeds, fungi and nematodes utilizing different extraction procedures and different assays. In order to improve feasibility, a special attention was paid to the evaluation of water extracts and other simply obtained plant derivatives, such as plant powder. It is easy to understand how crucial it could be to find active molecules soluble with water if we consider which are the main dispersing methods in agriculture. Hence, the experimentation about water extracts followed the entire doctoral experience, and this prospective focused the attention from the very beginning, leading many of the trials that were set, starting from some preliminary results obtained by the Agricultural, Food and Forest Sciences Department, University of Palermo.

Phytotoxic activity

Water extract

In vitro trials

Environmental controlled assays were meant to evaluate the inhibitory effects of the aqueous extracts of the donor plants, *A. arborescens*, *L. camara*, *M. azedarach* and *R. coriaria*, both pure (100%) and in 50% mixture, on seeds germination and initial growth of target plants, *Eruca vesicaria*, *Brassica napus*, *Araujia sericifera* and *Plantago indica*. Plant material was collected and adequately dried

before being ground, hence the extraction took place. A thin layer of filter paper, moistened with a defined quantity of extract or mixture, was placed in a Petri dish, then seeds were laid down in it, and incubated in a dark/light alternation conditions at constant temperature. Number of germinated seeds, shoots and roots elongation were recorded on a daily basis. Results showed a remarkable inhibition of germination of the tested extract, both pure and in mixture, exception made for *P. psyllium*, where it was noticed a stimulation of the germination.

Pot culture

In order to carry out the experimentation in a condition similar to the natural one, the previous *in vitro* experience was followed by a pot experiment, focused on the biological effect of pure extracts from the donor plants, *A. arborescens*, *L. camara*, *M. azedarach* and *R. coriaria*, on seeds germination and initial growth of *E. vesicaria*. Number of germinated seeds, shoots and roots elongation were recorded on a daily basis. Despite a good degree of agreement with the results obtained from the *in vitro* tests, these experiments highlighted the existence of a species-specific response. In detail, a reduction in the percentage of seeds germination after treatment with *A. arborescens* and *M. azedarach* extracts was noticed, as well as a significant delay in the mean seeds germination time in pots treated with *R. coriaria* extracts.

The aforementioned results, *in vitro* and pot experiments, were published in 2017 in the Allelopathy Journal with the title "*Herbicidal potential of aqueous extracts from Melia azedarach L., Artemisia arborescens L., Rhus coriaria L. and Lantana camara L.*"

Open field trial

This *in vivo* experiment was arranged in the Experimental Farm "*Sparacia*", with the aim of testing two of the most effective aqueous extracts, based on the previous findings, against weeds in open field conditions. Plant material was collected and adequately dried before being ground, hence the extraction took place. Extracts were then stored at zero degrees before delivering, which took place in two different times (February and April 2016) within properly set experimental plots of *Triticum durum* (cv. *Valbelice)*. Plots were surveyed periodically, from sowing to harvesting. Emerged weeds were counted and classified. General growth conditions of the crop were recorded. *A. arborescens* seemed to be the most active in weed suppression.

Raw powder

Greenhouse experiment was carried out at the Harper Adams University, UK, with the aim of reproducing natural occurrence of litter leaching against weeds. In particular, it was meant to investigate the inhibition effect of the leaches on seeds germination of *Alopecurus myosuroides* and

Sinapis arvensis. So that, aerial parts of the donor plants, *A. arborescens*, *D. viscosa* and *R. coriaria*, were dried, finely ground and sieved. This approach was based on the relatively simple preparation, with no solvent required, as well as for the good reactivity of the powder tested against other organisms. Raw powder, obtained as such, was mixed with the soil, and different amounts of raw powder represented different treatments. Number of germinated seeds was surveyed constantly during the experiments, while dry weight of the aboveground produced biomass was recorded at the end of the trial. The main outcome of these experiments confirmed the inhibition effect of *A. arborescens* against both target plants, at the highest concentrations.

Phytotoxicity-guided fractionation

This experimental activity was carried out at the Agricultural Research Service (ARS) of the United States Department of Agriculture (USDA). The protocol of extraction, separation, purification and identification of active compounds, guided by the bioassay response, was followed for each of the investigated plant species: *A. arborescens*, *D. viscosa* and *R. coriaria*. Different solvents were used in order to extract different classes of compounds. So that, dried and finely ground plant materials were undergone to the solid/liquid extraction, followed by the liquid/liquid ones. Hence, fractionation proceeded by mean of column chromatography technique. Each step of this protocol included a new set of bioassays which drove the process. Two kinds of assay were performed: the first one, ran at each step of the separation process, consisted in the assessment of the inhibition of seeds germination of the target plants: *Lactuca sativa* and *Agrostis stolonifera*; the second one, at the end of the process, on *Lemna aequinoctialis*, helped to quantify the minimum amount of pure compound needed to obtain the required effect. As a result, two lignans with strong phytotoxic activity were identified from *A. arborescens*: Ashantin and Sesamin.

The results of this experiment were published in 2018 on Natural Product Communications with the title "*Phytotoxic Lignans from Artemisia arborescens*"

Fungitoxic activity

Fungitoxicity-guided fractionation

This experimental activity was carried out at the Agricultural Research Service (ARS) of the United States Department of Agriculture (USDA). The donor plants and the experimental procedure were the same as those followed in the above described phytotoxicity-guided fractionation. Direct bioautography technique was applied at each step of the separation process, consisting in the assessment of growth inhibition of selected fungal species: *Botrytis cinerea*, *Colletotrichum acutatum*, *C. fragariae*, *C. gloeosporioides*, *Fusarium oxysporum* and *Phomopsis obscurans*; clear zones of

fungal growth inhibition on the TLC plate indicated the presence of antifungal constituents in each extract or pure compound.

As a result, three lignans with strong fungitoxic activity were identified from *D. viscosa*: Carabrone, Isocostic acid and Tomentosin.

In vitro experiment

Starting from the previously obtained results from *D. viscosa*, a new set of trials was carried out at the Plant Pathology laboratories of the SAAF Department (University of Palermo). This time, the inhibition was tested on a new pool of target fungi: *Aspergillus brasiliensis*, *A. flavus*, *A. fumigatus*, *A. luchuensis*, *Fusarium lycopersici*, *Botrytis cinerea*, *Ganoderma resinaceum* e *Laetiporus sulphurous*. The *in vitro* trial consisted in the assessment of inhibition of fungal growth on two conditioned substrates, one with *D. viscosa* leaves powder and one with *D. viscosa* water extract. Unexpectedly, substrate mixed with powder markedly inhibited fungal growth almost in every case, while water extract had a markedly lower effect. Only *B. cinerea* growth was not much affected by the treatments.

Nematicidal activity

A preliminary study on nematicidal activity of *D. viscosa* water extract was carried out in the laboratories of Crop and Environment Research Centre (CERC) at Harper Adams University. Various concentrations of the extract were tested against juvenile stage of potato cysts nematodes (PCN): *G. rostochiensis* e *G. pallida*. Visual assessment was performed, and number of dead and alive nematodes recorded. Results showed that a weak nematicidal effect at the highest concentration, but additional research needs to be done for a better understanding.

Conclusions

These studies carried out on the biocidal activities of *A. arborescens*, *D. viscosa* and *R. coriaria* have given a contribution to the enhancement of the scientific knowledge about the possibility to use plant-derived products for agricultural pest management. Results obtained clearly shown how *A. arborescens* extracts have interesting prospective in weed control, while *D. viscosa* expressed a strong inhibitory activity on fungal growth and seems to be feasible for deeper investigation against nematodes. Despite encouraging preliminary results, *R. coriaria* did not show any particular biocidal properties. A high species-specificity of the biocidal effects has emerged, since the aforementioned effects proved to be highly variable, both in direction and in intensity, according to the donor-target combination. Of course, this field of study is huge, and many efforts are further required before practical utilization of these compounds.

2 Introduction

Since the dawn of agriculture, field management is characterized by the continuous struggle of farmers against the multitude of limiting factors that hamper crop production. Crops compete with weeds for space, water, nutrients and light, but they also have to deal with insects, fungi, nematodes, bacteria and viruses, living in the same area, whose competition for food/energy requires putting in place big efforts. Weeds and pests control has a big impact on farming routine, absorbing a relevant part of time and resources. In this sense, the so-called "green revolution" was more than an evolutionary step, rather representing one of the biggest changes of humanity. Chemistry met the agricultural needs, mainly in terms of fertilizers, herbicides and pesticides, and a flourishing age started with abundant yield from healthy crops. Since then, the use of agrochemicals seemed the only possible answer to protect crops, but after many decades of their intense and uncontrolled use, new doubts have risen. New studies have stated how a prolonged use of chemicals, which are massively released in the environment, produces day by day accumulation phenomena and extensive environmental degradation. According to their chemical nature, such substances may lead to various levels of soil, groundwater and air pollution. Their massive presence may also affect food chain, and a prolonged exposure may result harmful for both livestock and human health (Ratnadass *et al.* 2012). Resistance phenomena to many commonly used chemicals are widespread, and, in addition, monoculture, currently practiced by modern agriculture, has led to a reduction of genetic variability of plants, ultimately selecting highly specialized and aggressive pathogens (Stukenbrock and McDonald 2008).

In this frame, the increasing demand for food, and the rising concerns for its safety, have pushed researchers to search for novel environmental-friendly strategies for pest control. Among these, an outstanding role is assumed by the search for novel active compounds, focusing on natural products as the widest source where to look at. Plants, indeed, have developed during their evolutionary process many strategies of adaptation to the environment, including the defending ability against herbivores, parasites and competing plants (Wink 1988, 2009). Most of these strategies can be translated in a pool of chemicals produced, that are released in the environment to accomplish their specific role. Plants produce two kinds of chemicals, which are commonly distinguished in primary and secondary metabolites. The first term refers to those compounds directly involved in plant growth, development and reproduction processes, hence underpinning basic functions of the plant. The second group includes all metabolites, usually species-specific (although there are certain similarities at genus level), that plants produce as a response to certain environmental stimuli. Secondary metabolites may be thought as the result of coevolution between plants and their biotic and abiotic

environment (Stavrianakou *et al.* 2006), and their presence reveals the strategies put in place against respective pests and competitors (Wink 2009). More than 20,000 secondary metabolites have been identified but this number keeps growing (Waterman 1992). From the chemical point of view, they belong to many chemical classes such as Alkaloids, Amines, Glucosinolates, Alkamides (with nitrogen), Terpenes, Saponins, Steroids, Phenylpropanoids, Coumarins, Lignans, Flavonoids, Tannins, Polyacetylenes, fatty acids, waxes, Polyketides, Carbohydrates, acids (without nitrogen) and others. There are many successful cases of secondary metabolites that have been discovered and, after being extracted from plants, have been applied in many different areas. Among insecticides it's worth recalling Pyrethrins, found in the flower head of *Chrysanthemum cinerariifolium (Trevir.) Vis.* (syn. of *Tanacetum cinerariifolium* (Trevir.) Sch.Bip.)and *Chrysanthemum coccineum* Willd. (syn. of *Tanacetum coccineum* (Willd.) Grierson) and accounted for about 25% of the worldwide insecticide market in 1998 (Kim *et al.* 2006). Other examples may be found in the field of herbicides discovery: Leptospermone, a β-triketone produced from *Leptospermum scoparium*, was the template for the synthesis of an analogue, Mesotrione, that due to its powerful herbicidal activity accounted for more than 50 countries sales for $400 million in 2017 (Carles, Joly, and Joly 2017).

Hence, the study about plant-derived product for pest control is a novel and exciting field of research. In this frame, this work was carried out with the main objective to contribute to the development of sustainable methods for crop protection, giving value to the local biological capital of the Mediterranean area through a detailed study on the biocidal activity of secondary metabolites produced by *A. arborescens*, *D. viscosa* and *R. coriaria*, with a view to their possible practical applications.

3 Selected biocidal plants

3.1 *Artemisia arborescens*

3.1.1 *Introduction*

Artemisia arborescens (Vaill.) L. belongs to the *Artemisia* (Vaill.) L. genus, the largest and most widely distributed genus of the *Daisy* family, *Compositae* (or *Asteraceae)*. Also known as *Silver sage*, *Large wormwood*, *Tree wormwood,* it is a perennial shrub, typical of Mediterranean wild flora, from 1 to 2 m tall, with silver grey-green, deeply divided leaves and clusters of inconspicuous yellow flowers that appear throughout late spring until the summer, depending on the environmental conditions. (Fascella *et al.* 2012).

A. arborescens is a nanophanerophyte (Bocchieri and Iiriti 2006, 2004). According to the Raunkiær classification of life forms, phanerophytes are trees or shrubs with dormant buds on branches freely projected into the air, while the root "nano" means that its size is lower than 2 m (Smith 1913).

The genus *Artemisia* comprises more than 500 species widespread in the temperate zones of Europe, Asia and North America (Bora and Sharma 2011; Sanz *et al.* 2008; Watson *et al.* 2002), with a few representatives in the Southern hemisphere (Valleás *et al.* 2001). Despite their bitter taste, mainly due to the high content in sesquiterpenes, some species provide food for wild animals including ungulates, rodents, birds and insects. Many *Artemisia* species are major cause of allergies in humans while all species produce aromatic oils, hence some are used as flavourings, hallucinogens, vermifuges, and pharmaceuticals, and some other are toxic (Watson *et al.* 2002). In natural and semi-natural pastures, several woody species, as *A. arborescens*, tend to replace desirable forage, under grazing pressure (Watson *et al.* 2002; Rühl and Pasta 2007). *A. arborescens* is a pioneer (sub)nitrophilous plant of thermo-xerophilous grasslands with a synanthropic character, hence its presence is often linked to disturbed and nutrient-rich conditions (Rühl and Pasta 2007) and is considered endemic of the Southern part of the Mediterranean area (Ornano *et al.* 2013). Such a xerophytic plant typically colonizes littoral rocks, cliffs and pastures forming upright covering formation of silvery foliage which disperse a mild camphor scent (Garcia *et al.* 2006; Motti and Bonanomi 2018), but can also be found inland where it grows on calcareous soils (Ornano *et al.* 2013).

A. arborescens plays an important ecological role as an host plant for various species of leafhoppers and aphids (Quartau 1996; Gish and Inbar 2006).

Figure 1 - Various images of A. arborescens. Up-left, aspect at flowering stage. Up-right, close-up on flowers. Down-left, inflorescence. Down-right, wild colony.

Artemisia essential oils have shown a different chemical profile depending on the genotype, the phenological stage and plant origins (Militello *et al.* 2012). Therefore, three main different chemotypes of *A. arborescens* have been identified according to the relative content of the essential oil major components, namely: a β-thujone/camphor type, found in Sardinia and Morocco; a chamazulene/camphor type, found in North-western part of USA and in Southern part of Italy including Calabria, Sicily and Aeolian Islands; a β-thujone/chamazulene type, found in Liguria, Sicily, Sardinia and Algeria (Dessí *et al.* 2001; Militello *et al.* 2012; Marongiu *et al.* 2006; Ornano *et al.* 2013).

3.1.2 Uses

As far as we know, folk medicine has used wild plants for medical purposes and *A. arborescens* was one of those. Although the plant has been known since antiquity as a contraceptive and abortifacient, it was also appreciated for its anti-inflammatory effects, mainly due to its content of chamazulenogenic compounds (Sacco *et al.* 1983). Nonetheless, many other applications have been reported, which used *A. arborescens* as a therapeutic resource in case of gastrointestinal disorders, respiratory complaints, dermatological problems, skeleto-muscular disorders, fever (Leonti *et al.*

2009; Sanna *et al.* 2015), diabetes (Leto *et al.* 2013), and also as an antispasmodic and antipyretic (Palmese *et al.* 2001). Many uses of *A. arborescens* are related to its antioxidant and antimicrobial activity (Carvalho *et al.* 2011; Bakkali *et al.* 2008; Saddi *et al.* 2007; Altunkaya *et al.* 2014; Younes *et al.* 2012; Sanna *et al.* 2015; Militello *et al.* 2011; Abad *et al.* 2012; Fascella *et al.* 2012; Dessí *et al.* 2001). Besides, as drought-resistant and nitrophilous plant, *A. arborescens* has been addressed to a recent interest even by the environmental engineering for phytorestoration (Cella and Collu 2004) and, due its hardiness, has been proposed as an ornamental plant to create hedges in low-maintenance gardens.

3.1.3 *Biological activities*

A. arborescens mainly owes the wide range of biological activities that raised its importance to its essential oil components. As a matter of fact, several investigations have been carried out in this direction, mainly in the countries where its presence is abundant and its use forms a part of local cultural heritage. These efforts have allowed to identify in its essential oil many chemical groups such as monoterpene hydrocarbons, oxygenated monoterpenes, sesquiterpene hydrocarbons, oxygenated sesquiterpenes, diterpenes and others (Militello 2012; Ornano *et al.* 2013). The main components which are used to distinguish among the different chemotypes are thujone, camphor and chamazulene (Figure 3). Chamazulene is an azulenic compound, degradation product of matricin (named after *Matricaria chamomilla L.*, chamomile), and is responsible

Figure 2 - Essential Oil distillation

for the blue color of the distillate (Ornano *et al.* 2013) (Figure 2). Many other compounds have been identified as well, such as germacrene-D, borneol, thujol, flavone, sabinene, terpinen-4-ol, β-cubebene, myrcene, linalool and others, whose abundance mostly depends on the Author and distillation method (Beyrouthy *et al.* 2011; Bora and Sharma 2011; Militello 2012; Ornano *et al.* 2013; Sacco *et al.* 1983)

Figure 3 - Chemical structures of (left to right) Camphor, Chamazulene and β-thujone

Academics have investigated *A. arborescens* extracts for several purposes. It has been demonstrated that *A. arborescens* essential oil possesses antifungal (*Rhizoctonia solani*), insecticidal (*Rhyzopertha dominica, Blattella germanica, Bemisia tabaci, Lymantria dispar* and *Mayetiola destructor*), aphicidal (*Aphis gossypii*) activity (Bouzenna and Krichen 2013; Yeom *et al.* 2015; Lamiri *et al.* 2001; Lai *et al.* 2006). Additionally, they were also effective against viruses and bacteria (*Lysteria monocytogenes* and *Herpes simplex virus*) (Abad *et al.* 2012), as well as nematodes (*Bursaphelenchus xylophilus*) (Kim *et al.* 2008). Although these results seem to support the use of *A. arborescens* essential oils against many pests and disease agents, so encouraging their use in agricultural and medical practice, many difficulties have to be solved because of their chemical instability in presence of air, light and even moisture which can determine a rapid degradation of some of their components. One important effort in this direction has been reported, with encouraging results, showing how the encapsulation of essential oils into solid lipid nanoparticles (SLN) can prevent their degradation and allow them to be released much slowly, stretching their endurance (Lai *et al.* 2006).

Finally, *A. arborescens* crude extracts have also demonstrated a good phytotoxic activity, making some of them eligible as potential bioherbicides (Dudai *et al.* 1999; Araniti *et al.* 2012; Labruzzo *et al.* 2018), and an effective repellent activity against apple codling moth neonates. The latter activity has been in part attributed to one of its constituent, thujone, which was demonstrated to have insecticidal properties, but several other constituents seem to play a role as well (Creed *et al.* 2015; Sangkyun *et al.* 1999).

3.2 *Dittrichia viscosa*

3.2.1 *Introduction*

Dittrichia viscosa Greuter (syn. *Cupularia viscosa* Godron & Gren., *Inula viscosa* (L.) Aiton) belongs to the *Daisy* family, *Compositae* (or *Asteraceae)* (Anderberg *et al.* 2005; Parolin *et al.* 2013; Pelser *et al.* 2010). Also known as *Woody Fleabane, Sticky fleabane* and *False Yellowhead*, it is a perennial shrub, native to the Mediterranean basin (Wang *et al.* 2004). *D. revoluta* (syn. of *Inula viscosa* subsp. *revoluta = D. viscosa* subsp. *revoluta*) exclusive of SW Portugal, and *D. orientalis* (syn.: *I. viscosa* var. *angustifolia*) distributed in the Eastern Mediterranean have been also classified (Brullo and de Marco 2000; Parolin *et al.* 2013).

Formerly belonging to the genus *Dittrichia* (Brullo and de Marco 2000) the so-called *Woody Fleabane* is classified as an Hemicryptophyte (Meletiou-Christou *et al.* 1998), that according to the Raunkiær system is an herbaceous perennials with dormant buds in the upper crust of the soil, just below the surface (Smith 1913). *D. viscosa* grows by marginal and ruderal environments, roadsides, damp habitats, dry riverbeds, abandoned fields or even urban areas, therefore is considered a synanthropic species (Wang *et al.* 2004; Anderberg *et al.* 2005; Parolin *et al.* 2013). Moreover, it has been classified as an alien invasive species in different parts of the world such as Australia (Queensland Government 2016), California, New Jersey, Pennsylvania (USDA 2009) and even in Belgium (Maise 2011) where it has been introduced and nowadays is included among invasive species in environmental protection guidelines.

D. viscosa has been described as a perennial plant with a woody base, erect, up to 130 cm high with serrulate oblong-lanceolate sticky leaves, hence its Latin name "*viscosa*". The green parts and the leaves are fully covered with glandular hairs which exude an intense-smelling oil, with an unmistakable odor (Cohen *et al.* 2006a; Nikolakaki and Christodoulakis 2004) (Figure 4).

Figure 4 - Various images of D. viscosa. Up-left, inflorescence with a butterfly. Up-right, inflorescence with a bee. Down-left, a plant at flowering stage. Down-right, close-up on leaves.

D. viscosa is mainly distributed in the Western Mediterranean areas such as Morocco, Algeria, Tunisia, Yugoslavia, Albania, Italy, France, Spain, Portugal but also in Greece and Bulgaria (Brullo and de Marco 2000; Parolin *et al.* 2013).

D. viscosa has two main growth periods. The first one spans from the end of March, when the plant starts sprouting, until May, when the main growth is completed, while starch accumulation in the entire plant keeps going until the end of June. The second growth period takes place from October to November, and is followed by the falling of leaves in December. Flower buds develop at the end of July and blooming lasts from August to October. The fruits ripe between October and November. Fruits are dispersed by the wind (Meletiou-Christou *et al.* 1998; Parolin *et al.* 2013). Due to its long-lasting flowering time throughout the dry period *D. viscosa* is an extraordinary entomophilous plant, which plays an important ecological role as it provides a shelter to different buds, mites, aphids and, especially important in agro-ecosystems, to several enemies of common crop pests (Parolin *et al.* 2013; Lykouressis *et al.* 2012; Perdikis *et al.* 2007; Mamoci *et al.* 2012). The biochemistry of its defensive mechanisms against herbivore insects is still unknown (Mamoci *et al.* 2012).

3.2.2 Uses

Since ancient times, *D. viscosa* has been used in folk medicine due to its antioxidant, anti-inflammatory (Marín *et al.* 2011), diuretic (Chahmi *et al.* 2015; Hernández *et al.* 2007), antipyretics, healing, antiseptic (Lauro and Rolih 1990) and anti-spasmodic (Tebbaa *et al.* 2011) properties.

According to its pioneer characteristics, which imply a natural ability to resist to extreme abiotic stresses such as a high level of metal pollutants and prolonged drought (Curadi *et al.* 2005; Conesa *et al.* 2011), *D. viscosa* has been considered a good candidate as a bio-accumulator for phytostabilization of metal polluted soils (Obeidy *et al.* 2016; Conesa *et al.* 2011; Martínez-Fernández *et al.* 2014), and also as a phytoextractor for phytorestoration of abandoned mines (Pistelli *et al.* 2017).

3.2.3 Biological activities

On the whole, *D. viscosa* is an interesting biological laboratory where the products are its secondary metabolites, mainly collected in the glandular hairs on its leaves. *D. viscosa* is a rich source of phenolics and terpenoids, which have been put in relation with the defensive role against phytopathogenic organisms and the allelopathic effects on neighbouring plants when leached to the soil (Inderjit and Duke 2003; Stavrianakou *et al.* 2006; Ceccherelli *et al.* 1985; Grande *et al.* 1992). Exudates also consist of several flavonoid aglycones (Wollenweber and Dietz 1981).

Chemical composition of this plant is correlated to its origin, hence to different pedoclimatic conditions, so that it is possible to notice dramatic qualitative and quantitative differences among individuals collected from different sites (Madani *et al.* 2016).

Plant compounds have been extracted in different ways and tested according to their ecological roles, being the subject of many reports and scientific papers, which have revealed some of its interesting features. As a matter of fact, extracts of *D. viscosa* have been pointed out as potential products for sustainable weed management, due to their phytotoxic nature (Omezzine *et al.* 2011; Dor and Hershenhorn 2012; Levizou *et al.* 2002), and also as an herbal source for fungicidal preparations against pathogens belonging to the families Oomycetes, Ascomycetes, and Basidiomycetes (Wang *et al.* 2004; Ghany and Ghany 2015; Cohen *et al.* 2006a). Furthermore, *D. viscosa* extracts have shown promising results against nematodes (Oka *et al.* 2001; 2006) and mites (Mansour *et al.* 2004; Merah and Djazouli 2016).

3.3 *Rhus coriaria*

3.3.1 *Introduction*

Rhus coriaria L. belongs to the *Anacardiaceae* family, which includes 60 genera and more than 600 species of shrubs and trees (Ahmad *et al.* 2013). Also known as *Sumac* (or *Sumaq*)*, Tanning Sumac* or even *Sicilian Sumac*, it grows all over the Southern Europe and Mediterranean coastline up to the Middle East (Moffett 2007; Giancarlo *et al.* 2006; Ahmad *et al.* 2013), often in marginal lands where, thanks to its shallow spreading root system, it prevents soil erosion (Ahmad *et al.* 2013). The name Sumac is often used to refer to the whole *Rhus* genus, which includes over 250 species of flowering plants spread in temperate and tropical region worldwide (Rayne and Mazza 2007). *R. coriaria* is the most representative species of its genus and is endemic to the Mediterranean and the South East of Anatolian Region of Turkey. Its name derives from *"sumâqâ"*, meaning red in Syriac (Moazeni and Mohseni 2012). *R. coriaria* is a shrub, 1-3 m in height, with imparipinnate leaves with 9-15 leaflets of different sizes. The inflorescence is a panicle, erect and compact with greenish white terminal flowers. The fruit is a red hairy flatten drupe containing a polished brown seed (Kizil and Turk 2010; Güvenç 1998; Ahmad *et al.* 2013; Andrés-Hernández and Terrazas 2009). Seeds are commonly dispersed by frugivorous birds which also contribute through their digestion to the softening of seed coat, hence facilitating their germination (Doussi and Thanos 1994).

Sumac belongs to the Phanerophyte caespitose (Sciandrello *et al.* 2014) which, according to Raunkiaer's classification of life forms, are plants with buds and branches freely projected into the air (Smith 1913), while "caespitose" refers to the behaviour of growing in small dense clumps or tufts. Although some Authors (Doussi and Thanos 1994) consider *R. coriaria* as a post-fire facultative seeder, that combines strategies by re-sprouting and recruiting new seedlings after fire, some others (Ne'eman *et al.* 1999) suggest that heat shock is necessary for fresh seeds in order to interrupt their dormancy, so that they classify it as a post-fire seeder. *R. coriaria* possesses hermaphrodite flowers pollinated by wind and insects (Zaitoun *et al.* 2007).

Figure 5 - Various images of R: coriaria. Up-left, ripe fruit. Up-right, green fruit. Down-left, phyllotaxis. Down-right, monospecific colony along the road.

3.3.2 Uses

Throughout millennia, Sumac has been used both for its nutritional quality, as a spice, condiment, appetizer, and souring agent; and for its medical values (Shabbir 2012). There are evidence of its use on "*De Materia Medica*" of Pedanius Dioscorides, a Greek physician who lived around 40-90 A.D. Dioscorides wrote about *Sumac* properties mainly as diuretic and anti-flatulent agent (Abu-Reidah *et al.* 2014). Depending on geographical origin and considered part of plant, *R. coriaria* has different popular uses, so that some Authors (Leto *et al.* 2013) report that the infusion of leaves was used for gingivitis, as antipyretic and febrifuge or for hyperhidrosis of feet; some others (Lev and Amar 2002) report the medical use of the seeds as perspiration stimulant, against diarrhoea and for cholesterol reduction. Moreover, in folk medicine, the amount of applications and uses of this plant is astonishing. Sumac is appreciated for its antifibrogenic, antifungal, anti-inflammatory, antimalarial, antimicrobial, DNA-protective (Chakraborty *et al.* 2009), antioxidant (Candan 2003; Ozcan 2003; Pourahmad *et al.* 2010), antithrombin, antitumorigenic, antiviral, cytotoxic, hypoglycaemic (Giancarlo *et al.* 2006), and leukopenic properties and
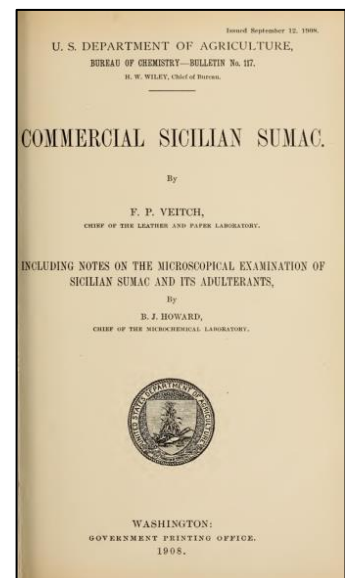
Figure 6 - Historic, archived document on leather tanning trade (U.S. Department of Agriculture 1908)

11

even for the treatment of indigestion, anorexia, diarrhoea, hemorrhagia and osteoarthritis (Moazeni and Mohseni 2012; Shabbir 2012; Rayne and Mazza 2007).

From the industrial point of view, *R. coriaria* has raised interest due to its increasing use in cosmetic and pharmaceutical industries as well as in preserving food (Ali *et al.* 2015; Gulmez *et al.* 2006), veterinary practices (Valiollahi *et al.* 2010; Gharaei *et al.* 2015) and preventing wood decay (Sen *et al.* 2009), while it was already well known in protein-based textile material and hide processing technology, particularly for its mordant dyeing in reddish and brownish (Kizil and Turk 2010; Bahar and Altug 2009; Abu-Reidah *et al.* 2014; Akçakoca Kumbasar *et al.* 2009).

3.3.3 *Biological activities*

So far, *R. coriaria* has been the object of several investigations which continue to open new pathways of research and possible applications. The study of ethnobotany of *Sumac* has leaded researchers to look thoroughly at its range of produced chemicals. So that, nutritionally and medically remarkable metabolites have been identified, such as tannins, phenolic acids, anthocyanins, organic acids and proteins stored in different parts of the plant (Shabbir 2012; Abu-Reidah *et al.* 2014).

The best-known class of compounds linked to *Sumac* are tannins (or tannoids). Tannins are widely distributed in plants, and their abundance is an important feature of *Rhus* genus. In plants they play a role of protection against herbivores, due to their astringent taste, and pests; moreover, they appear to have an effect on plant growth regulation. Tannins can be divided into two sub-classes: hydrolysable and condensed ones; *R. coriaria* is one of the major commercial source for hydrolysable tannins (Sarıözlü and Kıvanç 2009; Abu-Reidah *et al.* 2014), especially of gallotannins. These are polymers formed when gallic acid esterifies and binds with the hydroxyl group (−OH) of a polyol carbohydrate such as glucose. The hydrolysable tannins are the Sumac colouring agents, hence natural constituents of historical leather and textile dyes (Ferreira *et al.* 2004; Akçakoca Kumbasar *et al.* 2009). In addition, the main fatty acids composition of *Sumac* seeds was determined by gas chromatography (GC) and reported as follows: linoleic (49.35-60.57%), oleic (24.58-32.05%), palmitic (8.32-13.58%), stearic (1.57-3.03%) and linolenic (0.46-0.74%) (Ünver and Özcan 2010).

R. coriaria has a wide range of biological activities. Many efforts have been carried out in order to exploit and transfer them to the specific sectors where their properties can be applied. Various formulations and various kinds of *Sumac* extracts have been tested against pests and pathogens with different effectiveness. As an

Figure 7 - Chemical structure of Gallic acid

example, both water and alcoholic extracts were found to be effective against both Gram positive and Gram negative bacterial strains (*Escherichia coli, Helicobacter pylori, Propionibacterium acnes,*

Pseudomonas aeruginosa, Salmonella spp.*,* S*taphylococcus aureus*, *Bacillus* spp, etc.) (Nasar-Abbas and Halkman 2004; Kossah *et al.* 2013; Kirmusaoğlu *et al.* 2012; Gulmez *et al.* 2006), as well as against many fungal species such as *Aspergillus niger, Candida albicans, Colletotrichum acutatum,* and others (Ertürk 2010; Rashid *et al.* 2018; Abu-Reidah *et al.* 2014). Some study also reported a slight antiviral activity of the aqueous crude extract of *Sumac* against HSV-1 (*Herpes simplex virus*) (Sökmen 2001; Monavari *et al.* 2007). Other fields of research are opening new pathways with interesting results, so that it was shown that *R. coriaria* seems to have mite repellent (Mansour *et al.* 2004) and herbicidal (Labruzzo *et al.* 2017) as well as protoscolicide (Moazeni and Mohseni 2012) properties.

4 Experimental Part

4.1 Phytotoxic activity

4.1.1 Water extracts

Suppression of weeds is crucial to achieve a good crop yield. At the meantime, the abuse of chemical products for crops protection has already shown all its limits, as it has been noticed in the last decades. These statements are cornerstones onto which the following experiments have been planned. Interest was focused onto the self-defence mechanisms of certain plants, mainly belonging to the wild Mediterranean flora, particularly on their production and release of hydrophilic phytotoxic compounds. Hydrophilic attribute may appear as a limitation of the investigation itself but it has rather been chosen on purpose, considering the simplicity on large-scale diffusion in agriculture.

This set of experiments has been planned as composed by three subsequent steps: the first one, *in vitro* experiment, was meant to investigate the phytotoxic potential of aqueous extract of Chinaberry (*Melia azedarach* L.), Tree Wormwood (*A. arborescens*), Sicilian Sumac (*R. coriaria*) and Lantana (*L. camara*) on seeds germination of four target plants, while the second one, *in vivo,* was performed in pots, testing seeds germination of *E. vesicaria* (L.) Cav. (syn. of *Eruca sativa* Mill.*)* treated with aqueous extracts of *A. arborescens*, *L. camara*, *M. azedarach*, and *R. coriaria*, and could be considered as a bridge to the third experiment. Indeed, this last one was carried out in field, by evaluating the behaviour of a *Triticum durum* (durum wheat) crop treated with aqueous extract of *A. arborescens* and *R. coriaria*, for two consecutive years.

As a matter of fact, the use of plant water extracts to control weeds is gaining growing attention in organic and environmentally-friendly agriculture, but still unexplored is the study of the effect that such extracts may exert on the yield of durum wheat.

4.1.1.1 Environment-controlled experiment

This study was carried out to evaluate the herbicidal potential of aqueous extract of *M. azedarach* L., *A. arborescens*, *R. coriaria* and *L. camara* - donor plants - both pure and in 50% mixture on seed germination of Rocket (*Eruca vesicaria (L.) Cav.*) and Rapeseed (*Brassica napus* L.), Bladderflower (*Araujia sericifera* Brot.) and Psyllium (*Plantago indica* L.) - target plants. The same pure extracts were also tested in pot culture on seeds germination of *E. vesicaria*.

Plant selection was made by looking at their biological activities according to the literature (Araniti *et al.* 2012a; Lungu *et al.* 2011; Militello and Carrubba 2016; Shapla *et al.* 2011; Talukdar 2013). The aerial parts of *A. arborescens* and *R. coriaria* were collected in the nearby of the Experimental Farm *"Sparacia"*, Department of Agricultural and Forest Sciences, University of Palermo, Cammarata,

Agrigento, Italy (37°38' N - 13°46' E), on full flowering stage, during the early autumn 2015, while full-ripe fruit of *M. azedarach* and flowers of *L. camara* were collected in the nearby of Palermo, Italy, in March 2016. Plant material was dried at room temperature. Seeds of target plants, namely *E. vesicaria* and *B. napus* L. (crops), *A. sericifera.* and *P. indica*L. (weeds), were collected from June to August 2015 from the nearby of *Sparacia* Farm. The experimental activity, carried out in Petri dishes and pot cultures, took place on 2016.

The extraction procedure was the same for each donor plant: 100g of finely ground dry matter were poured with 1L of DDI water, 1:10 (w/v), and left on a stirring plate at constant agitation for 8hrs. Afterward, the aqueous extract was obtained by gravitational filtering of the mixture through Whatman #4 filter paper, then stored at 0°C until use. Once the extracts were prepared as explained above, 5ml were taken off and dried, kept into the oven at 105°C for 24hrs in order to obtain the exact concentration (% DW basis) of each extract which resulted to be as follows: *M. azedarach* 1.676%; *A. arborescens* 1.028%; *R. coriaria* 0.875%; *L. camara* 0.403%.

Petri dish bioassay consisted of 25 seeds of target plant (*E. vesicaria, B. napus, A. sericifera, P. indica*) laid onto a filter paper disk soaked with 5ml of each of 11 total treatments, as listed below:

- 4 treatments were 100% pure extracts
    - *A. arborescens,*
    - *L. camara,*
    - *M. azedarach,*
    - *R. coriaria*,
- 6 treatments were 50/50 (v/v) mixtures, derived by every possible combination between donor plants extracts:
    - *A. arborescens + L. camara;*
    - *A. arborescens + M. azedarach;*
    - *A. arborescens + R. coriaria;*
    - *L. camara + M. azedarach;*
    - *L. camara + R. coriaria;*
    - *M. azedarach + R. coriaria.*
- 1 negative control with DDI water.

4.1.1.2 *Petri-plate bioassays*

Petri dishes of 9cm of diameter were used for this assay; each one was filled with a filter paper disk soaked as described above and, after seeds placement, positioned into a growth chamber at 20°C ±1 with 14/10 (light/dark) hours cycles. Germinated seeds were counted on a daily basis. Roots and shoots were measured as well. A complete randomized design was set with 4 repetitions per treatment

(including control).

Germination percentage was determined by using the formula of Ranal and De Santana, 2006:

$$G = \left(\Sigma_1^k n\right) / 25 \times 100$$

Where, G: germination percentage, n: number of daily germinated seeds, k: last day of germination.

Mean germination time (MGT) was calculated as follows:

$$MGT = \Sigma_1^k (n \times g) / N$$

Where, n: Number of fully germinated seeds per day, g: number of days from the start of trial, N: total number of germinated seeds till the last day of germination (day k).

Phytotoxic level (PL) of treatments was assessed by comparing them with control, particularly based on roots and shoots measurements of seedlings, with the following formula:

$$PL = (lt - lc) / lc \times 100$$

Where, lt and lc are root and shoot lengths (cm) reached at removal time in treated and control seedlings, respectively.

4.1.1.3 Pot culture

A completely randomized design with 4 replicates was set to evaluate the efficacy of the donor plants extracts against *E. vesicaria* seedlings. This *in vivo* trial provided for 4 treatments and 1 control as listed below:4 treatments with aqueous extracts of:

- *A. arborescens,*
- *L. camara,*
- *M. azedarach,*
- *R. coriaria,*

1 negative control with DDI water.

The trial was made in pots (5 x 5 x 6 cm) filled with 100g clayey soil [Clay 41,2%; Silt 22,0%; Sand 36,8%; pH 8,06; EC 0,168 mS; total CaCO3 10,29%; active CaCO3 5,00%; Organic Matter 2,9%; Total N 0,07%; Extractable P2O5 0,0061%, Exchangeable K2O 0,032%; Cl 0,016%] collected from the Experimental Farm "*Sparacia*", Cammarata (AG, Italy). Soil was dried and sieved with a 0.2mm mesh, hence pots were filled and watered with DDI water as much as to reach the field capacity. After all the excessive water was drained from the bottom, 25 seeds of *E. vesicaria* were sown about 1 cm in depth. Two days before and 2 after sowing, 20ml of each treatment, including control, was added to each corresponding pot. Thereafter each pot received 20ml of DDI water every 2 days until the end of the trial, which was 14 days after emergence. Seedlings emergence was recorded on a daily basis. At the end of the trial, seedlings were gently removed from the soil and their height (mm) as well as root length (mm) were measured. Germination percentage (G%), Mean Germination Time (MGT) and phytotoxicity level (%) of extracts were calculated as previously described for the in-vitro assays.

4.1.1.4 Open field trial

This *in vivo* trial was aimed at testing aqueous extracts of two selected donor plants, *Artemisia arborescens* and *R. coriaria*. Selection was based on the results obtained in a first field trial performed in the preceding year (Carrubba *et al.* 2016), where these plants had proved deserving further studies. The experiment was arranged in the Experimental Farm "*Sparacia*", Cammarata (AG), Italy, on a crop of *Triticum durum* cv. *Valbelice* (0111 x BC5), obtained in 1992 by the Department of Agricultural, Food and Forest Sciences of the University of Palermo. Aerial parts of the selected donor plants were collected from wild populations growing in the nearby of Altofonte (Palermo), Italy, then dried at room temperature for at least 5 days, and finely ground. Hence, plant material was soaked with DDI water at the ratio of 1:10 (w:v) and left in continuous stirring at 70 rpm for at least 10 hrs. Gravitational filtration with filter paper (Whatman #4) was performed to eliminate the solid fraction. The obtained aqueous extract was placed in a refrigerator and kept at 4 °C until use. Five treatments were tested, which included the two aqueous extracts (4 L m^{-2}) of *A. arborescens* and *R. coriaria*, plus two negative controls: the first with only water (4 L m^{-2}), in order to verify if the additional amount of water contained in the extracts could have a stimulating effect on plant growth (both wheat and weeds), and to allow separating this effect, if any; the second negative control was set without any treatment (untreated). One positive control was additionally managed with a chemical herbicide, by supplying on selected plots a dose equivalent to 1.5 L ha^{-1} of a mixture of mesosulfuron-methyl 3% + iodosulfuron-methyl-sodium 0.6% + Mefenpir-diethyl 9% (ATLANTIS®, Bayer AG, Leverkusen, Germany). The preceding crop was Berseem clover (*Trifolium alexandrinum* L.). Tillage was performed by means of one summer soil work, at 25-30 cm in depth, followed by two shallower works. Sowing was made mechanically on December 22, 2015 by distributing the seeds at a soil depth of approximately 5 cm, on rows 30 cm apart, in order to obtain a seeding density of 350 viable seeds per m^2 (approximately 200 kg ha^{-1}). At sowing time, 1.5 t ha^{-1} of diammonium phosphate (18/46) was distributed for fertilization. Next, 1.1 t ha^{-1} of urea (46) was spread when the crop had reached the phase of full tillering (Zadoks *et al.* 1974).

The experimental plots were arranged in the field according to the above-mentioned randomized design with three repetitions per treatment. They were sized 1.67 x 1.20 m, with an area of 2 m^2. To avoid any drift phenomena between the plots, they were separated each other by a buffer area, 50 cm wide.

Treatments were performed twice for each plot during crop cycle, applying 4 L m^{-2} of each extract, prepared as above described. The first treatments (including chemical) were applied after 57 days after sowing (DAS), when the durum wheat plants were at the stage of 2 to 3 leaves unfolded, whereas the second application of water extracts was distributed after 105 DAS, as the crop was reaching the stem elongation stage (Zadoks *et al.* 1974).

Starting from the day of the first treatment, crop surveys were made regularly until harvest time; in each survey, the height of wheat canopy was measured, and on a randomly chosen, 50-cm long, row segment within each plot, the number of retrieved wheat and weeds stems were recorded, further categorizing weeds into monocots and dicots. Each plot was hand-harvested separately on June 27, 2016, with the exclusion of the above-mentioned border areas; the total obtained biomass (wheat and weeds) was sorted by botanical species and weighed, and in wheat, the number of spikes per unit area (m^2) was measured. Number of spikelets per spike and weight of seeds per spike were measured on a representative sample of 30 spikes per plot (including controls, and thousand seeds weight (TSW; g) was taken on a representative sample of kernels per each plot.

4.1.1.5 Statistical analyses

Survey data of *in vitro* and in pots experiments were analysed using SAS 9.2 software (SAS Institute Inc., Cary, NC, USA, 2002). Before the analysis, seed germination percentages were transformed in angular values by the formula:

$$X = \arcsin\sqrt{(\%)}$$

while zero values were transformed by the formula:

$$X = \arcsin\sqrt{(0,25/n)}$$

where n being fixed to 25, i.e. the common denominator of all fractions (Steel and Torrie 1980).

Data obtained from both the above experiments were separately submitted to ANOVA (procedure GLM), according to a completely randomized design with four repetitions. In both datasets, the analyses were run for every target species and Dunnett's test was used to separate all means from the respective controls (Steel and Torrie 1980; Gomez and Gomez 1984). Furthermore, with the purpose to detect any significant differences between the major experimental groups (different plant extracts in purity or mixed), an Orthogonal Contrast (OC) test was performed within each target species (Steel and Torrie 1980; Gomez and Gomez 1984).

Data obtained from the *open field* experiment were submitted to one-way ANOVA.

4.1.1.6 Results & Discussion

4.1.1.6.1 Petri dish Assay

Treatments markedly inhibited seeds germination in *E. vesicaria*, *B. napus* and *A. sericifera* exception made for *P. psyllium*, where the comparison "control vs all extracts" was statistically not significant (Figure 8 and Table 1). The Orthogonal Contrasts analysis for this species (Table 1) attributed this behaviour to significant positive effects of 50% mixtures on seeds germination, whereas, the pure extracts were inhibitory to germination. This behaviour of *P. psyllium* may probably be due to the special features of seed germination in this species, in which a hydrophilic mucilage is released by seed coats, with many eco-physiological roles including facilitation of seed hydration (North *et al.* 2014; Western 2012). Although mucilage release is mostly under genetic control (Western 2012; Baykan Erel *et al.* 2012), several environmental factors, including chemicals, may trigger this action. It is likely, therefore, that in our experimental conditions the combined action of the two mixed compounds induced mucilage release from the *P. psyllium* seeds, enhancing germination percentage.

Table 1 - Results of ANOVA, mean values by groups ± standard deviations and results of OC analysis for germination percentages (G%) in seedlings of four target species treated with different plant extracts, pure and in mixture.

G (%) Source of variation	Eruca versicaria	Brassica napus	Araujia sericifera	Plantago indica
Total DF	43	43	43	43
Plant extracts (PE)	$F_{(10, 33)}=25.54^{***}$	$F_{(10, 33)}=35.02^{***}$	$F_{(10, 33)}=46.71^{***}$	$F_{(10, 33)}=16.53^{***}$
Mean values by groups and Orthogonal Contrasts (O.C.)				
Control	92.0 ± 9.38	37.0 ± 5.20	60.0 ± 4.90	6.0 ± 4.90
All extracts	21.3 ± 15.98	10.4 ± 8.33	25.7 ± 17.73	10.2 ± 7.85
Control *vs* all extracts	$F_{(1, 33)}=183.37^{***}$	$F_{(1, 33)}=98.83^{***}$	$F_{(1, 33)}=96.83^{***}$	$F_{(1, 33)}=3.21^{\,n.s.}$
All pure extracts	31.8 ± 20.66	1.75 ± 4.24	33.25 ± 22.17	1.5 ± 4.21
Control *vs* all extracts	$F_{(1, 33)}=124.01^{***}$	$F_{(1, 33)}=209.15^{***}$	$F_{(1, 33)}=64.03^{***}$	$F_{(1, 33)}=6.64^{*}$
All mixtures	14.3 ± 4.46	16.2 ± 4.54	20.7 ± 11.53	16.0 ± 2.58
Control *vs* mixed	$F_{(1, 33)}=202.51^{***}$	$F_{(1, 33)}=37.32^{***}$	$F_{(1, 33)}=108.23^{***}$	$F_{(1, 33)}=21.88^{***}$
Pure extract *vs* mixed	$F_{(1, 33)}=20.47^{***}$	$F_{(1, 33)}=219.83^{***}$	$F_{(1, 33)}=12.60^{**}$	$F_{(1, 33)}=151.08^{***}$
A pure	16.0 ± 11.31	0.0	31.0 ± 3.32	0.0
A mixed	13.7 ± 4.46	16.3 ± 5.02	18.0 ± 8.56	16.3 ± 3.04
A pure vs A mixed	$F_{(1, 33)}<1^{n.s.}$	$F_{(1, 33)}=92.92^{***}$	$F_{(1, 33)}=13.54^{***}$	$F_{(1, 33)}=60.31^{***}$
L pure	51.0 ± 9.11	7.0 ± 5.92	59.0 ± 5.20	6.0 ± 6.63
L mixed	15.7 ± 3.82	15.7 ± 4.15	13.3 ± 4.99	15.3 ± 2.21
L pure vs L mixed	$F_{(1, 33)}=29.48^{***}$	$F_{(1, 33)}=17.51^{***}$	$F_{(1, 33)}=133.33^{***}$	$F_{(1, 33)}=18.07^{***}$
M pure	11.0 ± 3.32	0.0	0.0	0.0
M mixed	13.0 ± 4.65	17.7 ± 3.82	25.3 ± 12.58	16.3 ± 1.97
M pure vs M mixed	$F_{(1, 33)}<1^{n.s.}$	$F_{(1, 33)}=102.35^{***}$	$F_{(1, 33)}=136.40^{***}$	$F_{(1, 33)}=60.58^{***}$
R pure	49.0 ± 11.79	0.0	43.0 ± 7.68	0.0
R mixed	14.5 ± 4.27	15.6 ± 4.33	27.6 ± 12.12	16.4 ± 2.67
R pure vs R mixed	$F_{(1, 33)}=28.17^{***}$	$F_{(1, 33)}=84.88^{***}$	$F_{(1, 33)}=19.26^{***}$	$F_{(1, 33)}=59.05^{***}$

Mean germination time (MGT) varied significantly among both target plants and between the extracts applied. A marked delay was noticed in *E. vesicaria*, treated with *A. arborescens* pure extract, as well as in *A. sericifera*, treated with *A. arborescens, R. coriaria* and *L. camara*, while *B. napus* seeds germination appeared to be stimulated when treated with pure extracts of *L. camara* compared to the control. Similarly, in *P. psyllium*, all mixed extracts significantly hastened seeds germination compared to the control (Figure 9). Both roots and shoots lengths were markedly affected by the

plants extracts (Figure 10 and Figure 11), but as seen before, it was strictly dependent upon the target species and extract applied. Moreover, in all experimental treatments, shoot and root lengths recorded were lower than controls, except where growth was stimulated, which was the case of *P. psyllium* seeds, treated with pure extract of *L. camara*, and, only in root length of *A. sericifera,* treated with pure extract of *A. arborescens*, *R. coriaria* and *M. azedarach + R. coriaria* mixture (Figure 10). Anyways, these stimulatory effects were not significant at Dunnett's test.
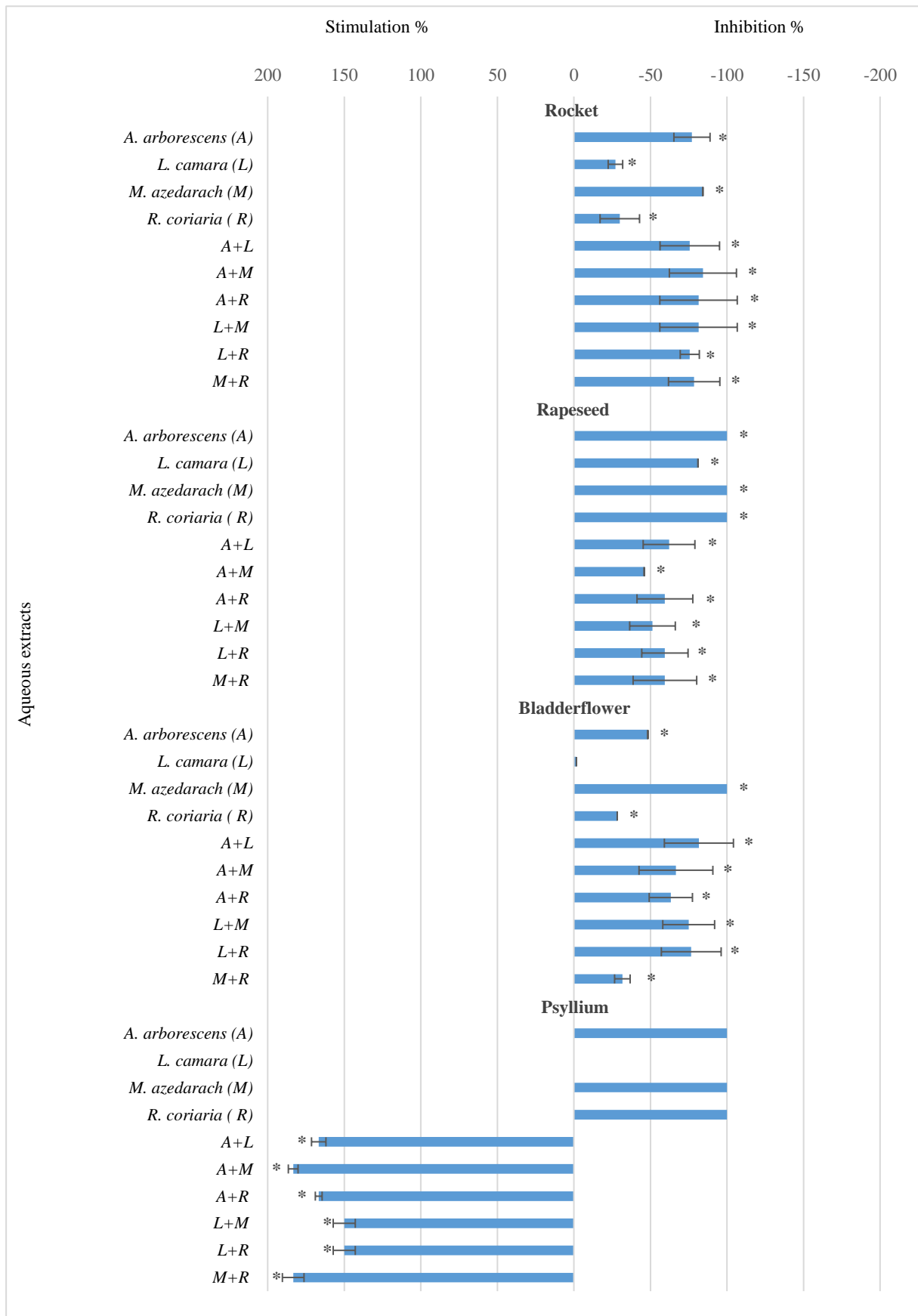
Figure 8 - Inhibition and stimulation effects (% over control) of pure and mixed aqueous extracts of A. arborescens (A), R. coriaria (R), L. camara (L) and M. azedarach (M) on seeds germination (%) of Rocket, Rapeseed, Bladderflower, and Psyllium. Mean values of four repetitions ± standard deviation.
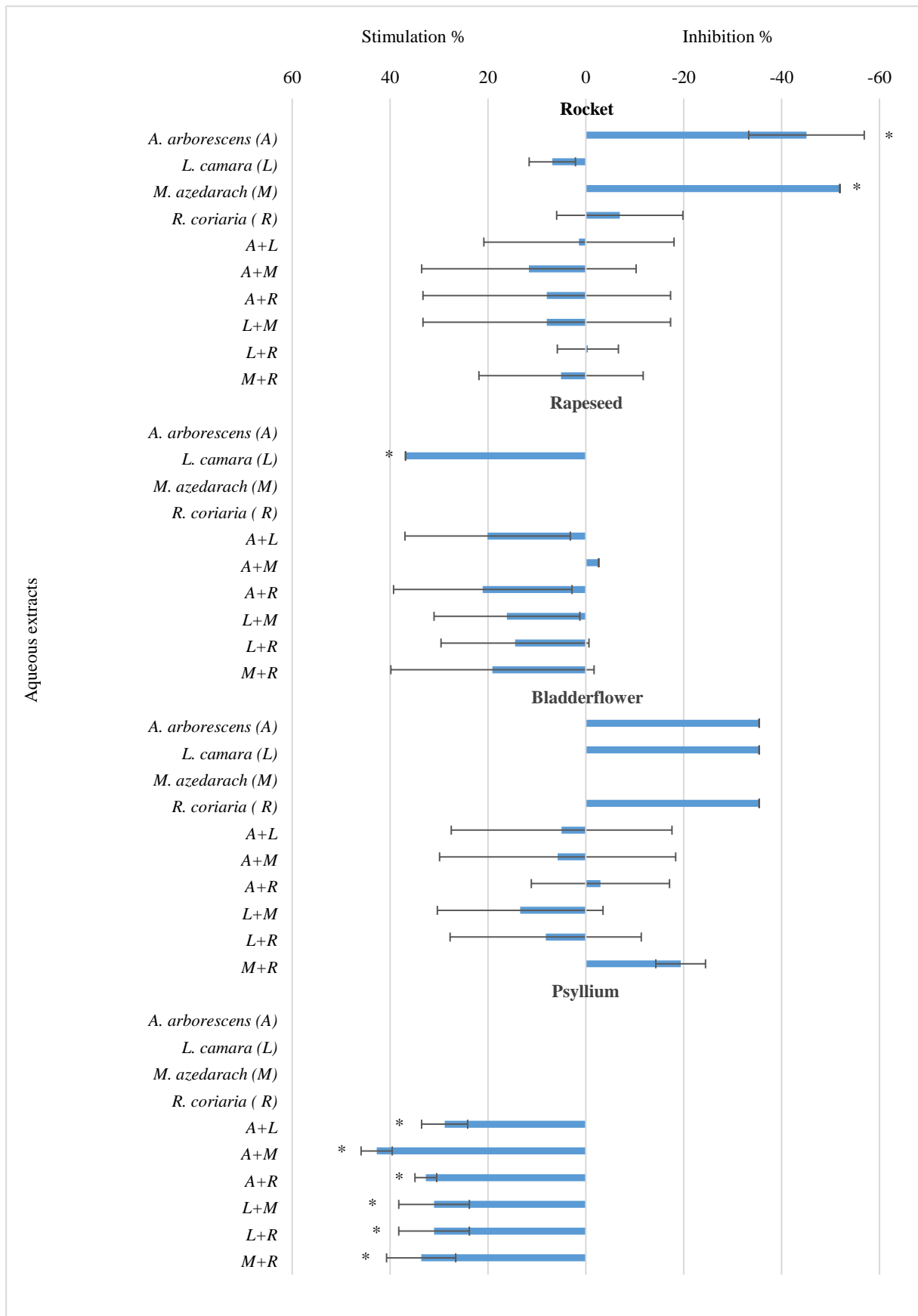
Figure 9 - Inhibition and stimulation effects (% over control) of pure and mixed aqueous extracts of A. arborescens (A), R. coriaria (R), L. camara (L) and M. azedarach (M) on Mean Germination Time (days) of Rocket, Rapeseed, Bladderflower, and Psyllium. Mean values of four repetitions ± standard deviation. Treatments marked with * were significantly different from the respective control at P ≤0.05 (Dunnett's test)
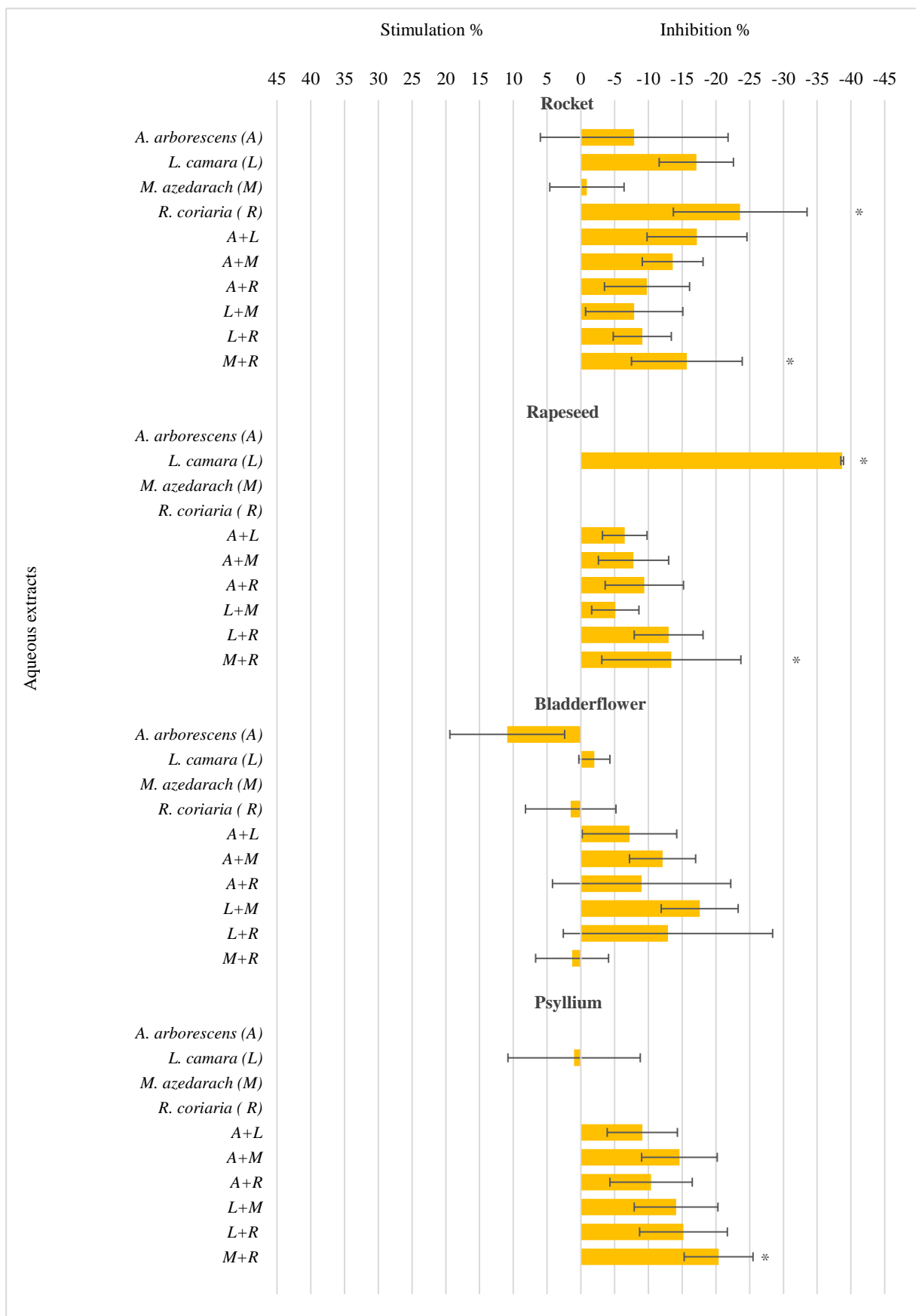
*Figure 10 - Inhibition and stimulation effects (% over control) of pure and mixed aqueous extracts of A. arborescens (A), R. coriaria (R), L. camara (L) and M. azedarach (M) on radicle length (cm) of Rocket, Rapeseed, Bladderflower, and Psyllium. Mean values of four repetitions ± standard deviation. Treatments marked with * were significantly different from the respective control at P ≤0.05 (Dunnett's test)*
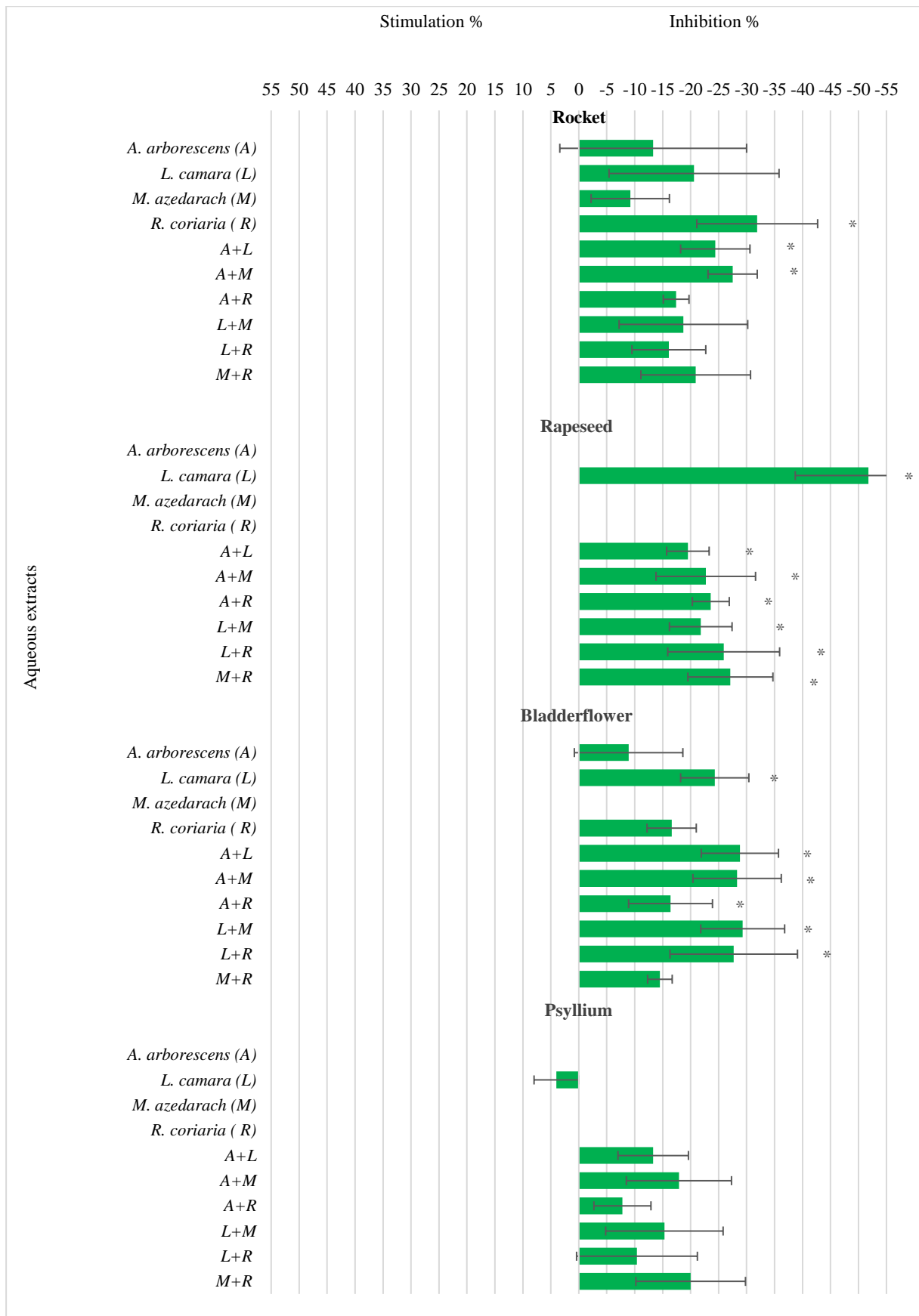
*Figure 11 - Inhibitory and stimulatory effects (% over control) of pure and mixed aqueous extracts of A. arborescens (A), R. coriaria (R), L. camara (L) and M. azedarach (M) on shoot length (cm) of E. vesicaria, B. napus, A. sericifera and P. psyllium. Mean values of four repetitions ± standard deviation. Treatments marked with * were significantly different from the respective control at P =0.05 (Dunnett's test)*

4.1.1.6.2 Pot culture assay

Similarities with *in vitro* experiment were noticed but still results were not identical (Figure 12). Pots treated with *A. arborescens* and *M. azedarach* extracts showed a statistically significant lower seeds germination percentage. Mean Germination Time sensibly increased, in other words seeds germination was delayed, in pots treated with *R. coriaria* extract while there was not such an evidence in pots treated with *A. arborescens* and *M. azedarach* extracts whose Mean Germination Time was not statistically different from the control.

Some interference between the soil and the aqueous extract could have occurred, activating germination in one case (*R. coriaria*) and diluting or detoxifying in the others (*A. arborescens* and *M. azedarach*), which could partially explain such results.
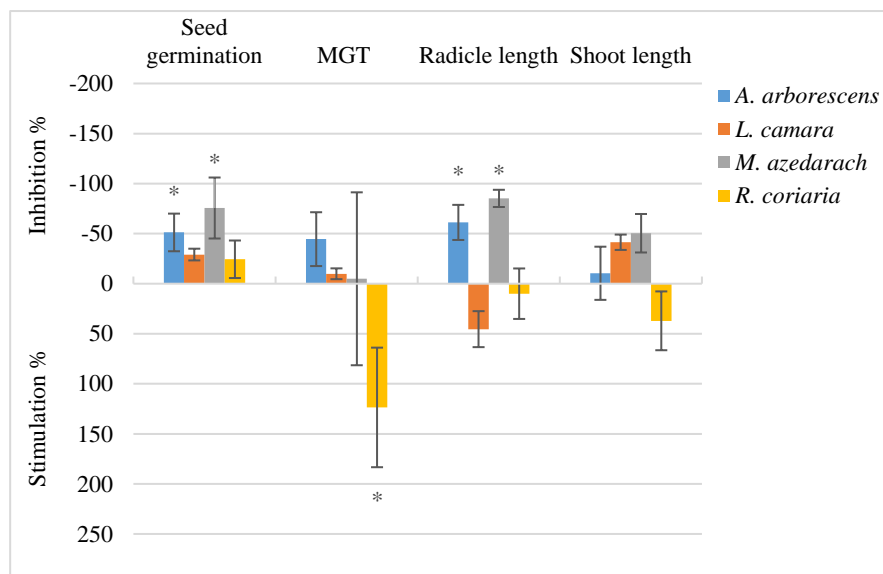
*Figure 12 - Inhibitory and stimulatory effects (% over control) of extracts of A. arborescens, R. coriaria, L. camara and M. azedarach on Seed Germination (%), Radicle length (cm) and Shoot length (cm) of E. vesicaria grown in pots. Mean values of four repetitions ± standard deviation. Treatments marked with * were significantly different from the respective control at P =0.05 (Dunnett's test).*

4.1.1.6.3 *Open field trial*

Figure n. 13 shows the trend of rainfall and temperatures recorded in the trial site throughout the experiment. Total rainfall amount (from December to June) reached 229 mm, rather uniformly distributed along the whole trial period. As usual in the trial site, the temperatures were fairly high,
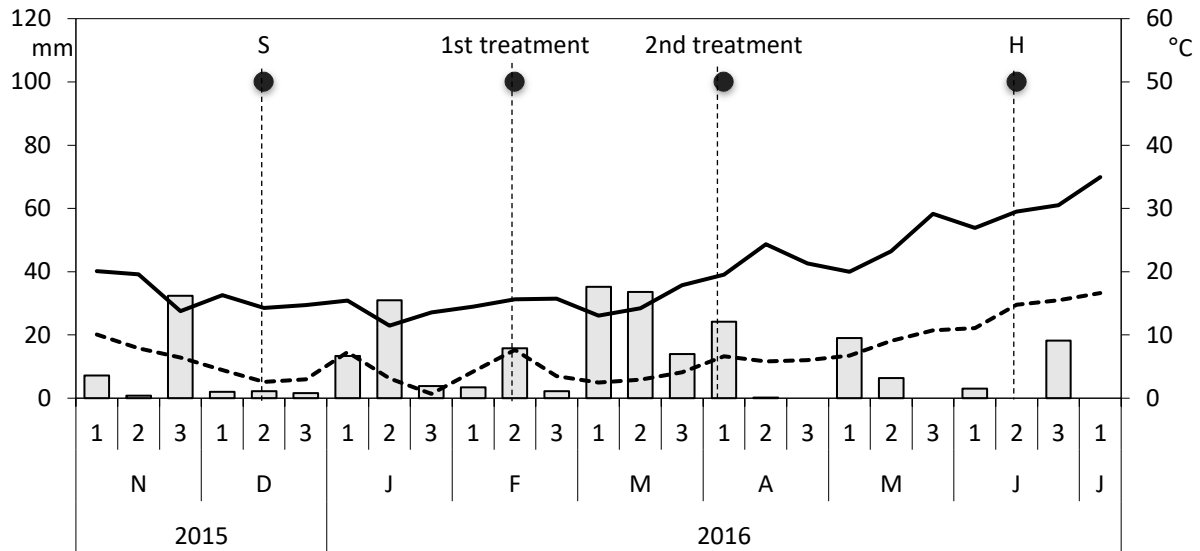
Figure 13 - Ten-day values of rainfall and temperatures recorded in 2015-16 at Sparacia (Cammarata, AG, Italy). Dots indicate the dates of sowing (S), weeding treatments and harvest (H)

with minimum values around 1 °C at the end of January, and maximum values spanning between 12 °C at the beginning of crop cycle and 30 °C close to harvest time.
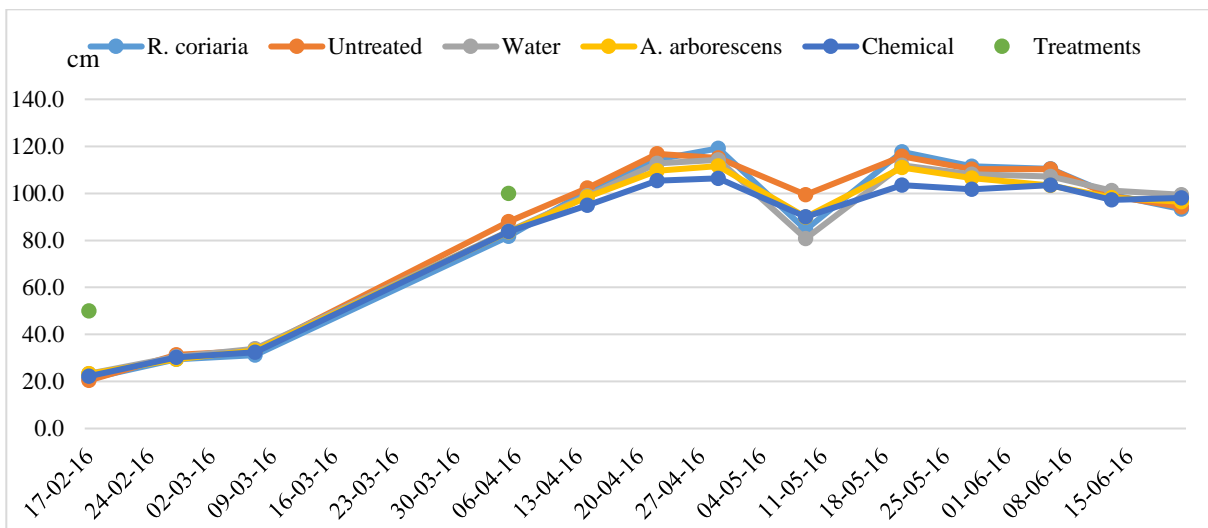
Figure 14 - Trend of mean height of canopy (cm) in durum wheat var. Valbelice treated with two water plant extracts, compared with two controls and one chemical weeding. Green dots indicate the dates of the treatments.

As shown in the graph in Figure 14, no evident difference showed up in the height values recorded throughout the trial on plants belonging to the diverse treatments. Plants grew regularly and

continuously until the end of April; thereafter, the heavy rains recorded in the first days of May caused plants lodging, and an apparent decrease of the height of wheat canopy. This phenomenon affected equally both treated and untreated plots, but the natural capability of durum wheat to recover from lodging, allowed plants to raise again within the following month. Hence, at harvest time, all plants reached height values slightly lower than 1 m, without exhibiting at ANOVA any significant difference (Figure 15). The graphs in Figure 15 also report the major yield traits recorded at harvest time on the experimental plots. Although in a few cases some differentiation between treatments seems to show up, it was not confirmed by the statistical analysis, and the productive behavior of wheat revealed to be unaffected by the distribution of any treatment.

The graphs in Figure 16 report the values of the count of weeds in all plots before and after the dates of the two treatments, showing both their total number and after sorting monocots and dicots. As shown, none of the experimental treatments, neither the chemical, was able to eradicate weeds completely, and a remarkable number of plants was noticed in all experimental plots, probably due to the heavy rainfalls that occurred after both treatments. The occurrence of a reduced number of monocots (mostly *Avena* spp.), in comparison with the untreated control, in the plots treated with the extracts of *A. arborescens,* although not supported by enough evidence so far, should be an interesting starting point for further researches.
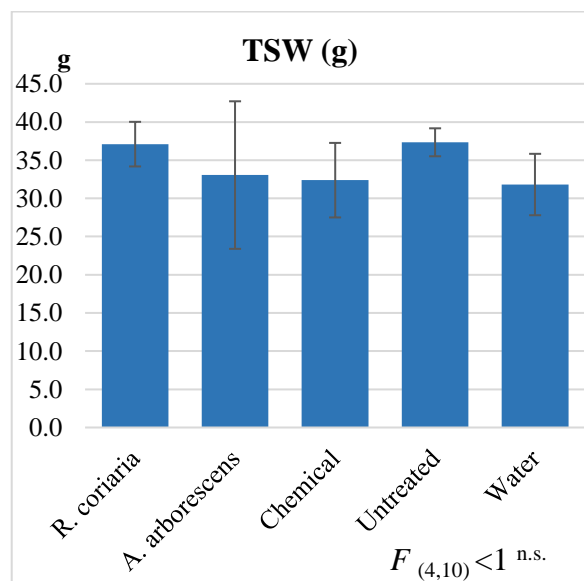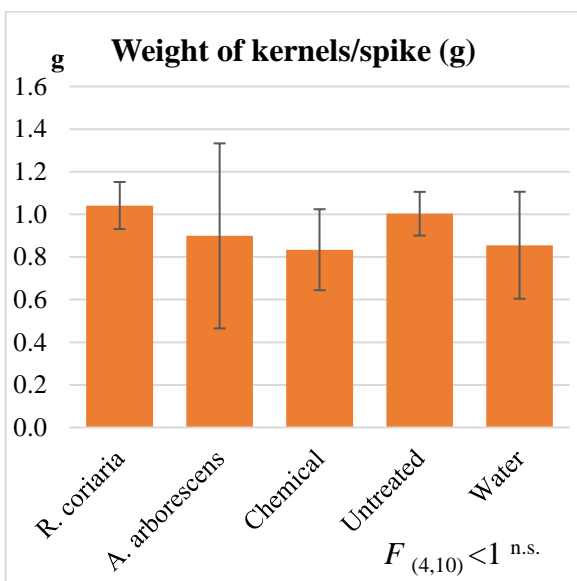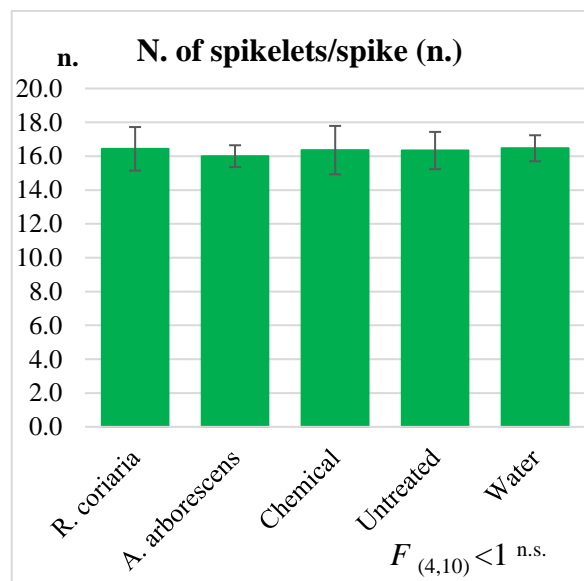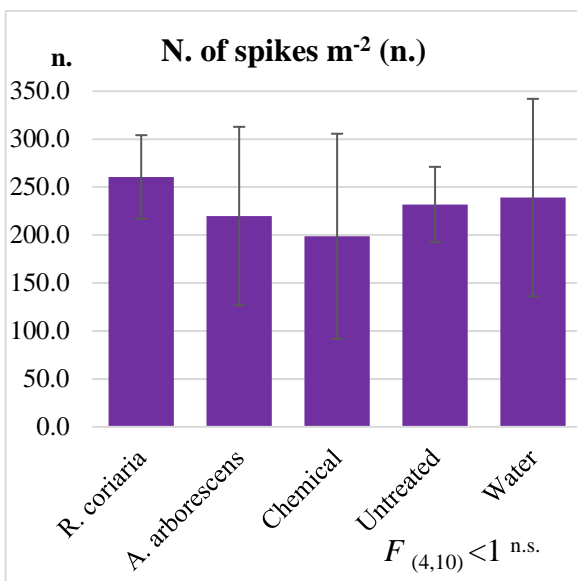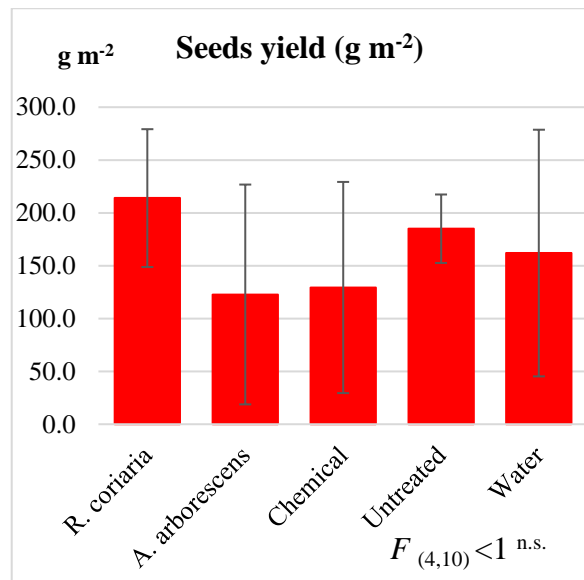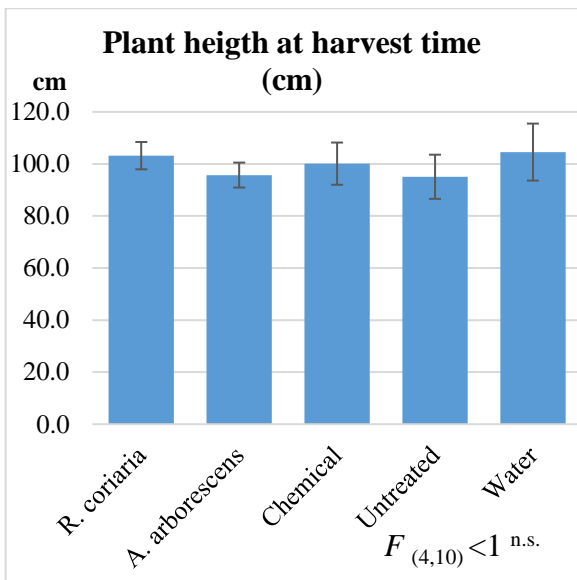
Figure 15 - Observed values of plant height at harvest time (cm), seeds yield (g m⁻²) and major yield parameters in durum wheat var. Valbelice treated with two water plant extracts, compared with two controls and one chemical weeding, and results of univariate ANOVA for each parameter.
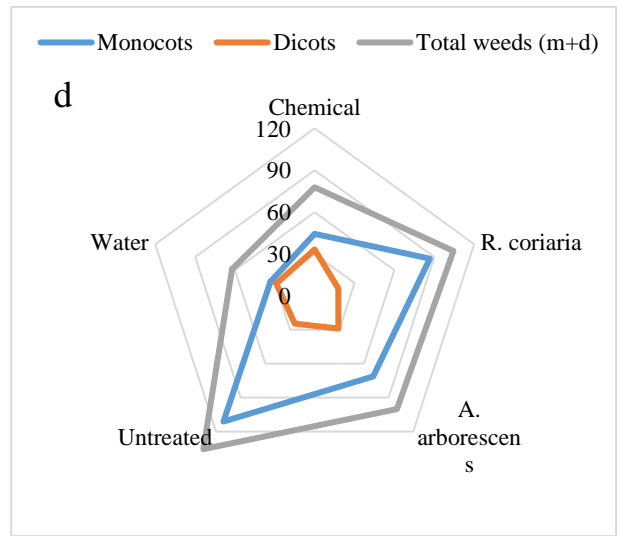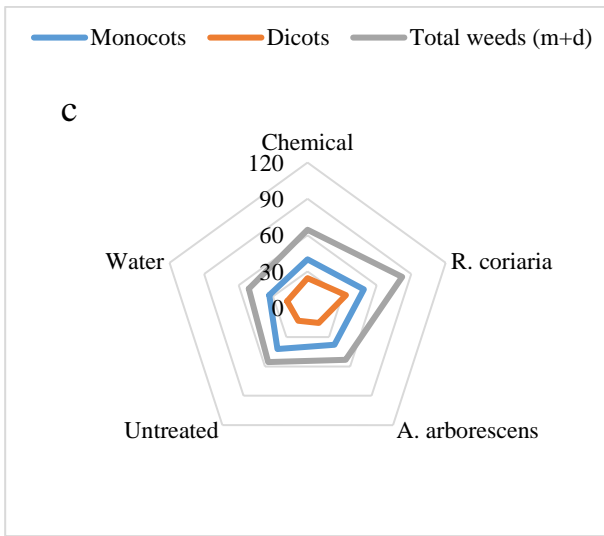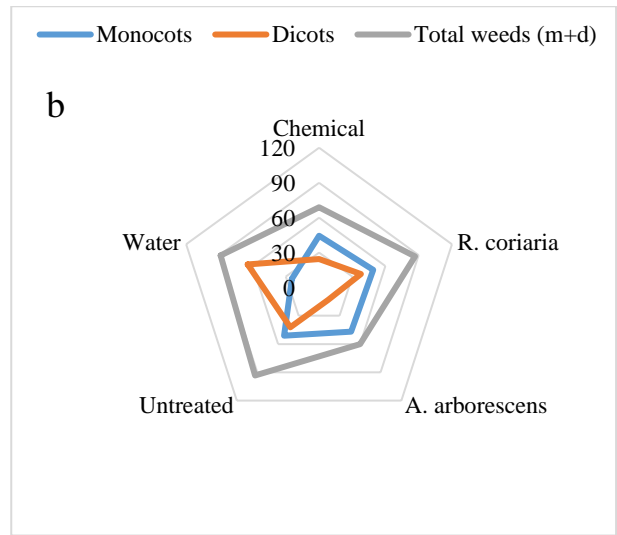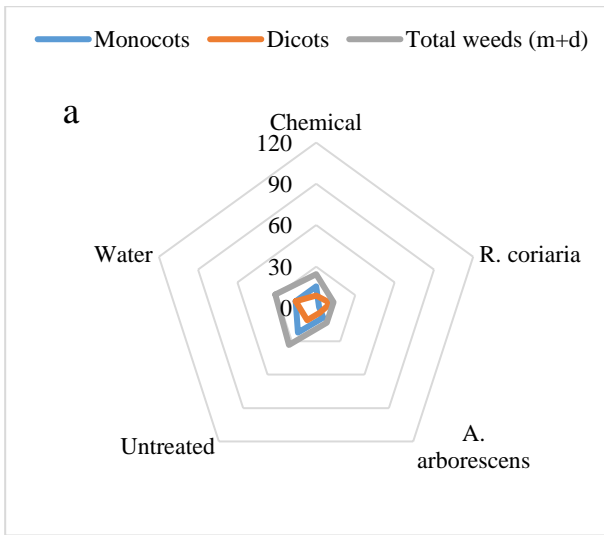
Figure 16 - Mean number of weeds m⁻² (total, only monocots and only dicots) counted in plots of durum wheat var. Valbelice treated with two water plant extracts (A. arborescens; R. coriaria), two controls (water; untreated) and one chemical weeding (Chemical), recorded on February 17th (a) and February 27th (b) 2016, and April 4th (c) and April 14th (d), 2016, i.e. before and after the two weeding treatments

4.1.2 Raw Powder

A simulation of naturally-occurring phenomena of leaching from leaves was carried out within the facilities of the Harper Adams University in Newport, Shropshire (UK). The aim of this investigation was to emulate natural occurrences in a controlled environment as a glasshouse. The idea was that leaching is one of the possible ways used by plants to control or suppress competitors in their surroundings. With this leading idea, trials were thought and planned, starting from the amount of plant material to test, back calculated from the retrievable quantity of water-soluble active compounds as obtained in previous studies (Militello and Carrubba 2016; *et al.* 2012b). Aerial parts of *A. arborescens*, *D. viscosa* and *R. coriaria* (donor plants) were finely ground and sieved by means of a 150μm mesh in order to maximize the solid contact surface. Two target plants were selected: *Alopecurus myosuroides*, also termed *Blackgrass*, and *Sinapis arvensis*, also known as *Charlock*; both are widespread weeds in UK crop fields, as well as in many other cropping systems throughout the world. *Blackgrass*, an annual monocot, is a major weed of cereal crops for most of the central and northern European Countries, and one of the most difficult to eradicate, due to its remarkable herbicide-resistance (Moss *et al.* 2007); *Charlock*, an annual dicot, is one of the most widespread weeds from Brassicaceae in cultivated lands of Europe and Canada, contributing to remarkable qualitative and quantitative yield losses in cereals (Dhima and Eleftherohorinos 2005).

Figure 17 - Alopecurus myosuroides

Figure 18 - Sinapis arvensis

Certified seeds of target plants were purchased from the commercial seed supplier Herbiseed Limited (Guildford, Surrey, UK). Seven treatments (T1 to T7) + Control (T0) were considered; each treatment corresponded to a different amount of donor plant powder, starting from the highest quantity (T1) to the lowest (T7), as described in Table 2.

Plant	Treatment	g
Aa – Dv – Rc	T1	1.0000
Aa – Dv – Rc	T2	0.5000
Aa – Dv – Rc	T3	0.2500
Aa – Dv – Rc	T4	0.1250
Aa – Dv – Rc	T5	0.0625
Aa – Dv – Rc	T6	0.0313
Aa – Dv – Rc	T7	0.0156
Ctrl	T0	0.0000

Two trials, one for *Charlock* and one for *Blackgrass*, with three repetitions each, were prepared (Table 3 and Table 4). Six 24-pots racks, one per repetition (3) per target plant (2), were used for the experiment. Treatments within each block were randomly assigned.

Table 3 - Charlock trial, distribution of treatments. The first number, from 1 to 24, is progressive from left to right, each corresponding to a different pot in the rack; Aa = A. arborescens, Dv = D. viscosa, Rc = R. coriaria; T1 to T7 are different treatments, while T0 is the negative (untreated) control

1st rep.					
1 Aa T2	2 Dv T4	3 Dv T7	4 Rc T7	5 Aa T5	6 Dv T5
7 Rc T2	8 Rc T1	9 Ctrl T0	10 Aa T4	11 Ctrl T0	12 Rc T6
13 Rc T3	14 Aa T3	15 Dv T1	16 Dv T6	17 Dv T3	18 Dv T2
19 Aa T7	20 Rc T5	21 Aa T1	22 Ctrl T0	23 Aa T6	24 Rc T4
2nd rep.					
1 Rc T2	2 Aa T4	3 Ctrl T0	4 Aa T3	5 Aa T1	6 Dv T5
7 Aa T5	8 Aa T2	9 Dv T2	10 Ctrl T0	11 Dv T4	12 Dv T3
13 Rc T7	14 Dv T7	15 Dv T1	16 Rc T1	17 Rc T3	18 Dv T6
19 Aa T6	20 Rc T6	21 Ctrl T0	22 Rc T5	23 Rc T4	24 Aa T7
3rd rep.					
1 Rc T2	2 Aa T1	3 Dv T4	4 Rc T4	5 Rc T3	6 Rc T1
7 Aa T7	8 Dv T6	9 Dv T7	10 Aa T5	11 Rc T7	12 Dv T3
13 Aa T4	14 Ctrl T0	15 Rc T5	16 Aa T2	17 Dv T2	18 Aa T3
19 Ctrl T0	3 Ctrl T0	21 Rc T6	22 Aa T6	23 Dv T1	24 Dv T5

Pots shape was truncated-square pyramid, and volume for each of them was approximately 90 cm^3. Every pot was half-filled (approx. 40 cm^3) only with soil while in the top half (approx. 50 cm^3) soil was mixed with plant powder at the rate of the corresponding treatment. Soil used was J Arthur Bower's John Innes No.2 Compost (© Westland Horticulture 2018). Ten seeds per pot were sown 1-cm deep and watered. Water supply was dispensed according to the evaporation occurred in a sealed pot placed on the same desk of the trial and filled with a known amount of water, refilling each evaporation loss on a daily basis.

The number of germinated seeds (N) was surveyed about every day (Figure 19 and Figure 20). The trial was considered over when no more seeds were germinating; aboveground biomass was harvested

and weighted, first fresh (FW) and then dry (DW, obtained after overnight treatment in stove at 105°C). Weight Index (WI), or specific weight, was calculated as follows:

WI = DW/N (g)   (1), where:

WI = weight index;

DW = dry weight (g) of the above ground biomass, measured on the target plant at 37 DAS (end of the experiment).

Table 4 - Blackgrass trial, distribution of treatments. The first number, from 1 to 24, is progressive from left to right, and is corresponding to a different pot in the rack; Aa = A. arborescens, Dv = D. viscosa, Rc = R. coriaria; T1 to T7 are different treatments, while T0 is the negative (untreated) control

1st rep.					
3 Ctrl T0	2 Dv T1	3 Aa T4	4 Rc T7	5 Dv T4	6 Aa T6
7 Aa T2	8 Aa T1	9 Dv T3	10 Dv T2	11 Ctrl T0	12 Rc T4
13 Dv T7	14 Aa T5	15 Rc T2	16 Aa T7	17 Ctrl T0	18 Rc T1
19 Rc T6	20 Rc T5	21 Dv T5	22 Dv T6	23 Aa T3	24 Rc T3
2nd rep.					
3 Ctrl T0	2 Dv T5	3 Aa T6	4 Aa T4	5 Ctrl T0	6 Ctrl T0
7 Dv T3	8 Dv T2	9 Rc T1	10 Aa T1	11 Dv T7	12 Rc T5
13 Aa T7	14 Dv T4	15 Rc T7	16 Rc T3	17 Dv T6	18 Aa T3
19 Rc T6	20 Dv T1	21 Rc T4	22 Rc T2	23 Aa T2	24 Aa T5
3rd rep.					
1 Rc T5	3 Ctrl T0	3 Rc T3	4 Rc T4	5 Dv T5	6 Rc T2
7 Aa T1	8 Ctrl T0	9 Aa T2	10 Aa T6	11 Aa T3	12 Dv T7
13 Rc T6	14 Aa T7	15 Ctrl T0	16 Rc T7	17 Dv T6	18 Dv T1
19 Aa T4	20 Dv T2	21 Dv T4	22 Rc T1	23 Aa T5	24 Dv T3

1st Rep.   2nd Rep.   3rd Rep.
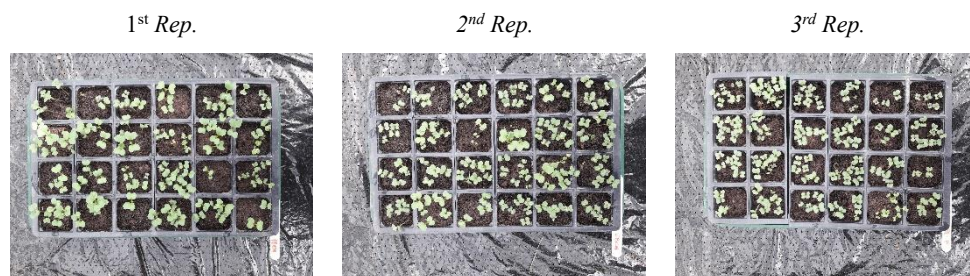
Figure 19 - 24-pots racks with sprouts of Charlock

1st Rep.   2nd Rep.   3rd Rep.

Figure 20 - 24-pots racks with sprouts of Blackgrass

4.1.2.1 Statistical analysis

Statistical analyses were carried out by mean of SPSS software (IBM Corp. Released 2016. IBM SPSS Statistics for Windows, Version 24.0. Armonk, NY: IBM Corp).

Analysis of variance (ANOVA) was performed in order to evaluate if any difference occurred between and/or within the groups. The values of number of shoots counted along the experiment, until its end (37 DAS – days after sowing) were transformed (Steel and Torrie 1980) to ensure the normal distribution according to the formula:

$$Y' = \sqrt{\left(Y + \frac{1}{2}\right)}$$ (2), where:

Y' = transformed data

Y = original data.

The same thing was done for the specific Weight Index (WI), obtained as already explained above, from the ratio between the dry weight of the above ground biomass (DW, produced by the target plant at the end of the experiment), and the number of shoots detected (N).

The assumption of normal distribution was tested by Shapiro Wilk's test ($p \le .05$)(Shapiro and Wilk 1965; Razali and Wah 2011) and by visual inspection of the histograms, normal Q-Q Plot and box plots, that showed that the residuals of the applied model were approximately normally distributed.

4.1.2.2 Result and discussion

4.1.2.2.1 Blackgrass: 37 days experiment

Table 5 reports the outcome from the application of ANOVA (GLM) on the number of shoots counted along the 37-days trial in all treatments. Twenty-two treatments were included in the model, i.e. 7 powder doses (from T1 - highest concentration to T7 - lowest concentration) for each of the 3 donor plants (Aa= *A. arborescens*; Dv= *D. viscosa*; Rc= *R. coriaria*), plus the untreated control (T0).

Table 5 - Blackgrass. Results of the ANOVA (General Linear Model) performed on the number of shoots (N) counted along the 37-days trial in all treatments.

Source of variation	Df	F
Treatments (T)	21	12.321***
Day (d)	17	21.974***
T x d	102	<1 n.s.
Error	900	
Total	1296	

*Significance of F values: ***= P $\le$ 0.001; ** = P $\le$ 0.01; * = P $\le$ 0.05; n.s. = not significant*

As shown, the outcome variable is highly significantly ($P \le 0.001$) influenced by both the treatments (T) and the day of observation (d), but the lack of significance of the interaction T x d clearly indicates

how these effects act additively and without any reciprocal dependence. The values of the above T x d interactions, that indicate the trend over time of number of shoots (N) per donor plant and treatment, are shown from Figure 21 to Figure 23. Although no significant difference was evidenced by the ANOVA, probably due to the wide variability within the samples, it is possible to make some overall observations: first, as expected, the number of shoots increased throughout the experiment timeframe and, second, different concentrations led to different responses.

Figure 21 - Blackgrass. Bar chart of averages by treatment and day of observation of number of shoots (N) from samples treated with A. arborescens. Red line represents the control. Error bars indicate standard deviations.

35

Figure 22 - Blackgrass. Bar chart of averages by treatment and day of observation of number of shoots (N) from samples treated with D. viscosa. Red line represents the control. Error bars indicate standard deviations.

36

Figure 23 - Blackgrass. Bar chart of averages by treatment and day of observation of number of shoots (N) from samples treated with R. coriaria. Red line represents the control. Error bars indicate standard deviations.
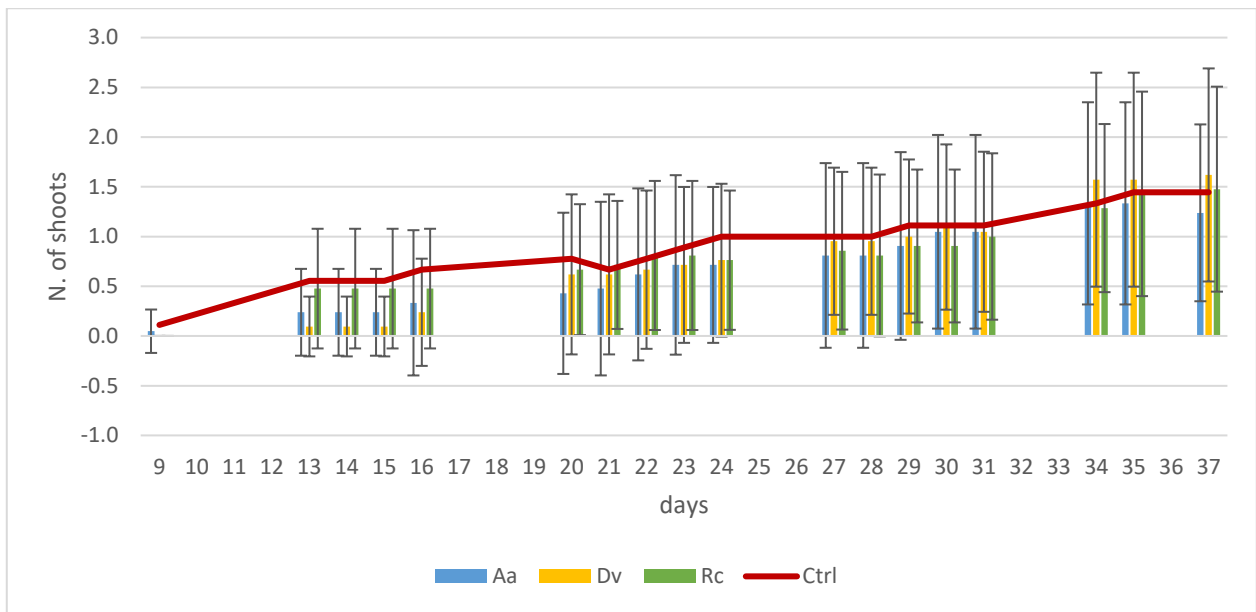
Figure 24 - Blackgrass. Average over time of total number of shoots (N) from samples treated with different donor plants, compared with the control (red line). For each value, vertical bars represent the standard deviation of the mean. Aa: A. arborescens, Dv: D. viscosa, Rc:R. coriaria.

The first statement is better explained by the observation of Figure 24. Independently upon the tested concentration, the number of shoots increased over time. Interestingly, throughout almost all observation days, the number of shoots detected in the controls was higher than that observed in the corresponding treatments. Only at the end of the experiment (days 34 to 37), a stimulating effect of *D. viscosa* started showing up, as shown by the corresponding values that in the graph are positioned above the control line.
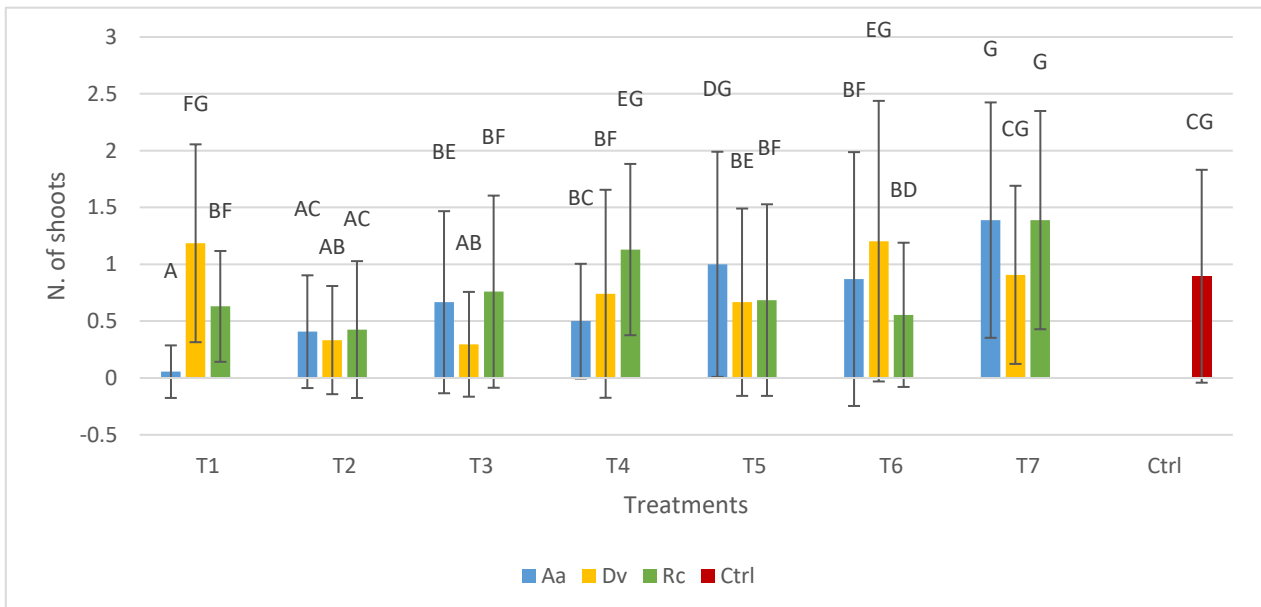
Figure 25 - Blackgrass. Mean values of number of shoots (N). Averages of all days of observation grouped by donor plant (Aa, Dv and Rc) and treatments (T1 to T7). For each value, vertical bars represent the standard deviation of the mean. Red bar represents the Control. Letters on top of the error bars (from A to G) correspond to homogeneous mean groups computed by Tukey's HSD test.

The second statement emerging from the ANOVA (Table 5) is related to the effect of treatments (T1 to T7) for each donor plant. As shown in Figure 25, different donor plants at different concentrations lead to different responses. An overall tendency to increase the number of shoots from the highest concentration (T1) to the lowest (T7) can be recognized; yet, this response is not the same for all three donor plants, and the highest number of shoots is found at T5, T6 and T7 for *A. arborescens, D. viscosa* and *R. coriaria*, respectively. At lower concentrations, an opposite effect of stimulation seems sometimes to take place (Figure 21, Figure 22 and Figure 23).

In order to achieve further information about the behaviour of the tested species subjected to the different treatments, a separate ANOVA was carried out on data obtained from each donor plant, and the means obtained from the seven treatments, averaged by day of observation, were compared to the corresponding values of the control by means of the Dunnett's test (Figure 26, Figure 27 and Figure 28).
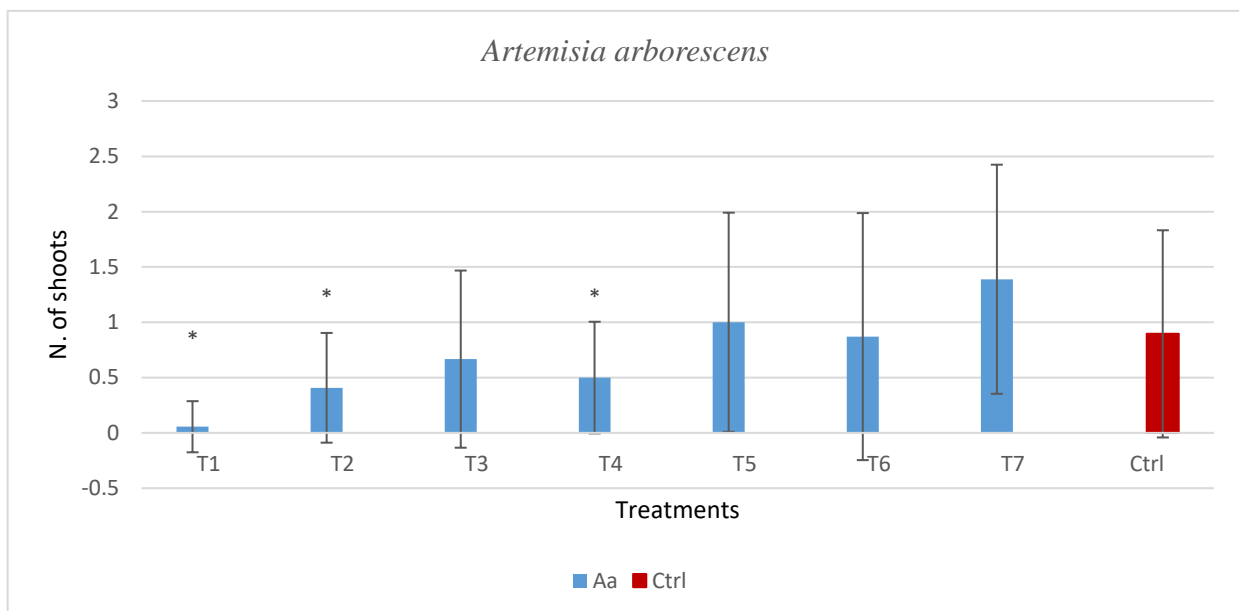
Figure 26 - Blackgrass. Mean values of number of shoots (N) of all observations recorded from samples treated with A. arborescens at different concentrations. $F_{(7,532)}= 14.333***$. Error bars represent standard deviation. Treatments marked with * are significantly different from the control at P $\leqslant 0.05$ (Dunnett's test)
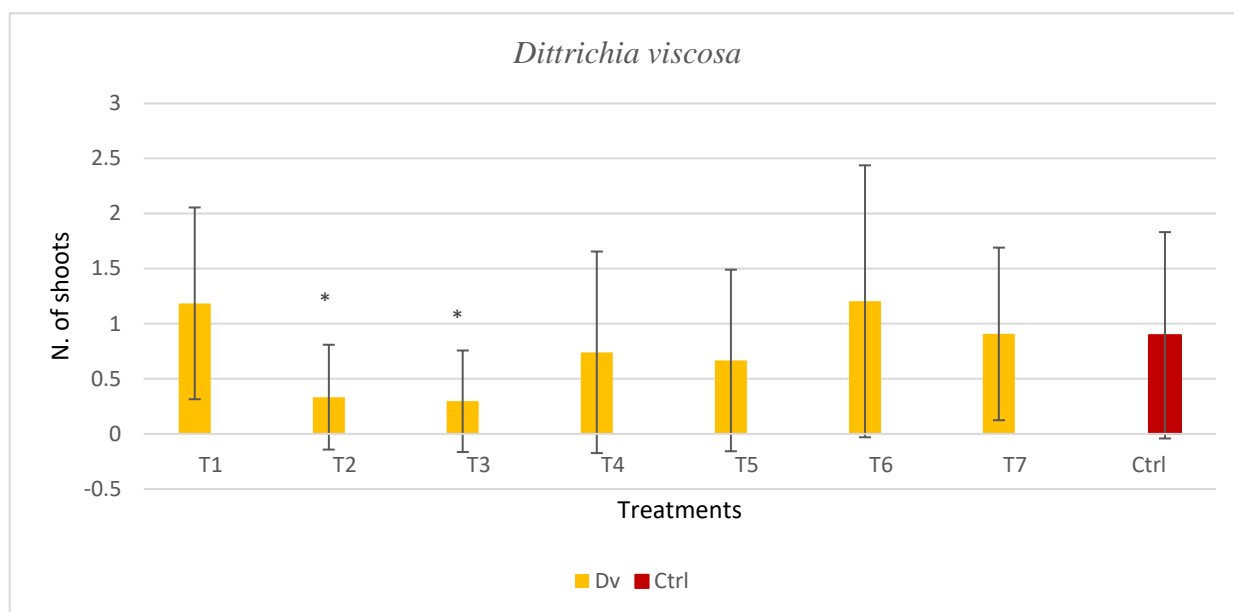
*Figure 27- Blackgrass. Mean values of number of shoots (N) of all observations recorded from samples treated with D. viscosa at different concentrations. F(7,532)= 9.306***. Error bars represent standard deviation. Treatments marked with * are significantly different from the control at P ≤0.05 (Dunnett's test)*

*Figure 28 - Blackgrass. Mean values of number of shoots (N) of all observations recorded from samples treated with R. coriaria at different concentrations. F(7,532)= 8.920***. Error bars represent standard deviation. Treatments marked with * are significantly different from the control at P ≤0.05 (Dunnett's test)*

As shown, in all donor species a significant (P ≤ 0.05) inhibition effect is expressed by the T2 treatments, whereas with varying concentrations, a different behaviour shows up according to the selected donor plant. Compared to the control, the treatments with a lower concentration (T5 to T7) seem to express a stimulating effect on shoots formation, especially evident in the T5 treatment for *A. arborescens*, in T6 for *D. viscosa* and in T7 for *R. coriaria*. Anyway, only in this last case this

behaviour is confirmed by Dunnett's test, whereas no others differentiate significantly from the control.

This variability in the response across concentration and day of observation may explain the overall response attributable to the donor plants, as reported in Figure 29. The mean number of shoots recorded from all experiments follows the trend Aa<Ctrl<Rc<Dv, and only *A. arborescens,* where three treatments out of seven showed a lower mean value than the control (Figure 26), confirmed its effectiveness.

Figure 29 - Blackgrass. Mean values of number of shoots (N) of all observations recorded from samples treated with A. arborescens (Aa), D. viscosa (Dv) and R. coriaria (Rc), averaged by concentrations and day of observation. F(3,1292)= 3.113*. Error bars represent standard deviation. Treatment marked with * is significantly different from the control at P ≤0.05 (Dunnett's test).

The same analyses were also performed to look at differences between and within the groups with reference to the Weight Index (WI). WI however did not give back any significant result, which means that once the sprouting started to take place no significant differences showed up in terms of produced biomass. Anyway, in most cases (exception made for the highest concentrations of *R. coriaria*), the mean Weight Index of the treated samples appeared to be lower than control. (Figure 30).
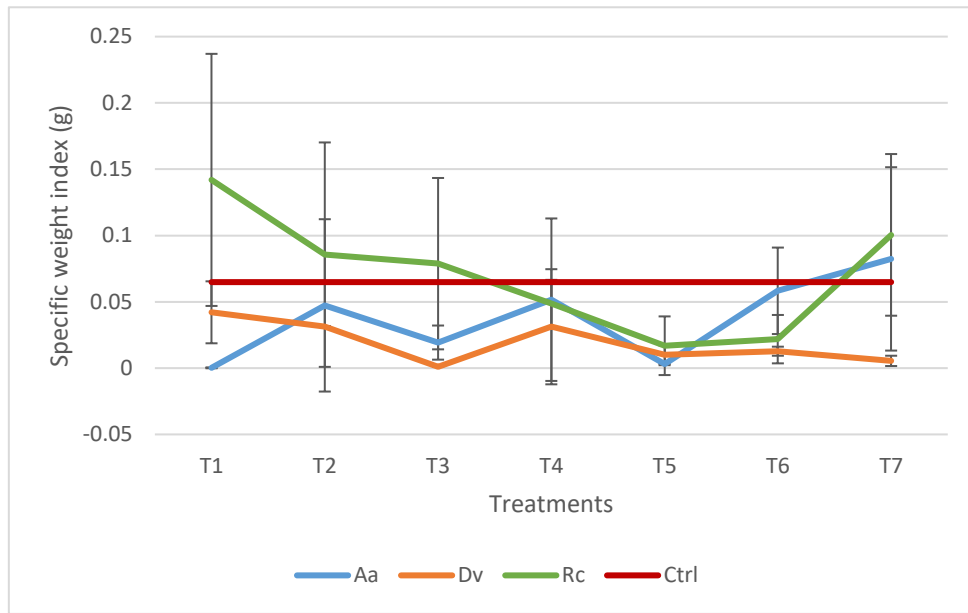
Figure 30 - Blackgrass. Average values by donor plant (Aa= A. arborescens; Dv= D. viscosa; Rc=R. coriaria) and treatment (T1 to T7) of the Weight Index of the shoots. Error bars represent standard deviations. Red line represents the control.

4.1.2.2.2 Charlock: 13 days experiment

Table 6 reports the outcome from the application of ANOVA (GLM) on the number of shoots counted along the 13-days trial in all treatments. Twenty-two treatments were included in the model, i.e. 7 powder doses (from T1 - highest concentration to T7 - lowest concentration) for each of the 3 donor plants (Aa= *A. arborescens*; Dv= *D. viscosa*; Rc= *R. coriaria*), plus the untreated control (T0).

Table 6 - Charlock. Results of the ANOVA (General Linear Model) performed on the number of shoots (N) counted along the 13-days trial in all treatments.

Source of variation	Df	F
Treatments (T)	21	5.866***
Day (d)	5	169.395***
T x d	105	1.049 n.s.
Error	300	
Total	432	

*Significance of F values: ***= P ⩽ 0.001; ** = P ⩽ 0.01; * = P ⩽ 0.05; n.s. = not significant*

As shown, the outcome variable is highly significantly ($P \leq 0.001$) influenced by both the treatments and the day of observation, but the lack of significance of the interaction T x d clearly indicates, once more, their additive effect in place of a reciprocal dependence. Although no significant difference was evidenced by the ANOVA, the values of the above T x d interactions, that indicate the trend over time of number of shoots (N) per donor plant and treatment, are shown from Figure 31 to Figure 33. As a general trend, it is possible to observe that, as expected and similarly to that obtained in Blackgrass experiment, the number of shoots increased throughout the experiment timeframe; yet, this increase

42

was much less evident than in Blackgrass, and on the 5th observation day the number of shoots had already reached a value pretty close to the final one (figure 34). .
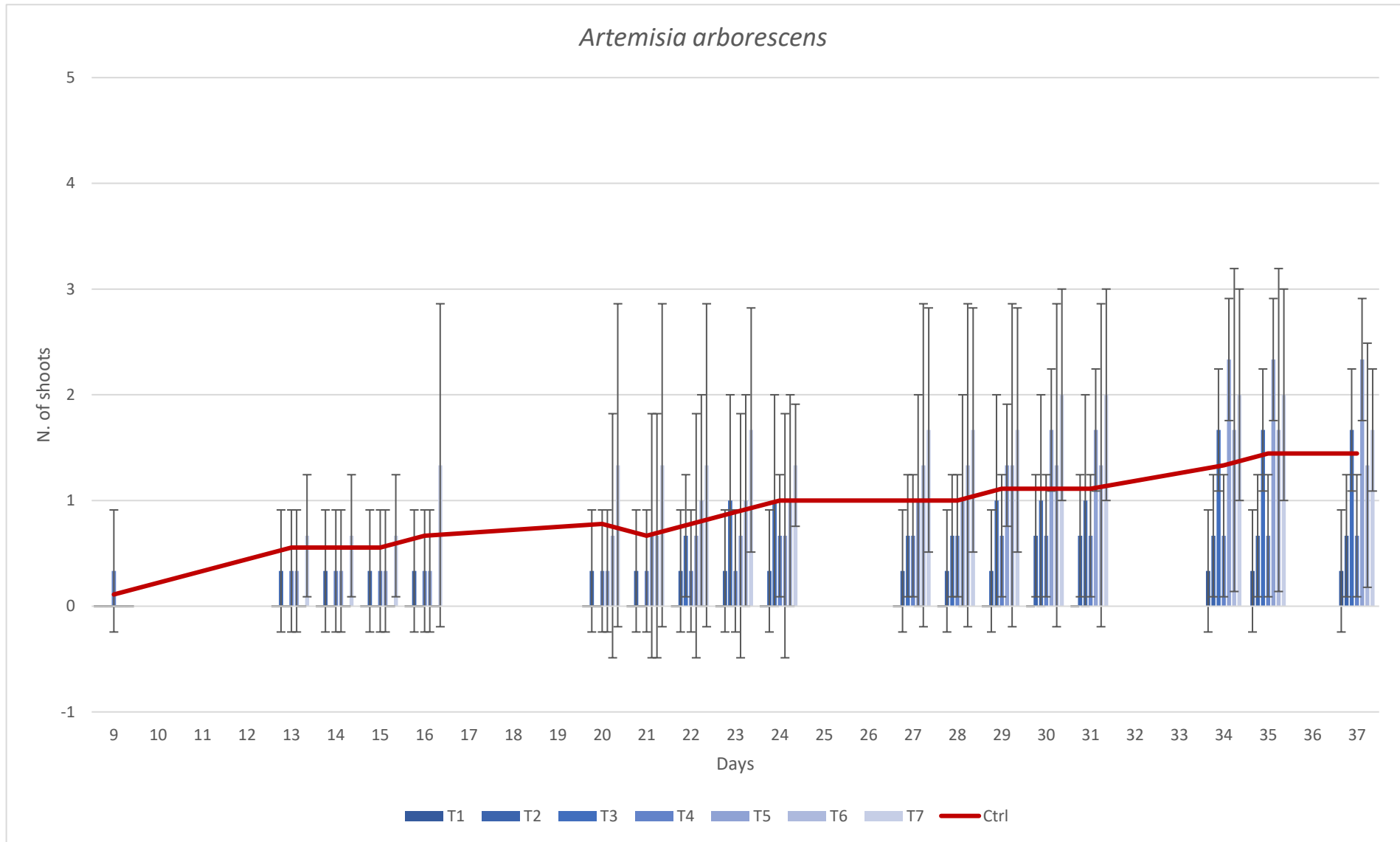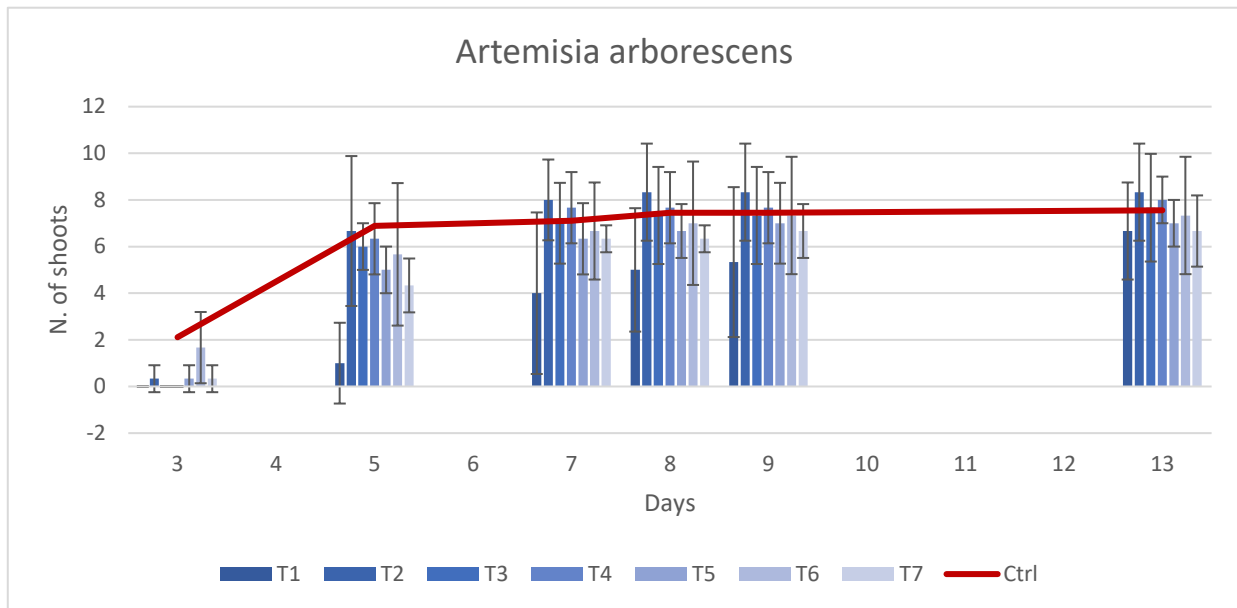

Figure 31 - Charlock. Bar chart of averages by treatment (T1 to T7) and day of observation of number of shoots (N) from samples treated with A. arborescens. Red line represents the control. Error bars indicate standard deviations.
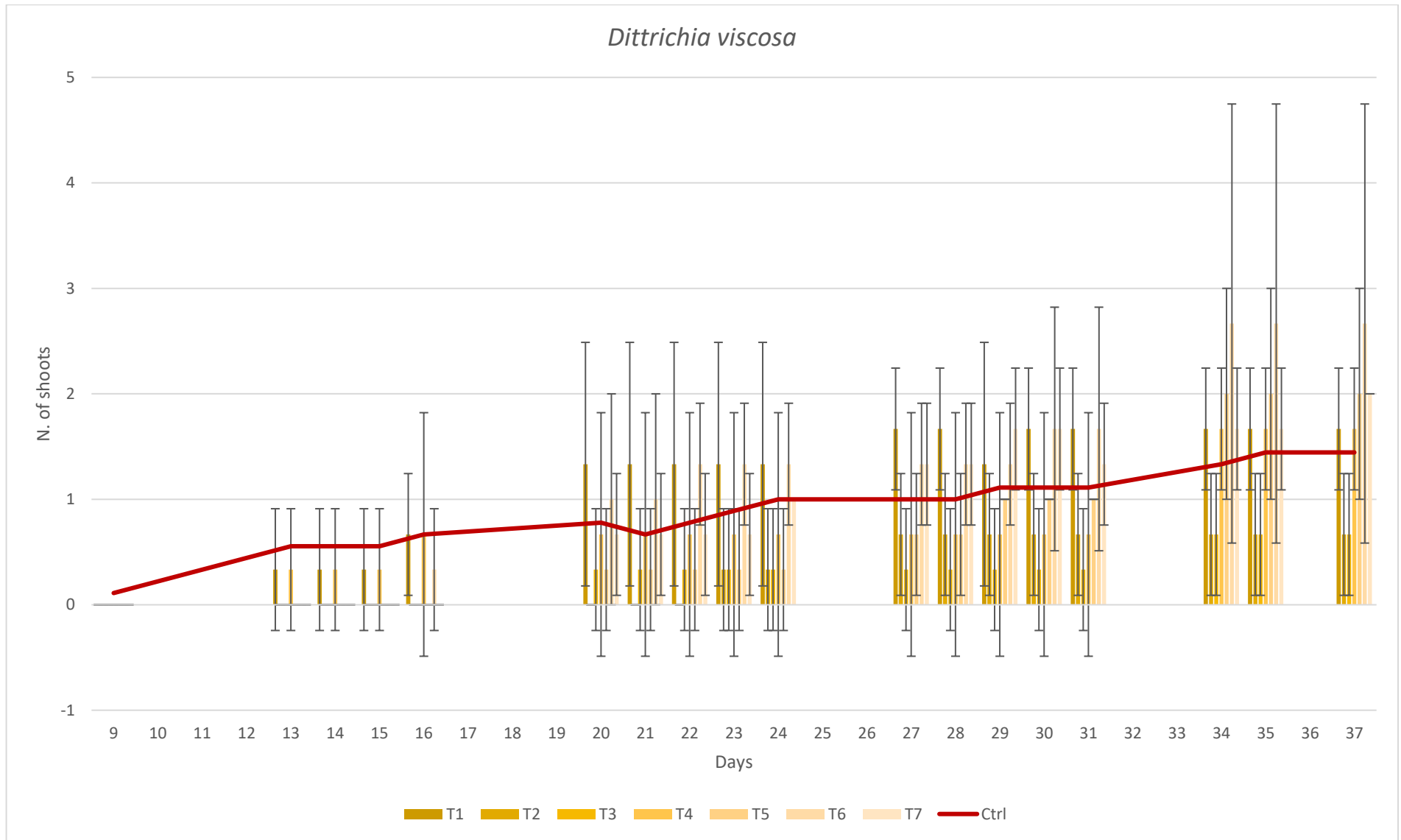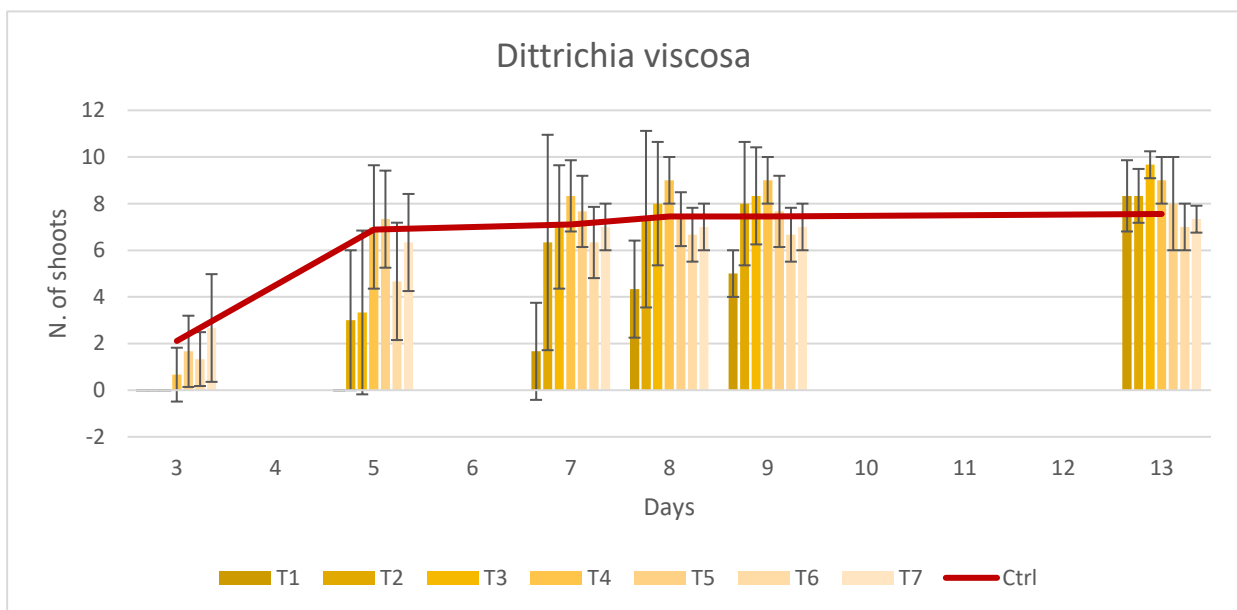

Figure 32 - Charlock. Bar chart of averages by treatment (T1 to T7) and day of observation of number of shoots (N) from samples treated with D. viscosa. Red line represents the control. Error bars indicate standard deviations.
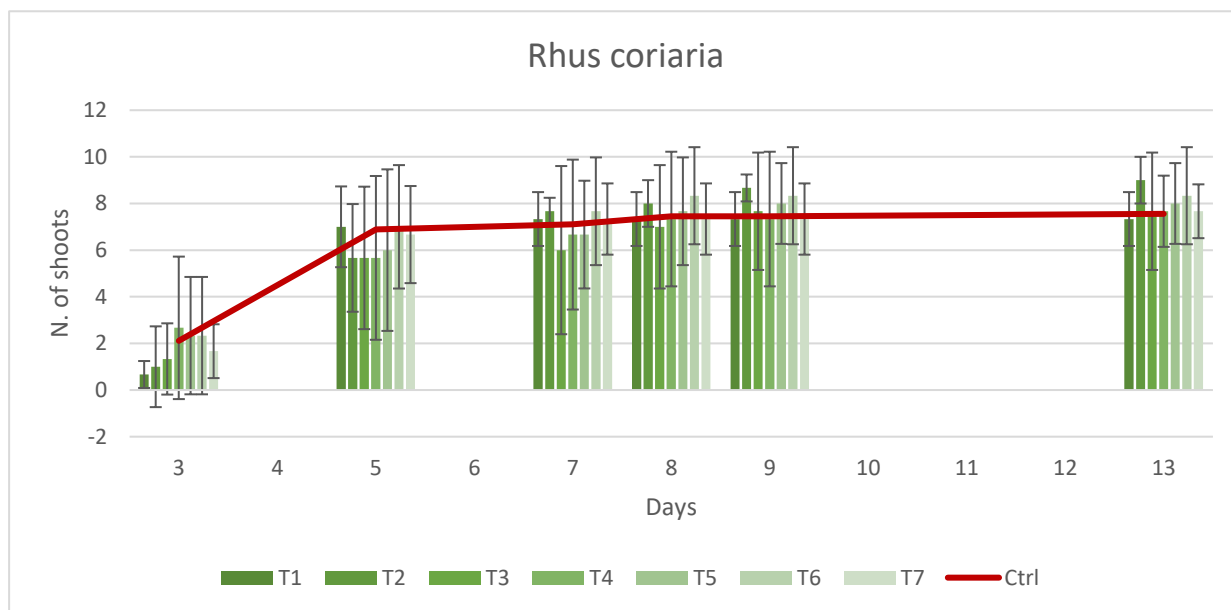
Figure 33 - Charlock. Bar chart of averages by treatment (T1 to T7) and day of observation of number of shoots (N) from samples treated with R. coriaria. Red line represents the control. Error bars indicate standard deviations.
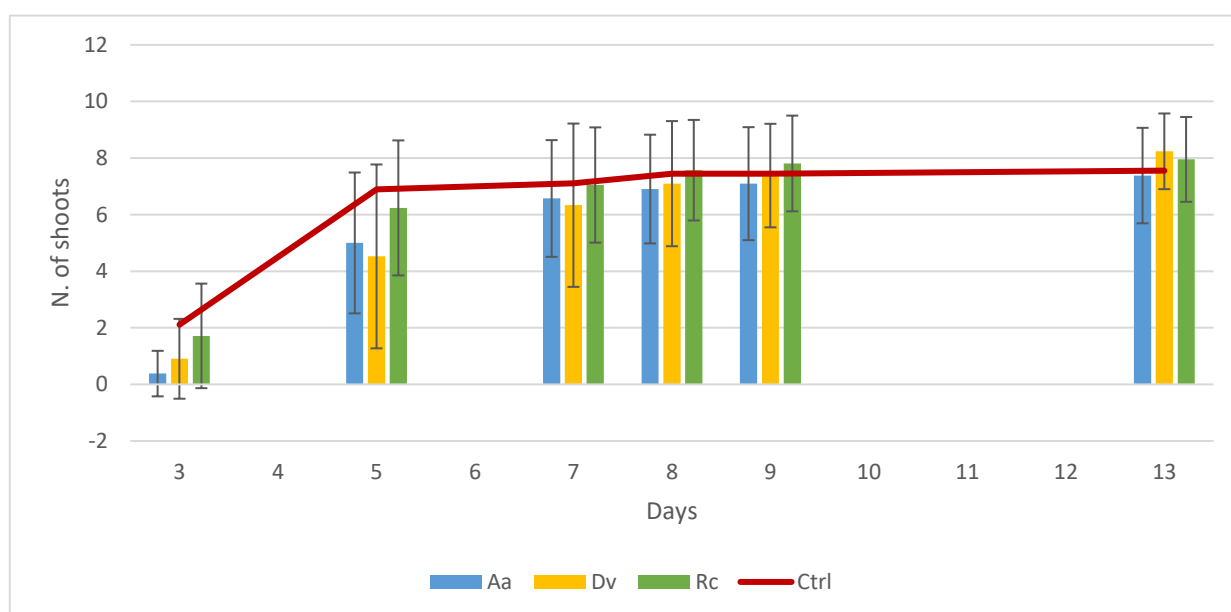
Figure 34 - Charlock. Average over time of total number of shoots (N) from samples treated with different donor plants, compared with the control (red line). For each value, vertical bars represent the standard deviation of the mean. Aa: A. arborescens, Dv: D. viscosa, Rc:R. coriaria.

Furthermore, the observation of Figure 34 clearly shows how, independently upon the tested concentration, until the 8th day after sowing, the number of shoots was higher in the controls than that observed on the same day in the treated pots. Only at the end of the experiment (day 13), a stimulating effect of *D. viscosa* and, slightly lower, of *R. coriaria* started showing up, as evidenced by the position of the corresponding values above the control line.

The second statement emerging from the ANOVA (Table 6) is related to the effect of treatments (T1 to T7) for each donor plant. As shown in Figure 35, different concentrations led to rather homogenous

44

responses, and the number of shoots was spanning between similar values across donor species and treatments. The only exceptions seem to be *A. arborescens* and *D. viscosa* at the highest concentration (T1) that expressed values significantly lower than the control.
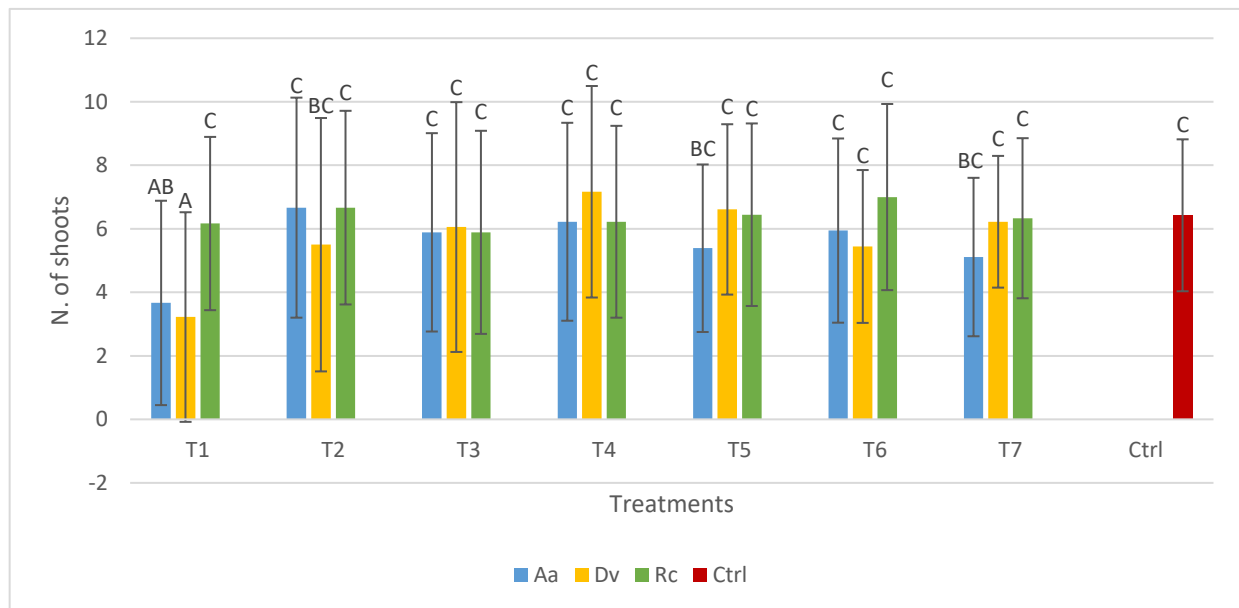
Figure 35 - Charlock. Mean values of number of shoots (N). Averages of all days of observation grouped by donor plant (Aa, Dv and Rc) and treatments (T1 to T7). For each value, vertical bars represent the standard deviation of the mean. Red bar represents the Control. Letters on top of the error bars (from A to C) correspond to homogeneous mean groups computed by Tukey's HSD test.

In order to achieve further information about the behaviour of the tested species to the treatments, a separate ANOVA was carried out on data obtained from each donor plant, and the means obtained from the seven treatments, averaged by day of observation, were compared to the corresponding values of the control by means of Dunnett's test (Figure 36, Figure 37 and Figure 38).
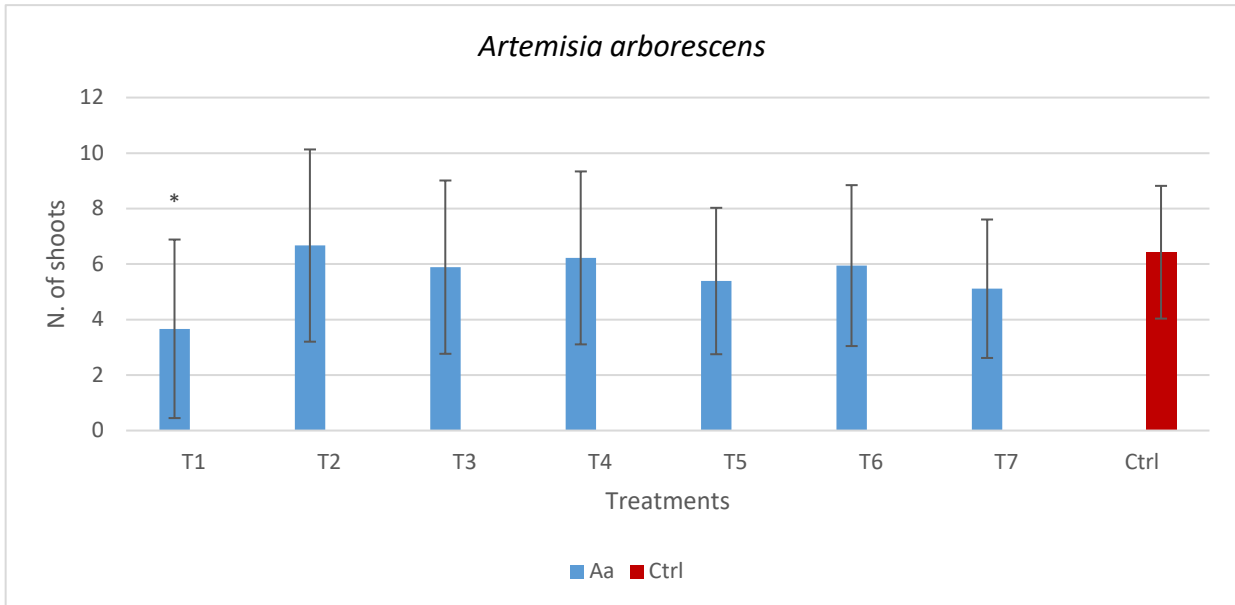
Figure 36 - Charlock. Mean values of number of shoots (N) of all observations recorded from samples treated with A. arborescens at different concentrations. $F_{(7,172)}= 2.147*$. Error bars represent standard deviation. Treatments marked with * are significantly different from the control at $P \leqslant 0.05$ (Dunnett's test)
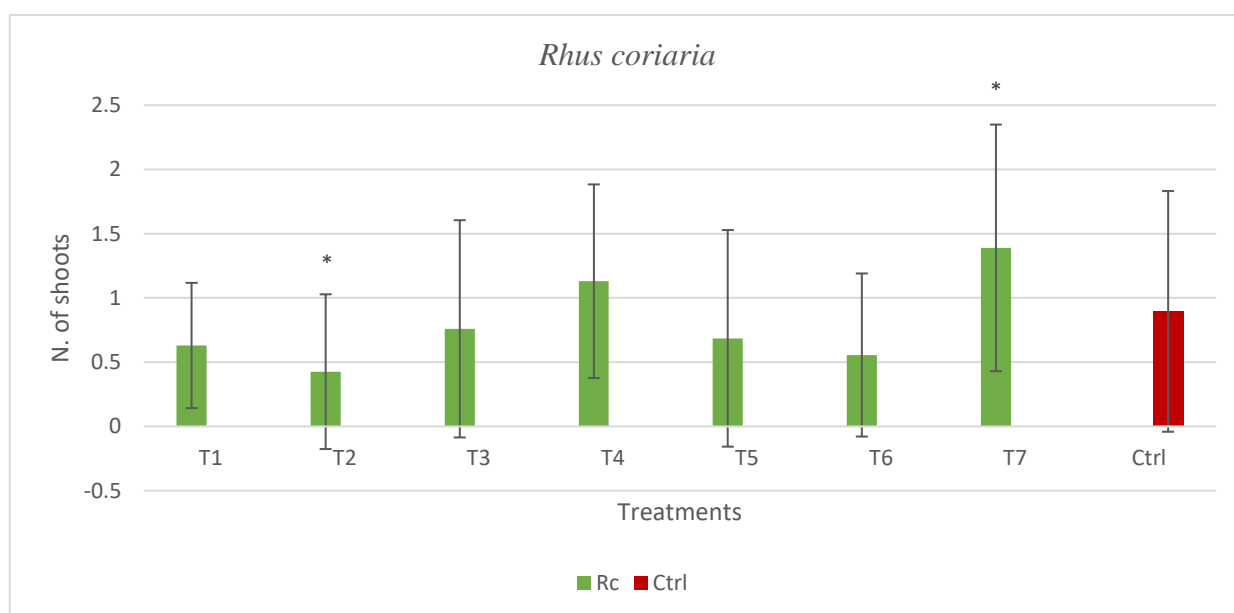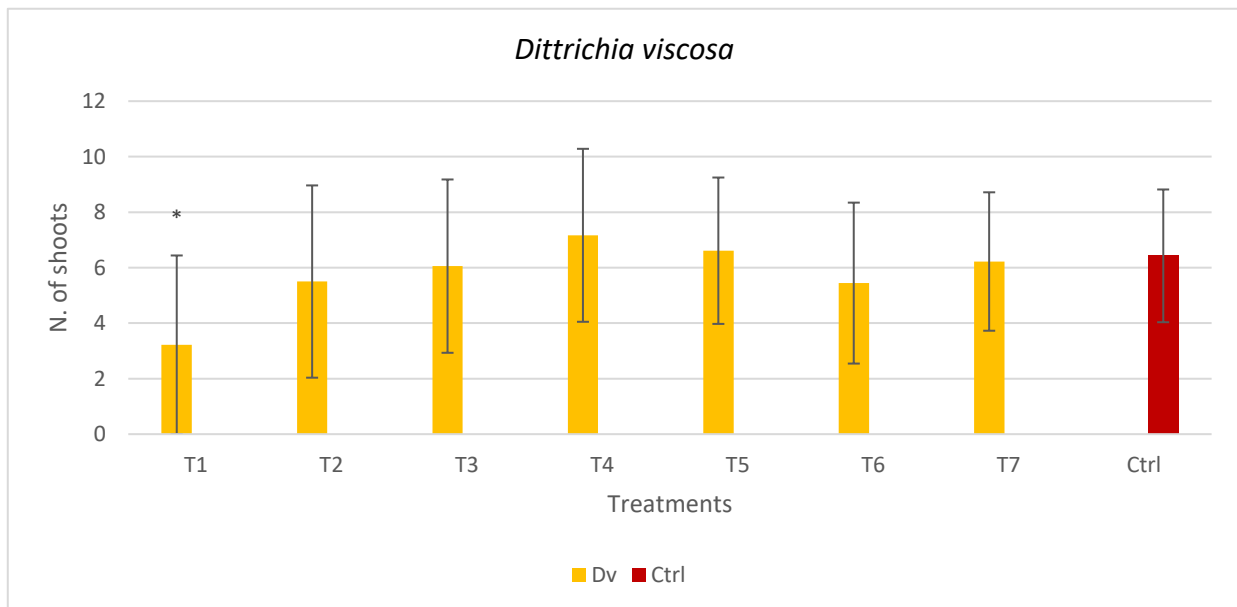

Figure 37- Charlock. Mean values of number of shoots (N) of all observations recorded from samples treated with D. viscosa at different concentrations. $F_{(7,172)}= 3.343*$. Error bars represent standard deviation. Treatments marked with * are significantly different from the control at $P \leq 0.05$ (Dunnett's test)
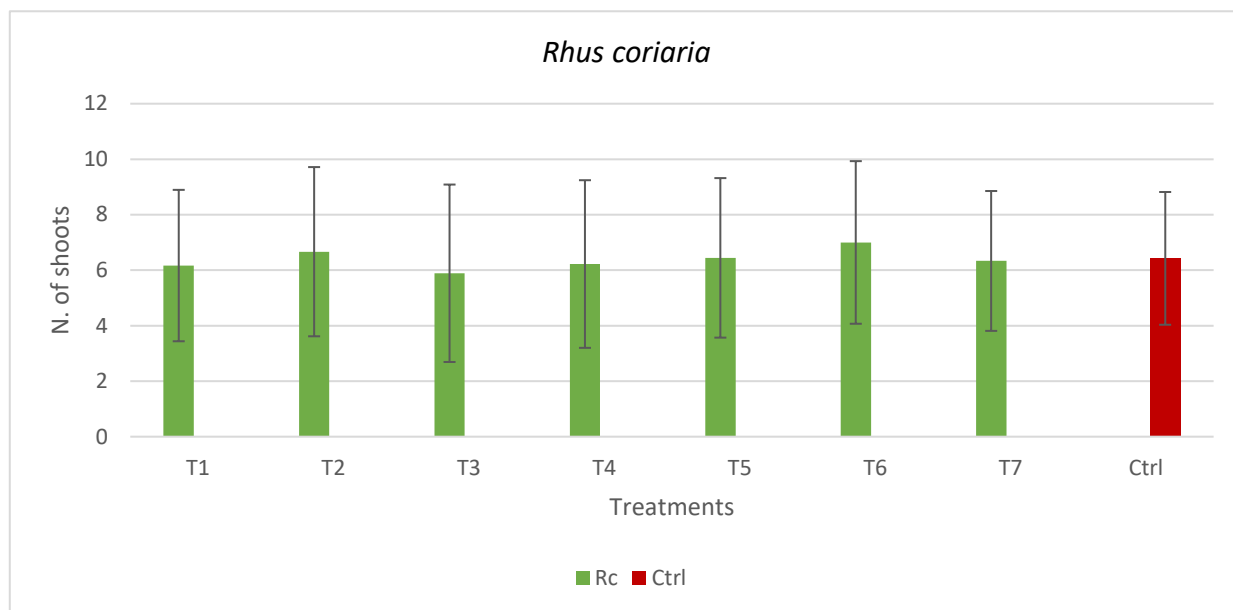
Figure 38 - Charlock. Mean values of number of shoots (N) of all observations recorded from samples treated with R. coriaria at different concentrations. F(7,172) >1^{n.s.}. Error bars represent standard deviation. Treatments marked with * are significantly different from the control at P ≤0.05 (Dunnett's test)

As shown, in two out of three donor plants, namely *A. arborescens* and *D. viscosa*, a significant (P ≤ 0.05) inhibition effect, also confirmed by Dunnett's test, is expressed by the T1 treatments. whereas with varying concentrations, a substantially homogenous behaviour shows up in the diverse donor plants. The mean number of shoots recorded from all experiments follows the trend Aa<Dv<Rc<Ctrl (Figure 39).
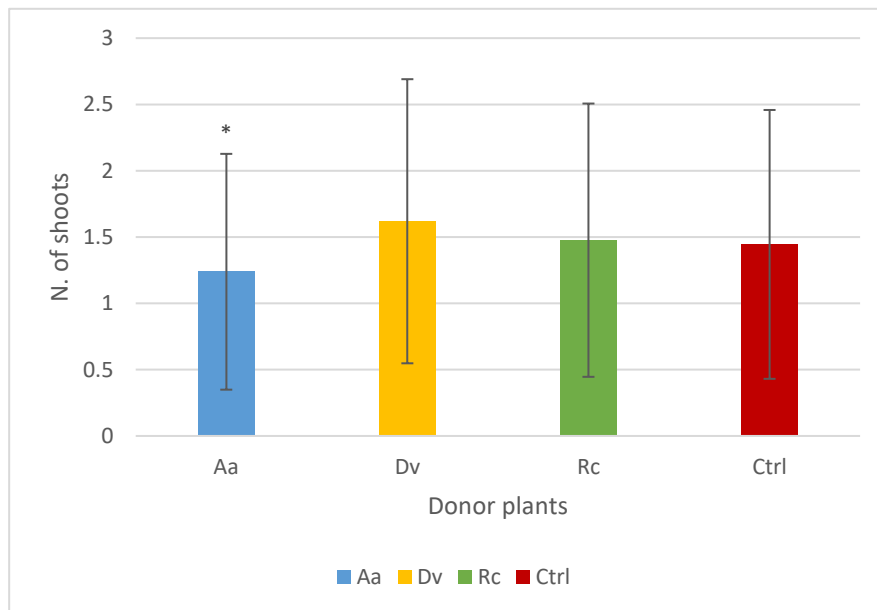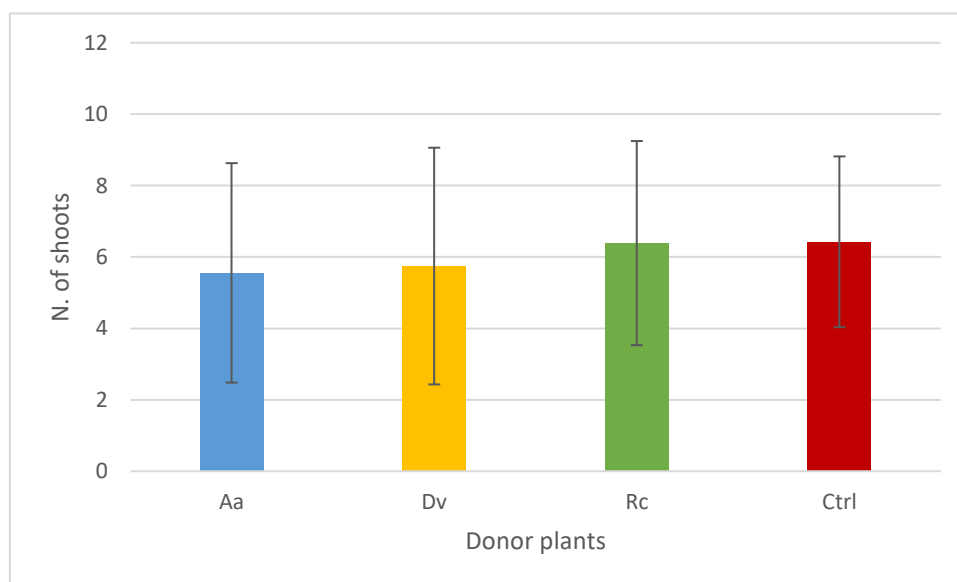

Figure 39 - Charlock. Mean values of number of shoots (N) of all observations recorded from samples treated with A. arborescens (Aa), D. viscosa (Dv) and R. coriaria (Rc), averaged by concentrations and day of observation. F(3,428)= 2.671*. Error bars represent standard deviation. Treatment marked with * is significantly different from the control at P ≤0.05 (Dunnett's test).

The same analyses were also performed to look at differences between and within the groups with reference to the Weight Index (WI), which did not highlight any significant effect of the samples related to the independent variables. Graphical support (Figure 40) on overall tendency displays how

generally treated samples produced less biomass compared to the control; in other words the size of the shoots collected from treated samples mostly seems to be smaller than the ones from control, exception made for the T5 treatment of *A. arborescens* which expressed a singularly high value. However, at the lowest concentrations the effects of *D. viscosa* and *R. coriaria* seem to disappear, aligning to the control.
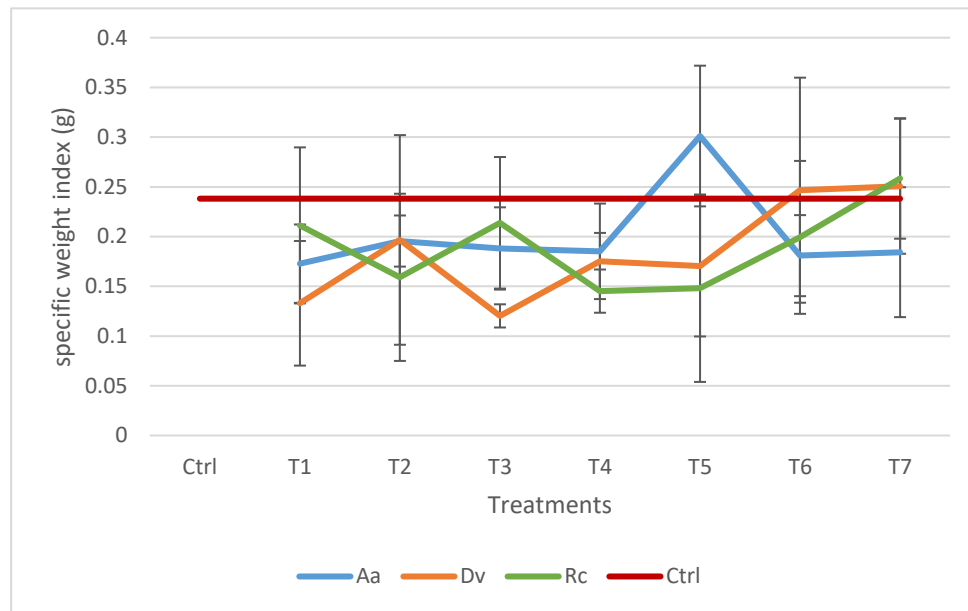
Figure 40 - Charlock. Average of the specific weights of the shoots produced from treated samples divided by donor plant. Error bars represent standard deviations.

In conclusion, both Blackgrass and Charlock experiments clearly indicate the occurrence of an inhibition effect, mainly observed at the highest concentration of finely ground plant material, in *A. arborescens* and secondly in *D. viscosa*, while *R. coriaria* seems to be less effective than the other donor species on Blackgrass seeds and almost ineffective on Charlock seeds. Anyway, this inhibition effect, when present, was mainly addressed to the germination of seeds rather than to the suppression of shoots growth after sprouting. An additional interesting outcome of the experiment was an occasional stimulation of seeds germination, seemingly dependent upon the combination of both concentration of the treatments and target plant seeds. These contrasting results could be maybe explained by the stimulation/inhibition pattern typical of the concentration-dependent allelopathic mechanisms (Khan *et al.* 2009; Mongelli *et al.* 1997; Labruzzo *et al.* 2017), but it clearly appears how these complex mechanisms are still far to be elucidated. However, as it was wished at the planning phase of the experiment to use as less inputs as possible to achieve some inhibition effect, the outcome confirmed this opportunity, especially in the case of *A. arborescens* and *D. viscosa.* Some additional effort has surely to be done to implement the degradation process. In fact, it has also to be said that the main limit of the experiments was the time, in that the release of produced allelochemicals, as it happens in nature, occurs throughout the season by means of the interaction of

biotic and abiotic factors, which provide to the decay of litter and to the transfer of the complexes and their derivatives, which sometime may have an even stronger allelochemical effect, downward through the soil by leaching (Wardle *et al.* 1998; Araniti *et al.* 2016). Lastly, the great variability and the lack of synchronicity of seeds sprouting, associated with an overall low germinability of seeds, complicated data interpretation. Probably, a pre-treatment of seeds, to rise the germination percentage, with a wider sample size could have helped to keep down the experimental variability and simultaneously to better define the line of tendency. Finally, the high amounts of powdered plant material that resulted to be necessary for achieving a biological effect could represent a limit for practical applications. However, although the massive use of plant material might not be feasible for large-scale agricultural systems, this might be different in urban areas, such as parks, gardens and flowerbeds, where the application of a thick layer of litter could be managed as a mulching technique, as already done with needle litter of pines.

4.1.3   *Phytotoxicity-guided fractionation*

Bio-guided fractionation is a typical protocol to isolate and purify a chemical compound from natural sources. It develops through a step-by-step separation process based on different physicochemical properties of the extracted components, assessing their biological activity and then proceeding with a new loop of separation-assessing (Malviya and Malviya 2017). In this study, carried out within the labs of the *Agricultural Research Service* of the *United States Department of Agriculture* (ARS-USDA), the bio-guided fractionation protocol was applied in order to identify secondary metabolites derived from *A. arborescens*, *D. viscosa* and *R. coriaria* extracts with phytotoxic activity. In particular, the aerial parts of each of those plants were collected in the nearby of Altofonte (Palermo), and dried at room temperature for one week, then stored in a cool and dry place before using. The flowchart of the extraction and assay processes is reported in Figure 50. Two hundred grams of plant material was finally ground, in order to maximize the solid-solvent contact area, then placed in a baker and poured with 2.5L of solvent, hence left onto a stirring plate for 60hrs. This first set of extractions, named Solid-Liquid extraction, was made in three following steps, each corresponding to a different solvent. So that, the first solvent used was dichloromethane (CH_2CL_2), also known as DCM; the second one was methanol (CH_3OH) and the last one was Distilled De-Ionized (DDI) water. Vacuum filtration (Figure 41) was performed with Whatman #2 filter paper in a Büchner funnel then extracts were dried by rotary evaporator (Figure 42) and nitrogen concentrator (Figure 43) while water was removed by freeze drying unit (lyophilizer) (Figure 44).

Figure 41 - vacuum filtration

Figure 42 - rotary evaporator

Figure 43 - Nitrogen concentrator
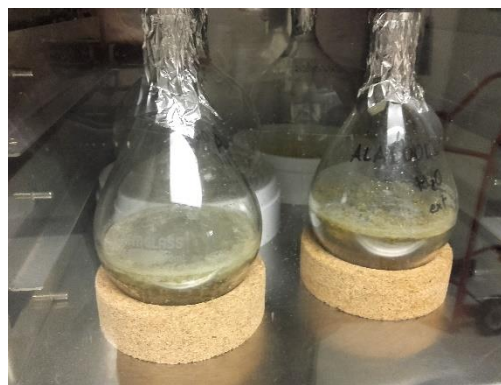
Figure 44 - Samples into the freeze-dryer unit

The so-obtained amount of extracts was collected in small vials and stored at 0°C until use. All three extracts were tested to assess the inhibition degree on seeds germination of two of the most common target plants used for phytotoxicity evaluation: a dicot, lettuce (*Lactuca sativa*), and a monocot, bentgrass (*Agrostis stolonifera*). Bioassay lasted 7 days and the outcome rating was based on a scale of 0 to 5, where 0 means no effect and 5 no growth or germination (Table 8). At the end of the assessment only *A. arborescens* DCM extract showed some relevant phytotoxic activity to be considered viable for further investigations.

Liquid-Liquid partitioning, based on different density of solvents and different affinity with the solutes, was performed. A modified Kupchan's extraction method (Kupchan *et al.* 1973)(Figure 46) was applied, hence 5g of DCM extract were dissolved in 200mL of MeOH/H2O, 9:1 (v/v) and placed in a separatory funnel (Figure 45), so for three times in a row 200mL of *n*-Hexane were poured into it, gently shacked and removed. Hexane extract was rotary evaporated.

Figure 45 - Separatory funnel during the partitioning process.
Detail of two separated solvents of different density

The partitioning kept going adding 57mL more of DDI water to make the solution MeOH/H2O, 70:30 (v/v). Therefore, three more extractions with Chloroform (CHCl3) were performed as for *n*-Hexane,

so they were mixed and rotary evaporated while the MeOH/H$_2$O fraction was firstly rotary evaporated and secondly lyophilized.
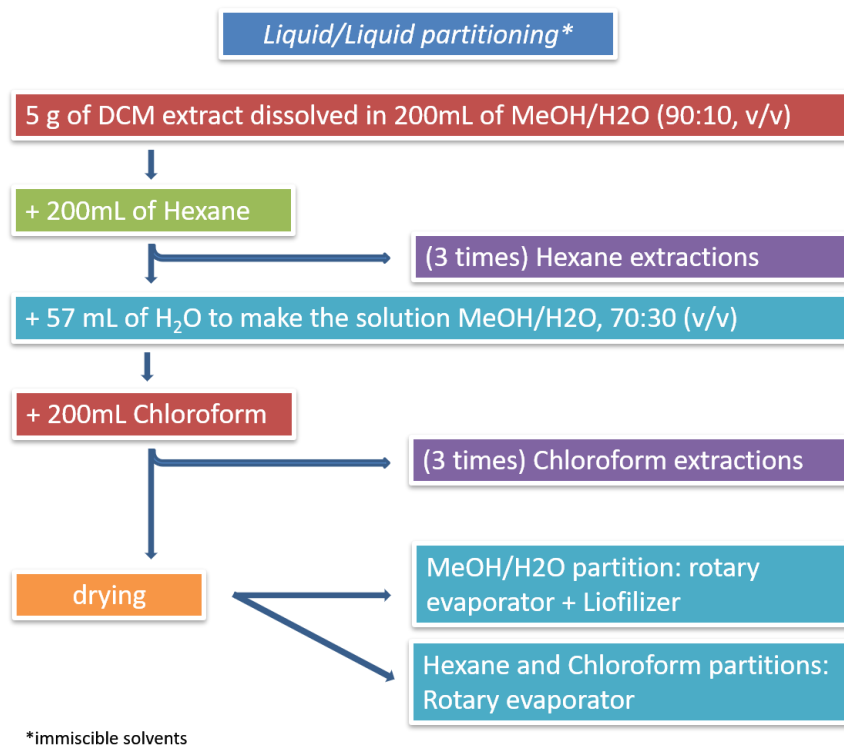
Figure 46 - Liquid/Liquid partitioning scheme

These three additional extracts were used for a second set of bioassays, performed as above, which showed that the chloroform extract was the most active.

At this point, a chromatography technique called Thin-Layer Chromatography was performed in few attempts aiming to find the best solvent system (Figure 47) to obtain a well-defined and separated spot, each one corresponding to a different compound with different distances from the base line.
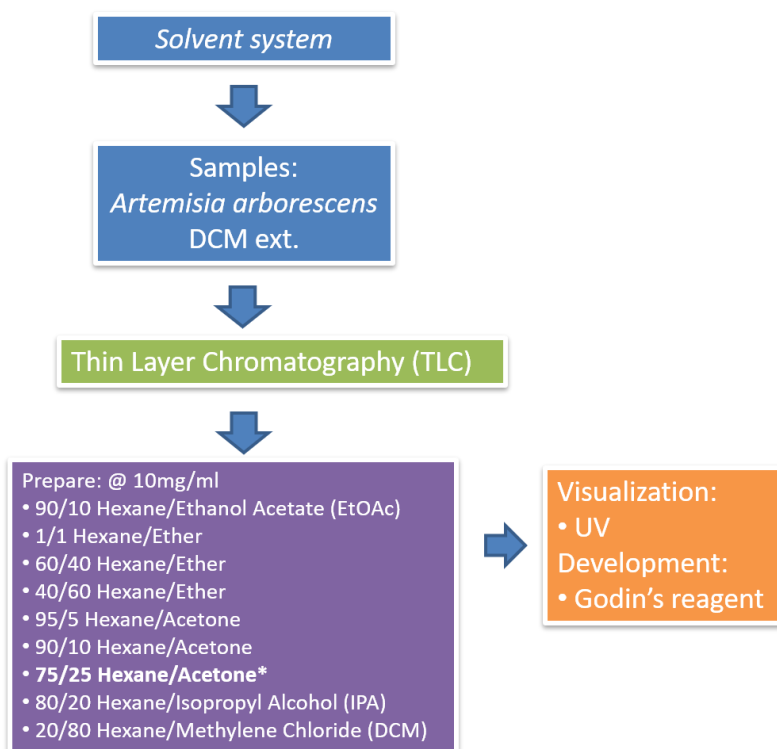
Figure 47 - Scheme of solvent system attempts

TLC is commonly used to separate components of a mixture based on the principle that a compound migrates with different speed according to its affinity for the mobile and stationary phases (Jork *et al.* 1990)

After each attempt, silica gel-coated TLC plate was placed under UV light, checking three different wavelengths (254, 302 and 365 nm), so each observed spot was marked with pencil. Godin's reagent (one volume of 1 % vanillin in Ethanol mixed with one volume of 3 % perchloric acid in water)(Godin 1954), sprayed on the surface of the TLC plate and then dried at 85°C, was used to develop the TLC plate in order to stain the spots (Figure 48).

Figure 48 - (left to right) Thin-layer chromatography; TLC plate after the elution; TLC plate under UV light.

Once found the best solvent system, this information was used to set the flash chromatography system, that was a Biotage, Inc. Isolera One pump equipped with a flash collector and a photo-diode array detector, through Cartridge SNAP 100g. Settings are as follows: running flow of 40 mL/min; step sequence, using *n*-Hexane:EtOAc, gradient beginning with 100:0 to 70:30 over 2400 mL, followed by 50:50 over 600 mL, then 0:100 over 400 mL. After few hours, from 1g of chloroform extract, 172 fractions in 22mL glass tube each, were collected and spotted in ascending order in a wide set of TLC plates in order to visualize the entire sequence of spots to merge fractions together based on the assumption that spots of the same colour and distances from the base line correspond to the same compound. Merging brought to obtain 13 fractions which were then bioassayed as above, and two of them (#3 and #8, Figure 49) resulted to have the most significant inhibition, especially on bentgrass, while a very weak activity was noticed on lettuce.
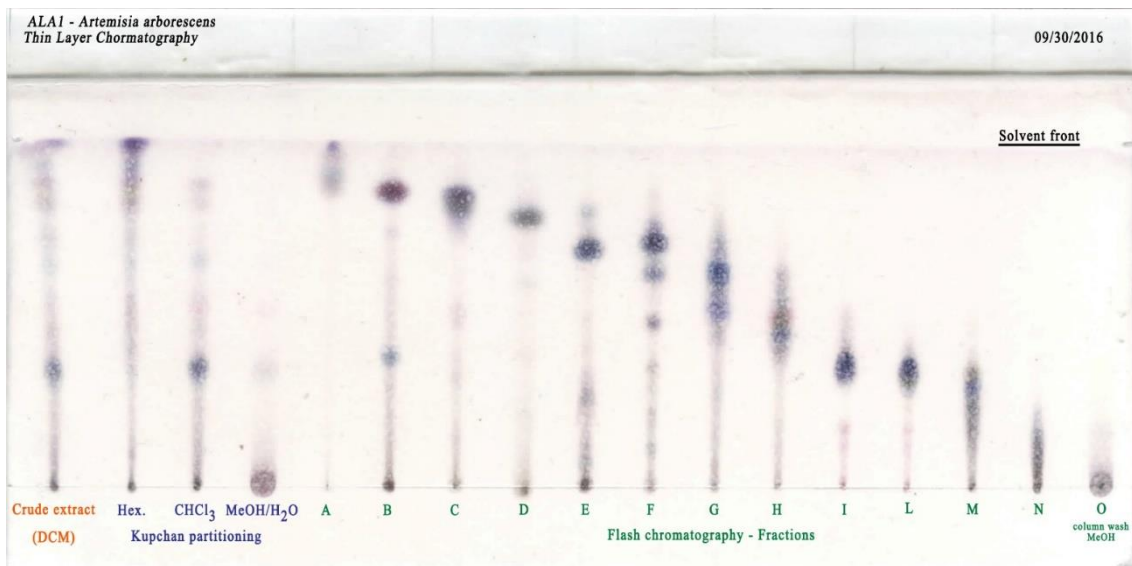
Figure 49 - TLC plate with all fractions eluted

The last two most active fractions where then analysed through NMR spectrometer Varian ANOVA 400 MHz and their spectra (H and ^{13}C) were recorded in CDCl$_3$ (Deuterated chloroform). Fractions were also analysed by GC/MSD on an Agilent Technologies 7890A GC system coupled to a 5975C Inert XL MSD. The GC, equipped with a DB-5 fused silica capillary column (30 m × 0.25 mm, film thickness of 0.25 μm) operated under the following conditions: injector temperature, 240°C; column temperature, 60–240°C at 3°C/min then held at 240°C for 5 min; carrier gas, He; injection volume, 1 μL (splitless injector). The MS mass range was from 40 to 650 m/z, a filament delay of 3 min, target TIC of 20,000, a prescan ionization of 100 μs, an iontrap temperature of 150°C, a manifold temperature of 60°C, and a transfer line temperature of 170°C. High-resolution mass (ESI-MS) spectra of isolated compounds in MeOH were acquired by direct injection of 20 μL of sample (approximately 0.1 mg mL-1) in a JEOL USA, Inc. (Peabody, MA) AccuTOF (JMS-T100LC).

At the end of the analysis, the two fractions were identified as pure compounds, namely two lignans: Sesamin (Figure 51) and Ashantin (Figure 52). Hence, for a more quantitative analysis of their phytotoxic effect they were bioassayed using *Lemna aequinoctialis Welw.* (syn. of *Lemna paucicostata Hegelm.*) as explained below.
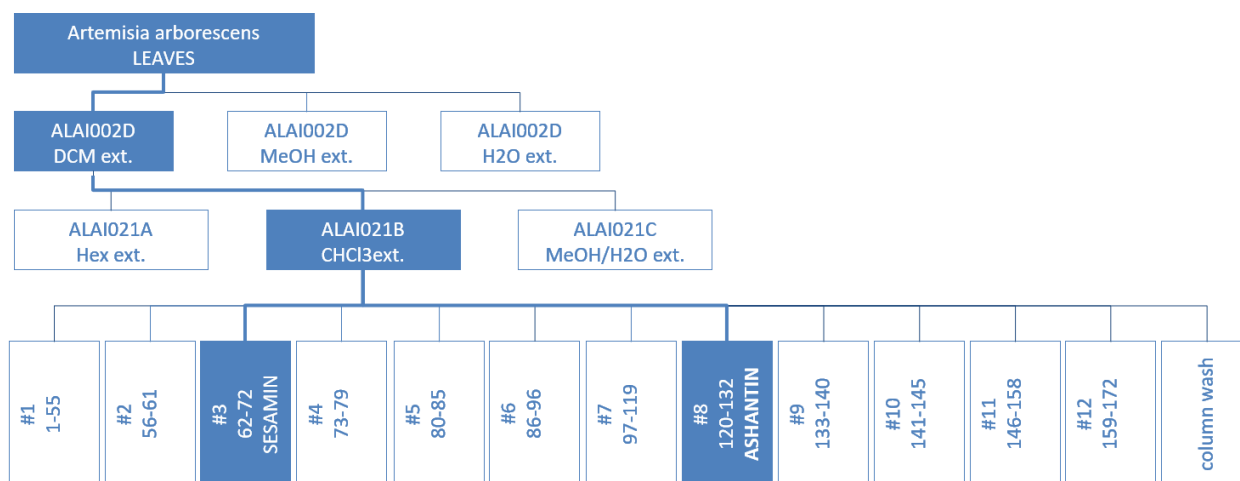
Figure 50 - A. arborescens flow chart of fractionation
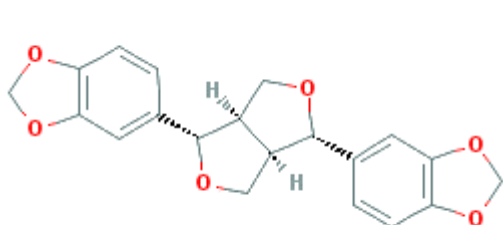
Figure 51 - Chemical structure of Sesamin
Formula: C20H18O6
Exact mass: 354.110338308
Molecular weight: 354.359
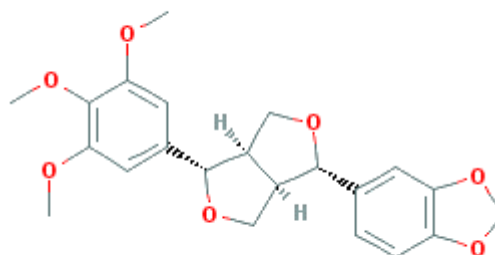
Figure 52 - Chemical structure of Ashantin
Formula: C22H24O7
Exact mass: 400.152203122
Molecular weight: 400.427

4.1.3.1   Bioassays description

4.1.3.1.1   *Lactuca sativa* and *Agrostis stolonifera*

Bioassay with both dicot and monocot was carried out slightly modifying Dayan *et al.* (2000) method. Certified seeds of lettuce (*Lactuca sativa L.*) and bentgrass (*Agrostis stolonifera L.*) were purchased. Seeds were sterilized with a sodium hypochlorite 10% solution for 10 minutes then thoroughly rinsed with DDI water. All assays were doubled in a sterile 24 well tissue-culture plate. Each sample well was filled with a filter paper disk (Whatmann #1, 1.5cm) soaked firstly with 180µL of DDI water and then with 20µL of appropriate dilution of the test solution, while control + solvent well contained 180µL of DDI water and 20µL of the solvent, sequenced as above (Figure 53). The whole operation was done in a sterile environment to avoid any chance of contamination. In each well an adequate amount of seeds was placed, i.e. 5 seeds for lettuce and, for bentgrass, enough seeds to cover the paper disk without overlapping (Figure 54). Parafilm was used to seal the lids, and then plates were incubated in Percival Scientific CU-36L5 at 26°C, under continuous light condition of 120 µmol s-1m-2 average light intensity (Figure 55). After 7 day of incubation a subjective ranking of plant growth was made based on a scale from 0 to 5, where 0 is for "no inhibition" (plants looked identical to the control), while 5 is for "no growth" (no seeds had germinated) (Figure 56).

Figure 53 - Preparation of the samples: soaking filter paper disks

Figure 54 - Preparation of the samples: distribution of target seeds

Figure 55 - Incubation of samples
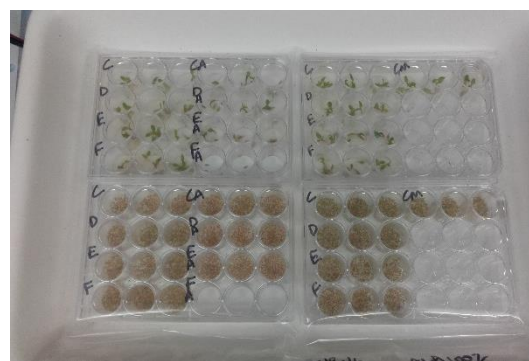
Figure 56 - Samples after 7 days of incubation

4.1.3.1.2 *Lemna aequinoctialis Welw.*

A more quantitative assay of isolated pure compounds was carried out slightly modifying Michel *et al.* (2004) method. The selected target plant was *L. aequinoctialis*, also called Duckweed, a monocotyledonous free-floating small plant, with exponential growth by clonal propagation, able to grow in nutrient solution under sterile environment, which makes it perfect for phytotoxicity assessment. Hence, duckweed was grown in Hoagland's N.2 Basal Salt Mixture (1.6 g/L), Table 7.

Table 7 - Hoagland's N2 Basal Salt Mixture

Nutrient	mg/L
KNO_3	1,515
KH2PO4	680
$MgSO_4 \cdot 7H_2O$	492
Na_2CO_3	20
$Ca(NO_3)_2 \cdot 4H_2O$	1,180
H_3BO_3	0.5
$ZnSO_4$	0.05
Na_2MoO_4	0.12
$MnCl_2$	0.47
$CoCl_2$	0.025
$CuSO_4$	0.025
Fe-EDTA	18.355

Thereafter, pH was adjusted to 5.5 using 1 N NaOH, then media was filter sterilized using 0.2μm filter and stored in sterile 1L bottles. Duckweed stocks from one or two three-frond plants, were firstly grown in 100mL of Hoagland's media in sterile baby food jars in a Percival Scientific CU-36L5 at 26°C, under continuous light condition of 120μmol $s^{-1}m^{-2}$ average light intensity, showing an approximately doubling time of 24 to 36 hours.
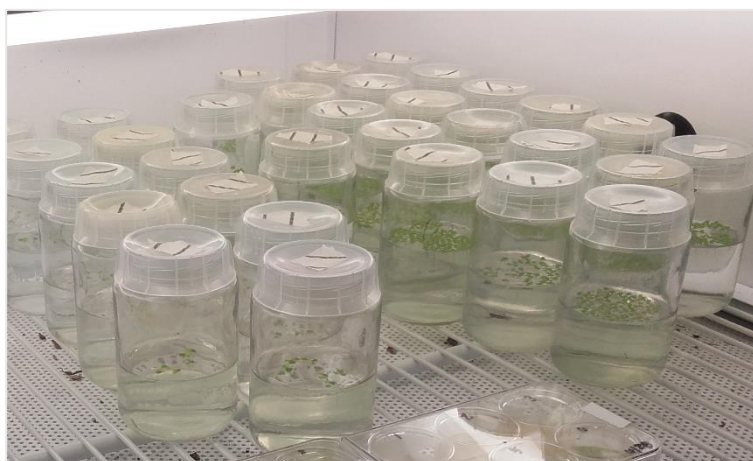
Figure 57 - L. aequinoctialis in incubation

Both screening and replicate series test were placed in sterile 6-well plates, each well containing 4950μL of Hoagland's media plus 50μL of water, or solvent, or the compound dissolved in the

appropriate solvent (at a concentration of 100x). Final concentration of the solvent was approximately 1% by volume.

Lemnatec Scanalyzer instrument was used to determine frond growth through a sequence of daily pictures, from day 0 to day 7, taken at each well and analysed by the software, which detects the fronds laying on the surface of the media in order to assess frond numbers, quantify colours and frond areas.

4.1.3.2 Results & Discussion

Although most of the fractions did show little phytotoxic activity, two of them, sesamin and ashantin (#3 and #8), strongly inhibited bentgrass seeds germination while poor activity was noticed towards lettuce (Table 8).

Table 8 - Sesamin and Ashantin bioassay ranking

Sample	Tested concentration	Solvent	Day	Bioassay ranking	
				Lettuce	Agrostis
Sesamin	1000 µM	10% acetone	7	0	3
Ashantin	1000 µM	10% acetone	7	1	4

After the first set of assays against bentgrass and lettuce, a more quantitative assay was carried out with duckweed. The averages of the three replicates were plotted along with the standard deviation. Results are shown as duckweed growth (% increased frond size) versus log concentration (µM)(Figure 58 and Figure 59).
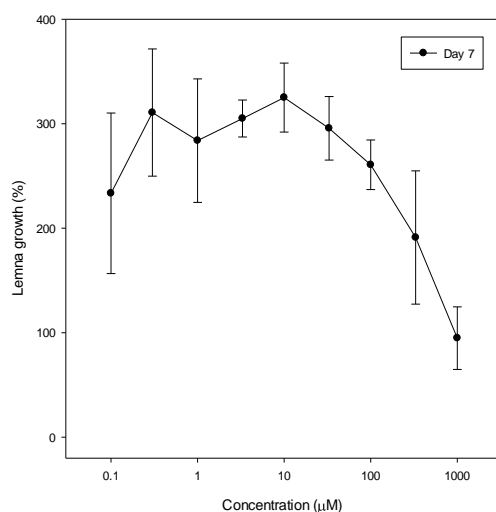
Figure 58 - Effects of increasing concentrations of sesamin on growth of L. aequinoctialis after 7 days of exposure. Bars represent the ± standard error of each mean
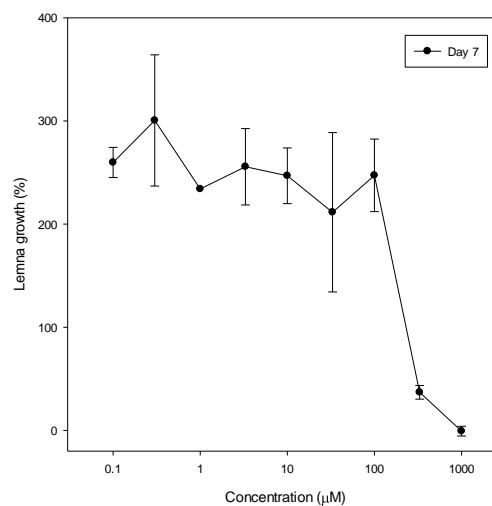
Figure 59 - Effects of increasing concentrations of ashantin on growth of L. aequinoctialis after 7 days of exposure. Bars represent the ± standard error of each mean

The half maximal inhibitory concentration (EC50) values recorded was ca. 401 and 224 µM for sesamin and ashantin, respectively. With this bioassay, these IC50 values are in the same range as those for the commercial herbicides naptalam (128 µM), glyphosate (388 µM), and clomazone (126 µM)(Michel *et al.* 2004). Sesamin has been previously reported in *A. arborescens* (Araniti *et al.* 2013,

2016; Greger 1981; Zarga *et al.* 1995) as well as Ashantin (Greger 1981; Tonelli *et al.* 2014). Although Sesamin was already reported for its phytotoxic activity (Greger 1981) no mention has been found about Ashantin.

Some other lignans are known to be phytotoxic. For example, the aryltetralin lignans podophyllotoxin, α-peltatin, and β-peltatin are active against both dicotyledonous and monocotyledonous plants by interfering with formation of mitotic microtubular organizing centers (Oliva *et al.* 2002). Ten lignans from *Helianthus annuus* were reported to be phytotoxic (Macías *et al.* 2004). Ashantin and sesamin showed good phytotoxic activity against bentgrass; further investigations are necessary about their mode of action, along with additional assays against monocot weeds.

4.2 Fungitoxic activity

4.2.1 Fungitoxicity-guided fractionation

Similarly, to what described for Phytotoxicity-guided fractionation in section 3.1.3, a bio-guided fractionation protocol was followed within the labs of the *Agricultural Research Service* of the *United States Department of Agriculture* (ARS-USDA), in order to identify secondary metabolites derived from *A. arborescens*, *D. viscosa* and *R. coriaria* extracts with fungitoxic activity. As in the previous experiment, the aerial parts of each of those plants were obtained from wild populations growing in the nearby of Altofonte (Palermo), and after drying at room temperature for one week, they were stored in a cool and dry place before using. The flowchart of the extractions guided by the assays is reported in Figure 50. Two hundred grams of plant material was finally ground, in order to maximize the plant-solvent contact area, then placed in a baker and poured with 2.5L of solvent, hence left onto a stirring plate for 60 hrs. This first set of extractions, named Solid-Liquid extraction, was made in three following steps, each corresponding to a different solvent. So that, the first solvent used was dichloromethane (CH_2CL_2), also known as DCM, the second one was methanol (CH_3OH) and the last one was Distilled De-Ionized (DDI) water. Vacuum filtration was performed with Whatman #2 filter paper in a Büchner funnel, then extracts were dried by rotary evaporator and nitrogen concentrator while water was removed by freeze drying unit (also known as lyophilizer). The extracts so obtained were collected in small vials and stored at 0°C until use. All three extracts were tested to assess the inhibition effect on a pool of selected fungi, specifically: *Botrytis cinerea, Colletotrichum acutatum, C. fragariae, C. gloeosporioides, Fusarium oxysporum* and *Phomopsis obscurans.* Bioassay lasted 7 days and rating was based on the clear area of inhibition around the spotted extracts, when active. At the end of the assessment only *D. viscosa* DCM extract showed some relevant fungicide activity to be considered viable for further investigations.

Liquid-Liquid partitioning, based on different solvents density and different affinity with the solutes, was performed. A modified Kupchan's extraction method (Kupchan *et al.* 1973) was applied, hence 5g of DCM extract were dissolved in 200mL of MeOH/H_2O, 9:1 (v/v) and placed in a separatory funnel, so for three times in a row 200mL of *n*-Hexane were poured into it, gently shacked and removed. Hexane extract was rotary evaporated.

The partitioning kept going adding 57mL more of DDI water to prepare the solution MeOH/H2O, 70:30 (v/v). Therefore, three additional extractions with Chloroform ($CHCl_3$) were performed as well as with *n*-Hexane, so they were mixed and rotary evaporated while the MeOH/H_2O fraction was firstly rotary evaporated and secondly lyophilized. These three additional extracts were used for a second set of bioassays, performed as above, which showed that the chloroform extract was the most

active.

At this point, a chromatography assay (Thin-Layer Chromatography - TLC) was performed in few attempts aiming to find the best solvent system to obtain a well-defined and separated spot, each one corresponding to a different compound with different distances from the base line.

TLC is commonly used to separate components of a mixture based on the principle that a compound migrates with different speed according to its affinity for the mobile and stationary phases (Jork *et al.* 1990). After each attempt, silica gel-coated TLC plate was placed under UV light, checking three different wavelengths (254, 302 and 365 nm), so each spot observed was marked with pencil. Godin's reagent (made of one volume of 1% vanillin in Ethanol mixed with one volume of 3% perchloric acid in water)(Godin 1954) sprayed on the surface of the TLC plate then dried at 85°C was used to develop the TLC plate in order to stain the spots. Once the best solvent system was found this information was used to set flash chromatography system: Biotage, Inc. Isolera One pump equipped with a flash collector and a photo-diode array detector, through Cartridge SNAP 100g. Setting are shown as follows: running flow of 40 mL/min; step sequence, using *n*-Hexane:EtOAc (Ethyl acetate), gradient beginning with 100:0 to 50:50 over 3000 mL, followed by 50:50 over 600 mL, then 0:100 over 900 mL. After few hours, from 1.30g of chloroform extract, 208 fractions in 22mL glass tube each, were collected and 5μL of each of them spotted in ascending order in a wide set of TLC plates in order to visualize the entire sequence of spots, to merge fractions together based on the assumption that spots of the same colour and distances from the base line correspond to the same compound (Figure 60 and Figure 61).

Figure 60 - racks of 22 mL glass tubes filled with fractions recovered after flash chromatography
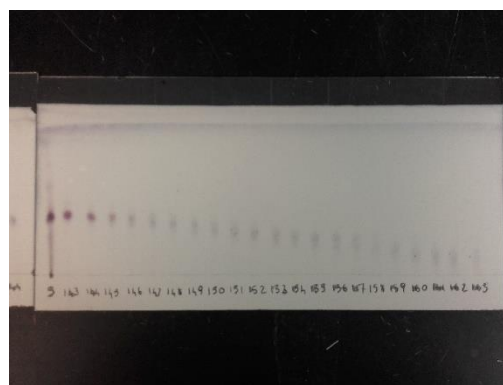
Figure 61 - sequence of spotted fractions developed on TLC plates

Merging brought to 11 fractions, which were then bio-assayed as above, resulted that only two of them (#4 and #8, Figure 50) had significant inhibition, against most of the selected target fungi.

These last two most active fractions where then analysed through NMR spectrometer Varian ANOVA 400 MHz and their spectra (H and ^{13}C) were recorded in CDCl$_3$ (Deuterated chloroform). Fractions were also analysed by GC/MSD on an Agilent Technologies 7890A GC system coupled to a 5975C

Inert XL MSD. The GC, equipped with a DB-5 fused silica capillary column (30 m × 0.25 mm, film thickness of 0.25 μm) operated under the following conditions: injector temperature, 240°C; column temperature, 60–240°C at 3°C/min then held at 240°C for 5 min; carrier gas, He; injection volume, 1 μL (splitless injector). The MS mass range was from 40 to 650 m/z, a filament delay of 3 min, target TIC of 20,000, a prescan ionization of 100 μs, an iontrap temperature of 150°C, a manifold temperature of 60°C, and a transfer line temperature of 170°C. High-resolution mass (ESI-MS) spectra of isolated compounds in MeOH were acquired by direct injection of 20μL of sample (approximately 0.1 mg mL-1) in a JEOL USA, Inc. (Peabody, MA) AccuTOF (JMS-T100LC).

At the end of the analysis, fraction #4 was identified as a pure compound, the Isocostic acid (Fontana *et al.* 2007; Hegazy *et al.* 2014) (Figure 51). The second isolated fraction (#8) went back through the flash chromatography system for a new round of purification. Settings were as follows: running flow of 40mL/min; step sequence, using *n*-Hexane:Et$_2$O (Diethyl ether), gradient beginning with 100:0 to 80:20 over 600mL, followed by 20:80 over 2000mL, then 0:100 over 400mL. After few hours, from 103.20mg of chloroform extract, 112 fractions in 22mL glass tube each, were collected and spotted in ascending order in a wide set of TLC plates as already mentioned above. Merging brought to 4 fractions which were then bio-assayed as above, resulted that only two of them (#7.2 and #7.4, Figure 50) had significant inhibition. Hence, they were analysed through the same equipment and setting as for the previous fraction #4, so that two compounds were identified: Carabrone (#7.2)(Figure 52) and Tomentosin (#7.4)(Figure 65).
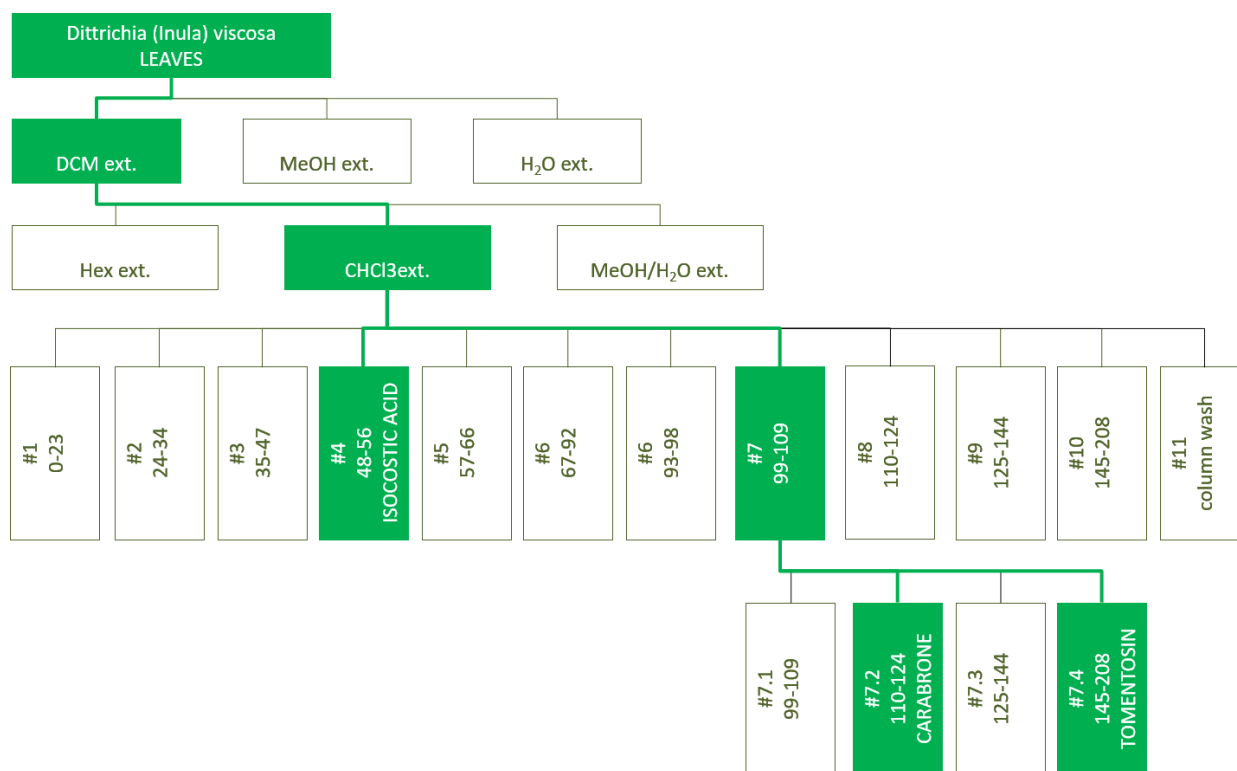
Figure 62 - flow chart of bio-guided fractionation of D. viscosa extracts
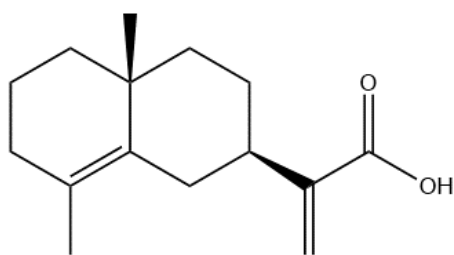
Figure 63 - Chemical structure of Isocostic acid

Chemical formula: C$_{15}$H$_{22}$O$_2$
Exact mass: 234.1620
Molecular weight: 234.3390
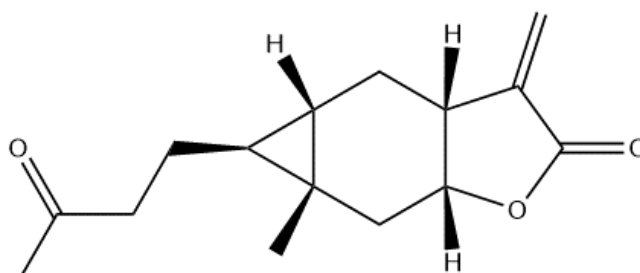
Figure 64 - Chemical structure of Carabrone

Chemical formula: C$_{15}$H$_{20}$O$_3$
Exact mass: 248.1412
Molecular weight: 248.3220
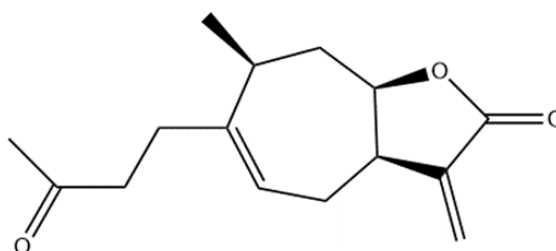
Figure 65 - Chemical structure of Tomentosin

Chemical formula: C$_{15}$H$_{20}$O$_3$
Exact mass: 248.1412
Molecular weight: 248.3220

4.2.1.1 Bioassays description: direct bioautography

4.2.1.1.1 Production of pathogenic fungi and inoculum preparation

Isolates of *Colletotrichum acutatum* Simmonds, *C. fragariae* Brooks, and *C. gloeosporioides* (Penz.) Penz. & Sacc. in Penz. were obtained from B. J. Smith, USDA-ARS, Small Fruit Research Station, Poplarville, MS. The three *Colletotrichum* species were isolated from strawberry (*Fragaria x ananassa* Duchesne (Duchesne ex Weston) Duchesne ex Rozier). *Botrytis cinerea* Pers. Fr was isolated from commercial strawberry. *Fusarium oxysporum* Schlechtend. Fr was isolated from orchid (*Cynoches* sp.). Fungal cultures were initiated on ½ strength potato dextrose agar (PDA, Difco, Detroit MI) from spores stored in sterile 10% glycerol RPMI (Roswell Park Memorial Institute, Gibco) buffer with MOPS (3-(N-morpholino) propane sulfonic acid) at -80°C. Fifty µL suspended spore solution was inoculated on PDA plate using crossed inoculation method. Inoculated plates were incubated at 24 ± 2°C under cool-white fluorescent lights (55 ± 5 µmols/m^2/s) with a 12h photoperiod. *Colletotrichum* cultures were subcultured or harvested from PDA every 7-10 days. Conidia were harvested by flooding plates with 3-5 mL of sterile distilled water and dislodging conidia by softly brushing the colonies with an L-shaped glass rod. Conidial suspensions were filtered through sterile Miracloth (Calbiochem- Novabiochem Corp., La Jolla, CA) spread on a sterile funnel to remove mycelia. Conidia suspensions were adjusted with sterile DDI water to a concentration of 1.0×10^6

CFU (Colony Forming Units)/mL. Conidia concentrations were determined photometrically from a standard curve based on the percent of transmittance (%T) at 625 nm. Conidial suspensions (Figure 69) of each fungal species were then adjusted to a concentration of 3.0×10^5 conidia/mL with liquid potato-dextrose broth (PDB, Difco).
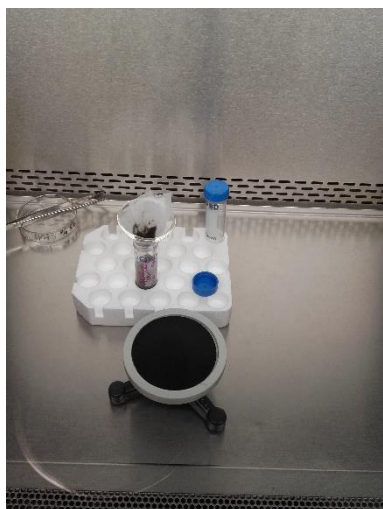
Figure 66 - flooding plates with conidia

Figure 67 - cool storage of conidia

Figure 68 - filtered conidia
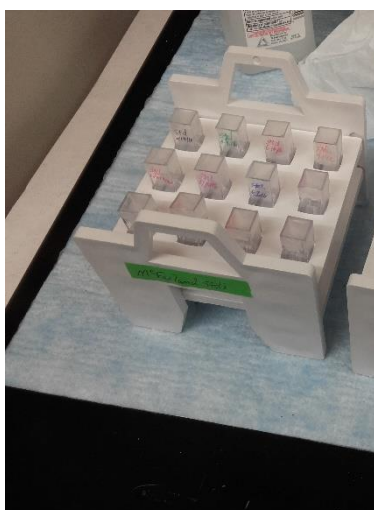
Figure 69 - conidia suspensions ready to concentration measurements

4.2.1.1.2 Direct bioautography

This method provides a simple technique to visually follow antifungal components through the separation process. Matrix, one-dimensional, and two-dimensional bioautography protocols on silica gel TLC plates with fungi as the test organisms are used to identify the antifungal activity according to published bioautography methods (Homans and Fuchs 1970; Wedge and Nagle 2000).

Direct bioautography is a successful technique to pre-screen large numbers of crude extracts (Wedge *et al.* 2009), or pure compounds (Tabanca *et al.* 2008). Bioautography is particularly important in evaluating lipophilic extracts and determining the number of active compounds in an extract (Wedge and Smith 2006; Wedge and Duke 2006).

Hence, stock solutions 1:10 (w/v) extract/solvent were previously prepared. Two different concentration of each extract, 10 and 100 µl, were tested on Si gel plates by calibrated capillary pipettes. After sample application and solvent evaporation to provide a solid amount of compound or pure compound applied, each TLC plate was subsequently sprayed with a spore suspension (3.0 x 105 spores/mL) of the fungus of interest and incubated in a moisture chamber for 4 days at 26°C with a 12h photoperiod. Clear zones of fungal growth inhibition on the TLC plate indicated the presence of antifungal constituents in each extract or pure compound.
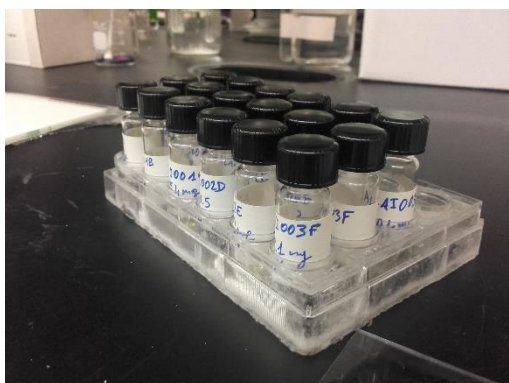
Figure 70 - Preparation of the samples: stock solutions of extracts

Figure 71 - Preparation of the samples: spotting crude extracts on TLC plates

Figure 72 - TLC plates prepared for the bioautography

Figure 73 - incubation of TLC plates into the moisture chamber
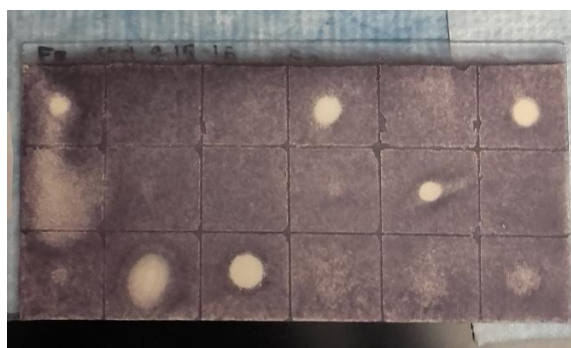
Figure 74 - Example of a TLC plate, 7 days after the inoculum.

4.2.1.2   Results & Discussion

Bioautography experiments using fungi were used to drive the bioassay guided fractionation and isolation of pure compounds Isocostic acid, Carabrone and Tomentosin. Means of inhibitory zone size and standard deviations, when available, were used to evaluate the antifungal activity of extracts and pure compounds tested at concentrations of 50, 100 and 200µM. Commercial technical grade Azoxystrobin, Captan, Benomyl and Cyprodinil (without formulation) were used as controls, tested at concentrations of 0.3, 3.0 and 30µM.

The results of this work confirmed previous findings (Cohen *et al.* 2006a) on fungitoxic activity of leaves extract of *D. viscosa*, this time applied on pathogenic fungi of crops and agents of food spoilage. Antifungal activity of Isocostic acid has little supporting references compared to its analogs, costic acid and several other eudesmane-type sesquiterpenes (Sanz *et al.* 1991; Zaki *et al.* 2017), already well known for their antifungal properties and also frequently reported, while Tomentosin and Carabron have been intensely studied (Cafarchia *et al.* 2001, 2002; Xie *et al.* 2015; L. Wang *et al.* 2017; H. Wang *et al.* 2014) and their fungitoxic activity well known. The three isolated compounds showed different inhibition/stimulation activity depending on both target fungus and concentration tested but also considering the times of the observation.

Although some encouraging effect of the extracts on *Botrytis cinerea* had been noticed during the fractionation process, they were not confirmed by the last step of the assay, where none of the pure compounds caused any inhibition, instead expressing an opposite stimulation effect. This behaviour could be partially explained by the activation of synergistic interactions of the phytochemicals (Einhellig 1995; Chou 2007; Ritz and Streibig 2014), which disappeared once each component was separated and acted individually (Figure 75, Figure 76 and Figure 78). Meanwhile, a good result was obtained by the treatments on *Colletotrichum fragariae*, where the inhibition was comparable with the standards, showing the following decreasing trend: Tomentosin>Carabrone>Isocostic acid (Figure 79 and Figure 80). Another good output came out from the tests against *Phomopsis obscurans*, which, according to its typical behaviour, required a longer timeframe compared to the other target fungi. In this case, all three pure compounds acted almost as effectively as the standards (Figure 85 and Figure 86). The other target fungi, *Colletotrichum acutatum, Colletotrichum gloeosporioides, Fusarium oxysporum* were variously affected by the treatments (Figure 77 and Figure 78; from Figure 81 to Figure 84). Forty-eight hours observations usually showed the most powerful effect, thereafter it decreased over time, as if fungi would succeed in adapting their metabolism to the substrates (Garnier *et al.* 2017; Huis In't Veld 1996).
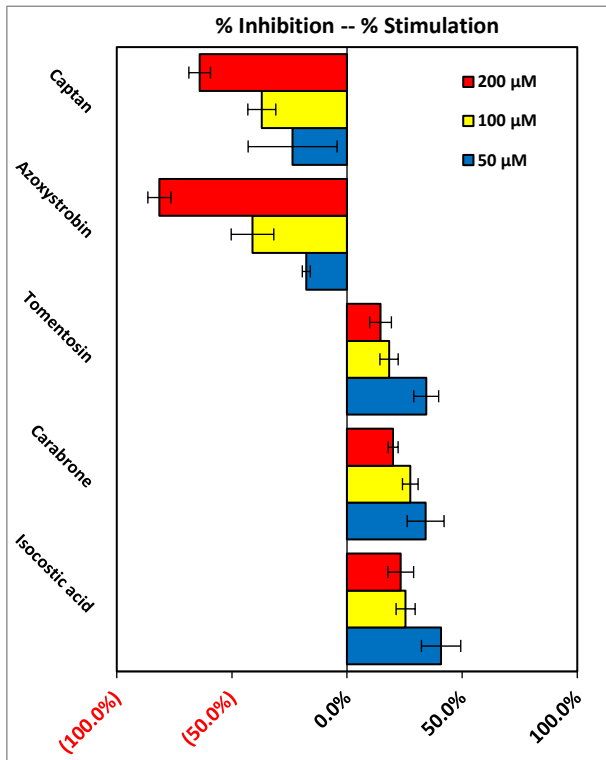
Figure 75 - Botrytis cinerea after 48h
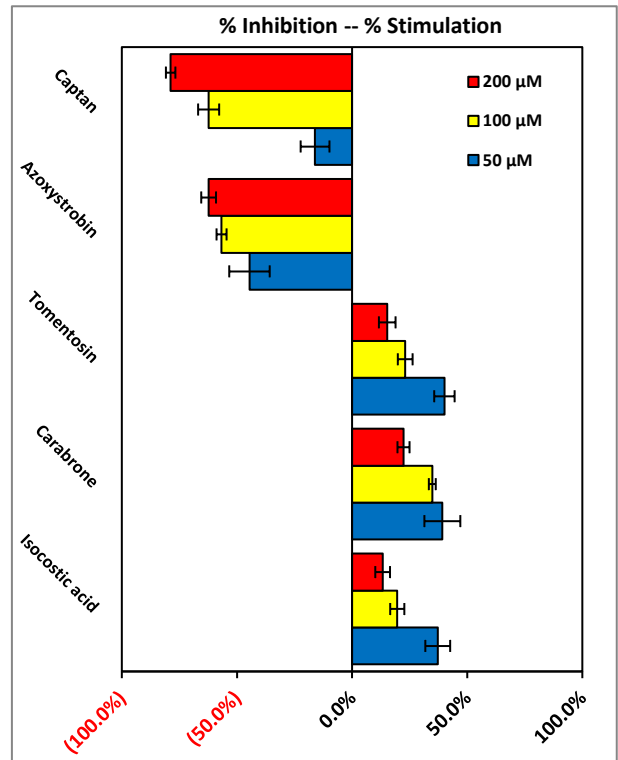
Figure 76 - Botrytis cinerea after 72h
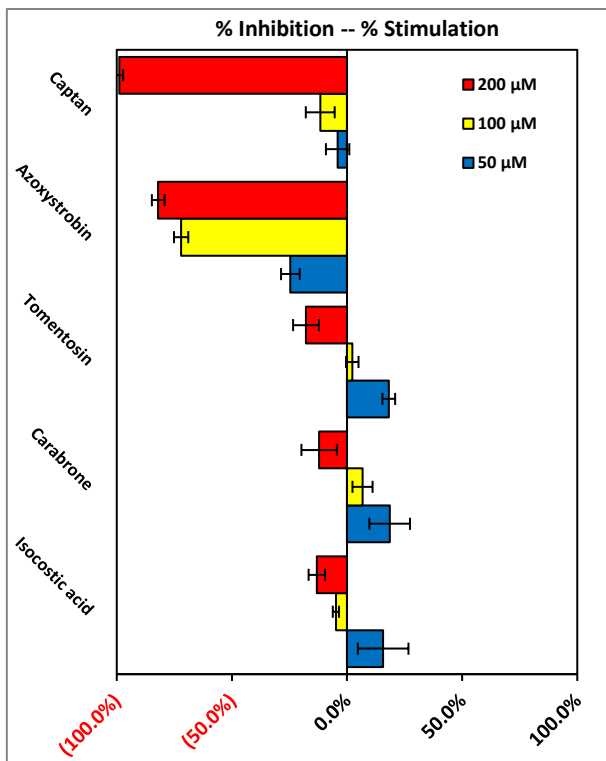
Figure 77 - Colletotrichum acutatum after 48h
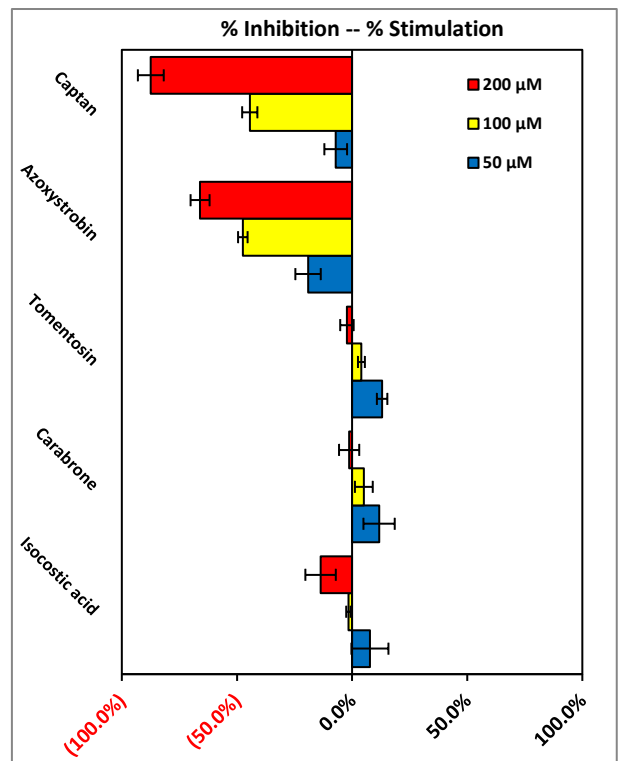
Figure 78 - Colletotrichum acutatum after 72h

67
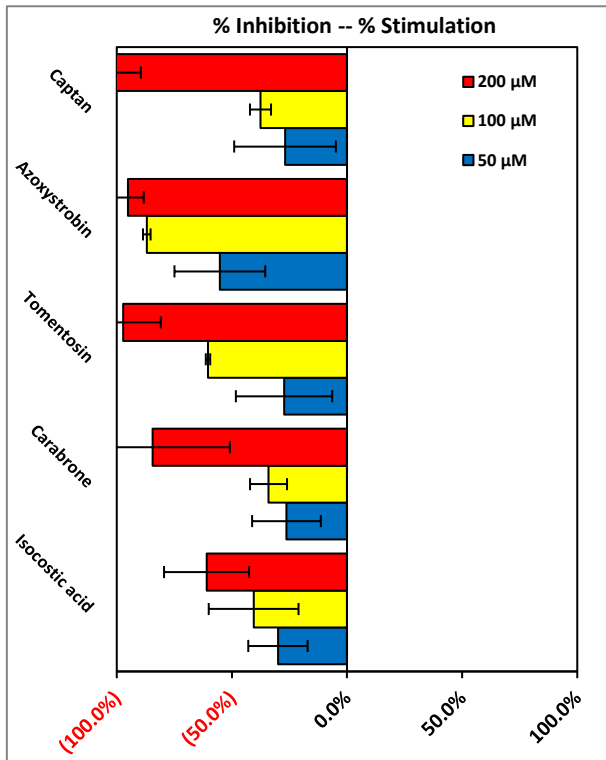
Figure 79 - Colletotrichum fragariae after 48h
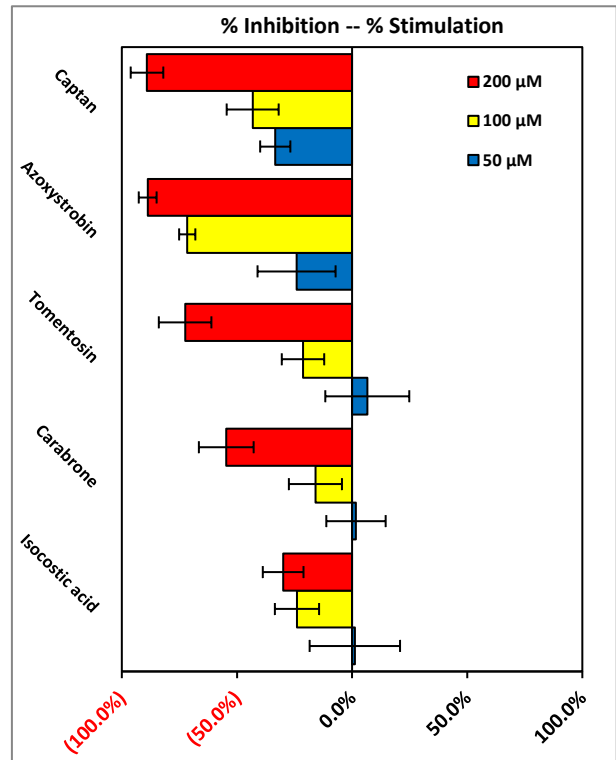
Figure 80 - Colletotrichum fragariae after 72h
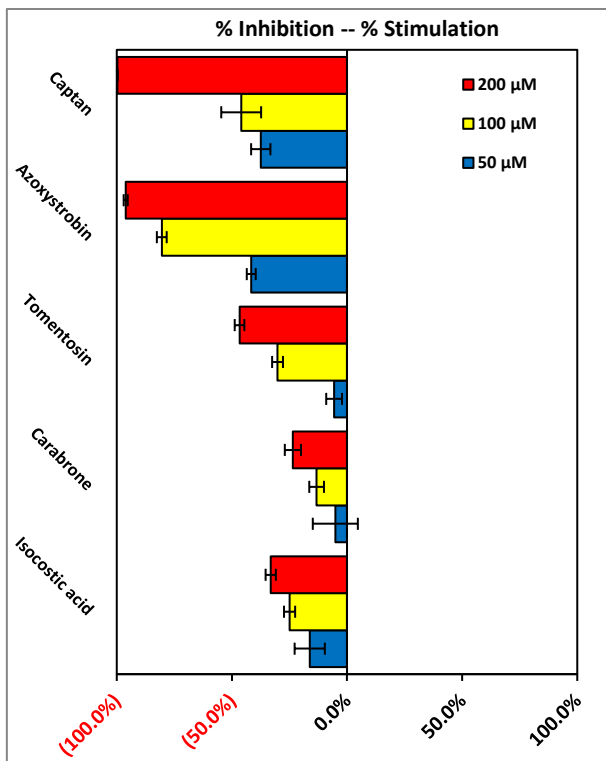
Figure 81 - Colletotrichum gloeosporioides after 48h
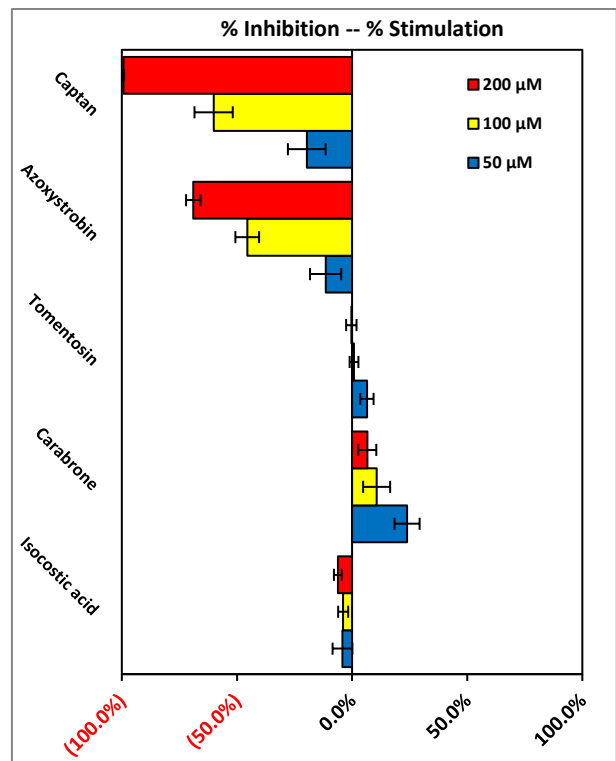
Figure 82 - Colletotrichum gloeosporioides after 72h

68
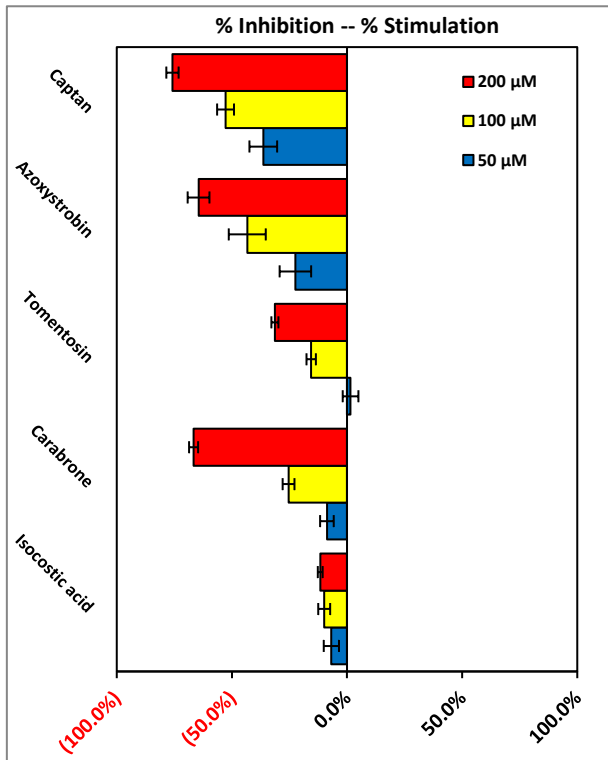
Figure 83 - Fusarium oxysporum after 48h
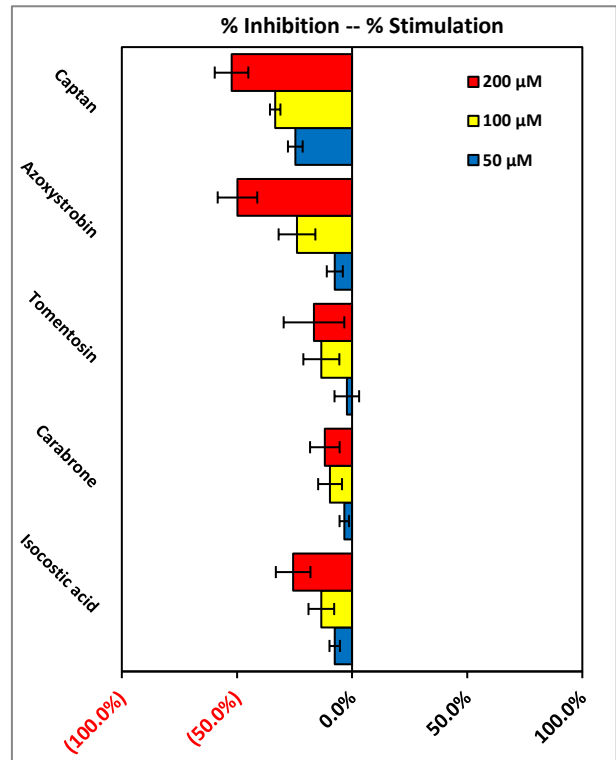
Figure 84 - Fusarium oxysporum after 72h
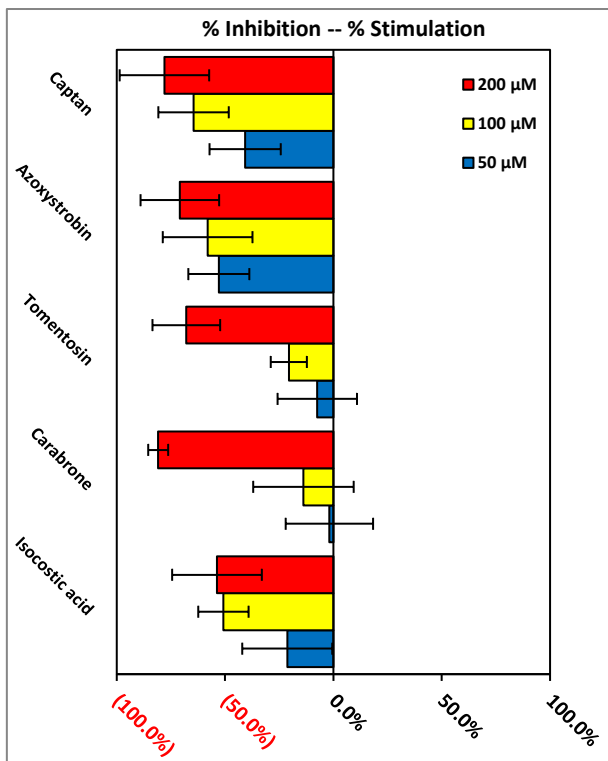
Figure 85 - Phomopsis obscurans after 120h
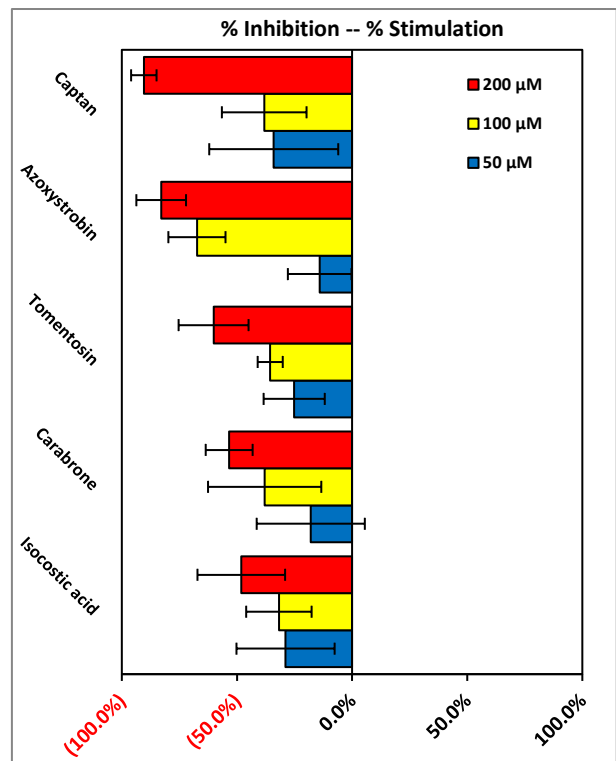
Figure 86 - Phomopsis obscurans after 144h

69

4.2.2   In vitro experiment

4.2.2.1   Introduction

Many studies (Maoz *et al.* 1999; Cohen *et al.* 2006a; Cafarchia *et al.* 2001; Haoui *et al.* 2016) have shown interesting fungicidal activities of *D. viscosa* leaves extracts against a number of pathogenic fungi (*Microsoposum canis*, *Trichophyton rubrum, Fusarium culmorum, Fusarium graminearum, Plasmopara viticola, Candida spp.)*. With the purpose to broaden previous findings to other targets, and put the bases for practical application, a new assay was set. The idea was meant to be targeted on some of the most economically important pathogens of crops and foodstuff and agents of wood decay, namely *Aspergillum, Fusarium, Botrytis, Ganoderma* and *Laetiporus*. *Aspergillum* and *Fusarium* species, belonging to the *Ascomycota* division, have a great economic and epidemiologic impact due to their ability to produce polyketide-derived mycotoxins; both species are very common components of the pathogenic microbial flora of many crops such as coffee, cotton, grapes, maize, peanuts, tree nuts, wheat, barley and other cereals, and may cause plant disease and rot of fruits, ultimately affecting the entire food chain (Palumbo *et al.* 2008). *Botrytis cinerea*, also belonging to the *Ascomycota* division, is an airborne plant pathogen that causes necrotic infections to over 200 different crops; many fungicides over the years have failed in contrasting it, due to its wide genetic plasticity. *B. cinerea* presently brings to massive losses in field and greenhouse crops, causing soft rotting and rotting of fruits and flowers by producing typical grey conidia (Williamson *et al.* 2007). In addition, two *Basidiomycetes* were taken into account, *Ganoderma resinaceum* and *Laetiporus sulphureus.* These are ligninolytic fungi with bracket-shaped fruiting body, that cause brown rot wood decay with two main impacts: on safety of urban trees and on the market value of timber stocks (Weber *et al.* 2004; Deflorio *et al.* 2008).

Knowing that most of the antifungal activity of *D. viscosa* extracts has been suggested to come from the lipophilic fraction (Cohen *et al.* 2006b), and taking into account the high amount of required inputs, in terms of energy, time and solvents, to process the plant material to obtain the extract, it was decided to try a faster and cheaper approach focusing on the plant crude powder. If effective, the use of raw powder instead of further processed items could minimize the inputs for a definitely more sustainable and broadly affordable development of the active product. Hence, the antifungal effect of *D. viscosa* raw powder was compared to that of its water extract, which was considered having a weak antifungal activity compared with the lipophilic part.

4.2.2.2   Materials and methods

Leaves of *D. viscosa* were collected from homogeneous wild populations growing in the nearby of Altofonte (Palermo) and dried for at least 24h at room temperature. The drying process was performed

under a fume hood, to ensure a constant airflow and facilitate the process of dehydration. Once dried, the plant material was easily crushed and pulverized using a common kitchen blender. The obtained powder was sieved by means of a 250μm mesh sieve and stored in cool and dry place until use. Water extracts were prepared by soaking 50g of finely sieved leaves of *D. viscosa* in 300ml of DDI water, and leaving the mass over 24h on stirring plate before gravity filtration with Whatman #2 filter paper. Water was removed by evaporation in oven for 24 hours at 40°C; the obtained product was collected and weighted, yielding 3.0929g of material. Eight fungi were selected as target, namely: *Aspergillus brasiliensis*, *A. flavus*, *A. fumigatus*, *A. luchuensis*, *Fusarium lycopersici*, *Botrytis cinerea*, *Ganoderma resinaceum* and *Laetiporus sulphureus*. The fungal species were grown in 9cm Petri dishes on Potato Dextrose Agar (PDA) medium for one week, to renew the colony from the slant tube where they had been stored after being isolated and identified. The PDA medium was prepared mixing 21g of PDA powder into 500ml of DDI water in a flask placed on a magnetic stirrer hotplate; agitation was kept until PDA powder was completely dissolved, then the mass was sterilized in autoclave at 121°C under 15psi of pressure for 1h. The chosen experimental design was a completely randomized scheme with three repetitions for each treatment, exception made for the control, that was repeated only twice.

Both treatments, *D. viscosa* raw powder (50g) and dried water extract (3.0929g, extracted from 50g of powder), were added to 500ml of PDA each, while negative control was set only using PDA (Table 9). After sterilization, the media were poured into the Petri dishes under sterile environment, and left to cool down and solidify before the inoculation.

Table 9 - In vitro evaluation of D. viscosa antifungal activity. Composition of media

Treatments	Preparation
Powder	500ml + 21g of PDA + 50g of *D. viscosa* plain powder
Water extract	500ml + 21g of PDA + 3.0929g of *D. viscosa* dried water extract
Control	500ml + 21g of PDA

Once the media were solidified and cooled at room temperature, a tiny piece of each target fungus from the renewed colony, including part of its growth medium, was placed in the middle of a Petri dish (Figure 87); lid was sealed with Parafilm, and the dishes incubated in a controlled environment chamber at 15°C.

The assessment was done at day 3, 6 and 9 after the inoculation, by measurement of the fungal growth diameter.
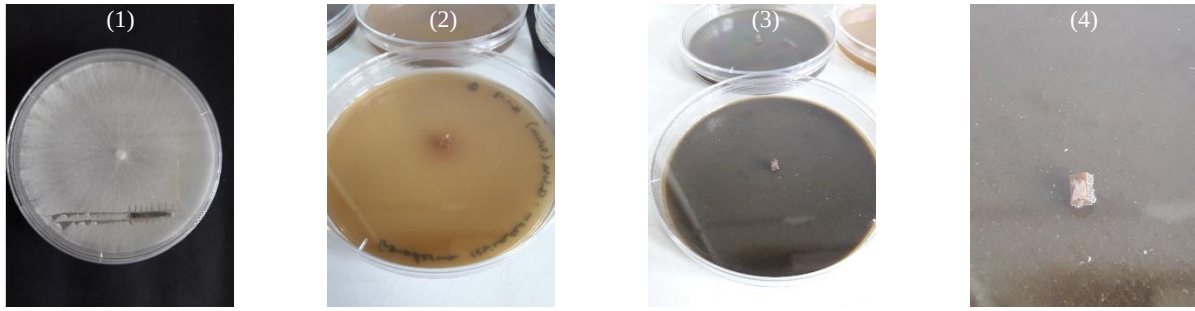
Figure 87 - Detail of the inoculation process. (1) taking out of the inoculum from the renewed colony; (2) laying down of the inoculum on PDA-water extract treatment; (3) laying down of the inoculum on PDA-powder treatment; (4) detail of inoculum onto the medium surface

4.2.2.3 Statistical analysis

Statistical analyses were carried out by mean of SPSS software (IBM Corp. Released 2016. IBM SPSS Statistics for Windows, Version 24.0. Armonk, NY: IBM Corp).

Analysis of variance (ANOVA) was performed in order to evaluate if any difference occurred between and/or within the groups. The diameter of growth (Dg) was measured at day 3, 6 and 9 after the inoculation and values recorded were transformed (Steel and Torrie 1980) to ensure the normal distribution according to the formula:

$$Y' = Log 10(Y + \frac{1}{2})$$ (2), where:

Y' = transformed data

Y = original data.

The assumption of normal distribution was tested by Shapiro Wilk's test ($p \leq 0.05$)(Shapiro and Wilk 1965; Razali and Wah 2011) and by visual inspection of the histograms, normal Q-Q Plot and box plots, showing that the after data transformation, residuals of the applied model were approximately normally distributed.

4.2.2.4 Results and discussion

Table 10 reports the outcome from the application of ANOVA on the diameter of growth measured during the assessment at day 3, 6 and 9, in all treatments. Two treatments were included in the model, i.e. powder dose (p) and water extract (w.e.) from leaves of *D. viscosa*, plus the untreated control (T0). As shown, the outcome variable was highly dependent ($P \leq 0.001$) on Treatments (T), day of observation (d) and species of fungi (F), as well as on all possible combination between them (all interactions were highly significant).

The graphs in figure 88 show the behaviour in time of all fungal species after in both treatments and in the untreated control. First, it is possible to observe how several fungal species did not grow whatsoever along the entire period of the experiment (9 days). This was the case in three out of eight strain of fungi treated with powder, namely *A. fumigatus*, *G. resinaceum* and *L. sulphurous*, and in

two out of eight strain of fungi treated with water extract, namely *G. resinaceum* and *L. sulphurous* (Figure 88). These cases of complete inhibition, due to a strong sensitivity of the targets to the treatments, represent an interesting result. Anyway, many other cases may be noticed in which an inhibitory effect of treatments shows up, as evidenced by the position of treatment curves that is always lying below the control. The strongest inhibition, from the beginning to the end of the experiment, has to be ascribed to the powder treatment which yielded the lowest means in all observed cases, while water extract appeared less powerful. Apart from this general trend, a great variability may be found in the specific response of each fungus to the treatments, also affected by the day of observation.

Table 10 - Results of the ANOVA (General Linear Model) performed on the transformed values of Diameter of growth (D) measured during the assessment at day 3, 6 and 9, in all treatments

Source of variation	Df	F
Treatments (T)	2	31135.107[***]
Day (d)	2	5764.940[***]
Fungi (F)	7	11103.112[***]
T x d	4	291.946[***]
T x F	14	2639.905[***]
d x F	14	481.784[***]
T x d x F	28	237.444[***]
Error	120	
Total	192	

*Significance of F values: ***= P $\leqslant$ 0.001; ** = P $\leqslant$ 0.01; * = P $\leqslant$ 0.05; n.s. = not significant*

Although a general inhibition effect of the treatments has been proved, an interesting phenomenon of adaptation to the conditioned substrate may be evidenced in *B. cinerea,* that, probably thanks to its wide genetic plasticity, at the end of the experiment reached in both treatments almost the same growth of the control.
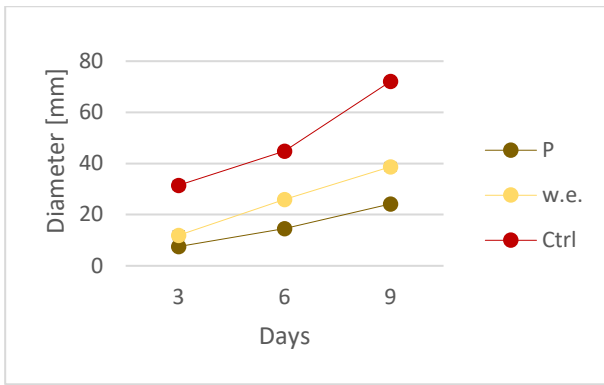
Aspergillus brasiliensis
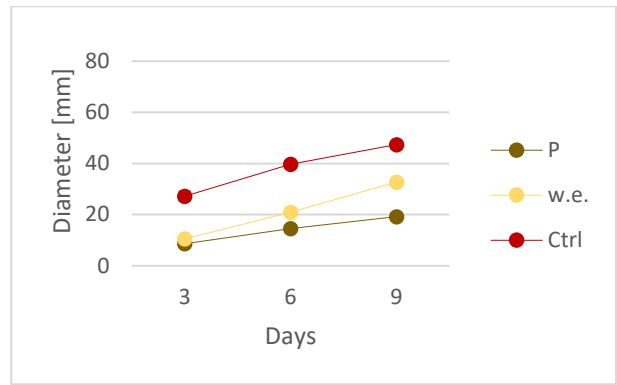
Aspergillus flavus
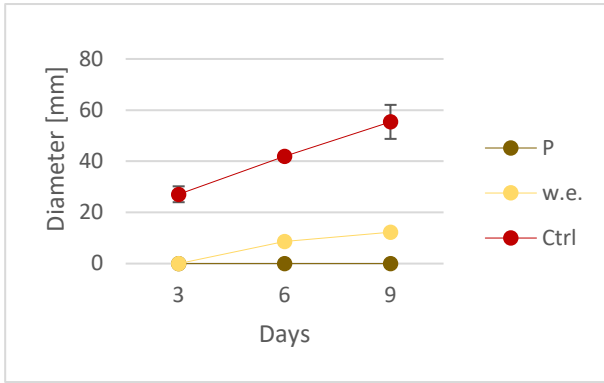
Aspergillus fumigatus
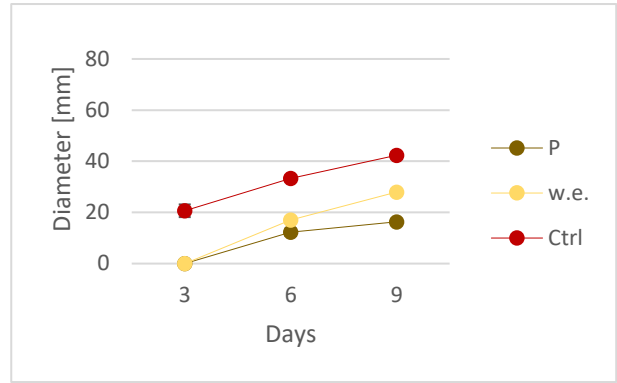
Aspergillus luchuensis
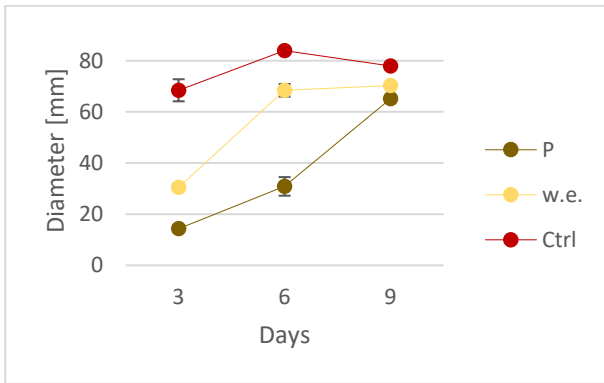
Botrytis cinerea
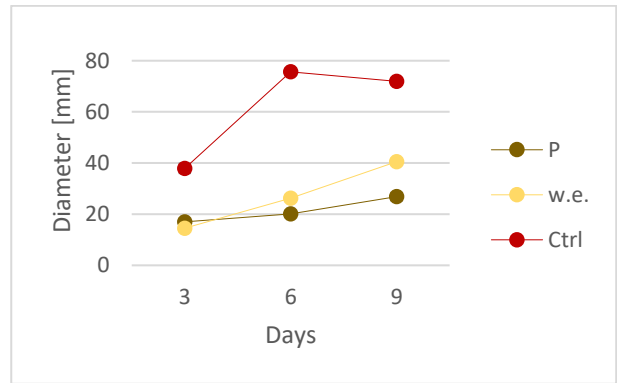
Fusarium lycopersici
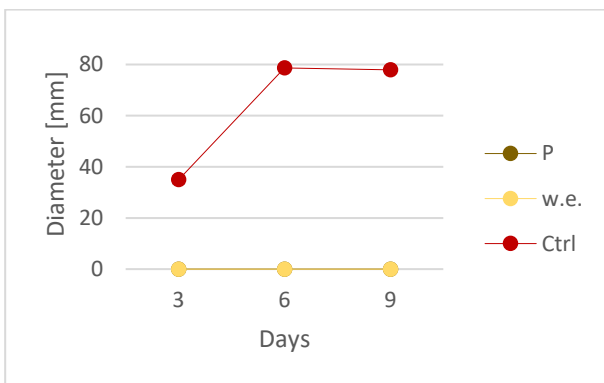
Ganoderma resinaceum
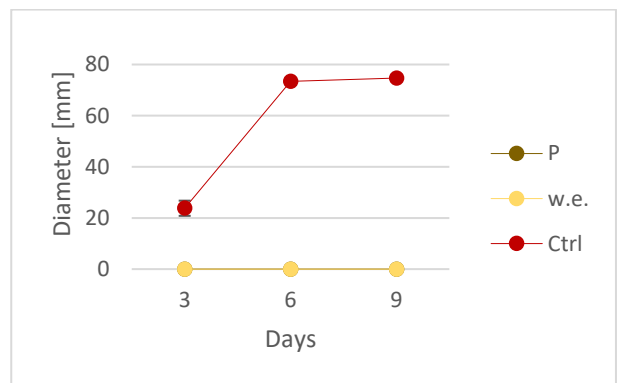
Laetiporus sulphureus

Figure 88 - Comparison of growth, over the time (days of observation), of different strain of fungi treated with D. viscosa powder (p) and water extract (w.e.) with not treated control (Ctrl). Error bars represent Standard deviations

4.3 Nematicidal activity

4.3.1 Dittrichia viscosa *water extract*

4.3.1.1 *Introduction*

Nematodes can be found as free-living organisms in a wide variety of habitats, such as in soil, marine sands, freshwater and muds, where they play an important role in nutrient turnover; they may also be found as parasites of almost every species of plant or animal, humans included. Phytonematodes parasitize plants seeking for food, which is basically cell contents, so that, infection usually come from the roots of the host plant causing abnormal growth and productivity, sometimes leading plant to death (Haroon *et al.* 2018).

Plant-parasitic nematodes cause an estimated annual mean crop yield loss from 10 to 15%, causing a monetary loss of about $ 78 billion worldwide(Lima *et al.* 2018).

Potato Cyst Nematodes (PCN - *Globodera rostochiensis* and *Globodera pallida)* cause the major yield losses in potato (*Solanum tuberosum*) and other members of *Solanaceae* family (Europe 2017). *G. rostochiensis* and *G. pallida* mainly differ in the colour of their early stage cysts, that is darker for *G. rostochiensis* and lighter for *G. pallida*. First reported in Germany in 1913, PCNs have spread all over Europe and overseas (Hockland 2002).

Above ground, PCNs symptoms are difficult to detect; they include poor growth, yellowing foliage, and wilting, while below ground the most common symptoms are a reduced size of tubers and excessively branched roots, which require the plant to be lifted for a visual assessment (Europe 2017). PCNs are obligated parasites. Juveniles emerge from the eggs, after having moulded within them, as a response to a stimulation from hosting plant exudates. Cysts, enlarged female body, provide shelter for hundreds of eggs which can remain dormant for decades before hatching and let juveniles migrate through the soil and invade the host root in the elongation zone, behind the root tip. PCN infestation is related to many biotic and abiotic factors but their reproductive potential still remains a key factor since number of eggs can be as big as $1500g^{-1}$ of soil in the most extreme situation (Moxnes and Hausken 2007).

During the last decades, the control of these phytoparasites has mainly relied on chemical treatments, such as fumigants (D'Addabbo *et al.* 2014). Among these, the well-known fumigant methyl bromide, banned in the EU because of several health and environmental issues. Nowadays, public concern for long-term risks linked to the prolonged exposure of agrochemicals have generated a new interest toward alternative pest control (Ghorbani *et al.* 2008).

4.3.1.2 Materials and methods

Previous studies (Oka *et al.* 2001, 2006) have shown interesting nematicide activities of *D. viscosa* leaves extracts against *Meloidogyne javanica*. As a first step to confirm previous findings even with other targets and put the bases for further investigations, a preliminary trial was set in order to evaluate its activity against PCNs. This trial was carried out in the lab of the *Crop and Environment Research Center* (CERC) at the Harper Adams University. First of all, 5g of finely ground leaves of *D. viscosa* were soaked with 200ml of DDI water and left over 48h on stirring plate before vacuum filtration with Fisherbrand® QT280 filter paper. Water was removed by freeze dryer unit and yield collected and weighted, recorded as 0.9557g. Thereafter, 7 stock solutions at different concentration were prepared (Table 11), weighting the sample and adding DDI water accordingly.

Table 11 - Evaluation of nematicidal activity of D. viscosa. Scheme of the tested stock solutions (treatments)

Plant specie	plant material	Solvent	Treatment	Concentration [g/ml]
D. viscosa	leaves	DDI water	T1	0.0400
			T2	0.0200
			T3	0.0100
			T4	0.0050
			T5	0.0025
			T0	0.0000

Secondly, cysts were separated from a soil sample, scrolling them on a paper sheet (Figure 89), based on the assumption that cysts, having a round shape, are more likely to roll over an inclined plane than the irregular soil particles; thereafter, cysts were visually selected under the microscope and collected one by one with tweezers (Figure 90).

Figure 89 - Soil sample including cysts

Figure 90 - Detailed view of cysts through 20x microscope lens

In order to hydrate the collected cysts, they were laid down in small tubes with a dense mesh on the bottom, to keep them in, and placed in separate wells of a 24-well plate poured with tap water (Figure 91). Lids were sealed with Parafilm and the dishes were incubated for 3 days at 20°C in a growth chamber.
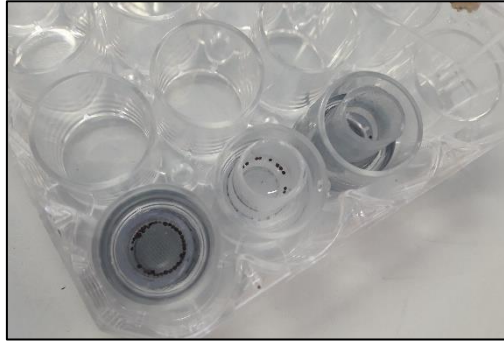
Figure 91 - Detail of cysts in water

After hydration of cysts, and eggs within them, the hatching-stimulation phase took place: water was replaced with diluted root potato leachate (RPL) in 1:4 (v/v), PRL:H_2O ratio and incubated. Dilution was previously prepared from filtered PRL of a second-stage potato seedling, bred in a pot with a collector of percolation at the bottom. PRL contains many hatching factors in it (Byrne *et al.* 1998), so that the exposure of eggs was kept for 10 days, considering as an average between *G. rostochiensis* and *G. pallida* hatching time (Devine and Jones 2001).

On the eleventh day after immersion on PRL, hatched nematodes were dispersed in the liquid, which was pipetted into a plastic tube, added with water up to reach 50ml. In order to estimate the number of nematodes dispersed, 5 times 10µl were took and spotted in a different region of a counting plate, then nematodes were counted under the microscope and recorded values were add up (i.e. $0 + 0 + 0 + 1 + 1 = 2$), so mean was calculated (i.e. $2 / 5 = 0.4$) and proportion applied to find out the approximate number of nematodes dispersed in the entire volume of 50ml ($0.4 / 10µl = X / 50000µl = (0.4 \times 50000) / 10 = 2000$ nematodes in 50ml of water). At this point, enough nematodes were available to start the trial which consisted in 5 treatments (T1:T5) (Table 11) plus Control (T0), with approximately 50 nematodes per unit, and 4 replicates.

Table 12 - Evaluation of nematicidal activity of D. viscosa. Distribution of the treatments within the replicates.

Rep. 1	Rep. 2	Rep 3	Rep. 4
R1T2	R2T0	R3T2	R4T1
R1T4	R2T1	R3T5	R4T4
R1T0	R2T3	R3T0	R4T3
R1T1	R2T2	R3T3	R4T5
R1T3	R2T4	R3T4	R4T2
R1T5	R2T5	R3T1	R4T0

The visual assessment of mortality took place at day 1, 2, 3 and 7 (Figure 92 and Figure 93).

Figure 92 - *Detail of visual assessment: counting plate under the microscope*

Figure 93 - *Juvenile-stage nematode*

The experiment consisted in placing 1ml from 50ml of water with dispersed nematodes in each counting plate with 1ml more of the appropriate treatment, which halved the prepared concentration (Table 13).

Table 13 - Evaluation of nematicidal activity of D. viscosa. Final tested concentrations (treatments)

Treatment	concentration [g/ml]
T1	0.0200
T2	0.0100
T3	0.00500
T4	0.00250
T5	0.00125
T0	0.0000

Visual assessment was performed after 24h, 48h, 72h and 7 days from the application of the treatments, when the number of dead and alive nematodes was recorded.

Unfortunately, due to the amount of dust in treatment T1, a proper assessment was impossible, so that the whole treatment was removed at the very beginning of the trial but left in place as shown in Table 12.

The number of dead (D) and alive (A) nematodes surveyed during the visual assessment were recorded and mortality index (MI), calculated as follow:

MI = D/p   (1), where:

D = number of dead nematodes detected;

p = entire population within the same counting plate.

4.3.1.3 Statistical analysis

Statistical analyses were carried out by mean of SPSS software (IBM Corp. Released 2016. IBM SPSS Statistics for Windows, Version 24.0. Armonk, NY: IBM Corp).

Analysis of variance (ANOVA) was performed in order to evaluate if any difference occurred between the treatments applied. Values of MI were transformed (Steel and Torrie 1980) to ensure the normal distribution according to the formula:

Y' = $log(Y + 1)$ (3), where:

Y' = transformed data

Y = original data.

The assumption of normal distribution was tested by Shapiro Wilk's test (p≤0.05)(Shapiro and Wilk 1965; Razali and Wah 2011) and by visual inspection of the histograms, normal Q-Q Plot and box plots, showing that after data transformation the residuals of the applied model were approximately normally distributed.

4.3.1.4 Results and discussion

Table 14 reports the outcome from the application of ANOVA (GLM) on the mortality index (MI) computed from dead and alive nematodes recorded during the observations. Five treatments were included in the model, i.e. 4 water extracts (from 21 - highest concentration to T5 - lowest concentration) of *D. viscosa*, plus the untreated control (T0).

Table 14 - Results of the ANOVA (General Linear Model) performed on the Mortality Index (MI) counted along the 7-days trial in all treatments.

Source of variation	Df	F
Treatments (T)	4	2.505*
Day (d)	3	1.519 [n.s.]
T x d	12	1.386 [n.s.]
Error	60	
Total	80	

Significance of F values: ***= P ⩽ 0.001; ** = P ⩽ 0.01; * = P ⩽ 0.05; n.s. = not significant

Even if this experiment was born as a preliminary trial to better understand if any further effort was worth to be done and, in that case, which settings should have been adjusted to carry out a more specific and quantitative one, some encouraging results came out. In fact, differences between the treatments, noticed during the observations, were confirmed by the GLM analysis. How it is noticeable on Figure 94 the best result was obtained at day 7 on T2; anyway, this preeminence was not confirmed by statistical analysis, and the lack of significance of the T x d interaction clearly demonstrates that the effect of treatments did not vary along the experiment. Interestingly, however, a significant effect of treatments showed up, and subsequent observation of data allowed to separate T2 and T5 treatments. The stronger effect of T2 compared to the other treatments is also visible on

Figure 95 which takes into account the mean values of all samples treated. Although, many interesting starting points came out from these results, yet the effects were far weaker from previous findings (Oka *et al.* 2001) on nematicide activity of *D. viscosa* water extracts. A few reasons may underlie, like the different target nematode, which could be less tolerant to the active compound and/or the different local pedoclimatic features, where the plant material was collected, which could have influenced the metabolic profile of the donor plants. Anyway, one more remark can be done about the solvent (water) of the extraction process, which, as it is well known, solubilize compounds with a strong polarity that might not be the case of those with a stronger activity, since the lipophilic compounds are basically avoided. Looking at the follow-up of this experiment, by taking into account the feasibility of any possible future application, it seems logical to take two possible directions, the first, could be to move on to a new target, which could be more sensible to the water extract, the second one, and probably the most acceptable, would be to focus to the main components of the metabolites profile of *D. viscosa*, such as costic/isocostic acid, carabrone and tomentosin and testing them both separately and in blends at various ratios in order to see if any interaction or synergy effects between them take place. Moreover, testing pure compounds helps to better quantify the minimum amount needed to kill the target as well as to define the minimum inhibition concentration (MIC).
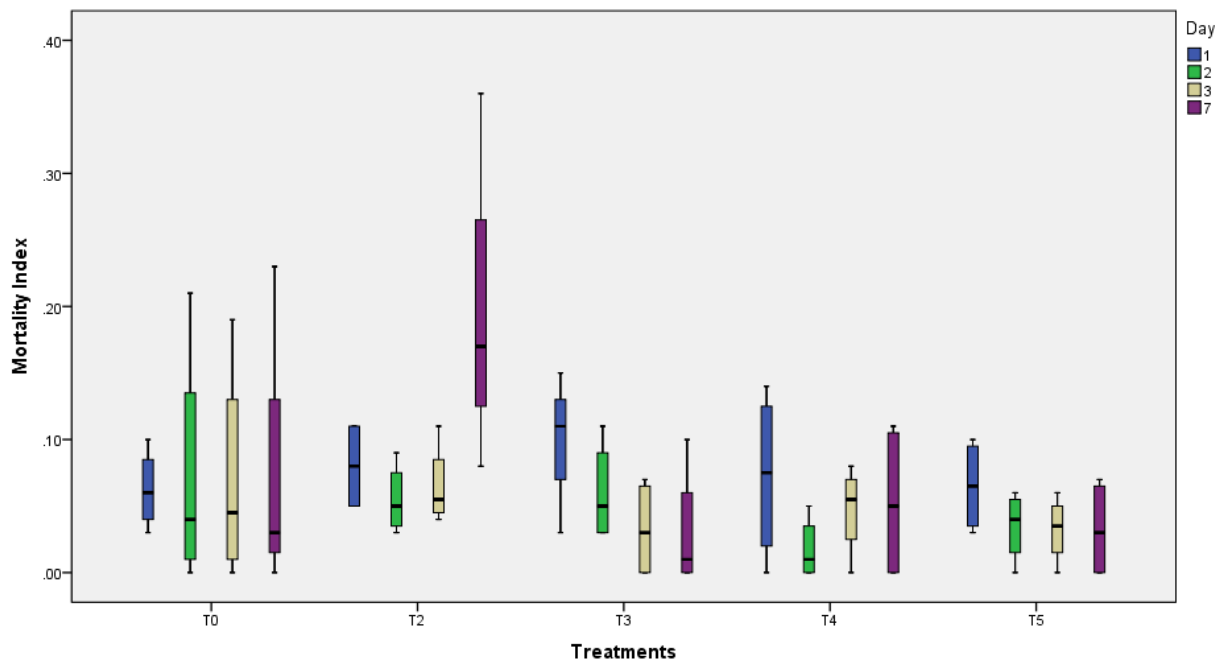
Figure 94 - marginal mean distribution, clustered by day of observation
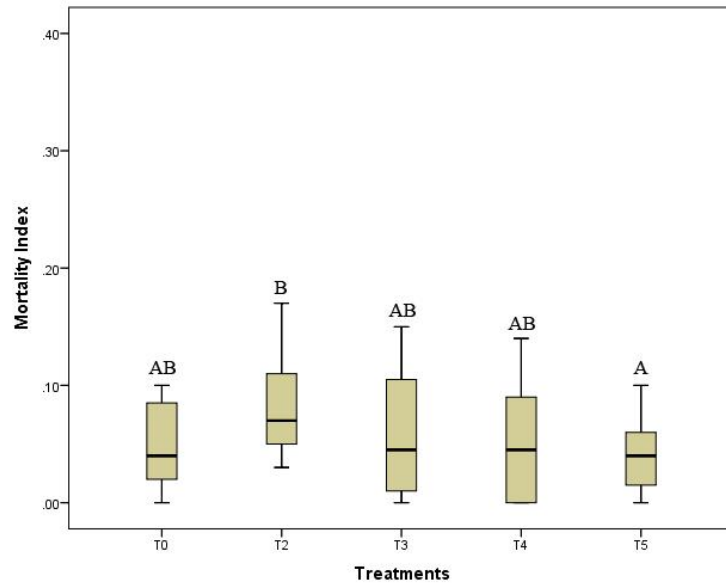
Figure 95 - Mean values of Mortality Index (MI) of all observations recorded from samples treated with D. viscosa at different concentrations. F(4,60)= 2.505*. Error bars represent standard deviation. Treatments marked with * are significantly different from the control at P ⩽0.05 (Dunnett's test)

5 Conclusions

This work, carried out over the past three years of the doctoral experience, was aimed to give a contribution to the enhancement of scientific knowledge about the biocidal potential of some wild Mediterranean plant species. A search in the worldwide literature about the use of plant-derived material for pest control shows that this research topic is usually explored through two different approaches. The first one mostly looks at detecting the single active molecules responsible for each demonstrated action, with the ultimate goal to synthetize them for a large-scale production. The separation of each component usually requires a large use of solvents - which is not really an "eco-friendly" procedure. New and more sustainable extraction methods, such as supercritical fluid extraction, are currently developing, but they are still expensive alternatives so far, and yet modelling is not that accurate if compared to the old but established one. The second approach takes into consideration the whole phyto-complex, i.e. the complex mixture of metabolites that may be found in plants as a consequence of the interaction between plants and their growth environment. Both views have pros and cons: while the first one moves from well-defined protocols and schemes to get fast results, the second approach takes in a greater consideration the occurrence of interactions - positive and negative - among the single chemical compounds. It is easy to argue that in this second case the final result, as it often happens in nature, may be far different from the simple addition of the effects achieved by each single detected compound. This higher complexity, and a consequent more troublesome interpretation of the research outcomes, is probably the major reason why this second approach to the studies about plant-derived extracts is much less used than the former one.

In this doctoral work several specific trials were performed, with the purpose to explore the activity of the selected plants towards different targets, also elucidating, when possible, the mechanisms underlying each specific action. A high species-specificity of the biocidal effects has emerged, since the aforementioned effects proved to be highly variable, both in direction and in intensity, according to the donor-target combination. As it was possible to understand from the various outcomes of the experimental activities, *A. arborescens* revealed an appreciable phytotoxic potential, which has been attributed to two of its secondary metabolites, Sesamin and Ashantin, that despite being found in many species of the genus *Artemisia*, haven't been tested before for their phytotoxic activity. Quite similar observations could be done about *D. viscosa,* which showed such a great effectiveness against a wide range of pathogenic fungi that it is hard to believe that there are no derived products already commercialized. Fungitoxic activity of *D. viscosa* is mainly ascribable to three of its secondary metabolites, Ashantin, Isocostic acid, and Tomentosin. As a remarkable breakthrough, raw powder, obtained from dried and finely ground aerial part of *D. viscosa*, appeared to have a good chance to be

a low-cost, ready-to-use, abundant and alternative solution for fruits and vegetables protection, especially in those cases where the use of chemicals is banned, such as organic farming. Slightly different is the case of its nematicide activity, which, differently from the above, may be better referred to the pure active compounds, to be thought as a potential alternative to chemicals on large-scale farming systems such as those including potato fields. Totally different conclusions came out from *R. coriaria* which, despite encouraging preliminary results about inhibition of seeds germination, did not confirm any substantial biocidal activity against targeted organisms. Although surely not exhaustive, this study has given a contribution to the enhancement of the scientific knowledge about the possibility to use plant-derived products for agricultural pest management. Of course, this research field of study is huge, and many efforts are further required before practical utilization of these compounds.

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7 Attachments

	Publication	**Editor**
I	Herbicidal potential of some water plant extracts	*International Congress of Aromatic and Medicinal Plants (Cipam) 2016*
II	Herbicidal potential of aqueous extracts from *Melia azedarach* L., *Artemisia arborescens* L., *Rhus coriaria* L. and *Lantana camara* L.	*Allelopathy journal 2017*
III	Quality Characteristics of Wholemeal Flour and Bread from Durum Wheat (*Triticum turgidum* L subsp. *durum* Desf.) after Field Treatment with Plant Water Extracts	*Journal of Food Science 2016*
IV	Phytotoxic Lignans from *Artemisia arborescens*	*Natural Product Communications 2018*

P227. Herbicidal potential of some water plant extracts

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Keywords: *allelopathy, bio-herbicides, crude extracts, weed management, Mediterranean plants*

Since pesticides have been introduced in agriculture, many environmental and human negative aspects have been observed, such as development of pest-resistant ecotypes, detrimental impacts on non-target organisms, environmental pollution and toxicity related to health hazards in humans and livestock. New policies are now moving to a safer environment-based and sustainable agricultural production. In response to this tendency, studies are focusing on natural compounds useful for crop protection, starting from essential oils and crude extracts of aromatic and medicinal plants. This issue is especially important for weed management, and a great interest is arising about the potential of such plants in controlling weed growth and development by means of allelopathic mechanism. Allelopathy is referred to any process, involving secondary metabolites released by plants, that influences the growth and/or development of biological systems in their nearby [1]. This work reports the results obtained from a preliminary evaluation of some plant water extracts (*Artemisia arborescens* (Vaill.) L., *Lantana camara* L., *Melia azedarach* L. and *Rhus coriaria* L.), alone and in 50% mixtures, in inhibiting germination and growth of several target species commonly found in Mediterranean agroecosystems: *Eruca sativa* Mill. and *Brassica napus* L. (crop species), *Araujia sericifera* Brot. and *Plantago psyllium* non auct. L., sensu L. (classed as weeds). Seed germination rate and velocity were measured in each treatment, along with the length variations of root and plumule of germinated seeds. The water extracts have shown in almost all experimental conditions an inhibitory effect of seed germination, but this effect was largely dependent on the combination target species-extract. In general, *B. napus* and *P. psyllium* showed the highest sensitivity to the treatments, so that three extracts on four (*A. arborescens, R. coriaria* and *M. azedarach*) totally inhibited seeds germination. The use of mixtures did not evidence synergistic effects compared to the pure extracts, and in one case (*P. psyllium*) they even exerted an overall enhancement of germination. Plant extracts showed high potentiality as a tool for environmentally friendly weed management strategies, to suggest in all cases when the recourse to chemicals is limited.

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Herbicidal potential of aqueous extracts from *Melia azedarach* L., *Artemisia arborescens* L., *Rhus coriaria* L. and *Lantana camara* L.

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ABSTRACT

In the search for new strategies for weed management in agricultural systems, a great interest is to use the plant extracts to replace or integrate, chemical weed control. Two experiments were done to test the effects of plant water extracts from Chinaberry (*Melia azedarach* L.), Tree Wormwood (*Artemisia arborescens* (Vaill.) L.), Sicilian Sumac (*Rhus coriaria* L.) and Lantana (*Lantana camara* L.) on seed germination of Rocket (*Eruca sativa* Mill.), Rapeseed (*Brassica napus* L.), Bladderflower (*Araujia sericifera* Brot.) and Psyllium (*Plantago psyllium* L). The water extracts (pure and 50% mixtures) from the donor species were applied on seeds of recipient plants. In second experiment in pots, these pure extracts were applied to test effects on the seed germination and seedlings growth of Rocket (*E. sativa*). The extracts inhibited the seed germination and this was dependent on the combined extracts of donor plants and target seeds. In the *in vitro* assay, pure extracts were more inhibitory than mixtures.

Key words: *Araujia sericifera*, *Artemisia arborescens*, bioherbicides, *Brassica napus*, crude extracts, *Eruca sativa*, *Lantana camara*, Mediterranean plants, *Melia azedarach*, *Plantago psyllium*, *Rhus coriaria*, seed germination, seedling growth, weed management.

INTRODUCTION

Worldwide, the weeds cause huge losses in crops yields (14). Presently, the most widespread method for weeds control is by synthetic herbicides. These have caused many problems viz., development of herbicide-resistant weeds ecotypes, detrimental impacts on non-target organisms, environmental pollution and toxicity related to health hazards in humans and livestock (15,26). However, the new agricultural production strategies are oriented to organic cropping, where the use of chemicals is not permitted (10,19). Hence, new policies are aiming to develop safer environment-based and sustainable agricultural production, that includes new methods for weed management and investigate the allelopathic potential of plant species for weed management (2,7). Allelopathy is a complex biological phenomenon, which involves release of secondary metabolites from plants, which influence the growth and/or development of plants in their vicinity (7,16,17). In a classical sense, allelopathy refers to *in vivo* plant-plant interactions that occur through specific chemical compounds (allelochemicals) produced and released by plants, both in natural and agricultural ecosystems. However, recently allelopathy definition has been

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Quality Characteristics of Wholemeal Flour and Bread from Durum Wheat (*Triticum turgidum* L subsp. *durum* Desf.) after Field Treatment with Plant Water Extracts

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Abstract: The use of selected plant water extracts to control pests and weeds is gaining growing attention in organic and sustainable agriculture, but the effects that such extracts may exert on the quality aspects of durum wheat are still unexplored. In 2014, 5 plant water extracts *(Artemisia arborescens, Euphorbia characias, Rhus coriaria, Thymus vulgaris, Lantana camara)* were prepared and distributed on durum wheat cv Valbelice to evaluate their potential herbicidal effects. After crop harvesting, the major physicochemical and technological parameters of wholemeal flours obtained from each treatment were measured and compared with those from chemical weeding and untreated controls. A baking test was also performed to evaluate the breadmaking quality. In wholemeal flours obtained after the treatment with plant extracts protein and dry gluten content were higher than in control and chemical weeding. Wholemeal flours obtained after chemical weeding reached the highest Mixograph parameters, and that from durum wheat treated with *R. coriaria* extract demonstrated a very high α-amylase activity.

We concluded that the treatments with plant water extracts may influence many quality traits of durum wheat. This occurrence must be taken into account in overall decisions concerning the use of plant extracts in pest and weed management practice.

Keywords: bread, cereal quality, Maillard reaction, phytochemicals, wholemeal flour

Practical Application: The results of this work may be useful for a proper use of natural products for weed management, in the frame of sustainable and organic cropping techniques.

Introduction

Growing knowledge on the environmental and health risks linked to the widespread use of synthetically derived products for pests and weed management has caused a general change in the behavior of many farmers, who are increasingly utilizing sustainable synthetic products or making environmentally friendly technical choices (Vyvyan 2002; Özçatalbaş 2014). In fact, in many countries (for example, in the European Union, where this topic is ruled by the EU Reg. 834/2007), the only allowed products for organic production are natural inorganic or plant-derived materials (Isman 2006). As a general rule, these products have a shorter shelf-life in comparison with the analogous synthetic products, as they show poor chemical stability to air, light, moisture, and high temperatures (Kühne 2008; Flamini 2012). Such a trait is typical of natural products. Furthermore, although there is no certainty that a "natural" product is also a "safe" product, they are not expected to pose a hazard to non-target organisms (including humans) or to the environment in most cases (Duke and others 2010). Hence,

the active compounds that are contained inside some plants seem to ensure compliance with the safety and low–persistence requirements that are so strongly advocated for. Currently, intense experimental activity is performed all over the world to identify plant products that meet strict safety requirements and have high efficacy (Isman 2006; Isman and Grieneisen 2014). Of course, plant extracts have a complex chemical nature, as they are composed of many different compounds whose presence in the final item may vary greatly according to the extraction method, the conditions of the starting plant material and so on (Verpoorte 1998). Obviously, to ascertain which specific compound is actually responsible for a given biological activity has great practical interest, and this approach has led to interesting results in a number of cases (Rates 2001; Copping and Duke 2007; Li and Vederas 2009; Duke and others 2010). However, in many cases, the search for a unique active product has led to the frustrating result that the effects shown for the whole plant extract were significantly different from its individual components. The occurrence of synergistic phenomena was advocated to justify this outcome. As such, a number of natural extracts are produced and used "as they are," by considering them as unique products mostly identified with their botanical source, rather than as the complex mixture that they actually are (Verpoorte 1998; Rates 2001; Copping and Duke 2007; Li and Vederas 2009; Appendino and Pollastro 2010). Of course, further improvement in the technical procedures for plant extraction, purification and analysis will allow some remediation for this problem.

Phytotoxic Lignans from *Artemisia arborescens*

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A systematic bioassay-guided fractionation of methylene chloride extracts of the aerial part of *Artemisia arborescens* was performed in order to identify its phytotoxic compounds. Two lignans were isolated, sesamin and ashantin, that inhibited growth of *Agrostis stolonifera* (bentgrass), a monocot, and *Lactuca sativa* (lettuce), a dicot, at 1 mg mL^{-1}. In a dose-response screening of these lignans for growth inhibition against *Lemna paucicostata* (duckweed), ashantin was the most active with an IC$_{50}$ of ca. 224 µM. The mode of action of these compounds is still unknown. In mosquito larvicidal bioassays the pure compounds sesamin and ashantin did not cause mortality at the highest dose of 125 mg/L against 1-d-old *Aedes aegypti* larvae. In bioautography bioassays for antifungal activity using *Botrytis cinerea, Fusarium oxysporum, Colletotrichum fragariae, Colletotrichum acutatum,* and *Colletotrichum gloeosporioides*, ashantin and sesamin were inactive at 5 µg and were therefore not subjected to additional screening in secondary antifungal assays.

Keywords: *Artemisia arborescens*, Allelopathy, Herbicidal, Phytotoxicity, Lactone, Lignans, Ashantin, Sesamin.

Artemisia are the most numerous species within the *Asteraceae* (*Compositae*) family [1, 2]. *Artemisia* includes more than 500 annual, biennial and perennial species which are herbs or small shrubs, mainly distributed in the temperate zones of the Northern hemisphere [3-5]. Known also as silver sage, large wormwood, tree wormwood and other common names, *Artemisia arborescens* (Vaill.) L. is a typical species of the Mediterranean wild flora which usually grows in full sun exposure and is very tolerant of heat and drought conditions. It is a perennial shrub from 1 to 2 m tall, with silver grey-green, deeply divided leaves and clusters of inconspicuous yellow flowers that appear throughout late spring until the summer, depending on the environmental conditions [6].

Secondary compounds of *A. arborescens* have antimicrobial, antiviral, pharmaceutical, insecticidal, and insect repellent activity [*e.g*., 7-12]. Relatively little research has been done on determination of phytotoxic compounds from *A. arborescens*. *A. arborescens* has been reported to produce phytotoxic compounds [13], but the exact compounds have not been reported. Only the identity of twenty compounds in the most phytotoxic fraction (*n*-hexane) of extracts of the plants shoots was provided. The phytotoxicity of any of the constituents alone was not provided. This was also the case in a study of the phytotoxicity of extracts of *A. arborescens* leaf litter by the same group [14]. No phytotoxicity bioassay-guided isolation of compounds from this species has been done that fractionates the activity to single compounds. This type of study has the potential to lead to the discovery of new compounds, and the genus *Artemisia* is known to have species-specific phytotoxins (*e.g*., artemisinin [15]) that would not be found by using only GC/MS or LC/MS to identify known compounds. The objective of this study was to find the most potent phytotoxins in *A. arborescens* with bioassay-guided isolation down to the single compound level.

Most of the fractions of the fractionation scheme had little phytotoxicity to lettuce or bentgrass. Fractions 3 and 8 had the strongest inhibition of bentgrass growth, while little effect was observed on lettuce. These fractions were found to be the lignans sesamin and ashantin (Figure 1), respectively. These purified compounds were assayed with a more quantitative bioassay with duckweed (Figures 2 and 3), yielding, IC$_{50}$ values of ca. 401 and 224 µM for sesamin and ashantin, respectively. With this bioassay, these IC$_{50}$ values are in the same range as those for the commercial herbicides naptalam (128 µM), glyphosate (388 µM), and clomazone (126 µM) [16].

Sesamin has been previously reported in *A. arborescens* [13, 14, 17-19] and ashantin has also been found in *A. arborescens* [17, 18]. Both compounds are found in several *Artemisia* species [17]. Araniti *et al*. [13, 14] reported sesamin in a phytotoxic hexane extract, but did not test its phytotoxicity alone. Sesamin has been previously reported as a phytotoxin. Tonelli *et al*. [18] reported sesamin and another lignan, kobusin, to be in a more phytotoxic fraction of *Virola sebifera* against lettuce. This conflicts with our finding of little activity of sesamin against lettuce, but their assay was done with a fraction containing both lignans, and there was no indication of what the concentration of sesamin was in their extract. We find no previous mention of ashantin as a phytotoxin.

Some other lignans are known to be phytotoxic. For example, the aryltetralin lignans podophyllotoxin, α-peltatin, and β-peltatin are active against both dicotyledonous and monocotyledonous plants by interfering with formation of mitotic microtubular organizing centers [20]. Ten lignans from *Helianthus annuus* were reported to be phytotoxic [21]. The results reported in the present paper add to what is known of the phytotoxicity of lignans. Ashantin and sesamin showed good phytotoxic activity against bentgrass; further investigations are necessary about their mode of action, along with additional assays against monocot weeds.