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Allelopathic effects of *Cynara cardunculus* L. extracts

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Ai miei genitori,
fonte inesauribile di sostegno e supporto

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Abstract

The present doctoral thesis aims to explore the potential use of Cynara cardunculus *L. leaf extracts for the biological control of weeds and pathogen microorganisms. In a first trial, the allelopathic effects of its leaf aqueous extracts on seed germination of six common weeds was demonstrated. Secondly, the set-up of the most efficient extraction method of its allelochemicals in terms of costs, yields and inhibitory activity was realized, selecting dried leaves as the best plant material and ethanol and ethyl acetate as the best solvents. Moreover, new C. cardunculus allelochemicals (cynaratriol, desacylcynaropicrin, 11,13-dihydro-desacylcynaropicrin and pinoresinol) were purified. Third, the effect of genotype, harvest time and light stress on the phytotoxicity, quantity and composition of sesquiterpene lactones in* C. cardunculus *leaf extracts was evaluated through a new UHPLC-MS/MS analysis method. Wild and cultivated cardoon showed the highest concentrations, while the April harvest revealed the best harvest time. Moreover, light stress stimulated the production of these allelochemicals. In a second trial, the effects resulting from three consecutive years of cultivation, in two different areas, with globe artichoke, cultivated and wild cardoon on the quali/quantitative weed soil seed bank and the changes in the eubacterial communities were studied. In both areas,* C. cardunculus *reduced weed seed bank in all treatments compared to controls. Nevertheless, the presence of cultivated cardoon had a negative influence towards* Bacillus subtilis *and a positive one on* Pseudomonas putida *and* Azospirillum brasilense*. Lastly, cultivated cardoon leaf extracts were assessed in vitro for the control of several*

microorganisms of agriculture and food interest. All the extracts showed an important antimicrobial activity.

Sommario

La presente tesi di dottorato si propone di esplorare l'uso potenziale di estratti fogliari di Cynara cardunculus *L. per il controllo biologico di malerbe e microrganismi patogeni. In una prima prova, sono stati valutati gli effetti allelopatici degli estratti acquosi fogliari sulla germinazione dei semi di sei piante infestanti. Successivamente, è stata realizzata la messa a punto del metodo di estrazione di allelochimici più efficiente in termini di costi, rese e attività inibitoria, individuando nelle foglie essiccate il migliore materiale vegetale e nell'etanolo e nell'acetato di etile i più efficaci solventi. Inoltre, sono stati purificati nuovi allelochimici di* C. cardunculus *(cinaratriolo, desacilcinaropicrina e 11,13-diidro-desacilcinaropicrina). Attraverso un nuovo metodo di analisi UHPLC-MS/MS, è stato altresì valutato l'effetto del genotipo, dell'epoca di raccolta e dello stress luminoso sulla fitotossicità, quantità e composizione dei sesquiterpeni lattonici negli estratti fogliari di* C. cardunculus. *Il cardo selvatico e coltivato hanno mostrato le maggiori concentrazioni, aprile si è rivelata la migliore epoca di raccolta e lo stress luminoso ha stimolato la produzione degli allelochimici. In una seconda prova, sono stati studiati gli effetti derivanti da tre anni consecutivi di coltivazione, in due aree diverse, di carciofo, cardo coltivato e selvatico sulla composizione quali/quantitativa della banca semi del terreno e i cambiamenti nelle comunità eubatteriche. In entrambe le aree,* C. cardunculus *ha ridotto la banca semi in tutte le tesi. Nondimeno, la presenza di cardo coltivato ha avuto*

un'influenza negativa sul Bacillus subtilis *e una positiva su* Pseudomonas putida *e* Azospirillum brasilense. *Infine, gli estratti fogliari di cardo coltivato sono stati valutati* in vitro *per il controllo di microrganismi di interesse agricolo e alimentare, mostrando un'importante attività antimicrobica.*

1. Allelopathy: Principles and Basic Aspects for Agroecosystem Control

This introduction has already been published in the following book chapter:

- Scavo, A., Restuccia, A., & Mauromicale, G. (2018a). Allelopathy: principles and basic aspects for agroecosystem control. In: Gaba S., Smith B., Lichtfouse E. (eds.) *Sustainable Agriculture Reviews 28*. Sustainable Agriculture Reviews, Springer, Cham, vol 28, pp 47–101.

1.1 Introduction

Allelopathy is an ecological phenomenon of most natural communities and agroecosystems, although it is often unrecognized. It has been observed how orchard replant problems, regeneration of forest species, occurrence of weed-free zones, dominance of exotic plants, spatial vegetation patterns, dynamics of communities, plant productivity and other ecological aspects are strictly linked to allelopathic mechanisms. Root exudates, upon release into the rhizosphere, also play an important role on soil microbial ecology, nutrients biogeochemical cycles and their uptake by plants. One of the most important examples is provided by the improvement of nitrogen use efficiency (NUE) through biological nitrification inhibition (BNI) made by *Nitrosomonas* spp. and *Nitrobacter* spp.

Modern agriculture has to deal with a rapid increase in world population, accompanied by a simultaneous decrease of the available resources. Therefore, in order to feed a growing population, agriculture has pursued the maximization of

yields. Cropping intensity resulted in a subsequent higher pest pressure on crops. Particularly, in order to eliminate the presence of weeds, insects and pathogens, synthetic chemicals were used indiscriminately. The wide use of herbicides, for example, resulted in increasing incidence of resistance in weeds to common herbicides, and in environmental pollution and human and animal health. Many of these problems may be effectively resolved through the manipulation of allelopathic mechanisms and their integration to traditional agricultural practices under Integrated Pest and Weed Management System (IPMS, IWMS). The most important modes by which allelopathy can be used in agroecosystems for sustainable crop production refers to (1) the selection of smothering crops, their breeding and inclusion in crop rotations; (2) the use of their residues as living mulches, dead mulches or green manure; (3) the selection of most active allelopathic compounds and their use as bioherbicides.

The term "allelopathy" is derived from the Greek words *allelon*, "of each other", and *pathos*, "to suffer" and literally means "*the injurious effect of one upon another*" (Rizvi *et al*., 1992). However, the term was coined for the first time by the Austrian plant physiologist Hans Molisch in 1937 in his book *Allelopathie* to include both harmful and beneficial biochemical interactions between all types of plants, including microorganisms.

In his first book, E. L. Rice excluded the beneficial effects, while reconsidered and accepted Molisch's definition in his second monograph: "*any direct or indirect harmful or beneficial effect by one plant (including microorganisms) on another through production of chemical compounds that*

escape into the environment". Both positive or stimulatory and negative or inhibitory effects are included in this definition. Rice's definition has been criticized by many authors because it refers to all types of interactions between plants (Watkinson, 1998). Instead, several other workers prefer to limit the use of the term to recognize only the negative effects, direct or indirect, produced by a plant, which is identify as the "donor" plant, on another plant called "target" or "afflicted" plant. According to the definition given by the International Allelopathy Society (IAS) in 1996, allelopathy includes "*any processes involving secondary metabolites produced by plants, microorganisms, viruses and fungi that influence the growth and the development of agricultural and biological systems (excluding animals), including positive and negative effects*" (Torres *et al*. 1996). Therefore, allelopathy includes plant-plant, plant-microorganisms, microorganisms-plant, microorganisms-microorganisms, plant-insect and plant-higher animal interactions.

1.2 History of allelopathy

The ability of some plant species to interfere with the germination, growth or development of other plant species has been well documented since antiquity, from more than 2000 years ago. The earliest observations on this phenomenon were made by Theophrastus (370–286 BC), a disciple of Aristotle and the father of botany, who around in 300 BC wrote in his two botanical works, *Historia plantarum* and *De causis plantarum*, about how chickpea (*Cicer arietinum* L.) "exhausted" the soil and destroyed weeds. He also reported the harmful effects of cabbage (the cabbage of

Theophrastus refers to something close to the wild form of *Brassica oleracea*, sometimes known as *Brassica cretica*, an edible, but bitter, herb of coastal regions richer in allelochemicals; Willis, 2007) on grapevine and proposed that these effects were caused by "odours" of cabbage plants.
The Greek author Bolos Demokritus (460–360 BC) of Mendes in Egypt, in his agricultural work *Georgics*, written around in 200 BC, suggested that trees may be died by sprinkling their roots with a mixture of lupine flowers soaked in hemlock juice.
Cato the Elder (234–140 BC), the famous Roman politician and writer who was a farmer in youth, and later Pliny the Elder (23–79 after Christ) in *Historia naturalis*, noted that walnut trees (*Juglans* spp.) were toxic to other plants and that both chickpea and barley (*Hordeum vulgare* L.) ruined cornlands (Weir *et al*., 2004). Pliny the Elder has been preceded bay Columella, who was a farmer in Cadiz. In his surviving works (*De rerum rusticarum* of 64 AD and *De arboribus*), Columella was the first who spoke about "soil sickness", described as a decrease in soil's fertility due to the repeated cultivation for more years of the crop on the same land.
In the seventeen century, both the English and Japanese literature shown cases of plants do not grow well in the presence of each other due to the production of toxic compounds, for example the Japanese red pine (*Pinus densiflora* Siebold & Zucc.) (Rice, 1984).
In 1804, the agronomist Young discovered that clover was apt to fail in some regions of England where it is cultivated constantly due to soil sickness, which accrues over time (Weston, 2005).

In 1832, the Swiss botanist De Candolle (1778–1841) proposed that such excretions of roots of some plants could injurious other plants and explain the exhaustion of soil. On the basis of De Candolle's suggestions, in 1881 Stickney and Hoy observed that vegetation under black walnut was very sparse, probably due to the high mineral requirements of the tree.

The interest in the field of allelopathy resumed in the twentieth century, thanks to the development of suitable techniques for the extraction, bioassay and chemical isolation of the involved substances (Willis, 2007). For example, in 1907, Schreiner and Reed for the first time isolated soil organic acids released from root exudates of certain plants that strongly inhibited the growth of some adjacent crops. In 1928, Davis was the first to extract and purify from the hulls and roots of walnut the juglone, 5-hydroxy-α-naphtaquinone (Rice, 1984).

In the period between 1960 and 1990, much progress has been made in the field of chromatography and spectroscopy, for the isolation and determination of the studied chemical compounds.

Whittaker and Feeny, in 1971, coined the term "allelochemicals". Chou and Waller (1983) describe the biochemical interactions between organisms, both inter- and intra-specific, as "allelochemical interactions".

1.3 Current status of allelopathy research

Despite the allelopathic interactions between plants were known since ancient times, the research on this topic has received great focus only on the end of the twentieth century. Specifically, the number of journal papers using the word

"allelopathy", as searched in the database Scopus®, has undergone an exponential growth since the 1970s with a rapid increased in the late 1990s (**Figure 1.1**).

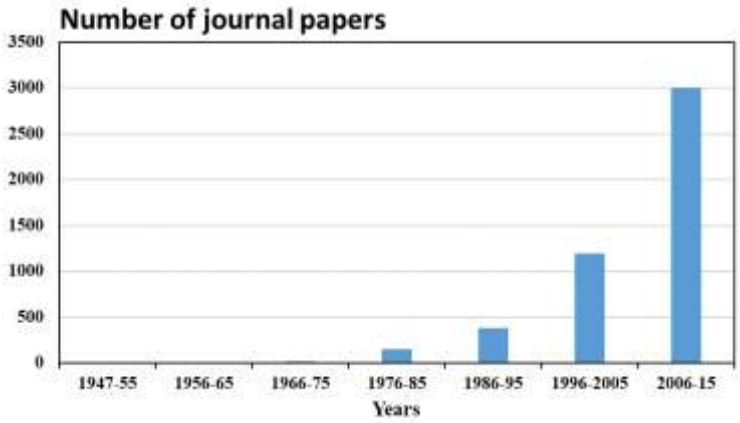


Figure 1.1 Number of journal papers accessed on Scopus® using the search term 'allelopathy or allelochemical' for each decade of the past 70 years. Note the high increase since the 1970s, particularly from the late 1990s.

Originally considered as a sub-discipline of chemical ecology, currently allelopathy embraces a broad range of disciplines: ecology, biochemistry, chemistry, physiology, agronomy, entomology, microbiology, forestry, soil science, proteomics, genetic, etc.

At least two reasons are involved for the slow progress in this area: one is the difficulty of designing field experiments about that unquestionably prove that a chemical produced by a plant directly affects a neighboring plant (Weir *et al*., 2004), the so-called "allelopathy paradigm". Another reason refers to the cases in which important papers on allelopathy have been discounted by later work (Duke, 2010).

However, great progresses have been made in recent years on the study of allelopathic activities and there are numerous examples of high profile researches who brought fame to this discipline. Indeed, in addition to the number of journal papers, it should be considered how changed the target of interested journal. In 1994 was found the *Allelopathy Journal*, the first journal exclusively devoted to allelopathy research; nowadays journals such as the *Journal of Chemical Ecology*, *Plant and Soil*, *Phytochemistry*, *Journal of Agricultural and Food Chemistry*, *Weed Science*, *Weed Research*, *Crop Protection*, etc. publish many papers on allelopathy. The most active country in allelopathy research are United States (with over 1000 works) and China (about 825), followed by India (394) and Japan (372) (source: Scopus®).

The increased interest of scientific community on allelopathy, has mean that in 1995 was founded the International Allelopathy Society (IAS) in India, which has a combined membership of over 1000 participants from over 50 countries. The Society hosts one meeting every three years. The first congress was held in Cadiz (Spain, in 1996) and the subsequent in Thunder Bay (Canada, 1999), Tsukuba (Japan, 2002), Wagga Wagga (Australia, 2005), Saratoga Springs (NY, USA, 2008), Guangzhou (China, 2011), Vigo (Spain, 2014), Marseille (France, 2017).

Several congresses and symposia were made in Europe and Asia and today exist regional organization focused on allelopathy topic, such as the Asian Allelopathy Society (AAS), or the European Allelopathy Society (EAS). Moreover, other scientific organizations, such as the American Chemical Society and the International

Association for Ecology have had allelopathy symposia as part of their programmes (Duke, 2010).

1.4 Terminology and classification

In his first book (1974), Rice reported Grümmer's terminology (1955, **Figure 1.2**) for chemical interactions between plants. Specifically, Grümmer recommended the terms:

(i) "antibiotic" to denote a chemical inhibitor produced by a microorganism that is toxic to other microorganisms;

(ii) Waksman's suggested term "phytoncide" for antimicrobial organic substances of plant origin;

(iii) Gaumann's term "marasmins" for compounds produced by microorganisms and harmful to higher plants;

(iv) the term "kolines" for chemical inhibitors produced by higher plants that are toxic to other higher plants species.

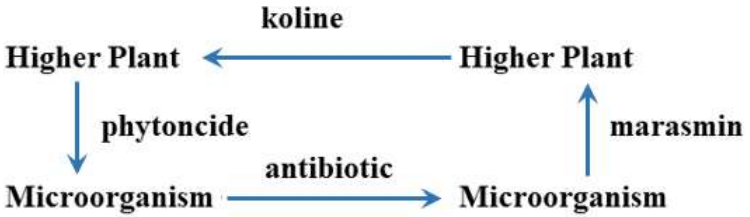


Figure 1.2 Terms for chemical agents that indicate the type of donor and receiver plants, as shown by the arrows (Einhellig, 1995, Modified).

However, these terms are rarely adopted by the authors, because compounds released from higher plants may be altered by microorganisms before the altered substance is contacted by another higher plant and it is very difficult to establish the source when a compound of any origin is

contacted through the soil medium the same compound. Besides, the same compund is likely to have multiple roles (Einhellig, 1995): in fact, there are marasmines that shows harmful effects on other microorganisms, kolines with antimicrobial activity and phytoncides that inhibit the development of plants. For these reasons, nowadays, the generic term "allelochemical" is adopted to denote the chemical compounds produced by plants, microorganisms, viruses and fungi involved in agricultural and biological ecosystems.

With the term "allelopathy" several authors prefer to refer only to detrimental effects, direct or indirect, of higher plants of one species (the donor) on another (the receptor) through the release into the environment of toxic chemical compounds, commonly defined "allelochemicals" (Putnam and Duke, 1978). Therefore, the detrimental effects can be direct or indirect and, about that, Aldrich (1984) describes two types of allelopathy: in the first case, the interference is caused by release into the environment of a compound that is active in the form in which is produced and released from the donor plant; this type is called true allelopathy. In the second case, the negative action is mediated by soil microorganisms that act on the decaying tissues of the donor plant or by enzymes that, after plant tissues destruction, interact with the pre-allelopathic substances by converting them into allelochemicals; this type is called functional allelopathy.

When a given species produces and releases allelochemicals that can cause damage to a different plant species, this phenomenon is called *heterotoxicity*; whereas, when its own germination and development is affected, this allelopathic

effect is called *autotoxicity* or *autoallelopathy* (Chon *et al*., 2006; Kruse *et al*. 2000; Miller, 1996).

1.4.1 Autotoxicity or autoallelopathy

Autotoxicity is an intraspecific form of allelopathy that was first described by Jensen *et al*. (1981) on alfalfa (*Medicago sativa* L.). In different cereals and vegetables crops, it was observed a significant reduction in both crop yield and quality due to the "soil sickness". This phenomenon occurs when the same crop (monoculture) or its relative species are cultivated on the same soil successively (Yu, 1999).

Autotoxicity has been studied mainly in the Cucurbitaceae family, for example in cucumber, melon and watermelon (de Albuquerque *et al*., 2010). Other autotoxic effects have been reported also in asparagus (*Asparagus officinalis* L.) (Hartung *et al*., 1990), rice (*Oryza sativa* L.) (Rice, 1979), grape (*Vitis* sp.) (Brinker and Creasy, 1988), wheat (*Triticum aestivum* L.) (Kimber, 1973; Lohdi *et al*., 1987), etc.

Probably autotoxicity derives from natural selection as result of controlling competition between older and younger plants for resources (e.g. light, water, nutrients, etc.) by maintaining a certain distance from them. In alfalfa, an autotoxic zone of about 20 cm radius exists around the old plants. Autoxicity is now identified as the most frequent cause of reseeding failure (Miller, 1996; Tesar, 1993; Webster *et al*., 1967).

Biotic and abiotic factors not only influence the production of autotoxins but modify their effects too (Yu, 2001). Autotoxicity involves many chemical compounds that are located in different quantities in the plant's tissues. For example, Miller (1996) founded that autotoxic effects of water extracts of plant parts of alfalfa in order are leaf > seed

> root > flower > stem. Generally, leaves present the highest concentration of allelochemicals, but often they are also released as root
exudates. In the soil, their movement is faster in sandy than in clay soils.

Autotoxicity primarily affects the seed germination and early root elongation, but in severe cases it may causes stand failure. Other typical symptoms are dwarfed, spindly, curling, yellowish-green seedlings with irregular brown-reddish to dark-brown lesions on the tap and lateral roots and only few effective nodules (Webster *et al*., 1967). However, as reported by Chon *et al*. (2006), there are ecological advantages linked to autotoxicity. For example, if seeds were not dispersed away from the parent plant leaving the new plant in position to compete for resources, it may encourages geographical distribution of the donor species, serves as an adaptation to induce dormancy and prevents decay of its seeds and propagules (Friedman and Waller, 1983).

Crop rotation is the best solution to avoid soil sickness. In particular, rotation intervals higher than 12-months are suggested because allowed natural decomposition of the chemicals. In light-textured soils, irrigation can leach the autotoxic chemical to shorten the rotation interval (Chon *et al.*, 2006).

Under field conditions, finding evidence of autotoxicity is not a problem of simple solution. In fact, despite many cases of auto-heterotoxicity have been reported in literature in laboratory conditions, autotoxicity is rarely present in natural ecosystems (Jackson and Willemsen, 1976; Wilson and Rice, 1968).

1.4.2 Allelopathy and competition

Allelopathy is a particular form of amensalism, a negative interaction between two species in which one organism is harmed or inhibited and the other is unaffected.

It is necessary to clarify the difference between the concept of allelopathy and competition to avoid misunderstandings. The first implies the release of inhibiting substances into the environment, whereas in the second a generic resource (e.g. water, light, minerals and space) is removed or reduced by another organism sharing the same habitat. In nature is difficult to separate these mechanisms: in fact, stress caused by competition increase the production of allelochemicals, while growth reduction caused by allelochemicals may reduce the competitive ability of inhibited plant. Besides, mechanisms of interference such as microbial nutrient immobilization, soil characteristics, climatic factors etc. can not be separated under field conditions. Therefore, competition and allelopathy undoubtedly interact in a highly synergism (Willis, 1994).

The difficulty in distinguishing and describing separately allelopathic effects from those of competition have induced Muller (1969) to propose the term "interference" to indicate the overall deleterious effects (allelopathy + competition) of one plant on another. According to Harper (1977), competition represents a physical interference, while allelopathy a chemical interference. For over 2000 years, allelopathy has been reported in literature with respect to plant interference (Weston, 2005). Nowadays, interference is a term widely used in the literature to denote "*the total adverse effect that plants exerts on each other when growing*

in a common ecosystem and it includes competition and allelopathy" (Zimdahl, 1999).

Like allelopathy, competition is difficult to demonstrate (Inderjit and Keating, 1999). While plant-plant resource competition has been readily accepted by biologists and ecologists, the same has not be the case for allelopathic interactions. According to Blum (2011), the difference in acceptance between competition and allelopathy was due to: (i) the modification in allelopathy definition over time; (ii) the lack in design bioassays of plant-plant allelopathic interactions; (iii) the forceful scepticism of several authors to plant-plant-allelopathic interactions and (iv) the higher standard of proof required for allelopathic interactions than those for competition.

One of the main challenges in the field of allelopathy is the separation of allelopathic effects from other mechanisms of plant interference, mainly competition (Singh *et al*., 2001). In recent years, several mathematical models were proposed to calculate the contribution of both allelopathy and competition to interference (An *et al*., 1993; Liu *et al*., 2005; etc.).

1.4.3 Establishing the proof of allelopathy

Although community's scientific attentions on allelopathy are continuously growing, its scientific evidence is not yet accepted by all scientists. This because its mechanisms are largely unknown, from production to release and fate of allelochemicals. Its comprehension is further complicated by vastness and chemical heterogeneity of the substances involved (Nelson, 1996). The amount of searches conducted until today on the topic, has induced researchers to assume that it is unusual for a single allelochemical product to be present, in field conditions, in sufficiently high amounts to

exteriorise significant effects (Einhellig, 1996). Besides, it seems that the effects are often caused by different compounds that act together additively or synergistically; but in some cases these compounds react antagonistically (Seigler, 1996). Allelochemicals are introduced into the environment together with a vast number of other compounds, since it is likely that synergistic effects enhance the observed activities (Putnam and Tang, 1986).

Moreover, allelochemicals production and release, their transport and transformation in soil and absorption in the receptor plant, as well as the plant's reaction to the compound, are highly dependent on environmental conditions. In many cases, stress conditions induce the donor plant to produce and release a higher level of allelochemicals and, in poor condition, neighbouring plants become more sensitive to these substances.

Establishing the proof of allelopathy and separating allelopathy from other mechanisms of interference such as competition is very difficult. In his book of 1977, page 494, Harper says: "*Demostrating this [toxicity in the field] has proved extraordinarily difficult- it is logically impossible to prove that it doesn't happen and perhaps nearly impossible to prove absolutely that it does*". Nowadays, the demonstration of allelopathy mechanisms has been achieved by creative experimentation and use of advanced biomolecular analytical techniques (Bais *et al*., 2003; Vivanco *et al*., 2004).

To establishing the cause-and-effect relationship in allelopathy, the following events must occur in sequence (Cheng ,1992):

1. a phytotoxic chemical is produced;

2. the chemical is transported from the producing organisms to the target plant;
3. the target plant is exposed to the chemical in sufficient quantity and for sufficient time to cause damage.

First of all, it is necessary to determinate if a plant is really allelopathic. Several types of clues can indicate if a species is allelopathic (Duke, 2015): (i) if an invasive plant eliminates most native plant species, probably it is allelopathic; (ii) sparse or no vegetation patterning around a particular species can indicate that it is allelopathic, e.g. black walnut; (iii) problems of "soil sickness" are often attributed to buildup of allelochemicals in the soil; (iv) knowledge that a plant species produces one or more potent phytotoxins can be a clue obtained from the phytochemical literature that the species might produce an allelochemical, e.g. the phytotoxic compound sorgoleone produced by all species of *Sorghum* spp.

Secondary, it is necessary to predict if a compound is an allelochemical. In fact, finding a phytotoxic compound in plants does not mean that the compound is necessary an allelochemical. The identification of a substance as allelochimical is actually dependent upon the context rather than on its biosynthetic origin (Berenbaum, 1995). According to Inderjit and Duke (2003), a compound may play several roles in nature, including that of an allelochemical, depending on the

organisms involved and on the specific environmental parameters affecting the organism. Thus, the exact same compound may sometimes be an allelochemical, and at other times or places play other roles. Modes of release, phytotoxic action, bioactive concentration, persistence and fate in the

environment, are all factors influencing the allelopathic nature of a compound, whereby a chemical does not act as an allelochemical in all situations.

Willis (1985) advanced six protocols required to demonstrate allelopathy, based on "Koch's postulates" (Williamson, 1990) for demonstrating that a disease is caused by an infectious agent:

(a) a pattern of inhibition of one species or plant by another must be shown;

(b) he putative aggressor plant must produce a toxin;

(c) here must be a mode of toxin release from the plant into the environment;

(d) there must be toxin transport and/or accumulation in the environment;

(e) the afflicted plant must have some means of toxin uptake;

(f) the observed pattern of inhibition cannot be explained solely by physical factors or other biotic factors, especially competition and herbivory.

As mentioned before, it is extremely difficult or impossible to follow these protocols in field conditions since biotic (e.g., soil microflora, root exudates of competitors) and abiotic (e.g., temperature fluctuations, water stress) factors strongly influence allelochemicals fate.

I consider Macias *et al*. (2007) guideline useful to refuting the cases of "suspected allelopathy":

(1) plant predominance/distribution/frequency cannot be explained solely on the basis of physical/biotic factors;

(2) the allelopathic plants (donors) should synthesise and release into the environment chemicals that must be or become bioactive;

(3) soil permanence and concentrations should be high enough to produce effects on the germination and/or growth of neughbouring plants, bacteria and/or fungi;
(4) uptake by the target plant and evidence of the detrimental/beneficial effects caused by the chemical/s.

1.5 Chemical nature of allelochemicals

According to Reese (1979), allelochemicals are "non-nutritional chemicals produced by one organism (plants, microorganisms, viruses and fungi) that affects the growth, health, behaviour or population biology of other species". Most of them are secondary metabolites (Whittaker and Feeny, 1971) and are produced as offshoots of primary metabolic pathways of carbohydrates, fats and amino acids. As secondary metabolites, they are of sporadic occurrence and do not play an obvious role in the basic metabolisms of organisms but serve for defensive adaptation. However, a significant role in allelopathy is also played by certain primary metabolites (Inderjit, 1999) as several free amino acids and organic acids.
Allelochemicals, even with a few exceptions, have basically four precursors: acetyl coenzyme A, shikimic acid, mevalonic acid and deoxyxylulose phosphate (**Figure 1.3**).
There are many thousands of such compounds, but only a relative limited number of them have been identified as allelochemicals (Rice, 1984). Allelochemicals consist of various chemical families. According to Whittaker and Feeny (1971), they could be classified into five major categories: phenylpropanes, acetogenins, terpenoids, steroids and alkaloids. Apart from the phenylpropanes and alkaloids,

which originate from amino acids, the rest generally originate from acetate.

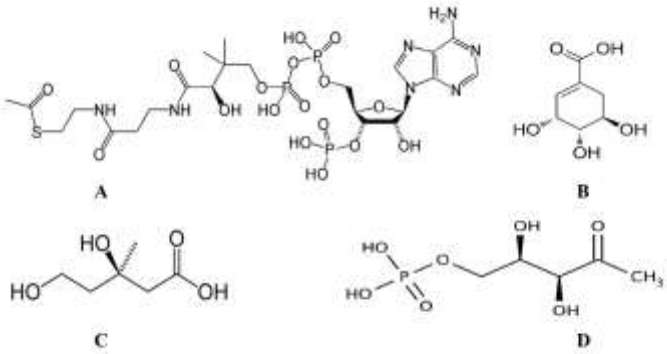


Figure 1.3 Allelochemical precursors: acetyl coenzyme A (**a**), shikimic acid (**b**), mevalonic acid (**c**) and deoxyxylulose phosphate (**d**) structural formula.

Based on the four precursors and on the different structures and properties of these compounds, Rice (1984) classified allelochemicals into 14 chemical classes plus a catchall category (miscellaneous):

a. simple water soluble organic acids, straight chain alcohols, aliphatic aldehydes and ketones;
b. simple unsaturated lactones;
c. long-chain fatty acids and polyacetylenes;
d. naphthoquinones, anthroquinones and complex quinones;
e. simple phenols, benzoic acid and derivatives;
f. cinnamic acid and derivatives;
g. flavonoids;
h. tannins;

i. terpenoids and steroids;
j. amino acids and polypeptides;
k. alkaloids and cyanohydrins;
l. sulphides and glucosides;
m. purines and nucleotides.

Plant growth regulators such as gibberellic acid, ethylene or salicylic acid, are also considered to be allelochemicals. Thanks to the progress of analysis technology in the last decades, it was possible to isolate and identify tens of thousands of allelochemicals and to perform sophisticated structural analysis of these molecules (Cheng and Cheng, 2015).

With a few exceptions, allelochemicals produced by higher plants and microorganisms usually arise through either the acetate or the shikimate pathway, or their chemical skeletons come from a combination of these two origins (**Figure 1.4**). Generally, plant phenolics originate from the shikimate pathway, while terpenoids from the mevalonate pathway, also known as the isoprenoid pathway. Several types of inhibitors, which originates from amino acids, come through the acetate pathway. Most of compounds that cause allelopathy were derived from amino acids, via the shikimate pathway (Rice 1984). Higher plants present two pathways for the formation of C5 terpenoid monomers, isopentenyl diphosphate: (i) the glyceraldehyde-3-phosphate/pyruvate pathway in the plastids, and (ii) the cytoplasmic acetate/mevalonate pathway (Lichtenthaler *et al*., 1997). However, the details of biosynthesis are not always known.

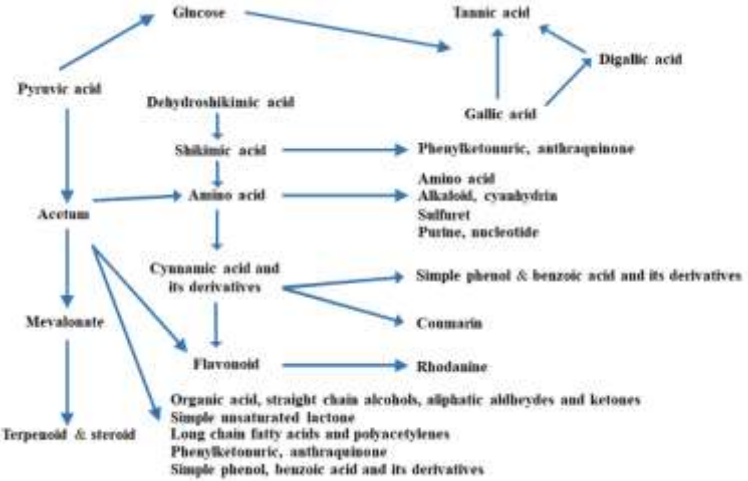


Figure 1.4 Acetate and shikimate pathway, the biosynthetic pathways of major allelopathic substances (Wang *et al*., 2006, Modified).

It is possible to group secondary metabolites into three main chemical classes: phenolic compounds, terpenoids and other compounds.

1.5.1 Phenolic compounds

Phenolic compounds fall within the class of most important and common plant allelochemicals in the ecosystem. As shown in **Figure 1.5**, they arise from shikimate and phenylalanine pathways (Harborne, 1989). Phenolic compounds consisting of a hydroxyl group (-OH) bonded directly to an aromatic hydrocarbon group. In the term "phenolic compound", structures with different degrees of chemical complexity are included, as shown in **Table 1.1**, according to the number of carbon atoms of the basic skeleton.

Structural diversity and intraspecific variability are the most significant characteristics of phenolic compounds (Hartmann, 1996). Besides, they are water soluble and could easily be leached by rain, whereas leaves are still attached to the plant or, thereafter, from leaf litter (Alsaadawi *et al*., 1985).

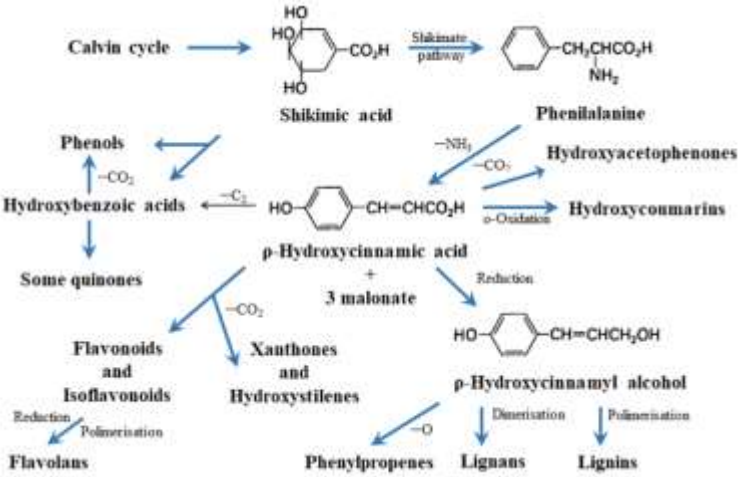


Figure 1.5 Biosynthetic origin of plant phenolics from shikimate and phenylalanine pathways (Harborne 1989, Modified).

1.5.1.1 Simple phenols

Simple phenols are all monomeric, consisting of only one aromatic ring (**Figure 1.6**). Probably, phenol is the precursor of all other phenolic compounds in plants. Schreiner and Reed (1908) reported that vanillin, vanillic acid (a benzoic acid) and hydrokinone are the most general simple phenols with growth-inhibiting allelopathic properties. In particular, hydrokinone is the aglycone of arbutin, and ρ-

hydroxybenzoic acid and vanillic acid are the most commonly identified benzoic acid derivatives involved in allelopathy. Hydrokinone, resorsinol and catechol, which are found in low concentrations in plants, are mostly secreted by insects as a defence mechanism against other insects and animals. Salicylic acid, on the other hand, possesses anaesthetic properties and is the active ingredient of Aspirin® (Pretorius and van der Watt 2011).

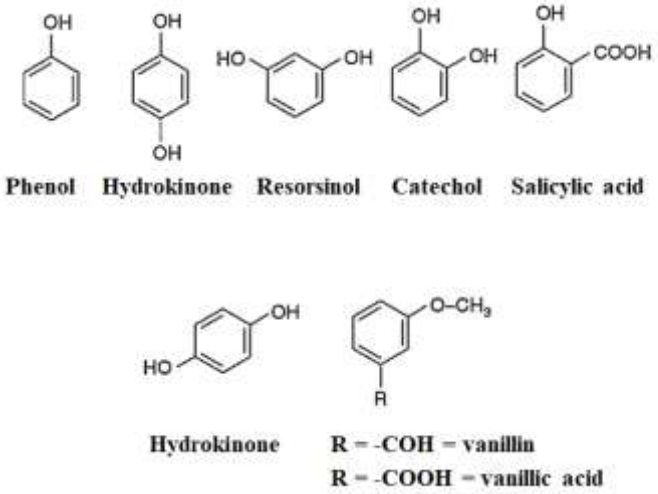


Figure 1.6 Some simple phenols with allelopathic potential (Pretorius and van der Watt, 2011, Modified).

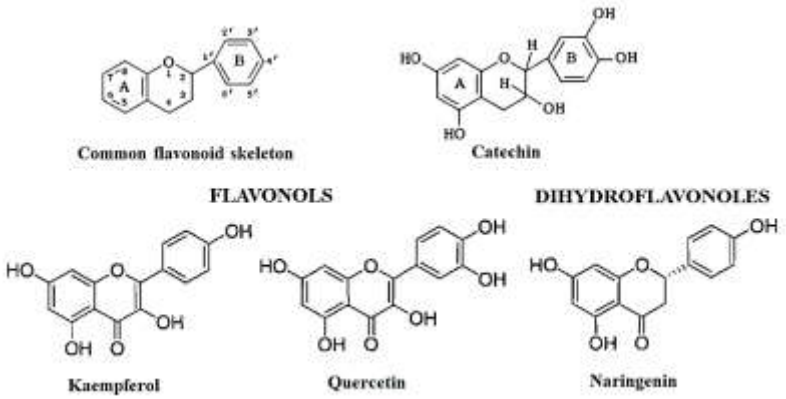


Figure 1.7 Some allelopathic flavonoids (Macias *et al*., 2007, Modified).

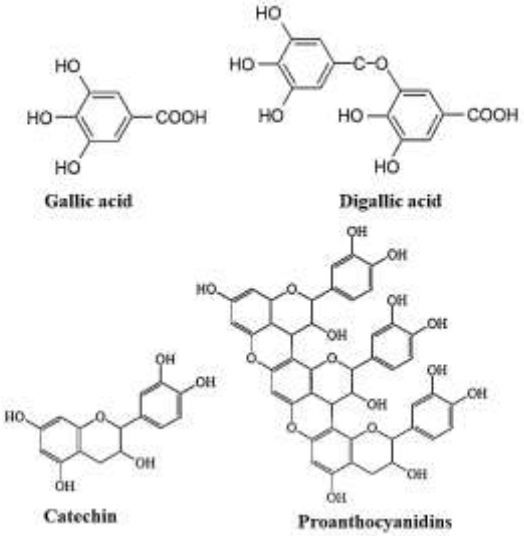


Figure 1.8 Some examples of hydrolyzable and condensed tannins.

Table 1.1 The major classes of phenolic compounds in plants (Source: Harborne 1980).

Number of carbon atoms	Basic skeleton	Class	Examples
6	C6	Simple phenols	Catechol, hydroquinone
		Benzoquinones	2,6-Dimethoxybenzoquinone
7	C6-C1	Phenolic acids	Gallic, salicylic
8	C6-C2	Acetophenones	3-Acetyl-6-methoxybenzaldehyde
		Tyrosine derivatives	Tyrosol
		Phenylacetic acids	p-hydroxyphenylacetic
9	C6-C3	Hydroxycinnamic acids	Caffeic, ferulic
		Phenylpropenes	Myristicin, eugenol
		Coumarins	Umbrelliferone, aesculetin
		Isocoumarins	Bergenon
		Chromones	Eugenin
10	C6-C4	Naphthoquinones	Juglone, plumbagin
13	C6-C1-C6	Xanthones	Mangiferin
14	C6-C2-C6	Stilbenes	Resveratrol
		Anthraquinones	Emodin
15	C6-C3-C6	Flavonoids	Quercetin, cyaniding
		Isoflavonoids	Genistein
18	$(C6\text{-}C3)^2$	Lignans	Pinoresinol
		Neolignans	Eusiderin
30	$(C6\text{-}C3\text{-}C6)^2$	Biflavonoids	Amentoflavone
n	$(C6\text{-}C3)_n$	Lignins	
	$(C6)_n$	Catechol melanins	
	$(C6\text{-}C3\text{-}C6)_n$	Flavolans (Condensed Tannins)	

1.5.1.2 Flavonoids

Flavonoids have a basic C6–C3–C6 skeleton (**Figure 1.7**) in which the A ring is of acetate origin and the B ring of shikimate origin (Neish, 1964). Flavonoids are the largest group of natural phenolic compounds in higher plants. In fact, more than 5000 different flavonoids have been described and it is estimated that about 2% of all carbon photosynthesized by plants is converted to flavonoids (Pretorius and van der Watt, 2011). They have roles associated with colour and pollination (e.g. flavones, flavonols, chalcones and catechins) and disease resistance, such as phytoalexins. They also have weak oestrogenic activity, for example the isoflavones (Macías *et al*., 2007). However, only a relative small number have been reported as toxins implicated in allelopathy. The most often cited are kaempferol, which is a yellow colour flavonol presents in apples, onions, citrus fruits, grape fruits etc., quercetin, naringenin, which is a dihydroflavon and ceratiolin, a non-phytotoxic dihydrochalcone present in the leaves of the dominant shrub of the Florida *Ceratiola ericoides* Michx.

1.5.1.3 Tannins

From the oxidative polymerization of catechins and flavan-3,4-diols derives condensed tannins, also called proanthocyanidins (PAs). When condensed tannins are hydrolysed by concentrated HCl, cyaniding chloride is formed. Another type of tannins are hydrolyzable tannins, which are derivate of gallic and *m*-Digallic acids hydrolysis (**Figure 1.8**). Some hydrolyzable tannins derive from a complex mixture of several phenolic acids, whereby many

types of hydrolyzable tannin molecules are possible (Rice, 1984).

1.5.1.4 Cinnamic acid and derivatives

Cinnamic acid and derivates arise from phenylalanine or tyrosine through the shikimic pathway (Neish, 1964; **Figure 1.9**). The first step of this pathway is catalyzed by the phenylalanine ammonia lyase (PAL), a widely distributed phenylpropanoid enzyme present in green plants, algae, fungi, and even in some prokaryotes (Hyun *et al*., 2011). They are phenyl propanoids containing 3-carbon side chain coupled to a phenol. They are formed in the biochemical route that yields lignin, the polymeric material that provides mechanical support to the plant cell wall (Xu *et al*., 2009). Cinnamic acid is a known allelochemical that affects seed germination and plant root growth and therefore influences several metabolic processes. Chlorogenic acids (CGAs) and isochlorogenic acid are formed as esters between different derivatives of cinnamic acid, caffeic acid specifically, and quinic acid molecules. They are components of the hydroxycinnsmic acids's classes. The main hydroxycinnamic acids with allelopathic properties are ferulic acid, caffeic acid, sinapic acid and ρ-coumaric acid.

1.5.1.5 Coumarins

Coumarins, classified as member of the benzopyrone family, all of which consist of a benzene ring joined to a pyrone ring, are lactones of o-hydroxycinnamic acid (Robinson, 1963). They are widely distributed in the Apiaceae, Rutaceae, Asteraceae and Fabaceae families. Coumarins, which are almost unknown in the animal kingdom, are present with high

frequency in the plant kingdom and occur in all parts of plants, depending on environmental conditions and seasonal changes. It is possible to classified four main coumarin sub-types: the simple coumarins, furanocoumarins, pyranocoumarins and the pyrone-substituted coumarins (**Figure 1.10**). The simple coumarins are the hydroxylated, alkoxylated and alkylated derivatives of the parent compound, coumarin, along with their glycosides (Jain and Joshi, 2012). Furanocoumarins consist of a five-membered furan ring attached to the coumarin nucleus, divided into linear or angular types with substitution at one or both of the remaining benzoid positions (Ojala, 2001). Umbelliferone, esculetin and scopoletin are the most widespread coumarins in nature.

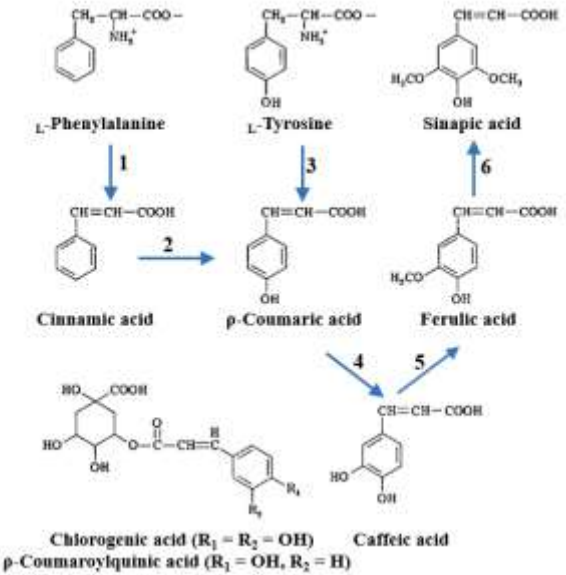


Figure 1.9 Examples of cinnamic acid derivatives with allelopathic potential (Rice, 1984, Modified).

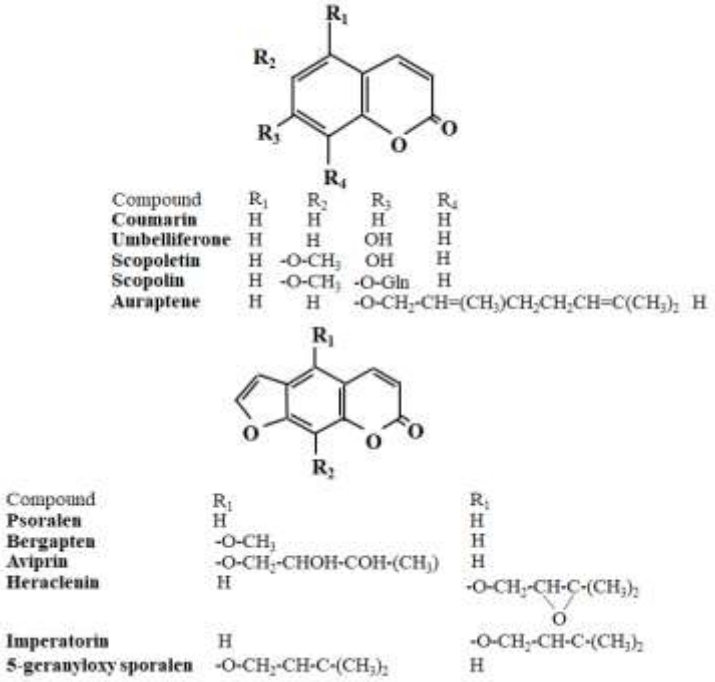


Figure 1.10 Structures of some allelopathic simple coumarins (up) and furanocoumarins (down) (Razavi, 2011, Modified).

1.5.1.6 Lichen metabolites

Among the second metabolites with allelopathic activities, there are a group of compounds never reported in higher plants: lichen metabolites. In fact, lichens produce secondary metabolites that are unique to the symbiosis (Romagni *et al*., 2004). Most of these compounds are aromatic and are derived from the polyketide pathway, with a few originating from the shikimic acid and mevalonic acid pathways (**Table 1.2**). Lichen metabolites can be divided into four main classes: depsides, depsidones, depsones and dibenzofurans (**Figure**

1.11). According to Rundel (1978), their ecological roles refer to the protection against damaging light conditions, chemical weathering compounds, allelopathic compounds and antiherbivore defence compounds.

Table 1.2 The major classes of secondary lichen metabolites (Source: Elix, 1996).

Biosynthetic Origin	Chemical Class	Examples
Polyketide	Depsides	Lecanoric acid
	Depsones	Picrolichenic acid
	Depsidones	Physodic acid
	Dibenzofurans	Pannaric acid
	Usnic acids	Usnic acid
	Chromones	Sordinone, Eugenitin
	Xanthones	Lichexanthone
	Anthraquinones	Emodin
Mevalonate	Diterpenes	16α-hydroxykaurane
	Triterpenes	Zeorin
	Steroids	Ergosterol
Shikimate	Terphenylquinones	Polyporic acid
	Pulvinic acid	Pulvinic acid

1.5.2 Terpenoids

Terpenoids or isoprenoids are secondary metabolites present in many organisms similar to the terpenes, from which they differ because the latter refers only to hydrocarbons. More than 50,000 terpenoids have been isolated from both terrestrial and marine plants, and fungi. After phenolics, they are the second largest group of secondary metabolites implicated in allelopathy. The class of the terpenoids presents a great variety of compounds in which the several structures, most of them multicyclic, differ from one another in their basic carbon skeletons and functional groups. All terpenoids

are based on a various but definite number of 5-carbon isoprene units, called also 2-methyl-1,3-butadiene. In plants, there are two independent metabolic pathways that create terpenoids: the classic mevalonic acid (MVA) pathway and the methylerythritol phosphate (MEP) pathway, also known as non-mevalonate pathway or mevalonic acid-independent pathway. The former occurs in the cytosol and produce also cholesterol; the latter takes place entirely in plastids. The MVA pathway provides the precursors for the biosynthesis of sesquiterpenes, phytosterols, brassinosteroids, and triterpenes (Newman and Chappell, 1999). Instead, the MEP pathway provides the C5-building blocks for the biosynthesis of carotenoids, chlorophyll, gibberellins, and monoterpene and diterpene specialized metabolites, which are exclusively or primarily produced in plastids (Lichtenthaler, 1999). In either pathways, terpenoids derives through the condensation of the end-products isopentenyl diphosphate (IPP) and its allylic isomer dimethylallyl diphosphate (DMAPP), giving geranyl pyrophosphate (GPP) (**Figure 1.12**). Isoprene is formed from DMAPP via the action of the enzyme isoprene synthase which catalyses elimination of diphosphate.
It is possible to classified terpenoids according to the number of isoprene units incorporated in the basic molecular skeleton (**Table 1.3**) (**Figure 1.13**).

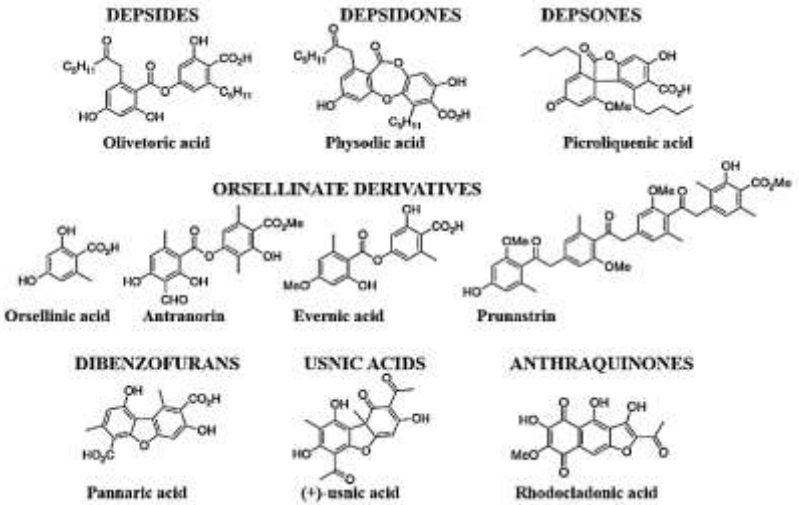


Figure 1.11 Lichen metabolites reported to have phytotoxic activity (Macías *et al*., 2007, Modified).

Table 3 Classification of terpenoids based upon the number of isoprene units.

Terpenoids	**Isoprene units**	**Number of carbon atoms**
Meroterpenoids	1	C5
Monoterpenoids	2	C10
Sesquiterpenoids	3	C15
Diterpenoids	4	C20
Sesterterpenoids	5	C25
Triterpenoids (es. sterols)	6	C30
Tetraterpenoids (es.carotenoids)	8	C40
Polyterpenoids (es. rubber)	many (> 100)	Polymer (> 500)

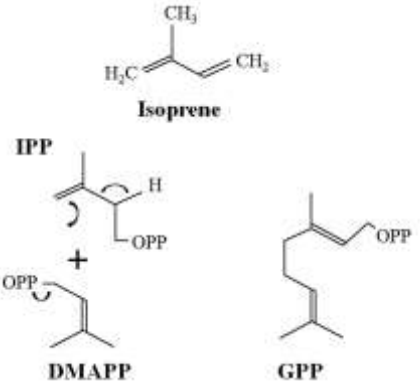


Figure 1.12 Isoprene (up) and some terpenoids precursors (down) molecular structural formula.

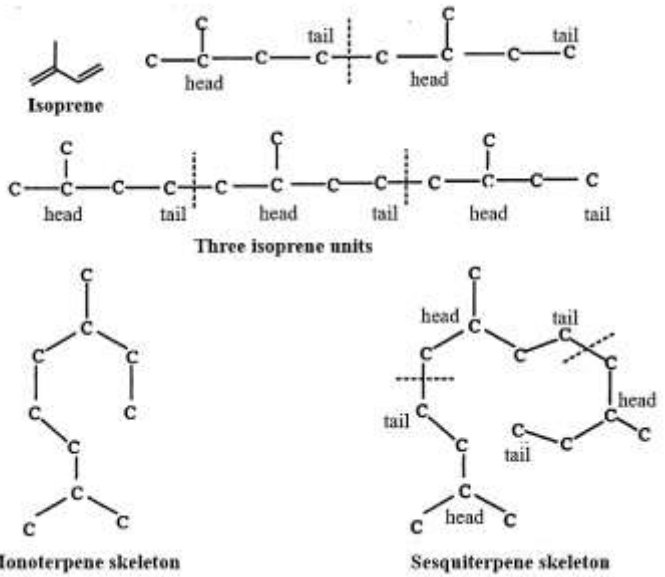


Figure 1.13 Arrangement of isoprene units in mono- and sesquiterpenoids (Bhat *et al*., 2005, Modified).

1.5.2.1 Monoterpenoids

Monoterpenoids, with sesquiterpenes, are the major components of essential oils. They are volatile compounds that have been described as the predominant terpenoid allelochemicals from higher plants. Most of them are inhibitors of seed germination and several microorganisms, mainly bacteria and fungi. Besides, they have been proposed as potential starting structures for herbicides (Vaughn and Spencer, 1993). For example, it can be observed the high structural similarity between monoterpenes 1,4- and 1,8-cineole and the herbicde cinmethylin (**Figure 1.14**). While a few, such as camphor or cineoles, occur in a near pure form, most of terpenoids occur as complex mixtures difficult to separate. Of the 14 most commonly occurring monoterpenes (α-pinene, β-pinene, Δ(3)-carene, d-limonene, camphene, myrcene, α-terpinene, β-phellandrene, sabinene, ρ-cymene, ocimene, α-thujene, terpinolene, and γ-terpinene), the first six are usually found to be most abundant (Shexia, 2012).

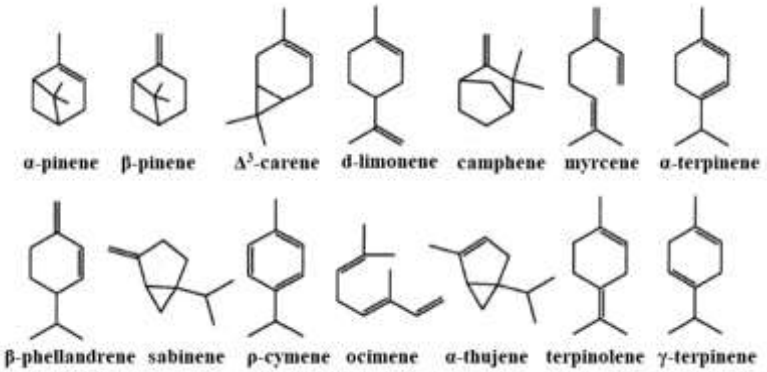


Figure 1.14 Chemical structures and name of monoterpenes (Shexia, 2012, Modified).

1.5.2.2 Sesquiterpene lactones

Sesquiterpenoids are the terpenoids with 15 carbons containing three isoprene units, plus a lactone ring. Their structures present several acyclic, mono-, bi-, tri-, and tetracyclic systems. They are present in high quantity in several plants, particularly in those of the Compositae family (Fraga, 2005). They have a wide range of biological activity, including plant growth-regulating, insect anti-feedant, anti-fungal, and anti-bacterial properties (Baruah *et al*., 1994; Picman, 1986). Some of the most common sesquiterpene lactones are artemisinin, isolated from the plant *Artemisia annua* L., and centaurepensin and cnicin, presents mainly in the members of Centaurea family (**Figure 1.15**).

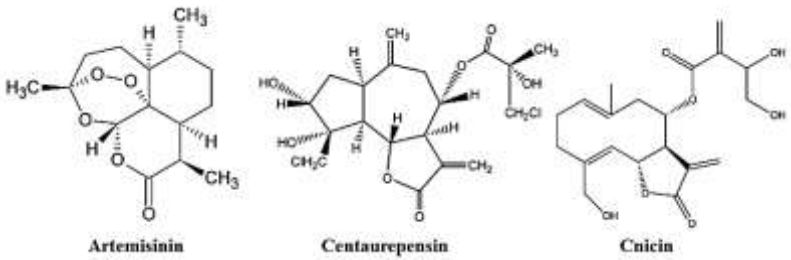


Figure 1.15 Chemical structures of some common sesquiterpene lactones.

Sesquiterpene lactones are important allelochemicals involving in the invasive potential of plant species, such as *Centaurea diffusa* Lam. (diffuse knapweed) and *C. maculosa* Lam. (spotted knapweed) in North America.

1.5.2.3 Diterpenoids

Diterpenoids have 20 carbon atoms and consist of four isoprene units. Such diterpenoids, such as giberrellins, act as important plant hormones and there are relatively few reported diterpenoid allelochemicals produced by plants.

The most famous diterpenoids are momilactones (**Figure 1.16**), rice diterpenes that are exuded from the roots of young rice seedlings due to the infection by blast fungus (*Pyricularia oryzae*) or irradiation with UV light (Cartwright *et al*., 1981). However, in literature momilactones are reported as phytoalexins, whereas momilactone A and B are the only rice diterpenoids identified as allelopathic agent.

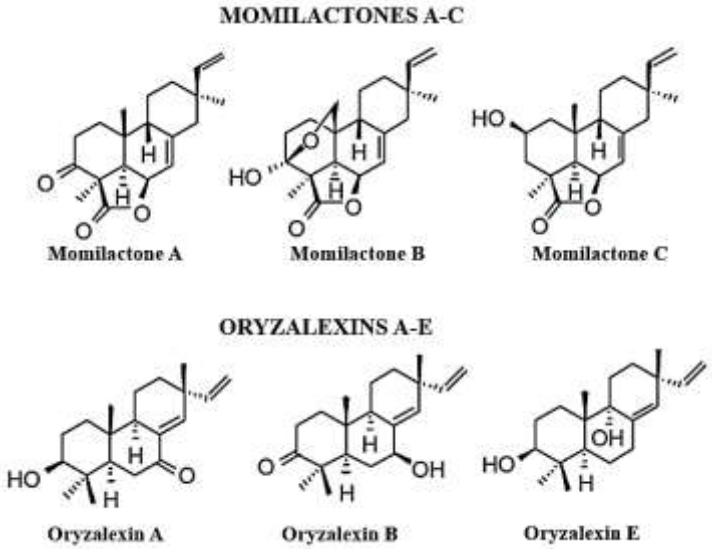


Figure 1.16 Rice allelochemicals: momilactones and oryzalexins (Macías *et al*., 2007, Modified).

1.5.2.4 Other terpenoids

Quassinoids are degradated triterpenes containing six isoprene units reported from the members of the Simaroubaceae family. They present a high structural complexity and, according to their basic skeleton, are categorized into five distinct groups: C-18, C-19, C-20, C-22 and C-25 types shown in **Figure 1.17**. Quassinoids present a wide range of biological activities including antitumor, antimalarial, anti-inflammatory, insecticidal, fungicidal and herbicidal. The first quassinoid identified as an allelopathic agent was quassin, a C20 type isolated from the quassia wood in Suriname (*Quassia amara* L.) by Clark's group in 1937. The most important quassinoid is ailanthone, an allelochemical produced by the tree-of-heaven (*Ailanthus altissima* (Mill.) Swingle) which inhibit the growth of other plants. Thanks to ailanthone, the *A. altissima* tree has become a strong invasive species in Europe (**Figure 1.18**).

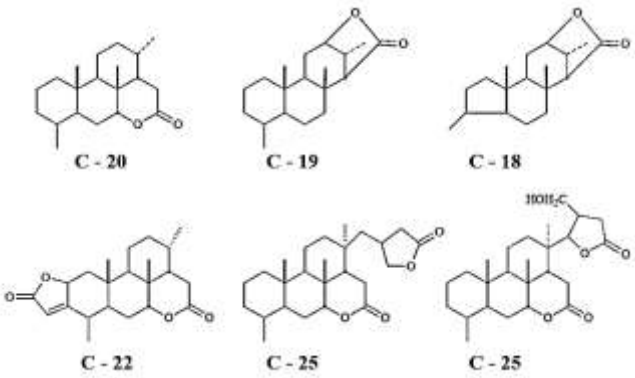


Figure 1.17 Skeleton of quassinoids (Guo *et al*., 2005, Modified).

Benzoxazinoids are hydroxamic acids produced by many species, mainly the plants of the Poaceae family (wheat, rye, maize) and in a few species of dicots. These compounds are very instable and, undergo hydrolysis, they contract into the corresponding benzoxazolinones (Macías *et al*., 2004). The most effective allelopathic compounds are DIBOA, DIMBOA and their breakdown products BOA and MBOA (Barnes and Putnam, 1987; Tabaglio *et al*., 2008).

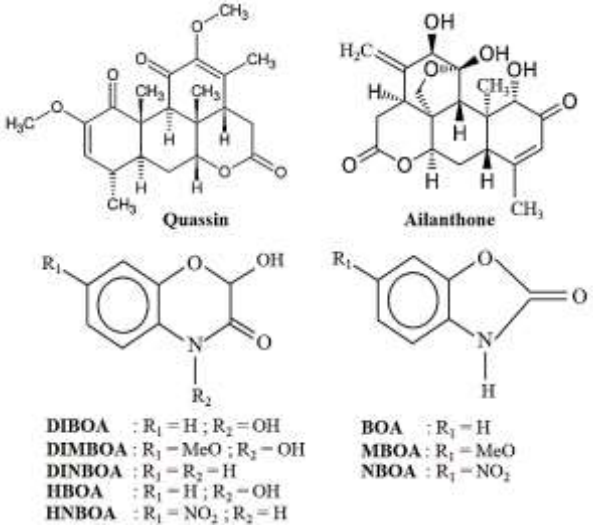


Figure 1.18 Chemical structures of some quassinoids and benzoxazinoids mentioned in the text.

Other important terpenoids with allelopathic potential belong to the chemical classes of glucosinolates and steroids. Glucosinolates are sulfur- and nitrogen-compounds that occur in most plants of the *Brassicales* order, e.g. in Brassicaceae and Capparidaceae families, with the role of defence against insects, herbivores and certain microbial

pathogens. They are degraded by the endogenous enzyme β-thioglucosidases, called myrosinase, into compounds such as isothiocyanates. Steroids are tetracyclic terpenoids containing 17 carbon atoms with only two methyl groups attached to the ring system. Very few are the steroids linked to plant-plant allelopathy, while there are several examples of antimicrobial activities such as the aglycones digitoxigenin produced by *Digitalis purpurea* L. and strophanthidin, produced by *Convallaria majalis* L. (Robinson, 1963).

1.5.3 Other compounds

1.5.3.1 Alkaloids

Alkaloids are organic compounds containing basic nitrogen atoms in the heterocyclic rings or in side chains. They present an enormous variety of structures and there is not a uniform classification of them. Alkaloids are produced by secondary metabolism of primary metabolites, usually amino acids. These compounds are produced by a large variety of organisms, including bacteria, fungi, plants, and animals. Plant alkaloids often demonstrate defensive activity against a wide variety of predators and competitors among microorganisms, fungi, viruses, invertebrate, vertebrate herbivores and plants (Blum, 2004). Today, more than 10,000 alkaloids are known, but only a small number of them present allelopathic activities. There are several works on the role of alkaloids as seed germination inhibitors (Evenari, 1949; Wink, 1983). Among allelopathic alkaloids there are papaverine, caffeine, emetine, gramine, etc. (**Figure 1.19**).

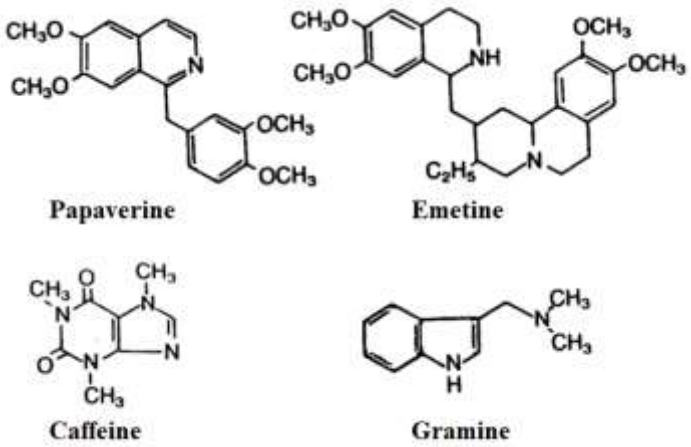


Figure 1.19 Chemical structures of some alkaloids identified as allelopathic agents.

1.5.3.2 Cyanogenic glycosides

Cyanogenic glycosides are glycosides consisting of a sugar group and a non-sugar group, called aglycone, in this case a cyanide group, which when enzymically hydrolyzed release cyanohydric acid (HCN), a compound extremely toxic. In most cases, hydrolysis is accomplished by the β-glucosidase, producing sugars and a cyanohydrin that spontaneously decomposes to HCN and a ketone or aldehyde (Francisco and Pinotti, 2000) (**Figure 1.20**).

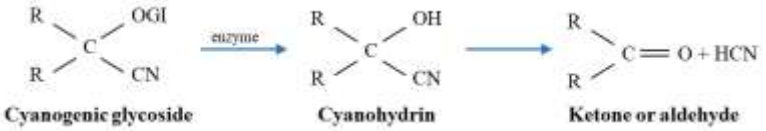


Figure 1.20 Pathway of release of HCN by cyanogenic plants (Francisco and Pinotti, 2000, Modified).

Cyanogenesis is a plant's protective mechanism against predators such as the herbivores. Cyanohydrins are very common in plant kingdom, but they were also founded in some species of ferns, fungi and bacteria (Harborne, 1972). The best known cyanogenic glycosides are dhurrin, which is present in sorghum (*Sorghum vulgare* Pers.) seedlings, amygdalin and prusanin, very common among plants of the Rosaceae, particularly the *Prunus genus* (**Figure 1.21**). These compounds are strong germination and growth inhibitors.

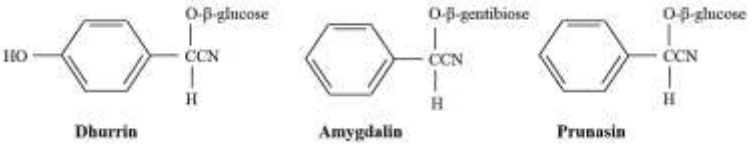


Figure 1.21 Chemical structures of some cyanogenic glycosides with allelopathic potential.

1.6 Modes of release of allelochemicals into the environment

Most of allelochemicals are distributed among many species belonging to several botanical families, but there are particular secondary metabolites that are restricted within a group of taxonomically related species. For example, salicacin, a phenol glucoside, is characteristic of the members of the Salicaceae family while benzoxazinoids are found mainly in the *Brassicales* order, and so on.

Plant allelochemicals are generally localized and sequestered in glandular or subepidermal layers (Ambika, 2013). They can be found, in different concentrations, in several parts of plants: leaves, stems, roots, rhizomes, seeds, flowers or

inflorescences, fruits and even pollen (Bertin *et al*., 2003; Gatti *et al*., 2004; Kruse *et al*., 2000). Generally, leaves represent the most consistent source of inhibitors for many plants but, in some cases, this is reversed with roots, which anyway are at least the second source of allelochemicals. Pollen also represent for many species, mainly in Poaceae and Asteraceae, an important source of allelochemicals. Much interesting results the work of Murphy (1999) on pollen allelopathy.
The presence of an allelopathic compound into a plant not necessarily imply a role on the ecosystem due to that compound. To exert an effect, the allelochemical must be released into the environment at a time when it can perform its inhibitory action (Sattin and Tei, 2001).
Plants release allelochemicals into the environment through four main pathways (**Figure 1.22**): (1) volatilization from living parts of the plant; (2) leaching from aboveground parts of the plant; (3) decomposition of plant material; (4) root exudation.
These pathways varie among species and according to the chemical nature of compounds.

1.6.1 Volatilization

Many plants release volatile inhibitory compounds under vapour form, mainly through leaves, in the atmosphere. Most of these volatile compounds are terpenoids, e.g. monoterpenoids, sesquiterpene lactones, etc. or hormones such as ethylene (CH2 = CH2), in this case released by fruits. Allelochemicals released by volatilization can be absorbed by plants directly from the atmosphere through gas exchanges or from soil, where they arrive due to rainfall or leaching. Many

works has been done on the allelopathic effects of volatile inhibitors (Chu *et al*., 2014; Nishida *et al*., 2005; Barney *et al*., 2005; Kong *et al*., 2002). Volatile allelochemicals are seed germination and growth inhibitors, present antimicrobial and antifungal activities and are involving in the "old field" succession. This mode of release generally shows its most significant ecological effects under arid and semiarid conditions (Rice, 1974).

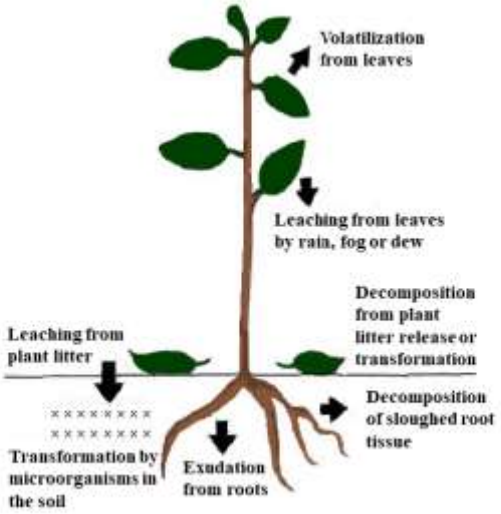


Figure 1.22 Major pathways of release of allelochemicals into the environment (Rice, 1984, Modified).

1.6.2 Leaching

Many kinds of allelochemicals have been identified in the leachates of plants. Leaching is the process that leads to the loss of chemical compounds from the aerial part of the donor plant or the ground litter by means the hydro-solubilization

made by rain, fog or irrigation. The quantity of leachates released depends on duration and amount of rainfall and on chemical nature of compounds. Water-soluble compounds are more leached than others. Among allelopathic leachates there are amino acids, phenolic compounds, terpenoids, alkaloids and fatty acids. They can derive from living or dead parts of plants. Buta and Spaulding (1989) founded that allelochemicals leached from excised leaves of tall fescue grass (*Festuca arundinacea* Schreb.) belong to three principal inhibitory compounds, abscisic acid, caffeic acid, and ρ-coumaric acid and founded that abscisic acid was the predominant inhibitor.

1.6.3 Root exudation

Like for volatilization, many researchers have studied the exudation phenomenon and discovered that living roots of many weed and crop species exude different types of organic compounds such as amino acids, carbohydrates, nucleotides, enzymes, steroids, terpenoids, tannins, fatty acids, alkaloids, vitamins and flavonoids. Root exudates play a fundamental role within the ecological succession of microorganisms in the rhizosphere and influence seed germination, root and shoot growth, nutrient uptake and nodulation (Inderjit and Weston, 2003; Pandya *et al*., 1984; Yu and Matsui, 1994). Besides, allelochemicals released by root in the rhizosphere influence resistance to pests and, inevitably, soil characteristic. For example, Hao *et al*. (2010) founded that rice exudates such as phenolic acids, sugars and free amino acids suppressed the Fusarium wilt of watermelon (*Fusarium oxysporum* f. sp. *niveum*), while those of watermelon significantly stimulated *Fusarium* spore germination and

sporulation. Root exudation is affected by a variety of factors including the age and species of plant, stress factors such as availability of moisture, temperature and light intensity, mineral nutrition, soil microorganisms (Hale *et al*., 1971). In cereals, it was observed that an amount between 5 and 21% of plant total photosynthates is released via root exudation (Haller and Stolp, 1985; Vivanco *et al*., 2002).

1.6.4 Decomposition of plant material

Considering that it is not easy to distinguish the different modes of release of allelochemicals in the environment, it is believed that decaying and leaching represent the main mode release of allelochemicals. The decomposition of plant residues adds a large quantity of allelochemicals into the rhizosphere (Goel, 1987). The process depends on the nature of plant residues, soil characteristics and it is closely associated with microbial activity, which is strongly influenced by temperature and soil water content. These products are converted by soil microflora from nontoxic compounds to toxic ones or into more biologically active products than the parents (Blum and Shafer, 1988). Generally, water-soluble inhibitors are easily leached out of plant litter after death when membranes lose their differential permeability (Rice, 1974). This pathway of release is often linked to autotoxicity probems such as the case of alfalfa (*M. sativa*), and it is strictly related to weed management with cover crops, green manure and intercropping.

1.7 Factors affecting allelochemical production

It is important to understand what means when a plant is "stressed". According to Levitt (1980), plant stress can be

defined as "*a state in which increasing external demands lead to the destabilization of plant functions, followed by a phase of normalization and improving of the resistance. If the plant is forced out of its tolerance limits and its acclimation capacity is over passed, the result can be a permanent damage or even plant death*". Different types of abiotic and biotic stress factors influence the quantity of allelochemicals released by the donor plant and the effect of an allelochemical on the target plant (Inderjit and Del Moral, 1997; **Figure 1.23**). Stress factors such as drought, irradiation, light, temperature, nutrient and water availability, diseases and pathogens, competitors, increase allelochemical production in a plant (Einhellig, 1996; Reigosa *et al*., 1999a, b). Allelochemical production is influenced also by morphological, physiological and ecological characteristics such as plant density, life cycle, plant age and habitat (Inderjit and Keating, 1999). The *stress hypothesis of Allelopathy* formulated by Reigosa *et al*. (1999a, b; Reigosa and Pedrol, 2002), states that allelopathy can appear and disappear in a place according to environmental changes, so that allelopathy becomes more important when and where plants are under stress.

Even though the production of allelochemicals in a plant can increase in response to stress, it is not clear whether a corresponding release of allelochemicals to the environment also occur (Einhellig, 1996; Inderjit and del Moral, 1997). However, the sensitivity of target plants to allelochemicals in general is affected by stress and typically it is increased (Einhellig, 1996; Reigosa *et al*., 1999a, b). The combination of several stress factors results in an increase of allelochemical concentrations in donor plants (del Moral,

1972). It is important to increase the research on the synergistic effects of stress factors because they generally occur in combinations under field conditions (Rice, 1974).

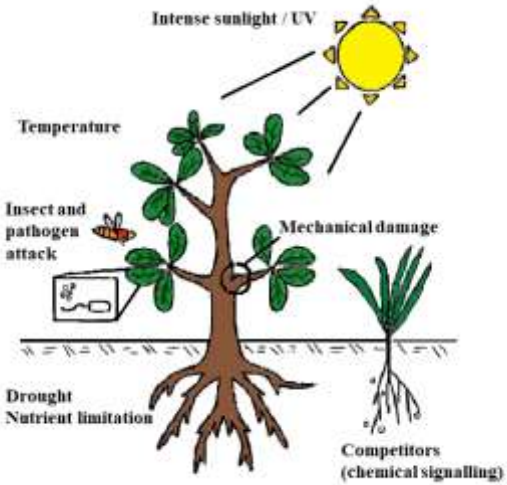


Figure 1.23 Abiotic and biotic stress factors affecting allelochemicals production (de Albuquerque *et al*., 2010, Modified).

1.7.1 Ligth

Amount, intensity and quality of light play an important role on the production of inhibitors. In fact, plants growing in greenhouse produce a less amount of allelochemicals than the same kinds growing out-of-doors (Rice, 1984).

Ionizing radiation increases the concentrations of phenolic allelochemicals in sunflower (*Helianthus annuus* L.) and tobacco (*Nicotiana tabacum* var. One Sucker) plants (Fomenko, 1968; Koeppe *et al*., 1970a; Rice, 1974).

Also the ultraviolet radiation, generally, increase the amounts of inhibitors produced by donor plants. Del Moral (1972) demonstrated that supplemental UV light increased amounts of total chlorogenic and isochlorogenic acids in sunflower.
Furness *et al*. (2008) reported increased allelopathic influence of houndstongue (*Cynoglossum officinale* L.) on some forage grasses. Li *et al*. (2009) found that the allelopathic potential of *Zanthoxylum bungeanum* on seed germination rate of alfalfa, lettuce and radish is improved under enhanced UV-B radiation and differed in relation to species.
It seems that visible light enhances the synthesis of inhibitory compounds in donor plants. Zucker (1963) was one of the earliest scientists who study the effects of visible light on allelochemical production. Kato-Noguchi (1999) reported that visible light may enhances allelopathic activity of germinating maize due to an increase in the level of DIBOA.
Allelochemical synthesis is influenced also by light quantity, as well as its quality. Photoperiod differently affects short-day plants and long-day ones (Zucker *et al*., 1965). In most cases, long days increase inhibitory compounds in donor plants regardless of the daylengths required for flowering (Rice, 1974).
Therefore, the amount of allelochemicals is generally greater during exposure of ultraviolet light and long daylength.

1.7.2 Temperature

In general, plants under thermal stress tend to produce more allelochemicals but, by contrasts, become more susceptible to them. Quantities produced are higher at lower temperatures, while high temperatures enhance allelochemical effects (Einhellig and Eckrich, 1984). Koeppe *et al*. (1970b) found

that chilling (8-9 °C) of tobacco plants increased the concentrations of total chlorogenic acids in old leaves, young leaves and stems, but decreased the concentration in the roots.

1.7.3 Water deficiency

Water deficiency, as well as all stress factors, result in increased concentrations of allelochemicals. Using NaCl in colture solution to cause water stress on sunflower plants, del Moral (1972) found that, after 31 days of treatment, the concentrations of total chlorogenic and isochlorogenic acids in roots, stems and leaves were increased (Rice, 1974). Among different irrigation levels, Ardi (1986) reported that inhibitory effects of purple nutsedge (*Cyperus rotundus* L.) on sweet corn (*Zea mays* L.) yield were reduced at the highest waters stress imposed. Tang *et al*. (1995), studying water deficiency on the allelopathic potential of purple nutsedge, found that both fresh and dry weights of its shoots and roots decrease with increasing waters stress (Inderjit and Keating, 1999). Oueslati *et al*. (2005) reported that barley (*H. vulgare*) autotoxicity increase under drought conditions.

1.7.4 Minerals availability

Many authors (Loche and Chouteau, 1963; Lehman and Rice, 1972; Mwaja *et al*., 1995; etc.) demonstrated that the mineral deficiencies of B, Ca, Mg, N, P, K and S, play an important role in the production of inhibitory compounds. This proves that the production of allelochemicals increases under nutritional stress condition.

Loche and Chouteau (1963) found increases in concentrations of scopolin and decreases in those of chlorogenic acid in calcium- and borum-deficient tobacco

leaves (Rice, 1974). Studying the effect of deficiency of N, K and P on phenolic content in sunflower, Lehman and Rice (1972) reported increased amounts of chlorogenic acid and scopoletin in old leaves, stems and roots of mineral-deficient plants than in controls. Mwaja *et al*. (1995) studied the effects of three fertility regimes (low, medium and high) on phytotoxicity, biomass production and allelochemical content in rye (*S. cereale*) and concluded that, despite the larger amounts of rye biomass, low fertilisation enhances the phytotoxicity and allelochemical content. Chamacho-Cristóbal *et al*. (2002) studied the effects of B deficiency on phenols and the activities of the enzymes involved in their biosynthesis in tobacco (*N. tabacum*) plants. They found a positive correlation between phenols concentrations and the activity of phenylalanine ammonia-lyase (PAL) after 5-7 days of B-deficiency. B deficiency, therefore, results in an increase of PAL activity and, in turn, in an enhancement of phenolic levels.

1.7.5 Soil characteristics

As suggested by Cheng (1989, 1992), the effectiveness of allelochemicals in soil is strictly influenced by physiochemical and biological soil factors.

In general, clay soils, which are characterized by high values of cation exchange capacity (CEC) and anion exchange capacity (AEC), adsorb more allelochemicals on the surface of their colloidal particles than sandy soils. Del Moral (1972), reported that fine-textured soils sorb more amounts of phenolic compounds than sandy-loam soils. Besides, allelochemicals persist for longer duration in clay soils (Einhellig, 1987). After soil adsorption, also the transport of

allelochemicals (i.e., the movement of allelopathic compounds from roots of donor plants to roots of target plants) depends on soil texture and chemical nature of inhibitors. Transport can be either through the air as vapour or in the soil solution (Cheng, 1992). The movement is faster in sandy than in clay soils (Jennings and Nelson, 1998).
The chemical characteristics of soil such as pH, organic carbon, nutrients available, ion exchange capacity, oxidation state, also play an important role on the fate of allelochemicals. For example, soil pH can affect the uptake and the immobilization of allelochemicals (Cheng, 1992). It must be considered that higher pH can stimulate microbial activity (Aarnio and Marikainen, 1994) and, in turn, allelochemical availability. Soil organic carbon can indirectly influence allelochemical stability and persistence (Huang *et al*., 1977; Lehman and Cheng, 1988). Organic matter, in fact, strongly enhance soil microbial activity, exerts a buffer capacity on soil pH and, thanks to its high ion exchange capacity, promote allelochemical sorption and retention. Lehman and Cheng (1988), studying the reactivity of phenolic acids in several soil, found that they are more stable in forest soils with high organic matter than in cultivated agricultural soils.

1.7.6 Biotic components

The expression of allelopathy may be influenced by a series of biotic factors such as diseases and pathogen attacks, weed competitors, interactions with herbicides, age of plant organs, plant density and habit (Einhellig, 1996).
Despite of detrimental effects, generally pathogens decrease the competitiveness and simultaneously increase the

allelopathic activity of their hosts (Mattner, 2006). In fact, as consequence of defensive adaptation, the attacks of phytophagous or plant-sucking insects and diseases cause a considerable increase in the release of allelopathic compounds.

Jay *et al*. (1999) reported that, because of infection with beet western yellows virus (BMYV), oilseed rape (*B. napus*) increased glucosinate concentrations in tissues by 14%. Woodhead (1981) found that sorghum plants infected with downy mildew (*Sclerospora sorghi* W. Weston & Uppal) or rust (probably *Puccinia purpurea* Cooke) or sorghum shoot fly (Atherigona soccata Rondani), increased phenolic concentrations. Soil in which rusted ryegrass (*Lolium perenne* L.) attacked by P. *coronata* Corda f.sp. *lolii* Brown was grown suppressed clover (*Trifolium repense* L.) biomass by 36% more in comparison with the direct effect of soil in which healthy ryegrass was grown in the greater rainfall areas of south-eastern Australia (de Albuquerque *et al*., 2010; Mattner and Parbery, 2001).

According to Belz (2007), weeds can elicit allelochemical biosynthesis in competing crops as well as insects or pathogens induce plant defences in attacked plants. The author reports examples about the exudation of three major allelochemicals in two allelopathic cultivars of rice (*O. sativa*) due to the presence of *Echinochloa crus-galli* (L.) Beauv. and the release of sorgoleone in a *Sorghum* hybrid after exposure to water-soluble root leachates released from *Abutilon theophrasti* Medik. She suggests that biotic-induced plant defences depends on a direct pest attack and on aerial or rhizosphere signals from healthy or attacked plants.

Lydon and Duke (1993) reported that herbicides, at both lethal and sub-lethal concentrations, influence allelochemical production by both direct and indirect effects. Many works has be done to determine the effect of herbicides on allelochemical biosynthesis. Winkler (1967) reported that levels of scopolin increased after spraying tobacco plants with maleic hydrazide. Lydon and Duke (1988) found that redroot piweed (*Amaranthus retroflexus* L.), ryegrass (*L. perenne*), soybean (*Glycine max* L.), velvet leaf (*A. theophrasti*) and yellow nutsedge (*C. esculentus*) presented high levels of shikimic acid and certain hydrobenzoic acids due to glyphosate treatments.

Many Authors agree in considering the age of plant organs as an important factor involved in the production of allelopathic compounds. Koeppe *et al*. (1969, 1970b) found that the age of both tobacco and sunflower leaves influenced the amounts of scopolin, chlorogenic and isochlorogenic acids. To improve weed management, it should be considered the age of donor plant at which release of allelochemicals starts (Inderjit and Keating, 1999). For example, Schumacher *et al*. (1983) discovered that wild oats (*Avena fatua* L.) become allelopathic against spring wheat (*T. aestivum*) at the four-leaf stage.

The influence of plant density of target species on the response to allelochemicals it is now widely accepted by the international scientific community. Weidenhamer *et al*. (1989), studying the density-dependent effects of varying amounts of gallic acid and hydroquinone on *Paspalum notatum* Flüggé and *S. lycopersicum* grown at different densities, found that the quantity of allelochemicals available

to each target species decreases with the increase in target species density.

1.8 Modes of action of allelochemicals

Understanding which compounds and which mechanisms of action are involved in allelopathy is important to develop predictive models (Inderjit and Duke, 2003). However, this is a question of not easy solution due to the great diversity of chemical families of allelochemicals and to the several molecular target site of phytotoxic compounds. Besides, is important to recognize that, in field situations, allelopathic activity is thought to be often due to joint action of mixtures of allelochemical rather than to one allelochemical (Einhellig, 1995). Since the visible effects of allelochemicals on plant processes are only secondary signs of primary changes (Winter, 1961), a clear separation of primary from secondary effects is very difficult.

Several Authors divides the mode of action of allelochemicals into indirect and direct action. The influence of secondary metabolites on soil properties and its microbial populations belong, for example, to indirect effects. According to Inderjit and Weiner (2001), indirect allelopathy could be due to (i) degraded or transformed products of released chemicals, (ii) effect of released chemicals on physical, chemical and biological soil factors and (iii) induction of release of biologically active chemicals by a third species.

Allelochemicals can alter:

a. cell division, elongation and structure;
b. membrane stability and permeability;
c. activity of various enzymes;

d. synthesis of plant endogenous hormones;
e. plant respiration;
f. plant photosynthesis and pigment synthesis;
g. protein synthesis and nucleic acid metabolism;
h. mineral uptake;
i. germination (of spores, seeds and pollen) and growth of target plant;
j. water balance of plant and conducting tissue.

These effects are rarely independent of each other. Rather, there is a closely relationship between them, since the same allelopathic compound can generates "multiple cascating effects" (**Figure 1.24**). Allelopathic effects are not only harmful. In fact, allelochemicals may have beneficial effects in response of their concentrations. A compound may be inhibitory at high concentration, stimulatory at low concentration, or have no effect at other concentrations (Ambika, 2013).

1.8.1 Inhibition of cell division, elongation and ultra-structure

Many works, since middle of twentieth century, have demonstrated that some allelochemicals could inhibit cell elongation, plant root elongation, cell cytology, and therefore affect the development of the whole plant (Li *et al*., 2010). In last decades, this topic has received great attention by researches (Burgos *et al*., 2004; Cheng *et al*., 2016; Grana *et al*., 2013; Hallak *et al*., 1999; Li *et al*., 1993; Sanchez-Moreiras *et al*., 2008; Vaughan and Ord, 1990;). Vaughan and Ord (1990) found that, at high concentrations (1mM), some phenolic acids inhibited cell division and affected the extension growth of the main root and the number of the

lateral roots of *Pisum sativum* cultured in a Hoagland nutrient solution under axenic conditions. Li *et al*. (1993) reported that coumarin significantly inhibited the root elongation of *Lactuca sativa* L. seedlings, reduced cellular activity and increased the thickness of the cortex cells. Hallak *et al*. (1999), studying the effects of sorghum root exudates on the cell cycle of *Phaseolus vulgaris* L., found that sorgoleone acts as a mitotic inhibitor reducing the number of cells in each cell division period and damaging tubulins. According to Burgoss *et al*. (2004), BOA and DIBOA reduced the regeneration of root cap cells and increased the width of cortical cells resulting in increased root diameter. Sanchez-Moreiras *et al*. (2008), investigating the effects of BOA on the root meristems of seedlings of lettuce, reported an inhibition of the mitotic process. Grana *et al*. (2013) reported that citral, a volatile monoterpene presents in the essential oils of several aromatic plants, has a strong long-term disorganising effect on cell ultra-structure in *Arabidopsis thaliana* L. seedlings. Cheng *et al*. (2016) observed that diallyl disulphide from garlic (*Allium sativum* L.), at lower concentrations (0.01–0.62 mM) significantly promoted root growth on tomato (*S. lycopersicum*), whereas higher levels (6.20-20.67 mM) inhibited root growth by affecting both the length and division activity of meristematic cells.

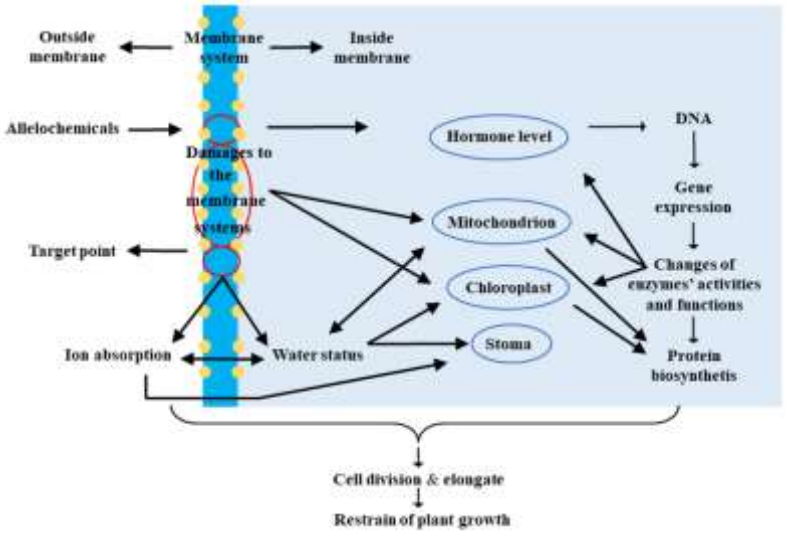


Figure 1.24 Possible mechanisms of action of allelochemicals in plants and their relationship. Allelochemicals, by altering the permeability of membranes, affect the electrochemical potential gradient across membranes. Once entered inside the cell membrane, allelochemicals cause damage at hormone, mitochondrion, chloroplast and stoma level. These effects are rarely independent of each other, but generally an allelopathic compound generates "multiple cascating effects" (Wang *et al*., 2006, Modified).

1.8.2 Interference with cell membrane permeability

Several allelopathic agents, especially phenolics, increase cell membrane permeability due to the inhibition of antioxidant enzymes (such as catalases and peroxidases) and to the increase of lipid peroxidation and free radicals level (the so-called reactive oxygen species or ROS) in plasma membranes. These changes in membrane permeability lead to a spillage of cell contents and, therefore, to a slow growth or death of plant tissues (Li *et al*., 2010).

Baziramakenga *et al*. (1995) reported the benzoic and cinnamic acids damages cell membrane integrity in intact soybean (*G. max* L. cv. Maple Bell) seedlings due to an increase of lipid peroxidation, which resulted from free radical formation in plasma membranes, inhibition of catalase and peroxidase activities, and sulfhydryl group depletion. Batish *et al*. (2006) found that 2-Benzoxazolinone (BOA) induces oxidative stress in in both leaves and roots of mung bean (*P. aureus*) as indicated by enhanced lipid peroxidation and accumulation of hydrogen peroxide (H_2O_2). Ladhari *et al*. (2014), studying the effects of aqueous (15 g/L) and methanol (6 g/L) extracts of *Capparis spinosa* L leaves. and *Cleome arabica* L. siliquae on lettuce, pointed out a disruption in membrane permeability revealed by a strong electrolyte leakage and a trigger in oxidative damage manifested by lipid peroxidation.

1.8.3 Interference with various enzyme activities

Many allelochemicals are known to interfere with the synthesis, functions, contents and activities of various enzymes (Cheng and Cheng, 2015). Allelopathic compounds affect the activity of enzymes such as pectolytic enzyme, cellulases, catalases, peroxidases, phosphorylases, ATPases, amylases, invertases, proteinases, decarboxylases, phosphatases, nitrate reductases, etc. (Rice, 1984). Several works were published in recent years on this topic (Cheng, 2012; Mahdavikia and Saharkhiz, 2016; Venturelli *et al*., 2015). Cheng (2012) found that diethyl phthalate inhibits glutamine synthetase isoenzymes in nitrogen for nitrogen assimilation and antioxidant enzymes in greater duckweed (*Spirodela polyrhiza* L.). Venturelli *et al.* (2015) reported

that cyclic hydroxamic acid (e.g., benzoxazinoids or benzoxazinones such as DIBOA and its methoxylated analogue DIMBOA) root exudates inhibit histone deacetylases both in vitro and in vivo and exert their activity through locus-specific alterations of histone acetylation and associated gene expression in *A. thaliana*. Mahdavikia and Saharkhiz (2016) studied the effects of peppermint (*Mentha piperita* L. CV. Mitcham) allelopathic water extracts on the morphophysiological and biochemical characteristics of tomato (*L. esculentum* Mill. CV. Rio Grande). They concluded that phenolic compounds, at the concentrations of 10% (v/v) extract, showed the maximum inhibitory effect on the amount of proline, soluble sugar and starch, as well as on the activities of tomato's antioxidant enzymes such as ascorbate peroxidase, catalase, peroxidase and superoxide dismutase.

1.8.4 Interference with synthesis of plant endogenous hormones

Plant endogenous hormones, also known as "phytohormones", are generally present at very low concentrations in plant tissues. Nowadays, they are classified into nine groups (auxins, cytokinins, gibberellins, abscisic acid, ethylene, brassinosteroids, jasmonates, salicylic acid and strigolactones), but more will probably be discovered. Several allelopathic compounds are structurally similar to plant hormones (Olofsdotter, 1988) and present similar mechanisms of action. Allelochemicals are able to reduce or inactive the physiological activity of phytohormones and to induce imbalances, thereby altering the normal growth and development of plants. Liu and Hu (2001), studying the effect

of ferulic acid (FA) on endogenous hormone level of wheat seedling**,** found that, at concentrations of 2.50mmol/L, FA has led to an accumulation of indolacetic acid, gibberellin and cytokinin, but the accumulation of these four hormones induced absisic acid increment. Brun *et al*. (1992) reported that some flavonoid aglycones inhibit polar auxin transport, inducing the formation of lateral roots and the suppression of ageotropic growth. However, a few of allelochemicals have phytohormones-protecting activity, mainly indoleacetic acid (IAA), by inhibiting the oxidation of IAA, which leads to the accumulation of auxin and therefore to a greater growth of plant (Andreae, 1952; Cvikrova *et al*., 1996; Mato *et al*., 1994; Sondheimer and Griffin, 1960).

1.8.5 Interference with respiration

Allelochemicals are able to inhibit most of the processes of respiration, from the O_2 uptake to the three phases of "dark" or mitochondrial respiration: the glycolysis, in which glucose is converted to pyruvate, the Kreb's cycle, which generates CO_2 and NADH and the electron transfer in the mitochondria or oxidatitve phosphorylation, which produce a large amount of ATP (Weir *et al*., 2004). However, allelochemicals can exert also positive effects on respiration, by stimulating the CO2 production (Lodhi and Nickell, 1973). Several allelopathic compounds affect mitochondrial respiration directly. Rasmussen *et al*. (1992) pointed out the disruption of mitochondrial functions and the block of electron transport in soybean and corn seedlings caused by sorgoleone. Rye's allelochemicals, BOA and DIBOA, are reported by Burgos *et al.* (2004) to reduce number of mitochondria, protein synthesis and lipid catabolism in cucumber seedlings. Hejl

and Koster (2004) found that juglone affects root oxygen uptake due to the disruption of root plasma membrane functions and, ate the concentrations from 10 to 1000 μM, significantly reduced H^+-ATPase activity in soybean and corn. Unfortunately, many of the allelochemicals effects on mitochondrial respiration are masked by photorespiration that occur in the chloroplasts (Weir *et al*., 2004).

1.8.6 Inhibition of photosynthesis and pigment synthesis

The adverse effects of allelochemicals on photosynthesis were demonstrated, but the detail mechanisms remain unknown. Allelochemicals can affect the three main process of photosynthesis (Zhou and Yu, 2006): i) the stomatal conductance and, thus, the gas exchanges between plant and atmosphere; ii) the "light reactions" with refer to the electron transport and iii) the "dark reactions", also known as the Calvin's cycle, for the carbon reduction.

One of the most important effects of allelochemicals on plant photosynthesis is represented by the acceleration of decomposition of photosynthetic pigments, mainly chlorophyll (Pan *et al*., 2015; Patterson, 1981; Sarkar and Chakraborty, 2015). Particularly, allelochemicals can reduce chlorophyll content by enhancing stimulation of Chl degradation, inhibition of Chl synthesis or interfering with the synthesis of porphyrin, which is the precursor of Chl biosynthesis, and porphyrin precursors (Proto, Mg-Proto and pchlide), through the inhibition of Mg-chelatase, the enzyme responsible for the conversion of Proto into Mg-Proto (Zhou and Yu 2006). Besides, Meazza *et al*. (2002) reported that sorgoleone strongly inhibits the enzyme ρ-hydroxyphenylpyruvate dioxygenase (HPPD), which

catalyze the biosynthesis of carotenoids, resulting in foliar bleaching.

There are many works demonstrating a decrease in leaf stomatal conductance due to allelochemicals treatments (Mishra, 2015; Rai *et al*., 2003; Yu *et al*., 2003, 2006;). Generally, the lower stomatal conductance is correlated with a reduction in CO_2 assimilation and intracellular CO_2 concentration (Zhou and Yu 2006). However, is difficult to demonstrate the effective correlation between allelochemicals and stomatal apertures, since opening and closing of stomata are influenced by a series of several factors such as water status of plant, mineral uptake, temperature, wind and relative humidity, photoperiodism, age of leaf, leaf area index, etc.

The most documented mode of action of allelochemicals on photosynthesis is represented by the inhibition of photosystem II (PSII) (Rimando *et al*., 1998). It is a specialized protein complex, localized in the thylakoid membranes of chloroplasts, that utilizes solar energy to drive the oxidation of water and the reduction of plastoquinone (PQ). Sorgoleone is reported to act in a similar way as triazine herbicides, by disrupting the electron-transfer chain between plastoquinone A (Q_A) and Q_B at the DI protein of PSII (Czarnota *et al*., 2001). In nature, there are several allelochemicals able to inhibit PSII, such as benzoxazolin-2(*3H*)-one (BOA), cinnamic acid (CA), capsaicin, the limonoid terpene odoratol and many quinones.

1.8.7 Inhibition of protein synthesis and nucleic acid metabolism

Several allelochemicals, mostly phenolics and alkaloids, can influence the protein biosynthesis and the nucleic acid metabolism. According to Wink and Latzbruning (1995), allelochemical alkaloids can inhibit protein biosynthesis and integrate with DNA. The Authors concluded that the degree of DNA intercalation is positively correlated with inhibition of DNA polymerase I, reverse transcriptase, and translation at the molecular level. Baziramakenga *et al*. (1997), studying the allelopathic effects of benzoic, *p*-hydroxy benzoic, vanillic, cinnamic, *p*-coumaric, and ferulic acids on nucleic acid and protein level in soybean seedlings, found that the incorporation of ^{32}P and ^{35}S-methionine into proteins was reduced by all phenolic acids, except for *p*-coumaric acid and vanillic acid at 125 μM.

Allelochemical may generate ROS (reactive oxygen species), such as superoxide anions (O_2^-), hydroxyl (OH^-) or hydroperoxyl (HO_2) radicals, that are able to affect membrane permeability, acid nucleic structure and protein synthesis, leading to cell death (Weir *et al*., 2004). Allelochemicals can interfere also with the gene expression (Fang et al. 2015; He *et al*., 2012; Ma *et al*., 2015), which is often induced in the receiver plants like a form of reaction to the donor plant's attack.

1.8.8 Interference with mineral uptake

It is important to recognize that the mineral salts amount absorbed by the root surface depends on several factors as the ion concentration, the soil pH, the ion availability in the volume soil and the ion requirements for the plant (Lambers

et al., 1998). Plants response to biotic and abiotic stress by altering their membrane properties. The modification of membrane activities is strictly correlated to important physiological processes such as cell elongation, seed germination, stomata opening and mineral uptake. Many works show as allelochemicals can affect the uptake of nutrients, which can be exhibited in the form of nutrient deficiency symptoms in growing plants and reduced plant growth (Brooker *et al*., 1992; Tharayil *et al*., 2009). It is known that allelopathic inhibition of mineral uptake results from alteration of cellular membrane functions in plant roots (Balke, 1985). Allelochemicals can: i) depolarize the electrochemical potential gradient across membranes, which guide the active absorption of mineral ions; ii) inhibit the activities of Na^+/K^+ by altering the permeability of membranes and, thus, the absorption and transport of mineral ions; iii) inhibit electron transport and oxidative phosphorylation, reducing the ATP content of cells; iv) stimulate the production of superoxide, hydrogen and hydroxyl radicals that cause a direct damage to the membrane, or accumulate free radicals that increase the lipid peroxidation in the plasma membrane inhibiting the correct nutrient uptake from these roots (**Figure 1.25**).

The interference of allelopathic compounds with mineral uptake depends on the chemical characteristics of allelochemicals and environmental conditions such as soil moisture, temperature, and especially pH. Particularly, this mode of action is reported to be concentration-dependent and ion- and species-specific (Inderjit and Keating, 1999).

Besides, some allelochemicals may act as natural chelators, enhancing the availability of minerals for the plant. Several

phenolic compounds are reported to bind with Fe, Mg, Al and Ca, and thus increase the availability of phosphate which otherwise forms complex with these metal ions (Appel, 1993). In soil with high concentrations of Al, the chelation of metal cations may increase the plant's resistance (Jabran *et al*., 2013).

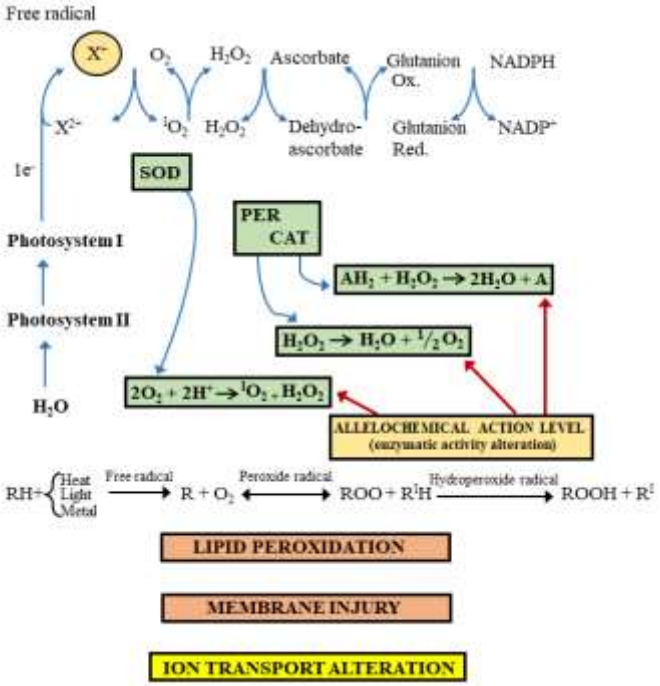


Figure 1.25 Possible allelochemical mode of action at enzymatic level with the subsequent ion uptake alteration. If the allelopathic compound causes an alteration in the activity of the enzymes implied in the oxygen metabolism, an accumulation of hydroperoxide radicals can occur. These radicals can be toxic to the membrane by a direct damage or by the formation of free radicals that induce an increase in lipid peroxidation (Sánchez-Moreiras and Weiss, 2001, Modified).

1.8.9 Interference with plant-water relationships

Many allelochemicals are able to affect water balance of the target plant by corking and clogging of xylem elements, reducing stomatal conductance of water, lowering water potential of plant and, thus, decreasing water uptake by roots. Barkosky and Einhellig (2003), investigating the effect of *p*-hydroxybenzoic acid (*p*HBA) on growth and plant-water relationships of soybean seedlings, found that, at concentration of 0.75 mM, *p*HBA had significantly lowered stomatal conductance, water potential and water use efficiency. The Authors concluded that the impact of *p*HBA on water relationships is an important mechanism of action causing a reduction in plant growth. Other phenolics compounds such as ferulic, *p*-coumaric, caffeic, hydrocinnamic, salicylic, *p*-hyroxybenzoic, gallic, and chlorogenic acids, as well as hydroquinone, vanillin, and umbelliferone altered normal water balance of target plant by reducing leaf water potential, turgor pressure, conductance, or changing tissue carbon-isotope ratio (Barkosky *et al*., 2000; Einhellig, 2004; Einhellig *et al*., 1985).

1.8.10 Inhibition of germination and interference with growth of plants

The inhibition of germination and the effects on growth of both crop and weed species are secondary expressions of primary effects such as the interferences with cell division and elongation, with cell membrane permeability and the alteration of plant respiration and photosynthesis. There is a large number of publications upon the effects of allellochemicals on growth and germination (Reigosa and Pazos-Malvido, 2007; Reigosa *et al*., 1999a; Scavo *et al*.,

2018b; Turk and Tawaha, 2003; Vokou *et al*., 2003; etc.). In recent years, new potential allelochemicals from several donor plants have been isolated and identified, and the number of works on this topic is continuously growing. Bioassays using Petri dishes are the commonest technique for proving allelopathic mechanism of action on seed germination and growth of target plants. Plant extracts are made from any part of the donor plant, commonly leaves and roots. They can be aqueous, hydroalcholic or fractions from different solvents (de Albuquerque *et al*., 2010).

In addition to seed germination, allelochemicals inhibit that of pollen grains and spores (Murphy and Aarssen, 1995; Roshchina and Melnikova, 1998; Roshchina, 2009). These three mechanisms of inhibition represent a means available for the species with high ecological potential to increase their environmental distribution.

1.9 Conclusions

The development of eco-friendly agricultural practices, which are able to increase crop production at the same time, represents the major challenge of new millennium agriculture. Allelopathic mechanisms are an important tool that may contribute to the improvement of the genetic diversity and maintenance of ecosystem stability. Allelopathy may be employed also in cropping systems for enhancing soil fertility and yields, as well as for weed and pest control through a chemical-free management. However, many aspects of the allelopathic phenomenon are still unknown. Therefore, the scientific community is called for further efforts to better understand the pathways for release of allelochemicals into the environment by the donor plant, the

effects of these inhibitory compounds on target plants physiological processes and on soil microbial population, and factors affecting their production. Only after acquiring a better knowledge of basic principles, it is possible to develop new strategies for weed and pest control in sustainable and organic farming agriculture.

2. Plant Allelochemicals: Agronomic, Nutritional and Ecological Relevance in the Soil System

This introduction was submitted on *Plant and Soil* as the following review now under revision:

- Scavo, A., Abbate, C., & Mauromicale, G. (2018c). Plant allelochemicals: agronomic, nutritional and ecological relevance in the Soil System. *Plant Soil*.

2.1 Introduction

In the last years, agriculture has dealt with an increasing environmental pollution mainly arising from two aspects linked by a common goal: the maximization of yields. On one side the irrational utilization of synthetic chemicals for weed and pest control in agroecosystems, on the other the mismanagement of fertilization, principally for what concern nitrogen. Allelopathy offered a new alternative for the development of eco-friendly agricultural practices, with the dual purpose of enhancing crop productivity and maintaining ecosystem stability (Scavo *et al*., 2018a). Allelopathy involves positive or negative effects of a plant (donor), including microorganisms, on neighbouring plants (receivers) through the release of chemical compounds into the environment, mostly in the soil. According to Inderjit and Weiner (2001), it is possible to distinguish between direct plant-plant allelopathic interference (allelopathy in the narrow sense) and indirect allelopathy. The first consist in the direct action of an allelochemical produced and released by a donor plant on receiver plants; the second represent the effects of allelochemicals on abiotic and biotic soil processes that affects other plants. Aldrich (1984) described these two

types of allelopathy as *true* and *functional* allelopathy. Most studies have focused their attention on direct allelopathy. However, it is impossible to separate direct from indirect allelopathic effects in field conditions, because many abiotic and biotic soil factors influence the fate of allelochemicals. Therefore, indirect allelopathic interactions can be considered more important in plant communities than direct ones (Inderjit and Weiner, 2001).

Allelochemicals released into the soil, can (i) degraded or transformed by soil microorganisms, (ii) induce a third species to produce another compound which interferes with donor plants and (iii) cause changes on soil abiotic factors that affect receiver plants.

It has been well documented how allelochemicals, in order to exert a phytotoxic effect, have to reach the root system of the target plant through the soil (Inderjit, 2001). However, the establishment of an allelopathic interference depends on several factors such as the concentration, the movement and the persistence of allelopathic compounds. In fact, allelochemicals are subjected to transformations by the complex chemical, physics and biological characteristics of the soil environment that determine their phytotoxic level (Blum, 2006; Cheng, 1992; Dao, 1987). Soil characteristics, especially the biological ones, vice versa may be affected by allelochemicals. Thus, a two-way relationship exists between them. However, these relationships were poorly investigated by the scientific community. Finding a linkage may help researches in studying and increasing the knowledge on the allelopathic behaviour of plants. Allelochemicals released into the rhizosphere exert a significant impact on nutrient availability, dynamics and uptake by plant. A broader

knowledge of the effects of plant allelochemicals on mineral nutrient soil cycles, heavy metal detoxification and nutrient solubility can enhance nutrient use efficiency through a reduction of their losses and develop a more efficient and sustainable fertilization technique.
This review focuses on the interactions between plant allelochemicals and soil physics, chemical and biological characteristics from an agronomic and ecological point of view, by reporting the literature available on this topic and pointing out both positive and negative relationships affecting allelochemicals phytotoxicity. Moreover, a balance of allelochemicals input and output in soil was realized for the first time. Besides, we highlighted the exudation process of allelochemicals and the transport mechanisms across plasma membranes. The influence of plant allelochemicals on mineral nutrition and the most important plant-soil-microorganism interactions were also discussed.

2.2 Balance of allelochemicals in soil

Donor plants release allelochemicals into the environment through volatilization from living parts of the plant, leaching from plant foliage, decomposition of plant material and root exudation (Scavo *et al*., 2018a). Except for volatilization, all the other pathways release allelopathic compounds into the soil. Once released by the donor plant, allelochemicals enter a complex plant-soil system in which diverse factors affect their availability, and consequently their effective influence on target plants (Blum *et al*., 1999; Kruse *et al*., 2000). This plant-soil system, in turn is influenced by several meteorological factors, demonstrating how complex the phenomenon of allelopathy is. As shown in **Figure 2.1**, in

addition to the chemical nature of the allelochemical produced, the phytotoxic activity of allelochemicals in soil is affected by climatic conditions (e.g. solar radiation, temperature, rainfall), soil factors (e.g. texture, pH, ion-exchange capacity, organic matter content, nutrient dynamics, moisture content and microbial ecology) and plant factors of both the donor and target plants (e.g. species, botanical variety, growth stages, plant parts, etc.).

In order to be adsorbed by the roots of target plant, allelochemicals may be present in soil solution (Kobayashi, 2004). Therefore, the key factor determining the phytotoxic activity of allelochemicals is their concentration in soil water. However, similar to herbicides, in the soil environment these compounds are subjected to retention, transformation and transport processes (Cheng, 1992; Weidenhamer, 1996). Retention is a physical process that consists in the interaction (frequently described as adsorption or sorption) between allelochemicals and soil particles (e.g., clays, Fe-, Al-, and Mn-sesquioxides, organic matter), water and/or air (Bezuidenhout and Laing, 2006; Cheng, 2010). In many cases, it is a dynamic and reversible process implying the mobility of allelochemicals in soil. Transformation is a positive or negative biochemical process, mainly operated by soil microorganisms, consisting in allelochemicals' conversion into more active, less active or entirely inactive compounds. This process leads to a reduction of the amount of the original allelochemical available for transport (Cheng, 1992). Transport, which consists in the allelochemical's movement in a soil, is strictly affected by the retention and transformation processes. The interaction of these processes is governed by the chemical nature of the allelopathic

compound, the organisms involved, the properties of the soil and the environmental conditions.

Understanding the concentration of available allelochemicals in soil is important, as well as for bioherbicides. The conceptual framework of **Figure 2.2** shows how the cycle of allelochemicals in soil dynamic is. Similar to herbicides, allelochemicals are continually removed and/or immobilised from the soil solution by leaching, microbial breakdown, adsorption to soil particles and plant uptake (Cheng, 1995; Inderjit *et al*., 2001; Weidenhamer, 1996). However, the behaviour of allelochemicals in soil is more complex than that of herbicides, because the first are continuously release from the donor plant with significant differences within plant organs (Abu-Romman, 2016; Iqbal *et al*., 2002; Suksungworn *et al*., 2016) and growth stages (Aslam *et al*., 2016).

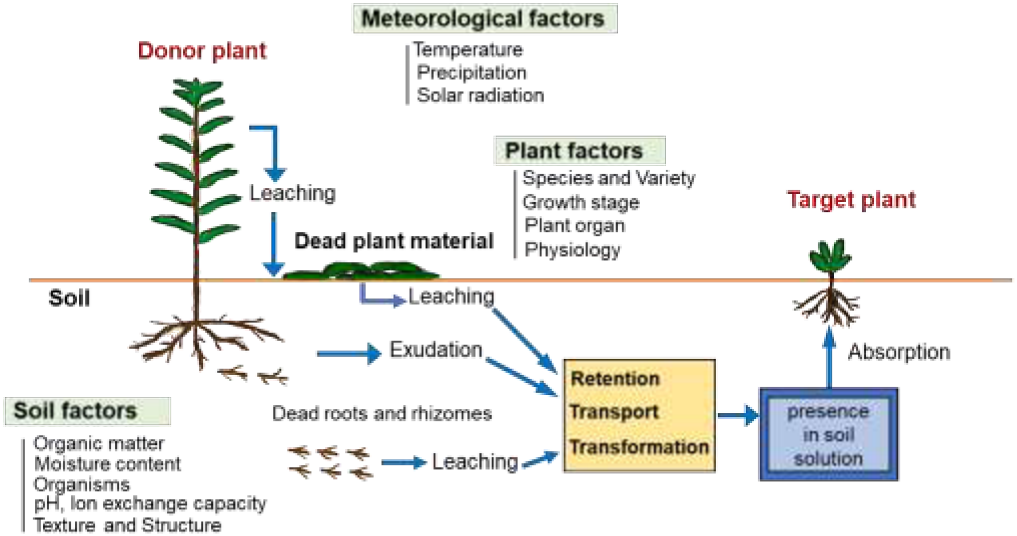


Figure 2.1 Influence of meteorological, soil and plant factors on the phytotoxicity of allelochemicals in soil (modified from Kobayashi, 2004). Different kinds of both meteorological and plant factors affect the quantity and quality of allelochemicals released by the donor plant. Once released into the soil system, several soil factors influence the retention, transport and transformation processes of allelochemicals in the soil and, thus, their presence in the soil solution in order to be absorbed by the target plant.

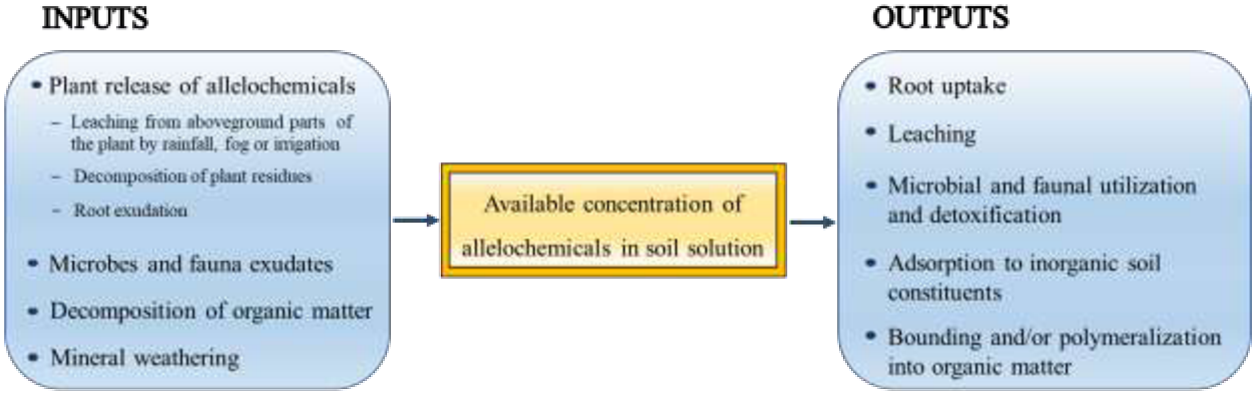


Figure 2.2 Balance of allelochemicals inputs and outputs in soil affecting their bioavailability in the soil solution and phytotoxic activity.

2.3 The root exudation

The root systems have a multitude of functions including anchorage of the plant and absorption of water and nutrients. In addition to these main functions, roots also are a site of photoassimilate storage and carbon reserves, phytohormone synthesis (e.g. auxins, cytokinins, abscisic acid, gibberellic acid, ethylene), synthetic activities (e.g. nitrogen fixation, synthesis of organic acids, etc.), and exudation of metabolites (Blum, 2006; Osmont *et al*., 2007). Over 10.000 allelochemicals have been estimated to be produced by higher plants, with a significant variability in their activity and mode of action in target plants (Weston *et al*., 2012). Living roots of many weed and crop species continuously produce and secrete both low- and high-molecular weight compounds into the rhizosphere in response to biotic and abiotic stresses (Bertin *et al*., 2003). The chemicals secreted into the soil by roots are broadly referred to as root exudates (Walker *et al*., 2003). Their quantity and quality are function of the plant species, cultivar, plant development stage, and environmental stress factors (Badri and Vivanco, 2009; Uren, 2000). Aulakh *et al*. (2001) found that rice exudation rates are, in general, lowest at seedling stage, increased until flowering but decreased at maturity. Sorghum and wheat root exudations decrease with plant age and increase when the soil is stressed by compaction, drought, and low nutrient supply (Weil and Brady, 2017). Lucas Garcia *et al*. (2001) reported a positively correlation between root exudation and root growth, indicating that actively growing root systems secrete more exudates. Besides, the nature of root surface morphology (e.g. suberized or unsuberized, with or without mycorrhizal hyphae, thickness of periderm, quantity and location of root

hairs, etc.) as well as the root system architecture (tap root system of dicotyledonous species or fibrous one of monocotyledonous plants, amount of root branching, number of lateral roots, etc.) are factors involved in determining the composition of exudates both quantitatively and qualitatively (Badri and Vivanco, 2009; Blum, 2006). Nevertheless, root exudation depends also on the root zone. The zone immediately behind the root tip is considered to be the major site of exudation (Pearson and Parkinson, 1961). The root cap (Curlango-Rivera *et al*., 2013; Hawes *et al*., 2012; Pina *et al*., 2016) and root hair cells (Czarnota *et al*., 2003a) are the root cells primarily involved in root exudation, followed by cortex and stellar cells (Pineros *et al*., 2000). It is clear that all these factors are strictly correlated, because the type of roots depends on plant age, season and soil conditions (e.g. texture, structure, temperature, water content, pH, etc.).

2.3.1 The rhizosphere and the importance of root exudates

The narrow region of soil matrix immediately surrounding the root and in which living roots secrete an enormous range of compounds is called "rhizosphere". This term was coined by Lorentz Hiltner (Hartmann *et al*., 2008) to describe the portion of soil where microorganism-mediated processes are under the influence of the root system (Berg and Smalla, 2009). Since that time, many definitions of rhizosphere have been suggested (Kennedy, 1998; McNear, 2013; Rovira and Davey, 1974; Uren, 2000). The rhizosphere, which can extend from about 0 to 2 mm or more (depending on the plant type, soil moisture and texture, and presence of mycorrhizae) away from the root surface, includes three zones (Lynch, 1987) (**Figure 2.3**): endorhizosphere, rhizoplane and

ectorhizosphere. The endorhizosphere refers to the internal zone and includes the apoplastic space between the root cortex and endodermis colonisable by microorganisms. Because the rhizosphere is defined as external to the root, Kloepper *et al*. (1992) and York *et al*. (2016) consider the term endorhizosphere improper. The rhizoplane, first defined by Clark (1949), represents the root surface including epidermis and mucilage. The ectorhizosphere is the soil layer surrounding the root.

The rhizosphere is a biologically active zone, influenced by root metabolic activities, strongly populated by microorganisms. For these reasons, its chemical, biological and physics characteristics differ drastically from those of bulk soil. It presents higher levels of cation exchange capacity, exchangeable base cations, base saturation, organic matter and carbon dioxide respect with the bulk soil (Gobran et al. 1998). The rhizosphere is characterized by gradients of its properties which change both in time and space.

The ubiquitous phenomenon consisting in the loss of carbon-containing compounds from plant roots into the rhizosphere is referred to as rhizodeposition (Doornbos *et al*., 2012; Jones *et al*., 2004). Root-derived compounds, generally called rhizodeposits, have been classified based on their mode of excretion and chemical composition into five predominate categories (Rovira, 1969):

1) root exudates: sugars, amino acids, organic acids, vitamins and hormones leaked through a passive process;

2) secretions: polymeric carbohydrates, enzymes and secondary metabolites secreted with the involvement of energy;

3) mucigel: newly removed cells of cellulose, pectin, starch, and lignin, secreted by the root cap as result of abrasive forces of the root movement through the soil;
4) lysates: shedding, wall and contents of sloughed-off cells as well as whole roots;
5) gases: carbon dioxide, ethylene, etc.

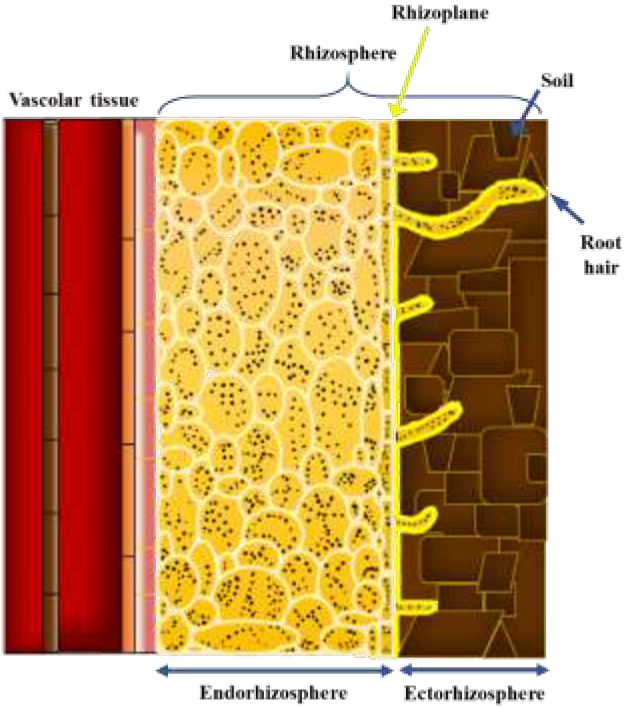


Figure 2.3 Scheme of the rhizosphere showing its three sections: the endorhizosphere, the rhizoplane and the ectorhizosphere (modified from McNear, 2013).

Therefore, root exudation is part of the rhizodeposition process. In addition to carbon-containing compounds derived from products of photosynthesis, plant roots release non-carbon-containing compounds such as the ion H^+, inorganic ions, water and electrons, although in lower quantities (Uren, 2000). However, it is the release of organic carbon that produces the most dramatic changes on the physics, biological and chemical nature of the soil (Jones *et al*., 2009). On average, 30 ± 60% of photosynthesized C is allocated by plants to the roots (Marschner, 1995). It is estimated that about 60% of photosynthesized C is transferred by annual plants to the roots, while up to 70% is allocated by woody plants. According to Marschner (1995), nearly 5 to 21% of all photosynthetically fixed carbon is released into the rhizosphere through root exudates by higher plants.

Root exudation involves two different processes: the excretion and the secretion (Bais *et al*., 2004a). The first consists in the release of metabolic wastes and mixture of compounds with unknown functions. The second, on the contrary, refers to substances with known functions. Root secretions are probably involved in external processes (e.g. nutrient acquisition), while excretions influence internal metabolic processes (Uren, 2000). Root exudates, which are a part of rhizodeposits, are generally grouped into low- and high-molecular weight compounds. Low-M_r compounds include amino acids, organic acids, sugars, phenolics, and other secondary metabolites and represent the majority of root exudates, whereas high-M_r compounds are represented by proteins, terpenoids, vitamins and polysaccharides (primarily mucilage) (Badri and Vivanco, 2009; Bais *et al*., 2006). An exhaustive list of root exudates has been well

reviewed in previous works (Bertin *et al*., 2003; Rovira, 1969; Uren, 2000). In literature, many evidences have been reported about the root exudation of allelochemicals, suggesting how this pathway of release represents the largest source of plant allelochemicals into the rhizosphere. Major allelochemicals released by plant through root exudation are listed in **Table 2.1**.

2.3.2 Cellular transport of root exudates and allelochemicals

Plant possess specific transport mechanisms within specialized plant cells to move root exudates as allelochemicals into the rhizosphere (Grotewold, 2004). For long time, root exudation has been considered only a passive process. Nowadays, we know that plants are able to actively secrete metabolites into the environment. The three passive pathways by which plant living roots release secondary metabolites out of the cell are diffusion, ion channels and vesicle transport (Bertin *et al*., 2003; Neumann and Romheld, 2001;), while the active secretion process involves the utilization of specific membrane-bound transport proteins embedded in the plasma membrane (**Figure 2.4**).

Table 2.1 List of major plant allelochemicals released into the rhizosphere through root exudation.

Chemical class	Allelochemicals	Donor plants	References
Alkaloids	α-tomatine	*Solanum lycopersicum* L.	1
	Hordenine, gramine	*Hordeum vulgare* L.	2
	Emetine	*Cephaelis ipecacuanha* (Brot.) L. Andersson	3
	Nicotine	*Nicotiana tabacum* L.	4
	8-hydroxyquinoline	*Centaurea diffusa* Lam.	5
Amino acids	Mugineic acid phytosiderophores	*Triticum aestivum* L., *T. durum*,	6
Benzoquinones	Sorgoleone, 5-ethoxysorgoleone, 2,5-dimethoxysorgoleone	*Sorghum halepense* (L.) Pers., *S. sudanense*, *S. vulgare*, *S. bicolor*, *S. bicolor* x S. *sudanense*	7
Benzoxazinoids	DIBOA (2,4-dihydroxy-(2*H*)-1,4-benzoxazin-3(4*H*)-one), DIMBOA (2,4-dihydroxy-7-methoxy-(2*H*)-1,4-benzoxazin-3(4*H*)-one), MBOA (6-methoxybenzoxazolin-2-one), BOA (benzoxazolin-2(3*H*)-one)	*Secale cereale* L., *T. aestivum*, T. *durum*, *T. spelta*, *Zea mays* L., × *Triticosecale* Wittm.	8
Coumarins	Cinnamic acid	*Cucumis sativus* L.	9
	Scopoletin, esculetin, coumarin	*Avena sativa* L.	10
	Umbelliferone	*A. sativa, Medicago sativa* L.	10, 11
	Esulone A, kansuinine B, 3,3′,4-tri-*O*-methylellagic acid	*Euphorbia esula* L.	12

Diterpenes	Momilactone A, momilactone B	*Oryza sativa* L.	13
Flavonoids	Quercetin	*Fagopyrum esculentum* Moench, *Miscanthus x giganteus*, *Lotus pedunculatus* Cav., *Glycine max* (L.) Merr., *M. sativa*, *Z. mays*	11, 14, 15, 16, 17, 21
	Luteolin, apigenin, luteolin- and apigenin derivatives	*M. sativa*, *L. pedunculatus*, *Pisum sativum* L., *Miscanthus x giganteus*	11, 15, 16, 18
	Kaempferol	*L. pedunculatus*, *M. sativa*	11, 16
	Naringenin	*L. pedunculatus, P. sativum*, *M. sativa*	11, 16, 18
	(±)-catechin	*Centarurea maculosa* Lam., *C. stoebe*, *C. diffusa*, *L. pedunculatus*, *Z. mays*	16, 19, 20, 21
Hydroxycinnamic acids and simple phenols	*p*-hydroxybenzoic, vanillic, *p*-coumaric, syringic, ferulic, caffeic	*A. sativa*, *O. sativa*, *T. aestivum*, *T. durum*,	10, 22
Isoflavonoids	(6a*R*,11a*R*)-maackiain, (6a*R*,11a*R*)- trifolirhizin, barbacarpan, formononetin, irilone	*Trifolium pratense* L.	23
Napthoquinones	Juglone	*Juglans nigra* L., *J. cinerea*, *J. regia*	24
Lignans	Pinoresinol	Several woody species (e.g. *Fraxinus excelsior* L.)	25
Quassinoids	Ailanthone, 2-dihydroailanthone	*Ailanthus altissima* (Mill.) Swingle	26
	Quassin, neoquassin	*Quassia amara* L., *A. altissima*	26
	Artemisinin	*Artemisia annua* L.	27

Sesquiterpene lactones	Dehydrocostus lactone, custunolide, tomentosin	*Helianthus annuus* L.	28
	Cnicin	*C. maculosa*	29
Sterols	Campesterol	*Arachis hypogaea* L.	30
	Stigmasterol	*A. hypogea*, *L. esculentum*,	30, 1
	β-sitosterol	*Sedum alfredii* Hance	31
Tannins	Ellagic acid	*E. esula*	12,
	Gallic acid	*C. sativus*, *F. esculentum*, *O. sativa*, *Aegiceras corniculatum* (L.) Blanco, *Miscanthus x giganteus*	9, 14, 15

1: Rial *et al*., 2018; **2**: Bouhaouel *et al*., 2018; **3**: Itoh *et al*., 1999; **4**: Zhao *et al*., 2013; **5**: Tharayil *et al*., 2009; **6**: Sugiura and Nomoto, 1984; **7**: Czarnota *et al*., 2003; **8**: Belz and Hurle, 2005; **9**: Yu *et al*., 2003; **10**: Pérez and Ormeño-Nuñez, 1991; **11**: Peters and Long, 1988; **12**: Qin *et al*., 2006; **13**: Kato-Noguchi *et al*., 2010; **14**: Kalinova *et al*., 2007; **15**: Técher *et al*., 2011; **16**: Steele *et al*., 1999; **17**: Graham, 1991; **18**: Makarova *et al*., 2016; **19**: Tharayil and Triebwasser 2010; **20**: Tharayil *et al*., 2009; **21**: Kidd *et al*., 2001; **22**:Wu *et al*., 2001; **23**: Liu *et al*., 2013; **24**: Bertin *et al*., 2003; **25**: Caligiani *et al*., 2013; **26**: Heisey, 1996; **27**: Li *et al*., 2014; **28**: Raupp and Spring, 2013; **29**: Kelsey and Locken, 1987; **30**: Thompson and Hale, 1983; **31**: Luo *et al*., 2017.

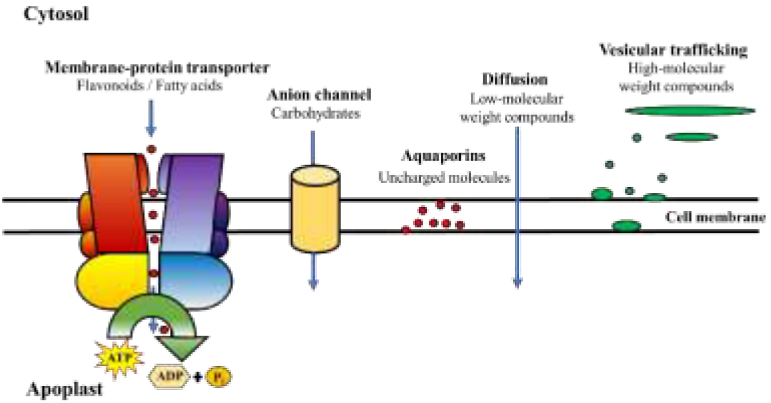


Figure 2.4 Pathways of release of root exudates into the rhizosphere through the plant cell membrane. Plants living roots can release secondary metabolites out of the cell primarily through three passive processes (diffusion, ion channels and vesicle transport) and an active secretion pathway involving the utilization of specific membrane-bound transport proteins (modified from Badri and Vivanco, 2009).

A compound, in order to be released from a cell, needs to cross at least one membrane and the cell wall (Weston et al., 2012). The cell membrane, called also plasma membrane, consists of an amphipathic phospholipid bilayer with embedded proteins and carbohydrate groups attached to lipids and proteins forming glycolipids or glycoproteins, respectively. Generally, cell membranes are highly impermeable to charged inorganic and organic substances such as ions and polar solutes (Blum, 2006). This prevents them from crossing plasma membranes unassisted. However, nonpolar and uncharged molecules, which are lipophilic compounds, are able to readily pass the lipid bilayer. Since most of allelochemicals are large charged molecules and ions, they are too polar to directly diffuse through the cell

membrane. Therefore, they need other transport pathways such as subcellular vesicles, channels and transporters, also known as carriers (Taiz and Zeiger, 2002; Walker *et al*., 2003).

Plants present membrane-protein transporters, also called carriers, which allow to actively secrete larger root exudates such as flavonoids and fatty acids. Among these membrane proteins there are the ATP-binding cassette (ABC) transporters, the multidrug and toxic compound extrusion (MATE) transporters, the aluminium-activate malate transporter (ALMT), and the major facilitator superfamily (MFS) (Weston *et al*., 2012). Membrane-protein transporters represent the most important secretion process involving the exudation of plant allelochemicals from root into the rhizosphere. Some examples are provided by benzoxazinoids from the Poaceae family, artemisinin, juglone and several phenolics and alkaloids. The intracellular transport of flavonoids, one of the most important chemical class comprising allelochemicals, and their exudation into the rhizosphere can occur via transporters of the ABC (Buer *et al.*, 2007) or MATE (Zhao and Dixon, 2009) families. The ABC transporters are an ancient superfamily of proteins, classified into 13 subfamilies, which can be found in all phyla (Higgins, 1992). They use the hydrolysis of ATP to power the transport of a broad range of compounds across cell membranes. Most of them are localized in the vacuolar membrane and play a wide variety of physiological roles such as the root exudation (Badri *et al*., 2008; Sugiyama *et al*., 2007). Sugiyama *et al*. (2007) indicated an ATP-dependent manner transport for the isoflavonoid ginestenin from soybean root vesicles. Many poacee species utilize YS1

(yellow stripe 1) and YS1-like (YSL) protein transporters to secrete and transport mugineic acid family phytosiderophores under Fe-deficiency (Senoura *et al*., 2017). MATE transporters, which are widely distributed in all kingdoms of living organisms, use an electrochemical gradient of H^+ or Na^+ to transport secondary metabolites from cells (Omote *et al.*, 2006). They are responsible for multidrug resistance (e.g. aluminium or tetramethylammonium tolerance) through the exudation of toxic metabolites and xenobiotics from cells. The ALMT genes are typical of plants and confer aluminium resistance by facilitating the efflux of malate anions (Ryan *et al*., 1995). Sasaki *et al*. (2004) found that the wheat gene ALMT1 and heterologous expression of ALMT1 in *Xenopus* oocytes, rice and cultured tobacco cells, encode a membrane protein for the efflux of malate from the root apices, avoiding aluminium toxicity in acid soils. The MFS proteins are the largest family of secondary transporters found in all phyla (Yan, 2013). They transport a wide spectrum of compounds (e.g. ions, carbohydrates, lipids, amino acids and peptides, nucleosides, etc.) across membranes.

Generally, the transport of high-M_r compounds by root cells is mediated by vesicles or specialized organelles (Battey and Blackbourn, 1993). Vesicle transport of secondary metabolites such as allelochemicals from the site of synthesis to storage compartments and to cell membrane for efflux is well characterized (Field *et al*., 2006; Robatzek, 2007). Probably, it is related to the necessity of separating from the cytoplasm and safely transporting allelochemicals, most of which are cytotoxic for the host cells (Weston *et al*., 2012). Flavonoids (e.g. luteonin, catechin, etc.), synthesized on the surface of endoplasmatic reticulum (ER), are separate from

the ER and transported by ER-originating vesicles that fuse to the cell membrane and release their contents (Walker *et al*., 2003). Bock *et al*. (2002) suggested a vesicle-mediated mechanism for the transport of the alkaloid berberine in different *Berberis* species and *Papaver somniferum* L. Moreover, it is thought that sorgoleone, the toxic benzoquinone produced by *Sorghum* spp., is synthesized on ER and Golgi bodies, transported through subcellular trafficking or to the plasma membrane for efflux, and exuded from living root hairs (Czarnota *et al*., 2003; Grotewold, 2001; Weston *et al*., 2012). Besides, vesicle transport is a mechanism of defence for plants, which react to pathogen infection by trafficking of antimicrobial compounds to the site of infection through subcellular-membrane vesicles and organelles such as the Golgi or ER-vesicles.

Membrane-protein transporters and subcellular vesicles are the most important mechanisms implicated in the translocation of allelochemicals across cell membranes into the rhizosphere. In addition to these processes, plants possess other passive pathways for the release of root exudates and secondary metabolites, even if little utilised because most of allelochemicals are complex-charged molecules. Diffusion is a passive process that consists in the transport of low-M_r compounds and uncharged molecules such as sugars, amino acids and phenolics through cell membranes (Sander and Bethke, 2000). It involves electrochemical positive concentration gradients between the cytoplasm of root cells and the soil which support the release of low-M_r compounds that, in the cytoplasmic pH of root cells, are negatively charged, and the uptake of cations from the outside of the cell (Bertin *et al*., 2003). Another diffusion process is represented

by aquaporins, also known as water channels. They are integral membrane proteins that facilitate the transport of water, and in some cases, small neutral molecules such as glycerol and urea (aquaglyceroporins) across cell membranes. Aquaporin permeability is regulated by phosphorylation (Assmann and Haubrick, 1996).
Ion channels are membrane protein complexes that allow the diffusion of ions and charged molecules across the cell membrane. This pathway of release does not need the input of ATP, but works through an electrochemical gradient. Ion channels are ion selective because are able to discriminate between size and charge of molecules. Anion channels are involved in the released of carbohydrates, specifically organic acids such as citrate, malate or oxalate into the rhizosphere (Jones and Darrah, 1995; Walker *et al*., 2003). Often organic acids, which are present at high concentrations in the cytoplasm of root cells, cannot diffuse across cell membranes under Al^{3+} toxicity. For example, maize (*Zea mays* L.) and wheat (*Triticum aestivum* L.) exudate citric, malic, and related organic acids through ion channels in response to high Al^{3+} concentrations (Ma *et al*., 2001).

2.4 Interactions between allelochemicals and soil characteristics

The behaviour of allelochemicals in soil is of central importance in determining their phytotoxic effects, particularly the adsorption-desorption balance, which influences the concentration of allelochemicals in soil water (Kobayashi, 2004). This balance is very dynamic and reversible. It is strictly influenced by soil physics (texture, structure, organic matter content, moisture and aeration),

chemical (reaction, ion exchange capacity, nutrient dynamics, O_2 and CO_2 concentrations), and biological characteristics (soil microorganisms). In the same way, plants can modify their rhizosphere characteristics through ion, H_2O and O_2 uptake and rhizodeposition (Darrah, 1991). A summary on the effects of soil characteristics on allelochemicals phytotoxicity is reported in **Table 2.2**. It is important to underline that the level of phytotoxicity is not affected by a single soil characteristic. They, on the contrary, are closely linked to each other and exert a multiple-effect on retention, transport and transformation processes of allelochemicals in soil.

2.4.1 Soil texture

Soil texture refers to the size of particles that make up a soil and to the particle-size distribution into textural classes. It has a large influence on water holding capacity, soil moisture, aeration and temperature, soil reaction, soil microbial communities, ion exchange capacity, nutrient retention, soil porosity, etc. Clay minerals such as kaolinites, vermiculites, smectites, etc. are the active portion of a soil, because chemical reactions occur on their surface. Soil texture strictly affects allelochemicals leaching and, consequently, their phytotoxic effects (de Albuquerque *et al*., 2011). Inderjit and Dakshini (1994) reported different amounts of *Pluchea lanceolata* (DC.) Oliv. & Hiern leaf leachates in four soil types (sandy loam, clay loam, silty loam, and sand). Jennings and Nelson (1998), studying in columns the influence of soil texture on alfalfa autotoxicity, found that chemicals moved through the Sarpy fine sandy loam (mixed, mesic Typic Udipsamments) faster than through the Carlow silty clay

loam (fine, smectitic, mesic Vertic Endoaquulls). According to del Moral and Muller (1970), *Eucalyptus camaldulensis* Dehnh. is more toxic on fine than on coarse soils. This is probably due to the high evaporation that concentrates allelochemicals near the soil surface, and to the low infiltration that prevents allelochemicals from leaching out of the rooting zone (Noy-Meir, 1973). Moreover, Rietveld *et al*. (1983) stated that in well-aerated and drained sandy soils, the microbial degradation of toxic compounds operated by aerobic microorganisms is accelerated. Goslee *et al*. (2001) reported that *Acroptilon repens* (L.) DC. became dominant faster and reached a higher proportion of the total biomass on fine- than on coarse-textured soils. El-Darier *et al*. (2014) found that *Medicago sativa* L. crude powder reduced the accumulation of total stem, leaf and root dry matter of *Vicia faba* L. more in clay than in sandy soils. On the contrary, Bouhaouel *et al*. (2018) pointed out that *Hordeum vulgare* L. subsp. v*ulgare* allelochemicals exuded by roots were more toxic in a sandy substrate. Also Shaukat *et al*. (2003) reported higher phytotoxic effects of *Conyza canadensis* L. shoot aqueous extracts in amendment sandy soils, followed by loamy sand and sandy loam soils, probably to the minimal adsorption on soil particles and to the low microbial and chemical degradation. According to Inderjit and Asakawa (2001), the higher inhibitory activity of plants in sandy soil is due to the nutrient deficiency characterizing these kinds of substrates, which stimulates the production and release of allelochemicals.

Results about the influence of soil texture on allelochemicals phytotoxicity are contradicting each other, since some authors found higher inhibitory activity in clay soils while

others in sandy substrates. We consider the former hypothesis more realistic, because the leaching of allelochemicals is the most important factor affecting their phytotoxic behaviour. However, most of experiments in literature were carried out in artificial substrates under laboratory conditions. The setup of long-term field experiments plays a key role for a better understanding on the effects of soil texture on allelochemicals phytotoxic potential.

2.4.2 Soil structure

Soil structure is the arrangement and organization of soil particles in the unit of soil particle density. Pagliai and Vignozzi (2002) defined soil structure as "the combination of different types of pores". Soil particles, particularly silicate clay, tend to bind together in aggregates. Soil aggregation generates porosity variability and regulates the ratios between solid, liquid and gaseous soil phases. Many agronomic functions depend on pore size distribution and shape (Ringroase-Voase and Bullock, 1984). Rhizodeposition promotes the formation of aggregates both directly and indirectly. Directly thanks to the adsorption of rhizodeposits (e.g. ions such as Ca^{2+}, Fe^{2+}, Al^{3+}, K^{+}, as well as mucillages and several organic acids) with colloids, and indirectly since root exudates are used as food by microorganisms, which play a key role in the aggregation process. Likewise, the availability of allelochemicals in the soil is affected by soil structure (Schmidt and Ley, 1999), mainly the retention process. A well-structured soil, for example, has a high cation exchange capacity, which decreases the leaching of allelochemicals operated by water. Size and shape of soil pores strongly affect allelochemicals adsorption. Jardine *et*

al. (1990), in fact, stated that solute concentrations in general is correlated to pore size. In particular, micropores have a greater retention capacity than meso- and macropores (Blum, 2006). Besides, soil structure can affect the transformation process carried out by microbial communities since it regulates soil porosity, the equilibrium between soil liquid and gaseous phases, as well as soil organic matter content. Moreover, the oxidation/reduction potential, also known as redox potential, strictly depends on the oxygen level in soil (Weil and Brady, 2017). Nevertheless, soil structure affects the release and spatial disposition of allelochemicals in soil by influencing the depth that roots can explore.

Although soil structure is a soil characteristic of central importance, its interactions with allelochemicals have been little investigated by the scientific community. Therefore, major efforts are requested by scientists to investigate the relationship between size and distribution of pores and channels with retention and transport processes of allelochemicals in soil.

Table 2.2 Positive (+) and negative (–) effects of soil characteristics on the allelochemicals phytotoxicity in soil.

Soil characteristic		Allelochemicals phytotoxicity	Type of interaction	References
Ion exchange capacity	High	–	A high ion exchange capacity means a higher retention of allelochemicals and, thus, a less bioavailability.	Belz *et al*., 2009; Inderjt and Bhowmik, 2004
	Low	+		
Organic matter content	High	–	Soil organic matter bonds allelochemicals and decreases their bioavailability and phytotoxicity. Moreover, it regulates soil pH, increase soil temperature and facilitates the chemical degradation carried out by microorganisms.	Dalton *et al*., 1989; Hess *et al*., 1992; Horrie *et al*., 1989
Reaction as pH	> 7	+	The different behaviour of plant allelochemicals in response to soil pH is explained by their different chemical structure and protonation status. Soil reaction, in fact, strongly affects the chemical transformation of allelochemicals into more or less toxic compounds.	Batish *et al*., 2007
	< 7	+		Norouzi *et al*., 2015
Structure	Well-structured	+	A well-structured soil presents a high porosity, an equilibrium between soil liquid and gaseous phases, and a high soil organic matter content, thus increasing the transformation process operated by microorganisms and decreasing allelochemicals leaching. Soil structure allows	Schmidt and Ley 1999

			the contact between allelochemicals and target plant roots due to the major spatial movement of roots.	
Texture	Clay	+	Clays, by decreasing water infiltration and increasing cation exchange capacity, reduce allelochemicals leaching. In sandy soils, aerobic microorganisms rapidly degrade allelochemicals.	del Moral and Mulle,r 1970; Goslee *et al*., 2001; Noy-Meir, 1973; Rietveld *et al*., 1983
	Sandy	+	Commons nutrient deficiencies characterising sandy-aerated soils stimulate allelochemicals production.	Inderjit and Asakawa, 2001

2.4.3 Soil organic matter content

Soil organic matter (SOM) represents the organic fraction of the soil solid phase, comprising about 2-3% of the total weight. SOM includes a complex mixture of many substances that, for simplicity, can be classified into five main classes: 1) *edaphon* (all the living organisms), 2) "fresh" organic matter, (e.g. plant litter and residues, root and leaf exudates, remains of soil organisms), 3) "labile" (= *easily altered*) humus (SOM with a carbon/ nitrogen ratio (C/N) of 15-20, 4) humus (SOM in which the humification process completely occurred and characterised by a C/N≈10).

Many evidences are reported about the influence of SOM on the availability of allelochemicals in soil, particularly on the adsorption-desorption process (Fageria, 2012; Inderjit, 2001; Vogel and Dawson, 1985). SOM, in fact, thanks to its high surface area and negative surface charges, contributes to enhance the cation exchange capacity in the same way as clays. Humin, humic and fulvic acids are estimated to account from 20 to 80% of the cation exchange capacity (Wagner and Wolf, 1998). From one side SOM can bond allelochemicals making them inactive and/or decreasing their bioavailability and phytotoxicity (Dalton *et al*., 1989). For example, Horrie *et al*., (1989) observed that *Cytisus scoparius* (L.) Link allelochemicals concentration was higher in soils with low SOM, inhibiting lettuce seedling emergence, while decreasing in soils with high SOM. In hot-semiarid soils, which generally present a low SOM (<1%), allelochemicals adsorption is low, promoting the spread and diffusion of allelopathic plants such as *Rhaponticum repens* (L.) Hidalgo and *Centaurea maculosa* Lam. (Grossl, 2008). Kulmatiski and Beard (2006) indicated that *C. maculosa* diffusion in the

field was lowered by adding activate carbon into the soil thanks to the sequestration of (±)-catechin. The activity of sorgoleone was reported to be decreased due to the high retention to SOM (Hess *et al*., 1992). On the other side allelochemicals, as well as heavy metals and cations such as Fe^{3+}, Al^{3+}, Mn^{2+} and Ca^{2+}, can be chelated by SOM in order to prevent their oxidation and increase their efficiency (Cheng, 1989; Jabran *et al*., 2013; Marschner, 1995). Moreover SOM, particularly humus, tends to give a darker colour to O and A horizons, which allows to increase the solar radiation absorption and, thus, the soil temperature (Blum, 2006; Fang *et al*., 2005). A higher soil temperature means a major microbial activity and, consequentially, more rapid transformation processes of allelochemicals. SOM exerts also a buffer power on soil pH by avoiding excesses of acidity or alkalinity (Jansen van Rensburg *et al*., 2009; McCauley *et al*., 2009).

2.4.4 Soil reaction

Soil reaction represents the degree of acidity, alkalinity or neutrality of the soil aqueous extract and it is expressed as pH. Soil reaction can affect the growth of both crop plants and weeds (Joe and Allen, 1980), the life of microorganisms (Rousk *et al*., 2009) and, mainly, the availability of nutrients (Härdtle *et al*., 2004). The influence of soil pH on allelochemicals is widely reported in literature (Batish *et al*., 2007; Borek *et al*., 1994; Norouzi *et al*., 2015). Soil reaction strictly affects the chemical transformation of allelochemicals into more or less toxic compounds. Borek et al. (1994) found that the enzymatic decomposition of sinigrin, a well know glucosinate produced by *Brassica* spp., operated

by the enzyme myrosinase (β-thioglucoside glucohydrolase) was strongly dependent on soil reaction. In particular, allylnitrile production was highest at pH 3.0, while at higher pH values (≈6.0) allyl isothiocyanate was the only sinigrin decomposition product. Also the degradation processes of benzoxazinoids in soil are under pH control. Niemeyer et al. (1982) documented an asymmetric bell-shaped curve of DIMBOA decomposition rates under a pH range, with a maximum around pH 9.0. Dayan (2006) reported a higher production of sorgoleone in *Sorghum bicolor* (L.) Moench seedlings grown in buffers as the pH decreased. Batish et al. (2007), studying the effect of *Chenopodium murale* L. residues on growth, nodulation and macromolecule content of chickpea (*Cicer arietinum* L.) and pea (*Pisum sativum* L.), pointed out that the pH of the residue-amended soil changed from neutral (6.85) to slightly alkaline (7.47) with 5-40 g residue kg^{-1} soil. The observed reduction in root, shoot length and dry matter accumulation in amended soils was accompanied to an increase in the SOM, electrical conductivity and available nitrogen. Norouzi *et al*. (2015) reported that the allelopathic effects of powdered below- and aboveground organs of alfalfa (*M. sativa*), sorghum (*S. bicolor*) and tobacco (*Nicotiana tabacum* L.) on several weeds increased in response to lower soil pH levels. At the same time, plants are able to modify rihizosphere pH through the exudation of allelochemicals, principally with the aim to increase nutrient availability.

2.4.5 Ion exchange capacity

In soil, both mineral (e.g. clay minerals as well as Fe-, Al- and Mn-oxides) and organic (humus) colloids present

negative or positive surface charges that allow to attract and hold with cations and anions (Lavelle and Spain, 2001). The ion exchange capacity represents the measurement of the total absorbed/desorbed ions per unit mass of soil. It is strictly influenced by soil texture, kind of clays, SOM, soil reaction, kind and concentration of ions, presence of ions of opposite charge. For example, the negative charges are more abundant in alkaline or sub-alkaline soil of semiarid regions, rich in 2:1 silicate clays, while positive charges predominate acid soils rich in 1:1 clays and Al- and Fe-oxides (Weil and Brady, 2017). In the first situation there is a prevalence of cation adsorption, in the second of anions. The ion exchange capacity includes the cation exchange capacity (CEC) and the anion exchange capacity (AEC). CEC is the number of readily exchangeable cations (e.g. Na^+, H^+, K^+, NH_4^+, Ca^{2+}, Mg^{2+}, Mn^{2+}, Al^{3+}, Fe^{3+}) neutralizing negative charges on soil surfaces (Rhoaeds, 1982). Soils can present CEC values between 6 and >40 meq/100 g. Generally, CEC is higher with high pH levels and clay soils. AEC is the same of CEC, expressed in terms of anions (e.g. $H_2PO_4^-$, NO_3^-, Cl^-, HPO_4^{--}, SO_4^{--}, PO_4^{---},), principally operated by Fe- and Al-oxides. AEC and CEC are inversely proportional. They are very important for the adsorption/desorption balance and, thus, for the retention and transport processes by affecting allelochemicals leaching and availability. Inderjit and Bhowmik (2004), evaluating the growth of cucumber (*Cucumis sativus* L.) and radish (*Raphanus sativus* L.) in two soils amended with different amounts of benzoic acid, reported a higher sorption of benzoic acid in the soil with higher clay content, SOM and CEC. Belz *et al*. (2009) found that parthenin degradation, which is the allelochemical

responsible for the invasive success of *Parthenium hysterophorus* L., is favoured in clay soils with high CEC. These two examples reported a lower allelopathic potential correlated to high CEC, probably due to the strong retention forces that prevent allelochemicals from coming in contact with target plants.

2.5 Mineral nutrients availability

Root exudation of allelochemicals into the rhizosphere exerts a strong influence on nutrient release, solubilization, mobilization and uptake by plants (Jabran *et al*., 2013; Yu and Matsui, 1997) (**Table 2.3**). It is known that mineral availability not only depends on their concentration, but also on their form. In soil, in fact, although a mineral is relatively abundant, it can be present in an unavailable form for the plant. This phenomenon is correlated mainly to the soil reaction. For example, P and Fe become insoluble in high pH levels in presence of calcium carbonate ($CaCO_3$), while Al^{3+} and Mn^{2+} precipitate in highly acid soils, often becoming toxic. Under nutrient deficiency, many plant species exudate allelochemicals in order to increase their availability (Jones and Darrah, 1994). One of the most important tools utilized by plants to increase their nutrition efficacy is the alteration of rhizosphere pH levels. According to *Bais et al*. (2006), root exudates can increase or decrease soil nutrient availability through two mechanisms: phytosiderophores (PS) and organic acid secretion.

Table 2.3 The role of soil allelochemicals in plant nutrition.

Nutrient soil condition	Allelochemicals role	References
Fe-deficiency	Phytosiderophores exudation from poaceae plant roots, formation of Fe^{3+}-phytosiderophores complexes, transport with YS1/YSL protein transporters across plasma membranes and improve available Fe^{2+} for plant.	Meda *et al*., 2007; Senoura *et al*., 2017; Ueno *et al*., 2007
Micronutrients deficiency	Exudation of mugineic acid family phytosiderophores to chelate metallic micronutrient and increase their solubility, availability and transport.	Suzuki *et al*., 2016; Tsai and Schmidt, 2017
P-deficiency	Organic acids secretion to acidified rhizosphere, block phosphate from Ca^{2+}, Fe^{3+} and Al^{3+}, and improve available P for plant.	Dakora and Phillips, 2002; Wang *et al*., 2014
Al^{3+} toxicity	Organic acids (e.g. citrate, malate, oxalate, etc.) and flavonoids exudation to chelate and detoxify soil aluminium.	Kochian *et al*., 2014; Valentinuzzi *et al*., 2016
High nitrification rate	Exudation of biological nitrification inhibition substances (BNIS) such as phenolics and terpenoids to inhibit the ammonium-oxiding bacteria enzymes.	Rice, 1984; Subbarao *et al*., 2009
	Allelochemicals exudation to inhibit soil N mineralization.	Dietz *et al*., 2013
High N volatilization losses	Allelochemicals exudation to inhibit the denitrification process of NO_3^- into N_2O and NO.	Ma, 2005
Nutrient availability and uptake by plant	Allelochemicals exudation to depolarize of cell membrane electrical potential, inhibit mitochondrial oxidative phosphorylation and electron transport, and alterate membrane permeability.	Balke, 1985

Several poaceae plants such as wheat, sorghum, oat and barley, under metallic micronutrient deficiency (mainly Zn^{2+}, Mn^{2+}, Fe^{3+} and Cu^{2+}), exudates metal-chelating amino acids called phytosiderophores (Sugiura and Nomoto, 1984). The most common example of plant PS utilization refers to Fe-deficiency. PS acts as chelators by forming organic complexes with Fe^{3+}, which is precipitated and insoluble in soils, and taking up the Fe^{3+}-phytosiderophore complex by Fe deficiency-inducible transporters of the YS1/YSL protein family, thus increasing its solubility and transport (Curie *et al*., 2001; Meda *et al*., 2007; Ueno *et al*., 2007). PS release and FePS uptake are under different genetic control among poaceae species (Römheld and Marschner 1990). Recently, Suzuki *et al*. (2016) reported the detection of 2'-deoxymugineic acid (DMA), a compound belonging to the mugineic acid family phytosiderophores (MAs) in the olive (*Olea europaea* L.) xylem sap, indicating for the first time the presence of PS in a non-graminaceous dicot plant. Nozoye *et al*. (2017) found that the biomass-energy crop *Erianthus ravennae* (L.) Beauv. secreted mugineic acid (MA) and deoxymugineic acid (DMA) under soil Fe-deficiency. Apart from PS in poaceae plants, dicots are also able to exudates phenolics compounds such as coumarins that work as metallic micronutrient chelators (mainly Al^{3+}, Fe^{3+} and Mn^{2+}) to avoid their deficiency in soil (Dakora and Phillips, 2002; Tsai and Schmidt, 2017).

Another important tool available for many plants to improve mineral nutrient acquisition refers to the organic acid secretion. Contrary to PS secretion, this mechanism provides a more importance on P availability than on micronutrient one (Dakora and Phillips, 2002). In soils, P availability

depends on pH level (it is very low in alkaline soils), concentrations of anions that compete with P ions for ligand exchange reactions and concentrations of metals Ca^{2+}, Fe^{3+} and Al^{3+} that can co-precipitate with P ions (Hinsinger, 2001). However, in the rhizosphere P availability is higher thanks to the localized acidification due to CO_2 or HCO_3^- secretion and exudation of organic acids such as malic or citric acids (Hoffland *et al*., 1989). Plant roots, under P- or Fe-deficiency, release citrate and other dicarboxylates to block phosphate from Ca^{2+}, Fe^{3+}, or Al^{3+} precipitates and release available P into the soil (Bais *et al*., 2006; Meda *et al*., 2007). The most known example of rhizosphere acidification due to allelochemicals exudation is provided by *Lupinus albus* L., which forms proteoid roots under P-deficiency in order to exudate a large amount of citrate and increase P solubilization (Johnson *et al*., 1996; Wang *et al*., 2014). Nevertheless, organic acid secretion by plant root is helpful in response to elevated Al^{3+} concentrations in the soil solution and as protection from Al^{3+} toxicity (Kochian *et al*., 2004). The Al-induced organic acid secretion is specie-specific, however citrate was found to be the most effective Al-detoxifying compound among different species (Kochian *et al*., 2004). Valentinuzzi *et al*. (2016) reported that *L. albus* exudes citrate and flavonoids as response to Al^{3+} toxicity. The mechanisms of Al^{3+} tolerance and P acquisition due to organic acid secretion into the rhizosphere are often linked because citrate, malate, oxalate, etc. chelate aluminium and mobilize phosphates (Chen *et al*., 2016).

Allelochemicals released by plants into the soil affect several phases of soil nitrogen cycle. One of the most important problems associated to intensive agriculture is related to the

environmental pollution caused by nitrogen leaching in groundwater. N leaching losses are estimated in a range of 20-100 kg ha^{-1} $year^{-1}$, depending on season and rainfall regime as well as on soil texture. The nitrification process consists in the transformation of ammoniacal N (NH_4^+) into nitric N (NO_3^-) through two phases:

1) NH_4^+ oxidation to nitrite (NO_2^-) carried out by the bacteria genera *Nitrosomonas*, *Nitrosococcus* and *Nistrosospira* (Teske *et al*., 1994);

2) NO_2^- oxidation to nitrate (NO_3^-) operated by the *Nitrobacter* genus (Both *et al*., 1992).

The regulation of nitrification, therefore, plays a key role for improving N-use efficiency in sustainable agriculture. It has been observed how different kinds of plant allelochemicals such as phenolics or terpenoids act as biological nitrification inhibition substances (BNIS) through the inhibition of the activities of enzymes (e.g. ammonium mono-oxygenase, hydroxylamine oxidoreductase, etc.) of ammonium-oxiding bacteria (Rice, 1984; Subbarao *et al*., 2009). Dietz *et al*., (2013) indicated that *Plantago lanceolata* L. allelochemicals such as aucubin, catalpol, and verbascoside, suppressed soil N mineralization due to the incorporation of leaf material into the soil. The utilization of BNIS could be an important tool in improving N-use efficiency of N fertilizers in agroecosystems by decreasing the nitrification rate (Jabran *et al.*, 2013). Moreover, allelochemicals can reduce N volatilization losses by affecting the denitrification process of NO_3^- into N_2O, as reported by Ma (2005) for wheat allelochemicals.

In addition to the increase of nutrient solubilization, availability and use efficiency, generally allelochemicals

decrease or inhibit mineral uptake by plants (Jabran *et al*., 2013). Several experiments demonstrated the inhibition of nutrient absorption in plants grown in association with other plants, due to the addition of specific allelochemicals in the nutrient solution (Abenavoli *et al*., 2010) or with plant residues and mulches leachates (Babu *et al*., 2013). Three main physiological processes are involved in the reduction of mineral nutrient uptake (Balke, 1985): 1) the depolarization of cell membrane electrical potential; 2) the inhibition of mitochondrial oxidative phosphorylation and electron transport, which means a decrease of the ATP content; 3) the alteration of membrane permeability. The inhibition of nutrient uptake is manifested through a reduction in mineral concentrations of plant tissues or a decrease of seedlings growth.

2.6 Interaction between allelochemicals and soil microorganisms

By definition, allelopathy implies the adding of inhibitory compounds into the environment, whereas in competition a generic resource (e.g. water, light, minerals and space) is removed or reduced by another organism sharing the same habitat (Scavo *et al*., 2018a). Moreover, allelopathy involves many kinds of interactions including plant-plant, plant-insects and plant-microorganism interactions, in which the allelopathic agent may be either the plant or the microorganism. Here we discuss only the plant-microorganism interactions with the plants as donors of allelochemicals. Many studies have been conducted about the *in vitro* antimicrobial activity of plant allelochemicals (Scavo *et al*., 2018d). However, mere presence of allelochemicals in

the donor plant and their phytotoxic and antimicrobial activities in artificial medium (e.g. agar) do not demonstrate an allelopathic activity in natural conditions (Romeo, 2000), because soil microorganisms consume a high quantity of organic molecules and, thus, inhibitory compounds may not accumulate to toxic levels.

The plant-soil system is a very complex environment populated by a wide range of microorganisms constantly interacting with plants through root exudates (Rouatt *et al*., 1960). Soil microorganisms comprise bacteria, fungi, actinomycetes, algae and prokaryotes. In total, the microbial communities in soil is estimated to exceed 10 billion g^{-1} of soil (Roselló-Mora and Amann, 2001). Within this value, bacteria represent the most abundant population, representing about 10^8 g^{-1} of soil with an extraordinary biodiversity (Wollum, 1998). Several factors such as crop management, soil texture and structure, soil pH, SOM and geographic location are involved in the determination of soil bacteria communities (Doornbos *et al*., 2012; Fierer and Jackson, 2006). Generally, bacteria abundance in the rhizosphere is reported to be higher than in root-free soil (Gamalero *et al*., 2004; Watt *et al*., 2006), and rhizosphere bacterial density follows the trend: basal region>bulk soil>apical region (Dennis *et al*., 2008). Several studies demonstrated differences in the composition of both bulk soil (Marilley *et al*., 1998) and between root zones (Marschner *et al*., 2002) in relation to rhizosphere bacterial communities. To proliferate and establish in the rhizosphere, bacteria must be able to utilize rhizodeposits, effectively colonize root surface and be able to compete with other organisms. Over the past twenty years, considerable progress has been made in the

understanding of the bacterial traits and genes involved in the rhizosphere colonization. The abilities of bacterial cells to move towards plant roots in response to carbon-containing compounds, also known as chemotaxis, and the consequent rapid growth, are important traits for bacterial species competitiveness in the rhizosphere (Dennis *et al*., 2010).
However, the number of species and their relative abundance within specific root zones is a poorly investigated aspect in rhizosphere microbial ecology. This is explained by the heterogeneity of rhizosphere microorganism abundance and distribution, which also varies considerably with respect to position along longitudinal root axes (Dennis *et al*., 2008). At the root base, in fact, bacterial communities have been observed to partially cover the rhizoplane, while in root apices they are present as clusters that occupy a relatively small proportion of the available root surface (Chin-A-Woeng *et al*., 1997). Moreover, each plant species cultivates a specific microbial rhizosphere community, both bacteria (Smalla *et al*., 2001) and fungi (Broeckling *et al*., 2008), and both in natural and agroecosystems (Broz *et al*., 2007), as reported by numerous studies carried out with the denaturing gradient-gel electrophoresis (DGGE) method. Plants affect and communicate with their microbial community through the release of specific root exudates. Rhizodeposits, including allelochemicals, are an important source of C for microorganisms, determining an increasing of microbial biomass around the roots. Besides, plants are able to modify the chemical composition of their root exudates once came in contact with microorganisms (De-la- Peña *et al*., 2008), in order to either recruiting beneficial bacteria or repressing pathogenic microorganisms (Doornbos *et al*., 2012).

As view for soil physics and chemical characteristics, a two-way relationship exists between plant-microorganism interactions. Once entered into the environment, persistence, bioavailability and biological activities of allelochemicals are strongly influenced by microbial communities. The microbial degradation/transformation of allelochemicals in soil determines the expression of allelopathy in many situations (Inderjit, 2001). The microbial degradation of a specific allelochemical depends on soil texture, structure, aeration, temperature, SOM and pH, as well as on the microbial species involved. For example, among different species of *Cephalosporium* genus (e.g. *C. furcatum*, *C. khandalense*, *C. nordinii* and *C. roseum*), *C. furcatum* presents the highest degrading capacity of ferulic acid (Rice, 1984). Nevertheless, the seasonal variation in the microbial population may influence the availability of allelochemicals. Abbate *et al*. (2005), carried out a study on the analysis of bacterial communities in the rhizosphere of transgenic rolABC citrange Troyer by using phenotypical testing (BIOLOG) and 16S rDNA gene-based molecular analysis (ARDRA; DGGE) to obtain a better understanding of the rhizosphere soil bacterial populations. ARDRA analysis showed that only minimal modifications occurred in the autotrophic ammonia oxidizer populations of rolABC citrange Troyer rhizosphere. The results obtained by molecular fingerprinting (DGGE) were in accordance with those obtained by metabolic fingerprinting (BIOLOG). Indeed, both methods showed that the structure of the bacterial rhizosphere communities of rolABC citrange Troyer was minimally altered. Howevers, the authors found that the eubacterial DGGE profiles reflected strong seasonal population shifts in the bacterial

rhizosphere community. Gyamfi et al. (2002) reported minor differences in the DGGE patterns of the eubacterial population associated with transgenic canola due to the seasonal variation. Dunfield and Germida (2003), studying the variation in the microbial community of a transgenic canola variety, found differences during the plant growing season while remaining stables after winter, concluding that the observed changes were temporary and did not persist in the next field season. On the other side, allelochemicals released by plants into the rhizosphere represent a source of energy for microorganisms and establish the nature of plant-microorganism interaction, which can be either beneficial and deleterious. Positive interactions affected by plant allelochemicals are represented by symbiotic associations with rhizobia, mycorrhizae and plant growth-promoting bacteria (PGPB), while negative ones include the associations with parasitic plants, herbivores and pathogenic microorganisms (Badri and Vivanco, 2009) (**Table 2.4**).

2.6.1 The role of allelochemicals in positive and negative plant-microorganism interactions

Positive plant-microorganism interactions include those with beneficial effects for the plant. The most important positive interaction is provided by the legume-*Rhizobium* symbiosis. This kind of association is very specific and each rhizobial strain nodulates a specific host legume. Chemical compounds responsible for this interaction are isoflavonoids such as daidzein, genistenin and luteolin (Perret *et al*., 2000; Peters *et al*., 1986; Sugiyama *et al*., 2007), whereas rhizobia produce lipochitooligosaccharides, called *nod*D, to communicate with the host plant (Phillips and Tsai, 1992). These compounds

exuded by legume roots govern the growth of rhizobial cells, their chemotaxis as well as the transcription of *nod* genes.

Mycorrhizal symbiosis represents another important positive plant-microorganism interaction. Arbuscular mycorrhizal fungi colonize the roots of a very wide range of plants in order to increase nutrient uptake, especially that of P, and enhance the plant health. Once perceived a chemical signal from the host plant, mycorrhizal fungi extensively invade its root tissues. P-availability is a key factor regulating the hyphal branching (Nagahashi and Douds, 1999). The hyphal branching of arbuscular mycorrhizal fungi is induced and stimulated by flavonoids (Buee *et al*., 2000) and, mainly, strigolactones such as sorgolactone, 5-deoxy-strigol and strigol (Akiyama *et al*., 2005). These compounds act at very low concentrations. Sorgolactone, for example, induces branching at a concentration as low as 10^{-13} M (Besserer *et al.*, 2006). Different plant species are reported to exude strigolactones: tomato (*Solanum lycopersicum* L.), sorghum (*S. bicolor*), maize (*Z. mays*), pearl millet (*Pennisetum glaucum* (L.) R.Br.), red clover (*Trifolium pretense* L.), cotton (*Gossypium hirsutum* L.), etc. (Awad *et al*., 2006; Cook *et al*., 1972; Rial *et al*., 2018).

PGPB are the rhizosphere bacteria involved in the promotion of plant growth. Only 1-2% of rhizosphere bacteria promote the plant growth (Antoun and Kloepper, 2001). The most important bacteria genera identified as PGPR are *Bacillus* and *Pseudomonas* spp. (Podile and Kishore, 2006). PGPB chemotaxis on root surface is influenced by root allelochemicals in order to attract these positive rhizoacteria when the plant is under stress condition (Somers *et al*., 2004). PGPB can affect plant growth both indirectly and directly.

The direct promotion is represented by the supply of nutrients and phytohormones such as auxins, cytokinins and gibberellins. The diazotroph *Azospirillum*, for example, aside from fixing nitrogen, secretes the above mentioned phytohormones for the host plant (Steenhoudt and Vanderleyden 2000). The indirect promotion is realized by increasing plant defensive capacity to phytopathogenic organisms (Van Loon, 2007) and tolerance to abiotic stresses. Many species of PGPB induce in different plant species (Bakker *et al*., 2003) the induced systemic resistance (ISR), which refers to the creation of a protective biofilm on plant roots with the aim to limit pathogen access (Bais *et a*l., 2004b). ISR is an important defensive mechanism for plant not only against soilborne pathogens, but also toward aboveground pathogenic microorganisms. In certain situations, above- and belowground parts of plants communicate to respond to pathogenic attack. When insect herbivores attack a plant, their roots produce (semi-)volatile organic compounds (VOCs) belonging terpenoids or thiophenes chemical classes (Vaughan *et al*., 2013) as cues for natural enemies of root herbivores (van Dam and Bouwmeester, 2016). Western corn rootworm (WRC) larvae induce maize roots to secret *(E)*-β-caryophyllene to attract an entomopathogenic nematode after feeding on maize's leaves (Rasmann *et al*., 2005). Hiltpold *et al*. (2015) indicated that the water exudates secreted by *P. sativum*, at low concentrations, attract beneficial entomopathogenic nematodes and stimulate their activity, while inducing reversible quiescence at high concentrations.

Since the wide literature on the *in vitro* antimicrobial effects of plant allelochemicals, appear reasonable that

allelochemicals exuded into the rhizosphere play inhibitory effects against pathogenic soil microorganisms. However, the role of allelochemicals in pathogenesis of root-infecting bacteria and fungi has not been fully appreciated, probably due to the inadequate methods available for analysis (Bais *et al*., 2006).

The association with parasitic plants such as *Striga* spp. and *Orobanche* spp. represents the most important example of negative plant-microorganism interaction. This type of interaction is mediated by the same mechanism and chemical compounds involved in the association with arbuscular mycorrhizal fungi (Badri and Vivanco, 2009). The latter process is stimulated under P-deficiency, inducing the secretion of strigolactones from donor plant roots. These compounds stimulate the colonization of host plant roots by promoting hyphal branching (Akiyama *et al*., 2005). Strigolactone and its derivatives, however, at the same time promote the parasitic plant infection by stimulating their seed germination through an increase of mitochondrial activity (Bouwmeester *et al*., 2007). In addition to strigolactones, also other compounds such as isoflavonoids, sorgoleone and the sesquiterpene lactones parthenolide and 3,5-dihydroxydehydrocostus-lactone have been reported to stimulate parasitic plant seed germination (Bouwmeester *et al*., 2003; Pérez de Luque *et al*., 2000). Moreover, strigolactone inhibition of arbuscular mycorrhizal shoot branching was reported (Gomez-Roldan *et al*., 2008).

The secretion of VOCs from plant roots can assume a negative aspect. Soil herbivores, in fact, seems to use VOCs to localize their host (van Dam and Bouwmeester, 2016). Eliers *et al*. (2016) reported that *Melolontha melolontha* L.

larvae use VOCs exuded by *Taraxacum* sect. *Ruderalia* Kirschner & al. to found it over a distance of several centimeters. A similar behaviour was observed n other insect herbivores such as *Diabrotica virgifera virgifera* involving the volatile compound *(E)*-β-caryophyllene (Robert *et al*., 2012).

Certain bacteria quench pathogen quorum-sensing capacity by degrading autoinducer signals, thereby blocking expression of numerous virulence genes (Morello *et al*., 2004). Examples of this inhibition have been found to exist in nature. Many Gram-negative bacteria utilize autoinducers such as N-acyl homoserine lactones (AHLs) to coordinate gene expression in a population density-dependent way. At low population densities, cells produce a basal level of AHL via the activity of AHL synthase. As cell density increases, AHLs accumulate in the growth environment. When a critical threshold concentration is reached, the AHL molecule diffuses into the cell and binds to its cognate receptor, which in turn activates or represses the coordinated expression of particular sets of genes that enhance the ecological competence of the bacterium (Fuqua *et al*., 2001). It is possible that plants can exude chemical compounds into the rhizosphere to take advantage of this bacterial communication system (Bais *et al*., 2006).

2.7 Future perspectives

Plants release a wide variety of chemical compounds into the environment either as defense mechanism against biotic and abiotic stress factors and as a tool to communicate with other plants, with soil microorganism and within the own plant. The progress in the analysis methodologies and technical

instrumentations occurred over the last years allowed the achievement of new knowledges on this topic. A better understanding of allelochemical behaviour in soil could positively be applied in agroecosystems for weed and pest control and integrated to traditional agricultural practices under Integrated Pest and Weed Management System (IPMS, IWMS). Allelopathic mechanisms can be effectively exploited for agroecosystem control in different modes. The most important refers to (1) the selection of smothering crops, their breeding and inclusion in crop rotations (Scavo *et al*., 2018e); (2) the use of their residues as living mulches, dead mulches or green manure; (3) the selection of most active allelopathic compounds and their use as bioherbicides (Scavo *et al*., 2018b, f, g). Nevertheless, allelopathy could be applied to manage nutrient soil dynamics, enhance plant nutrient use efficiency and avoid heavy metal-toxicity phenomena. However, many aspects of these interactions are unknown. It is now understood that allelochemicals, once released into the rhizosphere, interact with the chemical, physics and biological characteristics of the soil system that determine their phytotoxic level by influencing the retention, transport and transformations processes. A major challenge for the scientific community is to investigate the influence of soil physics and chemical characteristics in field conditions over long-term experiments, particularly for what concern the role of soil texture and structure on allelochemicals phytotoxicity. The complex of plant-microorganism interactions in the rhizosphere represent the area requesting major studies to better understand the aboveground chemical communication and the physiological processes involved in both positive and negative interactions with microorganisms. Moreover, the

knowledge on root exudates chemistry is currently high, with hundreds of allelochemicals identified in the last decades. Unfortunately, their transport processes through plasma membrane need more attention, with the aim to clarify the behaviour of allelopathic plants and control the genes involved for breeding programs. Given the complexity of the soil system and the high heterogeneity of soils in different environments, the challenge for researches appear harder compared to other scientific areas. Therefore, a great effort is requested for the scientific community, with the involvement of multidisciplinary research groups. Skills from botany, agronomy, biology, chemistry, ecology, soil chemistry, etc. are needed.

Table 2.4 The positive and negative role of allelochemicals in plant-soil-microorganism interactions.

Type of interaction	Allelochemicals role	References
Positive interactions		
Legume-*Rhizobium* symbiosis	Isoflavonoids exuded by legume roots enhance the growth of rhizobial cells, their chemotaxis and the transcription of *nod* genes.	Peters *et al*., 1986; Sugiyama *et al*., 2007
Arbuscular mycorrhizal associations	Under P-deficiency, different plant species exude strigolactones into the rhizosphere to stimulate the hyphal branching of mycorrhizal fungi.	Akiyama *et al*., 2005; Buee *et al*., 2000
Plant growth-promoting bacteria (PGPB)	Under stress conditions, plant allelochemicals exuded into the rhizosphere promote and attract PGPB chemotaxis on root surface.	Somers *et al*., 2004
Induced systematic resistance (ISR)	Several PGPB species, thanks to a chemical communication with plant via allelochemicals exuded into the rhizosphere, induce the creation of a protective biofilm on plant roots with the aim to limit pathogen access.	Bais *et al*., 2004b; Bakker *et al*., 2003
Plant (semi-)volatile organic compounds (VOCs) – herbivore insects	When insect herbivores attack a plant, their roots produce different kinds of VOCs as cues for natural enemies of root herbivores.	Hiltpold *et al*., 2015; Vaughan *et al*., 2013; van Dam and Bouwmeester, 2016

Negative interactions

Parasitic plant associations	Under P-deficiency, mycorrhizal fungi induce the secretion of strigolactones from donor plant roots. These compounds stimulate the colonization of host plant roots by promoting hyphal branching. Strigolactones, however, at the same time promote the parasitic plant infection by stimulating their seed germination through an increase of mitochondrial activity.	Bouwmeester *et al*., 2003, 2007
	Strigolactone and its derivatives, at certain concentrations, inhibit arbuscular mycorrhizal shoot branching.	Gomez-Roldan *et a*l., 2008
Plant VOCs – herbivore insects	Certain soil insect herbivores use VOCs released by plants into the rhizosphere to localize their host.	van Dam and Bouwmeester, 2016
Quorum sensing inhibitors	Plants can exude chemical compounds into the rhizosphere to interfere and take advantages from the quorum sensing capacity of bacteria.	Bais *et al*., 2006; Fuqua *et al*., 2001

3. *Cynara cardunculus* L.

3.1 Historical background

Cynara cardunculus L. is a known species since ancient times. Its origin is associated with Arabs, who played a key role for its diffusion across the Mediterranean Basin. All the current names commonly used to indicate the plant, «carciofo» for Italians, «alcachofa» for Spanish, «alcachofra» for Portugesues, derive from the Arabic خرشوف (kharshuf) through the Hispanic Arabic «al-harsúf». The English name «artichoke», the French «artichaut» and the German «artischocke» find their origin in the old Italian «articiocco» (Skeat, 1887), demonstrating the importance of Italy for its diffusion around Europe (Sonnante *et al*., 2007).
The earliest observations about this plant belong to Greek writers. The genus name *Cynara* probably come from the Greek Κυον (Kyon = dog) to associate its spines to a dog's teeth (Craig, 1858), while the species name *cardunculus* came from the Latin *carduus* + *-nculus* (=little cardoon). Generally, Greek writers such as Hesiod (800–700 BC) and Theophrastus (400–300 BC) used the word σκόλυμος (*scolymus*) to indicate other thistles than *C. cardunculus* (e.g. *Scolymus hispanicus* L.). Also, the classic Roman writers Columella (1st century AD, in *De rerum rusticarum*) and Pliny the Elder (23–79, in *Historia naturalis*) wrote about a generic *cinara* defining it as *hispida* (spiny). By reading them, in 1832 the Swiss botanist De Candolle (1778-1841) stated that cultivated globe artichoke was unknown in classical times. Afterwards, Foury (1989) suggested that globe artichoke cultivation started around the 1st century AD, when its domestication was probably ongoing but not yet

accomplished (Sonnante *et al*., 2007). According to Mauro *et al*. (2009), Sicily represents one of the centres of globe artichoke domestication, which probably occurred between 9th and 15th century in family or monastery gardens, while cardoon domestication has been realized in Spain and France (Sonnante *et al*., 2007).

3.2 Botanical classification

C. cardunculus is an ancient complex species, member of the Asteraceae family, *Cynara* genus, and native to the Mediterranean Basin. According to Fiori's classification, this species includes three botanical varieties: the globe artichoke [*C. cardunculus* L. var. *scolymus* (L.) Fiori], the cultivated cardoon (*C. cardunculus* L. var. *altilis* DC.) and their common progenitor wild cardoon [*C. cardunculus* L. var. *sylvestris* (Lamk) Fiori] (Rottemberg and Zohary, 1996). *C. cardunculus* presents a diploid chromosome number (2n = 2x = 34), and their three botanical varieties are mostly cross-pollinated and cross- compatible with one another (Basnizki and Zohary, 1994), producing fertile inter-taxon F1 hybrids. For many years, the globe artichoke was considered as an independent species indicated as *C. scolymus* L. Thanks to the isoenzymatic relationships (Rottemberg and Zohary, 1996) and the genetic and molecular (Lanteri *et al*., 2004; Mauro *et al*., 2009) studies, however, Fiori's classification was validated. In addition to *C. cardunculus*, the *Cynara* genus comprise other six wild species: *C. syriaca* Boiss., *C. cornigera* (Lindely) (syn. *C. sibthorpiana* Boiss.), *C. algarbiensis* Cosson, *C. baetica* (Sprengel) Pau (syn. *C. alba* Boiss.), *C. humilis* L. and *C. cyrenaica* (Maire & Weiller) (Rottenberg and Zohary, 1996; Wiklund, 1992). All the

members of the genus are herbaceous perennial plants characterised by spiny leaves and heads.

Figure 3.1 The four main types of globe artichoke, grouped on the capitulum shape basis. A= "Thorny"; B= "Violetti"; C= "Romaneschi"; D= "Catanesi".

3.2.1 Globe artichoke

Globe artichoke is the most known *C. cardunculus* botanical variety. The adjective "globe" refers to the edible part of the head, the immature inflorescence called capitulum, used as both fresh and processed food worldwide. As the other members of the species, globe artichoke is a perennial plant that can be reproduced by achenes (dry indehiscent fruits), by division of rooted basal stem portions or, usually, by *ovoli* (semi-dormant offshoots originating from underground buds). The former plants present a tap root system, while the latter ones have a fibrous root system with many adventitious roots that enlarge over time. The Mediterranean Basin is

characterised by a large presence of autochthonous landraces. Porceddu *et al*. (1976) divides globe artichoke germplasm into four distinct groups (Spinosi, Violetti, Romaneschi and Catanesi) (**Figure 3.1**), but only about a dozen varieties have any commercial significance (Basnizki and Zohary, 1994).
About 80-85% of the total plant and 60% of the whole head weight is a vegetative non-consumed portion. However, it is rich in nutraceutical and antioxidant compounds mainly represented by polyphenols (Lombardo *et al*., 2009, 2015; Pandino *et al*., 2012a), which can be used for cosmetic, industrial and pharmaceutic applications (Lattanzio *et al*., 2009; Pinelli *et al*., 2007). Alternative uses of globe artichoke refer to the production of minimally processed products ready-to-use (Licciardello *et al*., 2017; Lombardo *et al*., 2017; Pandino *et al*., 2017), oil, inulin (Lattanzio *et al*., 2002), vegetable rennet for traditional cheeses as well as for ornamental destinations (Lanteri *et al*., 2012).

3.2.2 Cultivated cardoon

Cultivated cardoon is a perennial geophyte C_3 plant characterised by a big and deep root system, a "ceppaia" (developed underground rhizome) differentiating lateral shoots commonly called "carducci" (which are able to form a new plant) and long leaves (more than 1 m) produced in high number (more than 40) (**Figure 3.2**). The crop shows its highest potential under the semi-arid zones of Mediterranean Basin, where it is able to photosynthetize in winter and uptake nutrient from deep soil layers (Mauromicale *et al*., 2014). Contrary to globe artichoke, which presents a high degree of heterozygosity, both cultivated and wild cardoon have a limited varietal diversity. Also, unlike to globe artichoke,

cultivated cardoon is usually gamically propagated by seeds sown in late spring as an annual crop. The crop is traditionally useable as a vegetable for human consumption for its enlarged bleached petiole of the leaves. Nowadays, it has been recognised as an energy crop since its large biomass (leaves, stems, flower heads and achenes) can be utilised as solid bio-fuel (Ierna *et al*., 2012) as well as for biodiesel or biomethane production (Mauromicale *et al*., 2014; Pesce *et al*., 2017) in alternative to cereals silage. Nevertheless, fresh biomass coming from cultivated cardoon could be used as green fodder for animal feed (Cajarville *et al*., 1999) and as an important source of polyphenols (Pandino *et al*., 2012b).

Figure 3.2 Cultivated cardoon development and high fresh biomass production (photo taken by Mauromicale, G.).

Figure 3.3 Wild cardoon with white-colored florets (photo taken by Mauromicale, G.).

3.2.3 Wild cardoon

Wild cardoon is the ancient progenitor of both *Cynara* cultivated forms and it is widespread among the Mediterranean and North American landscapes, where has become an invasive weed during centuries. It is a non-domesticated robust perennial plant, characterized by its rosette of large spiny leaves, branched flowering stems and blue-violet flowers (Portis *et al*., 2005) (**Figure 3.3**). According to Wiklund (1992), wild cardoon presents two subspecies: subsp. *cardunculus* and subsp. *flavescens* Wikl., difficulty distinguishing each other and coexisting in Sicily (Pignone and Sonnane, 2004). Wild cardoon, likely globe artichoke and cultivated cardoon, presents a high content of

phenolics compounds, especially in the leaves (Pandino *et al*., 2012b), and is usable for biomass energy and biocarburants production (Mauromicale *et al*., 2014; Pesce *et al*., 2017).

3.3 Diffusion

The globe artichoke plays a key role for the agricultural economy of Mediterranean Countries, where produce 1,124 t year^{-1} and hold about 80% of the world's globe artichoke growing areas (96,204 ha), with nearly three-quarters share of the output (FAOSTAT, 2018) (**Figure 3.4**). Italy is the most important globe artichoke world producer, accounting a production of ~370 t year^{-1} and a harvested area of ~39,338 ha (**Figure 3.5**). Sicily presents both the highest harvested area (14,260 ha) and production (138,2 t year^{-1}), about 37% of the total Italian production, followed by Puglia and Sardinia.

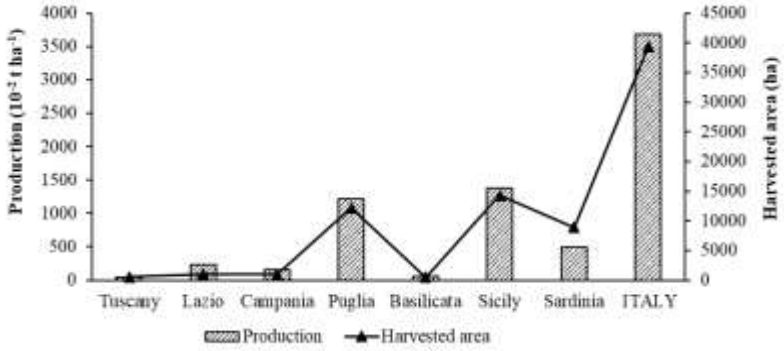


Figure 3.3 Globe artichoke annual production and harvested areas (at least 400 ha harvested) in Italian regions. Considered period 2018 (ISTAT 2018).

Other important producer countries are Peru, Argentina, China and USA. However, it is important to underline how traditionally producing countries such as France, Greece, Spain and Italy show a decreasing trend, while other countries, among which Egypt stands out, have increased their production in the last few years.
Regarding cultivated cardoon, unfortunately there are not official statistical analysis about its production and diffusion. It shows a regional importance and the area devoted to this crop is localized in South Europe countries (officially about 2-3000 ha, though this value is underestimated), mainly in France, Spain, Italy and Greece (Ierna and Mauromicale, 2010).

3.4 Polyphenols and sesquiterpene lactones

C. cardunculus, particularly the globe artichoke, is well known since ancient times for its beneficial effects on human health and for this reason it is widely adopted as functional food in the human diet. The beneficial effects are related to the high content of phenolics compounds in the heads and capitula, particularly caffeoylquinic- and dicaffeoylquinic acid derivatives, as well as luteolin-and apigenin derivatives (Pandino *et al*. 2012a, 2015). Not only edible parts of the plant, but also leaves, bracts and stems are a good source of flavones and caffeoylquinic acids (Pandino *et al*., 2013). Considering that the management of this waste material, which represents an important portion of the plant, is a major problem for industrial processors, its utilization as source of antioxidant and nutraceutical compounds for pharmacology and industry allows to profit by the whole plant. Many biotic (e.g. genotype, plant age, plant part, harvest time, etc.) and

abiotic (quantity and intensity of solar radiation, temperature, fertilization management and other agricultural practices) factors affect the polyphenolic profile and concentration in *C. cardunculus* (Lombardo *et al*., 2009, 2010, 2018). The plant produces these compounds as defence mechanism against oxidative damages caused by free radicals (Racchi *et al*., 2002).

The Asteraceae family, in addition to polyphenols, is an important natural source of characteristics terpenoids commonly used as taxonomic markers: the sesquiterpene lactones. *C. cardunculus*, particularly, presents a high content of these lipophilic constituents in the inflorescences, bracts and mainly in the leaves. Sesquiterpene lactones possess a wide range of biological activity such as phytotoxic (Rial *et al*., 2014), antimicrobial (Schinor *et al*., 2004), fungicidal (Wedge *et al*., 2000), cytotoxic and anti-cancer (Zhang *et al*., 2005). Among theme, cynaropicrin is reported to be the major sesquiterpene lactone in both globe artichoke and cultivated cardoon. Other important sesquiterpene lactones identified in *C. cardunculus* extracts are desacylcynaropicrin, grosheimin, desoxygrosgheimin, aguerin B, etc. Rial *et al*. (2014, 2016a) indicated these compounds as the principal responsible of C. *cardunculus* phytotoxic activity.

EXPERIMENTAL PART

4. Presentation of the Research Project

The PhD research project is organized on the assumption that allelopathic extracts from *C. cardunculus* can be employed for the biological control of weeds, plant pathogens and microorganisms of food interest. Particularly, the aim of this research is to investigate the allelopathic effects of *C. cardunculus* var. *sylvestris* (wild cardoon), var. *altilis* (cultivated cardoon) and var. *scolymus* (globe artichoke) leaf extracts on seed germination and seedlings growth of several common weeds in Mediterranean agroecosystems. In addition, the field allelopathic activity of *C. cardunculus* botanical varieties on weed seedbank, and the inhibitory activity on postharvest microorganisms and plant pathogens were also studied. Final goal is the identification of the inhibitory compounds responsible of globe artichoke, cultivated and wild cardoon allelopathic properties, for the future development and commercial applications of products representing a safer alternative to chemical fungicides, bactericides and herbicides.

With reference to leaf extracts, experimental objectives of the following research project are:

- investigation on the methodology for extraction of allelochemicals;
- isolation, identification and purification of allelochemicals by HPLC;

Regarding the weed control, the research project involves:

- laboratory bioassays for germination tests (with Petri dishes);
- laboratory bioassays for growth tests;

- field bioassays for the determination of the allelopathic effects on weed soil seedbank.

Fungi and bacteria control will be investigated through:

- *in vitro* antimicrobial activity, expressed as the diameter of the inhibition zones, through the agar well diffusion method;
- *in vivo* curative and preventive activity on aubergine burgers.

Therefore, the experimental part will be articulated into three main parts:

1) Allelopathic effect of *C. cardunculus* leaf extracts for weed control.
2) Field allelopathic activity of *C. cardunculus* L. to reduce size and composition of soil weed seed bank.
3) Antimicrobial activity of *C. cardunculus* leaf extracts.

5. Allelopathic Effect of *C. cardunculus* Leaf Extracts for Weed Control

5.1 Allelopathic effects of *Cynara cardunculus* L. leaf aqueous extracts on seed germination of some Mediterranean weed species

The following work has been already published as:

- Scavo, A., Restuccia, A., Pandino, G., Onofri, A., Mauromicale, G. (2018b). Allelopathic effects of *Cynara cardunculus* L. leaf aqueous extracts on seed germination of some Mediterranean weed species. *Ital J Agron 13*, 119-125.

5.1.1 Introduction

The presence of weeds causes serious losses to the agricultural production, both in quantitative and qualitative terms, because they constantly compete spatially with crop plants, limiting the available amount of nutrients, light and moisture. Weeds are very good colonisers, reproduce faster, produce a large number of small seeds with very prolonged viability in soil and survive in the most adverse situations, becoming part of the persistent soil seedbank. On average, weeds cause a yield crop reduction estimated around 34% (Oerke, 2006). The increase in world population and the simultaneous decrease of the available resources, have led agriculture to an indiscriminate use of synthetic herbicides for weed management. This wide usage has led to serious problems, such as the evolution of herbicide resistant weed populations and the negative impacts on environmental,

human and animal health (Jabran *et al*., 2015). Therefore, there is an urgent need to explore eco-friendly strategies for weed control.

Putnam and Duke (1978) were the first ones to assess the possibility of using allelopathic crops for weed management in agriculture, minimising the serious problems of environmental impact. Allelochemicals includes terpenoids, N-containing compounds and phenolic compounds and can be found in different parts of plant: leaves, stems, roots, rhizomes, seeds, flowers and even pollen (Bertin *et al*., 2003; Kruse *et al*., 2000). In contrast to a high proportion of synthetic agrochemicals, allelochemicals are biodegradable, mostly water-soluble and consist of non-halogenated molecules (Bhowmik and Inderjit, 2003). Besides, they present a wide chemical diversity, are selective (Dayan *et al*., 2012) and, thus, may offer new mode of actions (Macias *et al*., 2007).

According to Rial et al. (2014), cardoon allelochemicals (primarily aguerin B, grosheimin and cynaropicrin) possess a strong phytotoxicity on the germination and growth of standard target species (tomato, lettuce, onion and watercress) and weeds (barnyardgrass and brachiaria). Moreover, the joint action of binary mixtures of aguerin B, grosheimin and cynaropicrin and one nonactive compound (11,13-dihydroxy-8-desoxygrosheimin) was also investigated on wheat coleoptide (Rial et al., 2016a). The three *C. cardunculus* botanical varieties (globe artichoke, cultivated and wild cardoon) are a good source of caffeoylquinic acids and flavones as reported in previous works (Lombardo *et al*., 2015; Pandino *et al*., 2012b; Schütz *et al*., 2004). In particular, their leaves have been shown to

represent a potentially productive source of polyphenols (Lombardo *et al.*, 2009, 2015), which have various industrial, pharmaceutical and cosmetic application (Lattanzio *et al*., 2009; Pinelli *et al*., 2007). Thanks to these compounds, the *C. cardunculus* species have been stimulated the scientific interest at the aim to evaluate these crops as promising sources of natural antioxidant for food and not food applications. Nevertheless, the potential use of *C. cardunculus* leaves extracts for weed management is at the beginning of investigation.

For this reason, the purpose of this study was to evaluate the possible effects of wild cardoon, cultivated cardoon and globe artichoke allelochemicals on seed germination and mean germination time of six common weeds in Mediterranean agroecosystems. Moreover, the autoallelopathic activity on wild cardoon was considered too.

5.1.2 Material and methods

5.1.2.1 Sampling of C. cardunculus *plant material and preparation of aqueous leaf extracts*

Fresh material was sampled from cultivated cardoon, wild cardoon and globe artichoke plants at the 25th visible leaves growth stage (November 2014), randomly, from a field crop located in the Catania University experimental station farms situated in Catania Plain [10 m (a.s.l.), 37° 25' N, 15° 30' E]. The three botanical varieties were at the same phenological stage. The extraction was carried out according to Sarkar *et al*. (2012). In the laboratory, the plant material from each botanical variety (approximately 1 kg of leaves) was washed, cut and ground. Then, a portion of each gross material was mixed with distilled water (1:10 w/v). The mixture was kept

under dark conditions for 48 h at room temperature and, then, filtered through filter paper (Whatman No.2) to eliminate the solid fraction. From this solution, two different concentrations (40 and 80%), already investigated in our preliminary studies, were obtained for each botanical variety: wild cardoon ecotype *Marsala* (CW 40 - CW 80), cultivated cardoon cultivar *Verde de Peralta* (CC 40 - CC 80) and globe artichoke cultivar *Violetto di Sicilia* (ART 40 - ART 80). Each extract was compared using distilled water as control (C). The prepared aqueous extracts were transferred into a falcon flask and stored in a refrigerator (3°C) for further use. In a submitted work, we identified by HPLC analysis the phytotoxic compounds of *C. cardunculus*, such as caffeoylquinic acids and flavones (*e.g.* chlorogenic acid, luteolin 7-*O*-glucoronide, luteoilin, apigenin 7-*O*-glucoronide, apigenin, *etc*.).

5.1.2.2 Weed seed collection

Mature seeds from adult plants were collected from six common Mediterranean weed species in natural ecosystems as well as in field crops (**Table 5.1**). Collection sites were made from natural populations in the Catania Plane of Sicily (Italy, lat. 37° 28' N, long. 14° 57' E, at an average altitude of 50-150 m a.s.l.). The climate of this area is of a Mediterranean type, with a long, hot and dry summer, mild winter and rain falling mostly from late autumn to early spring. Daily mean temperature during the year ranges from 8.5°C to 26°C: minimum temperature is around 0°C, while maximum can peak at over 35°C. Annual precipitation is about 500 mm (Cristaudo *et al*., 2007).

After collection, seeds were cleaned, kept in paper bags and

dry-stored at room temperature until germination tests were performed. Seeds of each species were selected through the use of a stereomicroscope to achieve a homogeneity of the lots for size and colour.

5.1.2.3 Germination tests

Germination bioassays were arranged using 5 mL of leaf aqueous extracts at 40 and 80% and distilled water to humidify a double layer of sterilised filter paper (Whatman No. 2). Petri dishes, hermetically sealed with parafilm to prevent evaporation of the solution, were stored in incubators at the optimal conditions of temperature and photoperiod for single weed species tested. Germination tests were performed in continuous darkness and at a constant temperature of 35°C for *Amaranthus retroflexus* L. (Cristaudo *et al*., 2007) and *Portulaca oleracea* L. (Singh, 1973), in continuous darkness and at a constant temperature of 20°C for *C. carduncul*us var. *sylvestris* (Lekić *et al*., 2011), while *Diplotaxis erucoides* (L.) DC. (Gresta *et al*., 2010) and *Lavatera arborea* L. were incubated in alternating light (dark/light cycle 12/12 h) at 20°C and 25°C respectively. Besides, *Brassica campestris* L. (Kondra *et al*., 1983) and *Solanum nigrum* L. (Taab, 2009) were incubated in alternating light (dark/light cycle 8/16 h) at 20°C and 25°C respectively. Incubators maintained the designated temperature to within ±1°C and they were equipped with Osram cool white fluorescent lamps with an irradiance of 25 μmol m^{-2} s^{-1}, 400–750 nm.

For each treatment, four replications of 25 seeds were placed separately in 9 cm diameter plastic Petri dishes, transparent for dark/light alternating conditions and wrapped in sheets of aluminium foil for complete darkness. During the counting

process, germinated seeds in continuous darkness treatments were manipulated under a green safelight (490-560 nm), while seeds in alternating photoperiod were counted during the 12-h light period (Cristaudo *et al*., 2016).
Germination was determined by counting and removing germinated seeds every 24 h. Germination was considered when the radicals were greater than or equal to 2 mm in length. All the determinations were performed twice and each value of a replicate is therefore a mean of the two readings.

5.1.2.4 Data analysis
The percentage of final germination (G %) was calculated as the ratio between the number of seed germinated and the total number of seeds used in each Petri dish. The corresponding proportions were analysed by way of a binomial generalised linear model with logit link (Sileshi, 2012). Plant species, allelopathic compounds and their interaction were included as factors in the model. Wherever necessary, contrasts between means were performed by using the procedures outlined in Bretz *et al*. (2011). The Mean Germination Times for each Petri dish were obtained by using the Kaplan-Meyer estimators (Onofri *et al*., 2010), together with mid-point imputation to comply with interval censoring (Law and Brookmeyer, 1992). Mean germination time (MGT) were analysed by using two-way ANOVA; a graphical inspection of residuals showed that no significant deviations with respect to the basic assumptions for ANOVA were found.
Allelopathic effect response index (RI) was calculated using the Equation (1) of Williamson and Richardson (1988):

$$RI = \begin{cases} 1 - \frac{C}{T} & if\ T \geq C \\ \frac{T}{C} - 1 & if\ T < C \end{cases} \quad (1)$$

where T is the seed germination (%) for the treated plants and C is the seed germination (%) for the corresponding control. RI ranges from –1 to +1, with positive values indicating the stimulation of germination by the aqueous extracts and negative values indicating the inhibition of germination, relative to the control.

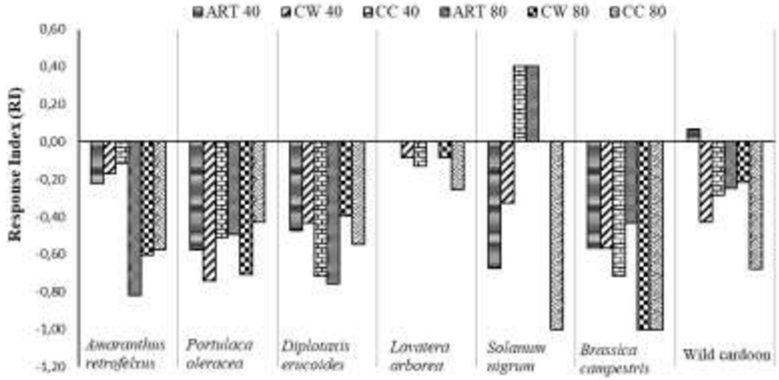


Figure 5.1 The influence of leaf aqueous extracts of *C. cardunculus* on the allelopathic effect Response Index (RI) in six weed species. The pooled standard error of the above means was 0.111. ART 40, globe artichoke extract 40%; CW 40, wild cardoon extract 40%; CC 40, cultivated cardoon 40%; ART 80, globe artichoke extract 80%; CW 80, wild cardoon extract 80%; CC 80, cultivated cardoon 80%.

Table 5.1 List of the plants used in the germination tests.

Common name	Scientific name	Family	Biological form	Corotype
Redroot pigwed	*Amaranthus retroflexus* L.	Amaranthaceae	T scap.	Cosmop.
Purslane	*Portulaca oleracea* L.	Portulacaceae	T scap.	Subcosmop.
White wall rocket	*Diplotaxis erucoides* (L.) DC.	Brassicaceae	T scap.	W-Medit. (Steno)
Tree mallow	*Lavatera arborea* L.	Malvaceae	H bien.	Steno-Medit.
Field mustard	*Brassica campestris* L.	Brassicaceae	T scap./H scap.	Medit.
Black nightshade	*Solanum nigrum* L.	Solanaceae	T scap.	Cosmop. (synanthrop.)
Wild cardoon	*C. cardunculus* L. var. *sylvestris*	Asteraceae	H scap.	Steno-Medit.

Table 5.2 Effects of leaf aqueous extract of *C. cardunculus* on seed germination (G%) of six weed species.

Leaf aqueous extract	*Amaranthus retroflexus*	*Portulaca oleracea*	*Diplotaxis erucoides*	*Lavatera arborea*	*Solanum nigrum*	*Brassica campestris*
Control	94.0 ± 2.37^{d}	61.0 ± 4.88^{d}	66.3 ± 5.29^{d}	47.0 ± 4.99^{a}	4.0 ± 2.26^{a}	8.8 ± 3.16^{a}
ART 40	73.0 ± 4.44^{c}	26.0 ± 4.39^{ab}	35.0 ± 5.33^{c}	47.0 ± 4.99^{a}	1.3 ± 1.32^{a}	3.8 ± 2.12^{a}
CW 40	78.0 ± 4.14^{c}	16.0 ± 3.67^{a}	37.5 ± 5.41^{c}	43.0 ± 4.95^{a}	2.7 ± 1.86^{a}	3.8 ± 2.12^{a}
CC 40	83.0 ± 3.76^{c}	30.0 ± 4.58^{bc}	18.8 ± 4.36^{ab}	41.0 ± 4.92^{a}	6.7 ± 2.88^{a}	2.5 ± 1.75^{a}
ART 80	17.0 ± 3.76^{a}	31.0 ± 4.62^{bc}	16.3 ± 4.12^{a}	47.0 ± 4.99^{a}	6.7 ± 2.88^{a}	5.0 ± 2.44^{a}
CW 80	37.0 ± 4.83^{b}	18.0 ± 3.84^{a}	40.0 ± 5.48^{c}	43.0 ± 4.95^{a}	4.0 ± 2.26^{a}	0.00
CC 80	40.0 ± 4.90^{b}	35.0 ± 4.77^{c}	30.0 ± 5.12^{bc}	35.0 ± 4.77^{a}	0.00	0.00

ART 40, globe artichoke extract 40%; CW 40, wild cardoon extract 40%; CC 40, cultivated cardoon 40%; ART 80, globe artichoke extract 80%; CW 80, wild cardoon extract 80%; CC 80, cultivated cardoon 80%. Values are given as means±standard error. $^{a\text{-}d}$Different letters indicate statistical significance for $P \leq 0.05$.

5.1.3 Results and discussion

Many works report the inhibition of seed germination in presence of plant allelochemicals (Reigosa and Pazos-Malvido, 2007; Sbai *et al*., 2016). *C. cardunculus* secondary metabolites, such as chlorogenic acid and luteolin 7-*O*-glucoronide, have been reported to show allelopathic activity on different crops (Abdul-Rahman and Habib, 1989; Hosni *et al*., 2013; Li *et al*., 1993). In this experiment, RI was significantly affected by the interaction of species and compound ($P=9.7\times10^{-12}$). The RI of *C. cardunculus* leaf aqueous extracts was negative in all weed species under study, except in *S. nigrum* (and wild cardoon) (**Figure 5.1**). This variability among weed species could be attributed to the different combination of allelochemicals profile present in each extract, as well as by their level. Our hypothesis is corroborated by Ambika (2013), who found as a compound may be inhibitory at high concentration, stimulatory at low concentration, or have no effect at other concentrations.

Regardless of the weed species, all extracts reduced weed seed germination if compared with control (**Figure 5.2**). The best result was obtained with CC 80, which inhibited the weed seed germination by about 64%. On the contrary, CC 40 showed the worst allelopathic effect by reducing only 26% final seed germination, as well as the effects of ART 40, CC 40 and CW 40 appeared less marked (**Figure 5.2**). Overall, our results reported that the concentrated extract (80%) had major negative effect on weed species germination than the diluted one (40%) (45.5 *vs* 33.3% respectively). Similar trend was noted by Chung and Miller (1995) on selected weed species treated with alfalfa (*Medicago sativa* ssp. *sativa* L.) residues.

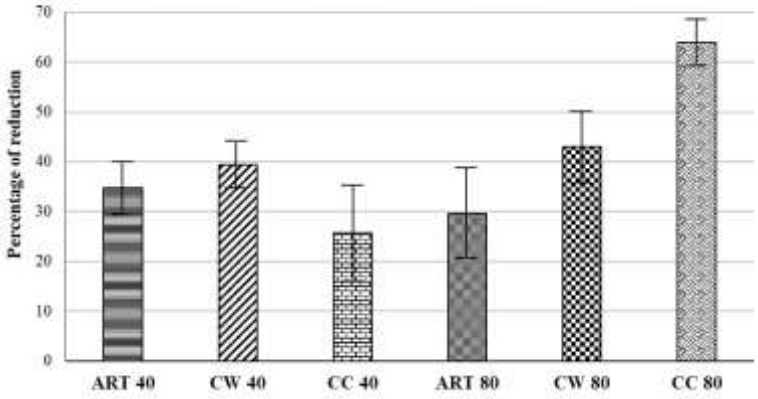


Figure 5.2 Percentage of reduction respect to control of weed seed germination in relation to extract of C. cardunculus. ART 40, globe artichoke extract 40%; CW 40, wild cardoon extract 40%; CC 40, cultivated cardoon 40%; ART 80, globe artichoke extract 80%; CW 80, wild cardoon extract 80%; CC 80, cultivated cardoon 80%.

In *A. retroflexus*, all aqueous extracts significantly lowered seed germination with a decrease, on average, of 58.1% as compared to the control. ART 80 resulted the most efficient, since allowed only 17% of seed germination (**Table 5.2**). Also in *P. oleracea* the allelopathic effect of the different solutions was significant, but not concentration-dependent. The percentage reduction of different types of extracts compared to the control was 42.5%. The lowest germination rates were obtained with CW 40 and 80 if compared with C (16 and 18% vs 61% respectively). These results are similar to many previous findings. According to Yarna *et al*. (2009), increasing of sorghum leaf extract concentration from 5 to 20% inhibited *A. retroflexus* germination from 70.76 to 92.77% and the germination time was extended too. Azizi

and Fuji (2006) found that germination of *A. retroflexus* and P. oleracea was completely inhibited at a concentration of 0.7% (v/v) and higher of *Eucalyptus globulus* Labill. essential oils. Besides, they found that the undiluted hydro-alcoholic extract of *Hypericum perforatum* L. and *Salvia officinalis* L. had a significant inhibitory effect on seed germination percentage for *A. retroflexus*, but not for *P. oleracea*. Dadkhah and Asaadi (2010) reported that foliar aqueous extract of *E. camaldulensis* Dehnh. not affected the germination percentage of *P. oleracea*, but severely reduced, especially at the higher aqueous extract concentration, the growth of young seedlings. Therefore, differently from other plant species, the *C. cardunculus* botanical varieties have a strong inhibitory effect on *P. oleracea*.

No studies about the allopathic effects on seed germination of *D. erucoides* have been published. Nevertheless, several works have been conducted upon the allelopathic activity of *D. erucoides* on field crops (Giordano *et al*., 2005; Qasem, 2007). In this experiment, germination of D. erucoides was significantly reduced by all aqueous extracts (66.3% control vs 29.1% average of aqueous extracts). The best results were obtained with ART 80 and CC 40 if compared with C (16.3% and 18.8% vs 66.3% respectively). However, the less marked effects were registered with CW 80. Results provide evidence of globe artichoke's foliar extracts strong allelopathic effect on *D. erucoides* seed germination. That is important under the applicative aspect because *D. erucoides* is one of the most harmful weed in Mediterranean environments.

Allelopathic effects of *C. cardunculus* extracts on *L. arborea* seed germination and mean germination time were not significant (**Table 5.2**). Therefore, *L. arborea* cannot be

considered a target plant for *C. cardunculus* foliar extracts. Also in *S. nigrum* and *B. campestris*, as well as in *L. arborea*, the germination percentage was not significantly affected by any extracts. The low seed germination percentages of *S. nigrum* and *B. campestris,* are probably due to the high seed dormancy of wild ecotypes. These results are in contrast with González *et al*. (1997) and Gao *et al*. (2009). The first found that the effects of six phenolics compounds obtained from the soil solution of nine pepper (*Capsicum annuum* L.) varieties on germination of *S. nigrum* were inhibitory only at a concentration of 10^{-2} M. The second reported that *B. campestris* seed germination and seed germination speed are strongly inhibited by *Hemistepta lyrata* Bunge water extract. *B. campestris* seed germination was inhibited (50%) also by aqueous extract of leaves of *Parthenium hysterophorus* L. at 2% concentration (Maharjan *et al*., 2007). Therefore, *S. nigrum* and *B. campestris*, are very sensitive to allelochemicals of some plant species, but not to *C. cardunculus* botanical varieties.

In addition to the reduction of germination, the delay in seed germination is crucial in weed control and can affect the ability of the seedlings to establish themselves in natural conditions (Chaves *et al*., 2001; Escurdero *et al*., 2000). **Table 5.3** shows how *C. cardunculus* extracts increased MGT of *A. retroflexus*, *P. oleracea* and *D. erucoides*. Since *S. nigrum* and *B. campestris* seeds showed low germination percentage values, their MGTs were not considered. In *A. retroflexus*, all aqueous extracts increased MGT (from 1.8 d of control to 5.9 d on average of the aqueous extracts) and the best results were obtained with CC 40, CC 80 and ART 80 (7.5 d, 7.0 d and 7.0 d respectively). Also in *P. oleracea*,

TMG was increased by all treatments (from 3.7 d of control to 5.4 d on average of treatments) and CW 40 showed the highest value (8.6 d). The most significant effect of *C. cardunculus* extracts on *D. erucoides* MGT was reached by using ART 80 and CW 80 (7.3 d and 7.2 d respectively).

Table 5.3 Effects of aqueous extract of *C. cardunculus* on mean germination time (MGT days) of four weed species.

Leaf aqueous extract	*Amaranthus retroflexus*	*Portulaca oleracea*	*Diplotaxis erucoides*	*Lavatera arborea*
Control	1.8^{a}	3.7^{a}	5.4^{ab}	6.9^{a}
ART 40	5.0^{bc}	4.2^{a}	2.3^{a}	7.3^{a}
CW 40	4.2^{ab}	8.6^{b}	4.5^{ab}	7.8^{a}
CC 40	7.5^{c}	4.3^{a}	5.4^{ab}	6.5^{a}
ART 80	7.0^{bc}	4.4^{a}	7.3^{d}	6.3^{a}
CW 80	5.0^{abc}	6.3^{ab}	7.2^{b}	8.6^{a}
CC 80	7.0^{bc}	4.4^{a}	6.1^{b}	8.2^{a}

ART 40, globe artichoke extract 40%; CW 40, wild cardoon extract 40%; CC 40, cultivated cardoon 40%; ART 80, globe artichoke extract 80%; CW 80, wild cardoon extract 80%; CC 80, cultivated cardoon 80%. Values are given as means with pooled standard error of a mean (SEM) =1.02 $^{a\text{-}c}$Different letters indicate statistical significance for $P \leq 0.05$.

In this experiment, the autoallelopathic effect on wild cardoon was also evaluated (**Table 5.4**). Our results showed that all the other leaf extracts, excluding ART 40, decreased the germination percentage if compared with C (19.6% *vs* 28% respectively). The most significant result (9%) was obtained with CC 80, followed by CW 40 (16%). Therefore, *C. cardunculus* L. shows an autoallelopathic capacity, although it is low. Autoallelopathy is probably a mechanism

that helps *C. cardunculus* invasion in natural ecosystem through maintaining seed dormancy when conditions are not conducive to growth or by increasing plant resistance to pathogens (Friedman and Waller, 1985).

Table 5.4 Autoallelopathic effect of leaf aqueous extract of *C. cardunculus* on response index (RI), on seed germination (G%) and mean germination time (MGT days).

Leaf aqueous extract	Wild cardoon		
	RI	G %	MGT d
Control		28.0 ± 4.49^{c}	9.8 ± 1.02^{ab}
ART 40	0,07	30.0 ± 4.58^{c}	9.9 ± 1.02^{b}
CW 40	-0,43	16.0 ± 3.67^{ab}	9.5 ± 1.02^{ab}
CC 40	-0,29	20.0 ± 4.00^{bc}	7.9 ± 1.02^{ab}
ART 80	-0,25	21.0 ± 4.07^{bc}	6.9 ± 1.02^{a}
CW 80	-0,21	22.0 ± 4.14^{bc}	9.3 ± 1.02^{ab}
CC 80	-0,68	9.0 ± 2.86^{a}	9.5 ± 1.02^{ab}

ART 40, globe artichoke extract 40%; CW 40, wild cardoon extract 40%; CC 40, cultivated cardoon 40%; ART 80, globe artichoke extract 80%; CW 80, wild cardoon extract 80%; CC 80, cultivated cardoon 80%. Values are given as means±standard error. $^{a\text{-}c}$Different letters indicate statistical significance for P≤0.05.

5.1.4 Conclusions

The present study exploited the allelopathic effect of leaf aqueous extracts of *C. cardunculus* on six common Mediterranean weeds. Overall, the inhibitory effect was concentration-dependent, even if different behaviour was observed among the considered weed seeds. These results are very promising in order to produce a bioherbicide based on *C. cardunculus* extracts. It will make possible to reduce the use of synthetic herbicides and, as consequence, should have

an impact positively on the environment and human health. Nevertheless, the trial was carried out *in vitro* conditions, thus, further investigations are needed to determine the effect of *C. cardunculus* allelochemicals in open field, in order to set up the best dose-response effect on common Mediterranean weeds. Future developments involve also an accurate study on the methodology for extraction of *C. cardunculus* allelochemicals, particularly on the utilisation of different solvents, on the stability of the extracts, as well as analysing the polyphenol profiles and investigating the phytotoxic effects of several genotype extracts from the three botanical varieties at different growth stages and stress conditions.

5.2 Leaf extracts of *Cynara cardunculus* L. var. *altilis* DC. as potential bioherbicide

The following work has been submitted on *Scientia Horticulturae* as follows:

- Scavo, A., Pandino, G., Restuccia, A., Mauromicale, G. (2018f). Leaf extracts of *Cynara cardunculus* L. var. *altilis* DC. as potential bioherbicide.

5.2.1 Introduction

Weeds are one of the most serious problem causing yield reductions both in organic and conventional farming systems. Their management has traditionally focused on direct chemical control. As the number of cases of herbicide-resistant weeds continues to increase, there is a need for new chemical classes of herbicides (Heap, 2014; Peterson *et al*., 2018). However, the number of synthetic chemicals with new target sites are decreasing dramatically (Soltys *et al*., 2013). Nowadays, the trend is to find a natural solution for weed control in agricultural field and, thus, the extraction of allelochemicals from plants could be useful for the production of bioherbicides (Dayan *et al*., 2012). According to Rice (1984), allelopathy includes any direct or indirect, harmful or beneficial, effect by one plant on another through the production of chemical compounds that are released into the environment. Allelochemicals can be introduced into the environment through volatilization, root exudation, foliar leaching, or residue decomposition. The manipulation of allelopathic mechanisms and their integration to traditional agricultural practices under Integrated Weed Management

System (IWMS) could represent a valid alternative to synthetic herbicides (Scavo *et al*., 2018a).
In a previous work we studied the allelopathic effect of globe artichoke [var. *scolymus* (L.) Fiori], cultivated cardoon and wild cardoon [var. *sylvestris* (Lamk) Fiori] leaf aqueous extracts (80 and 40%) on seed germination of six common Mediterranean weed species (Scavo *et al*., 2018b). The results showed mean reduction of about 41% in germination of all test species relative to the controls and the best result was obtained with the leaf extracts (80%) of cultivated cardoon. Rial *et al.* (2014) found that the secondary metabolites involved in cardoon phytotoxicity were the sesquiterpene lactones cynaropicrin, grosheimin and aguerin B.
Keeping in mind previous results, where the best ones were obtained with leaf extracts of cultivated cardoon, the aim of this work was to compare the inhibitory activity of leaves (fresh, dried and lyophilized) of this plant treated with three different solvents (bidistilled water, 70% methanol and 80% ethanol) in presence/absence of citric acid (20%) on four cosmopolitan weed species highly widespread in the Mediterranean Basin. In addition, it was evaluated the content and quality of caffeoylquinic acids and flavones in cultivated cardoon extracts in order to better understand the relationship (if any) between inhibitory activity and the polyphenol profile of *C. cardunculus*. In particular, it was analysed the polyphenol profile of cultivated cardoon leaves, because it showed a better performance compared both to globe artichoke and wild cardoon in our previous experiments.

5.2.2 Material and methods

The experiments were set up in a completely randomized design with four replications and repeated twice, as performed in most of allelopathy bioassays based on seed germination (Jefferson and Pennacchio, 2003; Turk and Tawaha, 2003), including: 3 leaf materials x 8 extracting solvents. In **Table 5.5**, all treatments are shown.

Table 5.5 Different leaf materials of cultivated cardoon and extracting solvent performed in this study.

Leaf material	Solvent	pH
Fresh	1) Bidistilled water	6.5*
Dried	2) Bidistilled water + 20% citric acid	6.2*
Lyophilized	3) 70% methanol	6.3*
	4) 70% methanol + 20% citric acid	6.2*
	5) 80% ethanol	6.6*
	6) 80% ethanol + 20% citric acid	6.3*
	7) Control-1	6.2
	8) Control-2	5.8

*Values are expressed as mean of the three leaf materials. Control-1: bidistilled water 100%; Control-2: bidistilled water/citric acid 80:20 (v/v).

5.2.2.1 Sampling of plant material and crop management

Sixty leaves were sampled from about fifty cultivated cardoon plants of 'Altilis 41', an open pollinated line selected by researchers at the University of Catania for biomass and biomolecules production. Leaves were harvested at the 25th visible leaves phenological growth stage (rosette developed) and leaves cover 50% of ground (code 35 according to the BBCH scale by Archontoulis *et al*., 2010). Plants were grown in the in the Catania experimental station [South Italy, 37° 25' N; 15° 30' E; 10 m a.s.l.], in a typic and/or vertic xerochrepts soil (Soil Survey Staff, 1999) with a clay texture. Soil

characteristics were as follows: sand 27%, clay 45%, silt 28%, organic matter 1%, total nitrogen 0.1%, available P_2O_5 10 ppm, exchangeable K_2O 210 ppm, and pH 7.2. Before planting, the field was ploughed to a depth of 30 cm, harrowed and a fertilizer rate of 50 kg N ha^{-1} (as urea), 80 kg P_2O_5 ha^{-1} (as double perphosphate) and 80 kg K_2O ha^{-1} (as potassium sulphate) was given. The local climate is semi-arid Mediterranean, with mild rain winters and hot dry summers. One hundred seedling bearing three leaves of 'Altilis 41' (about 40 days after seed germination) were transplanted into the field in early-September at the rate of 1 plant m^{-2}, using an inter- and intra-row spacing of 1.25 and 0.80 m. The expanded plot was kept weed- and insect-free by spraying oxyfluorfen and imidacloprid, respectively, when required.

5.2.2.2 Preparation of leaf extracts

In the laboratory, a sample of approximately 4 kg of randomly fresh leaves were washed, cut and ground. A portion was air dried in an oven at 45 °C up to constant weight and another one was freeze-dried. A portion of each material was mixed (1:10 w/v) with three different solvents: bidistilled water, 70% methanol (MeOH) and 80% ethanol (EtOH). Then, the mixtures were kept under dark conditions for 72 h at room temperature (20°C ± 1) and filtered through filter paper (Whatman No. 2) to eliminate the solid fraction. The MeOH and EtOH solutions were evaporated at 35 °C by use of rotary evaporator (Laborata 4000, Heidolph, Germany), and the residue was dissolved with bidistilled water. In addition, a portion of each extract was acidified with citric acid (20%) to test the effect (if any) of pH on the efficient of leaf extracts. The prepared extracts were transferred into a

falcon flask and stored in a refrigerator (3°C) for further uses. Each extract was compared using bidistilled water as Control 1 and bidistilled water/citric acid, 80:20 v/v as Control 2.

5.2.2.3 Seed collection

Mature seeds from plants of four common Mediterranean weed species were collected: *Amaranthus retroflexus* L., *Portulaca oleracea* L., *Stellaria media* (L.) Vill. and *Anagallis arvensis* L. The first two species are summer annual weeds, whereas the other ones are winter annuals. Collection sites of *A. retroflexus*, *P. oleracea* and *A. arvensis* were made from natural populations in the Catania Plain (latitude 37°28'N, longitude 14°57'E, at an average altitude of 50–150 m a.s.l.). Instead, collection site of *S. media* was made in Calatabiano (South Italy, latitude 37°49'N, longitude 15°13'E, at an altitude of 50 m a.s.l.). In both areas the climate is of a Mediterranean type, with a long, hot and dry summer, mild winter and rain falling mostly from late autumn to early spring. Daily mean temperature during the year ranges from 8.5°C to 26°C: minimum temperature is around 0°C, while maximum can peak at over 35°C. Annual precipitation is about 500 mm (Cristaudo *et al*., 2007).

After collection, seeds were cleaned, kept in paper bags and dry-stored at room temperature (20°C ± 2°C) until germination tests were performed. Mature seeds of each species were selected through the use of a stereomicroscope to achieve a homogeneity of the lots for size and colour.

5.2.2.4 Germination tests

Four replicates of 25 seeds were imbibed in 9 cm Petri dishes on two Whatman papers No. 2, with 5 mL of leaf extracts,

and incubated for 15 days at the optimal conditions of temperature and photoperiod for single weed species tested. Germination tests were performed in continuous darkness and at a constant temperature of 35°C for *A. retroflexus* and *P.oleracea*, while *S. media* and *A. arvensis* were incubated in alternating light (dark/light cycle 14/10 h) at 17°C. Petri dishes, hermetically sealed with parafilm to prevent evaporation of the solution, were transparent for dark/light alternating conditions and wrapped in sheets of aluminium foil for complete darkness. Incubators maintained the designated temperature to within ±1°C, and they were equipped with Osram cool white fluorescent lamps with an irradiance of 25 μmol m^{-2} s^{-1}, 400–750 nm.
During the counting process, germinated seeds in continuous darkness treatments were manipulated under a green safelight (490–560 nm), while seeds in alternating photoperiod were counted during the 12-h light period (Cristaudo *et al*., 2016). Germination was recorded by counting and removing germinated seeds every 24 h. Germination tests were considered ended when no seeds have germinated for 3 consecutive days. Seeds were considered germinated when the radicle protruted through the seed coat by 2 mm. All the trials were performed twice.

5.2.2.5 Identification and quantification of compounds

5.2.2.5.1 Reagents and Solvents

Reagents and solvents were purchased from VWR (Leighton Buzzard, UK) and were of analytical or HPLC grade. Apigenin 7-*O*-glucoside, apigenin, luteolin 7-*O*-glucoside, luteolin, 5-*O*-caffeoylquinic acid (chlorogenic acid), cynaropicrin and hesperetin were obtained from

Extrasynthese (Lyon, France), cynarin (1,3-di-*O*-caffeoylquinic acid) was from Roth (Karlsruhe, Germany). Milli-Q system (Millipore Corp., Bedford, MA) ultrapure water was used throughout this research.

5.2.2.5.2 HPLC analysis

Each extract (20 µL), excluding those with citric acid to avoid interferences in the chromatograms profile, was analysed using a series 1200 HPLC (Agilent Technologies, Palo Alto, USA) equipped with ChemStation software (version: B.03.01), a model G1379B degasser, a model G1312B binary gradient pump, a model G1367C thermoautosampler, a model G1316B column oven, and a model G1315C diode array detection system. Separations were achieved on a Zorbax Eclipse XDB-C_{18} (4.6 × 50 mm; 1.8 lm particle size), operated at 30°C, with a 0.2 µm stainless steel in-line filter.

For caffeoylquinic acids and flavones, the mobile phase was 1% formic acid in water (solvent A) and in acetonitrile (solvent B) at a flow rate of 0.5 mL min^{-1}. The gradient started with 5% B to reach 10% B at 5 min, 40% B at 20 min, 90% B at 25 min, 90% B at 29 min (Lombardo *et al*., 2015).

For the cynaropicrin, it was adapted the method proposed by Menin *et al*. (2012). The mobile phase was 0.1% formic acid in water (solvent A) and in acetonitrile (solvent B) at a flow rate of 0.3 mL min^{-1}. The gradient started with 5% B to reach 10% B at 10 min, 30% B at 25 min, 40% B at 30 min. Chromatograms were recorded at 240, 254, 280, 310 and 350 nm from diode array data collected between 200 and 600 nm. Identification of single compounds was by their retention times, both UV and MS spectra, and by data available in the literature (Schütz *et al*., 2004; Wang *et al*., 2003).

Quantification was performed using a calibration curve of the available standards. In particular, mono and dicaffeoylquinic acids were calculated using chlorogenic acid and cynarin as references, respectively. Monosuccinyl-dicaffeoylquinic acids were calculated using cynarin as reference. Apigenin and luteolin conjugates were quantified as apigenin-7-*O*-glucoside and luteolin-7-*O*-glucoside, respectively. All data presented are mean values ± standard error of three independent experiments and expressed as mg kg^{-1} of dry matter (DM).

5.2.2.6 Data analysis

The following indexes were calculated:

– the percentage of final germination (G %), calculated as the ratio between the number of seed germinated and the total number of seeds used in each Petri dish. the Mean Germination Time (MGT), calculated following the equation (1) of Ranal *et al* (2009):

$$MGT = \sum_{i=1}^{k} ni\ ti \,/\, \sum_{i=1}^{k} ni \qquad (1)$$

where n_i and t_i are respectively number of germinated seeds in the i^{th} time and the time from the start of the experiment to the i^{th} observation; k= last time of germination

5.2.2.7 Statistical analysis

Data from both G% and MGT were log-transformed to achieve a normality distribution, then were submitted to the Bartlett's test for the homogeneity of variance and the independence of cases was tested through a graphical inspection of residuals, which showed no significant

deviations. After verified the basic assumptions, all data were subjected to analysis of variance (ANOVA). Differences with $p<0.05$ were considered significant and Tukey's HSD test were conducted for mean multiple comparison. Data for each species were treated separately. Two factorial two-way ANOVA models were used: one model with leaf material and extraction solvents as main factors to analyse the effects of solvent and leaf material on the average of the four weed species; another model considering the species and the solvents as fixed factors to investigate the behaviour of each weed species to all treatments.

5.2.3 Results and discussion

5.2.3.1 Evaluation of leaf extracts phytotoxicity on weed seed germination

The inhibition of seed germination by allelochemicals is as a secondary expression of primary effects such as the interferences with cell division and elongation, as well as with cell membrane permeability (Scavo *et al*., 2018a).

Cultivated cardoon leaf extracts inhibited the germination of the four weed species (**Figure 5.3**). Our data revealed that lyophilized leaf extracts permitted only the 3.2% of seed germination as compared to 73% of Control 1 and 57% of Control 2, while the least effective with 16% were fresh leaves. These results suggest how lyophilized leaves appeared to be the best material for the production of cultivated cardoon bioherbicides. Nevertheless, the lyophilization process has an important cost impact on the production. Considering the good phytotoxic activity of dried leaves on weed seed germination and a lower cost management than the lyophilization, this material could be a

proper compromise to produce bioherbicides. In relation to the solvent, all the extracts significantly reduced seed germination on the average of four weed species, compared to both Controls (**Figure 5.4**). In particularly, the ethanolic extracts completely inhibited weed seed germination, while bidistilled water was the least effective with respect to the other ones. Similar trends were reported by many authors (Azizi *et al*., 2008; Franco *et al*., 2015; Reichel *et al*., 2013), who found more inhibitory effects of hydroalcoholic extracts compared to aqueous ones. Furthermore, the use of citric acid in the extracts appeared to respond positively because all solvents showed a better performance, even if it was statistically significant only for bidistilled water. Moreover, the effect of citric acid was species-dependent. In *A. retroflexus* had no effect on seed germination, while showed inhibitory effects on *P. oleracea* (72 and 40% in Control 1 and Control 2, respectively) and *S. media* (99 and 55% of Control 1 and Control 2, respectively). On the contrary, it exerted a stimulatory activity on *A. arvensis* (20 and 35% of Control 1 and Control 2, respectively). Also in literature both stimulatory (Cotrufo, 1963) and inhibitory (Esen *et al*., 2009) effects of citric acid have been reported. In this experiment, it always decreased the level of seed germination when combined with cultivated cardoon extracts. **Figure 5.5** showed the influence of solvent, in the average of all treatments, on weed seed germination. In *A. retroflexus* was observed the highest percentage of seed germination (37%), followed by *S. media* (27%). Their higher seed germination values are explained by their controls (100% and 98% of Controls 1 for *A. retroflexus* and *S. media*, respectively), which contribute to enhance the total seed germination. The

low seed germination value of *A. arvensis* (20% for Control 1 and 35% for Control 2) is probably due to the seed dormancy of wild ecotypes, in agreement with a previous result (Lipp and Ballard, 1963).

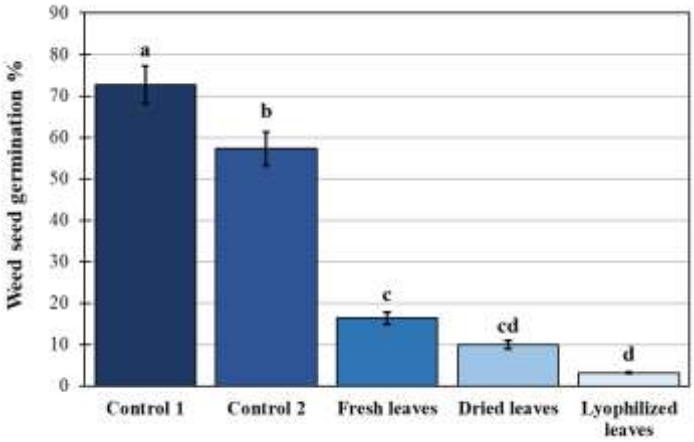


Figure 5.3 The influence of cultivated cardoon leaf material on weed seed germination (average of four weed species). Note: Control 1: Bidistilled water 100%; Control 2: Bidistilled water/citric acid 80:20 (v/v). Each bar means±standard error of untransformed data. Different letters indicate statistical significance for $P \leq 0.05$ from transformed data.

As shown in **Table 5.6**, the choice of the solvent on its own explained much of the weed seed anti-germination capacity of cultivated cardoon extracts. In particular, the contribution of solvent on the inhibitory activity of extracts was greater than the leaf material for G in all weed species, while its contribution for MGT was higher only for *S. media* and *A. arvensis*. In addition, the MGT was also more affected by the interaction 'solvent x leaf material' respect to the G.

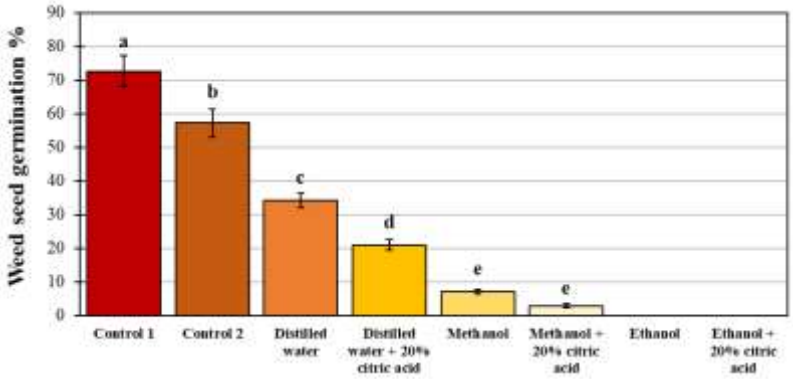


Figure 5.4 Weed seed germination (%) of different types of cultivated cardoon leaf extracts in relation to the solvent (average of four weed species). Note: Control 1: Bidistilled water 100%; Control 2: Bidistilled water/citric acid 80:20 (v/v). Each bar means±standard error of untransformed data. Different letters indicate statistical significance for $P \leq 0.05$ from transformed data.

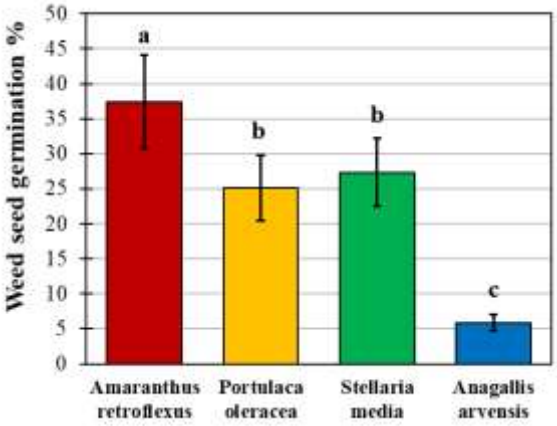


Figure 5.5 The influence of solvent on weed seed germination in relation to weed species (average of all treatments). Note: Each bar means±standard error of untransformed data. Different letters indicate statistical significance for $P \leq 0.05$ from transformed data.

All leaf extracts from cultivated cardoon showed inhibitory effects on seed germination of each weed species under study, but with different behaviour (**Table 5.7** and **5.8**). In *A. retroflexus*, all extracts significantly reduced seed germination, compared to 100% of the control 1 and 99% of Control 2 (**Table 5.7**). Aqueous and methanolic extracts in presence of citric acid resulted less inhibitory respect to those in its absence (44% *vs*. 36% and 12 *vs*. 9% of seed germination, respectively). The best perfomance was obtained by lyophilized leaves, which permitted only 4% of seed germination, followed by dried leaves (10%). Both fresh and lyophilized extracts reduced the germination of *P. oleracea*, while the dried one appeared to have a promoting effect. Also for *P. oleracea* the highest allelopathic effect was caused by lyophilized leaves, which permitted only 5% of seed germination, followed by fresh leaves (14%). In both *S. media* and *A. arvensis*, methanolic and ethanolic extracts completely inhibited seed germination, but also aqueous ones showed a strong inhibitory activity (**Table 5.8**). In *S. media*, the inhibitory effect of aqueous extract was significantly increased by adding citric acid (48 *vs*. 12% of weed seed germination, respectively). Nevertheless, the lowest germination rates were obtained with dried and lyophilized leaves if compared with Control 1 (4 and 5% *vs*. 99% respectively). In *A. arvensis*, the aqueous extracts could be used against the weed seed germination, since allowed only 3 and 4 % (bidistilled water and bidistilled water with citric acid, respectively) of seed germination. No studies about the allopathic effects on seed germination of *S. media* and *A. arvensis* have been published yet. Nevertheless, several works have been conducted upon the allelopathic activity of

S. media (Inderjit and Dakshini, 1998) and *A. arvensis* (Rebaz *et al*., 2001; Salam *et al*., 2011) on field crops.

The inhibitory activity was not expressed only in terms of reduction of seed final germination percentage, but it was also accompanied by a significant slowing of the germination process expressed through an increase in MGT. In our study, cultivated cardoon extracts increased MGT in all weed species considered, compared to controls (**Tables 5.9** and **5.10**). Both aqueous and methanolic extracts significantly affected the MGT, mainly the methanolic extract in *A. retroflexus* and *P. oleracea*, and the aqueous one in *S. media* and *A. avensis*. Nevertheless, MGT of the weed species under study was not increased by adding citric acid. The increase of weed MGT in presence of plant allelochemicals is widely reported (Angelini *et al*., 2003; Bahuguna *et al*., 2014; Mubeen *et al*., 2012). Bahuguna *et al*. (2014) reported an MGT value of 5.94 days for mustard seeds treated with 100% walnut leaf extract, compared to 4.99 days of the untreated control (0%). Mubeen *et al*. (2012) outlined MGT values of 8.29 and 9.17 days for *Eleusine indica* (L.) Gaertn. seeds treated with sorghum and sunflower water extracts, respectively, compared to 4.78 days with distilled water. Probably, the allelopathic effect on germination speed is due to the interference that block or delay the progress of metabolic processes during the germination process (Ferreira and Borguetti, 2004).

Table 5.6 *F*–values as absolute value and sum of squares (in brackets) of main factors and their interactions resulting from analysis of variance in weed species.

Source of variation	Degree of freedom	*Amaranthus retroflexus*		*Portulaca oleracea*	
		G	**MGT**	**G**	**MGT**
Solvent (S)	7	141^{***} (78)	74^{***} (18)	158^{***} (86)	25^{***} (15)
Leaf material (L)	2	25^{***} (14)	163^{***} (41)	16^{***} (8)	75^{***} (42)
(S) x (L)	14	15^{***} (8)	159^{***} (40)	12^{***} (6)	76^{***} (43)

Table 5.6 (Continue)

Source of variation	Degree of freedom	*Stellaria media*		*Anagallis arvensis*	
		G	**MGT**	**G**	**MGT**
Solvent (S)	7	161^{***} (90)	159^{***} (70)	211^{***} (97)	249^{***} (37)
Leaf material (L)	2	9^{***} (4)	3^{NS} (1)	5^{NS} (2)	201^{***} (30)
(S) x (L)	14	11^{***} (6)	67^{***} (29)	3^{NS} (1)	219^{***} (33)

Note: Values are given as *F* of Fisher. G: percentage of final germination; MGT: mean germination time. *** indicates significant at $P \leq 0.001$, and NS, not significant.

Table 5.7 Effects of leaf extracts of cultivated cardoon on seed Germination (%) of two spring-summer weed species.

Weed species	Solvent	Leaf material		
		D	**F**	**L**
Amaranthus retroflexus				
	Bidistilled water	10.7 ± 0.9	97.3 ± 2.7	0
	Bidistilled water + 20% CA	18.7 ± 0.9	93.3 ± 1.3	18.7 ± 1.7
	Methanol	28.0 ± 2.1	0	0
	Methanol + 20% CA	2.7 ± 0.3	24.0 ± 2.0	8.0 ± 0.6
	Ethanol	0	0	0
	Ethanol + 20% CA	0	0	0
	Mean	**10 b**	**36 a**	**4 c**
	Control-1 100.0			
	Control-2 99.0 ± 1.2			
	LSD interaction $_{(P\leq 0.05)}$ 0.04			
Portulaca oleracea				
	Bidistilled water	80.0 ± 4.0	48.0	21.3 ± 1.5
	Bidistilled water + 20% CA	14.7 ± 1.2	34.7 ± 3.4	10.7 ± 0.9
	Methanol	57.3 ± 1.3	0	0
	Methanol + 20% CA	0	0	0
	Ethanol	0	0	0
	Ethanol + 20% CA	0	0	0
	Mean	**25 a**	**14 b**	**5 c**
	Control-1 72.0 ± 4.0			
	Control-2 40.0 ± 6.7			
	LSD interaction $_{(P\leq 0.05)}$ 0.02			

Note: Values are given as means ± standard error. D: dried leaves; F: fresh leaves; L: lyophilized leaves; CA: extracts acidified with 20% citric acid; Control-1: bidistilled water 100%; Control-2: bidistilled water/citric acid 80:20 (v/v). Different letters indicate statistical significance for $P \leq 0.05$.

Table 5.8 Effects of leaf extracts of cultivated cardoon on seed Germination (%) of two autumn-winter weed species.

Weed species	Solvent	Leaf material		
		D	**F**	**L**
Stellaria media				
	Bidistilled water	25.3 ± 1.8	93.3 ± 3.5	26.7 ± 2.2
	Bidistilled water + 20% CA	0	33.3 ± 1.8	4.0 ± 0.3
	Methanol	0	0	0
	Methanol + 20% CA	0	0	0
	Ethanol	0	0	0
	Ethanol + 20% CA	0	0	0
	Mean	**4 b**	**14 a**	**5 b**
	Control-1 98.7 ± 1.3			
	Control-2 55.0			
	LSD interaction $_{(P\leq0.05)}$ 0.03			
Anagallis arvensis				
	Bidistilled water	2.7 ± 0.3	5.3 ± 0.6	0
	Bidistilled water + 20% CA	0	10.7 ± 0.7	0
	Methanol	0	0	0
	Methanol + 20% CA	0	0	0
	Ethanol	0	0	0
	Ethanol + 20% CA	0	0	0
	Mean	**0.4 b**	**2.6 a**	**0**
	Control-1 20.0 ± 2.3			
	Control-2 35.0 ± 4.2			
	LSD interaction $_{(P\leq0.05)}$ 0.01			

Note: Values are given as means ± standard error. D: dried leaves; F: fresh leaves; L: lyophilized leaves; CA: extracts acidified with 20% citric acid; Control-1: bidistilled water 100%; Control-2: bidistilled water/citric acid 80:20 (v/v). Different letters indicate statistical significance for $P \leq 0.05$.

Table 5.9 Effects of leaf extracts of cultivated cardoon on Mean Germination Time (MGT days) of two spring-summer weed species.

Weed species	Solvent	Leaf material		
		D	F	L
Amaranthus retroflexus				
	Bidistilled water	7.8 ± 0.6	2.5 ± 0.2	–
	Bidistilled water + 20% CA	4.6 ± 0.1	3.4 ± 0.1	5.9 ± 0.5
	Methanol	12.3 ± 0.6	–	–
	Methanol + 20% CA	2.3 ± 0.3	5.4 ± 0.4	6.9 ± 0.1
	Ethanol	–	–	–
	Ethanol + 20% CA	–	–	–
	Mean	**6.7 a**	**3.8 c**	**6.4 ab**
	Control-1 1.5 ± 0.1			
	Control-2 2.1			
	LSD interaction $_{(P\leq0.05)}$ 0.76			
Portulaca oleracea				
	Bidistilled water	2.5 ± 0.1	1.7 ± 0.1	4.0 ± 0.3
	Bidistilled water + 20% CA	2.6 ± 0.3	2.0 ± 0.2	3.6 ± 0.2
	Methanol	5.9 ± 0.4	–	–
	Methanol + 20% CA	–	–	–
	Ethanol	–	–	–
	Ethanol + 20% CA	–	–	–
	Mean	**1.8 b**	**1.8 b**	**3.8 a**
	Control-1 1.8 ± 0.2			
	Control-2 3.0 ± 0.1			
	LSD interaction $_{(P\leq0.05)}$ 0.44			

Note: Values are given as means ± standard error. D: dried leaves; F: fresh leaves; L: lyophilized leaves; CA: extracts acidified with 20% citric acid; Control-1: bidistilled water 100%; Control-2: bidistilled water/citric acid 80:20 (v/v). Different letters indicate statistical significance for $P \leq 0.05$.

Table 5.10 Effects of leaf extracts of cultivated cardoon on Mean Germination Time (MGT days) of two autumn-winter weed species.

Weed species	Solvent	Leaf material		
		D	**F**	**L**
Stellaria media				
	Bidistilled water	12.1 ± 0.7	6.1 ± 0.1	10.1± 0.7
	Bidistilled water + 20% CA	–	8.9 ± 0.3	3.7 ± 0.3
	Methanol	–	–	–
	Methanol + 20% CA	–	–	–
	Ethanol	–	–	–
	Ethanol + 20% CA	–	–	–
	Mean	**12.1 a**	**7.5 b**	**6.9 c**
	Control-1 3.9 ± 0.2			
	Control-2 8.0 ± 0.3			
	LSD interaction $_{(P\leq0.05)}$ 0.75			
Anagallis arvensis				
	Bidistilled water	7.3 ± 0.7	6.9 ± 0.5	–
	Bidistilled water + 20% CA	–	9.3 ± 0.2	–
	Methanol	–	–	–
	Methanol + 20% CA	–	–	–
	Ethanol	–	–	–
	Ethanol + 20% CA	–	–	–
	Mean	**7.3 b**	**8.1 a**	**–**
	Control-1 5.0 ± 0.2			
	Control-2 9.2 ± 0.3			
	LSD interaction $_{(P\leq0.05)}$ 0.55			

Note: Values are given as means ± standard error. D: dried leaves; F: fresh leaves; L: lyophilized leaves; CA: extracts acidified with 20% citric acid; Control-1: bidistilled water 100%; Control-2: bidistilled water/citric acid 80:20 (v/v). Different letters indicate statistical significance for $P \leq 0.05$.

Apart from *C. cardunculus*, no other evidences are reported about the involvement of allelopathy in the *Cynara* genus. Contrariwise, Asteraceae is a well-known botanical family involving many species with allelopathic properties. Some of the most important are sunflower (Macias *et al*., 2002), *Artemisia annua* L. (Lydon *et al*., 1997), *Helianthus tuberosus* L. (Tesio *et al*., 2011), and several weeds such as *Parthenium hysterophorus* L. and *Ambrosia polystachya* DC. (de Miranda *et al*., 2014), *Tagetes erecta* L. and *Bidens Pilosa* L. (Campbell *et al*., 1982), etc.

5.2.3.2 HPLC Analysis

According to Rice (1984), allelopathic compounds are plant secondary metabolites able to reduce or inactive the physiological activity of phytohormones such as gibberellins or to affect the activity of specific enzymes such as amylases and proteinases, which are necessary for seed germination. The results outlined above clearly indicate that cultivated cardoon leaf extracts have a high allelopathic effect on seed germination of both spring-summer and autumn-winter weeds. Thus, we decided to isolate and identify the secondary metabolites (phenolic acids, flavones and cynaropicrin) of aqueous, methanolic and ethanolic extracts responsible for the phytotoxic activity. The qualitative and quantitative profile of identified compounds (cynaropicrin, caffeoylquinic acids and flavones) was in agreement with previous studies (Pandino *et al*., 2011, 2015; Pinelli *et al*., 2007) and varied in relation to the extracting solvent (Table 7). The highest total measured polyphenols was found in the MeOH extract (14569 mg kg^{-1} of DM), followed by EtOH one (10454 mg kg^{-1} of DM), whereas the aqueous extract showed the lowest

content (2723 mg kg^{-1} of DM). In accordance to Pinelli *et al*. (2007), the main compounds detected here were caffeoylquinic acids, with 1,5-*O*-dicaffeoylquinic acids and chlorogenic acid. In particular, the concentration of chlorogenic acid was 513, 3397 and 6318 mg kg^{-1} of DM in bidistilled water, EtOH and MeOH extracts, respectively. In the alcoholic extracts, luteolin 7-*O*-glucuronide was the highest luteolin derivative compound (1894 and 2272 mg kg^{-1} of DM in EtOH and MeOH extracts, respectively), and apigenin 7-O-glucuronide was the main apigenin derivative compound (877 and 1150 mg kg^{-1} of DM in EtOH and MeOH extracts, respectively). Apigenin derivatives, however, were absent in aqueous extracts. Besides, the highest cynaropicrin content was found in the methanolic extract (158 mg kg^{-1} of DM), followed by ethanol (107 mg kg^{-1} of DM) and bidistilled water (54 mg kg^{-1} of DM).

Chlorogenic acid represents one of the most abundant phenolic acids in several plant extracts and its phytotoxic and allelopathic activity on different crops is reported in literature (Abdul-Rahman and Habib, 1989; Li *et al*., 1993). Several *C. cardunculus* phenolic compounds such as cynaropicrin, dicaffeoylquinic acid derivatives, luteolin 7-*O*-glucuronide, luteolin and apigenin 7-*O*-glucoside are known to possess allelopathic effects against weed and crop species. Rial *et al*. (2014) reported that cynaropicrin was very active (from 70 to 90% of inhibition) at 10^{-3}M on shoot length and root length of lettuce, cress, tomato, barnyardgrass and brachiaria. Beninger and Hall (2005) found that luteolin 7-*O*-glucuronide isolated from *Chrysanthemum morifolium* L, significantly reduced the frond number and chlorophyll content of *Lemna gibba* L. at 0.2 and 2.0 mM. Chlorogenic

acid and dicaffeoylquinic acid derivatives exuded by the roots of *Ipomoea batatas* (L.) Lam., inhibited radicle growth of *Panicum milliaceum* L. at 5 and 10 mM (Harrison et al. 2008). Hosni *et al*. (2013) reported that chlorogenic acid, luteolin and luteolin 7-*O*-glucoside, present in the aqueous extract of *C. coronarium* (Garland) suppress the germination and reduce the seedling growth of *Sinapis arvensis* L. and *Phalaris canariensis* L

Overall, the HPLC analysis outlined here a week relation between dose and allelopathic effect, because a major polyphenol content in the leaf extracts (methanolic) did not respond positively with a great inhibition weed germination. Probably, the higher inhibitory effects of ethanolic extract here observed was due to a wide polyphenol profile, such as apigenin or a major content of 1,5-*O*-dicaffeoylquinic acid, which all together have a better synergistic action on the weed species under study, respect to the methanolic extract.

According to our data, cultivated cardoon leaf extracts showed a significant allelopathic effect on seed germination of common weed species (*A. retroflexus*, *P. oleracea*, *S. media*, *A. arvensis)* in Mediterranean agroecosystems. Dried leaves could represent a valid leaf material for the potential production of bioherbicides, both under economically aspect and extraction yield. Based on the inhibition of seed germination, solvent's efficiency was ethanol > methanol > bidistilled water. Moreover, we have demonstrated that polyphenols accumulate mainly in methanolic extracts, with significant differences based on the extracting solvent. Nevertheless, this difference is not correlated with a better performance on the inhibition of weed species under study. In the future, we think that prospective research activities will

be focused on the evaluation of the phytotoxic effects of *C. cardunculus* for other weeds species of Mediterranean Basin and/or of different world areas.

Table 5.11 Caffeoylquinic acids, flavones and cynaropicrin (mg kg^{-1} of DM[(1)]) in dry leaf extracts of cultivated cardoon ('Altilis 41') in relation to the extracting solvent. Different letters within each row indicate statistical significance at $P \leq 0.05$. Each value represents the mean of $n=3$ ± standard error.

Compound	Solvent		
	Bidistilled	*Ethanol*	*Methanol*
5-*O*-caffeoylquinic acid	51.3 ± 2.0 c	340.0 ± 9.9 b	632 ± 9.8 a
1,5-*O*-dicaffeoylquinic acid	119.3 ± 33.3 c	230.5 ± 14.9 a	206.4 ± 3.2 b
Monosuccinildicaffeoylquinic acid	37.6 ± 10.5 b	36.4 ± 3.0 b	59.4 ± 0.1 a
Total caffeoylquinic acid	**208.2 c**	**606.9 b**	**897.8 a**
Luteolin 7-*O*-glucoronide	10.9 ± 4.1 c	189.4 ± 5.6 b	22.7 ± 0.8 a
Luteolin 7-*O*-malonylglucoside	nd[(2)]	50.5 ± 1.1 b	83.3 ± 1.3 a
Luteolin	53.2 ± 3.6	nd	nd
Total Luteolin	**64.1 c**	**239.9 a**	**106 b**
Apigenin 7-*O*-glucoside	nd	45.4 ± 3.6 b	61.7 ± 0.4 a
Apigenin 7-*O*-glucoronide	nd	87.7 ± 8.3 b	115.0 ± 2.2 a
Apigenin malonylglucoside	nd	62.0 ± 16.5 b	72.1 ± 0.4 a
Apigenin	trace	3.8 ± 1.2	nd
Total Apigenin	**trace**	**195.1 b**	**248.8 a**
Cynaropicrin	54 ± 4.0 c	10.7 ± 15.0 b	15.8 ± 2.0 a
Total measured polyphenols	**272 c**	**1046 b**	**1253 a**

[1)]DM = dry matter; [(2)]nd = not detected

5.3 The extraction procedure improves the allelopathic activity of cardoon (*Cynara cardunculus* var. *altilis*) leaf allelochemicals

The following work is accepted for publication by *Industrial Crops and Products* as follows:

- Scavo, A., Rial, C., Molinillo, J.M.G., Varela, R.M., Mauromicale, G., Macías, F.A. (2018g). The extraction procedure improves the allelopathic activity of cardoon (*Cynara cardunculus* var. *altilis*) leaf allelochemicals. *Ind Crop Prod* (In Press.).

5.3.1 Introduction

In cropping systems, weeds are the first factor affecting both crop yield and quality. Appleby *et al*. (2000) estimated the annual global economical loss caused by weeds at more than U.S. $100 billion dollars, with their control global cost running into $ billions (Kraehmer and Baur, 2013). In order to achieve the maximization of yields, modern agriculture indiscriminately adopted synthetic chemicals to eliminate the presence of weeds, accompanied by consequent ill effects on soil, water, humans and animal health, as well as an increasing incidence of resistance in weeds (Scavo *et al*., 2018a). Therefore, the search for alternative weed management strategies more economically and environmentally-sustainable is by now of central importance. A natural and environment-friendly strategy for weed control is provided by the manipulation of allelopathic mechanisms between crops and weeds. Allelopathy refers to both direct or indirect, harmful or beneficial, effects by a donor plant (including microorganisms) on a target species through the

production of chemical compounds that escape into the environment (Rice, 1984). These compounds, known as allelochemicals, are good candidates for the development of bioherbicides (Macías *et al*., 2007).

Cultivated cardoon leaves are a good source of polyphenols such as chlorogenic acid, luteolin- and apigenin derivatives (Pandino *et al*., 2012b; 2015), and sesquiterpene lactones such as cynaropicrin, aguerin B and grosheimin (Rial *et al*., 2014). In a preliminary study, Scavo *et al*. (2018b) evaluated the phytotoxic potential of the three *C. cardunculus* botanical varieties on seed germination of some common Mediterranean weed species, and cultivated cardoon resulted the most efficient. Nevertheless, the phytotoxic activity of these compounds has been well documented (Hosni *et al*., 2013; Rial *et al*., 2014; 2016a; Scavo *et al*., 2018f).

However, researches conducted on this topic were carried out with different extraction methodologies of cultivated cardoon allelochemicals. Moreover, the whole spectrum of cultivated cardoon phytotoxic compounds has not completely investigated. Since extraction methodology, analytical techniques and experimental application procedures are all factors that could affect the phytotoxicity of a chemical (Zhu *et al*., 2011), it is important to set up the best methodology for the extraction and analysis of cultivated cardoon leaf allelochemicals.

Therefore, our goals were to select the most efficient extraction method of cultivated cardoon phytotoxic compounds in terms of costs, yields and inhibitory activity, and to bioprospect the widest possible spectrum of its extracts.

5.3.2 Material and methods

5.3.2.1 Experimental design

In order to set up the most efficient system for the extraction of cultivated cardoon phytotoxic compounds, methodologies proposed by Scavo *et al*. (2018f) and Rial *et al*. (2014) were compared, with some modifications. Therefore, two principal groups of extracts were investigated (**Table 5.12**). For what concern Group 1 extracts, following the procedure describe by Scavo *et al*. (2018f), three dilutions (80, 40 and 20%,) of aqueous, methanolic and ethanolic extracts were initially evaluated in the coleoptile bioassay; the ethanolic extract was found to be the most active. In a second step, and with the purpose to compare the activities of both extraction methodologies, their phytotoxic activity was also performed at three concentrations (800, 400 and 200 ppm) and the ethanolic extract confirmed to show the highest phytotoxic activity. Moreover, the aqueous extract was purified with a liquid-liquid separation to afford two fractions: **H2O-R1** (water) and **H2O-R2** (EtOAc). Both fractions were bioassayed using the etiolated coleoptile test; fraction H2O-R2 showed the highest activity. In Group 2, the direct ethyl acetate extract from leaves was further investigated by removing the chlorophylls, in order to increase the yield of the extract. Two fractions were obtained: **EtOAc-WC** (EtOAc extract without chlorophylls) and **chlorophyll fraction**. Since aqueous extract and its fraction H2O-R2, methanolic, ethanolic and ethyl acetate extracts were the most active in the wheat coleoptile bioassay, they were evaluated through the weed phytotoxicity bioassay.

5.3.2.2 Chemicals

Organic solvents (methanol, ethanol, ethyl acetate, dichloromethane, chloroform, *n*-hexane, acetone, and acetonitrile) were HPLC grade and were purchased from Fischer Chemicals (Geel, Belgium). For NMR spectroscopy (Merck, Darmstadt, Germany), chloroform-D1 deuteration degree min. 99.8% and methanol-D4 deuteration degree min. 99.8% were used. Water was type I obtained from an Ultramatic system from Wasserlab (Barbatáin, Spain).

Table 5.12 Experimental design for extraction methodology of cultivated cardoon phytotoxic compounds performed in this study.

Group 1	**Group 2**
1) H_2O extract	1) Direct EtOAc extract from leaves
2) H_2O fraction of aqueous extract (H_2O-R1)	2) EtOAc extract without chlorophylls (EtOAc-WC)
3) EtOAc fraction of aqueous extract (H_2O-R2)	3) Chlorophyll fraction
4) MeOH/H_2O (70:30 v/v) extract	
5) EtOH/H_2O (80:20 v/v) extract	

5.3.2.3 NMR and HPLC analysis

For Nuclear Magnetic Resonance (NMR), 1H NMR and 13116 C NMR spectra were recorded at 400 and 500 MHz spectrometers (Agilent, Santa Clara, CA, U.S.A.) in a Varian INOVA spectrometer, using $CHCl_3$-d_1 and MeOH-d_4 as solvents. The resonances of residual chloroform and methanol at δ_H 7.25 and δ_H 3.30 for ^1H signals, and δ_C 77.00 and δ_C 49.00 for ^{13}C signals, were used as internal references. Chromatographic determinations were performed on a HPLC

Merck-Hitachi system model LaChrom (Tokio, Japan) equipped with a L-7100 Pump, a L-7490 LaChrom Detector and a D-7000 Interface. A semi-preparative column (LiChrospher 10 μm 250−10 mm Si60, Merck) with a LiChrospher Si60 (Merck) guard column, a normal phase analytical column (Luna silica (2) 10 μm 100Å 250−4.60 mm, Phenomenex, Torrance, CA, U.S.A.) and a reverse phase analytical column (Gemini C18 10 μm 250−4.5 mm, 110A RP-18, Phenomenex) with a SecurityGuard Cartridges Gemini RP-18 (Phenomenex) guard column were used. Thin layer chromatography (TLC) analyses were carried out using aluminium Silica gel 60 F_{254} and aluminium Silica gel 60 RP-18 F_{254S} sheets (Merck, Darmstadt, Germany).

5.3.2.4 Plant material, crop management and extract preparation

Leaves of cultivated cardoon cv. Altilis 41, selected by Catania 130 University within a breeding program on *C. cardunculus*, were collected randomly during November 2016 from about sixty plants at the 25th visible disease-free leaves growth stage in the Catania University experimental station situated in the Catania Plain [10 m (a.s.l.), 37° 25^{I} N, 15° 30^{I} E]. The local soil is a calcixerollic xerochrept (Soil Survey Staff, 1999) and the climate comprises mild-wet winters and warm-dry summers. Within each row, each plant was separated from its neighbour by 0.80 m and the inter-row spacing was 1.25 m; the result was a planting density of one plant per m^2. Starter fertilisation was applied before awakening with 80, 180, and 150 kg ha^{-1} of N, P_2O_5 and K_2O, respectively. On both seasons, further two N applications (as ammonium nitrate) were applied at a rate of 80 kg ha^{-1} on

early November and late-February, respectively. Irrigation was supplied. The plots were maintained weed- and insect-free by spraying oxyfluorfen and imidachloprid, respectively, when required. Fresh leaves were washed, air dried in an oven at 45 °C up to constant weight and powdered in an industrial mill. In **Figure 5.6**, a schematic representation of the different extraction procedures is reported.

For what concern extracts of Group 1, 70 g of dried leaves were soaked in 700 mL of water, methanol/water (70:30, v/v), and ethanol/water (80:20, v/v) for 72 h at room temperature (20°C ± 1) in the dark. The resulting mixtures were filtered through a layer of filtered paper (Whatman N° 2) and the solvents were evaporated to dryness by use of rotary evaporator (IKA RV 10 basic, VWR, U.S.A.) under vacuum below 40°C to give 10.01 g, 15.19 g and 12.25 g of aqueous, MeOH and EtOH extract, respectively. The residues were dissolved in 700ml of water and then diluted at three different concentrations (80, 40 and 20%) with water. The phytotoxic activity of these extract was evaluated through the etiolated wheat coleoptile bioassay. Since their high concentration, and to compare both extractions methodologies, in step two they were bioassayed at 0.8, 0.4 and 0.2 mg/mL, like extracts of Group 2.

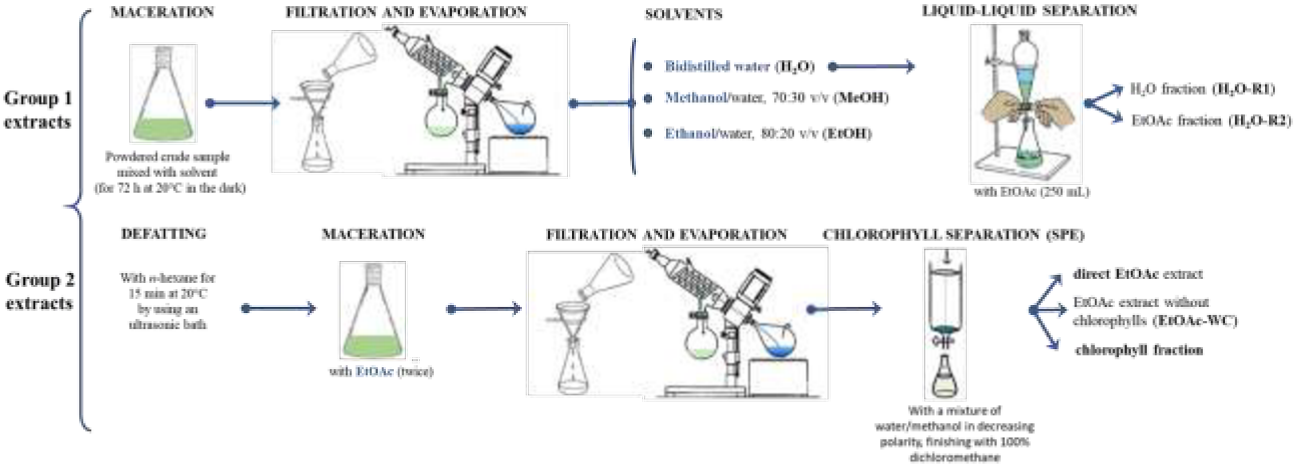


Figure 5.6 Schematic representation of different extraction methodologies performed in this study.

Regarding Group 2 extracts, 15 g of powdered dried leaves were first extracted with 250 mL of *n*-hexane at room temperature using an ultrasonic bath (360 W, J.P. Selecta, Barcelona, Spain) for 15 minutes, in order to remove oils, greases and waxes (degreasing). Then, the same leaves were air dried in an oven at 35 ºC x 24 h and the residues (14.40 g) re-extracted with 250 mL of EtOAc twice. The mixtures were filtered, and the solvent eliminated on a rotary evaporator at 35 °C, giving 617.8 mg of EtOAc extract. In addition, since the EtOAc extract was rich in chlorophylls, which were not the substances of interest, it was decided to remove them through reversed-phase separation in a 200−30 mm column filled with RP 18 of silica (Merck, Darstadt, Germany) as stationary phase and a mixture of water/methanol in decreasing polarity (from 100:0 to 0:100, v/v, in 20% increments), finishing with 100% dichloromethane as the mobile phase. Two fractions were obtained from 200 mg of the EtOAc extract: EtOAc-WC (175.7 mg, without chlorophylls) and the chlorophyll fraction (25.4 mg). These fractions, together with the direct EtOAc extract from leaves, were bioassayed with the coleoptile bioassay and the first one resulted the most active.

5.3.2.5 Isolation of compounds

Since the EtOAc extract was previously partitioned (Rial *et al.*, 2014), the aqueous extract was subjected to further purification in two separate steps with the aim to isolate, purify and evaluate other secondary metabolites involved in cultivated cardoon phytotoxic activity.

In step one, the crude aqueous extract was partitioned by means of liquid-liquid separation with EtOAc (250 mL x 3)

and then dried by reduced pressure evaporation to afford H_2O-R1 (9.1679 g, 100% water) and H_2O-R2 (842.1 mg, 100% EtOAc). The latter fraction was subjected to normal phase liquid column chromatography (500−60 mm) filled with Geduran Si 60A silica (300 g) from Merck (Darmstadt, Germany) as the stationary phase and a step gradient of four solvents [step 1 was 100% *n*-hexane (600 ml); step 2 was a linear gradient *n*-hexane/ethyl acetate of increasing polarity from 50 to 100% in ethyl acetate increasing 10% each time (400 mL); step 3 was 100% acetone (500 mL); step 4 was 100% methanol (500 mL)] as the mobile phase. Seven fractions were obtained (A−G) after comparison by normal phase TLC with a mixture of *n*-hexane/ethyl acetate 30:70 as the mobile phase. Fraction C (61.4 mg) showed to be mostly **1**. Fraction E (87.1 mg) was separated by HPLC using an isocratic mixture of chloroform/methanol (90:10 v/v, flow 3 mL/min, LiChrospher SI 60 normal phase semipreparative column, retention time 14 min) to yield **2** (30.8 mg). Fractions D (81.3 mg, 5 mg/injection) was separated by HPLC (LiChrospher SI 60 normal phase semipreparative column) using chloroform/methanol (95:5 v/v, flow 3 mL/min) to afford 6 fractions (D1−D6). Fraction D−5 (14.7 mg, 1 mg/injection) was purified by HPLC (Luna silica (2) normal phase analytical column) using *n*-hexane/acetone (75:25 v/v, flow 1 mL/min) to afford 2 fractions (D5.1−D5.2). Fraction D5.1 (9.2 mg, 1 mg/injection) was further purified by HPLC (Gemini C18 reverse phase analytical column) using a mixture of water/acetonitrile/methanol (70:20:10 v/v, flow 1 mL/min) to yield **2** (3.5 mg, retention time 5.2 min). Fraction D−6 (14.8 mg, 1 mg/injection) was purified by HPLC (Luna silica (2) normal phase analytical column) using *n*-

hexane/acetone (70:30 v/v, flow 1 mL/min) to afford 4 fractions (D6.1−D6.4). Fraction D6.4 (9.0 mg, 1mg/injection) was further purified by HPLC using the same procedure as described above for compound **3** to yield the epimeric mixture **4** (3.6 mg, retention time 4.7 min). Fraction B (14.7 mg, 1 mg/injection) was separated by HPLC (Luna silica (2) normal phase analytical column) using *n*-hexane/acetone (70:30 v/v, flow 1 mL/min) to yield **5** (3.5 mg, retention time 17.7 min). The purity and the structures of the isolated compounds were confirmed by the ^{1}H and ^{13}C NMR spectra and are shown in **Figure 5.7**.

5.3.2.6 Wheat coleoptile bioassay

Phytotoxic activity of different extracts and isolated compounds was first tested through the wheat coleoptile bioassay, a rapid and sensitive protocol for general bioactivity evaluation (Hancock *et al*., 1964). Seeds from *Triticum aestivum* L. cv. 'Catervo' were used for the coleoptile bioassay, which was carried out following Macias *et al*. (2000). All manipulations were performed under a green safelight (Nitsch and Nitsch, 1956). Both extracts and compounds were predissolved in dimethyl sulfoxide (DMSO, 0.1% v/v) and diluted in phosphate-citrate buffer containing 2% sucrose (Nitsch and Nitsch, 1956) at pH 5.6 to the final bioassay concentration (800, 400 and 200 ppm for extracts and fractions, and 10^{-3}, $3 \cdot 10^{-4}$, 10^{-4}, $3 \cdot 10^{-5}$ and 10^{-5} M for compounds). The commercial herbicide Logran® was used as positive control (Macias *et al*., 2000), while the buffer described above as negative control. 2 mL of crude extracts, fractions and pure compounds were added to test tubes (three tubes per dilution) following the placement of five coleoptiles

in each test tube, and they were placed in the dark at 25 ºC and 6 rpm in a roller tube apparatus for 24 h. The assay was made in duplicate. The coleoptile elongations were measured automatically by generation of their digital images, which were processed in a Photomed Equipment Software.

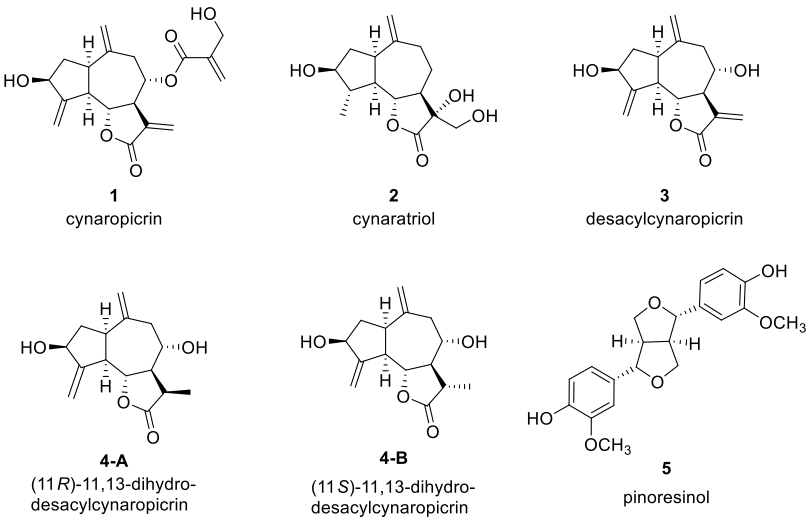


Figure 5.7 Chemical structures of compounds isolated from cultivated cardoon EtOAc fraction of aqueous extract: cynaropicrin (**1**), cynaratriol (**2**), desacylcynaropicrin (**3**), (11*R*)-11,13-dihydro-desacylcynaropicrin (**4A**), (11*S*)-11,13-dihydro-desacylcynaropicrin (**4B**) and pinoresinol (**5**).

5.3.2.7 Weed phytotoxicity bioassay

Six common weed species in Mediterranean Basin, both mono- and dicotyledons, were selected to test the phytotoxic activity of crude extracts, fractions and pure compounds: *Amaranthus retroflexus* L., *Portulaca oleracea* L., *Stellaria media* L., *Anagallis arvensis* (L.) Vil., *Echinochloa crus-galli* L. and *Lolium perenne* L. *E. crus-galli* and *L. perenne* seeds

were purchased from Herbiseed (Twyford, U.K.), while the others were collected in the Catania Plain (latitude 37°28'N, longitude 14°57'E, at an average altitude of 50–150 m a.s.l.), characterized by a Mediterranean clime with daily mean temperature of 8.5–26 °C during the year and year mean annual precipitation of about 500 mm (Cristaudo *et al*., 2007). After collection, seeds were cleaned, kept in paper bags and dry-stored at room temperature (20°C ± 2°C) until tests were performed. Seeds of each species were selected with a stereomicroscope to achieve a homogeneity of the lots for size and colour.

Four replicates of 20 seeds for each species were imbibed in 50 mm Petri dishes, on a sheet of Whatman No. 1 filter paper. Extracts were dissolved in 2-[*N*-morpholino]ethanesulfonic acid (MES) at 10^{-2} M and 1 M NaOH buffer (pH 6.0). Then, these solutions were diluted with 5 µL/mL of DMSO to obtain 800, 400 and 200 ppm concentrations for extracts or fractions. Parallel controls were also carried out as described before for wheat coleoptile bioassay. 1 mL of solutions above described were applied to each Petri dish, which successively were hermetically sealed with parafilm to prevent evaporation of the solution and incubated in a Memmert ICE 700 controlled environment chamber. Tests were performed in continuous darkness at 25 °C for *A. retroflexus*, *P. oleracea*, *L. perenne* and *E. crus-galli*, while *S. media* and *A. arvensis* were incubated in alternating light (dark/light cycle 14/10 h) at 17°C. Petri dishes were transparent for dark/light alternating conditions and wrapped in sheets of aluminium foil for complete darkness. Moreover, bioassays took 6 days for *A. retroflexus*, *L. perenne* and *E. crus-galli*, 9 days for *P. oleracea*, 11 days for *S. media* and 13 days for *A. arvensis*.

Then, all Petri dishes were frozen at –10 °C x 24 h to avoid subsequent growth during measurements process. Germination rate, root length ad shoot length were recorded through a Fitomed system (Rial *et al*., 2016b).

5.3.2.8 Data and statistical analysis

Statistical analysis of wheat coleoptile and weed phytotoxic bioassays data was carried out using Welch's test, with significance fixed at 0.01 (a) and 0.05 (b). Data are expressed as percentage difference from the control, with positive values representing stimulation and negatives representing inhibition. The half maximum inhibitory concentration (IC_{50}) values for compounds were calculated from non-linear regression with the PRISMA 6 package (PRISMA 6.0, GraphPhad software, San Diego, CA, USA.)

5.3.3 Results and discussion

Regarding the different studies published about the phytotoxic activity of *C. cardunculus* extract, it is crucial to set up the most efficient system for the extraction of cultivated cardoon phytotoxic compounds. For this aim, the methodologies proposed by Scavo *et al*. (2018f) and Rial *et al.* (2014) were compared. Dried leaves were chosen instead of lyophilized ones because of their good phytotoxic activity on weed seed germination and lower cost management compared to the lyophilization (Scavo *et al*., 2018f). Yields of crude extracts and their fractions ((g extract/ g dried leaves)·100) were 14.3 % for water, 1.2% for the H_2O-R1, 13.1% for the H_2O-R2, 21.7% for MeOH, 17.6% for EtOH, 4.1% for EtOAc, 3.6% for EtOAc-WC, and 0,5% for the chlorophyll fraction.

In a first step, the two different concentrations used by both authors were compare. The results of Group 1 extracts are reported in **Figure 5.8**. All the extracts were active on wheat coleoptile elongation. In particular, extracts utilized by Scavo *et al*. (2018f) significantly reduced wheat coleoptile elongation (more than 75%) among their profiles, and the EtOH extract resulted the most active at the three dilutions tested (80, 40 and 20%), with values of 100, 98 and 93%, respectively. The MeOH extract showed an intermediary behaviour, with an inhibitory activity ranged from 88 to 95%. The aqueous extract also showed high activity levels, inhibiting 95, 82 and 76% at 80, 40 and 20%, respectively. In view of the results, the concentrations used by Scavo *et al*. showed so high activity that they are not useful to compare extract due to they showed an inhibition close to 100% in all concentrations. For this reason, the extracts obtained by both methodologies were evaluated at 800, 400 and 200 ppm for an adequate comparison. These extracts also showed good phytotoxic levels (**Figure 5.8** and **5.9**). The EtOH extract inhibited 92, 80 and 48% the growth of etiolated coleoptiles with 800, 400 and 200 ppm, respectively. The aqueous and MeOH extracts inhibited 55 and 54%, respectively, the coleoptiles elongation at 800 ppm.

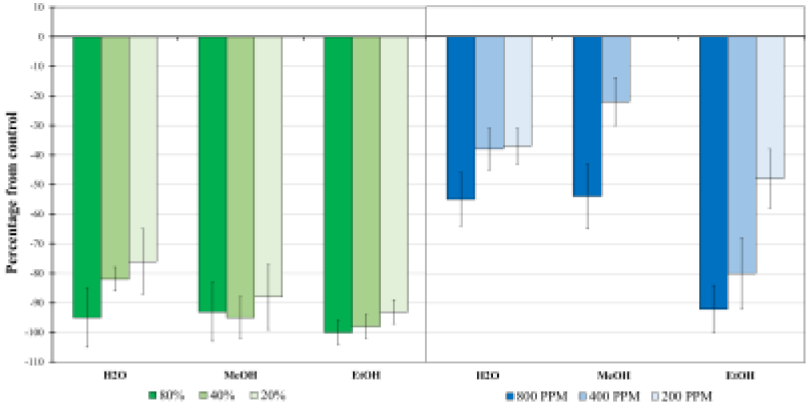


Figure 5.8 Effects of three crude cultivated cardoon leaf extracts (aqueous, methanolic and ethanolic) on wheat coleoptile elongation. Values are expressed as percentage difference from control. Each bar means ± standard deviation.

Since ethyl acetate-and water-extracts were active and TLC analysis showed different compositions, together with its low environmental impact, the aqueous extract was partitioned with liquid-liquid separation. The H_2O-R1 and H_2O-R2 fractions were chromatographed by normal phase TLC with a mixture of *n*-hexane/ethyl acetate 30:70 as the mobile phase and major compounds were detected in the latter fraction. Both fractions were bioassayed (**Figure 5.9**) and the etiolated coleoptile was significantly inhibited by H_2O-R2 (71 and 63% at 800 and 400 ppm, respectively), while the H_2O-R1 did not show any inhibitory activity. In a previous work, Rial *et al*. (2014) found that cardoon ethyl acetate extract was the most active fraction both in wheat coleoptile and phytotoxicity bioassay. In the present work, we further investigated the ethyl acetate extract by evaluation of its

inhibitory activity with (EtOAc) and without (EtOAc-WC) chlorophylls (**Figure 5.9**). The direct EtOAc extract and EtOAc-WC showed the most consistent profiles, with inhibiting values at 400 and 200 ppm of 100 and 93%, respectively, and 97 and 81%, respectively. The chlorophyll fraction did not show phytotoxic effects. Considering that EtOAc extract and EtOAc-WC showed similar activity values, as well as the higher yield and the lower cost management, the direct EtOAc extract from leaves was selected for the weed phytotoxicity bioassay.

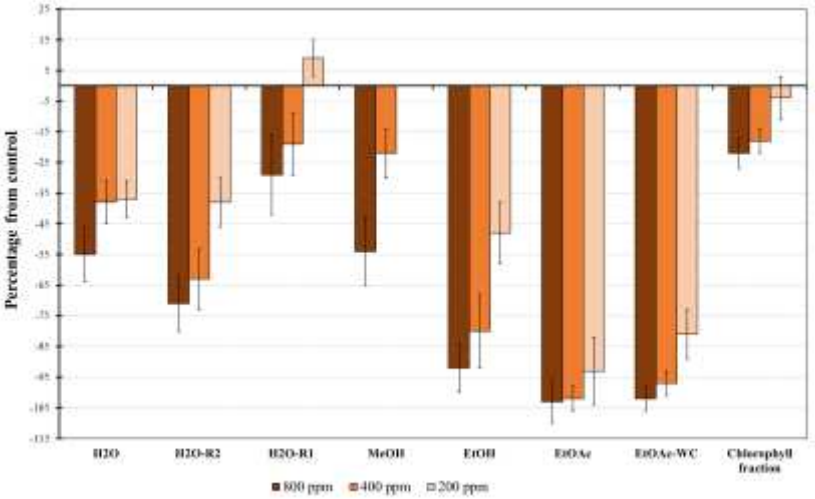


Figure 5.9 Effects of five crude cultivated cardoon leaf extracts (aqueous, methanolic, ethanolic and ethyl acetate) and fractions on wheat coleoptile elongation. Values are expressed as percentage difference from control. Each bar means ± standard deviation. H_2O, MeOH, EtOH, EtOAc: aqueous, methanolic, ethanolic and ethyl acetate extracts, respectively; H_2O-R2: ethyl acetate fraction from aqueous extract; H_2O-R1: polar (H_2O) fraction from aqueous extract; EtOAc-WC: ethyl acetate extract without chlorophylls.

Therefore, aqueous extract and H_2O-R2, methanolic, ethanolic and ethyl acetate extracts were evaluated in the phytotoxicity bioassay at the same concentrations previously described on six weeds widespread in the Mediterranean Basin: *A. retroflexus*, *P. oleracea*, *S. media*, *A. arvensis*, *E. crus-galli* and *L. perenne* (**Figure 5.10**–**5.12**). On the average of all weed species, root length was the most affected parameter and germination the least influenced, as previously reported by several authors with the same bioassay for other plant species (da Silva *et al*., 2017; Rial *et al*., 2018). In particular, the inhibition of root length was higher than of Logran for all species, with values close to 90% at 800 ppm for EtOH and EtOAc extracts. Germination was significantly affected only in *P. oleracea*, mainly by the EtOH extract for all the tested concentrations (31% of average inhibition), and in *L. perenne*, reaching inhibiting values of 24 and 21% for the EtOH extract and H_2O-R2 at 800 ppm, respectively. Shoot length, as well as root length, was affected by the EtOH and EtOAc extracts at both 800 and 400 ppm in all tested species.

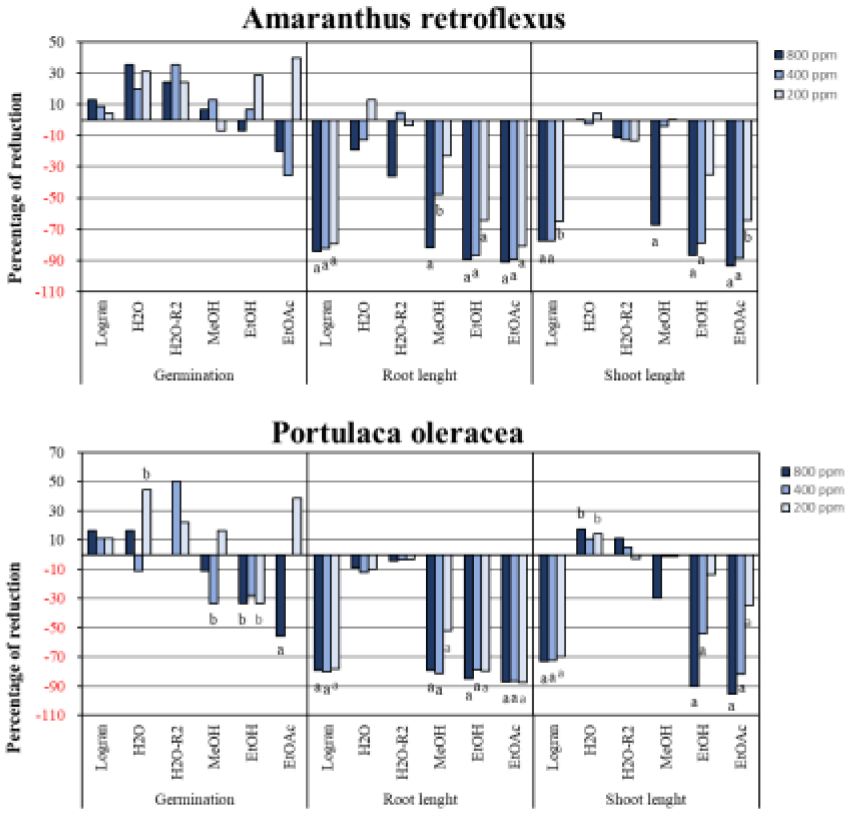


Figure 5.10 Effects of aqueous and its ethyl acetate fraction, methanolic, ethanolic and direct ethyl acetate extracts from cultivated cardoon leaves on the germination and growth of *Amaranthus retroflexus* and *Portulaca oleracea*. The commercial herbicide Logran® was used as a positive control. Values are expressed as percentage difference from control. a and b indicate significance for $P < 0.01$ and $0.01 < P < 0.05$, respectively.

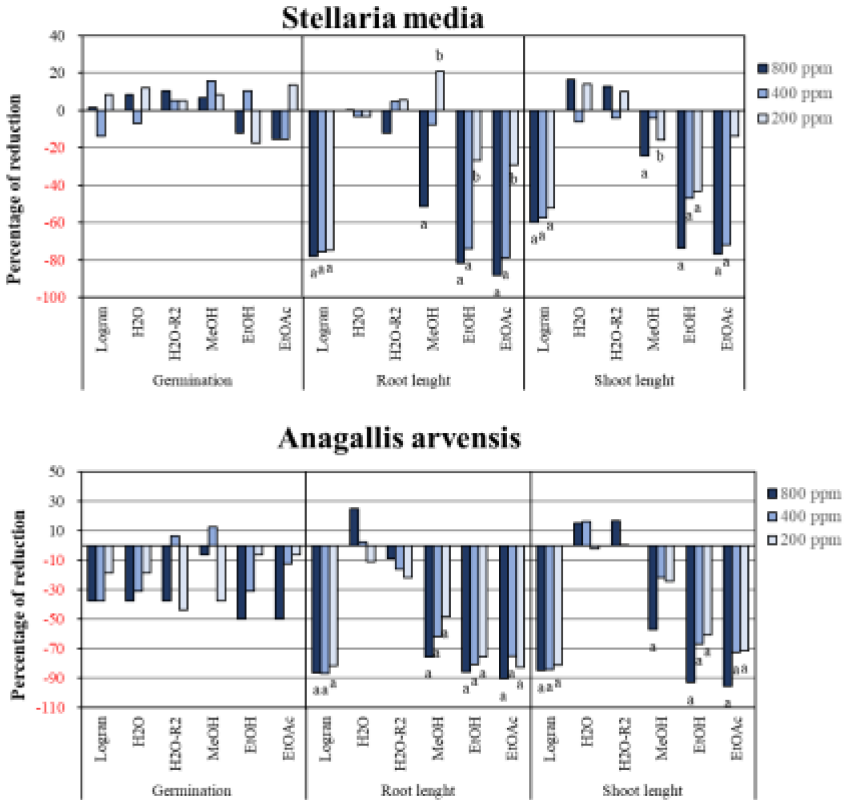


Figure 5.11 Effects of aqueous and its ethyl acetate fraction, methanolic, ethanolic and direct ethyl acetate extracts from cultivated cardoon leaves on the germination and growth of *Stellaria media* and *Anagallis arvensis*. The commercial herbicide Logran® was used as a positive control. Values are expressed as percentage difference from control. a and b indicate significance for $P < 0.01$ and $0.01 < P < 0.05$, respectively.

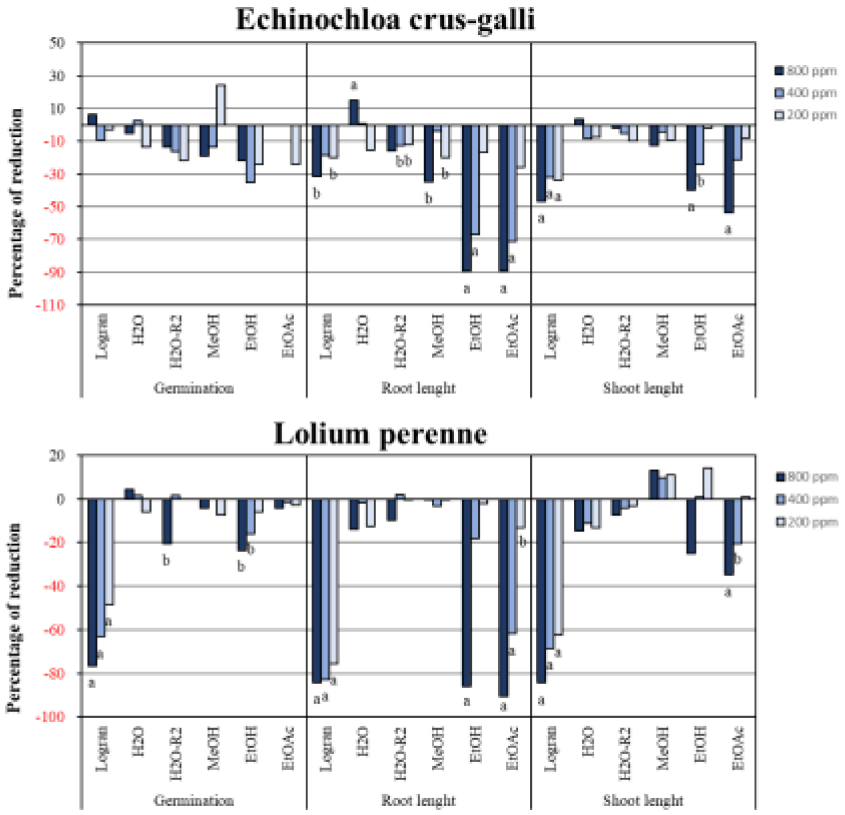


Figure 5.12 Effects of aqueous and its ethyl acetate fraction, methanolic, ethanolic and direct ethyl acetate extracts from cultivated cardoon leaves on the germination and growth of *Echinochloa crus-galli* and *Lolium perenne*. The commercial herbicide Logran® was used as a positive control. Values are expressed as percentage difference from control. a and b indicate significance for $P < 0.01$ and $0.01 < P < 0.05$, respectively.

Furthermore, in order to investigate other fractions with different polarity of cultivated cardoon extracts, H_2O-R2 was chromatographed in column chromatography and HPLC analysis. Cynaropicrin (**1)** (61.4 mg), previously isolated by Rial *et al*. (2014) was also identify in fraction C and four compounds were isolated and purified (**Figure 5.7**): the sesquiterpene lactones cynaratiol (**2**), desacylcynaropicrin (**3**), (11*R*)-11,13-dihydroxy-desacylcynaropicrin (**4A**) and (11*S*)-11,13-dihydroxy-desacylcynaropicrin (**4B**) as a 1:1 mixture of epimers as was evident from the ^{1}H NMR spectrum, and the lignan pinoresinol (**5**). All the identified compounds are typical of the Asteraceae family. Cynaratriol (**2**) has been previously isolated in *C. cardunculus* (Bernhard *et al*., 1979; Shimizu *et al*., 1988); pinoresinol (**5**) was reported in globe artichoke (Abu-Reidah *et al*., 2013; Gouveia and Castilho, 2012), but not in cultivated cardoon. Compounds **3** and **4** were reported here for the first time in this species. Except for pinoresinol (Macias *et al*., 1999), the phytotoxic activity of these compounds is unknown. For this reason, compounds **2**, **3**, **4** and **5** were assayed in the wheat coleoptile bioassay at 10^{-3}, $3{\cdot}10^{-4}$, 10^{-4}, $3{\cdot}10^{-5}$ and 10^{-5} M (**Figure 5.13**). Desacylcynaropicrin (**3**) and pinoresinol (**5**) showed the highest activity, inhibiting the wheat coleoptile by 96 and 83% at 10^{-3} M, respectively, and 56 and 37% at $3{\cdot}10^{-4}$ M, respectively. Their activity decreased with concentration. Compound **4** was active only at the first dilution (10^{-3} M, -59%), while pinoresinol (**5**) did not show significantly phytotoxic activity. IC_{50} values were: compound **3**, 216.1 μM mL^{-1} (R_2 = 0.9642); compound **4**, 991.4 μM mL^{-1} (R_2 = 0.9924); compound **5**, 444.0 μM mL^{-1} (R_2 = 0.9791); Logran®, 39.66 μM mL^{-1} (R_2 = 0.9505). Since compound **2**

not reached an inhibiting value of 50%, it not presents an IC_{50} value. All compounds showed IC_{50} values higher than Logran®, demonstrating their lower activity respect to that of the commercial herbicide. Desacylcynaropicrin (**3**) presents the lowest IC_{50} value, confirming that it was the most active compound, followed by pinoresinol (**5**). The presence of these compounds together with cynaropicrine, which is the most abundant, explains its phytotoxicity (Rial *et al*., 2014) (**Figure 5.13**). It is important to underline that allelopathic compounds rarely act alone, but generally the phytotoxic activity is due to the synergistic effects of mixture of compounds (Einhellig, 1995; Inderjit *et al*., 2002). Rial *et al*. (2016a) studied the joint action of 17 binary mixtures from three *C. cardunculus* active sesquiterpene lactones (cynaropicrin, grosheimin and aguerin B) and one non-active compound (11,13-dihydroxy-8-desoxygrosheimin), obtaining 25 additive interactions, 7 synergistic interactions and 2 antagonistic interactions. These results, together with the different concentrations, could explain the differences in the phytotoxic activity between extracts and pure compounds, as well as their high IC_{50} values.

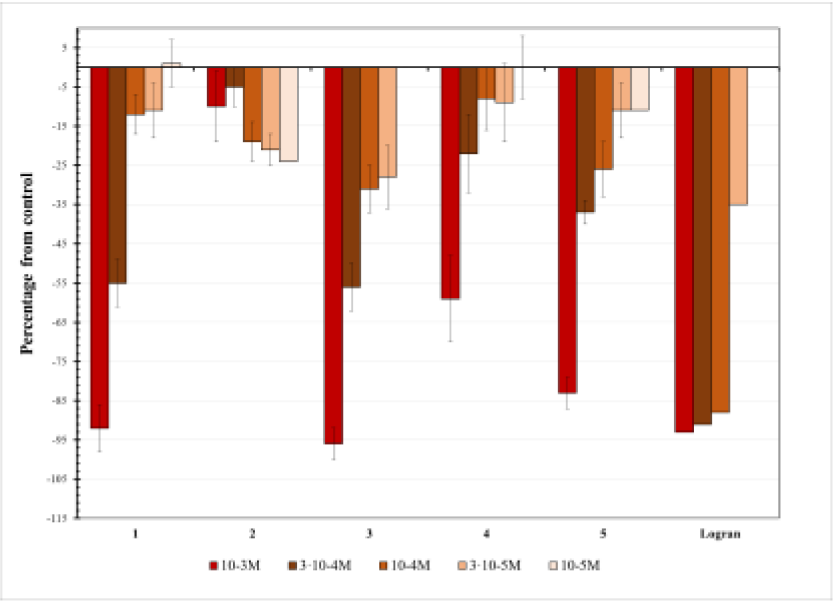


Figure 5.13 Effects of commercial herbicide Logran® and compounds cynaropicrin (**1**), cynaratriol (**2**), desacylcynaropicrin (**3**), 11,13-dihydro-desacylcynaropicrin (**4**), and pinoresinol (**5**) on wheat coleoptile elongation. Values are expressed as percentage difference from control. Each bar means ± standard deviation.

5.3.4 Conclusions

The extraction methodology of cultivated cardoon allelochemicals was investigated in this study by comparing different extracting procedures. Ethanolic and direct ethyl acetate from leaves were the most active extracts both on wheat coleoptile bioassay and weed germination and growth. Ethyl acetate extract was the most promising extract in view of its activities, even with the dilutions. Ethanol extract could also be selected for its high yield even showing a slightly lower activity than EtOAc extract. The sesquiterpene lactones

cynaratriol, desacylcynaropicrin and 11,13-dihydro-desacylcynaropicrin were reported in cultivated cardoon for the first time. Cynaropicrin, desacylcynaropicrin and pinoresinol showed the highest inhibitory activity on wheat coleoptile elongation, suggesting their influence on cultivated cardoon allelopathic potential, while cynaratriol was inactive. These results and the fact that cynaropicrin was extracted with water as mayor compound, could explain the activity showed by the aqueous extract. This is of central importance for the setup of the best extraction method of cultivated cardoon allelochemicals in terms of cost, yield and phytotoxic activity, and for a better understanding of its allelopathic behaviour.

5.4 Influence of genotype and harvest time on sesquiterpene lactone profile of *Cynara cardunculus* L. leaf extracts

5.4.1 Introduction

Several works have been carried on the allelopathic activity of *Cynara cardunculus* L., especially on cultivated cardoon. Its allelopathic potential is due mainly to the presence of sesquiterpene lactones (STLs), particularly cynaropicrin, grosheimin and aguerin B (Rial *et al*., 2014), as well as polyphenols such as chlorogenic acid, luteolin- and apigenin derivatives (Scavo *et al*., 2018f), which are present in high quantities in the leaves (Pandino *et al*., 2015). In a previous work, Scavo *et al*. (2018g) isolated three STLs (cynaratriol, desacylcynaropicrin and 11,13-dihydro-desacylcynaropicrin)

and a lignan (pinoresinol) from the ethyl acetate fraction of the aqueous extract in cultivated cardoon. Desacylcynaropicrin, 11,13-dihydro-desacylcynaropicrin and pinoresinol showed inhibitory effects on wheat coleoptile elongation, while cynaratriol was inactive. While the leaf polyphenol composition of *C. cardunculus* has been widely reported in literature (Pandino *et al*., 2011, 2015), only few works have been published on the STL profile (Rouphael *et al*., 2016; Seaman, 1982; Shimoda *et al*., 2003). STLs are characteristic compounds of the Asteraceae family derived from the farnesyl diphosphate (FPP) and represent the major lipophilic constituents in cultivated cardoon leaves (95 g kg^{-1} dry weight) (Ramos *et al*., 2013). In addition to the phytotoxic activity, they possess fungicidal (Wedge *et al*., 2000), antimicrobial (Schinor *et al*., 2004) and anti-cancer activity (Zhang *et al*., 2005).

Genotype and plant age are two important factors that should be considered in the better understanding of the allelopathic potential of *C. cardunculus* (Scavo *et al*., 2018a). The influence of genotype is widely reported in literature for several crop species such as rice (Kabir *et al*., 2010; Amb and Ahluwalia, 2016), sorghum (Alsaadawi *et al*., 1986), rye (Reberg-Horton *et al*., 2005), sunflower (Alsaadawi *et al*., 2012), etc. Many evidences were also reported about the influence of plant or organ age on the allelopathic effects of one plant (Schumacher *et al*., 1983; Wardle *et al*., 1993). Pandino *et al*. (2013), studying the quali-quantitative composition of polyphenols in globe artichoke tissues from November to April, reported that the highest polyphenol content for leaves was detected in the February-April harvest period. Considering the effects of genotype and harvest time

on *C. cardunculus* polyphenol profiles, our hypothesis is that these biotic factors could affect the STL concentration in *C. cardunculus* leaves and, thus, its phytotoxic activity.
This trial aims to examine the quantity and composition of STLs in *C. cardunculus* leaves as affected by genotype and harvest time, through a new UHPLC-MS/MS analysis method. Moreover, the leaf ethanolic extracts of six different genotypes obtained from three harvest times were evaluated on the wheat coleoptile elongation to find a correlation between the inhibitory activity and the STL profile.

5.4.2 Material and methods

5.4.2.1 Experiment design, plant material, field sampling and crop management

The experiment was set up in a completely randomized design with four replications including the six genotypes reported in **Table 5.13**. 'Naro 2' is a landrace from small-holdings grown in central Sicily, 'VSB3' is a clone selected from the most popular Sicilian varietal type 'Violetto di Sicilia'; 'Marsala' and 'Valparaiso' were obtained from a native stand in Marsala (Western Sicily) and Valparaiso (Chile), respectively; 'Altilis 41' is a synthetic cultivar developed by the University of Catania for biomass and bioactive molecules production; 'Bianco gigante' is an Italian commercial cultivar. Both latters are cultivated cardoons.
Plants were grown in the Catania experimental station [South Italy, 37° 25^{I} N; 15° 30^{I} E; 10 m a.s.l.] in a typic vertic and/or xerochrept soil (Soil Survey Staff, 1999) with clay texture. Soil characteristics were as follows: sand 27%, clay 45%, silt 28%, organic matter 1%, total nitrogen 0.1%, available P_2O_5 10 ppm, and pH 7.2. The local climate is semi-arid

Mediterranean, with mild rainy winters and hot dry summers. Prior to planting, the field was ploughed to a depth of 30 cm, harrowed and a fertilizer rate of 50 kg N ha^{-1} (as urea), 80 kg P_2O_5 ha^{-1} (as double perphosphate) and 80 kg K_2O ha^{-1} (as potassium sulphate) was given.
One hundred seedling bearing three leaves of cultivated and wild cardoon or fifty sprouting "ovoli" (semi-dormant offshoots) were transplanted into the field at the end of August at the rate of 1 plant m^{-2}, using an inter- and intra-row spacing of 1.25 and 0.80 m, respectively. The expanded plot was kept weed- and insect-free by spraying oxyfluorfen and imidacloprid, respectively, when required. Fifty full-expanded leaves were randomly sampled at the phenological growth stage reported in **Table 5.13** in the centre of each plot. Leaves from each genotype were harvested three times during the growing season from November to April. The genotypes, the harvest times and the corresponding phenological growth stages according to the BBCH scale proposed by Archontoulis et al. (2010) are reported in **Table 5.13**.

5.4.2.2 Meteorological conditions

Meteorological data were measured during the leaf development (from October to April) by a meteorological station (Mod. Multirecorder 2.40; EGT, Florence, Italy) sited within 250 m of the experimental field. Air temperature (minimum, maximum and mean) and global radiation were recorded every hour. Mean air temperature fell in December and January (11 and 12.8°C, respectively) and then rose to 16.5°C in April (**Figure 5.14**). Within 306 mm of total precipitation occurred during the growing season, the lowest level experienced in April (5.4 mm).

Table 5.13 Genotypes and harvest time with corresponding phenological stages performed in the study.

Genotype	Botanical variety	Harvest time	Phenological stage[a]
Naro 2 VSB3	var. *scolymus* (L.) Fiori	November 13th, 2017	rosette developed and leaves cover 50% of ground (code 35)
Marsala Valparaiso	var. *sylvestris* (Lamk) Fiori	January 9th, 2018	30% of the maximum leaf mass reached (code 43)
Altilis 41 Bianco gigante	var. *altilis* DC.	April 14th, 2018	50% of the maximum leaf mass reached (code 45)

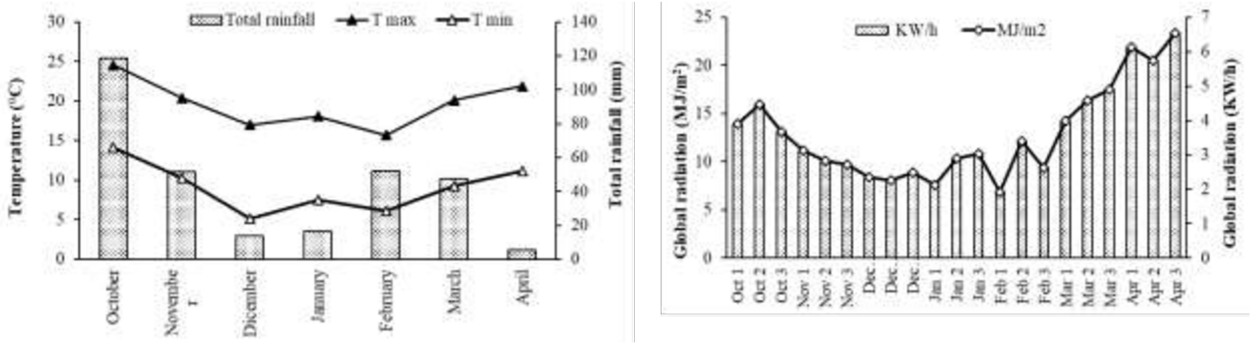


Figure 5.14 Meteorological condition during the growing season (October-April) in the Catania experimental station. 1: from 1st to 10th; 2: from 11th to 20th; 3: from 21st to 28th, 30th or 31st.

5.4.2.3 Reagents

The STLs cynaropicrin, aguerin B, grosheimin and 11,13-dihydroxi-8-desoxygrosheimin were obtained following the procedure previously described by Rial *et al*. (2014). Desacylcynaropicrin, 11,13-dihydro-desacylcynaropicrin and cynaratriol were isolated according the methodology describe by Scavo *et al*. (2018g). Santamarine was synthesized as reported previously by Lu and Fischer (1996). Molecular structures of compounds analyzed in this study are shown in **Figure 5.15**. The solvents for UHPLC-MS/MS analysis and the extractions were UHPLC-grade. Methanol (MeOH), ethanol (EtOH), dichloromethane and formic acid were obtained from Fischer Chemicals (Geel, Belgium). Water for LC-MS/MS analysis was type I obtained from an Ultramatic system from Wasserlab (Barbatáin, Spain). Ammonium formate was obtained from Sigma-Aldrich (St. Louis, MO, USA).

5.4.2.4 Leaf extract preparation

Leaf extracts were prepared according to the methodology proposed by Scavo *et al*. (2018g) with some modifications. 12 g of powdered dried leaves were mixed in 250 mL of ethanol/water (80:20, v/v) in an agitator incubator (Thermo Fisher Scientific Inc., USA) at 100 rpp for 72 h at room temperature (25 °C ± 1) in the dark, and then filtered through filter paper (Whatman N° 2). Solvents were evaporated by using a rotary evaporator (Laborata 4000, Heidolph, Germany) at 35 °C and the residues were re-dissolved with water. Since EtOH extracts were very rich in chlorophylls, it was decided to remove them by solid phase extraction (SPE) to facilitate the UHPLC-MS/MS analysis. Extracts,

previously dissolved in 1 mL of 100% MeOH and 1 mL of water, were passed through Strata-X 33 μm Polymeric Reversed Phase cartridges (200 mg/ 3mL, Phenomenex, CA, USA) using a vacuum manifold system (Phenomenex, CA, USA). Cartridges polymers were activated with 4 mL of 100% MeOH and 4 mL of water. Then, samples were load in the cartridges and were eluted with 100% MeOH (4 mL).

5.4.2.5 Wheat coleoptile bioassay

The inhibitory activity of the different extracts was evaluated by using the wheat coleoptile bioassay at 800, 400 and 200 ppm plus a negative control (0 ppm) represented by a phosphate-citrate buffer containing 2% sucrose (Nitsch and Nitsch, 1956) at pH 5.6, and the commercial herbicide Logran® as positive control. The experiment was carried out following Macias *et al*. (2000), with three replicates per dilution in duplicate.

5.4.2.6 UHPLC-MS/MS analysis

5.4.2.6.1 Multiple Reaction Monitoring

The compound-dependent parameters for each standard and the internal standard (IS) were optimized by direct infusion to the mass spectrometer to achieve maximum multiple reaction monitoring (MRM) signal intensities for the ammonium adduct $[M + NH_4]^+$. The parent or precursor ions, the fragments obtained by MRM analysis and the collision energy to achieve each fragmentation are provided in **Table 5.14**.

Table 5.14 Genotypes and harvest time with corresponding phenological stages performed in the study.

Compound	t_R(min)	Quantifier ion (m/z)			Qualifier ion (m/z)		
		Q1 Precursor ion	Q3 Product ion	C.E [a] (eV)	Q1 Precursor ion	Q3 Product ion	C.E. [a] (eV)
1	5.47	283	229	8	283	247	8
2	5.99	299	235	8	299	263	5
3	6.17	301	265	4	301	247	4
4	6.31	280	227	9	280	217	8
5	8.36	281	263	3	281	149	10
6	9.4	364	227	10	364	245	5
7 (IS)	9.67	266	231	6	266	185	10
8	9.87	348	227	9	348	245	6

[a] C.E.: Collision Energy

Table 5.15 Analytical characteristics of the chromatographic method.

Compound	intercept	Slope	regression coeff.	LOD µg/L	LOQ µg/L	repeatability RSD %[a]		Intermediate precision RSD %[a]	
						Area	t_R	Area	t_R
1	0.0448	1.0165	0.9940	5.51	18.36	7.6	1.0	9.2	1.3
2	-0.0852	0.1786	0.9986	3.10	10.34	5.1	1.1	7.4	1.5
3	-0.1051	0.9524	0.9983	2.16	7.20	4.6	1.1	4.3	1.63
4	-0.0413	0.6373	0.9995	2.77	9.22	5.2	1.1	8.3	1.4
5	-0.3687	4.8176	0.9990	2.32	7.73	7.0	1.2	8.9	1.4
6	-0.0587	0.8484	0.9997	0.09	0.28	5.0	02	7.1	0.2
8	-0.0430	0.7351	0.9977	0.04	0.14	7.8	0.1	8.9	0.1

[a]RSD %, relative standard deviation

5.4.2.6.2 Calibration curves

Each standard was dissolved in MeOH to achieve a stock standard solution concentration of 1000 mg/L. The external calibration curve for all the standards were prepared by the serial dilution of these stock solutions as follows: 11,13-dihydro-desacylcynaropicrin from 50 to 0.025 mg/L (11 levels), 11,13-dihydroxi-8-desoxygrosheimin from 50 to 0.25 mg/L (8 levels), cynaratriol from 50 to 0.1 mg/L (9 levels), desacylcynaropicrin from 50 to 0.05 mg/L (10 levels), grosheimin from 50 to 0.05 mg/L (10 levels), cynaropicrin from 50 to 0.25 mg/L (8 levels) and aguerin B from 50 to 0.1 mg/L (9 levels). Also, santamarine the IS was dissolved in MeOH to achieve a concentration of 1000mg/L, and it was added to all samples to give a final concentration of 1 mg/L. Santamarine is a characteristic sesquiterpene lactone of Asteraceae (da Silva *et al*., 2017; Romo de Vivar and Jiménez, 1965; Seaman, 1982), with a structure and molecular weight similar with the sesquiterpene lactones of interest, whose synthesis has been describe (Lu and Fischer, 1966) and it isn't present in *C. cardunculus* extract. For these reasons santamarine was selected as IS for the quantitation. A 5-µL aliquot of each standard solution was injected three times onto the UHPLC column. The calibration curve was made by plotting the peak area ratio (y) of standard to IS versus the ratio of their concentrations (x). The curve was fitted to a linear function with a weight of 1/nx ($R^2 > 0.99$), being "n" the calibration level. The compounds in the sample were determined by their peak area ratio with respect to the internal standard and by reference to the standard curve. All samples were filtered using a PTFE syringe filter (0.22 µm) prior to analysis and they were stored at –80 ºC.

5.4.2.6.3 Sample preparation

Extracts were dissolved with MeOH to achieve a ratio of 1/1 g/L and IS was also added to give a final concentration of 1 mg/L. A 5-µL aliquot of each sample was injected three times onto the UHPLC column. All samples were filtered using a PTFE syringe filter (0.22 µm) prior to analysis and they were stored at –80 ºC.

5.4.2.6.4 UHPLC-MS/MS method

Samples were analyzed on a Bruker EVOQ Triple Quadrupole Mass Spectrometer with an atmospheric pressure chemical ionization source (APCI) in positive mode. Samples were separated using a Kinetex 1.7 µm C18 (100 × 2.1 mm, 1.7 µm particle size) (Phenomenex, Torrance, CA, USA) maintained at 40 ºC. The mobile phase consisted of solvent A (water, 0.1% formic acid, 2mM ammonium formate) and solvent B (MeOH, 0.1% formic acid, 2mM ammonium formate) and the flow rate was set to 0.3 mL/min. The optimized linear gradient system was as follows: 0−1 min, 20% B; 1−3 min, to 25% B; 3−7 min, 25% B; 7−8 min, to 70% B; 8−9 min, to 100% B, 9-12 min, 100% B, 12-12.5 min, to 20% B, 12.5-15.5 min, 20% B. The autosampler was set at 7 °C to preserve the samples. The injection volume was 5 µL. The instrument parameters were as follows: spray current 100 µA, cone temperature 300 ºC, cone gas flow 20 psi, heated probe temperature 250 ºC, heated probe gas flow 40 psi, nebulizer gas flow 60 psi and collision pressure 2.0 mTorr.

5.4.2.6.5 Method validation

The validation of the method was carried out following ICH recommendations (ICH, 2005) (selectivity, linearity, precision of the instrumental system (repeatability and intermediate precision), detection and quantitation limits, and stability). The analytical properties of the calibration curves prepared are provided in **Table 5.15**. The quantitation (LOQ) and detection (LOD) limits were determined by nine replicate analyses and were measured as 10 and 3 times the standard deviation of baseline noise respectively. The precision of the method was studied in an intra- and interday assay (n = 9) for each compound. Repeatability was assessed by analyzing replicates of the same sample with all standards. The method was found to be precise with RSD values below of 5% in all cases. Intermediate precisions were below 10% for all analytes injected on three different days. The chromatographic separation of standards is shown in **Figure 5.16**.

5.4.2.7 Data and statistical analysis

Calibration curves and data acquisition of STLs were processed with the software MS Data Review (Bruker Chemical Analysis). The homogeneity of variance was verified with the Bartlett's test and the independence of cases by graphically inspecting the residuals. Afterwards, all data were subjected to analysis of variance (ANOVA). A two-way ANOVA (6 genotype x 3 harvest time) for STL profile, and a three-way ANOVA (6 genotype x 3 harvest time x 4 extract concentration) for wheat coleoptile bioassay were applied. In both cases, means were separated using the Student–Neuman–Keuls test only when the *F* test of the ANOVA for

treatments and interactions was significant at the ≤0.05 probability level. For what concern the wheat coleoptile bioassay, statistical analysis was carried out using Welch's test, with significance fixed at 0.01 (a) and 0.05 (b). The positive control was also included in the statistical analysis as a further concentration (0 ppm). Wheat coleoptile data are expressed as percentage difference from the control.

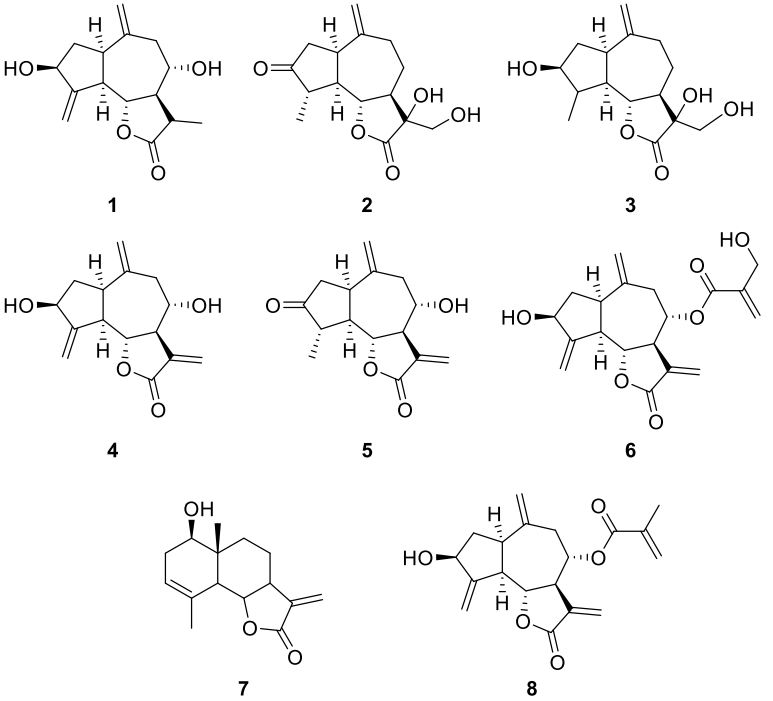


Figure 5.15 Structures of the sesquiterpene lactones analyzed by UHPLC-MS/MS: (**1**) 11,13-dihydro-desacylcynaropicrin, (**2**) 11,13-dihydroxi-8-desoxygrosheimin, (**3**) cynaratriol, (**4**) desacylcynaropicrin, (**5**) grosheimin, (**6**) cynaropicrin, (**7**) santamarin and (**8**) aguerin B.

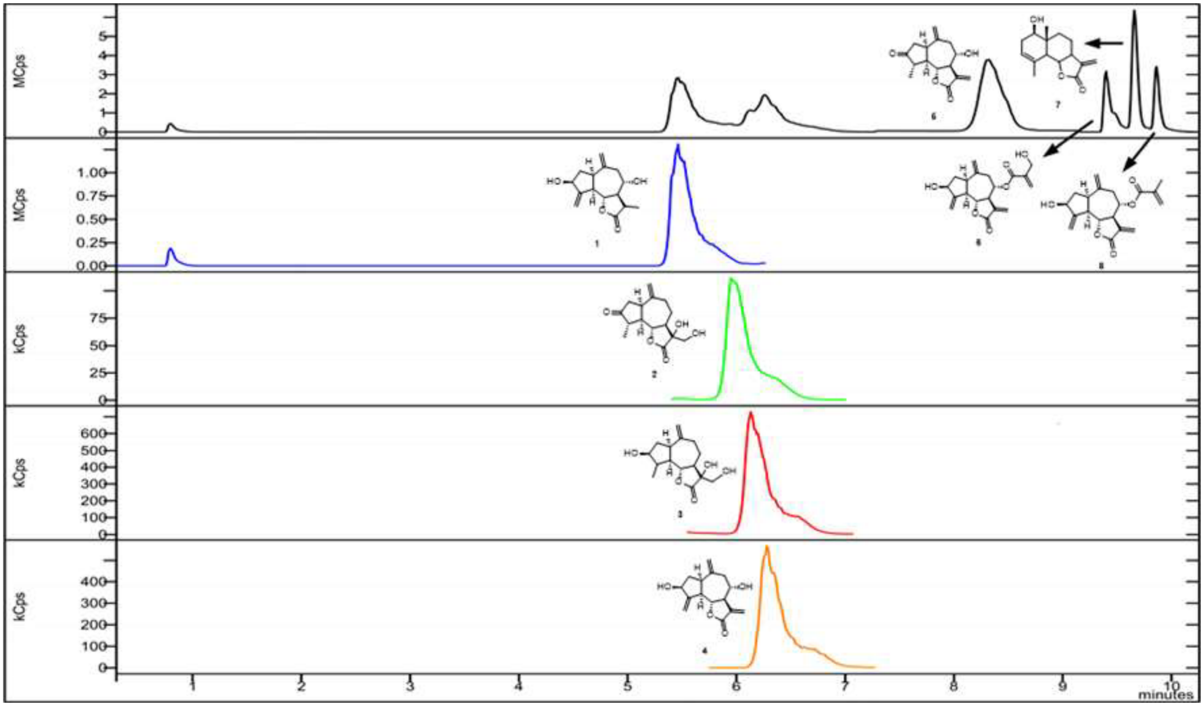


Figure 5.16 Chromatographic separation of standards.

5.4.3 Results and Discussion

Yields of different *C. cardunculus* extracts [(g extract/ g dried leaves)·100] is shown in **Table 5.16**. Yields increase with the growing season and reach the maximum value in the April harvest. The April harvest of 'Valparaiso' showed the highest yield (37.2), followed by the same harvest of 'Marsala'. Similar yield extracts were observed by Ramos *et al*. (2013) in cultivated cardoon leaves.

5.4.3.1 Sesquiterpene lactone content and profile

This study provides a quali-quantitative evaluation of the STL profile of globe artichoke, wild and cultivated cardoon leaves of six genotypes and three harvest times. 7 STLs were identified from UHPLC-MS/MS analysis (**Figure 5.15**). The harvest time accounted for 53% of the variance on the total STL concentration, calculated as the sum of individual STL contents determined by UHPLC. The "genotype x harvest time" interaction was also significant, showing that the total concentration of STLs of the six genotypes significantly varied in relation to the harvest time (**Table 5.17**). Regarding the composition of profiles, the contribution of harvest time on the variance was greater in all compounds, except for cynaratriol (F=2421), grosheimin (F=2455) and desacylcynaropicrin (F=1566). **Figures 5.17** and **5.18** show the "genotype x harvest time" interaction. Cynaropicrin and cynaratriol were the predominant STLs among the different extracts, while the other compounds were detected in very low amounts. The total concentrations were generally higher in April, except for 'Naro 2', 'Marsala' and 'Bianco gigante'. In fact, the highest total concentration was detected in the April harvest of 'Valparaiso' (150 mg/L), followed by the

same harvest of 'Altilis 41' (96 mg/L) (**Figure 5.17**). Cultivated cardoon (var. *altilis*) showed the highest amount of STLs among the three botanical varieties, followed by wild cardoon (var. *sylvestris*) and globe artichoke (var. *scolymus*). Cynaropicrin was the principal compound, ranging from 3.7 mg/L (in the January harvest of 'Marsala') to 120.4 mg/L (in the April harvest of 'Valparaiso'), and showed a similar trend to the total. The April harvest in 'Altilis 41' showed the highest cynaratriol concentration (39 mg/L), while it was absent in 'Marsala' as well as in both November and January harvests of 'VSB3'. Among the minor STLs, aguerin B was the most abundant, particularly in 'Altilis 41' (6.8 mg/L in April) and 'Valparaiso' (5.5 mg/L in April), while grosheimin concentration was higher in the globe artichokes (**Figure 5.18)**. Among 11,13-dihydroxy-8-desoxygrosheimin and 11,13-dihydro-desacylcynaropicrin, the April harvest of 'Altilis 41' confirmed to show the greatest concentration, with values of 2 mg/L and 0.15 mg/L, respectively

Informations about the STL content in *C. cardunculus* are limited and focused mainly in globe artichoke genotypes (Rouphael *et al*., 2016; Shimoda *et al*., 2003). Here we presented, for the first time, the STL profile from different genotypes belonging to all *C. cardunculus* botanical varieties among different growth stages. Rouphael *et al*. (2016) and Eljounaidi *et al*. (2015) reported grosheimin and its derivativs as the most abundant STLs in globe artichoke leaves, while our results are in agreement with Ramos *et al*. (2013), who indicated cynaropicrin as the predominant STL in cultivated cardoon.

The allelopathic potential is not always the same during the growing season, but the age of donor plant is an important

aspect affecting its phytotoxicity. Concentration of allelochemicals in plant tissues plays a key role in determining its allelopathic activity. Koeppe *et al*. (1970c) and Weston *et al*. (1989) documented that concentrations of allelochemicals in living plants decrease with increasing age of donor plant, while our results evidenced an opposite trend as reported by Reinhardt *et al*. (2006) and Koeppe *et al*. (1976). Is interesting to underline that STL variations during the different phenological stages of *C. cardunculus* are similar to those reported by Pandino *et al*. (2013) for polyphenols, indicating that in the February-April period the concentration of allelochemicals in leaf tissues is the highest. The month of April experienced an increase of temperature and both photoperiod and solar radiation. This combination, probably, contributed to stimulate the production of STLs in *C. cardunculus* leaves.

5.4.3.2 Wheat coleoptile elongation

In order to evaluate the inhibitory activity variations among the genotypes and harvest times under study, all the extracts were assayed using the wheat coleoptile bioassay, which allows to rapidly test the general phytotoxicity in presence of many treatments. The bioassay was performed at the *C. cardunculus* extract concentration of 0, 200, 400 and 800 ppm. Therefore, the "extract concentration" factor was also analysed. Results from three-way ANOVA are shown in **Table 5.18**. All factors and their interactions, including the "genotype x harvest time x concentration" interaction, significantly influenced wheat coleoptile elongation. However, variance was more heavily affected by extract

concentration (90.4%), which on its own explained much of the allelopathic potential.

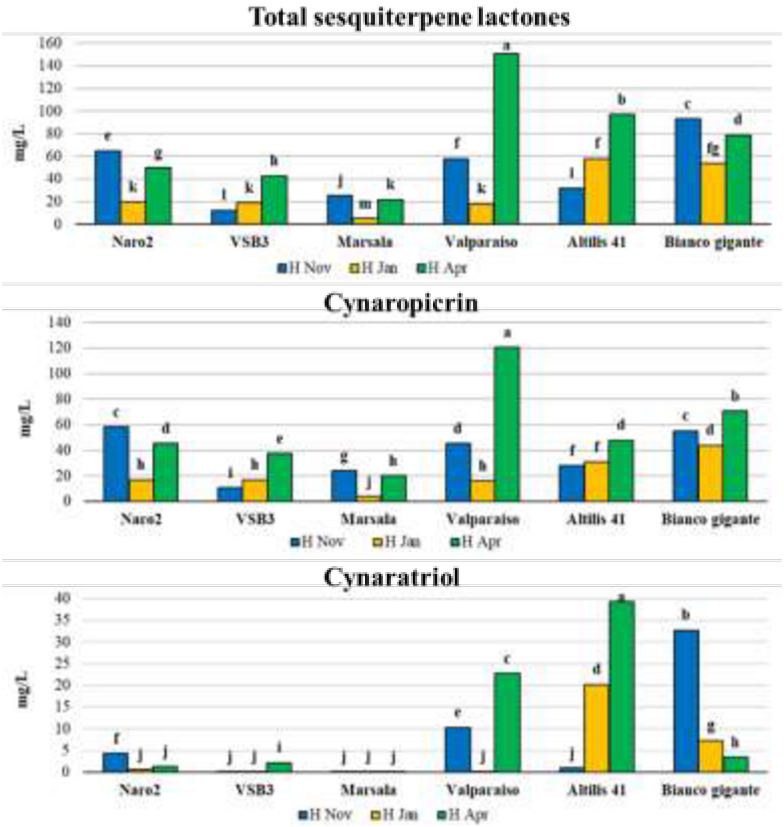


Figure 5.17 Quantification of total sesquiterpene lactone, cynaropycrin and cynaratriol content from six genotypes and three harvest times of *C. cardunculus* leaf extracts.
H Nov: harvest time at November 13th; H Jan: harvest time at January 9th; H Apr: harvest time at April 14th. Different letters indicate statistical significance for $P \leq 0.05$.

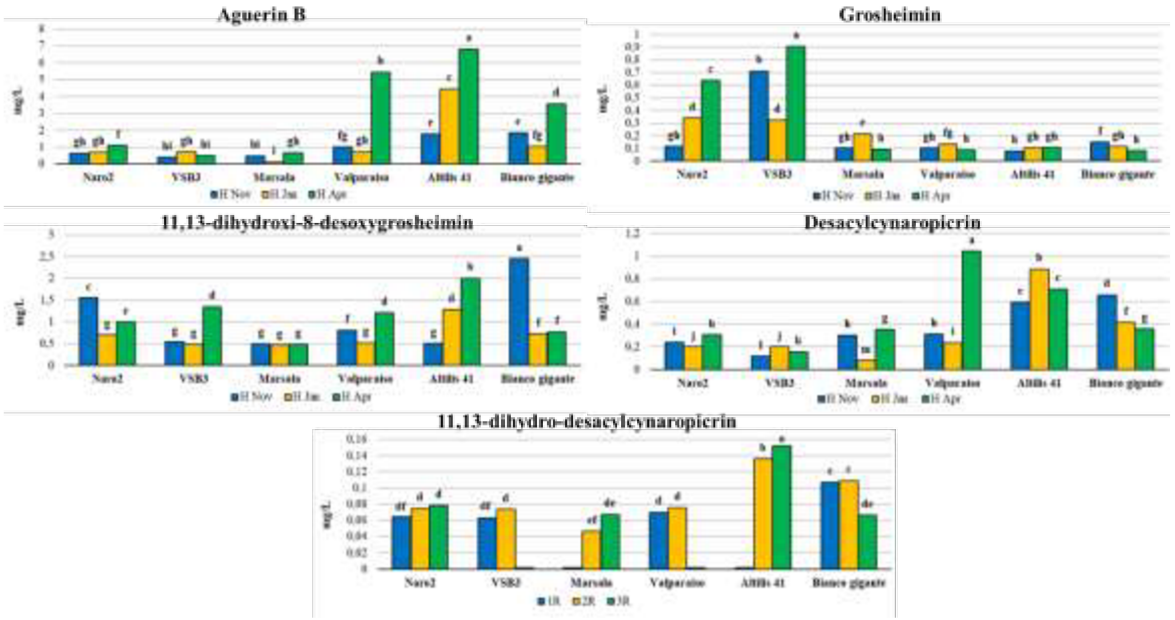


Figure 5.18 Quantification of minor sesquiterpene lactones (aguerin B, grosheimin, 11,13-dihydroxi-8-desoxygrosheimin, desacylcynaropicrin and 11,13-dihydro-desacylcynaropicrin) content from six genotypes and three harvest times of *C. cardunculus* leaf extracts.
H Nov: harvest time at November 13th; H Jan: harvest time at January 9th; H Apr: harvest time at April 14th. Different letters indicate statistical significance for $P \leq 0.05$.

Table 5.16 Yields of leaf extracts [(g extract/ g dried leaves)·100] obtained from *C. cardunculus*.

Globe artichoke extracts	Harvest time	Yield (%)	Wild cardoon extracts	Harvest time	Yield (%)	Cultivated cardoon extracts	Harvest time	Yield (%)
Naro 2	H Nov[a]	14.38	Marsala	H Nov	15.7	Altilis 41	H Nov	14.1
	H Jan[b]	15.97		H Jan	15.5		H Jan	16.9
	H Apr[c]	33.5		H Apr	34.5		H Apr	19.7
VSB3	H Nov	17.1	Valparaiso	H Nov	15.8	Bianco gigante	H Nov	15.2
	H Jan	14.5		H Jan	15.3		H Jan	14.8
	H Apr	21.9		H Apr	37.2		H Apr	24.1

Table 5.17 *F*–values of main factors and their interactions resulting from analysis of variance in sesquiterpene lactone concentration.

Source of variation	df	TOTAL STLs	Cynaropicrin	cynaratriol	aguerin B	grosheimin	11,13-dihydroxi-8-desoxygrosheimin	desacyl-cynaropicrin	11,13-dihydro-desacyl-cynaropicrin
Genotype (G)	5	1160^{***}	669^{***}	2421^{***}	519^{***}	2455^{***}	171^{***}	1566^{***}	56^{***}
Harvest time (T)	2	1892^{***}	1310^{***}	858^{***}	911^{***}	431^{***}	259^{***}	419^{***}	73^{***}
(G) x (T)	10	504^{***}	281^{***}	1697^{***}	181^{***}	455^{***}	231^{***}	517^{***}	72^{***}

Values are given as *F* of Fisher. df: degrees of freedrom. *** indicates significant at $P \leq 0.001$.

Table 5.18 *F*–values as absolute value and sum of squares (in brackets) of main factors and their interactions resulting from analysis of variance in wheat coleoptile elongation.

Source of variation	df	Wheat coleoptile elongation
Genotype (G)	5	52.6^{***} (4.7)
Harvest time (T)	2	34.8^{***} (3.1)
Concentration (C)	3	1028^{***} (90.4)
(G) x (T)	10	6.4^{***} (0.5)
(G) x (C)	15	7.1^{***} (0.6)
(T) x (C)	6	7.1^{***} (0.6)
(G) x (T) x (C)	30	1.4^{**} (0.1)

Values are given as *F* of Fisher. df: degrees of freedrom. *** and ** indicate significant at $P \leq 0.001$ and $P \leq 0.01$, respectively.

All the extracts were very active on wheat coleoptile elongation (**Figure 5.19**). Inhibitory activity was significantly higher with increasing concentration for all the six genotypes. On average, 800 ppm showed the greatest allelopathic activity compared to 400 and 200 ppm, reducing 65, 45 and 29% coleoptile elongation, respectively. Most of researches conducted on this topic reported a major allelopathic activity in relation to extract concentration (Chung and Miller, 1995; Scavo et al., 2018d). Harvest time influenced both concentration and genotype. The effect of concentration was higher in the April harvest, with values of 75, 51 and 32% for 800, 400 and 200 ppm, respectively. On the average of all genotypes, the November harvest showed a greater allelopathic effect than the January one, inhibiting 62, 46 and 25% the wheat coleoptile at 800, 400 and 200 ppm, respectively. Regarding the genotype, 'Valparaiso' showed the best profile reducing by 55% the coleoptile elongation on the average of all harvest times, followed by 'Marsala' (54%).

Therefore, wild cardoon was the most allelopathic *C. cardunculus* botanical varieties, while globe artichoke was the least active, with values of 46 and 29% for 'Naro 2' and 'VSB3', respectively, on the average of all harvest times. As view for concentration, the inhibitory activity was higher in the April harvest in all six genotypes.

Wheat coleoptile data are strictly correlated to the STL profiles analysed. The April harvest, in fact, which showed the highest allelopathic activity, was also richest in total STL, cynaropicrin, cynaratiol and aguerin B concentration (**Figure 5.17** and **5.18**), followed by November and January harvests. Moreover, 'Valparaiso' resulted to be the most allelopathic genotype, probably due to the greater STL concentration, particularly of cynaropicrin, representing the most responsible STL involved in *C. cardunculus* allelopathic activity, as well as to the higher yield extract. On the contrary, globe artichoke was the least effective as confirmed by the lower STL concentration. The allelopathic potential, however, not only depends on the total STL or single compound concentration, but on the qualitative composition of STL profiles, because generally allelochemicals act in synergism (Rial *et al*., 2016a).

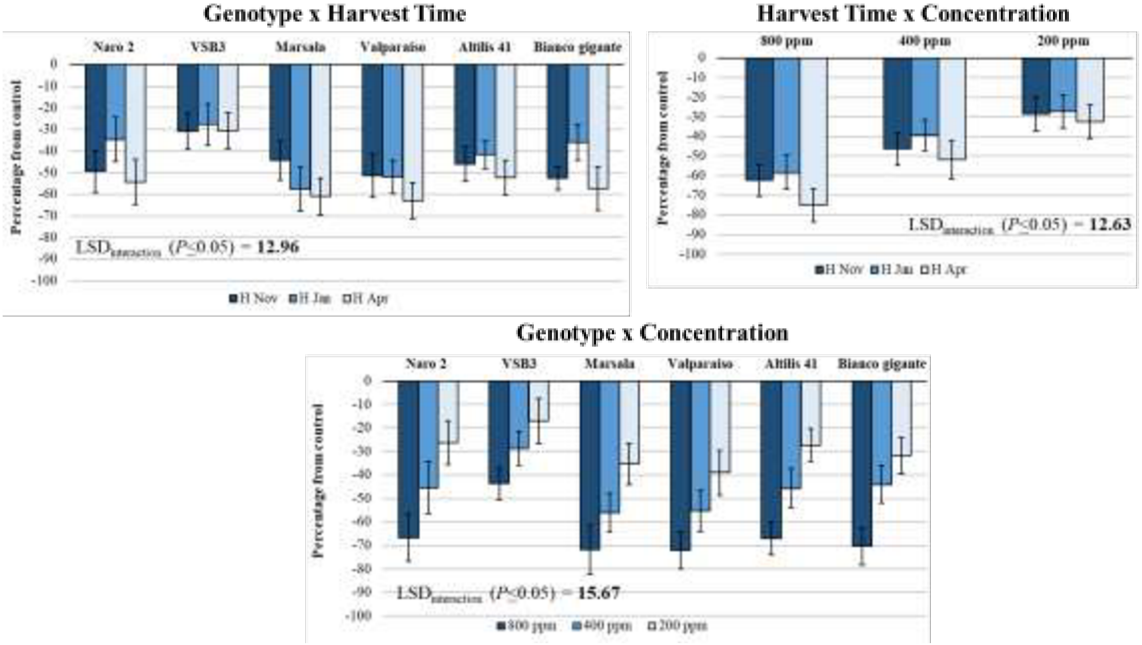


Figure 5.19 Effect of "genotype x harvest time", "harvest time x concentration" and "genotype x concentration" interactions from six genotypes and three harvest times of *C. cardunculus* leaf extracts on wheat coleoptile elongation. H Nov: harvest time at November 13th; H Jan: harvest time at January 9th; H Apr: harvest time at April 14th. Values are expressed as percentage difference from control. Each bar means ± standard deviation.

5.4.4 Conclusions

A new UHPLC-MS/MS analysis method for *C. cardunculus* STL detection was carried out. The qualitative and quantitative composition of STLs in *C. cardunculus* leaves as affected by genotype and harvest time was studied to find a correlation with the allelopathic activity. The April harvest showed both the highest STL concentration and phytotoxic effect on wheat coleoptile elongation, followed by November and January harvests. Cynaropicrin was the most abundant STL detected among the six genotypes under study, followed by cynaratriol and aguerin B. The highest amount of STLs was found in both cultivated cardoons, while 'Valparaiso' showed the greatest quantity among genotypes. The same results were confirmed in the wheat coleoptile bioassay, where 'Valparaiso' resulted the major allelopathic genotype. Globe artichoke was the worst botanical variety both in STL concentration and phytotoxic activity. Moreover, extract concentration exerted a strong influence on the allelopathic potential, with a higher inhibitory activity with increasing concentration for all the genotypes. Therefore, a close relationship exist was found between the phytotoxic activity and the STL profiles. These results are very important in identifying the best genotype and the most appropriate harvest time for a future bioherbicide production from *C. cardunculus* leaf extracts.

5.5 Genotype and harvest time affected the allelopathic activity of *Cynara cardunculus* L. extracts on *Amaranthus retroflexus* L. and *Portulaca oleracea* L.

5.5.1 Introduction

Cynara cardunculus L. is a complex C_3 species, member of the Asteraceae family, including three botanical varieties (Rottenberg and Zohary, 1996): the globe artichoke [var. *scolymus* (L.) Fiori], the cultivated cardoon (var. *altilis* DC.), and their progenitor wild cardoon [var. *sylvestris* (Lamk) Fiori].

In previous works, the allelopathic activity of *C. cardunculus* was investigated (Scavo *et al*., 2018b, f, g). Its phytotoxicity is due to the presence in the leaves of sesquiterpene lactones (STLs) mainly represented by cynaropicrin (Rial *et al*., 2014, 2016a), and polyphenols such as chlorogenic acid, luteolin and apigenin derivatives (Pandino *et al*., 2011, 2013; Scavo *et al*., 2018f). The phenolic profile of *C. cardunculus* is strongly affected by its genetic background and harvest time. Pandino *et al*. (2011) reported that globe artichoke leaves are richer in luteolin derivatives, while cultivated and wild cardoon present a higher content of apigenin derivatives. The February-April period is indicated by Pandino *et al.* (2013) as the optimal harvest time with the highest concentration of polyphenols in globe artichoke leaves. The effect of genotype and harvest time on STL quantity and profile was also previously studied, reporting the April harvest and wild cardoon var. Valparaiso as the best harvest time and genotype, respectively, in terms of STL concentration.

The objective of this research was to assess the effect of genotype and harvest time on *C. cardunculus* phytotoxicity by evaluating the allelopathic activity of its leaf extracts on the seedling growth of two cosmopolitan weed species.

5.5.2 Material and methods

5.5.2.1 Field experiment design, plant material and crop management

Leaves from six genotypes belonging to the three *C. cardunculus* botanical varieties were harvested three times during the growing season from November to April. The experimental design, the crop management as well as the genotypes with the corresponding phenological stages are reported in paragraph 5.4.2.1.

5.5.2.2 Meteorological conditions

Meteorological data are reported in paragraph 5.4.2.2.

5.5.2.3 Leaf extract preparation

Leaf extracts were prepared according to the methodology proposed by Scavo *et al*. (2018g). Removal of chlorophylls was in agreement with paragraph 1.2.3. Extracts were dissolved in 2-[*N*-morpholino]ethanesulfonic acid (MES) at 10^{-2} M and 1 M NaOH buffer (pH 6.0). Then, these solutions were diluted with 5 µL/mL of DMSO to obtain 800, 400 and 200 ppm concentrations for extracts or fractions. A phosphate-citrate buffer containing 2% sucrose at pH 5.6 (Nitsch and Nitsch, 1956) was used as negative control (0 ppm), while the commercial herbicide Logran® was adopted as positive control.

5.5.2.4 Weed phytotoxicity bioassay

Amaranthus retroflexus L. and *Portulaca oleracea* L. seeds were collected in the Catania Plain, cleaned from inert material, hard and broken seeds as well as from seeds of other species, and selected by using a stereomicroscope to homogenize the lots for size and color. Then, seeds were kept in paper bags and dry-stored at room temperature (20°C±2°C) until tests were performed.

Tests were performed under a completely randomized design with four replicates of 20 seeds. Experiment was repeated twice. Seeds were put into 50 mm Petri dishes on a sheet of Whatman No. 1 filter paper. Each Petri dish, previously imbibed with 1 mL of extracts, was hermetically sealed with parafilm to prevent evaporation of the solution, wrapped in sheets of aluminium and placed into a Memmert ICE 700 incubator in continuous darkness at 25 °C for both species. After 6 days for *A. retroflexus* and 9 for *P. oleracea*, Petri dishes were frozen at –10 °C x 24 h to avoid subsequent growth during measurements process. A Fitomed system was used to record root length ad shoot length (Rial *et al*., 2016b).

5.5.2.5 Data and statistical analysis

After verifying the homoscedasticity through Bartlett's test, a three-way analysis of variance (ANOVA) considering the genotype, the harvest time and the concentration as fixed factors was applied to all data, included the positive control (0 ppm). The Student–Neuman–Keuls test was used to separate means when the *F*-test was significant for at the 0.05 probability level. Root and shoot length data were analyzed using Welch's test, with significance fixed at 0.01 (a) and

0.05 (b). Their data are expressed as percentage difference from the control.

5.5.3 Results and discussion

5.5.3.1 Root length

In agreement with da Silva *et al*. (2017), Rial *et al*. (2018) and Scavo *et al*. (2018g), root length was the most affected parameter in both weed species (**Figure 5.20** and **5.21**). The interaction "genotype x harvest time x concentration" was significant both in *A. retroflexus* (*F*=5.5) and *P. oleracea* (*F*=1.9). However, in the former the contribution of harvest time on the inhibitory activity of extracts was greater than the genotype (*F*=127.6 *vs*. 55.5, respectively), while the latter showed an opposite trend (**Table 5.19**). As view for seed germination percentage, the choice of concentration on its own explained much of the allelopathic activity, contributing for 83.9% and 84.8% on the analysis of variance for *A. retroflexus* and *P. oleracea*, respectively.

In *A. retroflexus*, April harvest showed the highest allelopathic activity in all genotypes by reducing 39% root length, compared to 22 and 18% of January and November harvests, respectively. In detail, April harvest of 'Bianco gigante' resulted the most efficient, with inhibiting values of 93% at 800 ppm with respect to 84% of Logran at the same concentration (**Figure 5.20**). April harvest of 'Bianco gigante' showed also the best profile, inhibiting root length by 72 and 29% at 400 and 200 ppm, respectively. Wild cardoon genotypes manifested a higher inhibition of root length than Logran (87 *vs*. 84%, respectively). Globe artichoke var. VSB3 was the worst genotype.

P. oleracea showed a very similar trend to *A. retroflexus*. Also in *P. oleracea*, in fact, the April harvest allowed the greatest inhibitory activity on the average of all genotypes and concentrations, reaching a value of 50%, followed by November (38%) and January (36%) (**Figure 5.21**). Extract from April harvest of 'Bianco gigante' confirmed to be the best, reducing by 61% root length on the average of concentrations. Like in *A. retroflexus*, all extracts were more efficient when applied at 800 ppm, except for April harvest of 'Valparaiso', which showed the highest allelopathic activity at 400 ppm (69%). Valparaiso extracts were significantly active at all harvest times. Logran showed a higher phytotoxicity on *P. oleracea* root length at all concentrations than all the other extracts. Nevertheless, 'VSB3' resulted the worst genotype, while 'Valparaiso' and 'Bianco gigante' the best ones.

Table 5.19 *F*–Values as absolute value of main factors and their interactions resulting from analysis of variance in weed species.

Source of variation	df	Amaranthus retroflexus		Portulaca oleracea	
		Root length	Shoot legth	Root length	Shoot length
Genotype (G)	5	55.5^{***}	39.9^{***}	28.0^{***}	6.8^{***}
Harvest time (T)	2	127.6^{***}	133.4^{***}	28.1^{***}	12.6^{***}
Concentration (C)	3	1289.0^{***}	226.5^{***}	411.3^{***}	58.1^{***}
(G) x (T)	10	16.9^{***}	9.9^{***}	4.4^{***}	1.9^{*}
(G) x C)	15	16.4^{***}	16.8^{***}	5.4^{**}	2.0^{*}
(T) x (C)	6	24.7^{***}	61.2^{***}	6.3^{***}	6.1^{***}
(G) x (T) x (C)	30	5.5^{***}	4.4^{***}	1.9^{**}	1.8^{*}

Note: Values are given as *F* of Fisher. df: degree of freedom. ***, ** and * indicate significant at $P \leq 0.001$, $P \leq 0.01$ and $P \leq 0.005$, respectively, and *NS*, not significant.

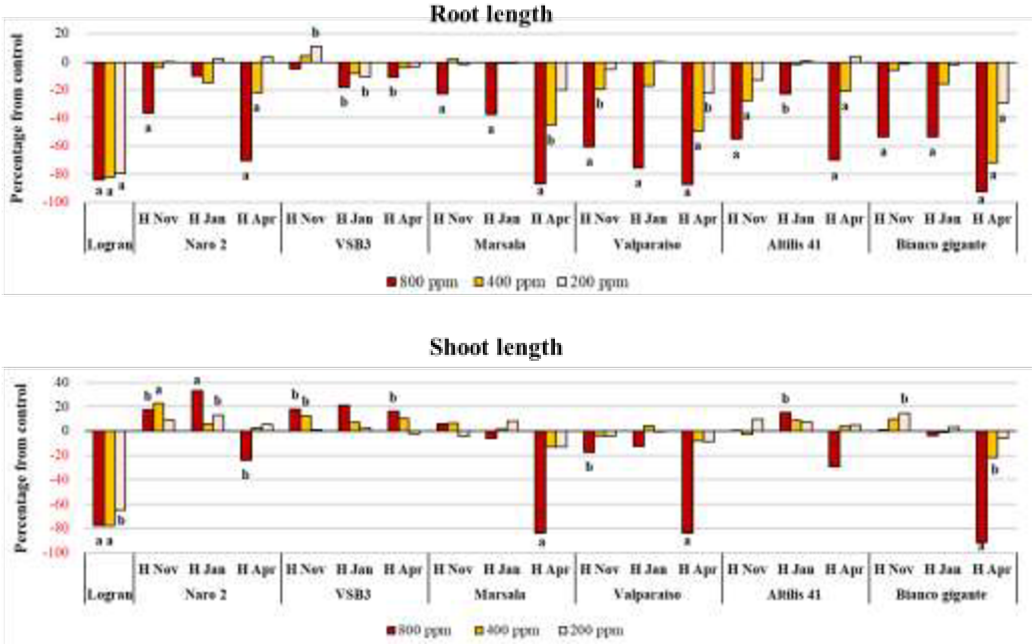


Figure 5.20 Effect of genotype, harvest time and concentration on the allelopathic activity of *C. cardunculus* extracts on the seedling growth of *A. retroflexus*.

Note: The commercial herbicide Logran® was used as a positive control. Values are expressed as percentage difference from control. a and b indicate significance respect to control for $P \leq 0.01$ and $P < 0.05$, respectively.

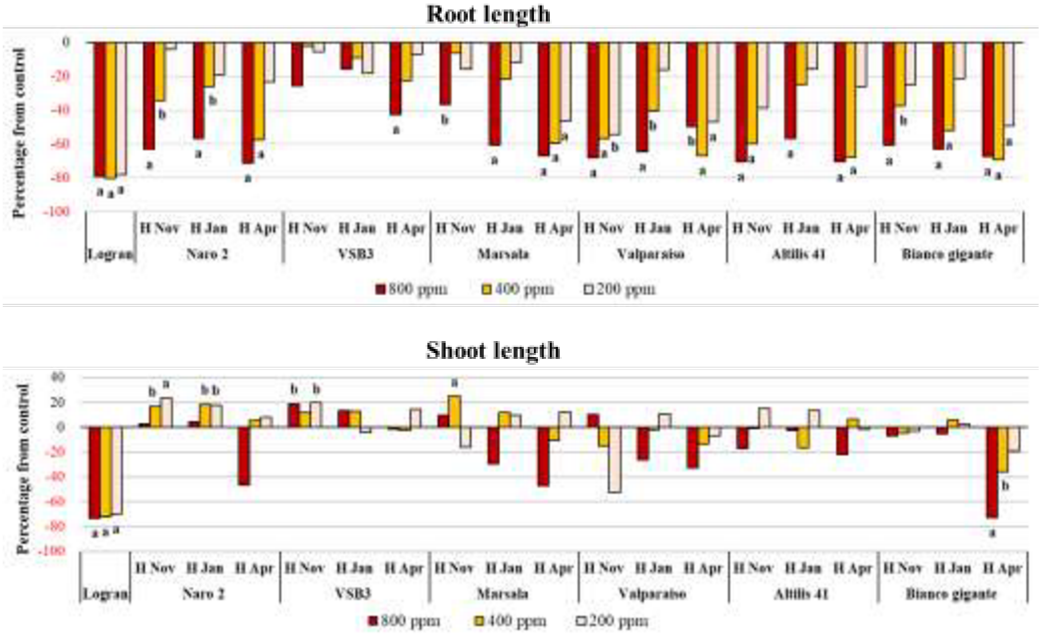


Figure 5.21 Effect of genotype, harvest time and concentration on the allelopathic activity of *C. cardunculus* extracts on the seedling growth of *P. oleracea*.

Note: The commercial herbicide Logran® was used as a positive control. Values are expressed as percentage difference from control. a and b indicate significance respect to control for $P \leq 0.01$ and $P \leq 0.05$, respectively.

5.5.3.2 Shoot length

Also for shoot length the interaction "genotype x harvest time x concentration" was significant both in *A. retroflexus* (F=4.4) and *P. oleracea* (F=1.8), with a prevalence of concentration on the analysis of variance (**Table 5.19**). Globe artichoke genotypes exerted stimulatory effects on both weed species, except for the April harvest of 'Naro 2', which inhibited by 24 and 46% at 800 ppm the shoot length of *A. retroflexus* and *P. oleracea*, respectively (**Figure 5.20** and **5.21**). In *A. retroflexus*, April harvest was the most inhibitory, showing higher values for 'Marsala' (84%), 'Valparaiso' (84%) and 'Bianco gigante' (92%) at 800 ppm than Logran (78%). Only the April harvest of 'Bianco gigante' significantly reduced *P. oleracea* shoot length, with values of 72 and 36% at 800 and 400 ppm, respectively.

5.5.3.3 Correlation between allelopathic activity and STL profile of extract

These results are strictly correlated to the STL profile of extracts, proving that STLs play a key role on the allelopathic activity of *C. cardunculus* (**Figure 5.17** and **5.18**). The higher allelopathic activity of extracts belonging from April harvest is corroborated by their STL profile. In fact, they are the richest in total STL, cynaropicrin, cynaratiol and aguerin B concentration. Globe artichoke resulted the worst botanical variety both in terms of phytotoxicity and STL concentration. Moreover, cultivated cardoon genotypes, which were very active on *A. retroflexus* and *O. oleracea* seedling growth inhibition, present a higher concentration of cynaratriol, aguerin B, 11,13-dihydroxi-8-desoxygrosheimin and 11,13-

dihydro-desacylcynaropicrin compared to the other genotypes.

5.5.4 Conclusions

The present study demonstrated the influence of genotype and harvest time on *C. cardunculus* allelopathic activity against two common weed species in the Mediterranean Basin. As observed in many other researchers conducted with this bioassay, root length was the most affected parameter and allelopathic activity grows with increasing of concentration. In both weed species, 'Bianco gigante' extracts belonging from April harvest showed the highest inhibitory activity, followed by 'Valparaiso'. The results obtained are heavenly correlated to extracts STL profiles in terms of quantity and quality

5.6 Effect of field light stress on sesquiterpene lactone composition and allelopathic activity of cultivated cardoon (*Cynara cardunculus* var. *altilis*) leaf extracts

5.6.1 Introduction

In previous researches, chemical compounds responsible for the allelopathic activity of *Cynara cardunculus* L. were discussed (Rial *et al*., 2014, 2016a; Scavo *et al*., 2018f, g). Besides, the best methodology for extraction of its allelochemicals in terms of cost management and allelopathic activity has been developed (Scavo *et al*., 2018f, g). *C.*

cardunculus phytotoxicity varies on the basis of genotype and leaf harvest time. Wild cardoon cv. Valparaiso showed the highest inhibitory activity on wheat coleoptile elongation, while cultivated cardoon cv. Bianco gigante more affected the seedgling growth of *Amaranthus retroflexus* L. and *Portulaca oleracea* L. In both cases, April harvest offered the best results on *C. Cardunculus* phytotoxic activity. Besides, *Scavo et al*. (2018b) reported cultivated cardoon as the best botanical variety in inhibiting seed germination of six common weed species in the Mediterranean Basin.

However, the allelopathic behaviour of a plant is the result of different abiotic and biotic stress factors, which influence the quantity of allelochemicals released by the donor plant and the effect of an allelochemical on the target plant (Inderjit and Del Moral, 1997; Scavo *et al*., 2018a). According to the "stress hypothesis of Allelopathy" formulated by Reigosa *et al.* (1999a), a stress condition generally increases the production of allelochemicals in the donor plant, as well as the sensitivity of the target plant. Quantity and quality of light is reported to affect the allelopathic behaviour in plants (Kato-Noguchi *et al*., 1999; Li *et al*., 2009; Scavo *et al*., 2018a). Thus, the aim of the present study was to evaluate the effect of light stress (by plant shading) under field condition on sesquiterpene lactone composition of cultivated cardoon leaf extracts and their allelopathic activity on wheat coleoptile elongation and on weeds germination and growth.

5.6.2 Material and methods

5.6.2.1 Experimental site design, crop management and leaf sampling

Experiments were carried out at the experimental station of Catania University [South Italy, 37° 25^{I} N; 15° 30^{I} E; 10 m a.s.l.] on cultivated cardoon cv. Bianco gigante, which in previous researches showed the highest phytotoxic activity. The local climate and soil characteristics are reported in paragraph 5.4.2.1. A completely randomized design with four replications, each of which comprised 50 plants, was adopted to study two imposed shading levels, removal of 0% (control) and 60% (light stress) of sunlight. Shading was imposed by erection of a black polyethylene net ("Ombra 60") at ~1.60 m above ground level from middle October to April. The effectiveness of the shading was tested on a weekly basis, both inside and outside the field experiment, using a solarimeter (Licor Line Quantum LQA, One Meter Sensing Length; LI-Cor Inc., Lincoln, NE, USA). The netting was extended down 0.30 m above ground level at each edge, in order to avoid lateral irradiations and to minimize the development of microclimate differences among main plots. The crop management was realized following paragraph 5.4.2.1. Two different harvest times were made: January 9th and April 14th, 2018. January harvest corresponds to 30% of the maximum leaf mass reached, code 43 according to the BBCH scale proposed by Archontoulis *et al*. (2010), while in April 50% of the maximum leaf mass is reached (code 45). Fifty full-expanded leaves were randomly sampled at the two phenological stages described first in the central part of each plot.

5.6.2.2 Meteorological conditions
See paragraph 5.4.2.2.

5.6.2.3 UHPLC-MS/MS analysis
List of reagents follows paragraph 5.4.2.3. Seven STLs were detected in cultivated cardoon leaves: cynaropicrin, cynaratriol, grosheimin, aguerin B, desacylcynaropicrin, 11,13-dihydro-desacylcynaropicrin, 11,13-dihydroxi-8-desoxygrosheimin and santamarin. Their molecular structures are shown in **Figure 5.15**. UHPLC-MS/MS analysis was carried out according to paragraph 5.4.2.6.

5.6.2.4 Leaf extract preparation
Leaf extract preparation was realized following Scavo *et al*. (2018f) and paragraph 5.4.2.4.

5.6.2.5 Wheat coleoptile and weed phytotoxicity bioassays
Wheat coleoptile and weed phytotoxicity bioassays were carried out as reported in paragraphs 5.4.2.5 and 5.5.2.4, respectively.

5.6.2.6 Statistical analysis
Bartlett's test was used to test for homoscedasticity, following which data were subjected to analysis of variance (ANOVA). A two-way ANOVA with shading level and harvest time as fixed factors was used for sesquiterpene lactone profile, while a three-way ANOVA model "shading level x harvest time x concentration" was employed to evaluate the allelopathic activity in both wheat coleoptile and weed phytotoxicity bioassays. Means of sesquiterpene lactone data were separated by the Student–Neuman–Keuls

test only when the *F*-test was significant at the 0.05 probability level. Weed phytotoxivity and wheat data were analyzed using Welch's test, with significance fixed at 0.01 (a) and 0.05 (b). Wheat elongation, as well as weed root and shoot length data are expressed as percentage difference from the control. 0 ppm was also included in the statistical analysis.

5.6.3 Results and discussion

Yields of extracts were [(g extract/ g dried leaves)·100] 14.8 and 24.1% for Control ('Bianco gigante' without stress), and 13.3 and 25.0 % for stressed 'Bianco gigante' in January and April harvest, respectively. In both cases April harvest led to greater yield extract than January, with the stressed genotype showing the highest yield. These yields are in agreement with Ramos *et al*. (2013).

5.6.3.1 Sesquiterpene lactone concentration and composition

The interaction "ligth stress x harvest time" was higly significant for all sesquiterpene lactones (STLs), suggesting how their concentration varied on the basis of both ligth stress condition and harvest time (**Table 5.20**). The latter more contributed than stress condition to the analysis of variance for the total concentration (*F*=637) and for all STLs.

Ligth stress significantly affected STL concentrations, with an increase of 16 and 34% in the April harvest of total and grosheimin, respectively, compared to Control (**Figure 5.22** and **5.23**). The highest increase was detected in cynaratriol (14.9 *vs*. 3.4 mg/L in April harvest of stressed and Control, respectively. An higher concentration in the Control was registered only in aguerin B. April harvest showed the highest concentration for both total and single STLs, except for

grosheimin, desacylcynaropicrin and 11,13-dihydro-desacylcynaropicrin Controls. The effect of harvest time was more marked if combined with the light stress. For istance, an increase of 210, 90 and 800% was registered going from January to April in the total, cynaropicrin and cynaratriol concentrations, respectively. This is probably due to the greater amount of time required for the ligth stress to take effect. In agreement with Ramos et al. (2013), cynaropicrin was the predominant STL of cultivated cardoon leaf extracts (70 and 73 mg/L in April harvest of Control and stressed, respectively), followed by cynaratriol (14.9 mg/L in stressed April harvest). Aguerin B was the major minor STL, even if with higher values in the Control (3.6 mg/L in April).

5.6.3.2 Wheat coleoptile bioassay

The ligth stress condition did not affected wheat coleoptile elongation, while the interaction "stress x harvest time x concentration" was significantly at the 0.05 probability level (**Table 5.21**). Extract concentration explained much of the inhibitory activity, contributing for 55.5% on variance.

Coleoptile's inhibition was marked for all extracts (**Figure 5.23**). Extract concentration more contributed to the inhibitory activity. 800 ppm, in fact, on the average of all treatments reduced by 69% coleoptile's elongation, compared to 46 and 31% of 400 and 200 ppm, respectively. Moreover, April harvest exerted an higher allelopathic activity compared to January, inhibiting by 64% vs. 33% on the average of all treatments. The ligth stress condition in the April harvest showed the best results, with values of 91, 77 and 46% at 800, 400 and 200 ppm, respectively, compared to 85, 54 and 33% of Control.

Table 5.20 *F*–values of main factors and their interactions resulting from analysis of variance in sesquiterpene lactone concentration.

Source of variation	df	TOTAL STLs	cynaropicrin	cynaratriol	aguerin B	grosheimin	11,13-dihydroxi-8-desoxygrosheimin	desacyl-cynaropicrin	11,13-dihydro-desacyl-cynaropicrin
Genotype (G)	1	12^{**}	33^{***}	41^{***}	278^{***}	5248^{***}	17^{**}	24^{**}	3^{NS}
Harvest time (T)	1	637^{***}	707^{***}	95^{***}	803^{***}	21780^{***}	270^{***}	193^{***}	0^{NS}
(G) x (T)	1	116^{***}	57^{***}	324^{***}	146^{***}	22311^{***}	176^{***}	301^{***}	31^{***}

Note: Values are given as *F* of Fisher. df: degrees of freedrom. *** and ** indicate significant at $P \leq 0.001$ and $P \leq 0.01$, respectively, and *NS*, not significant.

Table 5.21 *F*–values as absolute value and sum of squares (in brackets) of main factors and their interactions resulting from analysis of variance in wheat coleoptile elongation.

Source of variation	df	Wheat coleoptile elongation
Stress (S)	1	2.0^{NS} (0.4)
Harvest time (T)	1	165.9^{***} (34.2)
Concentration (C)	3	268.9^{***} (55.5)
(S) x (T)	1	17.9^{***} (3.7)
(S) x (C)	3	1.4^{NS} (0.3)
(T) x (C)	3	25.0^{***} (5.1)
(S) x (T) x (C)	3	3.7^{*} (0.8)

Note: Values are given as *F* of Fisher. df: degrees of freedrom. ***and * indicate significant at $P \leq 0.001$ and $P \leq 0.05$, respectively, and *NS*, not significant.

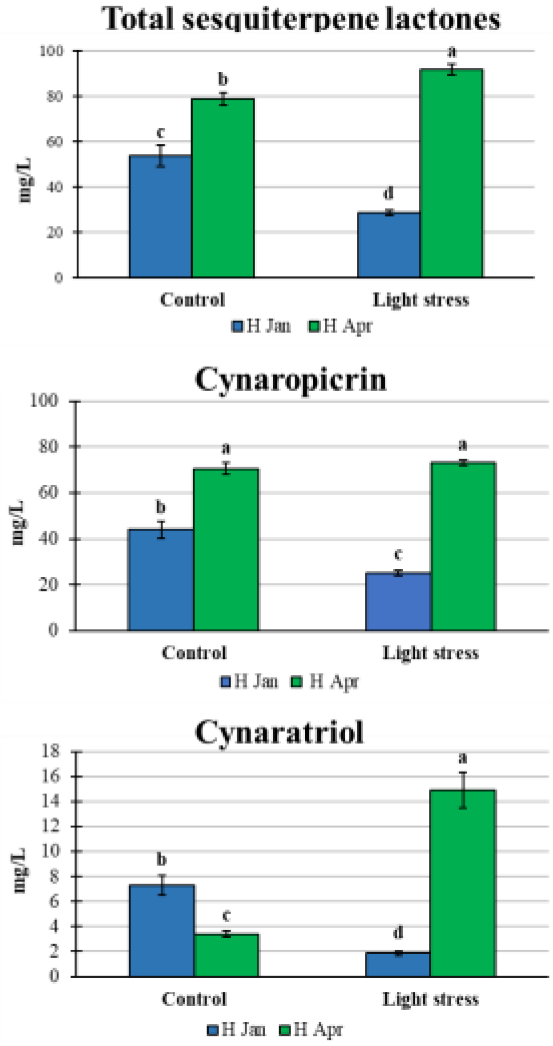


Figure 5.22 Quantification of total sesquiterpene lactone, cynaropycrin and cynaratriol content from cultivated cardoon leaf extracts in two different harvest times.
Control: 'Bianco gigante' without light stress. H Jan: harvest time at January 9th; H Apr: harvest time at April 14th. Different letters indicate statistical significance for $P \leq 0.05$.

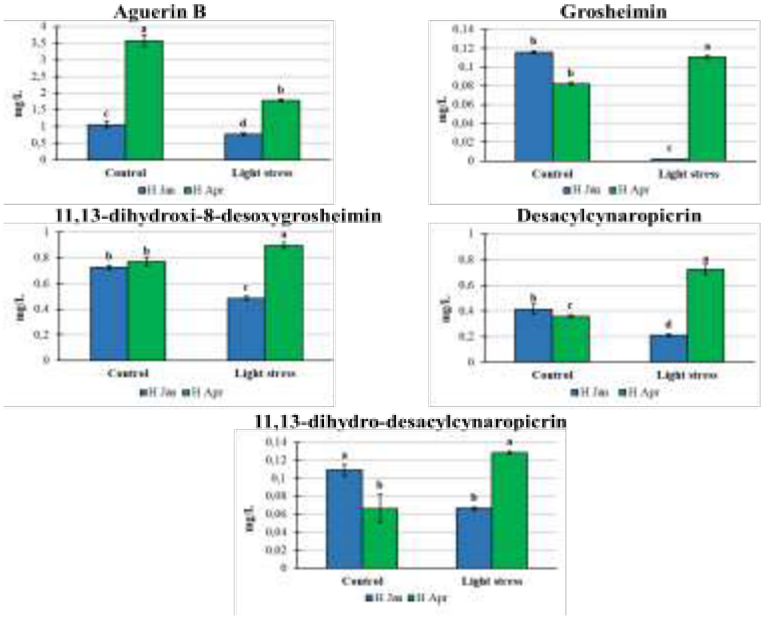


Figure 5.22 Quantification of minor sesquiterpene lactones (aguerin B, grosheimin, 11,13-dihydroxi-8-desoxygrosheimin, desacylcynaropicrin and 11,13-dihydro-desacylcynaropicrin) content from cultivated cardoon leaf extracts in two different harvest times.
Control: 'Bianco gigante' without light stress. H Jan: harvest time at January 9th; H Apr: harvest time at April 14th. Different letters indicate statistical significance for $P \leq 0.05$.

These results are corroborated by STL profile. During April, the plant accumulates more STLs in the leaves, resulting in a more pronounced phytotoxicity. A similar trend was also observed for polyphenols such as chlorogenic acid, luteolin- and apigenin derivatives (Pandino et al. 2013), which show allelopathic activity (Scavo et al. 2018a). Nevertheless, a prolungated ligth stress condition was found to stimulate the production of STLs in the leaves, and enhance the allelopathic activity at the same time. Probably, the thermal trend and the increase of temperatures from January to April influenced the synthesis of STLs. In fact, the combination of several stress factors results in an increase of allelochemical concentrations in donor plants (del Moral, 1972).

5.6.3.3 Weed phytotoxicity bioassay

The interaction "stress x harvest time x concentration" was not significant for all parameters under study in both species (**Table 5.22**). In agreement with previous results with the same bioassay and target species, seed germination percentage was the least affected parameter. All concentrations were significantly lower compared to the positive control (0 ppm). In both *A. retroflexus* and *P. oleracea*, 800 ppm resulted the most efficient in reducing seed germination, even if not statistical differences were observed with respect to the other concentrations (**Figure 5.24**).

Regarding root length, the interaction "harvest time x concentration" was significant in both weed species, with a higher contribute of concentration to variance. In both weeds the allelopathic activity significantly increases with increasing concentrations (**Figure 5.25**). Moreover, April

confirmed to be the most appropriate period for harvest time, since it significantly stimulates the production of STLs and phytotoxicity independently from concentration. A reduction of 14 and 37% in *A. retroflexus* root length was observed going from January to April at 800 and 400 ppm, respectively. A similar trend was observed for *P. oleracea*.

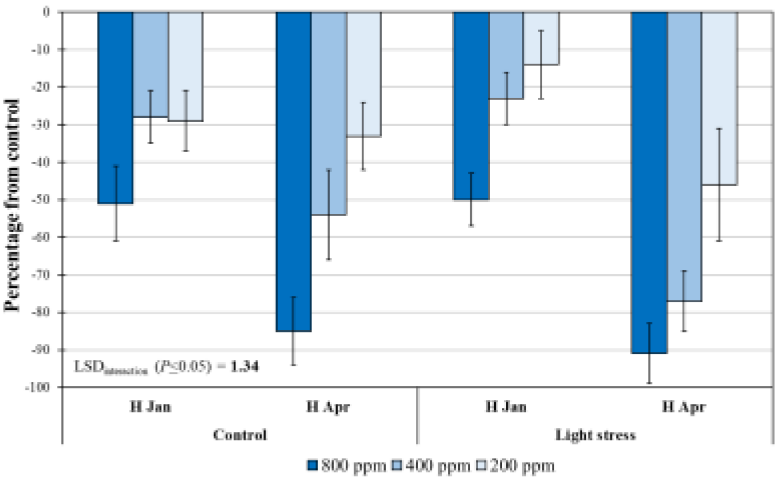


Figure 5.23 Effect of light stress condition in two different harvest times on the allelopathic activity of cultivated cardoon on wheat coleoptile elongation.
Control: 'Bianco gigante' without light stress; H Jan: harvest time at January 9th; H Apr: harvest time at April 14th. Values are expressed as percentage difference from control. Each bar means ± standard deviation

Table 5.22 *F*–Values as absolute of main factors and their interactions resulting from analysis of variance in weed species.

Source of variation	df	*Amaranthus retroflexus*			*Portulaca oleracea*		
		Germination	**Root length**	**Shoot legth**	**Germination**	**Root length**	**Shoot length**
Stress (S)	1	1.5^{NS}	0.4^{NS}	1.4^{NS}	3.9^{NS}	0.6^{NS}	0.6^{NS}
Harvest time (T)	1	0.02^{NS}	190.5^{***}	228.3^{***}	0.3^{NS}	18.6^{***}	60.5^{***}
Concentration (C)	3	10.4^{***}	589.1^{***}	219.8^{***}	11.0^{***}	193.9^{***}	35.3^{***}
(S) x (T)	1	1.3^{NS}	3.8^{NS}	0.3^{NS}	1.4^{NS}	0.3^{NS}	0.05^{NS}
(S) x C)	3	0.9^{NS}	0.5^{NS}	1.1^{NS}	1.3^{NS}	2.4^{NS}	1.7^{NS}
(T) x (C)	3	0.9^{NS}	35.0^{***}	116.9^{***}	0.1^{NS}	7.0^{***}	10.2^{***}
(S) x (T) x (C)	3	0.2^{NS}	2.6^{NS}	2.4^{NS}	0.5^{NS}	0.2^{NS}	0.1^{NS}

Note: Values are given as *F* of Fisher. df: degree of freedom. *** indicates significant at $P \leq 0.001$, and *NS*, not significant.

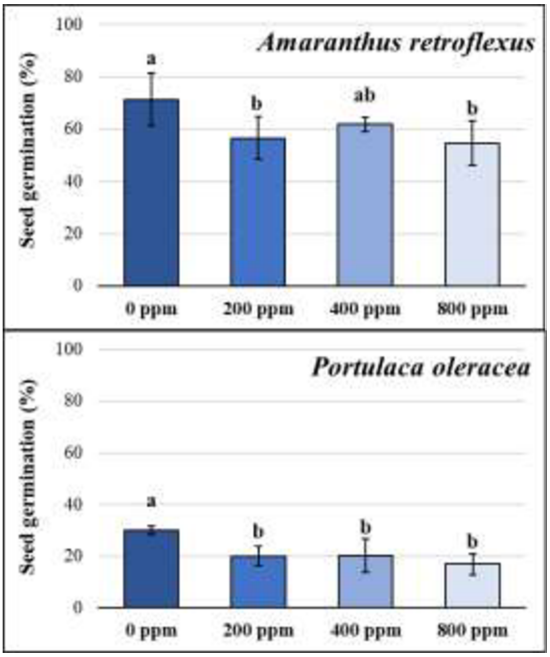


Figure 5.24 Effect of concentration on the allelopathic activity of cultivated cardoon extracts on *A. retroflexus* and *P. oleracea* seed germination percentage.
Note: Different letters indicate statistical significance for $P \leq 0.05$. Each bar means ± standard deviation.

A markedly effect of harvest time on the analysis of variance was registered for *A. retroflexus* and *P. oleracea* shoot length (**Table 5.22**). The interaction "harvest time x concentration" was highly significant in both species. Results were very similar to previous researches, with not statistical differences found among concentrations (**Figure 5.26**). April harvest always resulted in a greater allelopathic activity compared to January. In particular, the highest difference was observed in 800 ppm of *A. retroflexus* (1.8 *vs*. 26.5 mm of shoot length

from April and January extracts, respectively), followed by 11.0 *vs*. 30.8 mm in *P. oleracea*.

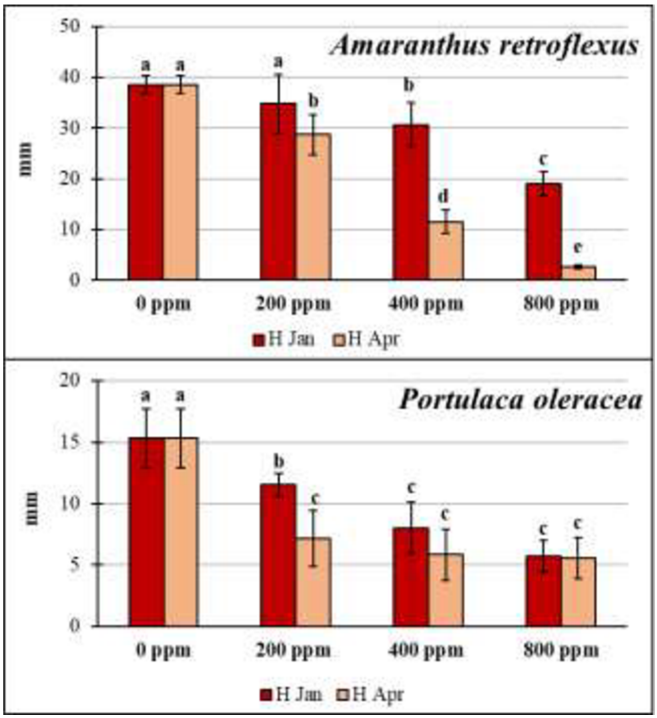


Figure 5.25 Effect of concentration on the allelopathic activity of cultivated cardoon extracts on *A. retroflexus* and *P. oleracea* root length. Note: Different letters indicate statistical significance for $P \leq 0.05$. Each bar means ± standard deviation.

5.6.4 Conclusions

In conclusions, the data obtained in the present research clearly indicated that light stress condition strongly improves the allelopathic activity in cultivated cardoon, mainly if

prolonged during time, thanks to the increase in both total and single STLs concentrations. Following previous foundings, cynaropicrin was the major STL detected. April resulted the best period stimulating cultivated cardoon phytotoxicity, which increase with increasing concentrations.

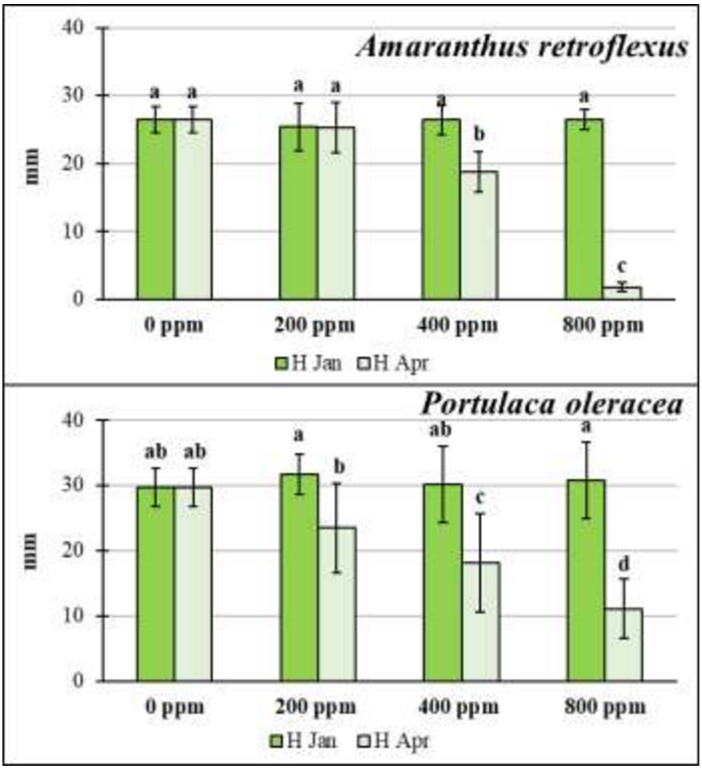
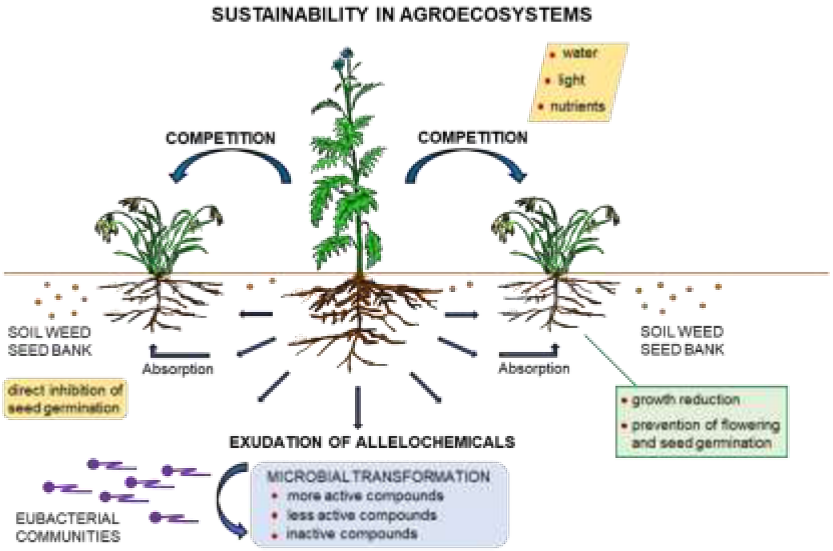


Figure 5.26 Effect of concentration on the allelopathic activity of cultivated cardoon extracts on *A. retroflexus* and *P. oleracea* shoot length. Note: Different letters indicate statistical significance for $P \leq 0.05$. Each bar means ± standard deviation.

6. Field Allelopathic Activity of *C. cardunculus* L. to reduce Size and Composition of Soil Weed Seed Bank

The present work is under review in *Agronomy for Sustainable Development* as follows:

- Scavo, A., Restuccia, A., Abbate, C., Mauromicale, G. (2018e). Potential use of field allelopathic activity of *Cynara cardunculus* L. to reduce size and composition of soil weed seed bank.



6.1 Introduction

In modern agriculture, due to the environmental impact of synthetic herbicides and the increasing incidence of

resistance in weeds, the search in eco-friendly strategies for weed control has become of central importance. In the last years, the environmental sustainability of weed management practices has become of central importance. The manipulation of the cultivated plant allelopathic mechanisms via crop rotation could represent the first step to reduce the soil seed bank in the agroecosystems, making it possible to use control weed means with low environmental impact.

The soil seed bank refers to the reserve of all viable (dormant as well as ready to germinate) weed seeds stored in the soil in a given area (Simpson *et al*., 1989). In agroecosystems, it is the primary source of new infestations of annual weeds and represents the majority of the weed species composition (Cavers, 1983). Soil seed bank characteristics play a fundamental role both on the weed populations that occur in a field and the subsequent weed management (Buhler *et al*., 1997). Since the real weed flora almost exclusively derives from the potential weed populations communities, knowing the seed bank size, composition, vertical distribution and dynamic plays a key role on the weed management. Thus, reducing the number of the weed seeds present in the soil seed bank is an important goal in the Integrated Weed Management System (IWMS; Menalled, 2008). In this way, preventive methods such as crop rotation are of central importance. Specifically, crop rotation is reported as the most important example for such allelopathic weed control (Farooq *et al*., 2011). Several crops have been reported to exert allelopathic effects on weeds when introduced within a crop rotation: rye (Schulz et al., 2013), sorghum (Weston *et al*., 2013), sunflower (Alsaadawi *et al*., 2012), etc. Moreover, crop rotation is associated with many benefits since can

reduce pest (both pathogens and weeds) pressure and soil erosion, improve the amount of soil organic matter and soil physical properties, reduce nutrient leaching, enhance soil fertility and, thus, increase yields (Lodhi *et al*., 1987).

Recently, the Asteraceae member *Cynara cardunculus* L. was studied for its allelopathic potential (Rial *et al*., 2014, 2016; Scavo *et al*., 2018b). The compounds responsible for this phytotoxic activity are sesquiterpene lactones such as cynaropicrin, aguerin B and grosheimin, and polyphenols such as chlorogenic acid, luteolin- and apigenin derivatives (Hosni *et al*., 2013; Lombardo *et al*., 2009; Pandino *et al*., 2012a). However, its allelopathic effects in field conditions are never been evaluated.

Several supports about the influence of soil microbial community on plant species abundance, seedling recruitment and seed bank dynamics have been provided (Chee-Sanford *et al*., 2006; Singh *et al*., 2004). Since seed bank and soil microorganisms are affected by the same soil physical, chemical and biological characteristics, it is reasonable to assert that changes in the seed bank are correlated to changes in the microbial community. The antimicrobial activity of *C. cardunculus* leaf extracts is well documented in literature (El Sohaimy *et al*., 2014; Kulić *et al*., 2008; Mossi and Echeverrigaray, 1999). Caffeoylquinic acids (Falleh *et al*., 2008) and flavonoids (Aljancić *et al*., 1999; Zhu *et al*., 2004) are the chemical compounds involved in its antimicrobial activity.

Our hypothesis is that allelochemicals from globe artichoke, cultivated and wild cardoon, which show phytotoxic and antimicrobial activity *in vitro* bioassays, could low weed seed bank after the repeated cultivation for several years on the

same field through their direct action and/or microbial transformation. For the first time, the effects resulting from a multidisciplinary research (agronomy, botany, soil microbiology) involving the repeated cultivation, in two different areas for three consecutive years, with globe artichoke, cultivated and wild cardoon, compared with a classical rotation wheat/faba bean and an olive grove, will be investigated by analyzing the quali/quantitative composition of soil seed bank and the changes in the eubacterial communities by denaturing gradient gel electrophoresis (DGGE) approach. Moreover, this work aims to evaluate for the first time the *in vitro* antibacterial activity of aqueous, methanolic and ethanolic leaf extracts of cultivated cardoon against three bacteria involved in the soil N-cycle.

6.2 Material and methods

6.2.1 Soil seed bank

6.2.1.1 Studied area and experimental design

The investigation on weed seed bank was carried out exploiting two different field-experiments: Experiment 1 was set up at the Agricultural Experimental Farm (AEF) of Catania University (Catania Plain, 37° 25^{I} N, 15° 30^{I} E; 10 m a.s.l.; Experiment 2 in Passitti (Catania Plain, 37° 26 ^{I} N, 14° 56 ^{I} E; 20 m a.s.l.). In both areas the climate is semiarid-Mediterranean, with a long, hot and dry summer, mild winter and rain falling mostly from late autumn to early spring. Daily mean temperature during the year ranges from 8.5°C to 26°C: minimum temperature is around 0°C, while maximum can peak at over 35°C. Annual precipitation is about 500 mm (Cristaudo *et al*., 2007). On the basis of Rivas-Martinez bioclimatic indexes, the sampling areas may be classified

within the thermo-Mediterranean upper bioclimatic belt, with inferior dry ombrotype (Brullo *et al*., 1996). The soils of the Catania Plain are alluvial, with medium fine-coarse texture (soils from clayey-loamy to sandy-loamy, from sandy to loamy-sandy and andic xerochrepts) (Fierotti, 1997). The soil type at the AEF is vertic xerochrepts (Soil Survey Staff, 1999) with clay texture (SISS, 1985). At the start of the experiment, the soil characteristics were as follow: sand (18%), silt (36%), clay (46%), limestone (4%), pH (8), organic matter (1.1%), total nitrogen (1.1 kg^{-1}), assimilable P_2O_5 (12 mg kg^{-1}), exchangeable K_2O (425 mg kg^{-1}), electrical conductivity (0.4 dSm^{-1}), whereas at Passitti the soil is classified as a Tipic Xero-fluvent (Soil Survey Staff, 1999) with a clay loam texture: sand (40%), silt (25%), clay (35%), limestone (2%), pH (7.9), organic matter (1.6%), total nitrogen (1.3 g Kg^{-1}), assimilable P_2O_5 (50 mg Kg^{-1}), exchangeable K_2O (425 mg Kg^{-1}), electrical conductivity (0.41 dSm^{-1}). In each field-experiment, we used three adjoin field plot (1000 m^{-2}) in the same area to guarantee pedoclimatic conditions as uniform as possible for the three crop rotations. Experiment 1 consisted of: i) a monoculture for three consecutive years (from 2014 to 2017) with globe artichoke; ii) a monoculture of cultivated cardoon for three consecutive years; iii) a classical rotation faba bean (*Vicia faba* L.) and wheat (*Triticum durum* Desf.) for four years. The choice of this control is explained since the grain legume/cereal rotation is the most common in the hot-arid zones of the Mediterranean Basin. The globe artichoke and cardoon plots were planted in August by "ovoli" (semi-dormant offshoots) or seeds (achenes), respectively using an inter- and intra-row spacing of 1.25 and 0.70 m. In second

and third year, the regrowth after the period of summer dormancy was carried out in August by irrigation. Seeds of faba bean and wheat were sown in November using a density of 40 and 400 seeds m^{-2}, respectively. Experiment 2 was carried out in: i) a monoculture for three consecutive years (from 2014 to 2017) with cultivated cardoon; ii) a monoculture of wild cardoon for three consecutive years; iii) an olive grove at the fourth year, a typical crop of the Mediterranean zones. Cardoon plots were planted and managed in the same way of those of Experiment 1. The olive trees, 3 years-old, were planted with a spacing of 4 m within rows x 6 m between rows (417 plants ha^{-1}). For all crops, the standard commercial practices were adopted. Weeds were mechanically controlled, for each season and rotation, by shallow hoeing (0.15 m deep) two times per year.

6.2.1.2 Soil seed bank sampling and seed identification

Soil samples were collected in May 2015 and 2017 (AEF) and May 2015 (Pasitti), to intercept all the winter-spring plant species, along the diagonals of each plot. Ten soil cores per plot (each of 0.75 dm^3) were collected with a metallic probe up to 15 cm depth. Soil samples were stored in paper bags for few days at 4 °C in the dark before being analysed to prevent seed germination prior to identification. Each soil sample was carefully mixed in laboratory and the inert fraction (stones, pebbles, etc.) was hand-removed, because it would render difficult their washing. For the direct seed extraction, seeds were separated from soil by washing. For an adequate representation of the entire soil core, four subsamples of 150 g for each plot were put into a metal tube with a removable cap, provided of steel mesh of 250 μm, and were washed

through an electric adjustable pressure (20-120 bar max) washer (Karcher, K 3500 model, Germany). Before seed extraction, soil samples from each core were pre-treated and mixed with 5 g of sodium hexametaphosphate solution for 20 min, to disperse readily the colloid matrix and facilitate the subsequent washing stages. The extracted fraction, composed of seeds mixed with mineral and organic particles, was transferred into Petri dishes and was air-died for 24 h for the hand separation; seeds were isolated from the remaining matter, then identified to species and counted. All the observations were performed using a MS5 Leica stereomicroscope (LeicaMicrosystems, Wetzlar, Germany). For the assessment of viability, the extracted seeds were submitted to the "seed crushing test" through slight finger pressuring (Vasileiadis *et al*., 2007). This procedure, although not rigorous as the tetrazolium test or germination test, is often used for seed bank because quicker and cheaper (Price *et al*., 2010). The values of viable seeds in each subsample were expressed as the number of seeds m^{-2} of surface area for each plot. Seeds were grouped in relation to botanical family and each species was assigned to a life-form category considering the Raunkiaer system (Raunkiaer, 1934). The nomenclature of species followed Conti *et al*. (2005).

6.2.2 Soil molecular analyses

6.2.2.1 DNA extraction

DNA was extracted directly from 250 µl of soil samples (Martin-Laurent *et al*., 2001). Samples were homogenised in 1 mL of extraction buffer [100 mM Tris, pH 8; 100 mM EDTA; 100 mM NaCl; 1% (w/v) polyvinylpyrrolidone; 2%

(w/v) sodium dodecyl sulphate] for 30 sec at 1600 rpm in a mini-bead cell disrupter. Cell debris was removed by centrifugation (5 min at 14 000 *g*). Proteins were eliminated after sodium acetate precipitation. Nucleic acids were precipitated with cold isopropanol, then washed with 70% ethanol. DNA extracts were purified with a polyvinylpyrrolidone spin column. The quality and the integrity of the DNA was checked by electrophoresis on 1% agarose gel.

6.2.2.2 Amplification of eubacterial 16S rDNA fragments for DGGE analysis

Eubacterial 16S rDNA was amplified using the primer sets GC-968f (5'- CGC CCG GGG CGC GCC CCG GGC GGG GCG GGG GCA CGG GGG GAA CGC GAA GAA CCT TA-3') and 1401r (5'-GCG TGT GTA CAA GAC CC-3'), as described by Felske *et al*. (1997) to obtain products of about 450 bp. The DNA template (80 ng) was amplified with 5 Units μl^{-1} Taq DNA Polymerase, 10 μM of each primer, 10 mM of each dNTP, 10 mM of $MgCl_2$, 500 $\mu g\ ml^{-1}$ of BSA and reaction buffer 1x (Invitrogen) in a final reaction volume of 50 μl. The PCR conditions were: 94 °C for 90 seconds, followed by 33 cycles at 95°C for 20 seconds, 56°C for 30 seconds, 72 °C for 45 seconds, and a final extension step at 72 °C for 7 minutes. Amplicons were analysed as described above.

6.2.2.3 Denaturing gradient gel electrophoresis (DGGE)

16S rDNA-DGGE was performed using the DCode System (Universal Mutation Detection System, Bio-rad). An amount of 300 ng of amplicons was loaded in duplicate (top filling

method) on 6% polyacrylamide gel (Acrylamide/Bisacrylamide, 40%, 37.5:1, Bio-rad) containing a denaturant gradient of 46-56% parallel to the electrophoresis direction made of urea and formamide (100% denaturant contains 7M urea and 40% formamide). Gels were electrophoresed at a constant temperature (60 °C) and voltage (75 V) for 16 hours, followed by 2 hours coloration using Sybr Green I nucleic acid gel stain 1:1000 diluted in the running buffer (FMC Bio Products, Rockland, ME USA). Bands were detected from digital images (Polaroid Gel Cam, Elect; Polaroid Type 667 Film ISO 3000) by UV light gel transillumination (λ 312 nm).

Bands to be sequenced were excised from the DGGE gels, placed in 50 µl sterile H_2O, and stored at -80 °C. Before PCR amplification, the samples were thawed for 1 h at room temperature, frozen again at -80 °C for 1 h, and finally thawed at 4 °C overnight to elute the DNA fragments. The eluted DNA (2 µl) was used as a template in PCR amplification with the primer set 968f - 1401r (without the GC-clamp).

6.2.3 In vitro *antibacterial activity of leaf extracts*

6.2.3.1 Sampling of plant material and preparation of leaf extracts

Leaves of cultivated cardoon cv. Altilis 41, which was selected in a breeding program of University of Catania on *C. cardunculus*, were collected randomly from about sixty plants at the 25th visible leaves growth stage in the Catania University experimental station farm. The extraction procedure was carried out following the procedure previously described by Scavo *et al*. (2018f). 35 g of dried leaves were macerated with 350 mL of bidistilled water (WE), a mixture

methanol/bidistilled water 70:30 v/v (ME) and ethanol/bidistilled water 80:20 v/v (EE) for 72 hours at 25 °C in the dark, and then filtered through filter paper (Whatman No.2) to eliminate the solid fraction. Methanolic and ethanolic mixtures were evaporated at 35 °C using a rotary evaporator (Laborata 4000, Heidolph, Germany) and the residues were re-dissolved with bidistilled water. The antibacterial bioassay was performed with three concentrations (100, 80 and 40%) for each extract diluted with bidistilled water. The prepared extracts were transferred into a falcon flask and stored at –20 °C for further uses.

6.2.3.2 Determination of the antibacterial activity

The antibacterial activity of cultivated cardoon leaf extracts was evaluated *in vitro* for the first time against the bacteria *Rhizobium leguminosarum*, *Sinorhizobium meliloti* and *Bacillus licheniformis*, selected within the microbial collection of Di3A (University of Catania).

Rhizobium leguminosarum is a well-known plant growth-promoting rhizobacterium (PGPR), which includes three biovars corresponding to the plants involved in the symbiosis: *R. leguminosarum* biovar *viciae*, *R. leguminosarum* biovar *trifolii*, and *R. leguminosarum* biovar *phaseoli* (Jordan, 1984). *Sinorhizobium meliloti* is a Gram-negative bacterium that nodulates the roots of alfalfa (*Medicago sativa* L.). Both bacteria play a key agronomic role for the survival of leguminous plants living in extreme conditions (e.g. arid regions), since the biological N_2 fixation (BNF) can achieves values of 200–300 kg N ha^{-1} per year for various legume crops and pasture species (Peoples et al., 1995). Kim *et al*. (2005) reported that the *Bacillus* strains were able to occur

simultaneously aerobic nitrification/denitrification. *Bacillus licheniformis* was found to convert ammonia to N_2 without the formation of nitrous oxide under aerobic conditions. Therefore, in removal of ammonia, the heterotrophic *Bacillus* strains present less complex metabolic pathways than autotrophs.

To evaluate the antibacterial activity of cultivated cardoon extracts, the agar well diffusion method was performed (Bauer *et al*., 1966; Pagliaruolo *et al*., 2016). 1 mL of a 24-h culture of the target bacterial suspensions (10^8 cells/mL) were reversed into sterile Petri dishes. Then, 20 mL of sterilized melted medium (~ 45 °C), such as Tryptone yeast extract agar for *R. leguminosarum* and *S. meliloti* and Nutrient agar for *Bacillus licheniformis*, were poured into the dishes and mixed until the media was solidified; holes of 6.3 mm diameter were aseptically pierced by means of sterile corks into the solidified media. 100 µL of tested extracts were introduced into the wells at the following concentrations, expressed as mg total polyphenols mL^{-1}: 0.3336, 0.278 and 0.139 for W; 1.7676, 1.473 and 0.7365 for ME; 1.2684, 1.057 and 0.5285 for EE. Since all the different dried extracts were redissolved in water before antibacterial assays, sterile bidistilled water was used as negative control. Plates were incubated at 28 °C, until visible growth of test microorganisms in the control plates. The diameter of the inhibition zones, including well diameter (expressed in mm), produced by the extracts against targeted bacterial species, was measured to quantify the antibacterial activity. The trial was repeated three times.

6.2.4 Data and statistical analysis

Data were submitted to the Bartlett's Test for the homogeneity of variance and then were subjected to analysis of variance (ANOVA). Means were separated on the basis of the Student–Neuman–Keuls test only when the *F* test of the ANOVA for treatments and interaction was significant at the 0.05 probability level. Data for each species were treated separately.

For what concern soil seed bank, a factorial two-way ANOVA model, for each experiment, was used to analyse the data, with cropping system and year as main factors. Besides, the differences in weed communities among cropping systems and year were estimated by using Sørensen's Similarity Indices (Magurran, 2004).

- The qualitative index was calculated using the equation:

$$[2C * (A + B)^{-1}]$$

where C is the number of species in common, A is the total number of species in one of the two situations compared, and B is the total number of species in the other situation.

- The quantitative Sørensen index, also known as Bray-Curtis similarity or Percentage similarity, was calculated as following:

$$[2N_t * (N_a + N_b)^{-1}]$$

where N_t is the sum of the lowest density values of the species common to the two situations compared, N_a is the sum of the density values of all species in one situation, and N_b is the sum of the density values of all species in the other situation. Sørenson's Similarity Indices range from 0 to 1, with high values indicating strong similarity between the situations compared.

6.3 Results and discussion

6.3.1 Soil seed bank size

In agricultural soils, seed bank size values ranging from near 0 to as much 1 million seeds m^{-2} have been reported (Fenner, 1985). The introduction of perennial crops in annual cropping systems contributes to decrease the soil seed bank of annual species over time (Hossain and Begum, 2015).

Regarding the Experiment 1, in 2015 the soil seed bank amounting to 9450 seeds m^{-2} in the control, was halved to 5050 seeds m^{-2} due to the repeated cultivation of globe artichoke, and even more reduced to 2600 seeds m^{-2} in the plot with cultivated cardoon (**Figure 6.1**). The same situation was found also in 2017, even with more differences: the amount of weed seeds in the control (12650 m^{-2}), compared to them of globe artichoke and cultivated cardoon, was 2.0 and 6.6 times higher, respectively. Regardless of the cropping system, the number of seeds in 2015 (6950 m^{-2}) decreased to 5700 m^{-2} in 2017. Therefore, the effect of the year on the analysis of variance was highly significant (F=18; p<0.001) (**Table 6.1**). The cropping system was also highly significant (F=318; p<0.001) (**Table 6.1**). In fact, on the average of the year of the experiment, the soil seed bank size of the control (11050 m^{-2}) significantly decreased to 5676 m^{-2} and 2250 m^{-2} in the plot cultivated with globe artichoke and cardoon, respectively. Moreover, the size of the soil seed bank was affected by the "cropping system x year" interaction (F=15; p<0.001) and the first gave the highest contribution to the interaction (**Table 6.1**). For what concern Experiment 2, the seed bank of control (olive grove) which amounted to 5333 seeds m^{-2}, was halved to 2600 and 2467 seeds m^{-2} due to the repeated cultivation of wild cardoon and cultivated cardoon,

respectively (**Figure 6.1**). Therefore, a reduction of the soil seed bank size was found in two different areas due to the repeated cultivation for three consecutive years of *C. cardunculus*, mainly the cardoons.

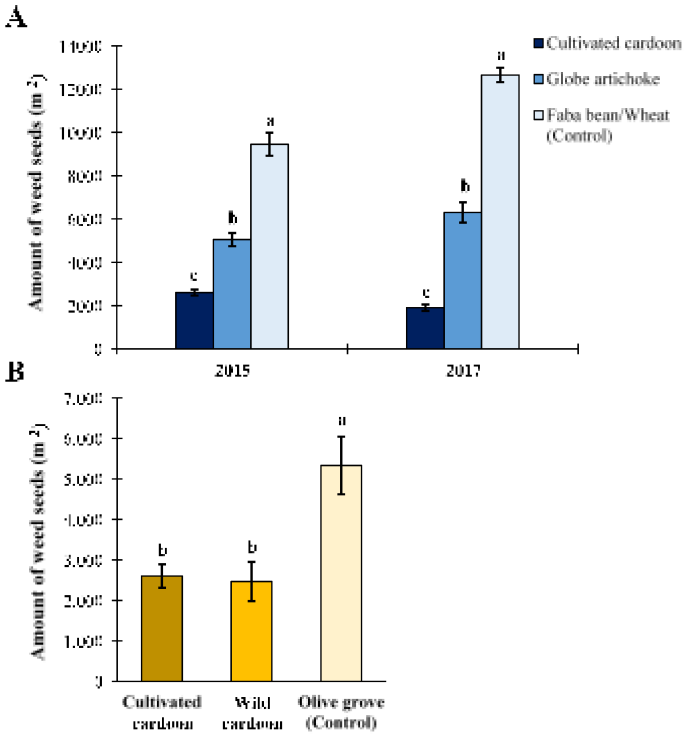


Figure 6.1 Amounts of weed seeds in the soil seed bank in Experiment 1 (A) and Experiment 2 (B).
Each bar means ± standard error. Different letters indicate statistical significance for $P \leq 0.05$.

Table 6.1 Analysis of variance for the effect of cropping system, year and their interactions that produced seed banks in Experiment 1.

Source of variation	df	SS	MS	F	*P*
Cropping system	2	314830000	157415000	318	0.00001
Year	1	9375000	9375000	18	0.0004
Cropping system x Year	2	15210000	7605000	15	0.0001
Error	18	8910000	495000		

Crop rotation is an important tool to lower weed pressure. Nevertheless, its contribution in agroecosystems is limited if left alone, becoming stronger when combined with other methods within an Integrated Weed Management Systems (Garrison *et al*., 2014). The results described above suggest the possibility to introduce cultivated cardoon or globe artichoke within a crop rotation for 1-2 years in order to decrease the size of soil seed bank. Other evidences are reported on the changes caused in weed populations by crop rotation, even after two years of crop sequences (Bellinder *et al*., 2004; Dorado *et al*., 1999). Many factors affect the quantity and quality of the weed seed bank under field conditions (Buhler *et al*., 2001). However, considering the similarities between the plots in terms of climatic conditions, soil characteristics and agronomic practices, appear reasonable to assert that the differences in the amount of weed seeds in the soil seed bank are related to the effects of globe artichoke and cultivated cardoon on the real and potential flora. These effects could be explained by two mechanisms: competition and allelopathy. *C. cardunculus* is an invasive perennial forb that, thanks to its high biomass production and well-developed root system, strongly competes with weeds for light, water and nutrient uptake (White and Holt, 2005).

Besides, its secondary metabolites released into the soil through leaching from leaves and ground litter, seem to exert an inhibitory effect on the weed population by preventing their flowering and seed germination. However, in field conditions is difficult to separate these mechanisms (Weidenhamer, 2006): stress caused by competition increase the production of allelochemicals, while growth reduction caused by allelochemicals may reduce the competitive ability of inhibited plant (Scavo *et al*., 2018a). These mechanisms are also involved in the invasive behaviour of wild cardoon in California grasslands (Marushia and Holt, 2006).

C. cardunculus can release allelochemicals into the rhizosphere through leaching from plant foliage, decomposition of plant residues on soil surface and exudation from roots. The first two pathways of release appear to play a more important role, since the polyphenol and sesquiterpene content is higher in the aboveground parts of the plant, particularly in the leaves (Lombardo *et al*., 2009; Pandino *et al*., 2012a). Once released into the rhizosphere, allelochemicals enter a complex plant-soil system in which different factors, both biotic and abiotic, affect their availability and, consequently, their effectiveness (Blum *et al*., 1999; Kruse *et al*., 2000). In order to manifest phytotoxic activity, allelochemicals should accumulate, persist at phytotoxic levels and come in contact with target plants (Inderjit and Nilsen, 2003). Furthermore, soil microorganisms significantly affect allelochemicals efficacy by converting them into more active, less active or entirely inactive compounds (Cipollini *et al*., 2012). Harper and Lynch (1982) suggested that the total allelopathic potential of plant residues is the result of water-soluble allelochemicals

released by residues prior to decomposition, as well as the insoluble allelochemicals released by microorganisms during decomposition.

6.3.2 Weed seed composition

In Experiment 1, a total of 25 weed species were identified in the 0-15 cm soil layer (**Table 6.2**). The seed bank was almost exclusively composed of herbaceous species, except for *Platanus orientalis* L. The analysis of the biological forms has pointed out a high percentage of therophytes (60%) and hemicryptophytes (24%,) while geophytes (12%) and phanerophytes (4%) were poorly represented. Moreover, 60% of total weeds found were annual, 12% biennial and 28% perennial, belonging to 18 botanical families: Amarantaceae, Asteraceae, Boriginaceae, Brassicaceae, Chenopodiaceae, Convolvulaceae, Fumariaceae, Lamiaceae, Oxalidaceae, Platanaceae, Polygonaceae, Portulacaeae, Primulaceae, Ranunculaceae, Rubiaceae, Scrophulariaceae, Solanaceae, Verbanaceae. The most representative families were Asteraceae (22%), Brassicaceae (17%) and Polygonaceae (11%). *Sinapis arvensis* L. was the most abundant species in the soil seed bank both in 2015 (9400 seeds m^{-2}) and 2017 (15000 seeds m^{-2}), followed by *Anagallis arvensis* L. (4600 seeds m^{-2}) and *Beta vulgaris* L. (1467 seeds m^{-2}) in 2015, and *Helminthotheca echioides* (L.) Holub (3000 seeds m^{-2}) and *Rumex* spp. (3000 seeds m^{-2}) in 2017. In Experiment 2, eleven taxa belonging to nine botanical families were identified (**Table 6.3**). A high percentage of therophytes (75%) was found like in the Experiment 1, followed by scapose hemicryptophytes (17%) and biennial hemicryptophytes (8%), while phanerophytes and geophytes

were absent. On the total of weeds found, 90% were annual and 10% biennial. *Amaranthus retroflexus* L. was the most the most common weed in the soil seed bank (2622 seeds m^{-2}), followed by *Portulaca oleracea* L. (733 seeds m^{-2}). In cultivated soils, generally, the seed bank is represented by a small number of dominant weed species and a big amount of species in low numbers (Cardina *et al*., 1991). According to Wilson (1988), the dominant species comprise from 70 to 90% of the total weed seed bank.

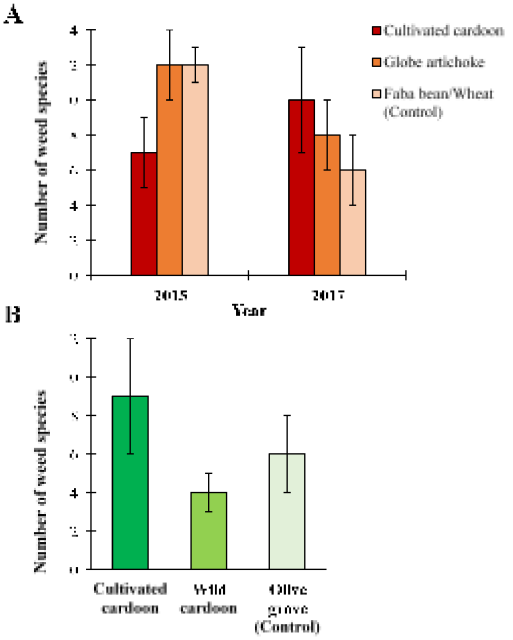


Figure 6.2 Number of weed species found in the soil seed bank in Experiment 1 (A) and Experiment 2 (B). Each bar means ± standard error.

Within the Experiment 1, in 2015 the cultivation of cardoon permitted the presence of seeds from only 7 weed species in

the soil seed bank, with respect to the 12 of globe artichoke and control (**Figure 6.2**). However, the number of weed species in the seed bank increased in 2017, except for globe artichoke which allowed to reduce to 8 species. Therefore, the permanence of globe artichoke for several years on the same field allows a reduction in the number of weed species. In Experiment 2, the highest number of weed species was found in the cultivated cardoon plot (9), followed by the olive grove (6) (**Figure 6.2**). The presence of wild cardoon for three consecutive years allowed the presence of only four weed species belonging to four botanical families, including *Portulacaceae* and *Amaranthaceae* with *P. oleracea* and *Amaranthus* sp. respectively, representing about 90% of the seed bank. This aspect is of central importance for the weed management, since a lower number of species gives the opportunity to focus on a specific control strategy.

6.3.3 Indices of similarity

Low qualitative and quantitative indices of similarity were determined between control and theses both in the same and different year (**Table 6.4**). It proves that the composition of the soil seed bank changes both qualitative and quantitative in the globe artichoke and cultivated cardoon plots, compared to the rotation wheat/fava bean. The smallest qualitative index was ascertained between the plot with cultivated cardoon in 2017 and the control in 2015 (0.18), while the highest between both globe artichoke and cultivated cardoon in 2015 (0.63). Quantitative index has reached the lowest values between both cultivated cardoon and control in 2017 (0.08), and between control in 2015 and cultivated cardoon in 2017 (0.09).

Table 6.2 Weed population under crop rotations in Experiment 1 classified into biological groups (BG) and ecophysiological groups (EG).

Weed species	BG	EG	2015			2017		
			Cultivated cardoon	Globe artichoke	Faba bean/Wheat (Control)	Cultivated cardoon	Globe artichoke	Faba bean/Wheat (Control))
Amaranthus sp.	Th	Su		●	●		●	
Anagallis arvensis L.	Th	Au-Wi	●	●	●	●	●	●
Beta vulgaris L.	Hr	Su	●	●		●	●	
Borago officinalis L.	Th	Sp-Su				●		
Capsella bursa-pastoris (L.) Medik. subsp. *bursa-pastoris*	Hr	In		●				
Centaurea napifolia L.	Th	Su		●	●			●
Convolvulus arvensis L.	G	In				●		
Cynara cardunculus var. *sylvestris* (Lamk) Fiori	Hr	Su				●		
Echium plantagineum L.	Hr - Th	Sp-Su	●					
Fallopia convolvulus (L.) Á. Löve	Th	Sp-Su	●	●	●	●	●	
Fumaria officinalis L.	Th	Sp-Su-Au			●			
Galium aparine L.	Th	Sp-Su						●
Helminthotheca echioides (L.) Holub	Th	Su-Au		●		●	●	●
Lamium amplexicaule L.	Th	In					●	
Oxalis pes-caprae L.	G	Au-Wi-Sp	●	●		●		
Platanus orientalis L.	Ph	Sp			●			
Portulaca oleracea L.	Th	Su		●	●			
Ranunculus arvensis L.	Th	Sp - Su					●	
Raphanus raphanistrum L.sub. *landra* (DC.) Bonnier & Layens	Th	Sp			●			

Rumex sp.	Hr	Sp - Su				•			•	
Sinapis arvensis L. subsp. *arvensis*	Th	Sp	•	•	•	•				•
Solanum nigrum L.	Th	In		•	•					
Sonchus sp.	G	In	•	•		•				
Verbena officinalis L.	Hr	Su-Au			•					
Veronica polita Fr.	Th	In								•

Note: Th: therophytes; Hr: hemicryptophytes; Ph: phanerophytes; G: geophytes; Su, Au, Wi, Sp: summer, autumn, winter, spring species; In: indifferent species.

Table 6.3 Weed population under crop rotations in Experiment 2, classified into biological groups (BG) and ecophysiological groups (EG).

Weed species listed	BG	EG	Cultivated cardoon	Wild cardoon	Olive grove (Control)
Amaranthus sp.	Th	Su	•	•	•
Beta vulgaris L.	Hr	Su	•		
Borago officinalis L.	Th	Sp-Su	•		•
Capsella bursa-pastoris (L.) Medik. subsp. *bursa-pastoris*	Hr	In			•
Fallopia convolvulus (L.) Á. Löve	Th	Sp-Su	•	•	•
Heliotropium europaeum L.	Th	Su	•		
Malva sylvestris L.	Hr	Sp-Su		•	•
Portulaca oleracea L.	Th	Su	•	•	•
Setaria sp.	Th	Su	•		
Sinapis arvensis L.	Th	In	•		
Solanum nigrum L.	Th	In	•		

Note: Th: therophytes; Hr: hemicryptophytes; Su, Sp: summer, spring species; In: indifferent species.

Table 6.4 Sørensen's qualitative and quantitative indices of similarity compared between all treatment combinations in Experiment 1.

		Qualitative index					
		2015			2017		
Year	**Crop**	**Cultivated cardoon**	**Globe artichoke**	**Faba bean/Wheat (Control)**	**Cultivated cardoon**	**Globe artichoke**	**Faba bean/Wheat (Control)**
2015							
	Cultivated cardoon		0.67	0.37	0.67	0.12	0.19
	Globe artichoke	0.63		0.25	0.47	0.32	0.12
	Faba bean/Wheat (Control)	0.32	0.58		0.09	0.07	0.72
2017							
	Cultivated cardoon	0.47	0.55	0.18		0.19	0.08
	Globe artichoke	0.27	0.40	0.30	0.44		0.11
	Faba bean/Wheat (Control)	0.31	0.33	0.22	0.38	0.29	
							Quantitative index

6.3.4 Soil molecular analyses

In order to verify the effects resulting from the repeated cultivation for three consecutive years with globe artichoke and cultivated cardoon, compared with a classical rotation wheat/faba bean, on soil eubacterial communities, molecular analysis was performed by PCR amplification of 16S rDNA genes from the total DNA of each sample and separation on parallel denaturing gradient gel by electrophoresis. **Figure 6.3** reports the DGGE results. In DGGE technique, each band corresponds to a specific microorganism and it is possible to cut the band from the gel and recover the related DNA for sequencing. Patterns proved to be very similar in terms of number and intensity of bands. However, the profiles showed the disappearance of some band and an intensification of others. In particular, the bands present in the soil control samples and indicated by the black box, became fainter in soil samples cultivated with globe artichoke, until to become very faint and maybe disappear in presence of cultivated cardoon. In parallel, the gel showed that the presence of cultivated cardoon had a stimulating effect towards two microorganisms of the bacterial community. In fact, the intensification of two different bands, indicated by blue and red boxes, has been detected in the gel of cultivated cardoon soil samples. The microorganisms were identified by Biodiversity s.r.l., Brescia, Italy, using NCBI library, as *Bacillus subtilis* (97%), *Pseudomonas putida* (98%) and *Azospirillum brasilense* (95%), respectively.

The permanence of cultivated cardoon for three consecutive years showed both toxic and stimulatory effects on soil microorganisms. In fact, on one side it had a negative influence towards *B. subtilis*, on the other side a positive one towards the beneficial soil bacteria *P. putida* and *A. brasilense*. The Gram-positive soil bacterium *B. subtilis*

encounters changing environmental conditions in its habitat. It is one of the best characterized bacteria and is used as a model organism for Gram-positive bacteria. *B. subtilis*is is a rod-shaped bacterium which produces endospores that allow the survival in extreme environmental conditions, including heat and desiccation (Härtig and Jahn, 2012). *P. putida* is a rod-shaped, flagellated, Gram-negative bacterium which is found in most of terrestrial and aquatic habitats in presence of oxygen. Its *optimum* is between 25-30 °C and can be easily isolated. *P. putida* presents several strains such as the KT244, and it is considered as a potential root colonizer for the rhizoremediation of pollutants and the biological control of pests. The peculiarity of this bacterium is due to the involvement of the most of its genes in the decomposition of aromatic or aliphatic hydrocarbons, which are dangerous chemicals caused by burning fuel, coal, tobacco and other organic substances (Espinosa-Urgel *et al*., 2000). *Azospirillum* spp. are soil bacteria able to promote the growth of 113 plant species belonging to 35 botanical families. These non-pathogenic and beneficial bacteria are ubiquitous in soils and colonize the roots of several plants. Moreover, *Azospirillum* spp. are microaerophilic bacteria able to fix nitrogen under free-living conditions and to navigate in chemical gradients, including oxygen (Alexandre, 2017).

The inhibition of *B. subtilis* by cultivated cardoon probably is caused by the secondary metabolites released into the rhizosphere. The transformation and/or degradation of plant secondary metabolites into more/less toxic compounds operated by soil microorganisms, is widely reported in literature. Souto *et al*. (2001), studying the allelopathic effects of tree species on some soil microbial populations and herbaceous plants, found that the proteolytic microorganisms

such as *Bacillus* spp. were negatively affected by *Eucalyptus globulus* Labill. and *Pinus radiata* D.Don.

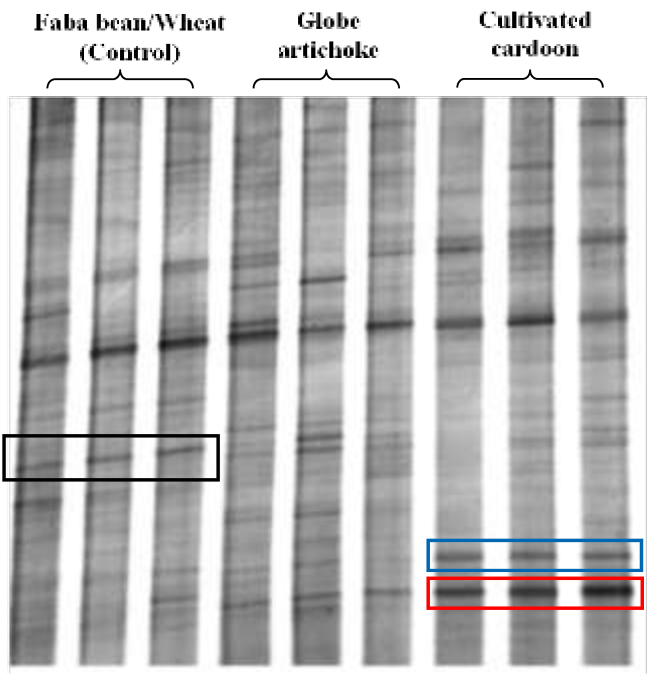


Figure 6.3 Denaturing gradient gel electrophoresis (DGGE) of soil eubacterial community in Experiment 1. Each plot/treatment is represented by three bands.

On the other hand, the positive effects of cultivated cardoon on *P. putida* and *A. brasilense* suggest how these microorganisms can use the secondary metabolites released by the plant for their growth and metabolism, preventing the accumulation into the rhizosphere. In according to our results, other authors reported that soil microbes are able to use and rapidly degrade many xenobiotic compounds including

pesticides, fungicides, and herbicides (Abbate *et al*., 2013; Blum, 1998; Chen *et al*., 2011).

6.3.5 In vitro *antibacterial activity*

The *in vitro* antimicrobial activity of cultivated cardoon leaf extracts against *R. leguminosarum*, *S. meliloti* and *B. licheniformis* is shown in **Table 6.5**. The obtained results showed a similar trend to those reported by DGGE. In fact, only the Gram-positive bacterium *B. licheniformis* was negatively influenced by ME and EE at all concentrations tested **(Figure 6.4)**. In particular, the ME showed a higher inhibitory activity (with inhibition haloes values of 23.5 and 22 mm at 1.7676 and 1.473 mg total polyphenols mL^{-1}, respectively) respect to EE (17.7 and 18 mm at 1.2684 and 1.057 mg total polyphenols mL^{-1}, respectively). Contrariwise, when tested at the lowest concentration the EE was able to more effectively control the growth of *B. licheniformis* compared to ME. The other two Gram-negative bacteria did not show any inhibitory effect. The higher resistance of Gram-negative bacteria to antibiotics and other chemicals with respect to Gram-positive one is explained by the presence of a double membrane surrounding each bacterial cell. In fact, although all bacteria have an inner cell membrane, Gram-negative bacteria present a unique outer membrane which excludes certain drugs and antibiotics from penetrating the cell. Kinkle *et al*. (1994), screening *Rhizobium* and *Bradyrhizobium* strains for resistance to tellurite, selenite, and selenite, reported high levels of resistance to the metals in *Rhizobium meliloti* and *Rhizobium fredii* strains.

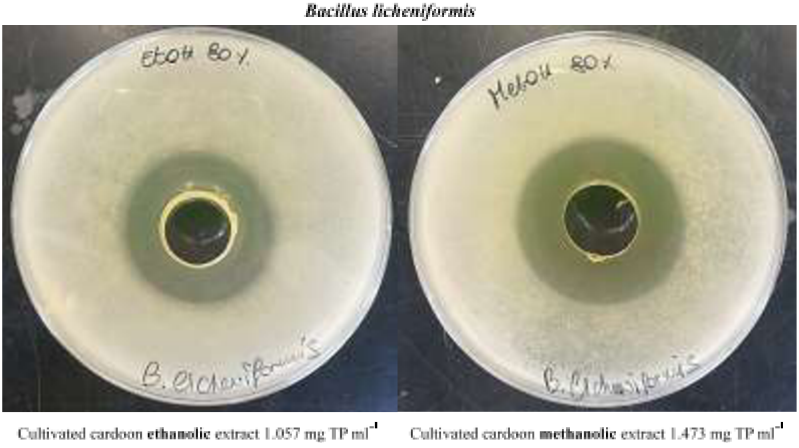


Figure 6.4 Diameter of inhibition of cultivated cardoon ethanolic and methanolic extracts on *Bacillus licheniformis*. TP: total polyphenols.

The results outlined above show how the repeated cultivation for three consecutive years of *C. cardunculus* reduce the size of soil seed bank by varying patterns of resource competition and allelopathic interactions. However, some of the most important negative effects associated with the maintenance of a single crop for several years on the same field are the reduction of nutrient availability, particularly that of nitrogen, and the imbalances in soil microbial community (Huang *et al*., 2003). Our results on the *in vitro* antimicrobial activity of leaf extracts, evidence that cultivated cardoon do not exert negative effects on *R. leguminosarum* and *S. meliloti*, which are two important bacteria involved in BNF.

6.4 Conclusions

In the present study, the allelopathic activity of *C. cardunculus* in field conditions was studied for the first time in a multidisciplinary approach. The presence of globe

artichoke, cultivated and wild cardoon for three consecutive years significantly and markedly reduced the amount of weed seeds in the soil seed bank of two different areas. Moreover, cultivated cardoon decreased the number of weed species compared to globe artichoke and a biennial rotation wheat/faba bean, showing the possibility to efficiently apply this strategy within the Integrated Weed Management System. The utilization of allelopathy via crop rotation revealed an important eco-friendly agricultural practice for the chemical-free weed management, with respect of environmental, human and animal health. Nevertheless, cultivated cardoon had a negative influence towards *B. subtilis*, while positive effects were registered on *P. putida* and *A. brasilense*. Besides, cultivated cardoon leaf extracts did not exert *in vitro* negative effects on *R. leguminosarum* and *S. meliloti*, while its methanolic and ethanolic extracts inhibited *B. licheniformis* at all concentrations tested. The microorganisms reported here are common in soil playing an important role on decomposition, biodegradation and nitrogen cycle. In fact, they can use a wide range of carbon sources, including molecules such as xenobiotics, which few other organisms can break down. Therefore, *C. cardunculus* plays a positive role on the rhizosphere microbial community. Our results suggest the advantages to introduce *C. cardunculus* within a crop rotation system in Mediterranean (or others) agroecosystems as indirect method to reduce the weed soil seed bank pressure without the adoption of chemical-synthesis herbicides and with respect of soil eubacterial communities, making possible eco-friendly management.

Table 6.5 Antibacterial activity of cultivated cardoon leaf extracts against *Rhizobium leguminosarum*, *Sinorhizobium meliloti* and *Bacillus licheniformis*.

Bacteria	Zones of inhibition (mm)*, **, ***								
	WE 0.3336 (mg TP ml^{-1})	**ME** 1.7676 (mg TP ml^{-1})	**EE** 1.2684 (mg TP ml^{-1})	**WE** 0.278 (mg TP ml^{-1})	**ME** 1.473 (mg TP ml^{-1})	**EE** 1.057 (mg TP ml^{-1})	**WE** 0.139 (mg TP ml^{-1})	**ME** 0.7365 (mg TP ml^{-1})	**EE** 0.5285 (mg TP ml^{-1})
Rhizobium leguminosarum	–	–	–	–	–	–	–	–	–
Sinorhizobium meliloti	–	–	–	–	–	–	–	–	–
Bacillus licheniformis	–	23.5	17.7	–	22	18	–	16	21

* Values are given as means of 3 replicates with pooled standard error of a mean (SEM) =0.04;
**100 µL of extract was applied to each well;
***WE: water extracts; ME: methanolic extracts; EE: ethanolic extracts; –no inhibition zone;
TP: total polyphenols.

7. Antimicrobial Activity of *C. cardunculus* Leaf Extracts

The results presented in this part of the work are composed of the following two articles:

- Scavo, A., Pandino, G., Restuccia, C., Parafiti, L., Cirvilleri, G., Mauromicale, G. (2018d). Antimicrobial activity of cultivated cardoon (*Cynara cardunculus* L. var. *altilis* DC.) leaf extracts against bacterial species of agricultural and food interest. *Ind Crop Prod* (In Press.).
- Mazzaglia, A., Licciardello, F., Scavo, A., Muratore, G., Mauromicale, G., Restuccia, C. (2018h). Effect of *Cynara cardunculus* extract on the shelf life of aubergine burgers. *Ital J Food Sci 1*, 9-23

7.1 Antimicrobial activity of cultivated cardoon (*Cynara cardunculus* L. var. *altilis* DC.) leaf extracts against bacterial species of agricultural and food interest

7.1.1 Introduction

Management of microorganisms, which can cause losses throughout the food chain or food-borne illness, by the use of natural antimicrobials have proved to be reliable alternative to chemical fungicides and bactericides (Akhtar *et al*., 2015; Aloui *et al*., 2014; Hintz *et al*., 2015; Kharchoufi *et al*., 2018a, b). The natural antimicrobial compounds occur mainly in plant kingdom as secondary metabolites, which play an important role against biotic and abiotic stressors

(Balasundram *et al*., 2006). The main secondary metabolites are the polyphenol compounds such as caffeoylquinic acids and flavonoids and the terpenoids, including sesquiterpene lactones, which have been shown a significant potential for insect control (Isman, 2006). Many of these secondary metabolites can act as herbicides, fungicides, bactericides and could be useful for disease control in agriculture (Dayan *et al*., 2009). The allelochemicals are in particular of sporadic occurrence and do not play an obvious role in the basic metabolism of organism, but serve for defensive adaptation (Scavo *et al*., 2018a). Therefore, the interest towards plant extracts, especially from vegetable byproducts or from non-food crops, has greatly increased in recent years (Gyawali and Ibrahim, 2014), both to bypass and even to reduce bacterial resistance to the antibiotics, probably by inducing some structural change in the resistant bacteria (Palaniappan and Holley, 2010).

The residues of *Cynara cardunculus* L., namely leaf and stem, represent 80-85% of the above ground biomass, and have a high content of flavones, caffeoylquinic acids and sesquiterpene lactones such as cynaropicrin, aguerin B and grosheimin (Llorach *et al*., 2002; Lombardo *et al*., 2018; Pandino *et al.,* 2011; Rial *et al*., 2014). *C. cardunculus* antimicrobial activity has been mainly reported for leaf extracts of globe artichoke (El Sohaimy, 2014; Mossi and Echeverrigaray, 1999; Zhu *et al*., 2004), while to the best of our knowledge the antimicrobial potential of cultivated cardoon leaf extracts is still poorly investigated (Falleh *et al*., 2008). Koubaa *et al*. (1999) reported that chlorogenic acid, cynarin, luteolin-7-*O*-rutinoside, and cymaroside, isolated by *C. cardunculus*, exhibited a relatively higher activity than

other compounds and were more effective against fungi than bacteria. The qualitative and quantitative profile of secondary metabolites in *C. cardunculus* is strictly influenced by the genetic background (Pandino *et al*., 2012a). In addition, cultivated cardoon produces a higher amount of aboveground dry biomass respect to globe artichoke and wild cardoon (Foti *et al*., 1999).

Since inexpensive and environmentally-sustainable recovery methods of bioactive compounds from plant materials should be preferred, especially for their use as food supplement/additive, the design of a suitable extraction method that permits to obtain food-grade extracts at acceptable yields, while safeguarding the precious bioactive features of *C. cardunculus* leaf components, becomes of substantial importance.

Therefore, this work aimed to evaluate *in vitro* the antibacterial activity of different extracts from cultivated cardoon leaves against several Gram positive and negative bacterial species of agriculture and food interest, as well as to investigate the correlation of the antimicrobial potential with the polyphenolic profile of the extracts.

7.1.2 Material and methods

7.1.2.1 Sampling of plant material and preparation of leaf extracts

Leaves were randomly sampled from sixty plants of 'Altilis 41' cultivated cardoon, selected by the University of Catania within a breeding program on *C. cardunculus*, at the 25th visible healthy leaf growth stage. This synthetic line is characterized by a considerable biomass production with a high incidence of the leaf blade respect to the central rib. Each

plot consisted of 30 plants, spaced 0.8 m apart with an inter-row spacing of 1.25 m. Crop management (fertilization, irrigation, weed and pest control) was performed according to standard commercial practices. In the laboratory, approximately 4 kg of randomly sampled fresh leaves were washed, cut, ground and air dried in an oven at 45 °C up to constant weight.

For extract preparation, we soaked dried leaves in bidistilled water, methanol (70%) and ethanol (80%) in the ratio 1:10 w/v. Then, the mixtures were kept under dark conditions for 72 hours at room temperature (20°C ± 1) and filtered through filter paper (Whatman No.2) to eliminate the solid fraction. The MeOH and EtOH solutions were evaporated at 35 °C by use of rotary evaporator (Laborata 4000, Heidolph, Germany) and the residue was dissolved in bidistilled water in order to maintain the same ratio. Yields, expressed as [(g extract/ g dried leaves)·100], were 13.7% for water, 23.1% for MeOH and 17.2% for EtOH.

7.1.2.2 Identification and quantification of compounds

7.1.2.2.1 Reagents and solvents

Reagents and solvents were purchased from VWR (Leighton Buzzard, UK) and were of analytical or HPLC grade. Apigenin 7-*O*-glucoside, apigenin, luteolin 7-*O*-glucoside, luteolin, 5-*O*-caffeoylquinic acid (chlorogenic acid), cynaropicrin were obtained from Extrasynthese (Lyon, France), cynarin (1,3-di-*O*-caffeoylquinic acid) was from Roth (Karlsruhe, Germany). Milli-Q system (Millipore Corp., Bedford, MA) ultrapure water was used throughout the experimental study.

7.1.2.2.2 HPLC analysis

Each extract (20 µL) was analysed using a series 1200 HPLC (Agilent Technologies, Palo Alto, USA) equipped with ChemStation software (version: B.03.01). Separations were achieved on a Zorbax Eclipse XDB-C_{18} (4.6 × 50 mm; 1.8 lm particle size), operated at 30°C, with a 0.2 µm stainless steel in-line filter.

For caffeoylquinic acids and flavones, the mobile phase was 1% formic acid in water (solvent A) and in acetonitrile (solvent B) at a flow rate of 0.5 mL/min. The gradient started with 5% B to reach 10% B at 5 min, 40% B at 20 min, 90% B at 25 min, 90% B at 29 min (Lombardo *et al*., 2015). For the cynaropicrin, it was adapted the method proposed by Menin *et al*. (2012). The mobile phase was 0.1% formic acid in water (solvent A) and in acetonitrile (solvent B) at a flow rate of 0.3 mL min^{-1}. The gradient started with 5% B to reach 10% B at 10 min, 30% B at 25 min, 40% at 30 min. Chromatograms were recorded at 280, 310 and 350 nm from diode array data collected between 200 and 600 nm.

Identification of single compounds was by their retention times, both UV and MS spectra, and by data available in the literature (Schütz *et al*., 2004; Wang *et al*., 2003). Quantification was performed using a calibration curve of the available standards. In particular, mono- and dicaffeoylquinic acids were calculated using chlorogenic acid and cynarin as references, respectively. Apigenin and luteolin conjugates were quantified as apigenin-7-*O*-glucoside and luteolin-7-*O*-glucoside, respectively. The analyses were carried out in triplicate (n=3) and the data, expressed as mg L^{-1}, are mean values ± standard error.

7.1.2.3 Target microorganisms

The antibacterial activity of cultivated cardoon leaf extracts was evaluated *in vitro* against the species *Bacillus megaterium*, *Bacillus subtilis*, *Bacillus cereus*, *Rhodococcus fascians*, *Listeria innocua*, *Staphylococcus aureus* (Gram positive) and *Xanthomonas perforans*, *Pseudomonas syringae* pv. tomato, *Escherichia coli*, *Salmonella enterica*, *Pseudomonas fluorescens* (Gram negative), selected within the microbial collection of Di3A (University of Catania).
The bacterial cultures were routinely maintained in Nutrient Agar (NA; CM0003, Oxoid, Basingstoke, UK) at 4 °C.

7.1.2.4 Determination of the antibacterial activity

The cardoon extracts were tested for the antibacterial activity on 24-h cultures of the target bacterial strains, at a concentration of 10^8 cells mL^{-1}, by the agar well diffusion method (Kharchoufi *et al*., 2018a). The assayed extract concentrations, representing 80 and 40% extract dilutions in sterile distilled water and expressed as mg total polyphenols mL^{-1}, were: 0.278 and 0.139 for W extract; 1.057 and 0.5285 for EtOH extract; 1.473 and 0.7365 for MeOH extract. Sterile distilled water was used as negative control. Plates were incubated at 30 °C, except for *P. syringae* and *X. perforans* that were incubated at 26 °C, until detectable bacterial growth in the control plates. The antibacterial activity was expressed as the width (cm) of the growth inhibition haloes around the well, produced by the extracts. The experiment was performed in triplicate.

7.1.2.5 Statistical analysis

All data were submitted to the Bartlett's Test for the homogeneity of variance and then were subjected to analysis of variance (ANOVA). Means were compared on the basis of the Student- Neuman-Keuls test only when the *F* test of the ANOVA for treatments and interaction was significant at the 0.05 probability level. A factorial three-way ANOVA model was used to analyse the data, with extraction solvent, extract concentration and bacterial species as main factors. For antibacterial activity, a two-way ANOVA (extraction solvent x extract concentration) was applied.

7.1.3 Results and discussion

7.1.3.1 Identification and quantification of extracted compounds

The qualitative and quantitative profile of the extracts changes in relation to the extracting solvent (**Table 5.11**). Eleven compounds were identified belonging to caffeoylquinic acids, flavones and sesquiterpene lactones. In particular, the main compounds were flavones such as apigenin and luteolin and their derivatives. Among detected caffeoylquinic acids, the monosuccinildicaffeoylquinic acid was found in contrast with Pandino *et al*. (2015). This discrepance could be related to the different age of leaves, since in this work we considered mature ones. The highest total measured polyphenols, as sum of identified compounds, was found in the MeOH extract (1253 mg L^{-1}), followed by the EtOH (1046 mg L^{-1}) and the W (272 mg L^{-1}) one. In accordance to a previous work (Pandino *et al*., 2011), the main compounds detected were 5-*O* caffeoylquinic and 1,5-*O*-dicaffeoylquinic acids. In particular, the concentration of

5-*O*-caffeoylquinic was higher in MeOH extract (632.0 mg L^{-1}) than both W and EtOH. On the contrary, the EtOH extract reported the highest 1,5-*O*-dicaffeoylquinic acid. In terms of flavones, we observed a profile similar to previous works (Pandino *et al*., 2015; Pinelli *et al*., 2007). In this work, the level of luteolin and its derivatives resulted higher than that of apigenin and its derivatives, in accordance to Pandino *et al*. (2013). The luteolin 7-*O*-glucuronide represented the major abundant luteolin derivative compound reaching 1894 mg L^{-1} in EtOH extract. Nevertheless, the major total content of luteolin compounds was recorded in EtOH extract (240 mg L^{-1}). Luteolin derivatives, however, were very poor in aqueous extract, while the apigenin derivatives were not found. Besides, the highest cynaropicrin content was found in the MeOH extract (15.8 mg L^{-1}), followed by EtOH (10.7 mg L^{-1}) and W (5.4 mg L^{-1}).

7.1.3.2 Antibacterial activity

The antibacterial properties of different extracts of cultivated cardoon leaves were assayed against Gram positive and negative strains. **Table 7.1** reports sizes of inhibition zones (clear zones around wells) produced by each extract towards targeted bacteria.

Cultivated cardoon extracts exhibited antagonistic activity against Gram positive and negative bacteria, although extraction solvent, extract concentration and bacterial species strongly affected the *in vitro* efficacy (**Table 7.2**). Extract concentration contributed more than the choice of the solvent on the extract antimicrobial activity in all bacterial species, except for *P. fluorescens*. In particular, extract concentration principally affected *R. fascians* (*F*=2160) and *B. megaterium*

(*F*=1936), while the highest extracting solvent values were found in *X. perforans* (*F*=543) and *P. syringae* (*F*=259). Moreover, the interaction 'solvent × extract concentration' was highly significant in all bacterial species, except for *B. cereus* and *S. aureus*. With regards to Gram positive bacteria, all the tested species were inhibited by the W extract at 0.278 mg total polyphenols mL^{-1}, with the exception of *B. subtilis* that was unaffected by the extract. The inhibition halo size varied from a minimum of 0.6 cm for *R. fascians* to a maximum of 0.8 cm for *B. megaterium* and *L. innocua* species (**Table 7.1**). No inhibition halo was observed for the tested Gram positive species when the W extract was applied at 0.139 mg total polyphenols mL^{-1}. MeOH and EtOH at the highest concentrations (1.473 and 1.057 mg total polyphenols mL^{-1}, respectively) were able to more effectively control the growth of all Gram positive species; for the methanol one the recorded inhibition haloes ranged from 0.8 cm for *B. subtilis* to 1.3 cm for *B. megaterium* and *B. cereus*, and for the ethanol one they varied from a minimum of 0.4 cm for *B. subtilis* to a maximum of 2.3 cm for *B. megaterium*.

When tested at the lowest concentrations, only MeOH and EtOH exerted an appreciable antibacterial activity (**Table 7.1**).

A different behaviour was observed for Gram negative bacterial species, in respect of which the spectrum of activity of the tested extracts was narrower. In particular, W extract showed antibacterial activity only against *P. syringae* and *X. perforans* (**Table 7.1**), when tested at the highest concentration. MeOH extract was not effective, even at the highest concentration, while EtOH extract showed detectable antibacterial activity both at 1.057 and 0.5285 mg total

polyphenols mL^{-1}. More specifically, EtOH extract at the highest concentration inhibited the growth of *P. syringae*, *X. perforans* and *P. fluorescens*, with inhibition haloes from 0.4 cm for *X. perforans* to 0.7 cm for *P. fluorescens*; EtOH extract at 0.5285 mg total polyphenols mL^{-1} was the only extract able to inhibit the growth of *P. fluorescens*, although with weaker efficacy (**Table 7.1**).

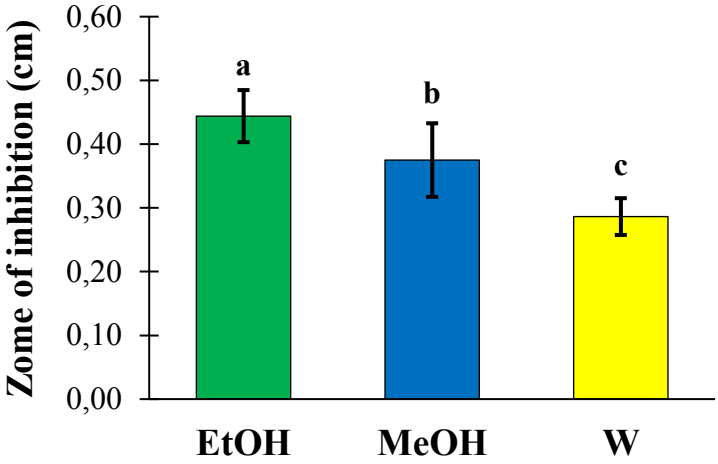


Figure 7.1 Zone of inhibition (cm) produced by cultivated cardoon extract in relation to the solvent (average of eleven bacteria). W: water extracts; MeOH: methanolic extracts; EtOH: ethanolic extracts.
Each bar means ± standard error. Different letters indicate statistical significance for $P \leq 0.05$.

Table 7.1 Antibacterial activity of cultivated cardoon leaf extracts.

	Zones of inhibition (cm)[*, **, ***]							
	Solvent (80%, v/v)				**Solvent 40%**			
Microorganisms	**W**	**MeOH**	**EtOH**	**Mean**	**W**	**MeOH**	**EtOH**	**Mean**
Bacillus cereus	0.7	1.3 ± 0.1	0.9 ± 0.1	**1**[b]	–	0.4 ± 0.1	0.2	**0.3**[a]
Bacillus megaterium	0.8 ± 0.1	1.3 ± 0.1	2.3 ± 0.1	**1.5**[a]	–	–	–	**–**
Bacillus subtilis	–	0.8	0.4 ± 0.1	**0.6**[c]	–	0.2 ± 0.1	0.1	**0.1**[b]
Escherichia coli	–	–	–	**–**	–	–	–	**–**
Listeria innocua	0.8	1.2	0.8 ± 0.1	**0.9**[b]	–	0.5	0.3	**0.4**[a]
Pseudomonas fluorescens	–	–	0.7 ± 0.1	**0.7**[c]	–	–	0.3 ± 0.1	**0.3**[a]
Pseudomonas syringae pv. *tomato*	1.2	–	0.6	**0.9**[b]	–	–	–	**–**
Rhodococcus fascians	0.6	1.2	1.2 ± 0.1	**1**[b]	–	–	–	**–**
Salmonella typhi	–	–	–	**–**	–	–	–	**–**
Staphylococcus aureus	0.7	1 ± 0.1	1.1 ± 0.1	**0.9**[b]	–	0.3 ± 0.1	0.5	**0.4**[a]
Xanthomonas perforans	1.5 ± 0.1	–	0.4	**0.9**[b]	–	–	–	**–**
Mean	**0.9**[b]	**1.1**[a]	**0.9**[b]		**–**	**0.3**[a]	**0.3**[a]	
LSD Interaction ($P \leq 0.05$)	0.1				0.07			

[*]Values, including diameter of the disk (6.3 cm), are the mean of 3 replicates and are given as means ± standard error. Different letters indicate statistical significance for $P \leq 0.05$.

[**]100 μL of extract was applied to each disk

[***]W: bidistilled water extracts; MeOH: methanolic extracts; EtOH: ethanolic extracts; –no inhibition zone

Table 7.2 *F*–values of main factors and their interactions resulting from analysis of variance in bacterial species.

Source of variation	df	*Bacillus cereus*	*Bacillus megaterium*	*Bacillus subtilis*	*Listeria innocua*	*Pseudomonas fluorescens*	*Pseudomonas syringae*
Solvent (S)	2	76^{***}	175^{***}	52^{***}	141^{***}	121^{***}	259^{***}
Extract concentration (C)	1	529^{***}	1936^{***}	52^{**}	840^{***}	37^{***}	778^{***}
(S) x (C)	2	4^{*}	175^{***}	18^{***}	15^{***}	37^{***}	259^{***}

Note: Values are given as *F* of Fisher. df: degrees of freedrom. NS: not significant; *** and * indicate significant at $P \leq 0.001$ and $P \leq 0.05$, respectively.

Table 7.2 (Continued)

Source of variation	df	*Rhodococcus fascians*	*Staphylococcus aureus*	*Xanthomonas perforans*
Solvent (S)	2	86^{***}	56^{***}	543^{***}
Extract concentration (C)	1	2160^{***}	324^{***}	1083^{**}
(S) x (C)	2	86^{***}	NS	543^{***}

Note: Values are given as *F* of Fisher. df: degrees of freedrom. NS: not significant; *** and * indicate significant at $P \leq 0.001$ and $P \leq 0.05$, respectively.

Regardless of the bacterial species and extract concentration, EtOH extract was significantly the most effective solvent against targeted bacteria, producing average inhibition haloes of 0.44 cm, followed by MeOH and W extracts (**Figure 7.1**). In addition, the highest concentrations of tested extracts revealed a significant efficacy (average inhibition haloes of 0.65 cm) when compared to the lowest concentrations (**Figure 7.2**).

The above-mentioned results are partially in accordance with the relatively few studies that are currently available. Falleh *et al.* (2008) compared phenolic contents and antibacterial activity of *C. cardunculus* L. leaf, seed and flower methanolic extracts; although the leaf and seed extracts had similar polyphenol content, only the leaf extract was more effective against Gram positive bacteria, mainly against *Staphylococcus epidermidis*, *S. aureus*, and *Micrococcus luteus*; the extract showed antibacterial activity also against *E. coli*, but not against *Salmonella thyphymurium*. Kukić *et al.* (2008) studied the antimicrobial properties of a residual water extract, obtained from involucral bracts of *C. cardunculus*, after extraction with EtOH and successive partition with $CHCl_3$, EtOAc and *n*-BuOH. The results showed that the EtOAc extract was the most effective, followed by the EtOH, $CHCl_3$, water and *n*-BuOH extracts; among pure compounds, previously isolated from *C. cardunculus* involucral bracts, luteolin possessed the highest antimicrobial activity. In the same study, the most resistant bacterial species was *S. typhimurium*, while *E. coli* was the most sensitive. With reference to the globe artichoke, Mossi and Echeverrigaray (1999) found that dichloromethane leaf extract totally inhibited the growth of Gram positive species

S. aureus, *B. subtilis* and *B. cereus*. Comparable results were also previously obtained with compounds isolated from globe artichoke leaves: chlorogenic acid, cynarin, luteolin 7-rutinoside and cynaroside, among bioactive isolated compounds, exhibited higher activities than other compounds and were more active against fungi than against bacteria (Zhu *et al*., 2004). Antimicrobial activity of apigenin, apigenin 7-glucoside, luteolin and other flavones has also previously been reported (Aljančić *et al*., 1999; Tshikalange *et al*., 2005).

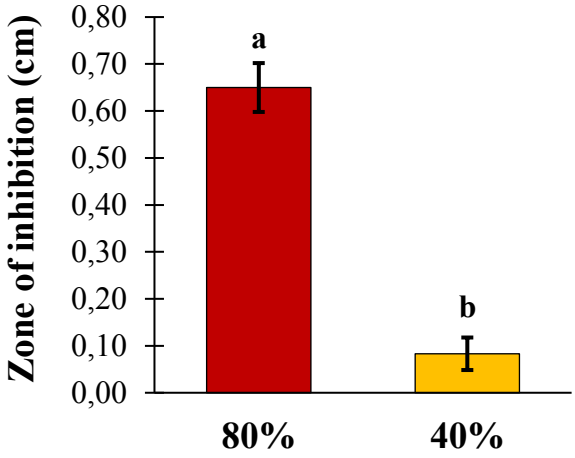


Figure 7.2 Zone of inhibition (cm) produced by cultivated cardoon extract in relation to the concentration (average of eleven bacteria). 80 and 40% refer to extract dilution.
Each bar means ± standard error. Different letters indicate statistical significance for $P \leq 0.05$.

Various workers have already shown that Gram positive bacteria are more sensitive to plant extracts as compared to

Gram negative bacteria. This different behaviour may be due to several possible factors, such as permeability barrier provided by the outer membrane in Gram negative bacteria or the presence in periplasmic space of enzymes that are able to break down molecules introduced from outside (Marasini *et al*., 2015; Parekh and Chanda, 2007).

The antibacterial activity of cultivated cardoon leaves may be related to the high level of phenolic components. Also, triterpenes and sesquiterpene lactones such as grosheimin, aguerin B and cynaropicrin, which is the most abundant lactone in *C. cardunculus* leaves, are reported to show antimicrobial activity (Kukić *et al*., 2008; Schinor *et al*., 2004; Yayli *et al*., 2009). Cynaropicrin, detected at higher concentrations in EtOH and MeOH extracts, respect to W one, has been defined as potent and irreversible inhibitor of the bacterial enzyme MurA, which is involved in the cytoplasmic biosynthesis of peptidoglycan precursors (Elsebai *et al*., 2016). Bachelier *et al.* (2006) firstly explained the antibacterial mode of action of sesquiterpene lactones on *Escherichia coli*; cynaropicrin forms covalent bonds with the thiol group of Cys115, through

Michael addition reaction. Referring to the structure-activity relationships with other investigated sesquiterpene lactones, the unsaturated ester side chain of cynaropicrin is particularly important for the inhibition of MurA, since it mimics the substrate phosphoenolpyruvate (PEP) (Bachelier *et al*., 2006).

The flavonoid apigenin, detected only in EtOH extract, has been demonstrated to possess antibacterial activity (Nayaka *et al*., 2014). Apigenin has been also validated as a natural antibiotic against quinolone-resistant bacteria (Morimoto *et*

al., 2015). Previous studies also showed the antibacterial activity of luteolin 7-*O*-glucoside, detected at the highest concentration in EtOH extract, in general more effective against Gram positive bacteria (Akroum *et al*., 2010); in addition, luteolin 7-*O*-glucoside was identified in *Lycopus europaeus* extract as one of the compounds responsible for antibacterial activity against clinical *S. aureus* strains (Fialová *et al*., 2015). Finally, caffeoylquinic acids, mainly found in cultivated cardoon EtOH extract followed by MeOH one, were previously identified from *Artemisia absinthium* as the antimicrobial compounds responsible for the growth inhibition of Gram positive pathogenic bacteria (Fiamegos *et al.*, 2011).

7.1.4 Conclusions

In this study, the *in vitro* antibacterial activity of cultivated cardoon leaf extracts was demonstrated on different Gram positive and negative bacterial species of agriculture and food interest. The obtained results suggest how the antibacterial activity of cultivated cardoon extracts varies among the extracting solvents with a major efficacy of EtOH, followed by MeOH and W extracts. However, the amount of polyphenols and cynaropicrin is not directly correlated with the higher antibacterial activity. Probably, the inhibitory activity of the tested leaf extracts is linked to the synergism of active compounds. According to our results, cultivated cardoon leaf could be a good candidate for the future development of a biobactericide for agriculture control and food preservation.

7.2 Effect of *Cynara cardunculus* L. extract on the shelf life of aburgine burgers

7.2.1 Introduction

Red meats are a rich source of animal fats that contain high amount of triglycerides, of saturated fatty acids as well as cholesterol. Recently, vegan substitutes for animal-based foods have become available; in particular, alternatives to cow's milk, including soy, almond and rice drinks. There are also yogurt, cheese, chicken and red meat vegan substitutes made of soy and/or other vegetable-based ingredients. Many of these products are made to resemble specific animal-based foods (McIlveen *et al.,* 1999). In the last years, the demand of consumers for veggie burgers has increased rapidly: in this context, vegetable burgers are convenient processed food products exclusively prepared from nonmeat ingredients (Adise *et al.,* 2015). Also, the demand for non-synthetic preservatives is increasing worldwide, such as antimicrobial compounds of natural origin, which should be not toxic for humans, environmentally safe, inexpensive and available in the market (Mohanka and Priyanka, 2014). There is growing interest in using natural antimicrobial compounds, especially extract from plants, for the preservation of foods. Among these, *Cynara cardunculus* L. leaves, characterized by a considerable presence of bioactive compounds, is widely recognized for medical purpose, and the potential use of their extracts to control the growth of food pathogenic and/or spoilage microorganisms is at the beginning of investigation (Zhu *et al.,* 2004).

For vegetable burgers, the cooking process should significantly reduce the number of vegetative microbial cells

and inactivate degradative enzymes. Consequently, spoilage of these products is primarily due to post-cooking contamination by microorganisms, whichcan be minimized by good hygiene and handling. The use of vacuum and long-term storage at refrigerated temperatures may promote the growth of psychrotrophic anaerobic/facultative anaerobic bacteria and yeasts allowing them to become dominantand deteriorate the product.
This research was intended to improve the sensory and microbiological shelf life of aubergine-based burgers with the addition of *Cynara cardunculus* extract characterized by a considerable presence of bioactive compounds.

7.2.2 Material and methods

Two concentrations of extract were used (1% and 3%) for the preparation of auberginebased burgers, indicated as Burger 1 and Burger 3, respectively. Burger samples prepared without *Cynara cardunculus* extract were taken as control (Burger C). Microbial load and sensory changes of vacuum-packed aubergine-based burgers were analyzed at the processing day (t0), after 30 (t1) and 105 days (t2) of cold storage (4±1°C).

7.2.2.1 Preparation of extract

The *Cynara cardunculus* extract was prepared according to Pandino *et al*. (2013).

7.2.2.2 Preparation of aubergine-based burgers

The following ingredients were added to prepare burgers for boiled and shredded eggplants: potatoes, onions, parsley, garlic, black pepper, nutmeg, dried tomato, thyme, salt, sugar, flour and maize starch, according to a consolidated industrial

receipt (Terranèo Emozioni Siciliane srl, Vittoria, RG). The mixture obtained was given the form of 100g burgers, which were subsequently fried in sunflower oil for 40 seconds, cooled, vacuumpacked and refrigerated at 4°C.

7.2.2.3 Sensory analysis

The sensory profile method UNI 10957 (2003) was used to measure any change in the sensory characters of samples, as a result of *Cynara cardunculus* extract treatment. Twelve judges were trained (ISO 8586, 2012) in 4 sessions to familiarize with scales and procedures. The evaluation sessions were conducted in a sensory laboratory (UNI EN ISO 8589, 2010) from 11:00 a.m. to 12:00 a.m. in individual booths illuminated with white light. Randomized samples were evaluated by assigning a score between 1 (absence of sensation) and 9 (extremely intense), using five attributes (colour, firmness, off-odour, offflavour and an overall assessment, expressed by considering all of the attributes). All data were acquired by a direct computerized registration system (FIZZ Byosistemes. ver. 2.00 M, Couternon, France). The sensory data for each attribute were submitted to one-way ANOVA by the software package Statgraphics® Centurion XVI (Statpoint Technologies, INC.) using samples as factors. The significance was tested by means of the F-test. To differentiate the samples, the mean values were submitted to the multiple comparison test using the least significant difference (LSD) procedure.

7.2.2.4 Microbiological analysis

Total mesophilic and psychrotrophic bacterial counts, yeast count were determined on the samples. An aliquot (10 g) of

burger was sterilely sampled from each package and homogenized with 90 mL of sterile physiologic solution in a Stomacher (Lab-Blender 400, Brinkmann, Westbury, NY, USA) for 30 s. The same diluent was used for subsequent decimal dilutions. The total mesophilic and psychrotrophic bacteria counts were performed on Plate Count Agar (PCA, Oxoid Ltd., Basingstoke, UK) with cycloheximide0.1% solution (Oxoid), incubated, respectively, at 32°C for 24-48 h and at 4°C for 10 d; yeast count was carried out on Sabouraud Dextrose Agar (SDA, Oxoid) supplementedwith chloramphenicol (0.1 g/L) incubated at 25°C for 48-72 h.
The microbiological counts, performed in triplicate, were expressed as log10 CFU/g of burger.

7.2.3 Results and discussion

7.2.3.1 Sensory analysis

Table 7.3 reports sensory attributes that significantly differentiated the burger samples during storage. The intensity (mean score) was reported only for the significantly different attributes.
All the attributes except for off-odour significantly differentiated the control burgers. At 105 days of storage there was a decrease of significative attributes. The attributes colour, firmness and overall significantly differentiated the Burger 1, while only the attribute colour and overall were significantly different for the Burger 3. Samples with 3% of extract had the highest intensity of overall score compared to the other samples at t1 and t2.

Table 7.3 Mean scores of the significant sensory attributes.

Sample	Attribute	t0	t1	t2
Burger C	Colour	5.50±1.73^{b*}	4.87±1.17^{b}	3.75±0.48^{a}
	Firmeness	5.87±1.69^{b}	5.25±0.83^{b}	3.50±1.50^{a}
	Off-flavour	5.12±2.03^{b}	4.87±1.90^{b}	2.87±0.78^{a}
	Overall	6.12±1.05^{b}	5.75±1.30^{b}	3.50±1.00^{a}
Burger 1	Colour	6.50±1.41^{b}	4.87±1.05^{a}	3.62±1.41^{a}
	Firmeness	6.50±1.58^{b}	5.12±0.78^{a}	3.87±1.05^{a}
	Overall	6.50±1.00^{c}	5.12±0.93^{b}	3.87±1.05^{a}
Burger 3	Colour	6.00±1.32^{b}	5.25±0.97^{ab}	4.12±1.27^{a}
	Overall	8.00±1.39^{b}	6.00±1.00^{a}	6.00±0.99^{a}

*Values marked with different letters in the same row are significantly different ($p \leq 0.05$) according to the LSD multiple comparison test.

7.2.3.2 Microbiological analysis

Mesophilic bacterial counts of control burger (Burger C), burger supplemented with 1% extract (Burger 1) and with 3% extract (Burger 3) are shown in **Table 7.4**. Psychrotrophic bacteria and yeasts were not detectable in any of the analyzed samples over the considered shelf life period.

The addition of *C. cardunculus* extract at 3% (v/w) completely inhibited the growth of mesophilic bacteria up to 30 d of refrigerated shelf life; in addition, the antibacterial effect of the extract persisted up to 105 d, since it significantly reduced mesophilic count by more than 1 log cfu/g, with respect to the unsupplemented burger sample

7.2.4 Conclusions

The present study showed the possibility of preparing a vegetable product without chemical preservatives. The addition of *Cynara cardunculus* extract has improved the sensory characteristics ensuring at the same time the microbiological stability of the product. As an additional

advantage, the use of *C. cardunculus* extract allows to improve the nutritional quality of the product, with special regards for the antioxidant potential.

Table 7.4 Microbial counts of different burger samples throughout the refrigerated storage.

	t0	**t1**	**t2**
		log CFU/g	
Mesophillic bacteria			
Burger C	2.53	3.36	2.91
Burger 1	1.95	2.59	2.66
Burger 3	nd	nd	1.85

nd: not detectable (below the detection limit of plate count technique)

Concluding remarks

The increasing demand for eco-friendly agricultural practices for weed and pest control, has led the scientific community to more investigate the utilization of allelopathic mechanisms and their integration into traditional agricultural practices under Integrated Pest and Weed Management System (IPMS, IWMS). Results from the present PhD research project clearly demonstrated the allelopathic activity of *C. cardunculus* extracts on weeds, plant pathogens and microorganisms of food interest as a chemical-free alternative management. Initially, *C. cardunculus* leaf aqueous extracts significantly decreased seed germination and mean germination time of six common weeds in Mediterranean agroecosystems. Then, the best methodology for allelochemicals extraction was carried out. Overall, dried leaves showed the most valid leaf material for the potential production of bioherbicides, both under economically aspect and extraction yield, while solvent's efficiency was ethanol > methanol > bidistilled water. Nevertheless, ethanolic and direct ethyl acetate from leaves were the most active extracts both on wheat coleoptile bioassay and weed germination and growth. Particularly, ethyl acetate extract was the most promising extract in view of its activities, even with the dilutions, followed by ethanol. HPLC and UHPLC-MS/MS analysis revealed that *C. cardunculus* leaf allelochemicals principally belongs to two different chemical classes: sesquiterpene lactones such as cynaropicrin, grosheimin and aguerin B on one side, and polyphenols such as caffeoylquinic acids and flavones on the other. Moreover, the sesquiterpene lactones cynaratriol, desacylcynaropicrin and 11,13-dihydro-desacylcynaropicrin were here reported in

cultivated cardoon for the first time. Cynaropicrin, desacylcynaropicrin and pinoresinol showed the highest inhibitory activity on wheat coleoptile elongation, suggesting their influence on cultivated cardoon allelopathic potential, while cynaratriol was inactive. In addition to extract concentration, both genotype, harvest time and light stress condition were found to affect sesquiterpene lactone amounts and profiles in *C. cardunculus* leaf extracts. The April harvest showed the highest concentration, with cynaropicrin resulting the most abundant sesquiterpene lactone in all genotypes. Cultivated cardoon 'Valparaiso' was the best genotype in terms of sesquiterpene lactone concentration, profiles and phytotoxic activity, while globe artichoke was the worst botanical variety. Furthermore, the introduction of globe artichoke, cultivated and wild cardoon within a crop rotation offered an important indirect method for weed control since it decreased (from -34 to -50%) the weed soil seed bank in two different areas. Besides, as revealed by denaturing gradient gel electrophoresis, the presence of cultivated cardoon had a negative influence towards the negative soil bacterium *Bacillus subtilis* and a positive effect on the positive bacteria *Pseudomonas putida* and *Azospirillum brasilense.* Finally, *C. cardunculus* leaf extracts showed a significant *in vitro* and *in vivo* antimicrobial activity on different Gram positive and negative bacterial species of agriculture and food interest.

As a whole, the results of this doctoral thesis encourage the advantages offered by *C. cardunculus* allelopathic properties for the future development of bioherbicides, biobactericides and biofungicides more respectful of environment, human and animal health.

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