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Herbicidal activity of Mediterranean essential oils and their effects on soil bioindicators

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Dedications

Every challenging work needs self-efforts as well as guidance of elders especially those who were very close to my heart

my humble effort I dedicate to my sweet and

loving guardian angels from family and

friends whose affection, love,

encouragement and prays of

day and night make me

able to get such

success and to

reach this

stage of

my

life

Amira Jouini

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Abstract

Environmental and health concerns caused for traditional crop protection systems have stimulated interest in alternative weed management strategies. Worldwide, efforts are being made to reduce the heavy reliance on synthetic herbicides that are used to control weeds. Natural herbicides based on allelopathic substances, such as volatile essential oils (EOs) extracted from plants, has been suggested to be one of the possible alternatives for achieving sustainable weed management. From one hand, EOs have shown ability to inhibit weeds seed germination and growth, on the other hand there is a lack of studies about the effects of such substances on soil microorganisms.

Therefore, in this thesis the phytotoxic and herbicidal activities of EOs extracted from Mediterranean plants were investigated for their potential use as natural herbicides in a sustainable weed management context. In addition, the effects of EOs, as well as, of other plant extracts, such as hydrolates, aqueous extracts and fresh leaves obtained from Mediterranean plants, were tested on soil microorganisms.

The donor species of EOs were selected based on previous experience of the research group and according to the current literature about the herbicidal activities of the secondary metabolites of these species or from species that are taxonomically closely related: *Eucalyptus camaldulensis* Dehnh., *Eucalyptus occidentalis* Endl., *Eucalyptus globulus* Labill., *Eucalyptus torquata* Luehm., *Eucalyptus lesoufii* Maiden, *Thymbra capitata* (L.) Cav., *Mentha* × *piperita* L. and *Santolina chamaecyparissus* L. The target weeds were two monocotyledons, *Avena fatua* L. and *Echinochloa crus-galli* (L.) P. Beauv, and two dicotyledons, *Portulaca oleracea* L. and *Amaranthus retroflexus* L., all them important weeds in Mediterranean crops.

EOs composition was analyzed by means of Gas Chromatography (GC) and Gas Chromatography-Mass Spectrometry (GC-MS). The in vitro assays were performed in germination chambers, to assess the effects of EOs on weed seed germination and seedling growth. The in vivo trials were conducted in greenhouse conditions, where EOs emulsified by Fitoil were applied on weed species by watering. Effects on weeds were evaluated by measuring these plant parameters variables: root, aerial parts, and total length of the plants, fresh and dry weight, efficacy of the treatments on each plant, and damage level. The study of EOs effects on soil microorganisms was carried out in a laboratory pot experiment, where soils were treated with EOs and leaf extracts and then incubated at room temperature (20-23°C).

Effects on soil microorganisms were measured by determining the main biochemical properties such as microbial biomass carbon and nitrogen, microbial respiration, and the relative abundance of the main microbial groups.

Results obtained from the *in vitro* bioassays revealed that all used EOs displayed of effectiveness against assayed weeds, controlling completely their germination process or reducing it and significantly inhibiting their seedling growth. Among them, *T. capitata* was the most effective. At lower doses, it blocked completely the seed germination of *A. retroflexus*, *P. olecerea, A. fatua* and *E. crus-galli*.

Greenhouse trials demonstrated herbicidal activity of *T. capitata*, *M. piperita* and *S. chamaecyparissus*, increasing their phytotoxicity with the dose. *T. capitata* was the most effective against all weeds at the maximum dose and *P. oleracea* was the most resistant weed. Soil microorganisms, after a transient upheaval period, induced by the addition of EOs, generally recovered their initial function and biomass. Only *T. capitata* EO at the highest dose did not allow soil microorganisms to completely recover their initial functionality.

Results of leaf extracts application on soil provided evidence that *Eucalyptus* leaves and their extracts (EOs, hydrolates and aqueous extracts), affected soil microbial community in different ways, and those effects were dependent on the *Eucalyptus* species.

So far, the results obtained make feasible to suggest EOs application as bio-herbicides in controlled environments, such as horticulture and in greenhouse conditions. However, the optimum dose of application must be determined, to control weeds and simultaneously, not negatively affect soil microorganisms. Nonetheless, further field research is necessary to completely understand the potential of EOs in field conditions, and it is required to develop an appropriate formulation to improve the persistence and penetrability of EOs and so increase their capacity to control weeds.

Resumen

Las preocupaciones ambientales y de salud causadas por los sistemas tradicionales de protección de cultivos han estimulado el interés en estrategias alternativas para el manejo de las malas hierbas. En todo el mundo se están haciendo esfuerzos para reducir la gran dependencia de los herbicidas sintéticos que se utilizan como principal método para el control de las plantas arvenses. Los herbicidas naturales basados en sustancias alelopáticas, como los aceites esenciales (AEs) extraídos de plantas, se han sugerido como una de las posibles alternativas para lograr un manejo sostenible de las arvenses. Por un lado, los AEs han mostrado capacidad para inhibir la germinación y el crecimiento de semillas de malas hierbas, por otro lado, hay una falta de estudios sobre los efectos de tales sustancias sobre los microorganismos del suelo.

Por lo tanto, en esta tesis se ha investigado la actividad fitotóxica y herbicida de los AEs extraídos de plantas mediterráneas para determinar su potencial como herbicidas naturales en un contexto de manejo sostenible de las malas hierbas. Además, se han ensayado los efectos de los aceites esenciales, así como de otros extractos de plantas como hidrolatos, extractos acuosos y hojas frescas obtenidas de plantas mediterráneas, sobre los microorganismos del suelo.

Las especies donadoras de AEs fueron seleccionadas en base a conocimientos previos del grupo de investigación, y de acuerdo con la bibliografía existente sobre la actividad herbicida de metabolitos secundarios de estas especies o de especies que están taxonómicamente estrechamente relacionadas: *Eucalyptus camaldulensis* Dehnh., *Eucalyptus occidentalis* Endl., *Eucalyptus globulus* Labill., *Eucalyptus torquata* Luehm., *Eucalyptus lesoufii* Maiden, *Thymbra capitata* (L.) Cav., *Mentha × piperita* L. y *Santolina chamaecyparissus* L. Como malas hierbas objetivo se seleccionaron dos monocotiledóneas, *Avena fatua* L. y *Echinochloa crus-galli* (L.) P. Beauv. y dos dicotiledóneas, *Portulaca oleracea* L. y *Amaranthus retroflexus* L., todas ellas importantes malas hierbas en cultivos Mediterráneos.

La composición de los AEs se analizó mediante Cromatografía de gases (CG) y Cromatografía de gases-Espectrometría de masas (CG-EM). Los ensayos in vitro se realizaron en cámaras de germinación, para evaluar los efectos fitotóxicos de los AEs sobre la germinación de las malas hierbas y el crecimiento de las plántulas. Los ensayos in vivo se realizaron en condiciones de

invernadero, los AEs se aplicaron emulsionados con Fitoil mediante riego. Los efectos sobre las malas hierbas se evaluaron midiendo las siguientes variables: longitud de la raíz, de la parte aérea y total de las plantas, peso fresco y seco, eficacia del aceite esencial en cada planta, y nivel de daño causado. El estudio de los efectos fitotóxicos de los AEs sobre los microorganismos del suelo se realizó en un experimento de laboratorio en macetas donde los suelos se trataron con los AEs y extractos de hojas y luego se incubaron a temperatura ambiente (20-23 ° C). Los efectos sobre los microorganismos del suelo se midieron determinando las principales propiedades bioquímicas como el carbono y nitrógeno de la biomasa microbiana, la respiración microbiana y la abundancia relativa de los principales grupos microbianos.

Los resultados obtenidos de los ensayos in vitro revelaron que todos los AEs mostraron efectividad contra las malas hierbas ensayadas, controlando completamente su germinación o reduciéndola e inhibiendo significativamente el crecimiento de las plántulas. Entre ellos, *T. capitata* fue el más eficaz. A las dosis más bajas, bloqueó completamente la germinación de semillas de *A. retroflexus*, *P. olecerea*, *A. fatua* y *E. crus-galli*.

Los ensayos en invernadero demostraron la actividad herbicida de *T. capitata*, *M. piperita* y *S. chamaecyparissus*, aumentando su fitotoxicidad con la dosis. *T. capitata* fue el AE más eficaz contra todas las malas hierbas a la dosis máxima y *P. oleracea* fue la especie más resistente. Los microorganismos del suelo, después de un período transitorio de agitación inducido por la adición de los AEs, generalmente recuperaron su función y biomasa iniciales. Solo el AE de *T.* capitata a la dosis más alta no permitió que los microorganismos del suelo recuperaran completamente su funcionalidad inicial.

Los resultados de la aplicación de extractos de hojas al suelo proporcionaron evidencia de que las hojas de eucalipto y sus extractos (AE, hidrolatos y extractos acuosos), afectaron a la comunidad microbiana del suelo de diferente modo, y sus efectos fueron dependientes de la especie de *Eucalyptus* considerada.

Hasta el momento, los resultados obtenidos permiten sugerir la aplicación de los Aes como bioherbicidas en entornos controlados, como en horticultura y en condiciones de invernadero. Se debe identificar la dosis óptima de aplicación para controlar las malas hierbas y simultáneamente, no afectar negativamente a los microorganismos del suelo. No obstante, se necesita más investigación de campo para determinar el potencial herbicidal de los AEs en condiciones de campo, y se debe desarrollar una formulación más apropidad para su aplicación,

de modo que se mejore su persistencia y penetrabilidad, y así se incremente su potencial para controlar las malas hierbas.

Resum

Les preocupacions ambientals i de salut causades pels sistemes tradicionals de protecció de cultius han estimulat l'interès per estratègies alternatives de gestió de les males herbes. A tot el món, s'estan fent esforços per reduir la gran dependència dels herbicides sintètics que s'utilitzen com a principal mètode per al control de les males herbes. Els herbicides naturals basats en substàncies al·lelopàtiques, com els olis essencials (OEs) extrets de plantes, s'han suggerit com una de les alternatives possibles per aconseguir una gestió sostenible de les males herbes. Per una banda, els OEs han mostrat capacitat per inhibir la germinació i el creixement de llavors de males herbes, però per altra banda, falten estudis sobre els efectes d'aquestes substàncies sobre els microorganismes del sòl.

Per tant, en aquesta tesi es van investigar les activitats fitotòxiques i herbicides dels OE extrets d'herbes mediterrànies per a un ús potencial com a herbicides naturals en un context de gestió sostenible de les males herbes. A més, es van provar els efectes d'aquests olis essencials i d'altres extractes vegetals, com hidrolats, extractes aquosos i fulles fresques obtingudes d'herbes mediterrànies sobre els microorganismes del sòl.

Les espècies donants d'OEs es van seleccionar bassant-se en la experiencia previa del grup d'investigació i en la bibliografía existent sobre les activitats biològiques dels metabòlits secundaris d'aquestes espècies o d'espècies taxonòmicament properes: *Eucalyptus camaldulensis* Dehnh., *Eucalyptus occidentalis* Endl., *Eucalyptus globulus* Labill., *Eucalyptus torquata* Luehm., *Eucalyptus lesoufii* Maiden, *Thymbra capitata* (L.) Cav., *Mentha* × *piperita* L. i *Santolina chamaecyparissus* L. Les males herbes objectiu seleccionades van ser dos monocotiledóneas, *Avena fatua* L. i *Echinochloa crus-galli* (L.) P. Beauv. i dos dicotiledóneas, *Portulaca oleracea* L. i *Amaranthus retroflexus* L., totes elles importants males herbes en cultius Mediterranis.

La composició dels OEs es va analitzar mitjançant Cromatografia de gasos (CG) i Cromatografia de gasos-espectrometria de mases (CG-EM). Els assajos in vitro es van realitzar en cambres de germinació per avaluar els efectes dels OEs sobre la germinació de les llavors de les males herbes i el creixement de les plàntules. Els assajos in vivo es van realitzar en condicions d'hivernacle, on es van aplicar per reg els OEs emulsionats amb Fitoil a les males herbes. Els efectes sobre les males herbes es van avaluar mesurant les variables: longitud de

l'arrel, de la part aérea i total de les plantes, pes fresc i sec, eficacia i nivell de dany. L'estudi dels efectes dels OEs sobre els microorganismes del sòl es van realitzar en un experiment en tiestos en condicions de laboratori, en el qual els sòls van ser tractats amb els OEs i els extractes de fulles i després van ser incubats a temperatura ambient (20-23 ° C). Els efectes sobre els microorganismes del sòl es van mesurar determinant les principals propietats bioquímiques, com la biomassa microbiana, el carboni i nitrogen, la respiració microbiana i l'abundància relativa dels principals grups microbians.

Els resultats obtinguts a partir dels assaigs in vitro van revelar que tots els OE estudiats van mostrar efectivitat contra les males herbes objectiu, controlant completament la seua germinació o reduint-la i inhibint significativament el creixement de les plàntules. Entre tots ells, *T. capitata* va ser el més eficaç. A dosis més baixes, va bloquejar completament la germinació de les llavors d'*A. retroflexus*, *P. olecerea*, *A. fatua* i *E. crus-galli.*

Els assaigs en hivernacle van demostrar l'activitat herbicida de *T. capitata*, *M. piperita* i *S. chamaecyparissus*, augmentant la seva fitotoxicitat amb la dosi. *T. capitata* va ser l'OE més eficaç contra totes les males herbes a la dosi màxima i *P. oleracea* va ser la mala herba més resistent. Els microorganismes del sòl, després d'un període de trastorn transitori induït per l'addició dels OEs, en general van recuperar la seva funció inicial i la seva biomassa. Només l'oli de *T. capitata*, a la dosi més alta no va permetre als microorganismes del sòl recuperar completament la seva funcionalitat inicial.

Els resultats de l'aplicació d'extractes de fulles al sòl van demostrar que les fulles d'eucaliptus i els seus extractes (OEs, hidrolats i extractes aquosos), afecten la comunitat microbiana del sòl de diferents maneres, i aquests efectes depenien de les espècies d'eucaliptus.

Fins ara, els resultats obtinguts fan possible suggerir l'aplicació d'EOs com a bioherbicides en entorns controlats com l'horticultura i en condicions d'hivernacle, però s'ha d'identificar la dosi òptima d'aplicació per controlar les males herbes i simultàniament, no afectar negativament els microorganismes del sòl. Tot i això, cal fer més investigacions de camp per entendre completament el potencial real dels OEs en condicions de camp, e cal trovar una formulació adecuada per millorar la persistència i la penetrabilitat del OEs, la cual cosa aumentarà la seua eficacia per al control de les males herbes.

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Preface

I Weeds

1 Definition and characteristics

What is a weed?

"Any plant or vegetation, fungi excluded, that interferes with human objectives"

European Weed Research Society (EWRS, 1986)

Are all wild plants growing in an agroecosystem "weed"?

Despite its general acceptance, the term weed is not easily defined. What some farmers consider a weed, others will find innocuous or even charming. And what one may call a weed in a soybean crop, another may call a wildflower in a forest setting. Our perceptions of what a weed is will vary based on location, plant species, population size, and other factors. In a farm, weeds are those plants that negatively affect crop production. First and foremost, weeds compete with market crops for resources, such as light, nutrients, and water, and potentially reduce crop yields. Weeds also lead to increased production costs: the costs of controlling them and the insects and diseases they harbor. During harvest, weeds can interfere with machinery and further reduce crop quality through contamination. Despite the lack of a clear definition for every circumstance, plants that fall into the weed category have shared characteristics that earn them the "weedy" distinction (Finney and Creamer, 2008).

Weed characteristics

Weeds are highly competitive, most weeds exhibit rapid seedling growth and an ability to reproduce when young, especially when they experience stress. Weeds mature quickly compared to most crop species, and many species thrive under a broad range of conditions. They can tolerate a wide range of adverse environmental conditions, such as drought stress and soil compaction. Weeds can scavenge and compete for resources, and they respond rapidly to favorable growing conditions. Furthermore, weeds have several characteristics that enhance reproductive capability:

- They reproduce by seeds or by vegetative propagation, or the most aggressive by both ways.
- They exploit different efficient mechanisms for seed dispersal.

- Some of them display self-compatibility (Zimdahl, 2018): a single propagule is enough to start a sexually reproducing colony of plants. Self-compatible flowering plants can usually produce seed without visits from specialized pollinating insects.
- They produce a great number of seeds. Examples of species that produce a great number of seeds per plant include redroot pigweed (*Amaranthus retroflexus*, 117,000 seeds per plant), common purslane (*Portulaca oleracea*, 52,000), shepherd's purse (*Capsella bursa-pastoris*, 38,000), common lambsquarters (*Chenopodium album*, 28,000), and yellow foxtail (*Setaria glauca*, 12,000) (Anderson, 1977).
- Weed seeds can also be dispersed across time through extended dormancy. A longevity study that involved 20 species of weed seeds buried in soil for more than 80 years (Darlington and Steinbauer, 1961), found that, after 20 years, 11 of the buried species were still viable; after 40 years, 8 were still viable [including purslane (*Portulaca oleracea* L.), redroot pigweed (*Amaranthus retroflexus* L.), shepherd's purse (Capsella bursa-pastoris (L.) Medik.), annual ragweed (*Ambrosia artemisiifolia),* and plantain (*Plantago major*)]; and after 80 years 3 species were still viable, specifically curly dock *(Rumex crispus*), common evening primrose (*Oenothera biennis*), and moth mullein (*Verbascum blattaria*).

2 Status quo of weed problems in agriculture

The global population is increasing daily and it is expected to reach 9.7 billion in 2050 and11.1 billion in 2100 (United Nation, 2017). Therefore, the area of usable farmland per person decreases from day to day, as more people need more food per head from less land (Hüter, 2011). In addition, because of various biotic factors (insects, fungi, bacteria, viruses, and weeds), food productivity by agriculture has decreased steadily (Mutlu *et al*., 2011). Therefore, maximizing world agricultural production depends greatly on the control of a wide variety of pests, including weed species, which can be defined as those undesired plants that interfere with human activity in agricultural and non-agricultural areas (Vyvyan, 2002). As an anthropologist term, they are plants which are called "out of place ". In the cut and thrust of ecosystem, there is no plant "out of place", but the reason of considering them so, is that, they create problems for agriculture concern: weeds have an impact on farmed species competing with them for moisture, light and nutrients (Randall, 2017). In cultivated areas they reduce the yield of the crops, pollute the cultivation area, and increase the seed bank, maintaining the problem in the following crops (Vyvyan, 2002). Weeds are considered the major problem in

agriculture fields because they bring about 34% of crop losses, compared to pathogens leading to 18% of crop losses (Oerke, 2006). For instance, in USA, weeds cause up to 12% loss in crop yield each year and cost nearly $15 billion (Pimentel *et al*., 2009). Weeds are responsible for 37–44% potential grain yield loss via competition for carbon dioxide, nutrients light and water in fields corn (*Zea mays* L.) in China (Wang *et al*., 2019).
In crop fields, yield losses to weeds have been estimated approximately 30% in maize and 14-30% in wheat (Bommarco *et al*., 2013).

Thus, they are often recognized as the most serious threat to organic crop production, and fear of ineffective weed control, are often perceived by farmers as one of the major obstacles to conversion from conventional to organic farming (Bàrberi, 2002).

Even, in weed-controlled systems, weeds can cause an approximate loss of 10% of the harvested final crop (Labrada *et al*., 1996). Furthermore, weed infestation does not only induce huge economic losses by reducing crop quantity yields, but also, generates, low-quality crop yields (Appleby *et al*., 2005).

3 Weed management and different control practices in agricultural crops

Weed control accounted for 46% of global pest management costs in 2005, accounting from the $33,600 million total spend (Agrow, 2006). Weed control can be achieved through several means such as:

<u>Cultural methods</u> consist of the modification of the environment where weeds grow so that the crop is reinforced or the competition of weeds with the crop is diminished. It includes crop rotation. Through long-term variations of crop species and planting times, rotations create a changing environment and prevent the dominance of a weed species. Researchers have compared emerged weed densities in test crops grown in rotation versus continually grown test crops. For most of the crops studied, weed densities were lower when a crop was grown in rotation (Melander *et al*., 2017). Other techniques are the use of fertilizers to favor cultivation or cover crops, the preparation of seedlings for transplantation, the management of adequate planting doses and irrigation, the preparation of a false seedbed, and the use of improved, highly adapted and resistant crop varieties. Those methods were criticized to be less effective and time consuming.

Physical and mechanical methods include any physical action that is carried out to destroy the weeds, such as hand weeding or with tools, mow or cut them, plow, harrow, hoe bury them or asphyxiate them. Flooding and burning are included in these types of practices. However, He *et al*. (2009) and Carvalledo *et al*. (2013) pointed out that those methods are a burden for farmers, because it is labor-intensive and time-consuming. In addition, those techniques are less effective since they could stimulate further weed emergence or allow recovery from damage. They consist in soil disturbance caused by working implements leading to serious problems on the root system of the crop and could bring new weed seeds close to the soil surface and may enhance soil N mineralization (Barberi, 2002; Hatcher and Melander, 2003).

Biological control of weed plants is based mainly on the fact that: 'most organisms have natural enemies that can destroy them'. Grazing animals, parasites, pathogens, and viruses of weeds have been used against them. The drawback of these methods is that the biological agent must be selectively managed, or the effect of the control will be more harmful than that of the weed.

Chemical control based on the use of synthetic herbicides remain the most common used method to fight weeds (Batish *et al*., 2004). An herbicide is composed of an active ingredient, co-formulants and adjuvants. The active ingredient is the chemical or biological substance that produces the toxic effect. The co-formulants are inert and have the purpose to reduce the concentration of the active principle. The coadjuvants, instead, are inactive substances that allow the herbicide to be better distributed on the surface of the plants, increasing their adhesion and persistence on plant species. The use of chemical herbicides has been raised since the development of the agro-chemical industry, during the two world wars. Between 1940 and 1970, great progress has been made in chemical products with the advent of the "Green Revolution" in Europe and the USA (Tilman *et al*., 2001).

Biotechnological control methods consist in using biotechnological tools as herbicide resistant crops by transgene technology, improvement of biocontrol agents, development of transgenic allelopathy in crops and characterization of weeds using molecular systematic. Plant transformation by transfer of cloned genes in susceptible plants through engineered vector technique is a popular method among those tools. In other words, this technology has the potential to make crops better competitors for weeds through improving competitive traits or making the crop more allelopathic. The advantage of herbicide resistance crops is increased crop yield due to broader spectrum of weed control and reduced crop injury. But precautions should be taken, whether herbicide resistance in weeds inviting shifting of weed flora or not.

This method is most used in USA than in Europe for the limitation of GMO use (Behrens *et al*., 2007; Duke *et al*., 2000).

4 Herbicide use and its impacts

4.1 Weed resistance

The overuse of synthetic herbicides by farmers during the last decades, application at high doses and repeatedly during cultivations, causes ecological consequences, since the biodiversity of farmland is reduced, facilitating changes in weed populations and the evolution of herbicide-resistant weed biotypes to multiple herbicide sites of action (Figure 1) and the appearance of resistant weed strains by crops (Figure 2) (Palumbi, 2001). The emergence of worldwide resistance (Figure 3) is linked to the repeated application of herbicides with the same mode of action and on the same crop, which imposes a selection of the most resistant individuals within the treated weed species, which were previously sensitive (Holt, 1992). In addition, many concerns have been expressed about herbicide potential health and environmental impact (Gitsopoulos *et al*., 2013).

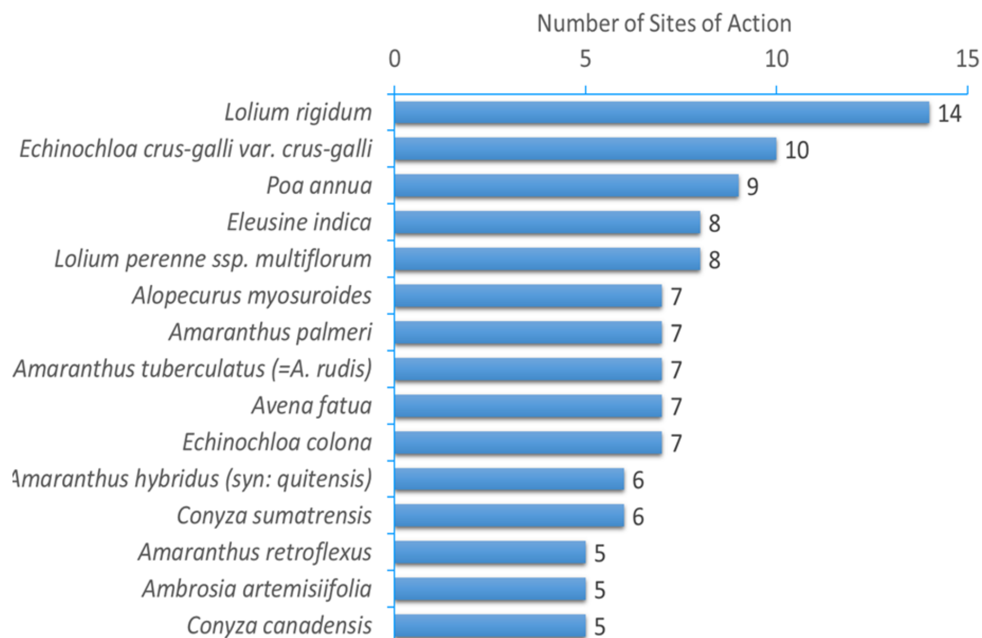

Figure 1. Weed species resistance to multiple herbicide site of action (modified from Weedscience.org, 2019).

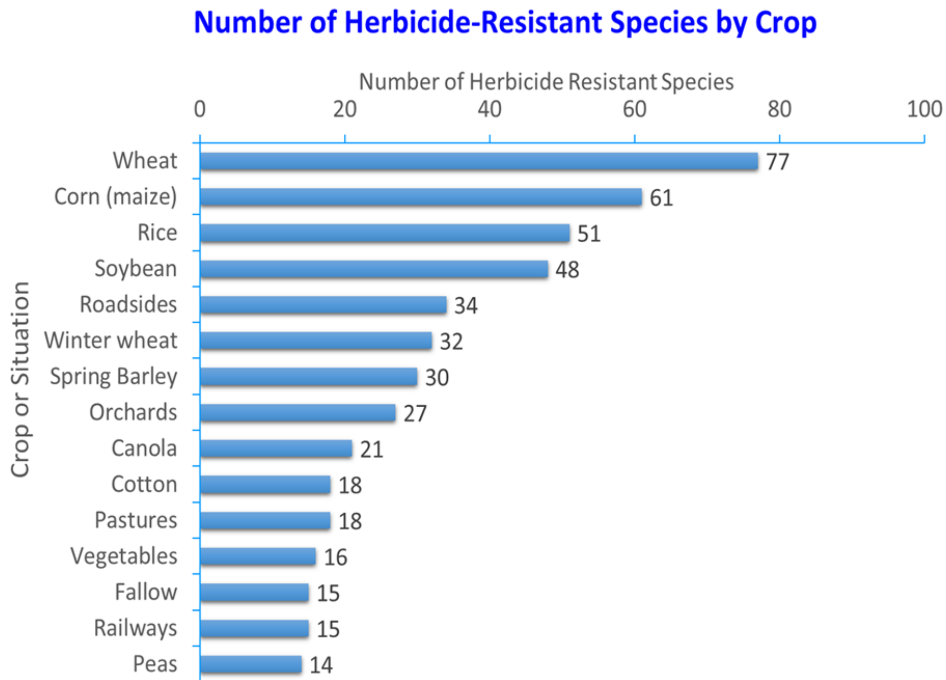

Figure 2. Number of herbicide-resistant species by crop (modified from Weedsciences.org, 2019).

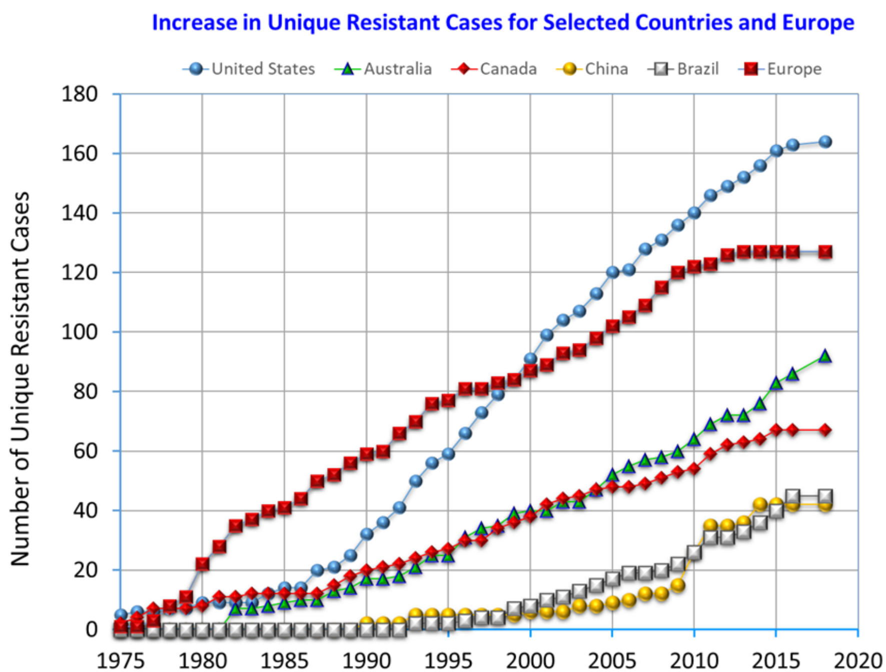

Figure 3. Increase in unique resistance in selected countries and Europe (modified from Weedscience.org, 2019).

4.2 Human health and environmental impacts

The integration of herbicides into the cycle of natural decomposition of organic compounds is largely unknown and problematic. It was due to the detection of the first resistance in the 50s in insects and agricultural pests, that becomes clear that the use of pesticides including herbicides could have serious harmful impacts such as toxicity, which is the ability of a substance to cause damage in living organisms. The toxic effects produced by herbicides can be immediate (acute toxicity) or cumulative (chronic toxicity), depending on the duration of exposure, the dose and the herbicide in question (Hager and Refsell, 2008).The exposure to residues in the environment, in the food, or in the water could trigger acute and chronic effects on human health, including eye and skin irritation, burns, acute and chronic neurotoxicity, cardiopulmonary problems, childhood methemoglobinemia, various types of cancer, particularly hematopoietic cancers, immune disorders, and fertility problems (Weisenburger, 1993). Furthermore, herbicides are moderately soluble in water and have relatively low adsorption coefficients in soil. Due to these properties, they can pollute the environment through agricultural runoff or leaching (Ren *et al*., 2011). Numerous investigations confirm the incidence (Holden *et al*., 1992; Walls *et al*., 1996), including impact on indigenous vegetation communities (Riemens *et al*., 2009).

4.3 Soil microbial community impacts

By the same token, herbicides also affect soil microorganisms. It has been determined that the number of substituents determines their toxic effects. The ability to capture electrons from substituents significantly influences the biological activity of herbicides, highlighting the electrostatic interactions between herbicide molecules and microorganisms (Nemes-Kósa and Cserháti, 1995). Soil microorganisms appear to be very suitable and sensitive early-warning indicators or predictive tools in soil health monitoring. Some herbicides have been found to inhibit the decomposition of cellulose in the soil. Also, repeated applications of herbicides could affect a shift in the microbial community structure. For example, fungi were most sensitive, being inhibited until the 40th day to combined prosulfuron + bromoxynil. The latter exerts an inhibitory effect on the activity of the dehydrogenase of about 80% for low concentrations and without any recuperation. This indicates that, despite the low quantities applied, herbicides had a deleterious effect on the activity and balance of the soil community and must be used with caution (Pampulha and Oliveira, 2006).

5 European Union (EU)legislations on plant protection products

In order to protect human health and the environment, the European Parliament and Council, regulates the use and commercialization of pesticides in order to reduce their risks, by establishing two important regulations: the Regulation (EC) No 1107/2009 of the European Parliament and of the Council of 21 October 2009 concerning the placing of plant protection products on the market, which repeals Council Directives 79/117/EEC and 91/414/EEC and the Directive 2009/128/EC of the European Parliament and of the Council of 21 October 2009 establishing a framework for Community action to achieve the sustainable pesticide use. At the same time, being involved and affected by pesticide use, generally, including herbicides, the society is becoming aware that new techniques must be used, that accomplish with the new regulations. They should be both effective, and conducive to the development of sustainable agriculture. The interest in sustainability increased after the term "sustainable development" was coined in 1987, in the report "Our Common Future", published by the United Nations and the World Commission on Environment and Development (Constance, 2010). Therefore, efforts are being made towards a search for chemicals that quickly break down in the environment and possess novel target sites. Natural plant products that play a variety of physiological roles possess these two properties and thus can serve as a source of novel herbicides (Weston and Duke, 2003). Thus, new technologies based on natural products as an alternative to synthetic chemical herbicides are being developed in combination with traditional cultural practices to control weeds, (Dayan *et al*., 2011).

Natural products are an interesting source for obtaining green herbicides, not only because of the great diversity and innovation of their formulas, but also because of the specific potential of their biological action and because of the reduced probability of producing accumulations of harmful waste in water and soils (Mercier, 2005).

6 Allelopathy and potential natural herbicides

Rice (1984) has defined allelopathy as any direct or indirect effect (stimulatory or inhibitory) of one plant (including microorganisms) on another, by releasing chemical compounds (allelochemicals) to the environment. Plants have their own defense mechanisms, not only against herbivorous predators but also against other plant species, in the fight for the colonization of space and the use of ecological resources. Besides, and according to the definition given by the International Allelopathy Society (IAS), Allelopathy is "*the science that*

studies any process involving secondary metabolites produced by plants, algae, bacteria and fungi that influence the growth and development of agricultural and biological systems" (Macías *et al*., 2008).

Allelochemicals can be released into the environment by numerous mechanisms: leaf volatilization, root exudation and leaching of leaves and plant debris in the soil by precipitation (Putnam, 1983). Allelochemicals that suppress or eliminate competent plants near the source plant have received special attention due to their potential as selective natural herbicides (Stonard and Miller-Wideman, 1995; Benner, 1996; Duke *et al*., 2000). The use of natural products as a reservoir of bioactive compounds has been widely exploited in medicine, the discovery and use of antibiotics being an example of this. The ethnobotanical approach, focusing on the study of plants traditionally used for medicinal purposes worldwide, demonstrates the importance of nature as a source of new drugs. This view has been considered only recently in agronomic studies (Macías *et al*., 2004).

Evidence of allelopathic interactions in nature caused by plants containing volatile allelochemicals have been frequently described (Chou, 1989; Azirak *et al*., 2008; Benvenuti *et al*., 2017). Most of the germination and growth inhibitors produced by perennial angiosperms identified by Rice (1984) are phenolic compounds or derivatives of cinnamic acid. That means allelopathic compounds can be used as a biological control on weeds and pests to decline the use of dangerous chemical products. Other authors also found coumarins, flavonoids, alkaloids, cyanglucosides, proteins and amino acids among the inhibitory compounds (Macías *et al*., 2008). To this list, terpenoids must be added, including volatile terpenes that are the main components of essential oils (EOs) (Fischer, 1986; Muller, 1965). Moreover, some EOs are classified as "Generally Recognized as Safe" (GRAS) by the US Food and Drug Administration (FDA).

Allelochemicals affect many physiological functions and biochemical reactions: enzymatic activities, cell division, cell elongation, membrane permeability and ion absorption. These chemical compounds, when released, are usually a mixture of many organic compounds that can exert toxic effects synergistically (Kalinova, 2010). On the other hand, when the amount of one active compound is very high within a given EO, it is also true that this EO compound alone could be even effective (Araniti *et al*., 2017, Vasilakoglou *et al*., 2013, Verdeguer *et al*., 2020). However, the details of the biochemical mechanism by which a compound exerts a toxic effect on plant growth are not yet well known (Batish *et al*., 2008).

7 Essential oils (EOs)

EOs are volatile, natural, complex compounds characterized by a strong odor and are produced by plants as secondary metabolites. They can be synthesized from the non-woody parts of all plant organs i.e. buds, flowers, leaves, stems, twigs, seeds, fruits, roots, wood or bark, and are stored in secretory cells, cavities, canals, epidermic cells or glandular trichomes, (Batish *et al*., 2008; Bakkali *et al*., 2008). They are extracted from various aromatic plants generally localized in temperate to warm countries like Mediterranean and tropical countries where they represent an important part of the traditional pharmacopoeia. They are liquid, volatile, limpid, and rarely colored, lipid soluble and soluble in organic solvents with a generally lower density than that of water. The extraction product can vary in quality, quantity and in composition according to climate, soil composition, plant organ, age, and vegetative cycle stage (Masotti *et al*., 2003; Angioni *et al*., 2006). So, in order to obtain EOs of constant composition, they must be extracted under the same conditions from the same organ of the plant which has been growing on the same soil, under the same climate and has been picked in the same season. In nature, EOs play an important role in the protection of the plants as antibacterial, antiviral, antifungal, insecticide and against herbivores by reducing their appetite for such plants. They also may attract some insects to favor the dispersion of pollens and seeds or repel undesirable others. At present, approximately 3000 EOs are known, 300 of which are commercially important especially for the pharmaceutical, agronomic, food, sanitary, cosmetic and perfume industries. EOs can contain about 20–60 components at quite different concentrations. They are characterized by one or many components at high concentrations (20–70%) compared to other components present in trace amounts. For example, carvacrol (30%) and thymol (27%) are the major components of the *Origanum compactum* EO, linalol (68%) of the *Coriandrum sativum* EO, α- and β-thuyone (57%) and camphor (24%) of the *Artemisia herba-alba* EO, 1,8-cineole (50%) of the *Cinnamomum camphora* EO, a-phellandrene (36%) and limonene (31%) of leaf and carvone (58%) and limonene (37%) of seed *Anethum graveolens* EO, menthol (59%) and menthone (19%) of *Mentha* x *piperita* EO. Generally, these major components determine the biological properties of them (Bakkali *et al*., 2008).

7.1 EOs as potential herbicides

The advantage of using EOs as herbicides is given by the joint action of several compounds present in them and not only by a single compound, whose quantity and persistence in the environment may be not enough to obtain a toxic response on its own. Furthermore, several

studies (Dudai *et al*., 1999; De Feo *et al*., 2002; Singh *et al*., 2003; Verdeguer, 2011) exhibited that numerous plant species possess significant germination and seedling growth inhibitory effects, and confirmed that plant EOs are a potential source of new herbicides. Tworkoski (2002) pointed out that EOs can be used as viable weed control technology under organic farming systems. More specifically, Dudai *et al*. (2000) showed that the EOs extracted from three species, *Cymbopogon citratus*, *Micromeria fruticosa* and *Origanum syriacum* were very active inhibitors of the germination. Likewise, Azirak and Karaman (2008), demonstrated that, the EOs from *Carum carvi*, *Mentha spicata*, *Origanum onites* and *Thymbra spicata* showed high inhibitory activity on the germination of seven weed seeds in vitro (*Alcea pallida*, *Amaranthus retroflexus, Centaurea salsotitialis*, *Raphanus raphanistrum*, *Rumex nepalensis*, *Sinapis arvensis* and *Sonchus oleraceus*). These oils reduced the growth of weed seedlings at low concentrations. The effectiveness of EO extracted from *Eucalyptus citriodora* has been proven as an alternative method to control *Phalaris minor* (Batish *et al*., 2007). EOs from various varieties of oregano (*Origanum* spp.) and basil (*Ocimum basilicum*) have been tested against *Echinochloa crus-galli* and *Chenopodium album* with some success (Vasilakoglu *et al.,* 2007). These EOs, mainly composed of *p*-cimene (20-25%), γ-terpinene (15-20%) and thymol (10-35%), have been patented for moss control (Dayan *et al*., 2009). *Leptospermum scoparium* leaf EO is composed of more than 70% sesquiterpenes (Christoph *et al*., 1999) and is rich in β-triketones (Hellyer, 1968; Douglas *et al*., 2004). Leptospermone, the most abundant triketone in this EO, causes bleaching of both broadleaf and narrowleaf weed leaves (Knudsen *et al*., 2000). Natural triketones have a structure like some synthetic herbicides, such as sulcotrione and mesotrione, and act on *p*-hydroxyphenylpyruvate dioxygenase (Lee *et al*., 1997; Dayan *et al.,* 2009).

II Soil

1 Definition

What is soil?

Several natural concepts lack a universally accepted definition. Soil is one of them. One of the reasons is its multifunctionality. Actually, land use conditioned the way soil was perceived. It was in the late 1880s that the Russian Vasilij V. Dokuchaev, the father of pedology, from the Greek pedon "soil" and logos "knowledge", gave dignity to soil as something with its own identity in the realm of natural objects. Dokuchaev, in fact, proposed a naturalistic concept of soil that prescinds from soil use. Essentially, he referred to the soil as a tridimensional entity located at earth's surface with morphology and unique physical, chemical and biological properties acquired by the interaction, through time, among living and dead organisms, rock, and climate on a given topographic position. Besides, the Russian Jacob S. Joffe, defined soil as a natural body, differentiated into horizons of mineral and organic constituents, usually unconsolidated, of variable depth, which differs from the parent material below in morphology, physical properties and constitution, chemical properties and composition, and biological characteristics (Joffe, 1936).

2 Soil quality

Throughout the time, many definitions of soil quality had been proposed. In 1984, Anderson and Gregorich defined soil quality as the sustained capability of a soil to accept, store and recycle water, nutrients, and energy. After that, in 1994, Doran and Parkin proposed a more objective definition of soil quality suggesting that it is the capacity of a soil to function, within ecosystem and land boundaries, to sustain productivity, maintain environmental quality and promote plant and animal health. Then, Karlen *et al*. (1997) assigned the ability of a specific soil to function within natural or managed ecosystem boundaries to sustain plant and animal productivity, maintain or enhance water and air quality, and support human health and habitation as a definition of soil quality. Nowadays, this definition is still accepted in the scientific literature, as dealing with the integrated view of soil quality. Keesstra *et al*. (2016) and Vogel *et al*. (2018) indicated that based on the integrative definition of soil quality, soils are no longer seen as a support for production only, but rather as a complex system interacting with the surrounding environment to provide various services. In view of soil multi-functionality, soil quality should be assessed because it expresses the capacity of soil to

function, sustain long-term crop production and to maintain a productive ecosystem services, environmental quality and biodiversity conservation (Bünemann *et al*., 2018). Soil quality can be assessed by measuring soil chemical, physical and biological parameters. Doran and Parkin (1996) have proposed a minimum data set to be used for soil quality assessment which includes physical (texture, rooting depth, infiltration rate, bulk density, water retention capacity), chemical (pH, total carbon, content, electrical conductivity, nutrients level) and biological (C and N microbial biomass, potentially mineralizable N, soil respiration) as basic indicators for an initial characterization of soil quality. Other supplementary soil properties could be used to evaluate soil quality based on specific climatic, geographic, and socio-economic conditions. Many authors (Klein *et al*., 1985; Nannipieri *et al*., 1990) have proposed several biological and biochemical parameters as sensitive parameters to the slight modifications that the soil can undergo under the action of any disturbing agent. Since soil microorganisms due to their quick metabolism can respond rapidly, they may reflect a hazardous environment and should be, therefore, preferentially considered when monitoring soil status (Laudicina *et al*., 2012). Furthermore, Bloem *et al*. (2005) suggested that national and international programs for monitoring soil quality should include microflora and respiration measurements and nitrogen mineralization, microbial diversity, and functional groups of soil fauna.

3 Microbial Biomass

Soil microbial biomass (MB) can be defined as the portion of soil organic matter that constitutes living microorganisms smaller than 5–10 μm^3 (Jenkinson and Ladd, 1981). Microbial biomass constitutes approximately 1–4% of the total organic carbon (MBC; Anderson and Domsch, 1989; Sparling, 1992) and 2–6% of the total organic nitrogen (MBN; Jenkinson, 1988). Typically, MBC ranges from 100 to >1000 mg C kg^{-1} of soil (klug, 1999). MB has a turnover time less than 1 year (Paul, 1984) and, therefore, responds to stress/disturbance factors more rapidly than the whole soil organic matter, the content of which may need decades to appreciably change. MB monitoring through the time is required to infer considerations on the changes in amount and nutrient content of the MB (Rice *et al*., 1996).

MB is typically measured indirectly using the following methods: fumigation incubation (FI), substrate-induced respiration (SIR), fumigation-extraction (FE) and/or measurement of ATP content (Jenkinson and Powlson, 1976; Anderson and Domsch, 1978; Jenkinson and Ladd, 1981; Brookes *et al*., 1985; Vance *et al*., 1987). These methods have greatly improved measurements of MB and its associated nutrient pools. However, Rice *et al*. (1996) recommend

the fumigation techniques since they are cheaper, easy to use, and facilitate assessment of the mineralizable C and N fractions of soil organic matter. Of the two fumigation methods, Klug, (1999) recommend the FE method as it is faster than FI and gives more reproducible results. FE involves two key steps: (1) destruction of microbial cell membranes by chloroform fumigation, and (2) extraction and analysis of cell constituents (Klug, 1999). MB acts as both a nutrient reservoir and as a catalyst for organic matter decomposition. Consideration of MB is crucial, therefore, to understand nutrient fluxes within and between ecosystems (Smith and Paul, 1990). Jenkinson *et al.* (1987) defined the MB as "the *eye of the needle through which all organic matter needs to pass".* MB is a sensitive indicator of toxicity attributable to pesticides, heavy metals, and other pollutants. Pollutants can affect microorganisms directly by causing toxic effects or indirectly by, for instance, decreasing the availability of substrates such as plant root exudates. Thus, the decreased energy available to the microbes could also result in a smaller population (Brookes, 1995; Kizilkaya *et al*., 2004; Perez-de- Mora *et al*., 2006).

4 Microbial Activity

Soil microbial activities are of critical importance for biogeochemical cycles. Microbial activity is regulated by many factors including nutrient, oxygen, water availability, temperature, and soil pH. Soil microbial activity can be measured under either field or laboratory conditions. In the field, variations in meteorological conditions during the experiment are inevitable. Furthermore, field measurements are often difficult to be interpreted. For example, soil respiration determined in the field is due to activity of microorganisms and other organisms such as macrofauna and plants, which vary significantly in different systems and throughout the season (Dilly *et al*., 2000). Laboratory procedures are usually carried out on sieved and stabilized soil samples at standardized temperature and water content. Such measurements generally include assays of enzyme activities, C and N mineralization. These, and eventually other microbial activity measurements, may be helpful to evaluate effects of soil management, land use and specific environmental conditions (Burns, 1977) on microbial activity. Laboratory methods allow the standardization of environmental factors and, thus, the comparison of results from soils of different geographical locations, environmental conditions, and even different laboratories. However, laboratory measurements generally represent microbial potential activities, as they are determined under optimized conditions (Nannipieri *et al*., 1990).

5 Carbon Mineralization (Soil Respiration)

Carbon dioxide (CO_2) release from soils, or soil respiration, is a multi-component process and, when it depends only on soil microorganisms, it is called “microbial respiration”. Hence, microbial respiration (MR), soil respiration minus root respiration, is a measure of the total metabolic activity of soil microorganisms that are decomposing organic matter (Haynes, 2005). MR, provides an indication of C available to soil microbes (Robertson *et al*., 1999) and can act as an index of soil organic matter quality (Haynes, 2005). MR is measured by incubating, in sealed chambers and at a constant temperature (generally 20 or 25°C), pre-conditioned live root-free soil samples at field-moisture level or re-wetted up to 40–60% of their water holding capacity (WHC). The CO_2 accumulated in the chamber headspace is then determined by different methods, i.e. by alkali trapping, gas-chromatography, or infrared-gas analysis. As it is evolved under controlled and optimal conditions, the amount of CO_2 efflux evolved during a short-term incubation (from 10 to 30 days) is also referred to as the potentially mineralizable C and is generally expressed in mg kg^{-1} of soil. The potentially mineralizable C generally coincides with the soil C fraction easily available to microflora. On the other hand, long-term incubations (up to several months) may supply information on C pools with a slow turnover. The short- and long-term release of CO_2 can, in fact, be used mathematically to indicate the functional pools of soil organic C commonly referred to as active (labile) and passive (recalcitrant) fraction soil organic C (Laudicina *et al*., 2012).

6 Microbial Community structure

The recent development of molecular and biochemical techniques has enabled a better understanding of microbial community structures in soil ecosystems (Kennedy and Gewin, 1997), with most of the species being unknown and unculturable (Torsvik *et al*., 1998). One widely used approach is the analysis of microbial phospholipid-linked fatty acid (PLFA) composition. In this method, microbial lipids are extracted from environmental samples in a phase mixture of chloroform, methanol, and water (Bligh and Dyer, 1959). Lipids associated with the organic phase are then fractionated into neutral, glyco- and phospholipids on silicic acid columns (Vestal and White, 1989). Finally, the phospholipids are subjected to alkaline methanolysis to produce fatty acid methyl esters (FAMEs) for gas chromatography analysis. Recently, less harsh methods have been developed. Hence, microbial fatty acids can be directly extracted from soil by a simple method that is easier and quicker (Hinojosa *et al*., 2005). It consists of a mild alkaline reagent to lyse cells (KOH in methanol) and release fatty acids from

lipids (ester-linked fatty acids; ELFAs) once the ester bonds are broken (Schutter and Dick, 2000). For instance, the ELFA method has been successfully used to characterize microbial communities in grass seed field soils and placed communities into groupings like those generated by a DNA-based method (Ritchie *et al*., 2000). Besides, some studies of Drijber *et al*. (2000) and Schutter and Dick, (2000) have outlined the effectiveness of the ester-linked fatty acid (ELFA) procedure for assessing community structure.

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Thesis structure and objectives

The thesis was accomplished from January 2017 until May 2020 within two departments:

1) Department of Agricultural, Food and Forestry Sciences, University of Palermo, Viale delle Scienze, Edificio 4, 90128 Palermo, Italy.

2) Institute of Mediterranean Agroforestry (IAM), Polytechnic University of Valencia, Camino de Vera s/n, C.P. 46022 Valencia, Spain

The thesis was arranged as a thesis of publications, including four chapters incorporated as the following order:

Chapter 1: Phytotoxic activity of seven essential oils extracted from Mediterranean species on weed seed germination and seedling growth

Aims: to assess the in vitro phytotoxic potential of EOs extracted from *T. capitata*, *M. piperita, S. chamaecyparissus*, *E. camaldulensis*, *E. occidentalis, E. lesouefii* and *E. torquata* against the target weeds *A. retroflexus, P. oleracea, A. fatua* and *E. crus-galli*.

Chapter 2: Soil bioindicators and herbicidal activity as affected by EOs extracted from three different Eucalyptus species applied in pre- and post- emergence.

This chapter was a preliminary experiment to figure out the adequate concentrations that should be applied in *in vivo* and to assay the suitable methodology in greenhouse conditions.

Aims: to test the effectiveness of three Eucalyptus species, namely *E. camaldulensis E. occidentalis* and *E. globulus* in controlling soil seed bank germination (pre-emergence) and the spontaneous plants weed flora of the soil (post-emergence) as well as their potential, on microbial biomass C, respiration and on the main microbial groups.

Chapter 3: Potential effects of essential oils extracted from Mediterranean aromatic plants on target weeds and soil microorganisms

Aims: To evaluate the herbicidal potential of *Thymbra capitata* (L.) Cav., *Mentha × piperita* L. and *Santolina chamaecyparissus* L. essential oils (EOs) on *Avena fatua* L., *Echinochloa crus-galli* (L.) P. Beauv, Portulaca oleracea L. and *Amaranthus retroflexus* L. and their effects on soil microorganisms.

This chapter is an accepted paper in Plants Journal.

Chapter 4: Effects of eucalyptus leaves and their extracts on soil microorganisms.

Aims: to assess the effects of Eucalyptus leaves and leaf extracts (Essential oils, hydrolats, Aqueous extracts) on soil microbial biomass C and N, microbial biomass activity, on the relative abundance of the main microbial groups as well as on microbial and metabolic quotients.

Chapter 1:

Phytotoxic potential activity of seven essential oils extracted from Mediterranean species on seed weed germination and seedlings growth

1 Introduction

Weeds cause serious problems in agricultural ecosystems because their competition with crops for water and nutrient uptake, light, and carbon dioxide, causing great losses in crops yield, up to 34% (Monaco *et al*., 2002; Oerke, 2006; Murphy *et al*., 2008). However, the overuse of synthetic herbicides to increase agriculture productivity has contributed in the emergence of weed resistance, gradual degradation of soil and adverse effects in environment, living organism and human health (Vyvyan, 2002). Therefore, environmental concerns, the great interests in sustainable and organic agriculture have stimulated the need to adopt alternative, natural and biodegradable tools, without negative impacts on human health and environment. (Dayan and Duke, 2010).

Secondary metabolites of plant species, such as volatile essential oils (EOs) and their constituents have been considered as a potential candidate to develop bioherbicides (Weston and Duke, 2003).

EOs are generally characterized by low toxicity, such as Citronella EO (Sharma *et al*., (2019) *Calamintha nepeta* and *Origanun virens* EOs (Arantes *et al*., 2019), *Mentha arvensis* and *Litsea cubeba* EOs (Wu *et al*., 2019) and *Eucalyptus camaldulensis* EO (Islam *et al*., 2014). As well as low persistence, since they contain a range of natural chemical compounds. Such diversity may lead to different modes of action, acting at different points of metabolism, so the probability that weeds develops resistant biotypes is lower than with synthetic herbicides, based on one active ingredient (Dayan *et al*., 2010). Particularly, terpenoids, monoterpenes and sesquiterpenes, are the main components of EOs and are often responsible for their plant inhibitory activity and phytotoxicity (Duke *et al*., 2002).

Many EOs demonstrated great species-specific phytotoxicity against seed germination, and seedlings growth (Azirak and Karaman, 2008). For instance, the phytotoxicity of *Eucalyptus* spp. EOs against *Parthenium hysterophorus*, *Cassia occidentalis*, *Echinochloa crus-galli* have been well documented (Batish *et al*., 2004; Singh *et al*., 2006). Furtherthemore, the EO extracted from *Eucalyptus camaldulensis* Dehnh., inhibited the germination and reduced the seedling growth of *Amaranthus hybridus* and *Portulaca oleracea* (Verdeguer *et al*., 2009) and the EO from *Eucaluptus citriodora* was effective to control the invasive noxious weed *Parthenium hysterophorus*, *Bidens pilosa*, *Amaranthus viridis*, *Rumex nepalensis* and *Leucaena leucocephala* (Singh *et al*., 2005; Setia *et al*., 2007; de Araújo-Filho *et al*., 2018). Also, *Eucalyptus tereticornis* shown a great phytotoxic potential (Kohli *et al*. 1998). Ramezani

et al. (2008) reported that EOs from *Eucalyptus nicholii* strongly inhibited the germination of *Amaranthus retroflexus*, *Portulaca oleracea* and *Acroptilon repens*. Besides, *Eucalyptus salubris* EO had the highest inhibition index for *Solanum elaeagnifolium* germination, root and shoot growth (Zhang *et al*., 2012).

EOs from *Eucalyptus* spp., which are composed of a variety of volatile monoterpenes such as 1,8-cineole, citronellol, citronellal, limonene, linalool and α-terpinene, have been found to be economically viable when they are used for crop protection, because *Eucalyptus* spp. are characterized by a high yield and high biomass productivity (Brooker and Kleinig, 2004; Batish *et al*., 2008). In addition, EO extracted from *Eucalyptus camaldulensis* Dehnh. (river red gum) is the highest commercialized in the world and it was investigated widely for its phytotoxic effects against many weeds (Green, 2002). However, EOs extracted from other eucalyptus species such as *Eucalyptus lesoufii* Maiden (goldfields blackbutt), *Eucalyptus torquata* Luehm. (coral gum) and *Eucalyptus occidentalis* Endl. (flat topped yate) have not been explored for its phytotoxic effects previously to our knowledge. However, *E. occidentalis* aqueous extract was investigated for its allelopathic effects against some species (Saadaoui *et al*., 2014).

In addition, EOs extracted from Lamiaceae have been proved by*in vitro* studies to be effective to inhibit seed germination (Angelini *et al*., 2003). Among this species, *Mentha x piperita* L. (peppermint), had revealed phytotoxic effects in several studies (Rolli *et al*., 2014; Mahdavikia and Saharkhiz., 2015 Campiglia *et al*, 2017) and *Thymbra capitata* L. (Cav) (thyme) (Verdeguer, 2011; Pirbalouti *et al*., 2013;). Likewise, EOs from Asteraceae showed great herbicidal potential (Benvenuti *et al*., 2017), including Santolina *chamaecyparissus* L. (cotton lavender, Asteraceae) whose herbicidal activity has been reported (Grosso *et al*., 2010).

Avena fatua L. (wild oat) is one of the most harmful, resistant grassy, weed of cereal crops in the world (Sharama and Born, 1978), together with *Echinochloa crus-galli* (L.) P. Beauv. (Barnyard grass), another monocotyledon serious weed of irrigated crops, especially rice. (Maun and Barrett, 1986; Chung *et al*., 2001). *Portulaca oleracea* L. (common purslane) and *Amaranthus retroflexus* L. (redroot pigweed) are two dicotyledons, annual weeds of tropical and subtropical crops and summer weds in Mediterranean crops, with an extensive world distribution. (Mitich, 1997; Weaver and McWilliams, 1980). All the above-mentioned weeds were selected in this work for being a major quality problem and a serious economic threat to crop yields.

Although, as above reported, some studies have shown that EOs extracted from *E. camaldulensis, M. piperita, T. capitata* and *S. chamaecyparissus*, may inhibit seed germination, their selectivity depends on the weed against they were applied. In addition, not all EOs exert the same effect on weeds at a given concentration. Hence, it is worthy to examine diverse sources of EOs and their phytotoxic potential to have the possibility to develop selective herbicides against specific weeds.

The aim of this work was to study the phytotoxic potential of EOs from *T. capitata*, *M. piperita, S. chamaecyparissus*, *E. camaldulensis*, *E. occidentalis, E. lesouefii* and *E. torquata* against the target weeds *A. retroflexus, P. oleracea, A. fatua* and *E. crus-galli* to determine if they could be a safer and effective alternative for weed management.

2 Materials and methods

2.1 Weeds

Seeds of *Portulaca oleracea* L. and *Avena fatua* L. were purchased from Herbiseed, (Reading, UK) in 2016 and 2017 respectively, and plants of *Echinochloa crus-galli* (L.) P.Beauv and *Amaranthus retroflexus* L. were collected from rice fields in Sollana (Valencia province, Spain) in September 2017. The plants were dried for 15 days at room temperature, afterwards seeds were extracted. Uniform mature and healthy seeds were selected and stored at room temperature until germination tests.

2.2 Essential oils

Fresh leaves of *Eucalyptus camaldulensis* Dehnh. and *Eucalyptus occidentalis* Endl. were collected in an afforested area near Agrigento (Sicily province, Italy) during November and December of 2017. The leaves of *Eucalyptus lesouefii* Maiden and *Eucalyptus torquata* Luehm. were collected during March, April and May from Gabes, located in the South of Tunisia in 2015.

The four EOs were extracted by hydrodistillation with a Clevenger type apparatus according to the standard procedure described in the European Pharmacopoeia (1975) and stored at 4 °C until their use. EOs extracted from *Thymbra capitata* L. (Cav), *Mentha* x *piperita* L. and *Santolina chamaecyparissus* L. were purchased respectively from Bordas (Sevilla province, Spain), Sigma-Aldrich (Darmstadt, Germany) and Ecoaromuz (Ademuz, Valencia province, Spain).

2.3 Phytotoxic assay

For the *in vitro* phytotoxicity tests, Twenty seeds of P. *oleracea* and A. *retroflexus*, ten of E. *crus-galli* and five of A. *fatua*, were selected and sowed in two layers of filter paper (73 g/m2) in Petri dishes (9 cm diameter) previously wetted with 5ml of distilled water, in case of the dicotyledons, and 6 ml for the monocotyledons. Different concentrations of the EOs were prepared and loaded on the inner side of other two layers of filter paper above the seeds. The controls were prepared with the same quantities of distilled water. For each concentration, five replicates were conducted for the dicotyledons and ten in the case of the monocotyledons. All the Petri dishes were sealed with Parafilm to reduce loss of moisture and release of EOs, then incubated in a germination-growth chamber from Equitec (Spain). According to previous assays, the germination conditions were alternated between 30.0 ± 0.1 °C, 16 h in light and 20.0 ± 0.1 °C, 8 h in dark for *A. retroflexus*, *P. oleracea* and *E. crus-galli*, while for *A. fatua* were set at 23.0 ± 0.1 °C, 8 h in light and 18.0 ± 0.1 °C, 16 h in dark. To evaluate the phytotoxic activity of the selected EOs, on germination and seedling length, data were recorded after 3, 5, 7, 10 and 14 days, by taking digital images of the Petri dishes, that were later processed with the software Digimizer v.4.6.1 (MedCalc Software, Ostend, Belgium, 2005-2016) to determine the seed germination percentage and the seedling length.

2.4 Gas Chromatography (GC)

EOs constituents were quantified by GC using a Clarus 500GC Perkin–Elmer apparatus equipped with a flame ionization detector (FID), and a capillary column ZB-5 (30 m x 0.25 mm i.d. x 0.25 m film thickness). The injection volume was 1 µl. The GC oven temperature was set at 60 °C for 5 min, with 3 °C increases per min to 180 °C, then 20 °C increases per min to 280 °C which was maintained for 10 min. Helium was the carrier gas (1.2 ml/min). Injector and detector temperatures were set at 250 °C. The percentage composition of the EO was computed from GC peak areas without correction factors by means of the software Total Chrom 6.2 (Perkinelmer inc., Wellesley, PA, USA).

2.5 Gas Chromatography-Mass Spectrometry (GC–MS)

EOs constituents were identified by gas chromatography coupled to mass spectrometry analysis using a Clarus 500 GC–MS from Perkin-Elmer Inc. apparatus equipped with the same capillary column, carrier, and operating conditions as described above for GC analysis. The temperature for the ionization source was set at 200 °C and an electron impact mode of 70 eV was employed. MS spectra were obtained by means of total ion scan (TIC) mode (mass range

m/z 45–500 uma). The total ion chromatograms and mass spectra were processed with the Turbomass 5.4 software (Perkinelmer inc.). Retention indexes were determined by injection of C8–C32 n-alkanes standard under the same conditions (Adams, 2007).

The EOs components were identified by comparison of their mass spectra with those of computer library NIST MS Search 2.0 and available data in the literature. Identification of the following compounds was confirmed by comparison of their experimental RI with those of authentic reference standards (Sigma-Aldrich, Darmstadt, Germany): α-pinene, β-pinene, camphene, myrcene, limonene, camphor, terpinolene, borneol, terpinen-4-ol and linalool.

2.6 Statistical analyses

All data were processed using the Statgraphics® Centurion XVI statistical package. A one-way ANOVA was performed. For the dicotyledons (*A. retroflexus* and *P. olecerea*) the ANOVA was performed for the germination and the total seedling growth and for the monocotyledons (*A.fatua* and *E. crus-galli*) the ANOVA was performed for the germination, and for coleoptile, radical and total seedling length. Percentage values were arcsin transformed. The means were compared using Fisher's least significant difference (LSD) test ($P < 0.05$).

3 Results

3.1 Chemical composition of the tested EOs

A total of 171 compounds were determined in all analyzed EOs, from which 30 (95.63%), 17 (92.53%), 15 (99.18%), 18 (96.11%), 17 (99.80%), 35 (99.66%) and 39 (98.95%) in *E. camaldulensis, E. lesouefii, E. occidentalis, E. torquata, T. capitata, M. piperita* and *S. chamaecyparissus* EO respectively (Table 1). Components were clustered by phytochemical groups in monoterpene hydrocarbons, oxygenated monoterpenes, sesquiterpene hydrocarbons, oxygenated sesquiterpenes, ketones and others, and listed according to the order of their elution on a methyl silicone HP-1 column.

In *E. camaldulensis* EO, the monoterpene compounds constituted 88.25% (49.32% the monoterpene hydrocarbons and 38.93% de oxygenated hydrocarbons, with p-cymene (38.83%), 1,8-cineole (18.67%) and cryptone (10.91%), as main compounds.

Regarding *E. lesoufii* EO, oxygenated monoterpenes (56.82%) were the main qualitative and quantitative phytochemical group found, being this EO rich in 1,8-cineole (53.42%). Oxygenated sesquiterpenes (33.45%) was the second most important group, with globulol

29.99 %, being the main compound in this fraction and the second more abundant constituent in the EO.

In *E. occidentalis* EO, the main phytochemical group was the oxygenated monoterpenes fraction, which accounted for 72.15%, represented essentially by 1,8-cineole (56.84%) and trans-pinocarveol (10.75%), followed by oxygenated monoterpenes (25.04%), with α-pinene (24.3%) as the most abundant component on this fraction, and second most abundant in the EO composition.

E. torquata EO, was characterized predominantly by ketones which constituted about 40% of the EO composition, mainly torquatone (39.76%), and a high fraction of oxygenated sesquiterpene (25.94%), among which γ-eudesmol (11.14%) and α-eudesmol (8.00%) were the most abundant. The compounds 1,8-cineole (13.31%) and trans-pinocarveol (4.22%) were the main constituents of the oxygenated monoterpene fraction (18.75%), while α-pinene 10.13 % constituted almost the totality of the monoterpene hydrocarbons group.

T. capitata EO was characterized by a high content of oxygenated monoterpenes (74.0%) with a high carvacrol percentage (72.30%), followed by monoterpene hydrocarbon fraction (22.5%), among which p-cymene (8.93%) and γ-terpinene (7%) were the main components. β-caryophyllene (3.14%) and caryophyllene oxide (0.14) were the only compounds detected in oxygenated and hydrocarbon sesquiterpenes, respectively.

The EO of *M. piperita* was outlined by a great amount of oxygenated monoterpenes (95.35%), characterized by menthol (51.8%) and menthone (20.5%). Lower percentage of sesquiterpene hydrocarbons (2.22%) and monoterpenes (1.95%) were detected.

Finally, the oxygenated monoterpenes (39.32%) were the most abundant fraction in *S. chamaecyparissus* EO, with 1,8-cineole (17.50%) as main compound. Other important constituents of this EO were, germacrene-D (12.60%), viridiflorol (13.56%) and 8-methylene-3-oxatricyclo [5,2,0,0(2,4)] nonane (12.24%).

Table 1. Chemical composition of essential oils extracted by hydrodistillation from *E. camaldulensis* (EC), *E. lesouefi* (EL); *E. occidentalis (*EO); *E. torquata* (ET); *T. capitata* (TC), *M. piperita* (MP) and *S. chamaecyparissus* (SC). KI, Kovats index.

Compounds	KI	EC	EL	EO	ET	TC	MP	SC
Monoterpene hydrocarbons (%)		**49.32**	**0.87**	**25.04**	**11.59**	**22.54**	**1.95**	**9.30**
Santolina triene	908	-	-	-	-	-	-	0.13
α-Thujene	930	1.13	-	-	-	0.89	0.01	-
α-Pinene	938	**3.93**	0.77	**24.3**	**10.13**	0.74	0.28	0.85
Thuja-2,4(10)-diene	947	0.13	-	0.16	-	-	-	-
Camphene	951	_	-	0.17	-	-	-	0.28
Sabinene	975	0.57	-	-	-	-	0.14	0.17
β-Pinene	978	3.79	-	0.41	0.08	0.29	0.43	**3.98**
Myrcene	991	0.65	-	-	-	1.95	0.01	-
α-Phellandrene	1004	-	-	-	-	0.16	-	-
γ-Terpinene	1016	-	-	-	-	**7.77**	0.13	1.18
α-Terpinene	1016	-	-	-	-	1.61	-	0.69
p-Cymene	1025	**38.83**	0.10	-	1.38	**8.93**	0.18	2.01
Limonene	1029	-	-	-	-	0.20	0.73	-
β-Phellandrene	1038	t	-	-	-	-	_	-
(Z)-β-Ocimene	1040	-	-	-	-	-	0.03	-
cis-Linalool oxide	1078	t	-	-	-	-	-	-
iso-Terpinolene	1087	-	-	-	-	-	0.02	-
p-Cymenene	1090	0.29	-	-	-	-	-	-
Oxygenated monoterpenes (%)		**38.93**	**56.82**	**72.15**	**18.57**	**73.98**	**95.35**	**39.32**
1,8-Cineole (eucalyptol)	1031	**18.67**	**53.42**	**56.84**	**13.31**	0.11	4.31	**17.50**
Artemisia ketone	1062	-	-	-	-	-	-	4.63
(Z)-Sabinene hydrate	1070	-	-	-	-	-	0.76	-
born	1097	-	-	-	-	0.77	0.09	0.42
α-Pinene oxide	1099	-	-	0.12	-	-	-	-
Linalool	1104	0.27	-	-	-	-	-	-
trans-Thujone	1117	0.20	-	-	-	-	-	-
endo-Fenchol	1118	-	-	0.10	-	-	-	-
α-Campholenal	1130	t	-	0.21	-	-	-	-
Nopinone	1141	t	-	-	-	-	-	-
Camphor	1142	-	-	-	-	-	-	4.03
trans-Pinocarveol	1143	0.72	1.07	**10.75**	**4.22**	-	-	0.17
Camphene hydrate	1153	-	t	-	-	-	-	-
Menthone	1154	-	-	-	-	-	**20.52**	-
(E)-Pinocamphone	1159	-	-	-	-	-	-	0.18
(Z)-Chrysanthemol	1162	-	-	-	-	-	-	3.80
Menthofuran	1163	-	-	-	-	-	5.21	-
neo-Menthol	1165	-	-	-	-	-	3.12	-
Pinocarvone	1166	0.26	0.21	3.12	0.91	-	-	-
Borneol	1168	-	-	-	-	0.16	-	1.11
Isoborneol	1170	-	-	0.25	-	-	-	-
(Z)-Pinocamphone	1172	-	-	-	-	-	-	2.03
Menthol	1175	-	-	-	-	-	**51.81**	-
Terpinen-4-ol	1177	2.21	0.46	-	-	0.37	0.67	2.69
iso-menthol	1182	-	-	-	-	-	0.60	-
Neoisomenthol	1187	-	-	-	-	-	0.08	-
α-Terpineol	1188	0.35	0.44	0.45	0.13	-	0.17	0.21
trans-ρ-Mentha-1(7),8-dien-2-ol	1191	-	0.54	0.31	-	-	-	-

Myrtenal	1192	0.59	-	-	-	-	-	1.31
Myrtenol	1193	-	-	-	-	-	-	1.07
Cryptone	1196	**10.91**	-	-	-	-	-	-
Verbenone	1198	0.13	-	-	-	-	-	0.16
m-Cumenol	1230	-	-	-	-	-	-	-
cis-p-Mentha-1(7),8-dien-2-ol	1233	-	0.49		-	-	-	-
Pulegone	1236	-	-	-	-	-	0.83	-
Cumin aldehyde	1245	2.73	-	-	-	-	-	-
Carvone	1249	1.27	-	-	-	-	-	-
Carvotanacetone	1250	-	-	-	-	-	-	-
Piperitone	1251	-	-	-	-	-	0.32	-
neo-Menthyl acetate	1273	-	-	-	-	-	0.16	-
p-Menth-1-en-7-al	1279	-	-	-	-	-	-	-
α-Terpinen-7-al	1288	0.23	-	-	-	-	-	-
Menthyl acetate	1291	-	-	-	-	-	**6.56**	-
Thymol	1292	-	-	-	-	0.27	-	-
Carvacrol	1300	0.05	-	-	-	**72.30**	-	-
iso-Menthyl acetate	1303	-	-	-	-	-	0.16	-
Methyl geranate	1324	-	0.19	-	-	-	-	-
4-hydroxy-Cryptone	1326	0.14	-	-	-	-	-	-
3-oxo-ρ-Menth-1-en-7-al	1340	0.20	-	-	-	-	-	-
Sesquiterpene hydrocarbons (%)		**0.56**	**1.39**	**1.07**	**0.00**	**3.14**	**2.22**	**21.78**
α-Ylangene	1373	-	-	-	-	-	-	0.08
α-Bourbonene	1381	-	-	-	-	-	0.17	-
β-Caryophyllene	1415	-	-	-	-	**3.14**	1.47	0.39
β-Farnesene	1454	-	-	-	-	-	0.02	-
allo-Aromadendrene	1457	-	-	1.07	-	-	-	**4.23**
Aromandendrene	1464	0.56	1.28	-	-	-	-	-
trans-Cadina-1(6),4-diene	1473	-	-	-	-	-	-	0.36
Germacrene-D	1477	-	-	-	-	-	0.42	**12.60**
β-Selinene	1491	-	-	-	-	-	0.13	-
Elixene	1492	-	-	-	-	-	-	2.80
Bicyclogermacrene	1499	-	0.11	-	-	-	-	-
γ-Cadinene	1509	-	-	-	-	-	-	0.32
δ-Cadinene	1519	-	-	-	-	-	-	1.00
Oxygenated sesquiterpenes (%)		**6.82**	**33.45**	**0.92**	**25.94**	**0.14**	**0.00**	**15.64**
Bornyl acetate	1283	-	-	-	-	-	-	0.08
Spathulenol	1447	**6.82**	-	-	1.29	-	-	1.42
Globulol	1559	-	**29.99**	-	3.08	-	-	-
Caryophyllene oxide	1577	-	-	-	-	0.14	-	0.19
Viridiflorol	1587	-	1.01	0.92	0.42	-	-	**13.56**
β-Oplopenone	1602	-	-	-	-	-	-	0.16
Spathulenol isomer	1616	-	-	-	-	-	-	-
β-Eudesmol	1629	-	1.74	-	**11.14**	-	-	-
Eremoligenol	1633	-	-	-	0.09	-	-	-
γ-Eudesmol	1634	-	0.71	-	1.41	-	-	-
iso-spathulenol	1640	-	-	-	-	-	-	-
Agarospirol	1644	-	-	-	0.51	-	-	-
α-Cadinol	1649	-	-	-	-	-	-	0.23
α-Eudesmol	1662	-	-	-	**8.00**	-	-	-

Ketones (%)		**0.00**	**0.00**	**0.00**	**40.01**	**0.00**	**0.00**	**0.00**
Apodophylene	1716	-	-	-	0.14	-	-	-
Torquatone	1833	-	-	-	**39.76**	-	-	-
demethyl-Isotorquatone	1881	-	-	-	0.11	-	-	-
Others (%)		**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.14**	**12.91**
1-Butanol. 2-methyl-, propanoate	973	-	-	-	-	-	-	0.20
1-Octen-3-ol	980	-	-	-	-	-	0.02	-
3-Octanol	995	-	-	-	-	-	0.07	-
iso-Amyl 2-methyl butyrate	1101	-	-	-	-	-	0.02	-
n-Amyl isovalerate	1106	-	-	-	-	-	0.04	0.48
8-methylene oxatricyclo[5,2,0,0(2,4)]nonane	1117	-	-	-	-	-	-	**12.24**
TOTAL IDENTIFIED (%)		**95.63**	**92.53**	**99.18**	**96.11**	**99.80**	**99.66**	**98.95**

In bold, the most significant compounds for each EO t<0.04

3.2 Germination trials

The germination of all the species assayed was significantly affected by the applied EOs, however, the most sensitive species to the EOs tested were *A. fatua* and *A. retroflexus* with 0.19 and 0.30 germination, respectively (Table 2), whereas *E. crus-galli* was significantly the most resistant weed, followed by *P. oleracea* which was the second less susceptible species.

Table 2. Overall essential oils germination effects per species.

Species	Germination (± SE)
Amaranthus retroflexus	0.30 ± 0.02 c
Portulaca oleracea	0.63 ± 0.02 b
Avena fatua	0.19 ± 0.01 d
Echinochloa crus-galli	0.78 ± 0.01 a

Germination data showed are the percentage values arcsin transformed.

6.1.1 A. retroflexus

All selected EOs at all the applied concentrations exerted significant phytotoxic effects on *A. retroflexus* seed germination as compared to the control (Table 3) The reduction of the germination appeared noteworthy already at the minimum concentration of all EOs. *T. capitata* EO provided the strongest activity, with a complete inhibition of germination at 1 µl/ml concentration *S. chamaecyparissus* and *E. camaldulensis* EOs reached the total germination inhibition at the dose of 2µl/ml and *M. piperita* EO at 4µl/ml. Moderate performances were recorded with *E. lesouefii, E. torquata* and *E. occidentalis* EOs which showed at 4µl/ml a germination reduction up to 4 %, 6% and 13% respectively (Table 3).

6.1.2 P. oleracea

The EO of *T. capitata* was the most effective, as it completely blocked *P. oleracea* seed germination at 2 µl/ml dose, also, it showed phytotoxic significant effects already at the minimum concentration tested at 0.125 µl/ml (Table 4). The rest of applied EOs did not reach the total inhibition even with the highest concentrations. However, they significantly reduced *P. oleracea* seed germination. At the same concentration, 8 µl/ml, *S. chamaecyparissus* showed a slightly greater inhibitory effect (97.36% of germination reduction) than *M. piperita, E. torquata* and *E. occidentalis* with 92.10 %, 91.35 % and 88.88 % of reduction, respectively. (Table 4).

6.1.3 A. fatua

A. fatua seed germination was completely blocked with all applied EOs at different extent. *T. capitata* and *M. piperita* showed the strongest phytotoxic effects, controlling completely *A. fatua* germination at 0.5 µl/ml, while all the other EOs, except *E. torquata* reached complete inhibition with 2 µl/ml. *E. torquata* showed germination stoppage with its highest dose 4µl/ml (Table 5).

6.1.4 E. crus-galli

In this set of trials, all EOs assayed exhibited significant reduction of the germination at their lowest dose 0.25 µl/ml. (Table 6). However, the phytotoxicity of *T. capitata* and *M. piperita* was highly remarkable, exhibiting a total suppression of *E. crus-galli* seed germination at the highest dose, 2 µl/ml. On the other hand, the rest of tested EOs could not block *E. crus-galli* germination. However, at the highest dose assayed, 2 µl/ml, they significantly reduced it by 28.08 % for *E. camaldulensis*, 21.34% for *E. lesouefii,* 25.84% for *E. torquata*, and 37.07% for *S. chamaecyparissus* (Table 6).

Table 3. *In vitro* effects of *E. camaldulesnis, E. lesouefii, E. occidentalis, E. torquata, T. capitata, M. piperita, and S. chamaecyparissus,* EOs against *A. retroflexus* seed germination

	Germination (% ± SE)						
Concentrations (µl/ml)	***E. camaldulensis***	***E. lesouefii***	***E. occidentalis***	***E. torquata***	***T. capitata***	***M. piperita***	***S. chamaecyparissus***
Control (0)	82.0 ± 3.4 a	82.0 ± 3.4 a	82.0 ± 3.4 a	82.0 ± 3.4 a	87.0 ± 2.0 a	87.0 ± 2.0 a	87.0 ± 2.0 a
0.125	-	-	-	-	62.0 ± 6.8 b	-	-
0.25	-	-	-	-	18.0 ± 1.2 c	-	-
0.5	4.0 ± 2.9 b	16.0 ± 4.3 b	63.0 ± 10.9 b	18.0 ± 5.6 b	6.0 ± 2.9 d	16.0 ± 7.9 b	14.0 ± 3.3 b
1	1.0 ± 1.0 b	14.0 ± 4.0 b	55.0 ± 3.5 b	14.0 ± 6.2 b	0.0 ± 0.0 e	8.0 ± 5.8 bc	4.0 ± 5.2 c
2	0.0 ± 0.0 b	10.0 ± 2.7 bc	26.0 ± 7.0 c	9.0 ± 2.4 b	-	1.0 ± 1.0 c	0.0 ± 0.0 c
4	0.0 ± 0.0 b	4.0 ± 1.9 c	13.0 ± 5.4 c	6.0 ± 4.0 b	-	0.0 ± 0.0 c	0.0 ± 0.0 c

Values are means ± standard error after 14 days of incubation. Within each EO, different letters in the same column indicate that mean are different at the 95% level of probability ($p < 0.05$) using Fisher's LSD test.

Table 4. *In vitro* effects of *E. camaldulesnis, E. lesouefii, E. occidentalis, E. torquata, T. capitata, M. piperita, and S. chamaecyparissus*, EOs against *P. oleracea* seed germination

	Germination (% ± SE)						
Concentrations (µl/ml)	***E. camaldulensis***	***E. lesouefii***	***E. occidentalis***	***E. torquata***	***T. capitata***	***M. piperita***	***S. chamaecyparissus***
Control (0)	81.0 ± 1.9 a	81.0 ± 1.9 a	81.0 ± 1.9 a	81.0 ± 1.9 a	76.0 ± 5.8 a	76.0 ± 5.8 a	76.0 ± 5.8 a
0.125	-	-	-	-	26.0 ± 4.8 b	-	-
0.25	74.0 ± 2.9 a	75.0 ± 2.2 a	73.0 ± 2.5 ab	66.0 ± 6.8 b	17.0 ± 4.1 bc	-	-
0.5	62.0 ± 4.4 ab	59.0 ± 3.7 b	69.0 ± 2.4 abc	58.0 ± 5.8 b	9.0 ± 2.9 c	61.0 ± 2.9 b	68.0 ± 3.4 ab
1	46.0 ± 9.3 b	54.0 ± 7.9 b	64.0 ± 5.5 bc	51.0 ± 4.6 bc	2.0 ± 1.2 d	54.0 ± 2.4 bc	56.0 ± 2.9 b
2	12.0 ± 6.5 c	27.0 ± 2.8 c	60.0 ± 4.5 c	42 ± 6.4 c	0.0 ± 0.0 d	39.0 ± 3.4 c	32.0 ± 5.4 c
4	4.0 ± 1.9 c	5.0 ± 2.7 d	35.0 ± 7.1 d	25 ± 3.5 d	-	13.0 ± 2.0 d	9.0 ± 2.9 d
8	-	-	9.0 ± 1.9 e	7.0 ± 2.5 e	-	6.0 ± 1.9 e	2.0 ± 1.2 e

Values are means ± standard error after 14 days of incubation. Within each EO, different letters in the same column indicate that mean are different at the 95% level of probability ($p < 0.05$) using Fisher's LSD test.

Table 5. *In vitro* effects of *E. camaldulesnis, E. lesouefii, E. occidentalis, E. torquata, T. capitata, M. piperita, and S. chamaecyparissus,* EOs against *A. fatua* seed germination

	Germination (% ± SE)						
Concentrations (µl/ml)	***E. camaldulensis***	***E. lesouefii***	***E. occidentalis***	***E. torquata***	***T. capitata***	***M. piperita***	***S. chamaecyparissus***
Control (0)	86.0 ± 5.2 a	86.0 ± 5.2 a	86.0 ± 5.2 a	86.0 ± 5.2 a	64.0 ± 5.8 a	64.0 ± 5.8 a	86.0 ± 5.2 a
0.125	-	-	-	-	56.0± 9.3 a	-	-
0.25	10.0 ± 4.5 b	-	-	-	14.0 ± 7.9 b	42.0 ± 9.6 b	-
0.5	6.0 ± 3.7 b	48.0 ± 7.4 b	24.0 ± 7.8 b	54.0 ± 9.3 b	0.0 ± 0.0 c	0.0 ± 0.0 c	28.0 ± 6.8 b
1	4.0 ± 2.7 bc	28.0 ± 6.8 c	18.0 ± 8.1 b	14.0 ± 4.3 c	0.0 ± 0.0 c	0.0 ± 0.0 c	4.0 ± 2.7 c
2	0.0 ± 0.0 c	0.0 ± 0.0 d	0.0 ± 0.0 c	2.0 ± 2.0 cd	-	0.0 ± 0.0 c	0.0 ± 0.0 c
4	0.0 ± 0.0 c	0.0 ± 0.0 d	0.0 ± 0.0 c	0.0 ± 0.0 d	-	0.0 ± 0.0 c	0.0 ± 0.0 c

Values are means ± standard error after 14 days of incubation. Within each EO, different letters in the same column indicate that mean are different at the 95% level of probability ($p < 0.05$) using Fisher's LSD test.

Table 6. *In vitro* effects of *E. camaldulesnis, E. lesouefii, E. occidentalis, E. torquata, T. capitata, M. piperita, and S. chamaecyparissus,* EOs against *E. crus-galli* seed germination

	Germination (% ± SE)						
Concentrations (µl/ml)	***E. camaldulensis***	***E. lesouefii***	***E. occidentalis***	***E. torquata***	***T. capitata***	***M. piperita***	***S. chamaecyparissus***
Control (0)	89.0 ± 3.5 a	89.0 ± 3.5 a	89.0 ± 3.5 a	89.0 ± 3.5 a	89.0 ± 3.5 a	89.0 ± 3.5 a	89.0 ± 3.5 a
0.25	80.0 ± 2.6 b	78.0 ± 2.0 b	81.0 ± 3.1 b	78.0 ± 2.0 b	50.0 ± 5.6 b	64.0 ± 4.5 b	77.0 ± 3.3 b
0.5	70.0 ± 3.7 bc	76.0 ± 3.1 b	76.0 ± 3.1 bc	72.0 ± 3.6 b	31.0 ± 6.7 c	37.0 ± 5.4 c	74.0 ± 3.4 b
1	67.0 ± 3.3 c	73.0 ± 3.3 b	73.0 ± 3.0 bc	68.0 ± 2.0 bc	13.0 ± 4.0 d	23.0 ± 6.8 d	69.0 ± 7.4 bc
2	64.0 ± 4.0 c	70.0 ± 3.0 b	66.0 ± 3.7 c	60.0 ± 2.8 c	0.0 ± 0.0 e	0.0 ± 0.0 e	56.0 ± 4.3 c

Values are means ± standard error after 14 days of incubation. Within each EO different letters in the same column indicate that mean are different at the 95% level of probability ($p < 0.05$) using Fisher's LSD test.

3.3 Seedling growth

6.1.5 A. retroflexus

All the applied EOs showed a significant inhibitory effect on seedling length with respect to the control. However, not all of them exerted significant phytotoxic effect at the minimum concentration. For the EOs that did not blocked seed germination with any concentrations, the maximum reduction of seedling length was found at the highest doses applied, 4 µl/ml, being 80.61% for *E. lesouefii*, 82.81% for *E. torquata* and 36% for *E. occidentalis* (Figure 1).

6.1.6 P. oleracea

In Figure 2 are shown results of seedlings length over time as affected by all the applied EOs. All of them exerted significant inhibitory effects on *P. oleracea* seedling growth with respect to the control at the minimum assayed concentration. At 4 µl/ml concentration *E. camaldulensis* attained 69% and *E. lesouefii* 77% of seedlings length reduction. At 8 µl/ml *S. chamaecyparissus* reached 86% of seedling length reduction followed by *E. torquata,* E. *occidentalis* and *M. piperita,* with reductions of 85%, 81 %, and 75%, respectively. The latter seems to be the more effective in inhibiting the germination than reducing seedlings length (Figure 2).

6.1.7 A. fatua

All assayed EOs have exerted significant phytotoxic effects on A. *fatua* seedling growth (Figure 3), coleoptile expansion (Table 7) and radicle elongation (Table 8) compared to the control at the lowest applied concentrations. Both *T. capitata* and *M. piperita* EOs, exerted the best performance by inhibiting 100 % seed germination (Figure 3), by blocking completely the coleoptile enlargement (Table 7) and the radical elongation (Table 8) at 0.5 µl/ml. No significant differences were noticed between the doses (0.125 and 0.25 µl/ml) on reducing radical length by *T. capitata* (Table 8). On the other hand, no significant differences within concentrations were observed, between total seedling length, coleoptile and radicle growth compared to the control for the rest of assayed EOs (Figure 3, Tables 7 and 8).

6.1.8 E. crus-galli

All assayed EOs have exerted significant phytotoxic effects on *E. crus-galli* seedling (Figure 4), coleoptile (Table 9) and radicle length (Table 10) of *E. crus-galli* compared to the control

at the lowest applied concentration 0.25 µl/ml. Only *T. capitata* and *M. piperita* achieved a total growth inhibition at the concentration 2µl/ml. At the same dose, *E. camaldulensis, E. torquata*, *E. lesouefii, S. chamaecyparissus* and *E. occidentalis* exhibited 67, 56, 54, 49 and 44% of total seedling growth reduction (Figure 4); 58, 53, 55, 53 and 35% inhibition of coleoptile length (Table 9), and 77, 59, 54, 44 and 56% diminution of radicle elongation, respectively (Table 10).

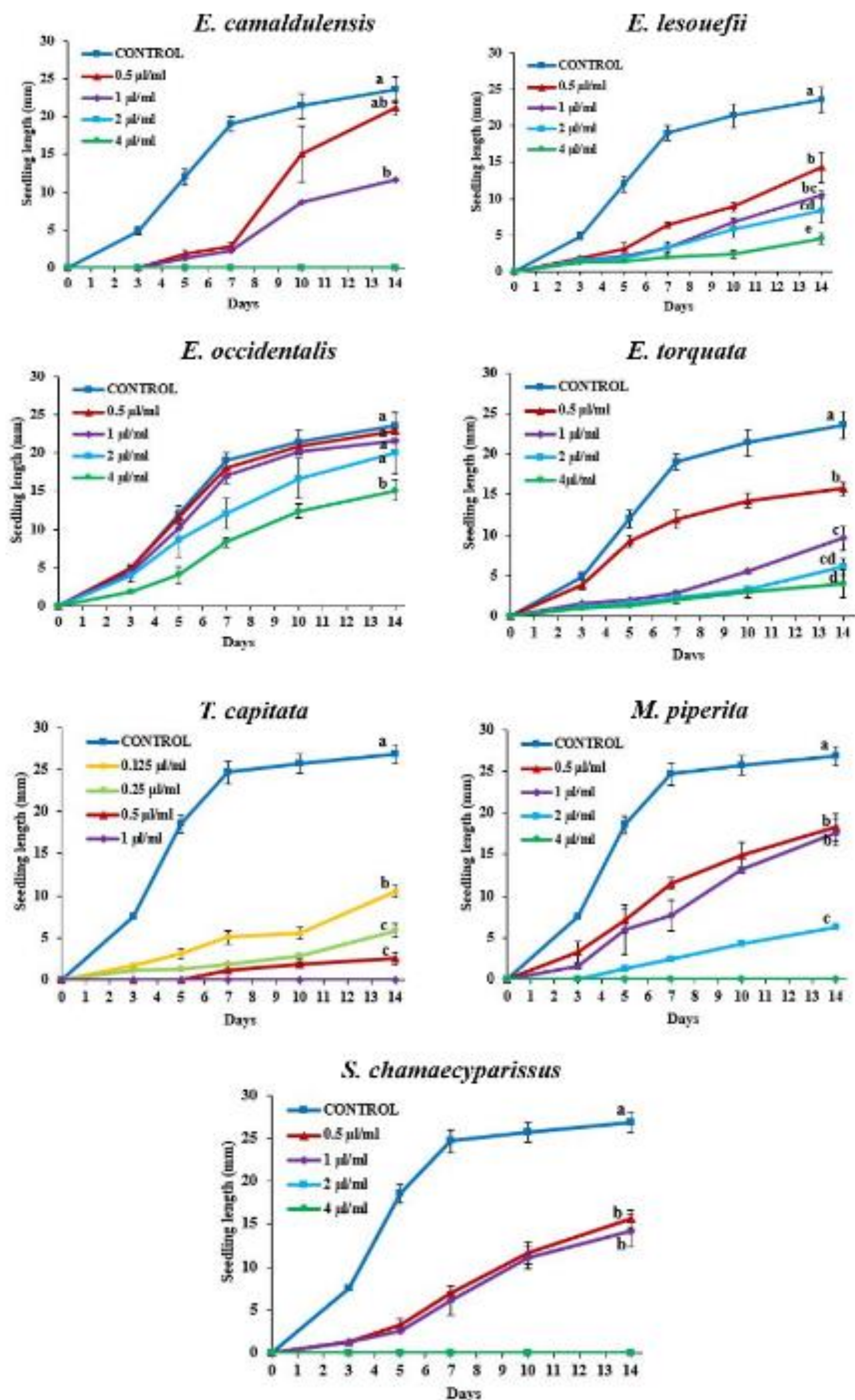

Figure 4. Seedling length (mm) (mean ± SE) measured for 14 days of *A. retroflexus* control or treated with EOs from *E. camaldulensis E. lesouefii,E. occidentalis, E. torquata, M. piperita, S. chamaecyparissus* at 0.5, 1, 2, 4 µl/ml and *T. capitata* at 0. 125, 0.25, 0.5, 1 µl/ml concentrations. For each EO, different letters indicate that means are different at the 95% level of probability ($p < 0.05$) using Fisher's LSD test.

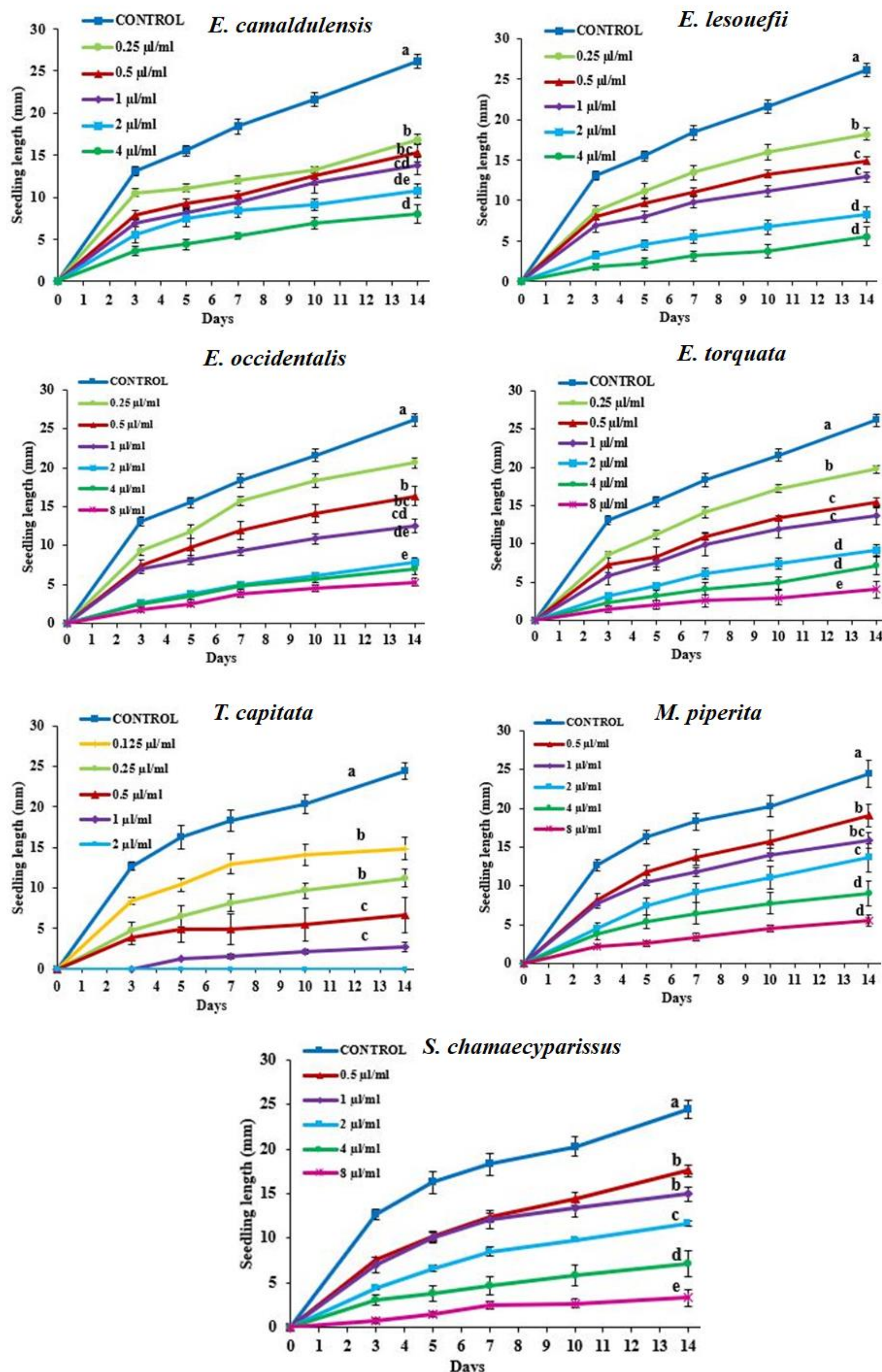

Figure 5. Seedling length (mm) (mean ± SE) measured for 14 days of *P. oleracea* control or treated with EOs from *E. camaldulensis and E. lesouefii* at 0.25, 0.5, 1, 2, 4 µl/ml concentrations, *E. occidentalis* and *E. torquata* at 0.25, 0.5, 1, 2, 4 ,8 µl/ml concentrations, *M. piperita* and *S. chamaecyparissus* at 0.5, 1, 2, 4, 8 µl/ml and *T. capitata* at 0.125, 0.25, 0.5, 1, 2 µl/ml concentrations. For each EO, different letters indicate that means are different at the 95% level of probability ($p < 0.05$) using Fisher's LSD test.

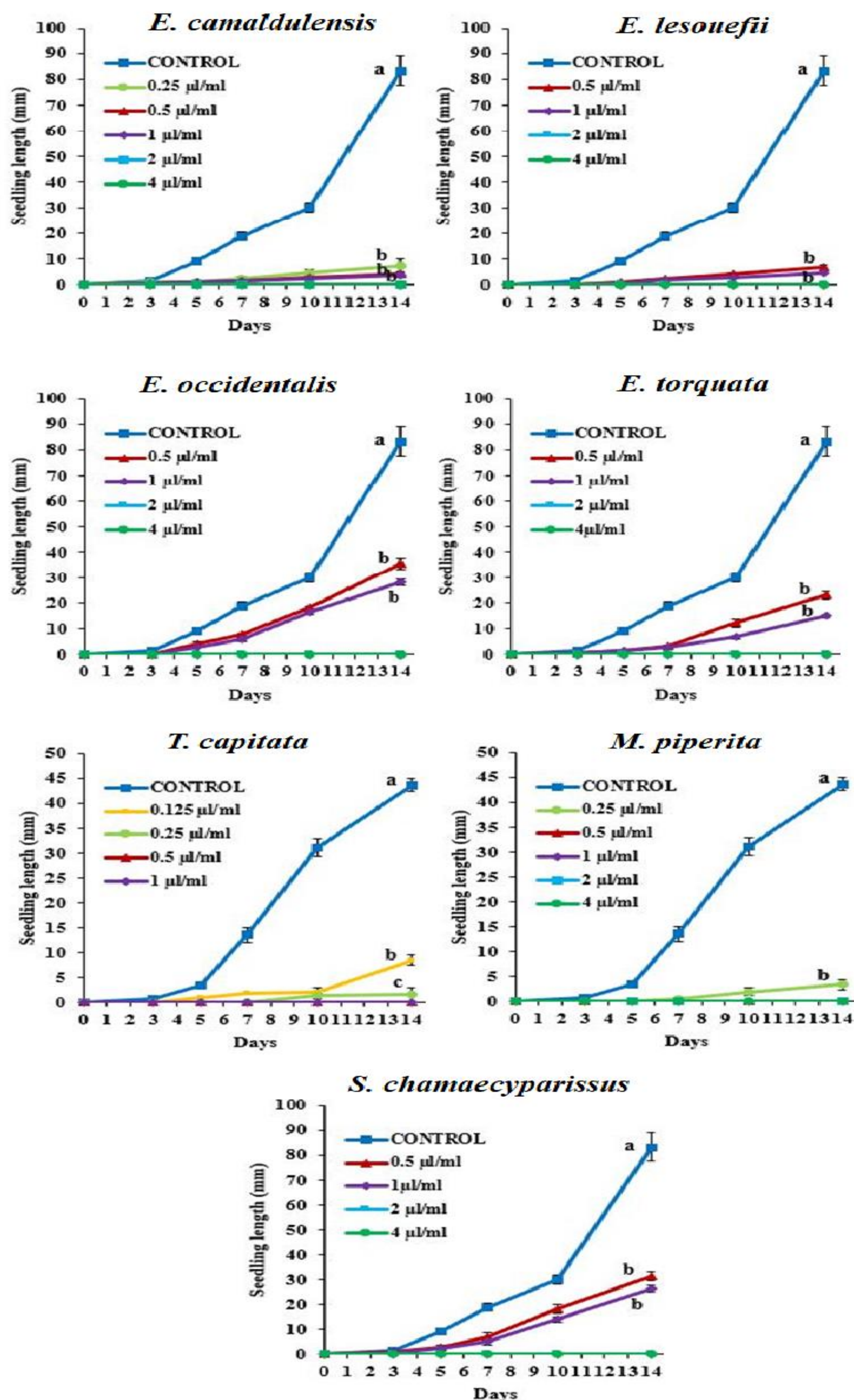

Figure 6. Seedling length (mm) (mean ± SE) measured for 14 days of *A. fatua* control or treated with EOs from *E. camaldulensis* at 0.25, 0.5, 1, 2, 4 µl/ml concentrations and *E. lesouefii* , *E. occidentalis, E. torquata* and *S. chamaecyparissus* at 0.5, 1, 2, 4 µl/ml concentrations*, M. piperita,* at 0.25, 0.5, 1, 2 µl/ml *and T. capitata* at 0.125, 0.25, 0.5, 1 µl/ml concentrations. For each EO, different letters indicate that means are different at the 95% level of probability ($p < 0.05$) using Fisher's LSD test.

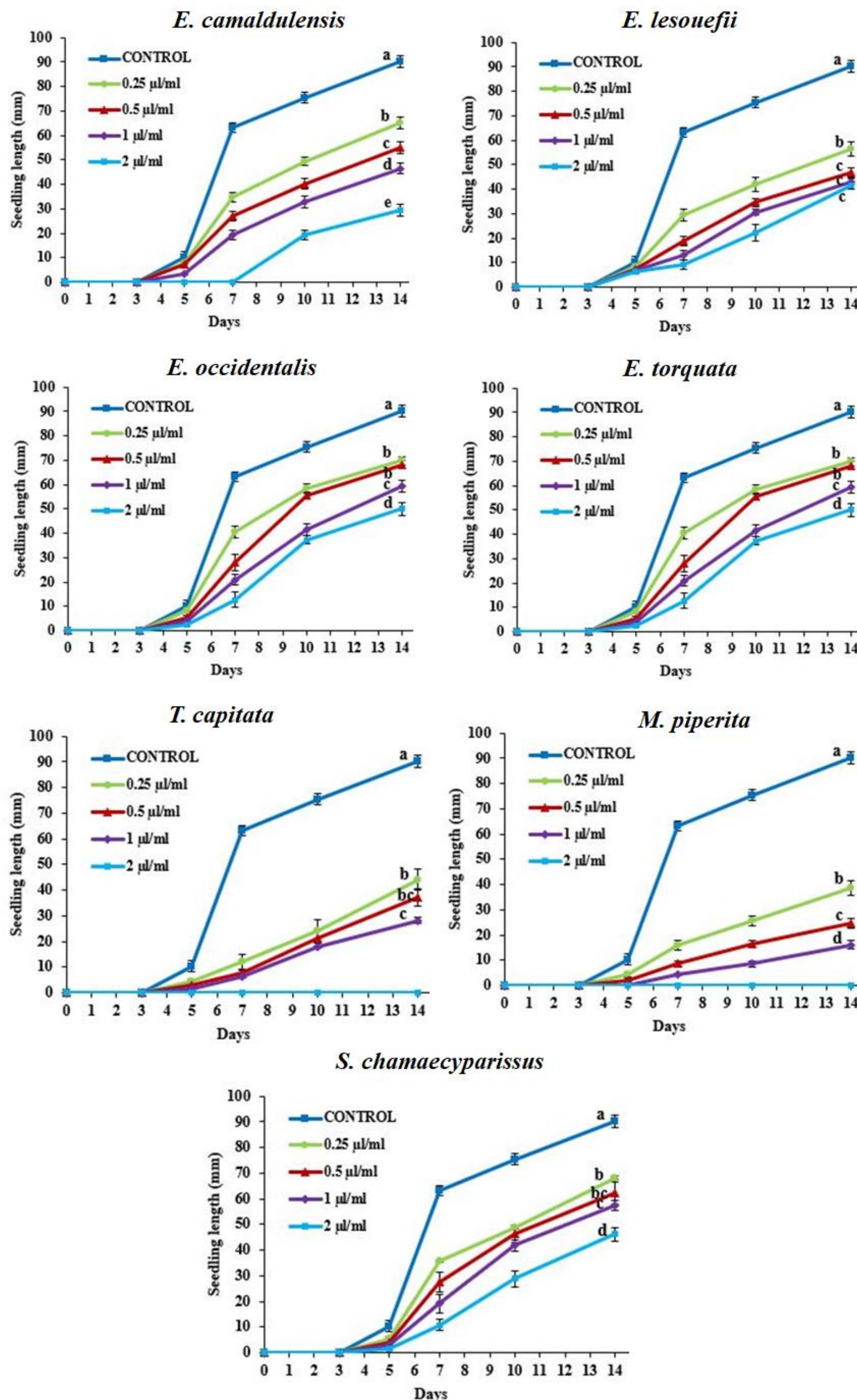

Figure 7. Seedling length (mm) (mean ± SE) measured for 14 days of *E. crus-galli* control or treated with EOs from *E. camaldulensis, E. lesouefii , E. occidentalis, E. torquata and S. chamaecyparissus , M. piperita, and T. capitata* at 0.25, 0.5, 1, 2 µl/ml concentrations. For each EO, different letters indicate that means are different at the 95% level of probability ($p < 0.05$) using Fisher's LSD test.

Table 7. *In vitro* effects *of E. camaldulesnis, E. lesouefii, E. occidentalis, E. torquata, T. capitata, M. piperita, and S. chamaecyparissus*, EOs against *A. fatua* coleoptile growth.

	Coleoptile length in mm (± SE)						
Concentrations (µl/ml)	***E. camaldulensis***	***E. lesouefii***	***E. occidentalis***	***E. torquata***	***T. capitata***	***M. piperita***	***S. chamaecyparissus***
Control (0)	45.25 ± 5.2 a	45.25 ± 2.90 a	45.25 ± 2.90 a	45.25 ± 2.90 a	22.52 ± 0.78 a	22.52 ± 0.78 a	45.25 ± 2.90 a
0.125	**	**	**	**	6.84 ± 1.43 b	**	**
0.25	5.27 ± 2.34 b	**	**	**	1.11 ± 0.48 c	1.82 ± 0.56 b	**
0.5	3.36 ± 0.46 b	4.00 ± 0.61b	18.38 ± 2.76 b	12.47 ± 0.98 b	**	**	15.83 ± 0.96 b
1	2.42 ± 0.28 b	2.26 ± 0.43b	14.56 ± 0.84 b	7.24 ± 1.07 b	**	**	12.79 ± 0.47 b
2	**	**	**	**	**	**	**
4	**	**	**	**	**	**	**

Values are means ± standard error after 14 days of incubation. Within each EO, different letters in the same column indicate that means are different at the 95% level of probability ($p < 0.05$) using Fisher's LSD test; *: indicates 0 mm in case of 0 germination

Table 8. *In vitro* effects *of E. camaldulesnis, E. lesouefii, E. occidentalis, E. torquata, T. capitata, M. piperita, and S. chamaecyparissus,* EOs against *A. fatua* radical growth

	Radicle length in mm (± SE)						
Concentrations (µl/ml)	***E. camaldulensis***	***E. leousoufii***	***E. occidentalis***	***E. torquata***	***T. capitata***	***M. piperita***	***S. chamaecyparissus***
Control (0)	37.93 ± 2.90 a	37.93 ± 2.90 a	37.93 ± 2.90 a	37.93 ± 2.90 a	21.12 ±1.23 a	21.12 ± 1.23 a	37.93 ± 2.90 a
0.125	**	**	**	**	1.55 ± 0.37 b	**	**
0.25	1.82 ± 0.31 b	**	**	**	0.39 ± 0.11 b	1.47 ± 0.43 b	**
0.5	1.73 ± 0.37 b	2.93 ± 0.58 b	17.10 ± 1.45 b	10.40 ± 1.17 b	**	**	15.54 ± 0.95 b
1	1.13 ± 0.20 b	2.59 ± 0.30 b	13.38 ± 1.05 b	14.00 ± 4.3 b	**	**	13.51 ± 0.81 b
2	**	**	**	**	**	**	**
4	**	**	**	**	**	**	**

Values are means ± standard error after 14 days of incubation. Within each EO, different letters in the same column indicate that means are different at the 95% level of probability ($p < 0.05$) using Fisher's LSD test; *: indicates 0 mm in case of 0 germination

Table 9. *In vitro* effects of *E. camaldulesnis, E. lesouefii, E. occidentalis, E. torquata, T. capitata, M. piperita, and S. chamaecyparissus*, EOs against *E. crus-galli* coleoptile growth

	Coleoptile length in mm (± SE)						
Concentrations µl/ml	***E. camaldulensis***	***E. leousoufii***	***E. occidentalis***	***E. torquata***	***T. capitata***	***M. piperita***	***S. chamaecyparissus***
Control (0)	51.28 ± 1.88 a	51.28 ± 1.88 a	51.28 ± 1.88 a	51.28 ± 1.88 a	51.28 ± 1.88 a	51.28 ± 1.88 a	51.28 ± 1.88 a
0.25	36.34 ± 1.59 b	28.92 ± 1.61 b	40.61 ± 0.65 b	27.37 ± 1.40 b	26.03 ± 1.59 b	27.46 ± 2.14 b	35.43 ± 2.62 b
0.5	31.37 ± 1.83 c	24.76 ± 0.81 c	39.49 ± 1.49 b	26.89 ± 0.72 b	22.56 ± 1.84 b	16.41 ± 1.42 c	31.32 ± 2.87 b
1	28.26 ± 1.47 c	24.53 ± 1.07 c	36.48 ± 1.86 bc	25.13 ± 0.90 b	15.50 ± 0.65 c	10.04 ± 0.99 d	30.42 ± 1.47 b
2	21.49 ± 1.68 d	23.47 ± 1.07 c	32.75 ± 1.87 c	24.16 ± 0.71 b	**	**	23.78 ± 1.20 c

Values are means ± standard error after 14 days of incubation. Within each EO, different letters in the same column indicate that means are different at the 95% level of probability ($p < 0.05$) using Fisher's LSD test; *: indicates 0 mm in case of 0 germination

Table 10. *In vitro* effects of *E. camaldulesnis, E. lesouefii, E. occidentalis, E. torquata, T. capitata, M. piperita, and S. chamaecyparissus,* EOs against *E. crus-galli radicle* growth

	Radicle length in mm (± SE)						
Concentrations µl/ml	***E. camaldulensis***	***E. leousoufii***	***E. occidentalis***	***E. torquata***	***T. capitata***	***M. piperita***	***S. chamaecyparissus***
Control (0)	38.81 ± 1.12 a	38.81 ± 1.12 a	38.81 ± 1.12 a	38.81 ± 1.12 a	38.81 ± 1.12 a	38.81 ± 1.12 a	38.81 ± 1.12 a
0.25	28.65 ± 1.07 b	27.45 ± 1.35 b	29.16 ± 1.72 b	23.59 ± 1.29 b	18.00 ± 2.28 b	11.18 ± 1.22 b	32.38 ± 1.06 b
0.5	23.64 ± 1.17 c	22.11 ± 1.47 c	28.32 ± 0.68 b	19.60 ± 1.22 c	14.60 ± 1.66 b	8.33 ± 0.81 bc	31.01 ± 1.77 bc
1	18.27 ± 1.35 d	18.95 ± 1.58 cd	22.90 ± 1.06 c	18.44 ± 0.75 cd	12.70 ± 0.60 b	6.09 ± 0.68 c	27.00 ± 1.54 c
2	8.68 ± 0.40 e	17.72 ± 0.74 d	17.29 ± 1.53 d	16.26 ± 1.01 d	**	**	22.34 ± 2.15 d

Values are means ± standard error each after 14 days of incubation. Within each EO, different letters in the same column indicate that means are different at the 95% level of probability ($p < 0.05$) using Fisher's LSD test; *: indicates 0 mm in case of 0 germination

4 Discussion

To our knowledge the current study is the first in which the phytotoxic effects of the EOs from the following eucalyptus species, *E. occidentalis, E torquata* and *E. lesouefii,* were tested. On the other hand, EOs from the other species have been demonstrated to have phytotoxic effects, inhibiting the germination and growth of some weeds, so they could be an alternative to synthetic herbicides, harmless to the environment and people's health. EOs from different aromatic plants belonging to *Lamiaceae, Compositae, Myrtaceae* and *Verbenaceae* families have been reported to have allelopathic properties (Dudai *et al*., 1999; Angelini *et al*., 2003; Azirak and Karaman, 2008; Shafique *et al*., 2011).
Our findings confirm that the phytotoxic effects of EOs are closely related to several factors, one of them is the species on which they are applied (Lee *et al*., 2002; Verdeguer 2011). All tested EOs exerted phytotoxic effects in some extent, they were generally selective according to the assayed weed. Their effectiveness on controlling seed germination followed this order: *T. capitata* > *E. camaldulensis* > *S. chamaecyparissus* > *M. piperita* > *E. lesouefii* > *E. torquata* > *E. occidentalis* for *A. retroflexus*; *T. capitata* > *E. camaldulensis* > *E. lesouefii* >*S. chamaecyparissus* > *M. piperita* > *E. torquata* > *E. occidentalis* for *P. oleracea; T. capitata* > *M. piperita* > *E. camaldulensis* > *S. chamaecyparissus* > *E. occidentalis* > *E. lesouefii* > *E. torquata* for *A. fatua; and T. capitata* > *M. piperita* > *S. chamaecyparissus* >*E. torquata* > *E. camaldulensis* > *E. occidentalis* > *E. lesouefii* for *E. crus-galli*. From an overall, point of view, the germination sensitivity of the examined weeds to inhibitory effect of assayed EOs, was different. The order of sensitiveness of the species for EOs was as follows: *A. fatua* >*A. retroflexus*> *P. oleracea* > *E. crus-galli* (Table 2). The high ability of *P. oleracea* and *E. crus-galli* in facing chemical-induced stress, adopting molecular, biochemical and anatomical strategies, is largely reported (Juliano *et al*., 2010; Malpassi., 2006; Norswothy and Riar *et al*., 2013; Mahdavikia and Saharkhiz, 2015).

Other factor is the employed doses of EOs. Overall, all tested EOs showed a dose-dependent effects although sometimes, no significant differences were noticed between the doses. The higher the applied dose was, the greater the inhibitory effect became. The most powerful were those, which showed great inhibitory activity against all the species tested even at low concentrations, as was the case of *T. capitata* in our study, since it totally blocked the germination of the four weed species tested at lower concentrations as compared to the rest of the assayed EOs and reduced drastically their seedlings length, by 90.56% for *A. retroflexus*

(0.5 µL/mL) (Figure 1), 89% for *P. oleracea* (1 µL/mL) (Figure2) 96.56% for *A. fatua* (with 0.25 µL/mL dose) (Figure 3), 68.7 % for *E. crus-galli* (1 µL/mL) (Figure 4). Similar results were found with *Mentha spicata, Origanum onites, Origanum vulgare* ssp. hirtum, which inhibited the germination of all tested weeds (*Amaranthus retroflexus*, *Echinochloa crus-galli*, *Portulaca oleracea, Setaria verticillata* and crops (tomato, cotton and rice) on which they were tested (Argyropoulos *et al*., 2008).

Moreover, the chemical composition is another factor closely related to EOs activity. Indeed, some authors have stated that oils rich in oxygenated monoterpenes are more active and phytotoxic than those that have a high content of hydrocarbon monoterpenes (Asplund, 1968; Vaughn and Spencer, 1993, Scrivanti *et al*., 2003; López *et al*., 2009). However, not all tested EOs have shown greater activity at a higher content of oxygenated compounds, *E. camaldulensis* and *E. torquata* EOs with high contents of monoterpenes hydrocarbons (49.32%) and ketones (40%) respectively (Table1), showed evenly phytotoxic effects, suggesting that the complex mixture of compounds present in each EO constrains their activities. The bioactivity of EO, normally attributed to the majority compounds, may also be due to the combined effects of various minor components or to the synergistic effects of some compounds (Barney *et al*., 2005; Koroch *et al*., 2007), *T. capitata* EO was characterized by a high content of carvacrol (72.30%), followed by *p*-cymene (8.93%). In literature, three chemotypes of *T. capitata* have been described, depending on the content of thymol (1), carvacrol (2) or both (3) (Bounatirou *et al*., 2007). Our tested EO was carvacrol chemotype (2), indicating a similar profile as reported in previous studies (Napoli *et al*., 2010; Ballester-Costa *et al*., 2013; Alam *et al*., 2014; Gagliano *et al*., 2019; Zaïri *et al*., 2019). The phytotoxicity of carvacrol as a single compound, as well as from *T. capitata* and other species EOs, have been largely reported. For instance, carvacrol was able to total suppress seed germination and seedling growth of *Amaranthus retroflexus*, *Chenopodium album*, and *Rumex crispus* (Kordali *et al*., 2008). Also, it led to a significant increase of ungerminated fresh seeds in all weed species (100% in *P. oleracea*, 98.7% in *E. crus-galli*, and 72% in *C. album*) (Angelini *et al*., 2003). Furthermore, Saoud *et al*. (2013) outlined that carvacrol (83.86%) from *T. capitata* EO inhibited the germination of *Sinapis arvensis*. *T. capitata* EO from populations growing in Tunisia with carvacrol content 69.15% reduced the germination of *Phalaris canariensis* and *Lolium rigidum* (Hanana *et al*., 2017). Likewise, *P. oleracea* germination was blocked at 0.5 and 1 µl/mL by *T. capitata* EO (carvacrol 77.02%) in Petri dishes assays (Verdeguer, 2011). Our results are according to other reports, in which oregano EO with 60.42% of carvacrol

content exhibited a total inhibition of *P. oleracea*, *L. multiflorum*, and *E. crus-galli* (Ibáñez and Blázquez, 2017). Other species, such as *S. rosmarinus*, *S. hortensis*, *T. vulgaris* and their phenolic compound (carvacrol) also demonstrated potent herbicidal effect on weed germination and seedling growth of various plant species (Angelini *et al*., 2003). Our results indicate that *T. capitata* EO is an effective broad-spectrum herbicide, due to its main component, carvacrol, as suggested by all the previously mentioned studies.

On the other hand, EO from *E. camaldulensis* was rich in *p*-cymene (38.83%) and 1,8-cineole (18.67%), being the most important constituents. Such results are coincident with EO composition of *E. camaldulensis* growing in Sardenia and in Turkey reported by Barra *et al* (2010) and Basak and Candan (2010) which found the same main compounds: *p*-cymene (42.7%) and 1,8-cineole (39.5%) for the EO from Sardenia, *p*-cymene (68.43%) and 1,8-cineole (13.92%) for the EO from Turkey. The phytotoxic effects of *E. camaldulensis* has been reported by controlling totally or reducing weed seed germination of many species such as: *Conyza canadensis*, *Parietaria judaica* and *C. album* (Verdeguer, 2011) and by inhibiting or reducing seedling growth of *Allium cepa*, *Spinacia oleracea*, *Lepidium sativa*, *Zea mays*, *Lycopersicon esculentum*, *Echinochloa crus-galli*, *Avena fatua* and *Rumex acetosella*. (Moradshahi *et al*., 2003). *E. camaldulensis* rich in 1,8-cineol (32.85%) and *p*-cymene (23.95%) from Turkey was able to control *Convolvulus arvensis, Melilotus offcinalis* and *Amaranthus retroflexus* in *in vitro* trials (Üstüner *et al*., 2018). However, the single compounds, *p*-cymene and 1,8-cineole were not suggested to be responsible for the phytotoxic and herbicidal effects. Since, in *in vitro* conditions, *p*-cymene as a single compound affected weakly the germination of *C. album*, *R. crispus* and *A. retroflexus*. In like manner, in greenhouse conditions, it did not exert any significant phytotoxic effect against the same plant species (Kordali *et al*., 2008). In addition, the inhibition index of 1,8-cineole on either germination or seedling growth of silverleaf nightshade was lower than that of the extracted eucalyptus spp EOs (Zhang *et al*., 2012). Nevertheless, Verdeguer *et al*. (2009) suggested according to other authors (Angelini *et al*., 2003) that 1,8-cineol is not the principal responsible for the allelopathic effect from *Eucalyptus* spp. EO. It has been suggested that the bioactive mechanism of *E. camaldulensis* EO against some weeds consists in decreasing the mitotic index in the apical meristem of *Allium cepa*, affecting Hill's reaction in the isolated chloroplasts of *Spinacia oleracea* and reduced the peroxidase activity in *Lepidium sativum*, *Echinochloa crus-galli, Avena fatua, Rumex acetosella, Zea mays* and *Lycopersicon esculentum* (Moradshahi *et al*., 2003), such several modes of action could not be associated to a single

major compound, but may result from the synergistic effects of several bioactive minor compounds.

M. piperita EO was constituted by menthol (51.81%) and menthone (20.52%) being the most abundant, followed by menthyl acetate (6.56%). These are the typical main components of peppermint EO that has been reported in previous investigations (Synowiec and Drozdek, 2016; Brahmi *et al*., 2017; Desam *et al*., 2017). The allelopathic potential of the EO from peppermint has been previously confirmed by some researchers (Campiglia *et al*., 2007; Dudai *et al*., 1999). Nevertheless, Fatemi *et al*. (2014) and Kamatou *et al*. (2013) corroborated that the biological properties of peppermint depend on its chemical composition, especially on its major components, menthol and menthone. The high percentage of menthol in peppermint oil confers its effectiveness in inhibiting ryegrass (62%) and wild mustard (44%) seed germination (Campiglia *et al*., 2007). Besides, *M piperita* EO showed strong phytotoxic effect against germination and seedling growth of some noxious weeds (Saharkhiz *et al*., 2010). The main component responsible for the depolarization of the membrane was menthol, followed by menthone. Withal, Mucciarelli *et al*. (2001) demonstrated that both menthol and menthone significantly reduced mitochondrial respiration in root cells. Finally, the knowledge of the phytotoxic effects of peppermint oil against weeds could be important for its development as bioherbicide, of broad-spectrum activity or selective.

Concerning *S. chamaecyparissus* EO, three components were the most abundant: 1,8-cineole (17.50%), viridiflorol (13.56%), and germacrene-D (12.60%), and 8-methylene-3-oxatricyclo [5,2,0,0(2,4)] nonane (12.24%). In our sample 1,8-cineole was the main component, as reported by Ahuja *et al*. (2004), Grosso *et al*. (2010) and Salah-Fatnassi (2017). However, variation in the number and abundance of identified compounds have been described in *S. chamaecyparissus* EOs from different origins, like Egypt (Aboutabl *et al*., 1987), France (Vernin, 1991), Italy (Tognolini *et al*., 2006) or Algeria (Nouasri *et al*., 2015). Grosso *et al*. (2010) proposed that *S. chamaecyparissus* EO can be a promising alternative to synthetic herbicides due to its potential herbicidal activities against the leaf growth of *L. perenne* as weed species and scarce and moderate damage to crops, especially on *L. sativa* seeds. Furthermore, seed germination inhibition of *P. oleracea* in Petri dishes was more efficient with *S. chamaecyparissus* EO than with the synthetic herbicide Agrocide that can persist in the soil for 54 days or Prowl with a half-life about 90 days (Grosso *et al*., 2010). Besides, *S. chamaecyparissus* EO rich in 1,8-cineole (24.8%) was able to control *P. oleracea* and *Vicia sativa* germination and reduced significantly shoot and root length. In addition, in greenhouse

conditions, Mercedes *et al*. (2020) demonstrated that *S. chamaecyparissus* EO was effective to control *Erigeron bonariensis*. The advantage of using volatile herbicides such as *S. chamecyparissus* EO is due to their low persistence in the field when compared with nonvolatile herbicides such as, for instance, Agrocide and Prowl (Grosso *et al*., 2010).

Compared with previous studies, the composition of our analyzed *E. torquata* EO sample, with torquatone (39.76%) and 1,8-cineol (13.31 %) being the predominant compounds, was similar to the same species from Australia: torquatone (42.0%), 1,8-cineole (11.2%) (Branska *et al*., 2005), but, differed greatly from the EO of *E. torquata* collected from Iran, being 1,8-cineol the main compound (Sefidkon *et al*., 2010). Regarding *E. ocidentalis* EO composition, 1,8-cineol (56.84%) was the most abundant compound along with α-pinene (24.3%), qualitative and quantitative differences were detected from the composition determined in studies conducted in Iran, in which the main constituents were t-cadinol (17.20%) and 1,8-cineol (15.50%) (Bande-Borujeni *et al*., 2018). Lastly, there are few studies about the chemical composition of *E. lesouefii* EO. Our findings revealed that *E. lesouefii* was the oil with the lowest content of monoterpene hydrocarbons (0.78%) as compared to the rest of studied EOs (Table 1). The major components were 1,8-cineole (53.42 %) and globulol (29.99%). This EO showed the modest phytotoxic effects compared to the rest of investigated EOs. However, it was able to totally inhibit the germination of *A. fatua*, one from the four weed species assayed. On the other hand, *E. torquata* suppressed the germination of *A. retroflexus*, *P. oleracea* and *E. crus-galli* by about 93, 91 and 33% respectively and reduced their seedlings length by about 83, 85 and 55% respectively, whereas *E. occidentalis* was capable to restrain seed germination of *A. rerflexus, P. oleracea* and *E. crus-galli* by 84, 89 and 26% respectively and to reduce their seedling length by 36, 80 and 44% respectively. Finally, *E. lesouefii* inhibited *A. retroflexus, P. oleracea and E. crus-galli* germination by 95, 94 and 21%, respectively and reduced their seedlings length by 80, 78 and 54%, respectively. So, the herbicidal effects of those EOs should be evaluated in greenhouse and field conditions to verify their potential use as natural herbicides.

Althoughnot much is known about the mechanism of the inhibitory activity on seedling growth of EOs and their compounds, yet inhibition of cell proliferation in root apical meristems has been discussed as one of the reasons for growth inhibition (Kaur *et al*., 2010). Golisz *et al*. (2008) noted that some allelochemicals can cause root cell death indirectly by facilitating the production of reactive oxygen species which may also act as signaling molecules leading to changes in hormonal balance during seed germination (Singh *et al*., 2005; Kaur *et al*., 2010;

Mutlu *et al.*, 2011). Many studies have revealed that phenolic compounds and volatile monoterpenes can seriously interfere with metabolic processes during germination, seedling growth and later growth stages (Kaur *et al.*, 2010; Scrivanti, 2010; Nourimand *et al.*, 2011; Poonpaiboonpipat *et al.*, 2013), leading to accumulation of lipid globules in the cytoplasm, reduction in some organelles, such as mitochondria, possibly due to inhibition of DNA and synthesis or disruption of membranes surrounding mitochondria and nuclei (Nishida *et al.*, 2005). Therefore, the secondary metabolites of plant species offer the organic potential to develop new herbicide formulations based on natural compounds, or as a guide towards identifying active components to obtain natural herbicides (Verdeguer *et al.*, 2011).

5 Conclusions

This research shows that there is a potential to control weed germination and seedling growth using natural compounds, since results clearly demonstrated that the tested EOs, to a different extent, were significantly effective against assayed weeds, controlling completely their germination process or reducing it and inhibiting significantly their seedling growth.

Among them, *T. capitata* was the most effective, exhibiting a very strong phytotoxic activity against all species tested. At lower doses, it blocked completely the seed germination of *A. retroflexus, P. olecerea, A. fatua* and *E. crus-galli.* This wide herbicidal action could be used for the development of broad-spectrum herbicides. The second most effective was *M. piperita* EO, which was capable to control completely the germination of all the weeds studied except *P. oleracea*, causing a severe reduction of their seedling length. *E. camaldulensis* and *S. chamaecyparissus* are the following in effectiveness being able to totally block the germination of 2 of the 4 investigated weeds at different concentrations: *A. retroflexus and A. fatua* and reducing their seedling length.

Finally, the three EOs from eucalyptus species which herbicidal activity was tested for the first time, *E. torquata, E. occidentalis* and *E. lesouefii* EOs, displayed lower phytotoxicity. They inhibit completely the germination of only the most sensitive weed *A. fatua*. They as well caused a significant decrease in seed germination and seedling growth of the rest of the weeds, suggesting a selective activity, so their possible use as selective bio-herbicides.

So far, the results obtained make feasible to suggest EOs application as bio-herbicides in controlled environments such as horticulture and in greenhouses. Nevertheless, more trials in real conditions are needed to completely understand the real potential of these EOs as bio-

herbicides. Persistence of EOs should be improved, these natural compounds being highly volatile and so less lasting in the environment. Nonetheless, further field research is necessary to develop an appropriate technology of EO application to control weed plant species.

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Chapter 2:

Soil bioindicators and herbicidal activity as affected by EOs extracted from three different Eucalyptus species applied in pre and post emergence.

1 Introduction

During the past years, an increasing demand for agricultural techniques respectful with the environment has led to reduce the reliance on synthetic pesticides and has encouraged the use of sustainable pest management techniques, according to the Directive 2009/128/EC, by which the European Union promoted integrated pest management to control pests, prioritizing techniques like cultural, mechanical or biological control methods, as an alternative to the use of synthetic pesticides (Durel *et al*., 2015). In this framework, sustainable weed control is one of the main challenges for integrated pest management, because of the weak presence of developed products on the market (Cordeau *et al*., 2016; Puig *et al*., 2018). Therefore, the investigation of new strategies based on the use of plant species able to produce and release phytotoxic compounds, could be an effective method to substitute synthetic herbicides (Benvenuti *et al*., 2017). Among these plants *Eucalyptus* spp. could offer a great opportunity (Singh *et al*., 2005; Zhao *et al*., 2007).

Eucalyptus genus (family Myrtaceae) is native of Australia, but it grows over a wide range of soils and climates. For this great adaptability, it has been introduced into North and South Africa, Asia, and Southern Europe (Spain, Portugal, and Italy) (Ahmad *et al*., 2005; Tolosana *et al*., 2010). *Eucalyptus* plantations are easily established and fast growing and can be highly profitable for the commercial production of timber and fiber products (Zhao *et al*., 2007). In Italy, about 60 species of *Eucalyptus* trees are distributed all-around, of these *Eucalyptus camaldulensis* Dehnh. (River red gum) is the most common species (Campisi *et al*, 2002). In Sicily, three species have naturalized: *E. camaldulensis, Eucalyptus occidentalis* Endl. (flat topped yate) and *Eucalyptus globulus* Labill (tasmanian blue gum) (Badalamenti *et al*., 2018).

Eucalytpus trees perform a variety of indirect services. They are tall and evergreen trees with fragrant foliage rich in oil glands, an excellent source of essential oils (EOs) which are a complex mixture of variety of monoterpenes and sesquiterpenes, and aromatic phenols. EOs from eucalyptus are among the most important commercially EOs in the world due to its wide range of biological properties, which could beexploitated for pest management (Green, 2002; Dhakad *et al*., 2018). In fact, several studies undertaken on Eucalyptus EOs showed their antimicrobial (Sartorelli *et al*., 2007; Gilles *et al*., 2010), antibacterial (Elaissi *et al*., 2011 and 2012 a and b), antifungal (Tolba *et al*., 2015), antioxidant (Tavakoli *et al*., 2017; Ray *et al*., 2018) and insecticidal (Batish *et al*., 2008; Filomeno *et al*., 2017) activities.

On the other hand, different species of the genus *Eucalyptus* have been the subject of several chemical, allelopathic and phytotoxic studies (Aragão *et al*., 2015; Batish *et al*., 2008; Kaur *et al*., 2012; Zhang *et al*., 2010, 2012, 2014). Setia *et al*. (2007) reported that volatile EOs from *E. citriodora* were phytotoxic to the germination and growth of several weed species, such as *Bidens pilosa*, *Amaranthus viridis*, *Rumex nepalensis* and *Leucaena leucocephala*. Similarly, Ramezani *et al*. (2009) reported that EOs from *E. nicholii* strongly inhibited the germination of *Amaranthus retroflexus*, *Portulaca oleracea* and *Acroptilon repens*. More specifically, *E. globulus* EO has been widely studied for its high content of allelochemicals and phytotoxic activity (Boulekbache-Makhlouf *et al*., 2013; Rassaeifar *et al*., 2013; Luís *et al*., 2016; Jaime and Ferrer, 2018). Also, *E. camaldulensis* EO inhibited the germination and growth of many weeds (Khan *et al*., 2009; Maaloul, 2019; Verdeguer *et al*., 2009 and 2020).

To our knowledge, no studies have been carried out before about the phytotoxic activity of *E. occidentalis* EO, although the phytotoxic potential of its aqueous extracts has been investigated (Saadaoui *et al*., 2014). Moreover, EOs are generally regarded as safer compounds because of their biodegradable nature (Isman, 2000), hence, they may serve as an excellent ecofriendly tool for weed management. It is thus worthwhile to explore *Eucalyptus* EOs as potential novel herbicides (Zhang *et al*., 2010). Therefore, the first aim of this study was to test the effectiveness of three *Eucalyptus* EOs, from the species *E. camaldulensis E. occidentalis* and *E. globulus* to control germination of weeds from the soil weed seed bank (applied in pre-emergence) and to control the spontaneous weeds emerged from the soil weed seed bank (applied in post-emergence).

Moreover, their *in-situ* application may alter soil microbial communities and affect soil biological equilibrium due to their possible accumulation in soil layers, thus influencing nutrient cycling (Meite, 2018; Salazar Ledesma *et al*., 2018; Shaheen *et al*., 2017).

Therefore, EOs impact on soil health is an emerging issue, since long term agricultural sustainability and productivity depends on maintaining favorable chemical, biological and physical features of the soil (Arias *et al*., 2005). As it is well known, soil is a heterogeneous mixture of organic and inorganic substances where chemical, biological and physical changes occur at the molecular level. So, a slight disturbance in its composition can result in a significant change in suitability for agricultural use. More specifically, soil microorganismsare the most important players within the ecosystem, for participating in the global nutrients cycle, decomposition of organic matter and release of nutrients, therefore they are crucial indicators

of soil health (Pankhurst *et al*., 1995; Bever, 2003). Thus, changes in microbial community size, community structure and activity can be used as early indicators of soil quality and health after being treated by *Eucalyptus* EOs (Acosta-Martínez *et al*., 2011; Brennan and Acosta-Martinez, 2017; Van Der Heijden *et al*., 2008).

Nevertheless, the second aim of the present study was to assess the potential of *E. camaldulensis, E. occidentalis* and *E. globulus*, on microbial biomass C, respiration and on the main microbial groups.

2 Materials and methods

2.1 Plant material and oil extraction

Fresh leaves of *E. camaldulensis*, *E. occidentalis* and *E. globulus* were collected from an afforested area near Agrigento (Sicily, Italy) (37° 28′ 10″ N; 13° 40′ 04″ E) on April 2017, and were subjected to hydro-distillation for 5 h, using a Clevenger apparatus. The EO yield was 0.21 %, 0.4%, and 0.4% (v/w) for *E. camaldulensis*, *E. occidentalis* and *E. globulus,* respectively. EOs were dried with anhydrous sodium sulphate and stored at 4 °C for further use. The yield percentage is expressed in (v/w): volume of oil obtained in milliliters, per grams of distilled plant x 100.

2.2 Chemical analysis of essential oils Gas chromatography–mass spectrometry (GC/MS)

An Agilent 6890 gas chromatograph instrument equipped with the mass spectrometer detector Agilent 5975 B was used for the chromatographic analyses. A fused silica capillary column SLB5MS (length 30 m, internal diameter 0.25 mm, 0.25 μm film thickness of silphenylene polymer equivalent in polarity to poly-5 % diphenyl / 95 % dimethyl siloxane phase) from Supelco, Italy, was the stationary phase. The injector in splitless mode had a temperature of 250°C. Experimental chromatographic conditions were as follows: Helium carrier gas at 1 ml/min; oven temperature program: 5 min isotherm at 40°C followed by a linear temperature increase of 4°C min-1 up to 200°C held for 2 min. MS scan conditions were: ionization technique, electronic impact (EI) at 70 eV, source temperature 230°C, interface temperature 280°C, mass scan range 33-350 m/z. The sample injected 1/50 diluted in pentane was 1 ml. For quantitative results, each sample was analyzed in GC-FID 2.23. The instrumental conditions for the gas chromatograph were the same as above reported. The FID detector was set at 250°C and 1ml of neat oil was injected. The quantitative composition was obtained by peak area

component was considered equal to 1 and three replicates of each sample were made. Internal standard was undecane. Identification of the individual components was based (i) on comparison of their GC retention indices (RI), determined relatively to the retention, time of a series of n-alkanes with linear interpolation, with those of authentic compounds, (ii) on computer matching with mass spectral libraries (NIST 05) and (iii) comparison with spectra of authentic samples or literature data.

2.3 Experimental set up

2.3.1 Pre-Emergence herbicidal tests

To test the potential effect of EOs on weeds from soil seed bank and on soil microorganisms, a short-term experiment in greenhouse conditions was set-up. The topsoil (0-5 cm) of an Inceptisol within the campus of Palermo University, never treated with synthetic herbicides, was used. Its main characteristics were sand 54.95 %, silt 18.85 %, clay 26.20 %, total organic carbon (TOC) 17.15 g kg^{-1}, pH 7.1, electric conductivity (EC) 0.65 dS m^{-1} and total carbonates 33.04 %. After sampling, the soil was air-dried and sieved at 1 cm. Subsequently, 500 g of this soil were placed in 20 aluminum trays (10 x 20 x 5 cm). The aliquots were brought up to 100 % of its water holding capacity (WHC) by adding 150 mL of tap water, followed by 70 mL of tap water containing 8 mL L^{-1} of each extracted EO. The amount of EO added were 112 µl 100 g^{-1} of soil for *E. camaldulensis* (EC)*, E. occidentalis* (EO) and *E. globulus* (EG), respectively. Considering that, EOs are immiscible with water, fitoil (Xeda, Italy) which is a biological adjuvant with 40% soybean oil, was used as emulsifier at a concentration of 1 ml L^{-1}. Two controls were used, the first with only water (Cw) and the second with water and Fitoil at the concentration of 0.1% (v/v) (Cf). Treatments were applied when the soils were bare before any weed seedlings sprouted. Four replicates per treatment were run, then placed in greenhouse for 30 days. During this period, soil humidity was weekly adjusted up to 50% of WHC by replenishing the water loss. At the 30th day, sprouted plants were cut, counted, identified, weighted, and dried at 100 °C to determine the following parameters: number of sprouted plants from each identified species, fresh and dry weight. Simultaneously, soils aliquots were sampled from the trays to determine the main soil microbial groups, and 20 g of each soil were incubated as described above in 200 mL jars at constant temperature (25.0 ± 0.5°C), to measure soil respiration rate (CO_2 emission) after 1, 3, 7, 10, 14, 18, 24 and 28 days of incubation. Water contents were added to maintain constant moisture after each gas sampling occasion.

2.3.2 Post-Emergence herbicidal test

For the post-emergence herbicidal test, the same soil for the pre emergence test was used. The soil was air dried, sieved at 1 cm and then aliquots of 500 g were placed in 20 aluminum trays (10 × 20 x 5cm) and incubated under greenhouse conditions. During incubation, the water content was kept at 50% of its WHC by irrigating it with tap water. After 20 days and when weed seedlings have emerged and were in the phenological stage of third leaf emerged, each EO was applied by spraying it with a manual trigger sprayer on the plants, at the same concentration used for the pre-emergence experiment, 112 µl 100 g^{-1} of soil for EC, EG and EO. The two controls Cw and Cf were used as well. Plants were repeatedly treated by the same concentration of EOs (112 µl 100 g^{-1} of soil for EC, EG and EO) three times, in alternating days for one week. This experiment was carried out in quadruplicate as well. After 15 days from the last application of the treatments, plants and soils were sampled in identic way as done for the pre-emergence experiment to determine also the following plants parameters: number of grown plants from each identified species, fresh and dry weight, and the following soil biochemical parameters: microbial biomass carbon (MBC), extractable C, main microbial groups. Soil respiration was determined by incubating 20 g of soil from each tray in 200 mL jars at constant temperature (25.0 ± 0.5°C) and sampling the CO_2 accumulated in the headspace of the jars at days 1, 3, 7, 10, 14, 18, 24 and 28 after incubation. Water contents were added to maintain constant moisture after each gas sampling occasion

2.3.3 Soil analyses

C mineralization was determined for both samples from postemergence and preemergence experiments, in the headspace of the incubated bottles as explained above by a gas chromatograph (Thermo Trace GC 90 GC, fitted with a thermal conductivity detected, Poropak Q column and using He as the carrier). After each CO_2 sampling, flasks were ventilated with fresh air for 30 min and then sealed again, after possible replenishment of lost soil moisture by distilled water. The C mineralization rate, expressed as mg CO_2–C kg^{-1} dry soil day^{-1}, was fitted to the following first-order decay function:

Mineralized $C = C_0 e^{-kt}$

where C0 is the biologically available C (mg kg^{-1}) at time zero (i.e. the intercept value), k is the decay rate constant, and t is the sampling incubation time. The total CO_2–C mineralized

over 28 days of incubation was calculated by the linear interpolation of two neighboring rates and the integration over time:

$$\text{Total C mineralized}= \sum_{i}^{n} [(r_i + r_{i+1}) * \frac{d}{2} + \cdots + (r_{n-1} + r_n) * \frac{d}{2}]$$

where i is the date of the first measurement of CO_2–C rate, n is the last day of measurement of CO_2–C rate, r is the CO_2–C rate expressed as mg CO_2–C kg^{-1} dry soil day^{-1}, d is the number of days between the two consecutive CO_2 rate measurements.

The soil microbial community structure of both experiments was analyzed by direct extraction of ester-linked fatty acids (ELFAs) according to the Schutter and Dick (2000) method. Briefly, 5 g of soil of each sample (fresh weight) were mixed with 15 ml 0.2 M KOH in methanol and 0.5 ml of internal standard (C19:0). Then, they were incubated at 37 ºC and shaken with vortex every 10-15 minutes for one hour, thus allowing the release and subsequent methylation of ELFAs. The soil pH was then neutralized by the addition of 3 ml 1.0 M acetic acid and fatty acid methyl esters (FAMEs) were extracted with 10 ml hexane. After being centrifugated at 1000-1500 rpm, the upper hexane layer was transferred to clean tubes and evaporated in a desiccating centrifuge. The dried samples were re-suspended in 200 µl hexane to be analyzed by a gas chromatograph (FOCUS™ GC, Thermo Scientific Inc., Waltham, MA USA) equipped with a flame ionization detector and a fused-silica capillary column Mega-10 (50 m x 0.32 mm I.D.; film thickness 0.25 µm). The GC temperature progression was initial isotherm at 115°C for 5 minutes, increase at a rate of 1.5 °C per minute from 115 to 230°C, and final isotherm at 230°C for 2 minutes. Both injection port and detector were set up at 250°C and helium at 1 mL min-1 in a constant flow mode was used as carrier. The injected volume was 1 µL (50:1 split ratio). Nonadecanoic acid methyl ester (19:0; cat no. N-5377, Sigma-Aldrich Co.) was used as internal standard for the quantification of FAMEs. The identification of the peaks was done by comparing the retention times of each FAMEs to known standards (Supelco Bacterial Acid Methyl Esters mix cat no. 47080-U and Supelco 37 Component FAME mix cat no. 47885-U). Fatty acids with less than 14 carbon atoms or more than 20 carbon atoms were excluded as considered originating from non-microbial sources. The FAs i15:0, a15:0, 15:0, i16:0, i17:0, 17:0, cy17:0,18:1ω7, cy19:0 were used to represent bacterial biomass while 18:2ω6,9 for fungal biomass. The FAs i15:0, a15:0, i16:0, i17:0 were chosen to represent Gram-positive bacteria (bac G+) while 16:1ω7, 18:1ω7, cy17:0 and cy19:0 for Gram-negative bacteria (bac G-) (Laudicina *et al.*, 2011). The summed mass of all the extracted ELFAs (nmol fatty acid g^{-1} soil) was used as indicator of Microbial biomass (MB) for postemergence experiment.

2.3.4 Statistical analysis

Trials were conducted in a randomized complete design with four replications. Plants data were evaluated for normality and homogeneity and then subjected to one-way ANOVA followed by Fisher's multiple comparison test (LSD intervals, Least Significant Difference, at $P<0.05$) for the separation of the means.

3 Results

3.1 Chemical composition of EOs

A total of 94 compounds were identified in the three tested EOs: 38 in *E. camaldulensis*, 29 in *E. occidentalis* and 27 in *E. globulus* (Table 1). The qualitative and quantitative composition of the three species, is reported in Table 1, in which the compounds were clustered in monoterpene hydrocarbons, oxygenated monoterpenes, sesquiterpene hydrocarbons, and oxygenated sesquiterpenes. The highest quantities of oxygenated monoterpenes (about 89%) were found in *E. occidentalis* EO, being 1,8-cineol the main compound (about 83%). Likewise, the other two EOs were also rich in oxygenated monoterpenes and in addition 1,8-cineol was also the most abundant compound (67% and 52% for *E. globulus* and *E. camaldulensis* EOs, respectively). *E. globulus* EO was characterized by the highest amount of monoterpene hydrocarbons (about 23%) represented mainly by α-pinene (about 22%), followed by *E. camaldulensis* EO (22% of monoterpene hydrocarbons) represented by 15% of α-pinene and 6% of *p*-cymene. *E. occidentalis* EO contained the lowest percentage of monoterpene hydrocarbons (about 10%), in which α-pinene accounted for 8%. Sesquiterpene hydrocarbons fraction was more abundant in *E. camaldulensis* EO than in the other EOs, with Aromadendrene (by about 3%) as the main compound of this fraction (Table 1).

Table 11. Chemical composition of essential oils from fresh and mature leaves of *E. camaldulensis* (EC), *E. occidentalis* (EO) and *E. globulus* (EG). KI, Kovats index.

Compounds	KI	Concentrations (% peak area)		
		EC	EO	EG
Monoterpene hydrocarbons		**22**	**10.03**	**23.18**
α-Pinene	939	**15.03**	**8.46**	**21.79**
α-Fenchene + Camphene	953	0.18	0.06	0.17
Thuja-2,4(10) -diene	957	0.11	0.05	0.10
Verbenene	967	-	-	0.05
β-Pinene	980	0.21	0.18	0.27
Decane	1000	0.05	0.02	-
α-Phellanderene	1005	0.06	0.07	-
p-Cymene	1026	**6.08**	1.10	0.73
δ-Terpinene	1062	0.12	0.04	0.02
p-Cymenene	1089	0.16	0.05	0.05
Oxygenated Monoterpenes		**74.3**	**89.26**	**75**
1,8-cineol (eucalyptol)	1035	**51.84**	**83.05**	**66.66**
Endo-fenchol	1112	0.31	0.08	0.14
cis-Para-menth-2-en-1-ol	1121	0.04	-	-
α-Campholenal	1125	0.20	0.10	0.11
trans-Pinocarveol	1139	**13.00**	**2.59**	**5.53**
Exo-methylcamphenilol	1155	0.06	-	-
Pinocarvone	1164	6.09	1.35	1.61
Borneol	1165	0.34	0.07	0.16
Pinocamphone	1173	0.05	-	-
Terpinen-4-ol	1175	0.20	0.13	0.05
p-cymen-8-ol	1183	0.09	-	0.01
trans p-mentha 1,7,8 dien-2-ol	1185	0.33	0.42	0.16
α-terpineol	1189	0.43	0.23	0.25
Myrtenal+Myrtenol	1193	0.34	0.08	0.10
Verbenone	1204	0.03	-	-
L-carveol	1210	0.05	-	-
trans-Carveol	1220	0.19	0.12	0.07
cis-p-Mentha-1(7),8-dien-2-ol	1225	0.21	0.26	0.11
d-Carvone	1234	0.05	0.05	-
Thymol	1290	0.30	0.12	-
3-p-Cymenol	1295	0.04	-	-
Sabynil acetate	1297	0.03	-	-
Carvacrol	1298	0.08	-	-
Exo 2-hydroxycineole acetate	1344	-	0.07	0.04
α-Terpinyl acetate	1400	-	0.54	-
Sesquiterpene hydrocarbons		**3.40**	**0.65**	**0.97**
β-Caryophyllene	1418	0.04	0.31	-
β-Gurjunene	1432	0.05	-	0.03
Aromadendrene	1439	3.06	0.20	0.85
α-Humulene	1450	-	0.14	-
Allo-Aromadendrene	1461	0.18	-	0.09
β-selinene	1490	0.07	-	-
Oxygenated sesquiterpenes		**0.30**	**0.06**	**0.85**
Selina-3,7(11) -diene	1542	-	-	0.72
Spathulenol	1576	0.30	-	-
Viridiflorol	1590	-	0.06	0.13
Total		**100**	**100**	**100**

3.2 Effects of pre-emergence treatment on plants

The results of the number of sprouted plants and their biomass parameters (fresh and dry weight) of the pre-emergence treatments are reported in Table 2. The number of identified species is reported in Table 3. The identified species were *Datura stramonium*, *Ecballium elaterium*, *Amaranthus retroflexus*., *Portulaca oleracea* and *Avena fatua.*

The three EOS tested did not show phytotoxic effects, as there were no significant differences in the number of sprouted plants among the different treatments with EOs and the controls (Table 2), only *E. globulus* EO reduced the number of sprouted plants significantly by 30% compared to Cw, but its number of plants was not significant different from Cf. Biomass plant parameters were not affected by any treatments. However, Cf declined significantly the dry weight compared to Cw. Regarding the number of identified species, it was halved by EG and EC (Table 2).

Table 12. Number of sprouted plants, their biomass (fresh and dry weight) and number of identified species in soil treated in pre-emergence with *E. camaldulensis* (EC), *E. occidentalis* (EO) and *E. globulus* (EG) essential oils.

Treatments	Number of Sprouted plants	Fresh weight (g)	Dry weight (g)	Number of identified species
Cw	27 ± 4 a	2.05 ± 0.39 a	0.18 ± 0.03 a	4
Cf	21 ± 1 ab	1.13 ± 0.22 a	0.08 ± 0.01 b	4
EC	25 ± 2 ab	1.48 ± 0.21 a	0.10 ± 0.02 ab	2
EO	23 ± 2 ab	1.67 ± 0.26 a	0.17 ± 0.03 a	4
EG	19 ± 1 b	1.60 ± 0.23 a	0.12 ± 0.02 ab	2

Reported results are means of four replicates ± standard error. Different letters in the same column indicate significant differences among treatments at $P<0.05$.

On the other hand, *E. camaldulensis* and *E. occidentalis* EOs did not show any significant differences from the controls on all identified species. While *E. globulus* EO exhibit significant phytotoxic effects on *A. retroflexus* compared to Cf, and on *P. oleracea* and *A. fatua* compared to Cw (Table3).

Table 13. Number of weeds from each identified species emerged from soil treated in pre-emergence with *E. camaldulensis* (EC), *E. occidentalis* (EO) and *E. globulus* (EG) essential oils.

Treatments	*Datura stramonium*	*Ecballium elaterium*	*Amaranthus retroflexus*	*Portulaca oleracea*	*Avena fatua*
Cw	2 ± 1 a	1 ± 1 a	0 ab	23 ± 4 a	1 ± 0 a
Cf	4 ± 1 a	0 a	1 ± 0 a	15 ± 0 ab	1 ± 0 ab
EC	5 ± 1 a	0 a	0 ab	20 ± 2 ab	0 ab
EO	4 ± 0 a	0 a	1 ± 1 ab	17 ± 2 ab	1 ± 1 ab
EG	5 ± 1 a	0 a	0 b	14 ± 1 b	0 b

Reported results are means of four replicates ± standard error. Different letters in the same column indicate significant differences among treatments at $P<0.05$.

3.3 Effects of post-emergence treatments in plants

Table 4 shows the results of the number of weeds grown and their biomass parameters (fresh and dry weight) of the post-emergence treatments (EC, EO, EG, EOs at 8µl mL^{-1}, two controls: Cw, water and Cf, water + Fitoil). Results of the number of identified weed species are presented in Table 5 Identified species were *Datura stramonium*, *Sonchus arvensis*, *Amaranthus retroflexus*, *Portulaca oleracea* and *Avena fatua*.

The highest number of grown plants was observed in Cf, which kept a number higher than all the other treatments, including the water control Cw. All used EOs significantly reduced the number of grown plants compared to Cf but no significant differences were observed between the treatments and Cw. No statistical differences were observed in plant biomass parameters between the treatments and the two controls. (Table 4).

Table 14. Effects of post-emergence treatments with 8 µL mL^{-1} of *E. camaldulensis* (EC), *E. occidentalis* (EO) and *E. globulus* (EG) EOs on the number of grown plants and their biomass parameters (fresh weight and dry weight) and number of identified species

Treatments	Number of grown plants	Fresh weight (g)	Dry weight (g)	Number of identified species
Cw	19 ± 3 ab	3.10 ± 0.32 a	0.23 ± 0.03 a	5
Cf	27 ± 1 a	3.72 ± 0.32 a	0.28 ± 0.03 a	5
EC	17 ± 2 b	2.49 ± 0.25 a	0.20 ± 0.02 a	3
EO	17 ± 2 b	2.80 ± 0.49 a	0.21 ± 0.05 a	3
EG	18 ± 1 b	3.29 ± 0.24 a	0.23 ± 0.02 a	3

Reported results are means of four replicates ± standard error. Different letters in the same column indicate significant differences among treatments at $P<0.05$.

D. stramonium was not present in the treatments with *E. occidentalis* and *E. globulus*, which showed significant differences with the controls (Table 5). The rest of the identified species were not affected by any EO (Table 5).

Table 15. Effects of post-emergence treatments with 8 µL mL^{-1} of *E. camaldulensis* (EC), *E. occidentalis* (EO) and *E. globulus* (EG) EOs on the number of the identified sprouted species: *Datura stramonium, Sonchus arvensis, Amaranthus retroflexus, Portulaca oleracea*.

Treatments	***Datura Stramonium***	***Sonchus arvensis***	***Amaranthus retroflexus***	***Portulaca oleracea***	***Avena fatua***
Cw	2 ± 1 a	1 ± 0 a	1 ± 1 a	14 ± 3 a	1 ± 0 a
Cf	2 ± 0 ab	2 ± 1 a	1 ± 0 a	22 ± 1 a	1 ± 1 a
EC	1 ± 0 abc	0 a	0 a	15 ± 2 a	1 ± 0 a
EO	0 c	1 ± 1 a	0 a	15 ± 2 a	1 ± 1 a
EG	0 c	2 ± 1 a	0 a	16 ± 1 a	1 ± 0 a

Reported results are means of four replicates ± standard error. Different letters in the same column indicate significant differences among treatments at $P<0.05$.

3.4 Effects of EOs on soil biochemical properties and main microbial groups

Carbon mineralization and the abundance of the main microbial groups were not affected by the application of EOs neither in pre-emergence (Table 6; Figure 1; Figure 2) nor in post-emergence (Table7; Figure 3; Figure 4). However, in post-emergence although elfas did not show significant differences, the B+/B- ratio was decreased by the EOs compared to the controls.

Table 16. Main microbial groups in nmol g-1 of soil treated in pre-emergence with *E. camaldulensis* (EC), *E. occidentalis* (EO) and *E. globulus* (EG) EOs

Treatments	Bacteria	Fungi	B+/B-	F/B
Cw	106.62 ± 8.82 a	34.48 ± 4.88 a	1.38 ± 0.01a	0.32 ± 0.02 a
Cf	97.65 ± 7.24 a	46.94 ± 1.11 a	1.35 ± 0.09a	0.48 ± 0.02 a
EC	96.91 ± 6.99 a	29.05 ± 2.90 a	1.34 ± 0.12a	0.30 ± 0.01 a
EO	96.50 ± 2.12 a	36.90 ± 2.98 a	1.65 ± 0.21a	0.38 ±0.04 a
EG	106.23 ± 4.56 a	39.14 ± 5.00 a	1.55 ± 0.01a	0.37 ± 0.03 a

Reported results are means of four replicates ± standard deviation. Reported results are means of four replicates ± standard deviation. Different letters in the same column indicate that means are different at the 95% level of probability ($p < 0.05$) using Fisher's LSD test

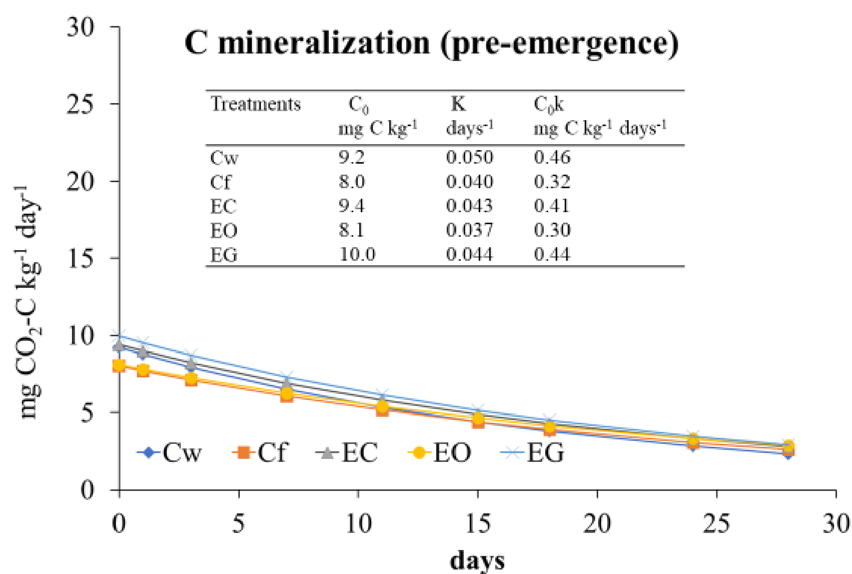

Figure 8. Microbial respiration rate fitted to the exponential first order decay function (Mineralized C = C_0 e-kt) and derived parameters (C_0, biological available C; k, turnover constant rate; C_0K, initial potential rate of C mineralization) determined on soil treated in pre-emergence with *E. camaldulensis* (EC), *E. occidentalis* (EO) and *E. globulus* (EG) essential oils. Reported results are means (n=4)

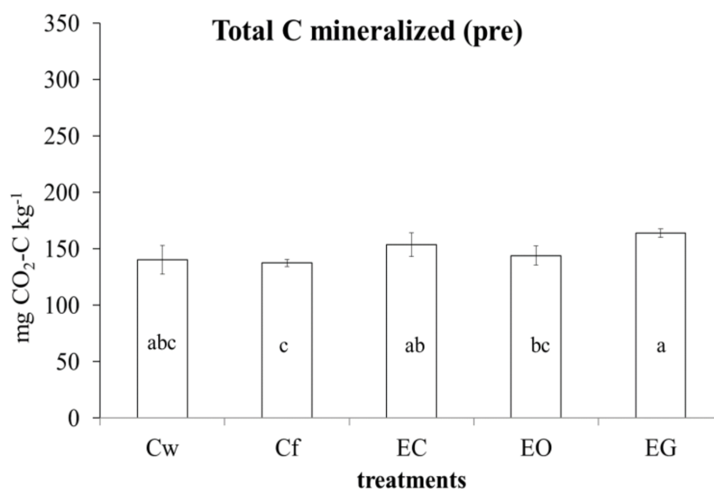

Figure 9. Total Carbone mineralized of soil treated in pre-emergence with *E. camaldulensis* (EC), *E. occidentalis* (EO) and *E. globulus* (EG) EOs. Reported results are means (n=4). Bars indicate the standard deviations.

Table 17. Main microbial groups and total elfas (ester linked fatty acids) in nmol g^{-1} of soil treated in post-emergence with *E. camaldulensis* (EC), *E. occidentalis* (EO) and *E. globulus* (EG) EOs

Treatments	Total elfas	Bacteria	Fungi	B+/B-	F/B
Cw	240.98 ± 5.68 a	97.98 ± 5.69 a	23.04 ± 1.36 a	4.00 ± 0.62 a	0.24 ± 0.00 a
Cf	175.01 ± 8.41 a	61.49 ± 9.30 a	22.37 ± 2.30 a	3.69 ± 0.50 a	0.37 ± 0.02 a
EC	182.86 ± 2.63 a	72.5 ± 7.71 a	15.72 ± 1.61 a	2.00 ± 0.01 b	0.22 ± 0.00 a
EO	183.87 ± 12.41 a	59.51 ± 12.12 a	18.02 ± 3.13 a	1.84 ± 0.05 b	0.31 ± 0.12 a
EG	204.81 ± 30.50 a	84.08 ± 18.05 a	18.97 ± 2.87 a	1.97 ± 0.44 b	0.23 ± 0.01 a

Reported results are means of four replicates ± standard deviation. Different letters in the same column indicate that means are different at the 95% level of probability ($p < 0.05$) using Fisher's LSD test

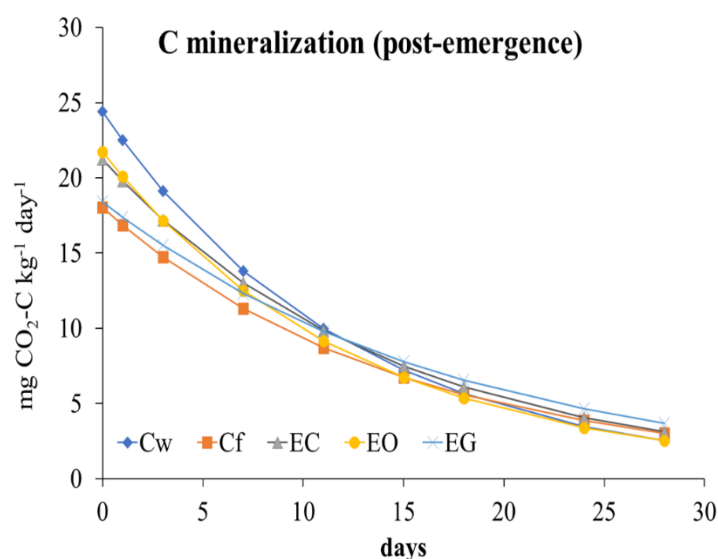

Figure 10. Microbial respiration rate fitted to the exponential first order decay function (Mineralized C = C_0 e-kt) and derived parameters (C_0, biological available C; k, turnover constant rate; C_0K, initial potential rate of C mineralization) determined on soil treated in post-emergence with *E. camaldulensis* (EC), *E. occidentalis* (EO) and *E. globulus* (EG) EOs. Reported results are means (n=4)

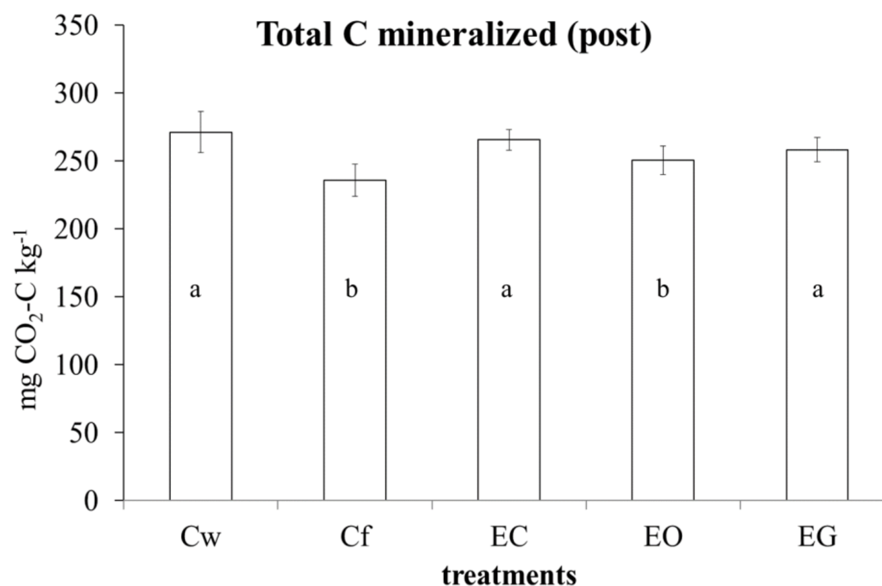

Figure 11.Total Carbon mineralized of soil treated in post-emergence with *E. camaldulensis* (EC), *E. occidentalis* (EO) and *E. globulus* (EG) essential oils. Reported results are means (n=4). Bars indicate the standard deviations.

4 Discussion

4.1 Percentage Yield and chemical composition of EOs

E. camaldulensis showed the lowest yield average 0.21% compared to the other two assayed EOs which provided the same value of the yield average 0.4%. Our tested species provided lesser yield than species reported in other studies: 0.71% was reported for *E. camaldulensis* EO from Spain (Verdeguer *et al*., 2009), 1.91% and 0.7% for *E. globulus* EO from Pakistan and Chile respectively (Ghaffar *et al*., 2015; Tampe *et al*., 2020), 1.1 % for *E. occidentalis* EO from Tunisia (Elaissi *et al*., 2010). The yields of *Eucalyptus* EOs from leaves depend on the species studied and vary from 0.10 to 9.0 % (Zangh *et al*., 2010). This fluctuation is due to tree age (De Andrade and Gomes, 2000), leaf age (Goguadze *et al*., 1986; Silvestre *et al*., 1997), altitude (Manian and Gopalakrishnan, 1995), season (Rafique and Chaudhary,1996), harvest time (Doran *et al*., 1996) and the different climatic conditions and the geographic parameters (Ghaffar *et al*., 2015).

Regarding the chemical composition, 1,8-cineole was the most abundant compound in all EOs tested. It was present in *E. camaldulensis* EO by about 52%, followed by α-pinene 15%. The *E. camaldulensis* EO composition from different origins had been widely reported and two groups were distinguished: those that contain 1,8-cineole and α-pinene as the main compounds, including *E. camaldulensis* from Nigeria, Egypt, Iran, Tunisia and Senegal (Oyedeji *et al*., 2000; Maximous, 2004; Sefidkon *et al*., 2006; Sebei *et al*., 2015; Ndiaye *et al*., 2017),

corresponding to our findings, and those that contain spathulenol, *p*-cymene and cryptone as main compounds and small quantities of 1,8-cineol, including *E. camaldulensis* from south Florida, Greece and Spain (Pappas and Sheppard-Hanger, 2000; Tsiri *et al*., 2003; Verdeguer *et al*., 2009). Similarly, EO from *E. globulus* was rich in 1,8-cineole (67%) and α-pinene (22%), such findings agreed with previous results of *E. globulus* EO composition from China, Pakistan, Iran, Chile and Ethiopia (Yang *et al*., 2004; Ghaffar *et al*., 2015; Rassaeifar *et al*., 2013; Tampe *et al*., 2020 ; Fikremariam *et al*., 2019). Also 1,8-cineol (83%) was the most abundant compound along with α-pinene (8%) in EO from *E. occidentalis*. The EO composition of *E. occidentalis* from Iran was determined and the main constituents were t-cadinol (17.20%) and 1,8-cineol (15.50%) (Bande-Borujeni *et al*., 2018). Overall, little differences were distinguished between the three extracted EOs chemical compositions.

4.2 Herbicidal activity

4.2.1 Pre-emergence treatments

In our pre-emergence bioassay, the results obtained with fitoil applied at 1µl/ml, in which no significant differences were observed in the germination and the fresh weight, corroborate the work of other authors, such as Verdeguer (2011) and García (2013). However, a significant reduction of the dry weight occurred with Cf and this could be explained by the fact that we did not consider the roots in the weight of the plants, since plants were cut from the trays to let the soil intact.

Since in this study treatments were applied on seeds from the soil seedbank and there were low presence in the used soil of the weeds *D. stramonium, E. elaterium*, *A. retroflexus* and *A. fatua,* solid conclusions about the results of the EOs against the germination of these weeds cannot be stablished. Only the results on *P. olecerea*, which was the most abundant weed on the trays are reliable. In previous assays, *P. oleracea* showed more resistance than other weeds to treatments to control its germination with EOs (Verdeguer, 2011).

EOs herbicidal potential is closely related to the species on which they are applied. *E. globulus* EO reduced *P. oleracea* germination significantly only respect to Cw. Azizi and Fuji (2005) demonstrated the phytotoxic effect of *E. globulus* EO on *P. olecerea* and other weeds like *A. retroflexus* and *A. fatua.* Rassaeifar *et al*. (2013) confirmed the allelopathic effect of *E. globulus* EO on seed germination and seedling establishment of other weed species such as: *Amaranthus blitoides* and *Cynodon dactylon*.

At the applied dose 8 µl ml $^{-1}$, *E. globulus* EO was only able to reduce the germination of *P. oleracea* with respect to Cw. However, greater effects could be expected with higher doses, since Ataollahi *et al*. (2014), explained that the higher was the concentration of *E. globulus* extracts, superior was the inhibition of germination and seedling growth of *S. nigrum*.

On the other hand, *E. camaldulensis* EO did not show any significant phytotoxic effects. In previous works, seed germination of *P. oleracea* was not inhibited by extract aqueous of *E. camaldulensis* (Dadkhah, 2013). Verdeguer *et al*. (2009) reported the phytotoxicity of *E. camaldulensis* EO which significantly decreased the germination of *Amaranthus hybridus* and *P. oleracea* in *in vitro* conditions. Dadkhah (2012) found that germination, growth, and photosynthesis of *A. retroflexus* were inhibited by aqueous extract of *E. camaldulensis.* In *in vitro* assays the phytotoxic effects of EOs against weed seeds germination are maximum because they are very close to the seeds they have to control. In *in vivo* conditions EOs volatilize and are not permanently in contact with the seeds, as they are in a Petri dish. So, to find phytotoxic effects in *in vivo* conditions the doses must be increased and the EOs formulated adequately to avoid quick volatilization.

To our knowledge, this is the first time that the herbicidal potential of *E. occidentalis* EO was tested *in vivo*. Although it contained the highest quantity of 1,8-cineol, it exhibited no effect at all. According to other authors (Angelini *et al*., 2003; Verdeguer *et al*., 2009), these findings suggest that 1,8-cineol is not the principal compound responsible for the allelopathic effect of *Eucalyptus* spp. EOs

To sum up, the weak or no effectiveness of the tested EOs could be related to the used dose, in the way that higher doses need to be tested, and to the sandy texture of the used soil since del Moral and Muller (1970) explained that herbal inhibition could not occur in sand, and the optimal conditions for the allelopathic effects were found in poorly drained soils, poorly aerated, superficial and with a high colloidal content, since they favored the accumulation of allelochemicals.

4.2.2 Post emergence treatment

In the post-emergence bioassay, the highest values of the number of grown plants were recorded in the controls, the one with fitoil above all, that apparently exhibited a stimulatory effect, as it was reported in Verdeguer *et al*, (2020).

The three sprayed EOs were able to reduce the number of grown plants significantly respect Cf (Table 4), so EOs could be counteracting that stimulatory effect of Cf. Therefore, the use of fitoil at the dose 1µl ml^{-1} could mask the activity of EOs. The reduction of the number of identified species confirm the inhibitory effect of the used EOs and could indicate that they were exercising a selective control over some species. Indeed, studies showed that the sensitivity of different species against allelopathic effects of EOs can be different (Dudai *et al*., 1999, Verdeguer, 2011). In like manner, Chu *et al*. (2014) proved that in nature, the inhibitory effects relating to allelopathy in *Eucalyptus urophylla* plantation vary according to the present species.

It is interesting to note that although treatments were not significant between them, *E. globulus* EO herbicidal activity against *Phaseolus aureus*, *Hordeum vulgare*, *Avena sativa*, *Echinochloa crus-galli* and *D. stramonium* was reported in Kohli *et al*. (1998) and Jaime *et al*. (2018). Likewise, *E. globulus* aqueous extract inhibited lettuce germination and radicle growth, displaying dose-dependent phytotoxic activity (Souto *et al*., 1994; Yamagushi *et al*., 2011; Puig *et al*., 2018). More specifically, α-pinene high content in *E. globulus* EO, suggest that it is related to its herbicidal effect. Indeed, many reports showed that α-pinene inhibited the root growth of the *Zea mays* (Scrivanti *et al*. 2003). De Martino *et al*. (2010) have demonstrated that α-pinene inhibited the germination and radical elongation of *R. sativus* and *Lepidium sativum*. Although the mode of action of EOs against germination still remains unclear, several studies showed that α-pinene and 1,8-cineole, the two main components of these EOs, act by increasing the lipid peroxidation, leading to a disruption of the membrane integrity, uncoupling the oxidative phosphorylation by acting as a classical protonophoric agent, and inhibiting the electron transfer chain (Dayan *et al*. 2000; Scrivanti *et al*., 2003). Other minor compounds may also contribute to the herbicidal effects with possible synergistic and antagonistic interactions among the components (Ben Ghnaya *et al*., 2013).

Lastly, at the same concentration of 8µl ml $^{-1}$, *E. occidentalis* EO sprayed in post-emergence showed weak herbicidal effect compared to no effect in the pre-emergency bioassay. It could be that this oil act better when it is sprayed on the plants than on seeds, or for the high susceptibility of *D. stramonium* to post-emergence herbicides as it is reported in Bowers and Baumn (1984) and Dobbels and Kapusta (1993).

4.2.3 Effects of EOs on soil biochemical properties and main microbial groups

The absence of effects on soil microorganisms following the application of Eucalyptus EOs in pre-emergence treatment may be ascribed to the low amount of EO applied per unit weight of soil. Indeed, being this the first experiment, such results were used to individuate a starting dose to be used for the following experiments.

Regarding the effect of essential EOs in post-emergence on soil microorganisms, although the amount of EOs applied was higher than that applied in pre-emergence since three application were done, the effect on soil microorganisms were scarce. Indeed, only the B+/B- ratio were decreased compared to the control. Such results may be ascribed to an increase of C availability by the addition of the assayed EOs (Fanin *et al.*, 2019).

5 Conclusions

Overall, all EOs at the tested dose of 8 µl mL^{-1}, showed a weak herbicidal effect or no effect at all. In pre-emergence assay, *E. occidentalis* and *E. camaldulensis* EOs were not able to inhibit the germination of any weed, while *E. globulus* showed a weak effect reducing *P. oleracea* germination, with significant differences with Cw but not with Cf and the other EOs. In post-emergence assay, *E. camaldulensis* lacked also of phytotoxicity and *E. occidentalis* and *E. globulus* showed weak herbicidal effects. On the other hand, such dose did not affect soil microorganisms. It is important to highlight that the weak herbicidal activity of the EOs tested could be due to the low applied concentration. More research is necessary, with higher concentrations and testing the EOs against different weed species to better understand their herbicidal potential and weed responses to their application.

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Chapter 3:

Potential effects of essential oils extracted from Mediterranean aromatic plants on target weeds and soil microorganisms

1 Introduction

One of the main challenges of the Agriculture of the 21st century is to increase crop production in a sustainable way, e.g. minimizing the use of pesticides (Villa *et al*. 2017). The widespread use of synthetic herbicides may lead to the accumulation of toxic residues in agricultural products and result in soil and groundwater pollution, development of weed resistance and adverse effects on human and animal health (Hatcher and Melander 2003; Hossard *et al*. 2017). Synthetic herbicides can be immobilized in soil by adsorption or binding to colloids (Kanissery *et al*. 2019) so affecting both soil organic matter turnover and microbial community composition (Haney *et al*. 2000; Lancaster *et al*. 2010; Ntalli *et al*. 2019). One potential fulfillment to the demand of alternative natural and safe products is the exploitation of renewable resources, such as medicinal and aromatic plants known for their allelopathic effects (Benvenuti *et al*. 2017; Della Pepa *et al*. 2019).

A variety of allelochemicals, including plant secondary metabolites, such as essential oils (EOs), have been proved to inhibit seed germination and weed growth (Della Pepa *et al*. 2019). EOs are suitable in sustainable and organic agriculture for their rapid decomposition in environment and volatile characteristics (El Gawad *et al*. 2019). Their effectiveness in the control of weeds lies on the joint action of an array of different compounds present in them, whose quantity and persistence in the environment may be not enough to inhibit seed germination and plant growth on its own (Araniti *et al*. 2013; Xianfei *et al*. 2007). However, when the amount of one active compound is very high within a given EO, it is also true that this EO compound alone could be even effective (Araniti *et al*. 2017, Vasilakoglou *et al*. 2013, Verdeguer *et al*., 2020). In addition, since EOs usually have various modes of action, it is more complicated for weeds to easily develop resistance against them. (Blazquez 2014; Sadgrove and Jones 2015). In fact, they can suppress the weed growth by affecting biochemical and physiological processes such as reducing cell survival, chlorophyll content, RNA contents, acid soluble carbohydrates and water-soluble carbohydrate (Araniti *et al*. 2018; Zhang *et al*. 2010). Despite their allelopathic potential, many EOs are classified as “Generally Recognized as Safe” (GRAS) by the US Food and Drug Administration (FDA) (Tworkoski 2002).

EOs extracted from Lamiaceae have been demonstrated in *in vitro* studies to be effective in inhibiting seed germination (Angelini *et al*. 2003). The most important species in this family, in terms of high economical value due to the great production of EOs, are *Mentha x piperita* L. (Peppermint) and *Thymbra capitata* L. (Cav) (synonym *Thymus capitatus* (L.) Hoffmanns. & Link) (Thyme) (Pirbalouti *et al*. 2013; XiaoHua *et al*. 2012). *Mentha x piperita* L. is a cultivated

natural hybrid of *Mentha aquatica* L. (water mint) and *Mentha spicata* L. (spearmint), both native species of the Mediterranean region. It is cultivated worldwide because its EO has antioxidant and antimicrobial activities and is used as eco-friendly pesticide (Gharib and Teixeira da Silva 2013; Okmen *et al*. 2017). Likewise, its phytotoxic activity has been demonstrated in several studies (Mahdavikia and Saharkhiz 2015; Rolli *et al*. 2014).

The species of *Thymus* genus, native of Southern Europe, North Africa, and Asia (Lawrence and Tucker 2002; Morales 2002), are largely used as medicinal plants (Barros *et al*. 2010). Due to the presence of polyphenols, *T. capitata* EO is used in food preservation (Solomakos *et al*. 2008) and has been demonstrated possessing antioxidant properties (Bozin *et al*. 2006). Moreover, the antimicrobial (Gagliano Candela *et al*. 2019; Küçükbay *et al*. 2014) and herbicidal activities (Verdeguer 2011; Verdeguer *et al*. 2020) of *T. capitata* EO have been verified. *Santolina chamaecyparissus* L. (cotton lavender) is an aromatic plant belonging to Asteraceae family. Its analgesic, bactericidal, fungicidal, vermifuge and vulnerary properties have been described (Cuellar *et al*. 1998). Furthermore, its herbicidal activity is well documented (Grosso *et al*. 2010). Although, as aforementioned, some studies have shown EOs extracted from *M. piperita*, *T. capitata* and *S. chamaecyparissus* may inhibit seed germination and weed growth, the majority of them have been conducted in *in vitro* conditions and against few weed species. Therefore, their selectivity towards some of the most widespread and troublesome weeds has yet to be investigated. On the other hand, even few studies are available about the effects of such EOs on soil microorganisms. Vokou and Liotiri (1999) found that EOs extracted from five different aromatic plants, not including those tested in this study, increased microbial respiration. Similarly, also EOs extracted from *Lavandula stoechas* L. increased microbial respiration as a result of bacteria growth stimulation (Vokou *et al*. 2002). Such results, however, are in contrast with those of Khare *et al*. (2019) who reported a decrease of microbial biomass and activity. Such few studies with even contrasting results demonstrated that, if EOs were deemed to be used in the field for an integrated pest management, further studies are needed to better elucidate their effects on soil microorganisms as playing pivotal roles in the soil organic matter turnover and nutrient cycling. In addition, not all EOs exert the same effect on weeds at a given concentration (Verdeguer *et al*. 2009; Verdeguer 2011).

Therefore, this study has two main purposes: the first, to assess the effectiveness of *M. piperita*, *T. capitata* and *S. chamaecyparissus* EOs to control some of the most troublesome weeds of many crops around the world such as *Avena fatua* L. (wild oat), *Echinochloa crus-galli* (L.) P. Beauv. (barnyard grass), *Portulaca oleracea* L. (common purslane) and *Amaranthus retroflexus* L. (redroot pigweed) and, the second, to assess the effect of these EOs on microbial

biomass C and N, respiration and on the relative abundance of main microbial groups. Such soil biochemical properties were selected because an increase or decrease, or a shift of the main microbial groups are easy to interpret in terms of substrates availability and stress/disturbance for soil microorganisms (Anderson, 2003; Kaur *et al*. 2005; Laudicina *et al*. 2012). The hypotheses tested in this study were that *T. capitata, M. piperita* and *S. chamaecyparissus* EOs are able to i) inhibit or, at least, to reduce weeds growth, ii) negatively affect soil microbial biomass C and N and microbial activity, iii) increase the bacteria to fungi ratio as the former are abler than fungi in using available C source, iv) as an overall effect, determine a stress/disturbance conditions for soil microorganisms.

2 Materials and Methods

2.1 Essential oils

Thymbra capitata (L.) Cav., *Mentha × piperita* L. and *Santolina chamaecyparissus* L. EOs were purchased from Bordas (Sevilla, Spain), Sigma-Aldrich (Darmstadt, Germany) and Ecoaromuz (Ademuz, Valencia, Spain), respectively. They were analysed by gas chromatography (GC) coupled with mass spectrometry (Clarus 500 GC–MS from Perkinelmer inc.) equipped with the capillary column ZB-5 (30 m × 0.25 mm i.d. × 0.25 µm film thickness). The injection volume was 1 µL. The GC oven temperature was set at 60°C for 5 min, with increases of 3°C per min up to 180°C, then increases of 20°C per min up to 280°C which was maintained for 10 min. Helium was the carrier gas at a flux of 1.2 mL min^{-1}. Injector and detector temperatures were set at 250°C. Ionization source temperature was set at 200°C and 70 eV electron impact mode was employed. MS spectra were obtained by means of total ion scan mode (mass range m/z 45–500 uma). The total ion chromatograms and mass spectra were processed with the Turbomass 5.4 software (PerkinElmer Inc., UK). Retention indexes were determined by injection of C_8–C_{32} n-alkanes standards under the same conditions. The EO components were identified by comparison of their mass spectra with those of computer library NIST MS Search 2.0 and available data in the literature. Identification of α-pinene, β-pinene, camphene, myrcene, limonene, camphor, terpinolene, β-thujone, borneol, terpinen-4-ol, bornyl acetate and linalool was confirmed by comparison of their experimental RI with those of the reference standards (Sigma-Aldrich). The quantification of the compounds found in each EO was performed by gas chromatography (Clarus 500GC, PerkinElmer Inc., UK) equipped with a flame ionization detector, the same column and operating conditions as described above for

the GC-MS. The percentage composition of each EO was computed from the GC peak areas by means of the software Total Chrom 6.2 (Perkin Elmer Inc., Wellesley, PA, USA).

2.2 Herbicidal activity of EOs against target weeds

Seeds of *Portulaca oleracea* L.*, Amaranthus retroflexus* L. and *Avena fatua* L. were purchased from Herbiseed (United Kingdom) in 2017, and seeds of *Echinochloa crus-galli* (L.) P. Beauv. were collected from rice fields in Sollana (Valencia, Spain) in September 2017. Seeds germination was achieved using a germination-growth chamber (Equitec, Spain). *P. oleracea*, *A. retroflexus* and E. *crus-galli* seeds were germinated using a 16h / 8h (light/dark) photoperiod, settling the temperatures at 30 ± 0.1°C and 20 ± 0.1°C for light and dark conditions, respectively. *A. fatua* seeds were germinated using 8 h (23.0 ± 0.1°C) / 16 h (18.0 ± 0.1°C) light/dark conditions. After germination (about one week), emerged seedlings were selected for uniformity in growth and individually transplanted in polypropylene square pots (8 x 8 x 7 cm) previously filled with a 2 cm drainage layer of perlite and a 5 cm layer of soil (220 g) collected in an organic citrus orchard (39º 37′ 24.8″ N, 0º 17′ 25.6″ W, Puzol, Valencia, Spain). Transplanted seedlings were then transferred in a glass greenhouse. Dates during which weeds were grown and greenhouse temperature and humidity conditions are reported in Table 1.

Table 1. Greenhouse temperature and relative humidity conditions during the experimental period.

Species	Date	Temperature (°C)			Relative Humidity (%)		
		Mean	Maximum	Minimum	Mean	Maximum	Minimum
A. retroflexus	30/08/2018-28/09/2018	26.8	38.0	20.0	71.3	89.0	31.14
P. oleracea	3/07/2018-22/07/2018	29.0	44.0	23.2	64.0	94.0	17.4
A. fatua	26/05/2018-24/06/2018	26.0	37.7	19.0	62.3	97.5	23.2
E. crus-galli	16/07/2018-25/07/2018	28.8	44.0	23.2	64.3	92.0	17.4

To each pot, 80 mL of water were added to bring the soil to 4/5 of its water holding capacity (WHC) and left overnight. The day after, 100% of soil WHC was reached by irrigation adding 20 mL of an emulsion containing a given EO (Ogg 1986, Potter *et al*. 2008). EO water emulsions were prepared using 0.5 mL L^{-1} of the emulsifier Fitoil (Xeda, Italy). Based on the results of previous studies (García-Plasencia 2013; Verdeguer 2011, Verdeguer *et al*. 2020), three different emulsions were prepared for each EO at the following concentrations:

- T. *capitata*: 4 (T1), 8 (T2), 12 (T3) µL mL^{-1};

- M. *piperita*: 12 (M1), 16 (M2), 20 (M3) µL mL^{-1};
- S. *chamaecyparissus*: 12 (S1), 16 (S2), 20 (S3) µL mL^{-1}

Also, two controls were established: the first irrigated only with water (Cw) and the second irrigated with water plus Fitoil at 0.5 mL L^{-1} (Cf). EOs treatments were applied when plants reached the phenological stage of 2-3 true leaves, corresponding to 12-13 BBCH (Biologische Bundesanstalt, Bundessortenamt and CHemical industry) scale for the monocotyledons *A. fatua* and *E. crus-galli*, and 3-4 true leaves, corresponding to 13-14 BBCH scale for the dicotyledons *P. oleracea* and *A. retroflexus*. Ten replicates per each treatment were performed. To evaluate any phytotoxic effect, photos of the plants were taken just after 24 and 48 h after the application of the treatments and then each 3 or 5 days for the whole experiment. At the end of the experiment, the entire plant from each pot was reclaimed by dipping in water the root apparatus to remove any soil residues and images of all plants were registered. The software Digimizer v.4.6.1 (MedCalc Software, Ostend, Belgium, 2005-2016) was used to process and analyse the images to determine total (TL), root (RL) and aerial part (APL) length of the plants and also the damage level (DL). The latter was assessed developing a damage scale for each species. The scale range was from 0 (no damage) to 4 (death of the plant) for the monocotyledons (*A. fatua* and *E. crus-galli*, Figures 1 and 2, respectively), and from 0 (no damage) to 3 (death of the plant) for the dicotyledons (*P. oleracea* and *A. retroflexus*; Figures 3 and 4, respectively). Fresh (FW) and dry weights (at 60°C for 48 h; DW) were also determined. The efficacy of a given EO was considered as its capacity to kill the plants and was assessed by attributing the value 0 if the plant was alive and 100 if the plant was dead.

Figure 12. Scale of damage level for the monocotyledon *Avena fatua*

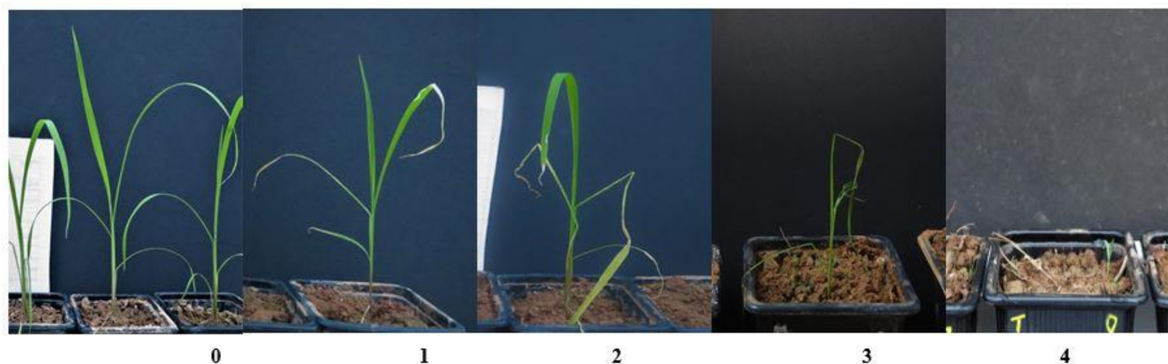

Figure 13. Scale of damage level for the monocotyledon *Echinochloa crus-galli*

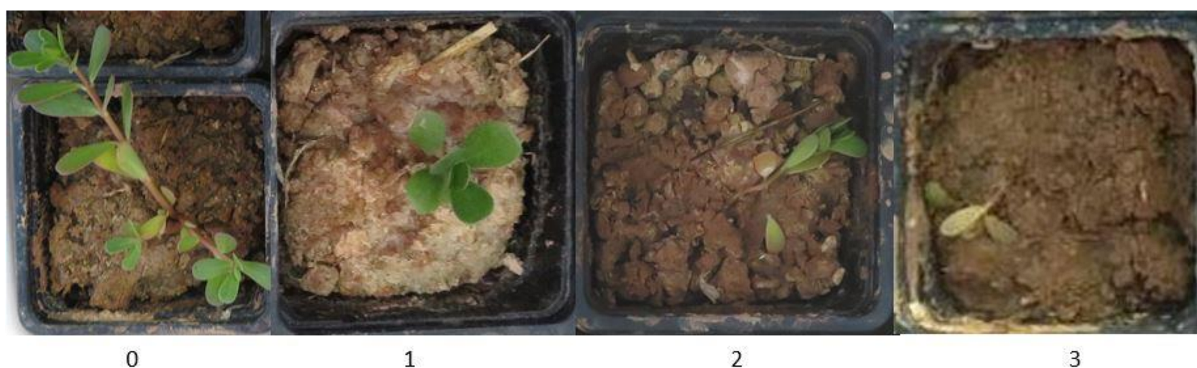

Figure 14. Scale of damage level for the dicotyledon *Portulaca oleracea*

Figure 15.Scale of damage level for the dicotyledon *Amaranthus retroflexus*

2.3 Effects of EOs on soil microorganisms

To test the effects of EOs on soil microorganisms, a short-term laboratory incubation experiment was set-up. The topsoil (0-15 cm) of a citrus [*Citrus sinensis* (L.) Osbeck] orchard never treated with synthetic herbicides was used. Its main characteristics were: sand 64.9 %, clay 15.9 %, organic carbon 2.3 %, pH 7.0, electric conductivity 0.1 dS m^{-1} and total nitrogen 1.2 g kg^{-1}. After sampling, the soil was air-dried and sieved at 2 mm. Aliquots of 350 g of soil were placed in 1L plastic bottles and moistened with only water up to 2/3 of 50% of its WHC.

Then, a volume of EO emulsion was added thus reaching the 50% of its WHC. The amounts of EO added were 31 (THY1), 62 (THY2) and 93 µL 100 g^{-1} (THY3) of soil for *T. capitata* treatment, and 93, 123 and 153 µL 100 g^{-1} of soil for both *M. piperita* (MNT1, MNT2, MNT3) and *S. chamaecyparissus* (SNT1, SNT2, SNT3) treatments. Two controls were also prepared: the first with only water (Cw) and the second with water and fitoil (Cf) at a concentration of 0.05% (v/v) to moist the soil. Four replicates per treatment were run. After the EOs addition, plastic bottles were incubated in the dark, at constant temperature (25.0 ± 0.5°C), for 56 days. During the incubation, water loss was monitored by weighing the bottles and eventually watering them with only water to maintain the soil WHC at 50%.

At days 7, 28 and 56, soils were analysed to determine some biochemical properties. The fumigation– extraction method (Vance *et al*. 1987) was used to assess microbial biomass C (MBC). Fulabilemigated and not fumigated soil sub-samples (15 g) were extracted with 0.5 M K_2SO_4, at a ratio of 1:4 (w/v). Total organic C in soil extracts was determined by hot digestion-oxidation (sulphuric acid-dichromate mixture). MBC was estimated as the difference between the organic C held in fumigated extract and that in not fumigated extract, multiplied by a conversion factor (k_{EC}) of 2.64. The K_2SO_4-extractable C of not fumigated soil was assumed as a proxy of the readily available C pool (Laudicina *et al*. 2013). Microbial biomass N (MBN) was calculated multiplying by 5 the difference between the ninhydrin reactive N determined on fumigated and not fumigated soil 0.5M K_2SO_4 extracts, respectively, according to (Joergensen and Brookes 1990). Concurrently, glass jars of 200 mL with 20 g of soil aliquots from each of the above treatment were incubated, in the dark and at 23-25°C, to determine microbial respiration. The CO_2 accumulated in the headspace of the glass jars at days 1, 4, 7, 10, 17, 23, 31, 39 and 53 was assessed by a gas chromatograph equipped with a thermal conductivity detector. At each CO_2 determination, jars were ventilated with fresh air for 30 min and then sealed again, after possible replenishment of lost soil moisture by distilled water. The C mineralization rate, expressed as mg CO_2–C kg^{-1} dry soil day^{-1}, was fitted to the following first order decay function (Riffaldi *et al*. 1996):

Mineralized C = $C_0\ e^{-kt}$

where C_0 is the biologically available C (mg kg^{1}) at time zero (i.e. the intercept value), k is the decay rate constant, and t is the sampling incubation day. The total CO_2–C evolved over 53 days of incubation was calculated by the linear interpolation of two neighbouring rates and the integration over time:

$$\text{Total C mineralized} = \sum_{i}^{n}[(r_i + r_{i+1}) * \frac{d}{2} + \cdots + (r_{n-1} + r_n) * \frac{d}{2}]$$

where i is the date of the first measurement of CO_2–C rate, n is the last day of measurement of CO_2–C rate, r is the CO_2–C rate expressed as mg CO_2–C kg^{-1} dry soil day^{-1}, and d is the number of days between the two consecutive CO_2 rate measurements. The specific respiration rate, or metabolic quotient (qCO_2), i.e. the amount of CO_2 emitted per unit of MBC per time unit, was calculated as mg CO_2–C g^{-1} MBC h^{-1}.
Fatty acids (FAs) were extracted from soils according to the modified Bligh and Dyer method (Wu *et al*. 2009). The fatty acid methyl esters (FAMEs) were detected by a gas chromatograph (FOCUS™ GC, Thermo Scientific Inc., Waltham, MA USA) equipped with a flame ionization detector and a fused-silica capillary column Mega-10 (50 m x 0.32 mm I.D.; film thickness 0.25 µm). The GC temperature progression was initial isotherm at 115°C for 5 minutes, increases of 1.5 °C per min up to 230°C, and final isotherm at 230°C for 2 minutes. Both injection port and detector were set at 250°C and helium at 1 mL min^{-1} in a constant flow mode was used as carrier. The injected volume was 1 µL (50:1 split ratio). Nonadecanoic acid methyl ester (19:0; cat no. N-5377, Sigma-Aldrich Co.) was used as internal standard for the quantification of FAMEs. Peak identification was done by comparing the retention times of each FAMEs to known standards (Supelco Bacterial Acid Methyl Esters mix cat no. 47080-U and Supelco 37 Component FAME mix cat no. 47885-U). Fatty acids with less than 14 carbon atoms or more than 20 carbon atoms were excluded as considered originating from non-microbial sources. The FAs i15:0, a15:0, 15:0, i16:0, i17:0, 17:0, cy17:0,18:1ω7, cy19:0 were used to represent bacterial biomass while 18:2ω6,9 for fungal biomass. The FAs i15:0, a15:0, i16:0, i17:0 were chosen to represent Gram-positive bacteria (bacG+) while 16:1ω7, 18:1ω7, cy17:0 and cy19:0 for Gram-negative bacteria (bacG-) (Laudicina *et al*. 2011).

2.4 Statistical analysis

Plant experiment was carried out with a completely randomized design with ten replicates. Biometric plant variables (TL, RL, APL, FW, DW) and DL data were evaluated for normality and variance homogeneity and then subjected to one-way ANOVA, followed by Fisher's multiple comparison test (LSD intervals, Least Significant Difference, at $P<0.05$) for the separation of the means in each species. A multifactor analysis of variance (ANOVA) was performed on efficacy including species and treatment as effects.
Reported soil data, referred to oven-dry soil (105 °C) weight, are the arithmetic means of four replicates. Before performing parametric statistical analyses, normal distribution and variance

homogeneity of the data were checked by Kolmogorov–Smirnoff goodness-of-fit and Levene's tests, respectively. Within each EO treatment, soil data were subjected to two-way ANOVA with EO dose (four levels; three EO doses and the control, Cf) and incubation day (three levels: days 7, 28 and 56) as factors. Within each EO type (THY, MNT, SNT), significant differences at $P<0.05$ among doses at the same incubation day and among incubation days at the same dose were assessed by the least significant difference (LSD) post-hoc test. All analyses were performed by Statgraphics Centurion version XVII.

3 Results

3.1 Essential oils composition

A total of 91 compounds (Table 2) were identified in the three tested EOs: 17 in *T. capitata*, 35 in M. *piperita* and 39 in *S. chamaecyparissus*. *T. capitata* EO was characterized mainly by a high content of oxygenated monoterpenes (74.0%) and monoterpene hydrocarbons (22.5%; Table 2). Among the oxygenated monoterpenes, carvacrol was the most abundant (72.3%; Table 2). Also *M. piperita* (95.3%) and *S. chamaecyparissus* (39.3%) EOs were particularly rich in oxygenated monoterpenes. Among them, menthol (51.8%) and menthone (20.5%) were the most abundant components in *M. piperita*, whereas 1,8-cineole (17.5%) in *S. chamaecyparissus* (Table 2).

Table 2. Chemical composition of essential oils extracted by hydrodistillation from *T. capitata* (TC), M. *piperita* (MP) and *S. chamaecyparissus* (SC). KI, Kovats index.

Compounds	**KI.**	**TC**	**MP**	**SC**
Monoterpene hydrocarbons (%)		22.54	1.95	9.30
Santolina triene	908	-	-	0.13
α-Thujene	930	0.89	0.01	-
α-Pinene	938	0.74	0.28	0.85
Thuja-2,4(10)-diene	947	-	-	-
Camphene	951	-	-	0.28
Sabinene	975	-	0.14	0.17
β-Pinene	978	0.29	0.43	3.98
Myrcene	991	1.95	0.01	-
α-Phellandrene	1004	0.16	-	-
γ-Terpinene	1016	**7.77**	0.13	1.18
α-Terpinene	1016	1.61	-	0.69
p-Cymene	1025	**8.93**	0.18	2.01
Limonene	1029	0.20	0.73	-
(Z)-β-Ocimene	1040	-	0.03	-
iso-Terpinolene	1087	-	0.02	-
Oxygenated monoterpenes (%)		73.98	95.35	39.32
1,8-Cineole	1031	0.11	4.31	**17.50**
trans-Pinocarveol	1037	-	-	0.17
Artemisia ketone	1062	-	-	4.63

(Z)-Sabinene hydrate	1070	-	0.76	-
born	1097	0.77	0.09	0.42
Camphor	1142	-	-	4.03
Menthone	1154	-	**20.52**	-
(E)-Pinocamphone	1159	-	-	0.18
(Z)-Chrysanthemol	1162	-	-	3.80
Menthofuran	1163	-	5.21	-
neo-Menthol	1165	-	3.12	-
Borneol	1168	0.16	-	1.11
(Z)-Pinocamphone	1172	-	-	2.03
Menthol	1175	-	**51.81**	-
Terpinen-4-ol	1177	0.37	0.67	2.69
iso-menthol	1182	-	0.60	-
Neoisomenthol	1187	-	0.08	-
α-Terpineol	1188	-	0.17	0.21
Myrtenal	1192	-	-	1.31
Myrtenol	1193	-	-	1.07
Verbenone	1198	-	-	0.16
m-Cumenol	1230	-	-	-
Pulegone	1236	-	0.83	-
Piperitone	1251	-	0.32	-
neo-Menthyl acetate	1273	-	0.16	-
p-Menth-1-en-7-al	1279	-	-	-
Menthyl acetate	1291	-	**6.56**	-
Thymol	1292	0.27	-	-
Carvacrol	1300	**72.30**	-	-
iso-Menthyl acetate	1303	-	0.16	-
Sesquiterpene hydrocarbons (%)		3.14	2.22	21.78
α-Ylangene	1373	-	-	0.08
α-Bourbonene	1381	-	0.17	-
β-Caryophyllene	1415	3.14	1.47	0.39
β-Farnesene	1454	-	0.02	-
allo-Aromadendrene	1457	-	-	4.23
trans-Cadina-1(6),4-diene	1473			0.36
Germacrene-D	1477	-	0.42	**12.60**
β-Selinene	1491	-	0.13	-
Elixene	1492	-	-	2.80
γ-Cadinene	1509	-	-	0.32
δ-Cadinene	1519	-	-	1.00
Oxygenated sesquiterpenes (%)		0.14	0.00	15.64
Bornyl acetate	1283	-	-	0.08
Spathulenol	1477	-	-	1.42
Caryophyllene oxide	1577	0.14	-	0.19
Viridiflorol	1587	-	-	**13.56**
β-Oplopenone	1602	-	-	0.16
α-Cadinol	1649	-	-	0.23
Others (%)		0.00	0.14	12.91
1-Butanol. 2-methyl-, propanoate	973	-	-	0.20
1-Octen-3-ol	980	-	0.02	-
3-Octanol	995	-	0.07	-
iso-Amyl 2-methyl butyrate	1101	-	0.02	-
n-Amyl isovalerate	1106	-	0.04	0.48
8-methylene-3-oxatricyclo [5,2,0,0(2,4)]nonane	1117	-	-	**12.24**

TOTAL IDENTIFIED (%)	**99.80**	**99.66**	**98.95**

In bold, the most significant compounds for each EO

3.2 Effect of EOs on target plants

3.2.1 Effects of EOs on A. retroflexus

The efficacy of EOs on *A. retroflexus* was the maximum for *T. capitata* and *M. piperita* EO at the two highest doses (Table 3). The only two treatments set up for *S. chamaecyparissus* EO (results of S1 treatment are not reported in Table 3 because of the lack of a sufficient number of plants to set up the treatment) also suggested an increase of the efficacy by increasing EO dose as efficacy increased from 60 for S2 to 90 for S3. However, regardless of EO type, alive plants showed plant biometric variables lower than those of the two controls (Table 3). The damage level (DL) was the maximum (3) for the two highest doses of *T. capitata* and *M. piperita* and close to them (2.8) for S3, also S2 presented higher DL than the two controls.

Table 3. Effects of *T. capitata* (T1, T2 and T3 are 4, 8 and 12 µL mL^{-1} doses of application), *M. piperita* (M1, M2 and M3 are 12, 16 and 20 µL mL^{-1} doses of application), and *S. chamaecyparissus* (S1, S2 and S3 are 12, 16 and 20 µL mL^{-1} doses of application) EOs applied against *A. retroflexus* on the efficacy (E), plant biometric variables [aerial part (APL), root (RL) and total length (TL), fresh (FW) and dry weights (DW)] and damage level (DL).

Treatment/Dose	E	APL cm	RL cm	TL cm	FW g	DW g	DL
Cw	0 d	9.9 a	7.4 a	17.3 a	0.48 a	0.09 a	0.2 c
Cf	0 d	11.4 a	6.5 a	17.9 a	0.46 a	0.08 a	0.1 c
T1	30 c	5.9 b	2.6 b	8.5 b	0.32 ab	0.04 b	1.3 b
T2	100 a	0 e	0 c	0 d	0 d	0 c	3.0 a
T3	100 a	0 e	0 c	0 d	0 d	0 c	3.0 a
M1	50 bc	5 bc	2.9 b	8.1 b	0.27 bc	0.04 b	1.6 b
M2	100 a	0 e	0 c	0 d	0 d	0 c	3.0 a
M3	100 a	0 e	0 c	0 d	0 d	0 c	3.0 a
S1	-	-	-	-	-	-	-
S2	60 b	2.9 cd	1.7 bc	4.5 bc	0.13 cd	0.02 bc	2.0 b
S3	90 a	0.8 de	0.4 c	1.2 cd	0.03 d	0 c	2.8 a

Reported results are means of ten replicates. Different letters along the column indicate significant differences among treatments at $P<0.05$

3.2.2 Effects of EOs on P. oleracea

Only *T. capitata* and *M. piperita* EOs were effective to control *P. oleracea* at the two highest doses, with the first EO the most effective. On the other hand, at the highest doses, all tested EOs affected the plant biometric variables (Table 4). The damage level followed the same pattern of efficacy so being significantly higher than the two controls only with the two highest doses of *T. capitata* and *M. piperita* EOs.

Table 4. Effects of *T. capitata* (T1, T2 and T3 are 4, 8 and 12 µL mL^{-1} dose of application), *M. piperita* (M1, M2 and M3 are 12, 16 and 20 µL mL^{-1} dose of application), and *S. chamaecyparissus* (S1, S2 and S3 are 12, 16 and 20 µL mL^{-1} dose of application) EOs applied against *P. oleracea* on the efficacy (E), plant biometric variables [aerial part (APL), root (RL) and total length (TL), fresh (FW) and dry weights (DW)] and damage level (DL).

Treatment/Dose	E	APL cm	RL cm	TL cm	FW g	DW g	DL
Cw	0 c	9.3 a	10.9 a	19.1 a	1.6 a	0.20 a	0 c
Cf	0 c	8.9 a	11.3 a	20.2 a	1.5 a	0.17 a	0 c
T1	0 c	8.3 ab	8.5 abc	16.9 ab	1.3 ab	0.14 ab	0 c
T2	40 b	4.9 cd	5.5 cd	10.4 de	0.7 c	0.07 c	1.2 b
T3	90 a	0.7 e	0.7 e	1.3 f	0.1 d	0 e	2.7 a
M1	30 bc	6.0 bc	6.1 bcd	12.1 bcd	1.0 bc	0.10 bc	0.9 bc
M2	40 b	5.1 cd	5.3 cd	10.7 cde	0.7 c	0.08 c	1.2 b
M3	40 b	3.0 de	2.8 de	5.7 ef	0.6 cd	0.05 ce	1.2 b
S1	0 c	8.0 ab	8.4 abc	16.4 abc	1.6 ab	0.16 a	0 c
S2	10 bc	7.9 ab	8.7 abc	16.7 abc	1.6 ab	0.16 a	0.3 bc
S3	30 bc	5.8 bc	6.1 bcd	11.9 bcd	0.9 bc	0.08 bc	0.9 bc

Reported results are means of ten replicates. Different letters along the column indicate significant differences among treatments at $P<0.05$.

3.2.3 Effects of EOs on A. fatua

This weed species was more effectively controlled by *T. capitata* and *M. piperita* EOs, with efficacy increasing with the dose. *S. chamaecyparissus*, although still significantly, was less effective but with S2 and S3 doses not differing between them (Table 5). It is to note that Cf stimulated fresh and dry plant weights.

Table 5. Effects of *T. capitata* (T1, T2 and T3 are 4, 8 and 12 µL mL^{-1} dose of application), *M. piperita* (M1, M2 and M3 are 12, 16 and 20 µL mL^{-1} dose of application), and *S. chamaecyparissus* (S1, S2 and S3 are 12, 16 and 20 µL mL^{-1} dose of application) EOs applied against *A. fatua* on the efficacy (E), plant biometric variables [aerial part (APL), root (RL) and total length (TL), fresh (FW) and dry weights (DW)] and damage level (DL).

Treatment/Dose	E	APL cm	RL cm	TL cm	FW g	DW g	DL
Cw	0 e	28.0 a	18.1 a	46.1 a	1.02 b	0.19 b	0.15 d
Cf	0 e	28.5 a	17.7 a	46.2 a	1.45 a	0.23 a	0.15 d
T1	80 abc	6.0 cd	3.3 c	10.4 cd	0.20 d	0.02 d	3.2 ab
T2	90 ab	2.4 cd	1.8 c	4.2 cd	0.09 d	0.02 d	3.6 ab
T3	100 a	0 d	0 c	0 d	0 d	0 d	4.0 a
M1	70 bc	5.0 cd	4.1 c	9.2 cd	0.09 d	0 d	3.0 b
M2	90 ab	1.4 d	0.5 c	1.9 cd	0.01 d	0 d	3.7 ab
M3	100 a	0 d	0 c	0 d	0 d	0 d	4.0 a
S1	30 d	16.3 b	9.3 b	25.6 b	0.57 c	0.09 b	1.4 c
S2	60 c	8.8 c	3.7 c	12.6 c	0.16 d	0.02 d	2.9 b
S3	60 c	5.0 cd	3.5 c	8.5 cd	0.06 d	0.01 d	3.1 ab

Reported results are means of ten replicates. Different letters along the column indicate significant differences among treatments at P<0.05.

3.2.4 Effects of EOs on E. crus-galli

T. capitata EO was the most effective to control this weed, reaching the full effectiveness at the highest dose. *M. piperita* EO showed similar performances to T2 and T3 at M2 and M3, respectively (Table 6). Otherwise, *S. chamaecyparissus* EO at the highest dose did not even reach half the effectiveness of the other two EOs. Also biometric variables of the remaining alive plants were affected by all EOs, being reduced strongly overall by *T. capitata and M. piperita* EOs at the highest doses and to a lesser extent by *S. chamaecyparissus* EO, although we have to consider that the doses the of *T. capitata* were the lowest. The damage level followed the same pattern of the biometric plant variables being on average higher in *M. piperita* followed by *T. capitata* and then by *S. chamaecyparissus* EO.

Table 6. Effects of *T. capitata* (T1, T2 and T3 are 4, 8 and 12 µL mL^{-1} dose of application), *M. piperita* (M1, M2 and M3 are 12, 16 and 20 µL mL^{-1} dose of application), and *S. chamaecyparissus* (S1, S2 and S3 are 12, 16 and 20 µL mL^{-1} dose of application) EOs applied against *E. crus-galli* on the efficacy (E), plant biometric variables [aerial part (APL), root (RL) and total length (TL), fresh (FW) and dry weights (DW)] and damage level (DL).

Treatment/Dose	E	APL cm	RL cm	TL cm	FW g	DW g	DL
Cw	0 d	28.6 a	20.8 a	49.5 a	1.20 a	0.15 a	0.10 f
Cf	0 d	27.3 ab	19.9 a	47.2 a	0.90 ab	0.11 ab	0.10 f
T1	10 cd	26.1 ab	17.2 ab	43.3 ab	0.91 ab	0.10 ab	0.9 e
T2	50 b	14.6 cd	9.9 cd	24.5 cd	0.40 cde	0.06 bcde	2.4 bc
T3	100 a	0 f	0 f	0 f	0 f	0 f	4.0 a
M1	40 bc	10.4 cde	7.8 cde	18.5 cde	0.21 def	0.03 def	1.8 cd
M2	50 b	6.1 def	5.6 def	11.7 def	0.16 ef	0.02 ef	3.7 ab
M3	90 a	2.3 ef	2.5 ef	4.8 ef	0.21 f	0 f	4.0 a
S1	10 cd	19.2 bc	13.1 bc	32.3 bc	0.70 bc	0.1 bc	0.8 de
S2	30 bcd	17.2 c	12.3 bc	29.5 bc	0.54 cd	0.1 bcd	1.8 cd
S3	40 bc	12.2 cd	6.9 cde	19.1 cde	0.32 def	0.04 cdef	2.3 bc

Reported results are means of ten replicates. Different letters along the column indicate significant differences among treatments at P<0.05.

3.2.5 Overall efficacy of EOs

Among the investigated species *A. retroflexus* and *A. fatua* were the most sensitive targeted weed species to EOs being the efficacy for them greater than 50, whereas *P. oleracea* and *E. crus-galli* were the least sensitive with an EOs efficacy lower than 40 (Table 7). Fitoil treatment (Cf) did not exert any phytotoxic effect on weeds since no statistical differences were observed with water control (Cw). All EO treatments, at different extent, significantly provoked weeds

death, with however *S. chamaecyparissus* EO at the lowest dose causing the least damage (Table 7). *T. capitata* and *M. piperita* EOs were the most effective in killing the targeted weeds.

Table 7. Overall essential oils efficacy per species and per treatment. Treatments were: *T. capitata* EO (T1, T2 and T3 are 4, 8 and 12 µL mL^{-1} doses of application), *M. piperita* EO (M1, M2 and M3 are 12, 16 and 20 µL mL^{-1} doses of application) and *S. chamaecyparissus* EO (S1, S2 and S3 are 12, 16 and 20 µL mL^{-1} doses of application).

Species	Efficacy
Amaranthus retroflexus	64 a
Portulaca oleracea	25 c
Avena fatua	52 a
Echinochloa crus-galli	38 b

Treatment/Dose	Efficacy
Cw	0 g
Cf	0 g
T1	30 ef
T2	70 bc
T3	97 a
M1	47 d
M2	70 bc
M3	82 ab
S1	13 fg
S2	40 de
S3	55 cd

Reported results are means of ten replicates. Different letters along the column indicate statistical differences among species or treatments at $P < 0.05$.

3.3 Effects of EOs on soil biochemical properties and on the main microbial groups

Regardless of incubation day, C_{extr} and MBN generally did not differ between Cw and Cf, while MBC and qCO_2 were higher in Cf than Cw (Table 8). Consequently, in Cw the MBC/MBN ratio was more than twice in Cf. The two controls did not differ in respiration rate (Table 8). Regarding the main microbial groups, few differences occurred between the two controls: bacteria did not significantly differ, while fungi and gram-negative bacteria were greater in Cf than in Cw but only at day 56. The above results indicated that fitoil alone exerted some effects on the soil biochemical properties. Thus, to isolate the effects due to solely the added EOs, we decided to compare the results of the EOs treated soil with the Cf control.

Table 8. Biochemical parameters and main microbial groups determined in soil irrigated with water (Cw) or with fitoil emulsion (Cf, 0.5 mL L^{-1}) during the incubation.

Treatment	**Water Control (Cw)**			**Fitoil Control (Cf)**		
Sampling day	**7**	**28**	**56**	**7**	**28**	**56**
Soil variables						
Cextr (mg kg^{-1})	136 Ba	152 Aa	69 Ab	171 Aa	164a A	81 Ab
MBC (mg kg^{-1})	219 Ac	316 Ab	460 Aa	141 Bb	169 Bb	246 Ba
MBN (mg kg^{-1})	14 Bb	21 Aab	28 Aa	20 Ab	28 Aab	36 Aa
MBC/MBN	15.9 Aa	15.5 Aa	16.2 Aa	7.0 Ba	6.1 Ba	6.9 Ba
Microbial respiration (mg CO_2-C kg^{-1} d^{-1})	15.5 Aa	11.9 Aab	8.7 Ab	14.3 Aa	11.4 Aab	8.8 Ab
Metabolic quotient (qCO_2; mg CO_2-C g^{-1} MBC h^{-1})	3.0 Ba	1.6 Bab	0.8 Bb	4.2 Aa	2.8 Aab	1.5 Ab
Bacteria (nmol kg^{-1})	65.2 Aa	73.5 Ba	66.8 Ba	76.5 Aa	84.7 Ba	82.4 Ba
Fungi (nmol kg^{-1})	13.8 Aa	18.6 Aa	2.3 Bb	13.3 Aa	18.8 Aa	17.3 Aa
Bacteria gram positive (nmol kg^{-1})	20.7 Ac	36.4 Ab	53.1 Aa	32.0 Ab	43.5 Aa	46.7 Aa
Bacteria gram negative (nmol kg^{-1})	44.4 Aa	37.1 Aa	13.7 Bb	44.5 Aa	41.2 Aa	35.7 Aa
Fungi/Bacteria	0.21 Aa	0.25 Aa	0.04 Bb	0.17 Aa	0.22 Aa	0.23 Aa
Bacteria Gram positive/ Gram negative	0.48 Ab	1.00 Ab	4.14 Aa	0.72 Aa	1.06 Aa	1.28 Bb

Capital letters indicate significant differences among the two controls at the same incubation day. Lower case letters indicate significant differences among incubation days within a given control. N = 4.

3.3.1 Effects of T. capitata EO on soil biochemical properties and main microbial groups

Extractable C was mainly affected by the interaction dose x day (77% of explained variance; Figure 5). During the incubation, it decreased in Cf and increased in soil treated with EO, especially with T1 and T2. MBC trend was opposite compared to Extractable C since throughout incubation it increased in Cf while decreased in EO treated soil at any dose. Dose and dose x day almost at the same extent affected MBC (Figure 5). MBC, regardless of incubation day, decreased with increasing EO dose (Figure 5). The interaction dose x day explained the greatest amount of variance of MBN as, during the incubation, it increased in Cf, did not change with T1 and T2 doses while decreased in THY3 (Figure 5). Thus, except for T1 and T2 doses, the trend of MBN was parallel to that of MBC. Cumulative CO_2 was not affected by *T. capitata* EO at any dose (Figure 5); in fact, although at the beginning of the incubation, CO_2 emission rate was higher in EO treated soils compared to Cf (and Cw), starting from day 23 the rate was inverted (Figure 6). The biologically available C at time zero (C_0), as well as the turnover constant rate, were higher in soil treated with EO than in Cf and proportionally increased with EO dose (Figure 6). The qCO_2 was significantly affected by both factors (Table 9); indeed, regardless of dose, it was the highest at day 7, whereas at any incubation day with the highest dose. Also, the main microbial groups were generally and similarly affected by both factors and their interaction. In comparison with Cf, *T. capitata* EO slightly decreased bacteria

especially at 28 days of the incubation, while fungi strongly decreased with THY3 (Table 9). As a consequence, the F/B ratio THY1 and THY2 at 56 days was much higher than Cf BacG+ decreased compared to Cf while BacG- did not differ so much, so that the ratio BacG+/BacG- at 28 days decreased compared to Cf.

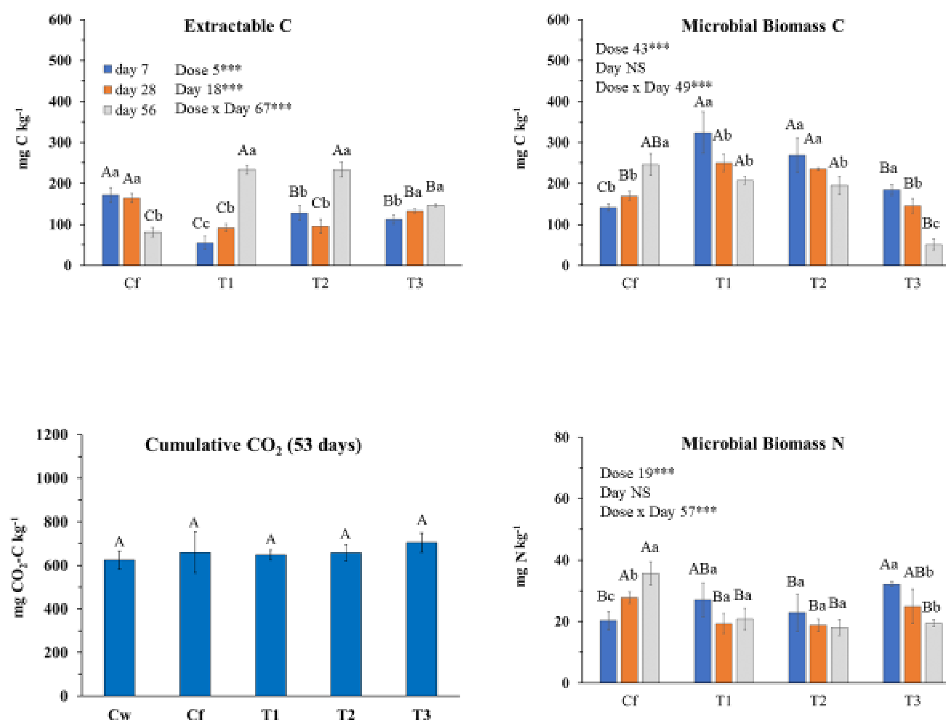

Figure 5. Biochemical soil variables determined at 7, 28 and 56 incubation days after *Thymbra capitata* L. (Cav) essential oil applied at different doses: Cf (0.03 µL Fitoil g^{-1} of soil and 0 µL EO g^{-1} of soil), T1 (0.03 µL Fitoil g^{-1} of soil and 0.31 µL EO g^{-1} of soil), T2 (0.03 µL Fitoil g^{-1} of soil and 0.67 µL EO g^{-1} of soil), and T3 (0.03 µL Fitoil g^{-1} of soil and 0.93 µL EO g^{-1} of soil). Reported results are means ±standard deviations (n=4). The percentage of variance explained by incubation day, dose of essential oil and by their interaction are also reported. Capital letters indicate significant differences among doses within the same incubation day. Lower case letters indicate significant differences among incubation days within the same dose. *, ** and *** indicate significant at $P<0.05$, $P<0.01$ and $P<0.001$, NS, not significant.

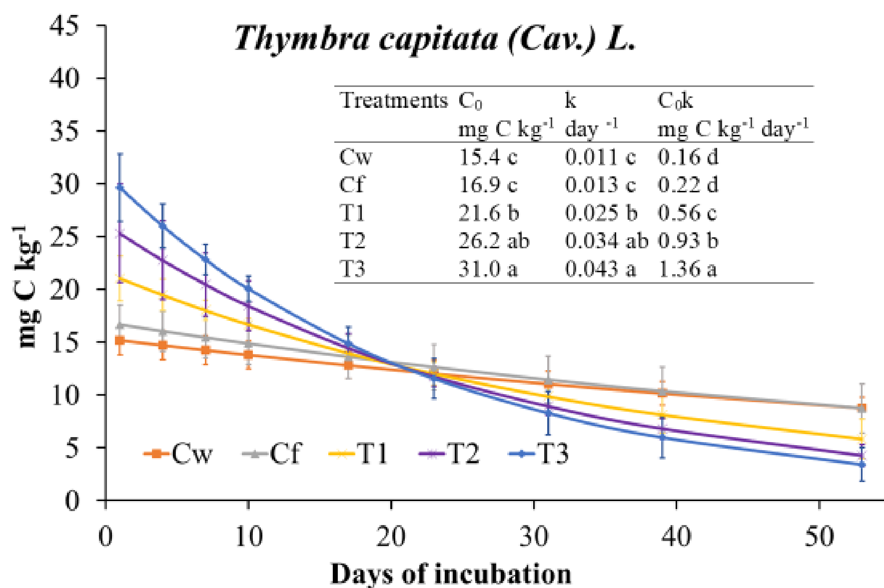

Figure 6. Microbial respiration rate fitted to the exponential first order decay function (Mineralized C = C_0 e^{-kt}) and derived parameters (C_0, biological available C; k, turnover constant rate; C_0k, initial potential rate of C mineralization) determined on soil treated with *Thymbra capitata* L. (Cav.) essential oil. Treatments were: Cw (0 µL Fitoil g^{-1} of soil and 0 µL EO g^{-1} of soil), Cf (0.03 µL Fitoil g^{-1} of soil and 0 µL EO g^{-1} of soil), T1 (0.03 µL Fitoil g^{-1} of soil and 3.1 µL EO g^{-1} of soil), T2 (0.03 µL Fitoil g^{-1} of soil and 0.67 µL EO g^{-1} of soil), and T3 (0.03 µL Fitoil g^{-1} of soil and 0.93 µL EO g^{-1} of soil). Reported results are means (n=4). Bars indicate the standard deviations. Lower case letters indicate significant differences among treatments.

Table 9. Metabolic quotient, main microbial groups (nmol g^{-1}) and percentage of variance explained by incubation day, dose of essential oil and by their interaction. Treatments were: *T. capitata* (THY1, THY2 and THY3 are 4, 8 and 12 µL mL^{-1} doses of application), *M. piperita* (MNT1, MNT2 and MNT3 are 12, 16 and 20 µL mL^{-1} doses of application) and *S. chamaecyparissus* (SNT1, SNT2 and SNT3 are 12, 16 and 20 µL mL^{-1} doses of application) essential oils; Cf, control with fitoil.

Treatment/Dose	Day	qCO_2	Bacteria	Fungi	BacG+	BacG-	F/B	BacG+/BacG-
Cf	7	4.2 a	76 a	13 b	32 a	44 a	0.17 a	0.72 a
Cf	28	2.8 b	85 a	19 a	43 a	41 a	0.22 a	1.06 a
Cf	56	1.5 c	82 a	17 ab	47 a	36 b	0.23 a	1.28 a
THY1	7	**2.4** Ca	67 Ba	15 Ab	30 ABa	37 Ba	0.22 Bb	0.81 Aa
	28	**1.8** Bb	**69** Aa	**16** Aab	**30** Aa	39 Aa	0.23 Ab	**0.77** Aab
	56	1.2 Bc	**16** Ab	20 Aa	**6** Ab	9 Ab	**1.31** Aa	0.71 Bb
THY2	7	**3.2** Ba	55 Ba	12 Ba	24 Ba	31 Ba	0.22 Bb	0.91 Ab
	28	**1.8** Bb	**64** Aa	**13** Ba	**27** Aa	36 Aa	0.20 Ab	**0.74** Ab
	56	**0.9** Bc	32 Ab	13 ABa	19 Aa	13 Aa	**1.04** Aa	1.55 Aa
THY3	7	**5.2** Aa	97 Aa	3 Cb	35 Aa	61 Aa	0.03 Ba	0.57 Aa
	28	2.7 Ab	**46** Bb	**4** Cb	**20** Bb	**27** Ba	0.08 Bab	**0.74** Aa
	56	**2.9** Ab	60 Aab	**9** Ba	20 Ab	40 Aa	0.18 Ba	0.69 Ba
Day		*59****	*16***	*12**	*NS*	*22***	*34***	*14**
Dose		*22***	*19***	*72****	*27***	*14**	*19***	*22***
Day x Dose		*14**	*29***	*6**	*26***	*24***	*31***	*29***
MNT1	7	**15.0** Aa	72 Aa	**7** Bb	**15** ABc	58 Aa	0.11 Bb	**0.28** Ac
	28	2.3 Bb	**73** Aa	**10** Bab	**32** Ab	41 Ab	**0.13** Cab	0.77 Ab
	56	1.9 Ab	58 Aa	**10** Ba	39 Aa	**20** ABc	0.18 Bb	2.56 ABa
MNT2	7	**15.8** Aa	59 Aa	11 Ab	**13** Bc	46 Aa	0.20 Aa	**0.31** Ac
	28	2.5 Bb	**62** Ba	**12** ABab	**30** ABb	**32** Bb	0.20 Ba	0.96 Ab
	56	**0.8** Bc	55 Aa	13 Ba	41 Aa	**15** Bc	0.24 Ba	2.81 Aa
MNT3	7	**10.3** Ba	66 Aa	14 Ab	**18** Ac	48 Aa	0.21 Ab	**0.39** Ac
	28	**2.0** Bb	**61** Bb	**15** Ab	**26** Bb	**34** Bab	0.25 Ab	**0.76** Ab
	56	**0.9** Bc	62 Aab	23 Aa	31 Aa	31 Ab	0.37 Aa	1.02 Ba
Day		*69****	*NS*	*18***	*51****	*53****	*24***	*52****
Dose		*7**	*44****	*54****	*23***	*5**	*45****	*5**
Day x Dose		*22***	*NS*	*15**	*NS*	*12**	*8**	*25***
SNT1	7	3.9 Ba	81 Aa	**27** Aa	**4** Bc	**78** Aa	**0.33** Aa	**0.05** Bc
	28	**1.6** Cb	**93** Aa	**23** Ab	44 Ab	49 Ab	0.24 Ab	0.93 Ab
	56	1.4 Ab	74 ABa	**21** Ab	68 Aa	**6** Bc	0.30 Aab	**11.29** Aa
SNT2	7	3.7 Ba	80 Ac	**19** Ba	**10** Ac	**70** Aa	**0.23** ABa	**0.15** Ab
	28	**2.0** Bb	90 Ab	17 Bab	39 ABb	51 Ab	0.19 Bab	0.76 Ab
	56	1.1 Ac	100 Aa	16 Bb	62 Aa	38 Ac	0.16 Bb	1.73 Ca
SNT3	7	**4.9** Aa	66 Ac	9 Ca	**10** Ac	56 Ba	0.14 Ba	**0.18** Ab
	28	3.3 Ab	86 Aa	**3** Cb	**36** Bb	50 Aa	**0.03** Cb	**0.72** Ab
	56	1.3 Ac	56 Bb	**2** Cb	48 Aa	**8** Bb	**0.03** Cb	**5.85** Ba
Day		*81****	*14**	*NS*	*72****	*61****	*5**	*40****
Dose		*7**	*25***	*82****	*NS*	*6**	*72****	*13**
Day x Dose		*NS*	*25***	*10**	*16***	*28***	*13**	*44****

Capital letters indicate significant differences among doses at the same incubation day within a treatment. Lower case letters indicate significant differences among incubation days at the same dose within a treatment. Numbers in bold indicate significant differences at P<0.05 compared to the fitoil control (Cf) at a given day. BacG+ and BacG- indicate gram positive and negative bacteria, respectively. *, ** and *** indicate significant at P<0.05, P<0.01 and P<0.001, NS, not significant. N = 4.

3.3.2 Effects of M. piperita EO on soil biochemical properties and main microbial groups

Incubation day more than EO dose affected almost all the investigated soil biochemical parameters (Figure 7); however, at the end of incubation (56 days) changes among Cf and EO treatments were negligible. At day 7 extractable C increased with the EO dose, then strongly decreased whereas MBC increased with incubation day at the two highest doses (Figure 7). MBN pattern was affected mainly by the interaction of the two tested factors. Indeed, during the first 28 days of the incubation it was affected only by the lowest dose which decreased it compared to the control. At day 56, it was positively affected only by the intermediate dose. Compared to Cf, CO_2 emission rates and, consequently, cumulative CO_2 were higher in EO treated soils but not significantly different occurred among them (Figure 7 and 8). Also, the C_0 and k parameters were the highest in EO treated soils with no difference among dose. The qCO_2 was mainly affected by incubation day (69% of variance explained) as it decreased during the incubation at any EO dose (Table 9). Notably, the qCO_2 at day 7 in EO treated soils was 2 to 3 times higher than in Cf, then drastically decreased with negligible differences among EO treatments and control. Both bacteria and fungi slightly decreased compared to the control at any EO dose (Table 9). BacG+ decreased more consistently than BacG- after EO application, so that, at the beginning of the incubation, the ratio BacG+/BacG- was lower than the control. At day 56, however, all the microbial groups did not significantly differ from the control.

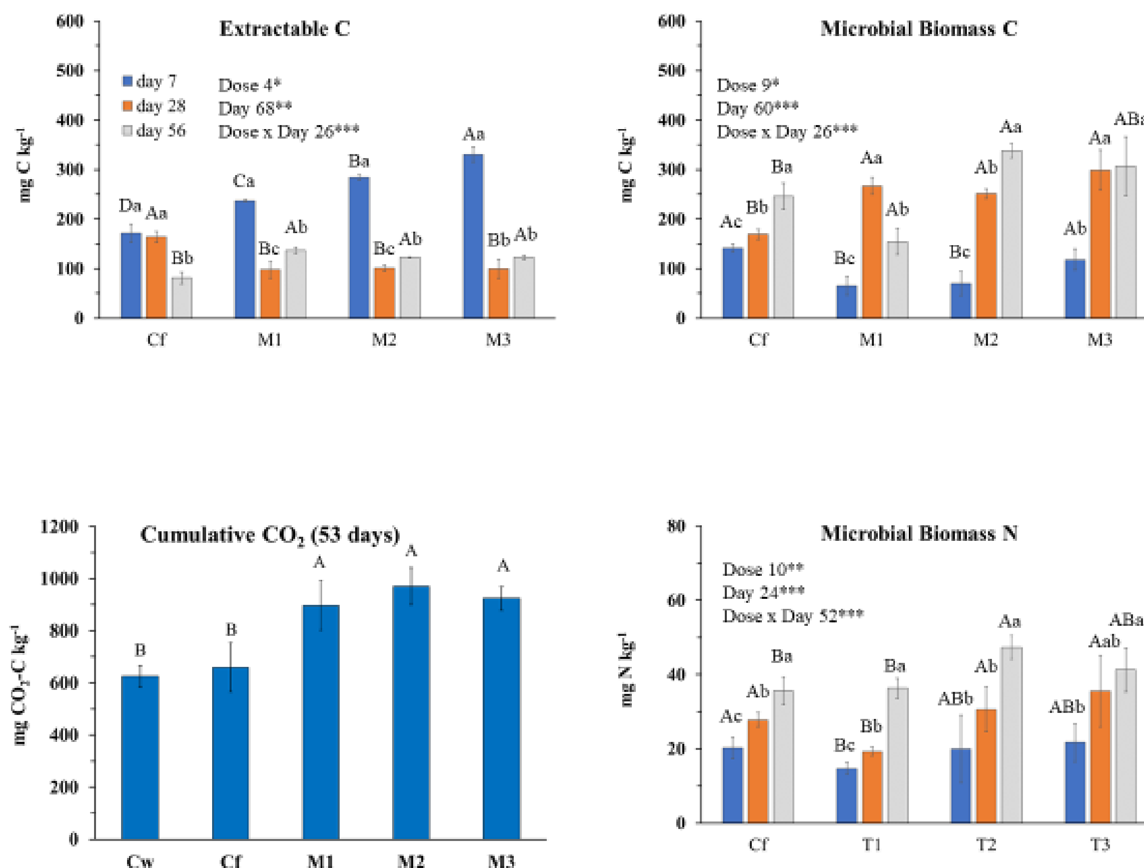

Figure 7. Biochemical soil variables determined at 7, 28 and 56 incubation days after *Mentha x piperita* L. essential oil applied at different concentrations: Cf (0.03 µL Fitoil g^{-1} of soil and 0 µL EO g^{-1} of soil), M1 (0.03 µL Fitoil g^{-1} of soil and 0.93 µL EO g^{-1} of soil), M2 (0.03 µL Fitoil g^{-1} of soil and 1.23 µL EO g^{-1} of soil), and M3 (0.03 µL Fitoil g^{-1} of soil and 1.54 µL EO g^{-1} of soil). Reported results are means ± standard deviations (n=4). The percentage of variance explained by incubation day, dose of essential oil and by their interaction are also reported. Capital letters indicate significant differences among doses within the same incubation day. Lower case letters indicate significant differences among incubation days within the same dose. *, ** and *** indicate significant at P<0.05, P<0.01 and P<0.001, NS, not significant.

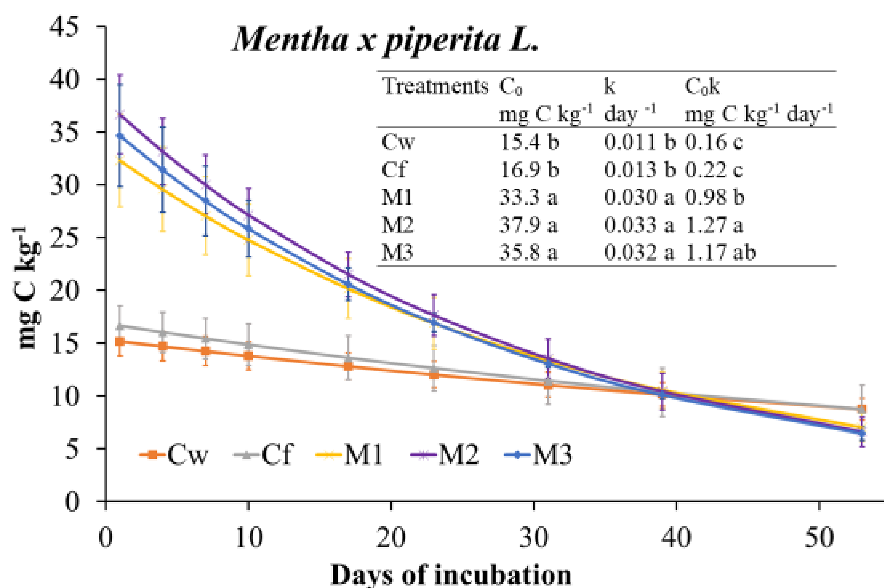

Figure 8. Microbial respiration rate fitted to the exponential first order decay function (Mineralized C = C_0 e^{-kt}) and derived parameters (C_0, biological available C; k, turnover constant rate; C_0K, initial potential rate of C mineralization) determined on soil treated with *Mentha x piperita* L. essential oil. Treatments were: Cw (0 µL Fitoil g^{-1} of soil and 0 µL EO g^{-1} of soil), Cf (0.03 µL Fitoil g^{-1} of soil and 0 µL EO g^{-1} of soil), M1 (0.03 µL Fitoil g^{-1} of soil and 9.3 µL EO g^{-1} of soil), M2 (0.03 µL Fitoil g^{-1} of soil and 1.23 µL EO g^{-1} of soil), and M3 (0.03 µL Fitoil g^{-1} of soil and 1.54 µL EO g^{-1} of soil). Reported results are means (n=4). Bars indicate the standard deviations. Lower case letters indicate significant differences among treatments.

3.3.3 Effects of S. chamaecyparissus EO on soil biochemical properties and main microbial groups

As in *M. piperita* treatment, incubation day more than EO dose affected investigated soil parameters in *S. chamaecyparissus* treatment (Figure 9). Extractable C decreased in all treatments during the incubation (Figure 9) and at day 7 it increased with the EO dose. Then, it slumped with negligible differences among EO doses and Cf. MBC at day 7 and 28 was higher in EO treated soils than Cf, also decreasing with the dose, whereas at day 56 any difference among Cf and treated soils disappeared. The trend of MBN was peculiar since at day 7, in EO treated soil and regardless of dose, it was about three times higher than in Cf, at day 28 it slumped in the presence of EO down to Cf value with no difference among doses, whereas at day 56, compared to Cf, it decreased with increasing EO dose (Figure 9). Cumulative CO_2 and other parameters related to C mineralization were higher in EO treated soils than Cf, with no significant difference among EO doses (Figures 9 and 10). The qCO_2

was mainly affected by incubation day (Table 9) since at any EO dose it decreased during the incubation, with generally no difference among treatments and the control. Fungi were strongly affected by the EO dose, since during the whole incubation they were increased by SNT1 in comparison to Cf, whereas at days 28 and 56 were lowered by SNT3 (Table 9). Total bacteria were not much affected by EO, whereas at day 7 BacG+ were decreased and BacG- increased; consequently, the BacG+/BacG- ratio strongly increased during the incubation in EO treated soil (Table 9).

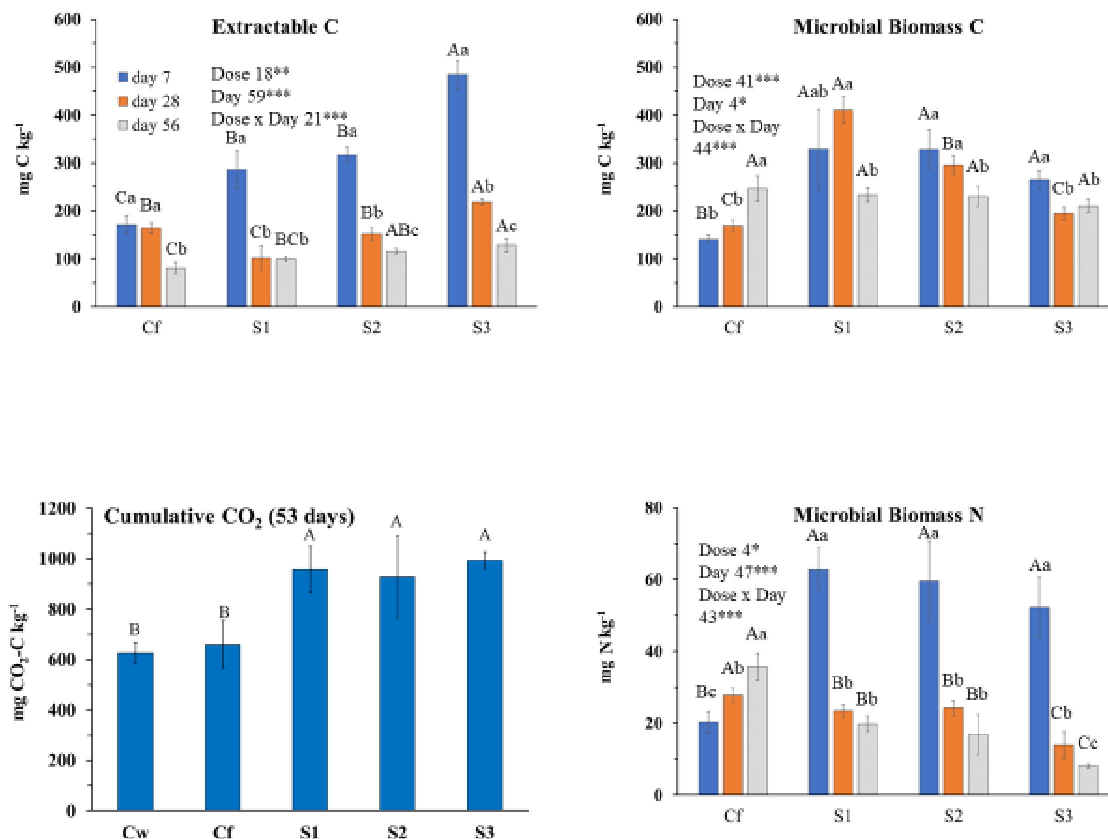

Figure 9. Biochemical soil variables determined at 7, 28 and 56 incubation days after *Santolina chamaecyparissus* L. essential oil applied at different concentrations: Cf (0.03 µL Fitoil g^{-1} of soil and 0 µL EO g^{-1} of soil), S1 (0.03 µL Fitoil g^{-1} of soil and 0.93 µL EO g^{-1} of soil), S2 (0.03 µL Fitoil g^{-1} of soil and 1.23 µL EO g^{-1} of soil), and S3 (0.03 µL Fitoil g^{-1} of soil and 1.54 µL EO g^{-1} of soil). Reported results are means±standard deviations (n=4). The percentage of variance explained by incubation day, dose of essential oil and by their interaction are also reported. Capital letters indicate significant differences among doses within the same incubation day. Lower case letters indicate significant differences among incubation days within the same dose. *, ** and *** indicate significant at P<0.05, P<0.01 and P<0.001, NS, not significant.

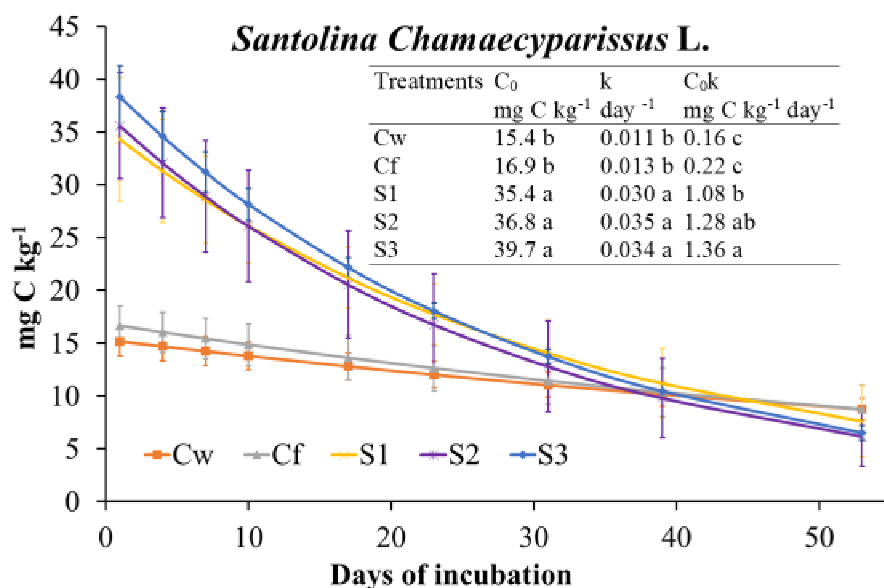

Figure 10. Microbial respiration rate fitted to the exponential first order decay function (Mineralized C = C_0 e^{-kt}) and derived parameters (C_0, biological available C; k, turnover constant rate; C_0K, initial potential rate of C mineralization) determined on soil treated with *Santolina chamaecyparissus* L. essential oil. Treatments were: Cw (0 µL Fitoil g^{-1} of soil and 0 µL EO g^{-1} of soil), Cf (0.03 µL Fitoil g^{-1} of soil and 0 µL EO g^{-1} of soil), S1 (0.03 µL Fitoil g^{-1} of soil and 9.3 µL EO g^{-1} of soil), S2 (0.03 µL Fitoil g^{-1} of soil and 1.23 µL EO g^{-1} of soil), and S3 (0.03 µL Fitoil g^{-1} of soil and 1.54 µL EO g^{-1} of soil). Reported results are means (n=4). Bars indicate the standard deviations. Lower case letters indicate significant differences among treatments.

4 Discussion

4.1 Herbicidal activity of *T. capitata*, *M. piperita* and *S. chamaecyparissus* EOs

To our knowledge this is the first study to test *in vivo* the herbicidal potential of *T. capitata, M. piperita* and *S. chamaecyparissus* EOs against the highly competitive and herbicide-resistant weeds *A. retroflexus*, *P. oleracea*, *A. fatua* and *E. crus-galli* (Heap 2020). The herbicidal activity of the tested EOs was dependent on the targeted weed species, the type of EOs (because of their different compositions) and the dose of application. The investigated target weed species showed an overall resistance to the tested EOs according to the following order: *P. oleracea> E. crus-galli>A. fatua >A. retroflexus*. The high ability of *P. oleracea* and *E. crus-galli* in facing chemical-induced stress, adopting molecular, biochemical, and anatomical strategies, is largely reported (Juliano *et al*. 2010; Malpassi 2006; Norswothy and Riar *et al*. 2013; Smith 2005). Anyway, such resistance is dependent on several ecological (e.g. genotype, ecotype) (Avila *et al*. 2005; Liu 2018) and non-ecological factors, such as the chemical used. For example, Norswothy and Smith (2005) reported that *P. oleracea* was significantly resistant

to the pre-emergence herbicide dimethenamid but extremely sensitive to pendimethalin. As well as synthetic herbicides, also natural compounds, such as terpenoids, could have a wide range of metabolic targets depending on their molecular structure (Chaimovitsh *et al*. 2010; Duke *et al*. 2004). For example, it has been reported that both the monoterpene citral and the sesquiterpene farnesene are able to alter the hormones balance and cell ultrastructure in seedlings of *Arabidopsis thaliana* (Araniti *et al*. 2016; Araniti *et al*. 2017; Graña *et al*. 2016), whereas the sesquiterpene *trans*-caryophyllene alter plant water status, photosystem II efficiency and may also inhibit the germination and growth of several weeds (Araniti *et al*. 2017). Overall, these findings suggest that EOs, being a complex mixture of different molecules with different modes of action, could represent an interesting source for the development of new multi-targeted bioherbicides due to their wide and versatile chemical composition. Therefore, it is crucial to know the chemotype from which EOs are extracted as well as their main compounds which characterize them.

In *T. capitata* EOs three chemotypes have been described: thymol, carvacrol, and thymol/carvacrol, each characterized by the predominance of the compounds (Bounatirou *et al*. 2007; Karousou *et al*. 2005; Miceli *et al*. 2006). The high abundance of carvacrol and the low amount of thymol, observed in the EO used in our experiments, suggest that it was a carvacrol chemotype. Similarly, the EO of *S. chamaecyparissus,* known to have several chemotypes particularly rich in camphor, borneol, 1,8-cineole and others (Garg *et al*. 2001), could be identified as an 1,8 cineole chemotype since this molecule was the most abundant. Finally, as largely reported (Brahmi *et al*. 2017; Synowiec *et al*. 2019), and confirmed by our analysis, the most abundant compounds characterizing the EO of *M. piperita* were menthol and menthone.

Regarding the dose of application, the herbicidal activity of EOs increased by increasing the dose. *T. capitata* EO was the most effective, but also *M. piperita* EO displayed good potential as natural herbicide, except against *P. oleracea*. On the other hand, *S. chamaecyparissus* EO revealed more selectivity being very effective especially against *A. retroflexus*. Therefore, although the latter EO was not as much potent as *T. capitata* or *M. piperita*, it could be interesting for the development of selective natural herbicides. With regard to the latter issue, it is important to highlight that tested EOs could be an important and sustainable tool for weed management, not only by killing target weeds but also reducing their ability to compete with the crops, as they were capable to reduce their vigour and growth. Many *in vitro* studies have shown that *T. capitata* EO was able to inhibit seed germination of several noxious weeds (García-Plasencia 2013; Hanana *et al*. 2017; Verdeguer 2011), such as *Erigeron bonariensis*,

one of the most important cosmopolite weeds especially in no-tilled soils with problems of resistance to glyphosate (Verdeguer *et al*. 2020). In addtiton, *T. capitata* has been described as an allelopathic species capable to reduce both germination and growth of neighbouring species (Katz *et al*. 1987). Such allelopathic activity has been ascribed to carvacrol, the main EO constituent (Katz *et al*. 1987, Saoud *et al*. 2013). Phytotoxicity of pure carvacrol stood out its extremely high herbicidal activity on *Amaranthus retroflexus* and *Chenopodium album*, which was greater than the commercial herbicide 2,4-D isooctyl ester (Kordali *et al*. 2008). Moreover, de Assis Alves *et al*. (2018) demonstrated that carvacrol evidenced a genotoxic effect, higher than glyphosate, altering the cell cycle in *Lactuca sativa* and *Sorghum bicolor* meristematic cells.

With regard to the herbicidal activity of *M. piperita* EO, previous studies confirmed the herbicidal potential of its EO against field bindweed (*Convolvulus arvensis* L.), purslane (*Portulaca oleracea* L.) and jungle rice (*Echinochloa colonum* L.), also suggesting its possible use in the formulation of natural herbicides (Mahdavikia and Saharkhiz, 2015). However, as *M. piperita* EO was phytotoxic for the crops on which was tested, it could be developed as non-selective broad-spectrum herbicide. Campiglia *et al*. (2007), conducting a pot experiment, found that *M. piperita* EO was effective in controlling the germination of *A. retroflexus*, *Sinapis arvensis*, and *Lolium* spp. These results are consistent with this work findings, confirming that *A. retroflexus* is a sensitive species to *M. piperita* EO. The herbicidal activity of *M. piperita* EO is to be ascribed to its ability to interfere with root plasma membrane. Indeed, Maffei *et al*. (2001) have demonstrated that menthol and menthone, the main compounds held in *M. piperita* EO, are responsible for the depolarization of the membrane potential on cucumber roots. In addition, Mucciarelli *et al*. (2001) demonstrated that both menthol and menthone were significantly reducing mitochondrial respiration in root cells. About the herbicidal potential of *S. chamaecyparissus* EO, few studies are available, many of which have been performed in *in vitro* conditions. Such studies support the selectivity of this EO depending on the species against it is applied. *S. chamaecyparissus* EO, rich in 1,8-cineole (24.8%), was tested on seed germination and root and shoot growth of four crops (*Zea mays*, *Triticum durum*, *Pisum sativum*, and *Lactuca sativa*) and two weeds (*Portulaca oleracea* and *Vicia sativa*). The germination of both weeds, wheat and lettuce was inhibited while it was less harmful for sweet corn and dwarf pea. In addition, it was more active on *P. oleracea*, reducing its shoot and root length, than on the crops (Grosso *et al*. 2010). On the other hand, *S. chamaecyparissus* EO from an industrial sample, with 1,8-cineole (9.8%) and 8-methylene-3-oxatricyclo [5.2.0.02,4] nonane (8.2%) as main compounds, showed a moderate phytotoxicity against the leaf growth

of *L. perenne*, but did not show negative effects against *L. sativa* seeds (De Elguea-Culebras *et al*. 2018).

4.2 Changes in soil biochemical properties and in main microbial groups following the addition of EOs

Due to the scarcity of studies dealing with the effects of tested EOs on soil biochemical properties and on the main microbial groups, the discussion of these results was carried out in comparison with occurring *in vitro* studies, when available, aimed at assessing the effects of a single or few key constituents held within the EOs.

4.2.1 T. capitata (THY) EO effects

The addition of THY EO stimulated soil microorganisms but in a different way, depending on the dose and incubation day. The increase of MBC, MBN and respiration, and the decrease of extractable C occurred immediately after the addition of the EO at the lowest dose, suggesting that the available C substrates already in soil, plus those added by EO treatment, were mainly immobilised by soil microorganisms and mineralised. On the contrary, by increasing the EO dose, at the beginning of incubation the stimulation concerned only microbial respiration. Towards the end of the incubation, the stimulation effects induced by the two lowest THY EO doses vanished, whereas the highest dose decreased both MBC and activity. These results suggested that THY EO at the highest dose killed part of soil microorganisms (MBC and MBN decreased) and that the surviving ones were not able to use the cytoplasmic materials released outside as demonstrated by the increase of extractable C and the low rate of respiration compared to the control. Overall, such results suggested that THY EO at the highest concentration may be deleterious for soil microorganisms and that such negative effects occur after about two months. Such findings agreed with those of Vokou and Liotiri (1999) who reported THY EO can be used as carbon source by soil microorganisms within one month since its supply. On the other hand, however, the reduction of microbial biomass agreed with Monokrousos *et al*. (2004) and Papatheodorou *et al*. (2002) who showed that the presence of THY plants strongly interfered with soil microbial biomass and activity.

The biocidal effect of THY EO may be attributed to its main component, the carvacrol, that is able to permeate and depolarize the cytoplasmic membrane of microorganisms, so releasing outside the cell membrane associated materials (Helander *et al*. 1998; Xu *et al*. 2008). At the beginning of incubation, the changes in MBC and respiration, on average, decreased the qCO_2 at the two lowest doses while increased it at the highest one towards the end of incubation.

Such changes may be attributed the increase of microbial biomass and the decrease of C substrates availability and the BacG+/BacG- ratio at the two lowest doses, whereas to the increase of C substrates availability and to the decrease of microbial biomass at the highest dose (Nannipieri *et al*. 2003; Vittori Antisari *et al*. 2016; Wardle and Ghani, 2018).

4.2.2 M. Piperita (MNT) EO effects

MNT EO had an immediate antimicrobial activity as evidenced by the decrease of the soil microbial biomass and by the increase of extractable C at day 7. However, the fraction of soil microorganisms that survived to such initial antimicrobial activity later was able to utilise the fresh organic C substrates coming form killed microorganisms, as confirmed by the remarkable increase of both microbial biomass and respiration rate but also by the biological available C. Such changes were confirmed by the initial huge increase of the qCO_2 indicating a strong stress/disturbance condition for soil microorganisms, which was followed by the return to qCO_2 comparable to control at the end of incubation, once the stress finished (Wardle and Ghani 2018).

The antimicrobial activity of MNT EO could probably be associated with its main constituent, menthol (51.81% in this study). Indeed, İşcan *et al*. (2002), using the bioautographic test, found that menthol was responsible for the antimicrobial activity against plant pathogenic microorganisms. However, also additive, synergistic, or antagonistic effects due to interactions among EO constituents, even those present at low concentrations, cannot be excluded (Xianfei *et al*. 2007; Zakarya *et al*. 1993). The later increases of MBC and MBN at day 28 and 56 were probably due to an adaptation to the compounds added by the EO and to the previous great amount of released organic C substrates which were immobilized by the surviving microbial biomass, so reducing the stress/disturbance conditions as evidenced by the decrease of qCO_2. Therefore, such results indicated that MNT EO provoked a transient negative impact on soil microorganisms and/or that soil microorganisms had high resilience capability (Allison and Martiny 2008). Also the effects on the main microbial groups were transient as, after a reduction occurred during the first 28 days of incubation that concerned both bacteria and fungi, at the end of the incubation the fungi/bacteria and the bacG+/bacG- ratios did not show significant differences compared to the control.

4.2.3 S. chamaecyparissus (SNT) EO treatment

SNT EO stimulated both soil microbial biomass and respiration, but up to 28 days of incubation. However, the simultaneous great increase of extractable C might indicate that immediately after its addition some microorganisms were killed and the released cytoplasmic materials promptly either immobilised or mineralized, as confirmed by the increase of respiration rate and biological available C.

The antimicrobial activity of SNT EO was likely due to one of its main constituents, the 1,8-cineole, that is well known for its antimicrobial activity (Khubeiz and Mansour 2016; Morcia *et al*. 2012). Furthermore, as already stated, the inhibitory activity might result from the interaction of its constituents, even those present at low concentrations. In fact, as demonstrated by Viljoen *et al*. (2003), 1,8-cineole in combination with camphor (4.03% in the SNT EO tested in this study) showed higher antimicrobial effects. The great stimulation induced by SNT EO, however, did not affect the qCO_2 since MBC and respiration rate were proportionally enhanced. Such stimulation effect was transient since at the end of the incubation both MBC and respiration were not significantly different from the control. On the other hand, MBN was significantly decreased at the end of the incubation suggesting, at least, an increase in the fungi to bacteria ratio since the first have higher MBC/MBN ratio than the latter (Cleveland and Liptzin 2007). PLFAs data partially confirmed such findings being the fungi/bacteria ratio higher than the control at the lowest EO concentrations and day 7. Moreover, PLFAs data put in evidence that the relative abundances of the main microbial groups at the end of the incubation rarely changed, with also no relapses on the microbial activity, so suggesting a good resilience of the soil microorganisms.

5 Conclusions

Several studies have been carried out on the phytotoxic activity of EOs against weeds and on their potential use as natural herbicides. Anyway, most of these works have been performed in *in vitro* experiments and not in microcosms that try to mimic the natural conditions. Moreover, in *in vitro* approaches seeds and/or seedlings are directly exposed to the EOs in sterile conditions, i.e. strongly reducing and/or retarding EOs transformation/degradation normally mediated by soil microorganisms. To our knowledge, this is the first time that the effects of essential oils from *T. capitata*, *M. piperita* and *S. chamaecyparissus* against targeted weeds and soil microorganisms have been studied with a more practical approach, i.e. *in vivo* conditions, monitoring their effects in order to know their real potential as an alternative to synthetic

herbicides, within a strategy of Integrated Weed Management, and analysing the benefits or disadvantages derived from their employment.

Results clearly demonstrated that tested EOs, at a different extent, were significantly effective against weeds, killing them completely or significantly reducing their growth parameters. Among them, *T. capitata* was the most effective, followed by *M. piperita*. Both EOs showed broad spectrum of activity, with *T. capitata* at the highest doses applied (12 µl mL^{-1}) killing plants of all weed species (100 efficacy), except for *P. oleracea* (90 efficacy). *M. piperita* at the highest dose (20 µl mL^{-1}) controlled completely (100 efficacy) *A. retroflexus* and *A. fatua* plants but showed 90 and 40 efficacy on *P. oleracea* and *E. crus-galli*, respectively. Although *S. chamaecyparissus* EO was less active compared with the other EOs, it displayed a very remarkable selective activity, being highly effective against *A. retroflexus* (90 efficacy at the highest dose, 20 µl mL^{-1}). It could be interesting to study it more profoundly as selective herbicide, while *T. capitata* and *M. piperita* could have a wider action, exhibiting excellent potential for the development of broad-spectrum herbicides. Anyway, a good natural herbicide besides to be effective, at the same time, should not have side effects on soil microorganisms. Here, results clearly demonstrated that, except for *T. capitata* EO at the highest concentration, which significantly increased the specific respiration rate, the other EOs generally stimulated soil biochemical properties, or their effect on them were transient. Furthermore, even when changes in the main microbial groups persisted, soil microbial activity was not irredeemably affected, suggesting that essential oils did not compromise the functional redundancy.

Another advantage in using these EOs from a conservationist point of view in agro-ecosystems could be that, as they are able to decrease the weed growth parameters, thus reducing their fitness and competitiveness, they could contribute to maintain a high biodiversity by not completely eradicating the weeds, instead giving the crop an opportunity to outcompete them.

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Chapter 4:

Effects of eucalyptus leaves and their extracts on soil microorganisms

1 Introduction

Eucalyptus is a large genus of the Myrtaceae family that includes 900 species and subspecies. In southern Europe (Italy, Spain, and Portugal) *Eucalyptus* spp. are the only large scale and economically successful short rotation tree plantations (Facciotto and Mughini, 2003; Tolosana *et al.,* 2010; Tomé *et al*., 2001). Evidence of the bioactivity of *Eucalyptus* leaves and leaves extracts have been widely reported and include antimicrobial, antiviral, fungicidal, insecticidal, anti-inflammatory, anti-nociceptive and antioxidant activities (Zhang *et al*., 2010; Li *et al*., 2012; Martins *et al*., 2013; Zhang *et al*., 2014). Moreover, *Eucalyptus* have showed strong phytotoxic potential due to the presence of a wide array of biologically active compounds, especially held in its essential oils (EOs) rich in terpenoids (Batish *et al*., 2008). Such an aspect is of great interest due to the increasing demand of natural herbicides in both developed and developing countries to be used in modern integrated pest management strategies and weed control in the context of sustainable and organic agriculture (Sodaeizadeh *et al*., 2009; El-Darier *et al*., 2018; Azad *et al*., 2020).

Puig *et al*., (2013) reported, in greenhouse conditions, the bioherbicide potential of *Eucalyptus* leaves incorporated into the soil as a green manure, pointing out different modes of action for target species and attributing the phytotoxic effects to the release of water-soluble compounds into the soil solution. Indeed, aqueous extracts of *Eucalyptus* fresh leaves and litter have been found to inhibit the growth of herbs in laboratory, greenhouse, and field experiments (Khan *et al*., 2008; Stanton *et al*., 2008; Zhang *et al*., 2014). For instance, (cold) aqueous extracts of *E. globulus* demonstrated a phytotoxic effect on germination and radicle growth of *Amaranthus retroflexus* and *Echinochloa crus-galli* (Puig *et al*., 2013). Khan *et al*. (2008) found that aqueous extracts from *E. camaldulensis* significantly inhibited weed germination and seedling growth. Also, the (hot) aqueous extract (hydrolate) of *Eucalyptus dundasii* obtained during steam distillation was reported to be phytotoxic on the germination and growth of *Lolium rigidum* and *Hordeum glaucum* (Wu *et al*., 2011). In addition, also EOs extracted from *Eucalyptus* leaves have shown strong phytotoxic effects against many weeds, such as *Parthenium hysterophorus, Cassia occidentalis, Echinochloa crus-galli, Bidens pilosa, Amaranthus viridis, Rumex nepalensis, Leucaena leucocephala, Casuarina pusilla* and *Leptospermum myrsinoides* (Singh *et al*., 2005; Batish *et al*., 2006; Setia *et al*., 2007). Hence, the phytotoxic activity of *Eucalyptus* leaves extracts obtained by different methods as well as the entire leaves suggests that they may be potentially used as bioherbicides. Also, the authors observed that in different afforested zones of *Eucalyptus* species growing in Sicily (Italy) large

areas of soil surface beneath *Eucalyptus* trees were completely bare or with scarce vegetation (Figures S1). However, such bioherbicide activity is dependent on Eucalyptus species since, vigorously vegetation can also be found (Figure S2). Such bare soil surfaces were found mainly beneath *E. occidentalis* and not under *E. camaldulensis*. In contrast, del Moral and Muller (1970) reported that in California vegetation growing close to *E. camaldulensis* was severely inhibited with annual plants rarely reaching maturity where the litter of *E. camaldulensis* accumulated.

However, applied *in situ*, leaves and leaves extracts could accumulate in the upper soil layers, and its leach or the surface runoff could influence soil properties by undergoing chemical processes such as hydrolysis, photolysis and chemical degradation and also interacting with soil microorganisms (Meite *et al*., 2018; Salazar-Ledesma *et al*., 2018; Shaheen *et al*., 2017). In fact, although hydrolysable phenolic compounds and tannins may have a short lifetime in soils, their effects or those of other compounds may outstay due to their hydrophobicity and oil vesicles prolonged integrity (Bernhard *et al*., 2003). The antimicrobial effects of *Eucalyptus* leaf extracts have been outlined in several studies (Delaquis *et al*., 2001; Takahashi *et al*., 2004; Somda *et al*., 2007; Sartorelli *et al*., 2007; Correa *et al*., 2019; Sabo and Knezevic, 2019; Tian *et al*, 2020). So, if the antimicrobial activity of leaf extracts injures soil microorganisms, soil processes mediated by them would be critically affected, as well.

Little research has been carried out about *Eucalyptus* leaf extracts effects on soil microorganisms. Some old studies indicated that *Eucalyptus* leaves contain toxic organic compounds (Rice, 1984) which may have a deleterious impact on soil microorganisms (Dellacassa *et al*., 1995; Animon *et al*, 1999), and others outlined that the allelochemicals released into soil during decomposition of *Eucalyptus* leaves had a toxic effect on soil microorganisms and may thus affect the nutrient cycling and hence soil fertility (Chander *et al*., 1995).

Such an aspect is of great environmental concern since soil microorganisms play a major role in soil fertility and resilience (Griffiths *et al*., 2008). Furthermore, soil biochemical properties are considered the most responsive bioindicators of soil quality (Laudicina *et al*., 2012). In addition, according to Powlson *et al* (1987), soil microbial biomass measurement can give an early indication of changes in total soil organic matter long before changes in total soil C and N can be reliably detected. Therefore, more studies would be needed to better elucidate the effects of *Eucalyptus* leaves and leaves extracts on soil microorganisms if they are thought to be used as natural herbicides. Thus, the aim of this study was to assess the effects of *Eucalyptus* leaves and leaves extracts on soil microbial biomass C and N, microbial biomass activity, on

the relative abundance of the main microbial groups as well as on microbial and metabolic quotients. Such biochemical soil properties were selected as they are early indicators of perturbation of soil microorganisms and soil functioning (Schloter *et al*. 2003; Anderson 2003; Kocak *et al*., 2016).

With these aims we focused on *E. camaldulensis* which is worldwide the most important and studied species (Sabo and Knezevic, 2019) and *E. occidentalis* of which, to our knowledge, its biological activity against soil microorganisms has never been investigated.

Specifically, a laboratory experiment was planned to test leaves and their extracts obtained by 1) hydrodistillation (EOs and hydrolates; the latter are the byproducts obtained by hydrodistilation of plant materials) and 2) aqueous extracts from both *E. camaldulensis* and *E. occidentalis.*

Figure S1. Bare soil under *Eucalyptus occidentalis*.

Figure S2. Vigorously vegetation under *Eucalyptus camaldulensis*.

2 Materials and methods

2.1 Plant extracts

The plant material used in this study consisted of leaves of *E. camaldulensis* and *E. occidentalis* randomly collected in an afforested area near Agrigento (Sicily province, Italy) during March 2019. The leaves were dried on paper towel at 40ºC for 48 h.

EOs and hydrolates were extracted from leaves with a 1:2 ratio (w/v) by 12 hours of hydrodistillation using Clevenger apparatus as described in the European Pharmacopeia (2007). After the extraction, EOs were dried with anhydrous sodium sulphate and stored at 4 °C for further use. Hydrolates were the water recovered after EOs hydrodistillation.

Aqueous extracts were obtained by soaking 200 g of Eucalyptus leaves from each species in 2 L of distilled water for 72 hours at 22-25ºC and filtered through Whatman 42 filter paper. Hydrolates, aqueous extracts and EOs were stored at -20°C in sterile containers until further use.

Finally, fresh leaves from the two species were dried at 40°C for 48 hours, then cut into pieces (1-2 cm^2), and stored at 4°C until further use.

2.2 Experimental design

A laboratory experiment was carried out, using a completely randomized design with 4 replicates. A citrus orchard soil was collected from the experimental field of the University of Palermo. The soil was air dried and then sieved at < 2 mm. The main characteristics of the soil were: sand 77.1 %, silt 17.6 %, clay 5.3 %, total organic carbon 21.8 g kg^{-1}, total carbonates 76 g kg^{-1}, pH 7.08, electric conductivity 0.3 dS m^{-1} and total nitrogen 0.98 g kg^{-1}. Aliquots of 350 g of soil were weighed in 1 L plastic bottles and treatments were applied as follows:

1) Cw, soil moistened up to 50% of its water holding capacity (WHC) with distilled water.

2) Cf, soil moistened up to 50% of its WHC with a water suspension at 0.05% (v/v) of Fitoil (Xeda, Italy; it is an emulsifier 40% soybean oil).

3) EC1 and EC2, soil moistened up to 50% of its WHC with *E. camaldulensis* EO emulsion to have 2.64 µL and 3.52 mL of EO g^{-1} of soil, respectively.

4) EO1 and EO2, soil moistened up to 50% of its WHC with *E. occidentalis* EO emulsion to have 2.64 µL and 3.52 mL of EO g^{-1} of soil, respectively.

5) LEC and LEO, soil was added with chopped leaves (dried at 40°C for 48 hours) of *E. camaldulensis* and *E. occidentalis* at a rate of 6.6 mg and 5.0 mg per g^{-1} of soil. The amount of chopped leaves added was calculated on the basis of the amount of litterfall present beneath *E. camaldulensis* and *E. occidentalis* during leaves sampling and that was 780 g and 575 g per m^{-2} of soil (average of 4 replicates), respectively.

6) HEC1 and HEC2, soil moistened up to 50% of its WHC with hydrolate of *E. camaldulensis* not diluted or diluted with water at 1:1 ratio (v/v), respectively.

7) HEO1 and HEO2, soil moistened up to 50% of its WHC with hydrolate of *E. occidentalis* not diluted or diluted with water at 1:1 ratio (v/v), respectively.

8) AEC1 and AEC2: soil moistened up to 50% of its WHC with aqueous extract of *E. camaldulensis* not diluted or diluted with water at 1:1 ratio (v/v), respectively.

9) AEO1 and AEO2: soil moistened up to 50% of its WHC with hydrolate of *E. occidentalis* not diluted or diluted with water at 1:1 ratio (v/v), respectively.

Table 1. Characteristics of hydrolates and aqueous extracts

Characteristics	**HEC**	**HEO**	**AEC**	**AEO**
Carbon (mg mL$^{-1)}$	1.9 c	1.4 d	9.4 a	4.4 b
Nitrogen (µg mL^{-1})	8.9 c	6.9 d	17.5 a	12.9 b
pH	5.35 b	5.84 a	4.25 c	4.17 c
EC (dS m^{-1})	1.6 d	1.5 c	2.6 a	1.6 b

After the application of all the treatments, soils were incubated in dark at room temperature (20-23°C) for 35 days and their humidity weekly adjusted up to 50% of WHC by replenishing the lost water. Concurrently, 20 g of soil of each treatment were incubated as above in 200 mL jars and sealed with a rubber stopper to determine microbial activity (CO_2 emission rate) during 36 days of incubation.

2.3 Leaf extract analyses

Reaction of hydrolates and aqueous extracts was determined on not diluted sample aliquots potentiometrically by using a glass membrane electrode. Also, electrical conductivity was determined on not diluted sample aliquots by using a digital conductivity meter. Total C and N were determined by acid dichromate oxidation and by micro-Kjeldhal method, respectively, on hydrolates and aqueous extracts filtered through Whatman 42 paper and diluted (1:1; v/v) with distilled water

EOs were analysed by gas impact mode was employed. MS spectra were obtained by means of total ion scan mode (mass range m/z 45–500 uma). The total ion chromatograms and mass spectra were processed with the Turbomass 5.4 software (PerkinElmer Inc., UK). Retention indexes were determined by injection of C_8–C_{32} n-alkanes standards under the same conditions. The EO components were identified by comparison of their mass spectra with those of computer library NIST MS Search 2.0 and available data in the literature. Identification of α-pinene, β-pinene, camphene, myrcene, camphor, borneol, terpinen-4-ol, and linalool was confirmed by comparison of their experimental RI with those of the reference standards (Sigma-Aldrich). The quantification of the compounds found in each EO was performed by gas chromatography (Clarus 500GC, PerkinElmer Inc., UK) equipped with a flame ionization detector, the same column and operating conditions as described above for the GC-MS. The percentage composition of each EO was computed from the GC peak areas by means of the software Total Chrom 6.2 (Perkin Elmer Inc., Wellesley, PA, USA) chromatography (GC) coupled with mass spectrometry (Clarus 500 GC–MS from Perkinelmer inc.) equipped with

the capillary column ZB-5 (30 m × 0.25 mm i.d. × 0.25 μm film thickness). The injection volume was 1 μL. The GC oven temperature was set at 60°C for 5 min, with increases of 3°C per min up to 180°C, and then increases of 20°C per min up to 280°C which was maintained for 10 min. Helium was the carrier gas at a flux of 1.2 mL min^{-1}. Injector and detector temperatures were set at 250°C. Ionization source temperature was set at 200°C and 70 eV electron

2.4 Soil analyses

Seven days after the application of the treatments and at the end of the experiment (day 35), incubated soils were analyzed to determine some biochemical properties. Microbial biomass C (MBC) was determined by the fumigation–extraction method (Vance *et al*., 1987). Fumigated and not fumigated soil samples were extracted with 0.5 M K_2SO_4 solution at a ratio of 1:4 (w/v). The total organic C in the extract was determined by dichromate-sulphuric acid digestion method and the C of microbial origin was obtained multiplying the differences between organic C in fumigated and not fumigated samples by 2.64. The concentration of K_2SO_4-extractable C from not fumigated samples was assumed as a proxy of available C for soil microorganisms (Laudicina *et al*. 2013). Microbial biomass N (MBN) was calculated by multiplying by 5 the difference between the ninhydrin reactive N determined on fumigated and not fumigated soil 0.5M K_2SO_4 extracts, according to (Joergensen and Brookes, 1990).

The CO_2 daily accumulated by incubated 20 g soil aliquots in the headspace of the bottles was determined by a gas chromatograph (Thermo Trace GC 90 GC, fitted with a thermal conductivity detected, Poropak Q column and using He as the carrier) at day 1, 5, 8, 12, 15, 22, 29, 36 during the incubation. After each CO_2 sampling, flasks were ventilated with fresh air for 30 min and then sealed again, after possible replenishment of lost soil moisture by distilled water. The C mineralization rate, expressed as mg CO_2–C kg^{-1} dry soil day^{-1}, was fitted to the following first-order decay function:

Mineralized C = $C_0\ e^{-kt}$

where C_0 is the biologically available C (mg kg^{-1}) at time zero (i.e. the intercept value), k is the decay rate constant, and t is the sampling incubation time. The total CO_2–C mineralized over 36 days of incubation was calculated by the linear interpolation of two neighboring rates and the integration over time:

Total mineralized C = $\sum_{i}^{n}[(r_i + r_{i+1}) * \frac{d}{2} + \cdots + (r_{n-1} + r_n) * \frac{d}{2}]$

where i is the date of the first measurement of CO_2–C rate, n is the last day of measurement of CO_2–C rate, r is the CO_2–C rate expressed as mg CO_2–C kg^{-1} dry soil day^{-1}, and d is the number of days between the two consecutive CO_2 rate measurements.

Fatty acids (FAs) were extracted from soils and analyzed according to the modified Bligh and Dyer method (Wu *et al.*, 2009). The fatty acid methyl esters were detected and quantified by a gas chromatograph (FOCUS™ GC, Thermo Scientific Inc., Waltham, MA USA) equipped with a flame ionization detector and a fused-silica capillary column Mega-10 (50 m x 0.32 mm I.D.; film thickness 0.25 µm). The GC temperature progression was initial isotherm at 115°C for 5 minutes, increase at a rate of 1.5°C per minute from 115 to 230°C, and final isotherm at 230°C for 2 minutes. Both injection port and detector were set up at 250°C and helium at 1 mL min^{-1} in a constant flow mode was used as carrier. The injected volume was 1 µL in a splitless mode. Nonadecanoic acid methyl ester (19:0; cat no. N-5377, Sigma-Aldrich Co.) was used as an internal standard for quantification of the fatty acid methyl esters (FAs). The identification of the peaks was based on comparison of retention times to known standards (Supelco Bacterial Acid Methyl Esters mix cat no. 47080-U and Supelco 37 Component FAME mix cat no. 47885-U). Fatty acids with less than 14 carbon atoms or more than 20 carbon atoms were excluded as considered originating from non-microbial sources. The FAs i15:0, a15:0, 15:0, i16:0, i17:0, 17:0, cy17:0,18:1ω7, cy19:0 were used to represent bacterial biomass while 18:2ω6,9 for fungal biomass. The FAs i15:0, a15:0, i16:0, i17:0 were chosen to represent Gram-positive bacteria (B+) while 16:1ω7, 18:1ω7, cy17:0 and cy19:0 for Gram-negative bacteria (B-) (Laudicina *et al.*, 2012).

2.5 Statistical analysis

Reported soil data, referred to oven-dry (105 °C) soil weight, are the arithmetic means of four replicates. Before performing parametric statistical analyses, normal distribution and variance homogeneity of the data were checked by Kolmogorov–Smirnoff goodness-of-fit and Levene's tests, respectively. Within each treatment (aqueous extract, leaf, essential oil, hydrolates) and within each incubation time (days 7 and 35), soil data were subjected to two-way ANOVA with eucalyptus type (two levels) and application dose (2 levels for aqueous extract, essential oil, hydrolate; 1 level for leaves) as factors. Significant differences at $P<0.05$ among eucalyptus type and application dose were assessed by the least significant difference (LSD) post-hoc test. All analyses were performed by Statgraphics Centurion version XVII.

3 Results

3.1 Chemical composition of EOs

The yield of EO was half in *E. camaldulensis* compared to that of *E. occidentalis* (2.1 mL and 4.3 mL kg^{-1} of leaves, respectively). A total of 30 compounds in *E. camaldulensis* and 15 in *E. occidentalis* were identified (Table 2) and grouped according to their chemical properties (Table1). *E. occidentalis* EO was characterized mainly by oxygenated monoterpenes (about 72%), of which 1,8-cineole (eucalyptol) was the main compound (57%, Table 2), and by oxygenated sesquiterpenes (25%) represented mainly by α-pinene (about 24%). *E. camaldulensis* EO consisted mainly of monoterpene hydrocarbons (about 49%), with *p*-cymene (about 39%) as main compound, and oxygenated monoterpenes (39%; Table 2), of which 1,8-cineole was the main component (nearly 19%; Table 1).

Table 2. Chemical composition of essential oils from *E. camaldulensis* (EC) and *E. occidentalis* (EO). KI, Kovats index.

Compounds	KI.	EC	EO
Monoterpene hydrocarbons (%)		**49.32**	**25.04**
α-Thujene	930	1.13	-
α-Pinene	938	**3.93**	**24.30**
Thuja-2,4(10)-diene	947	0.13	0.16
Camphene	951	-	0.17
Sabinene	975	0.57	-
β-Pinene	978	3.79	0.41
Myrcene	991	0.65	-
p-Cymene	1025	**38.83**	-
β-Phellandrene	1038	T	-
cis-Linalool oxide	1078	T	-
p-Cymenene	1090	0.29	-
Oxygenated monoterpenes (%)		**38.93**	**72.15**
1,8-Cineole (eucalyptol)	1031	**18.67**	**56.84**
α-Pinene oxide	1099	-	0.12
Linalool	1104	0.27	-
trans-Thujone	1117	0.20	-
endo-Fenchol	1118	-	0.10
α-Campholenal	1130	T	0.21
Nopinone	1141	T	-
trans-Pinocarveol	1143	0.72	**10.75**
Pinocarvone	1166	0.26	3.12
Borneol	1168	-	-
Isoborneol	1170	-	0.25
Terpinen-4-ol	1177	2.21	-
α-Terpineol	1188	0.35	0.45
trans-ρ-Mentha-1(7),8-dien-2-ol	1191	-	0.31
Myrtenal	1192	0.59	-
Cryptone	1196	**10.91**	-
Verbenone	1198	0.13	-
Cumin aldehyde	1245	2.73	-
Carvone	1249	1.27	-
α-Terpinen-7-al	1288	0.23	-
Carvacrol	1300	0.05	-
4-hydroxy-Cryptone	1326	0.14	-
3-oxo-ρ-Menth-1-en-7-al	1340	0.20	-
Sesquiterpene hydrocarbons (%)		**0.56**	**1.07**
allo-Aromadendrene	1457	-	1.07
Aromandendrene	1464	0.56	-
Oxygenated sesquiterpenes (%)		**6.82**	**0.92**
Spathulenol	1447	**6.82**	-
Viridiflorol	1587	-	0.92
Total identified (%)		**95.63**	**99.18**

In bold, the most significant compounds for each EO **t< 0.03**

3.2 Effects of eucalyptus leaves on soil biochemical properties

Extractable C was doubled by the addition of eucalyptus leaves compared to Cw. At day 7, extractable C did not differ between the two treatments, whereas at day 35 it was the highest in LEO (Figure 1). At both incubation days, also MBC was increased by the two treatments, with no differences between them, compared to Cw, but the increase was more consistent, about 40%, at day 7. Within each treatment, from day 7 to day 35, MBC decreased by 35% in Cw and LEC, whereas by 50% in LEO. MBN was affected only by LEO at day 7 when it was higher than other treatments. Microbial respiration was greatly stimulated by eucalyptus leaves. During the first 10 day of incubation, C mineralization rate was greater in LEC compared to LEO; then, C mineralization rate was similar in LEC and LEO but still greater than in the control (Figure 1). Also, the biological available C and the turnover rate constant was greater in treated soil being the highest in LEC, whereas the total C mineralized did not differ (Figure 1). Both bacteria, specifically the gram-negative ones, and fungi increased following the addition of leaves. At day 7, bacteria were increased only by LEC and fungi by LEO. The fungi to bacteria ratio was increased only by LEO whereas LEC, as well as LEO at day 35, decreased the bacG+/G- ratio (Table 3).

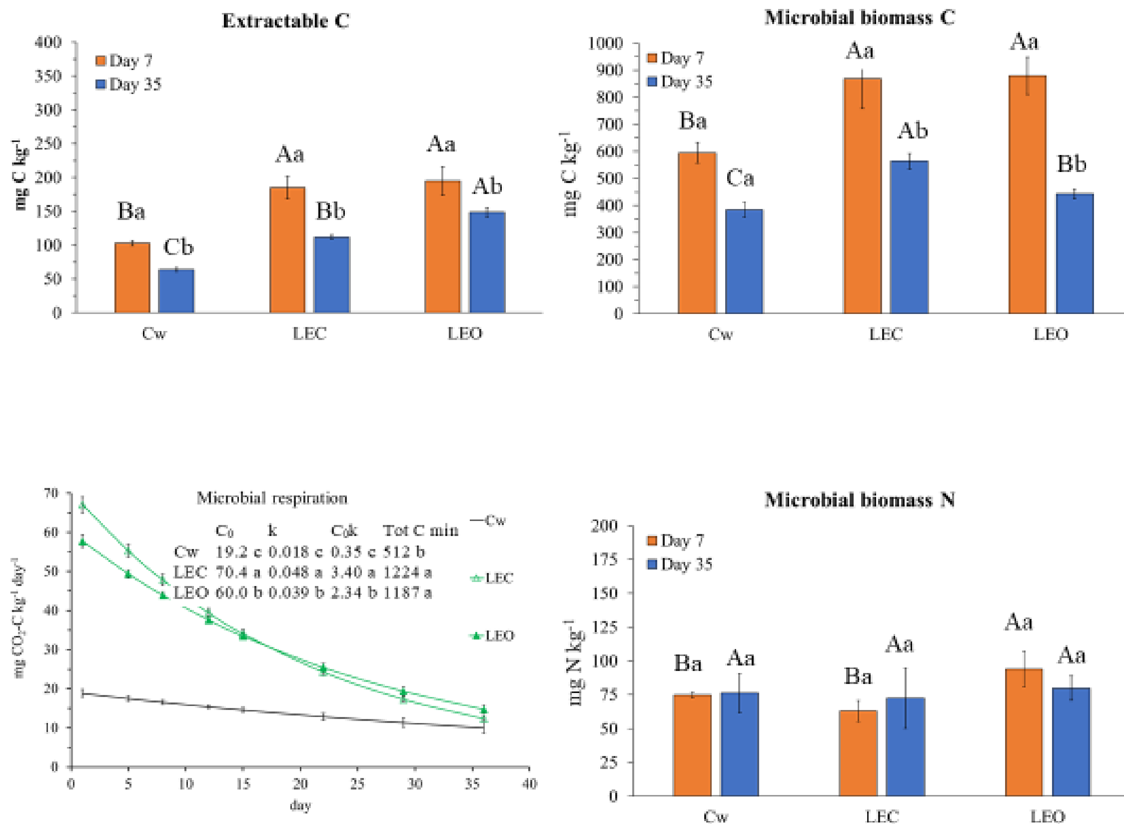

Figure 1. Biochemical soil variables determined at 7 and 35 incubation days after moistening the soil up 50% of its water holding capacity with distilled water and adding E. *camaldulensis* (EC) and E. *occidentalis* (EO) leaves. Treatments were: LEC and LEO, soil was added with chopped leaves (dried at 40°C for 48 hours) of *E. camaldulensis* and *E. occidentalis* at a rate of 6.6 mg and 5.0 mg per g^{-1} of soil. The amount of chopped leaves added was calculated on the basis of the amount of litterfall present beneath *E. camaldulensis* and *E. occidentalis* during leaves sampling and that was 780 g and 575 g per m-2 of soil (average of 4 replicates), respectively. Microbial respiration rate fitted to the exponential first order decay function (Mineralized C = C_0 e^{-kt}) and derived parameters (C_0, biological available C; k, turnover constant rate; C_0k, initial potential rate of C mineralization). Reported results are means ±standard deviations (n=4). Capital letters indicate significant differences among doses within the same incubation day. Lower case letters indicate significant differences between the two-incubation days at the same treatment. Numbers in bold indicate significant differences with the control.

Table 3. Main microbial groups in soil moistened up to 50% of its water holding capacity with distilled water and in which were added *E. camaldulensis* (EC) and *E. occidentalis* (EO) leaves. Treatments were: LEC and LEO, chopped leaves (dried at 40°C for 48 hours) of *E. camaldulensis* and *E. occidentalis* were added to the soil at a rate of 6.6 mg and 5.0 mg per g-1 of soil. Capital letters indicate significant differences among treatments within the same incubation day. Lower case letters indicate significant differences between the two incubation days at the same treatment. Numbers in bold indicate significant differences with the control

Treatments	day	Microbial biomass	Bacteria	Fungi	BacGp	BacGn	F_B	Bp_Bn
Cw	7	401 a	120 a	16 a	53 a	50 a	0.14 a	1.05 a
Cw	35	541 a	119 a	14 a	61 a	50 a	0.13 a	1.27 a
LEC	7	**820** Aa	**251** Aa	27 Aa	74 Aa	**169** Aa	0.12 Aa	**0.56** Aa
LEO	7	**798** Aa	184 Aa	**45** Aa	73 Aa	**100** Aa	**0.25** Aa	**0.74** Aa
LEC	35	**872** Aa	**230** Aa	**36** Aa	98 Aa	**124** Aa	0.16 Aa	0.83 Aa
LEO	35	676 Ba	**174** Ba	**45** Aa	69 Ba	**92** Ba	**0.26** Aa	**0.75** Aa

3.3 Effects of aqueous extracts on soil biochemical properties

Aqueous extracts of eucalyptus increased extractable C compared to the control at both sample days. AEC increased extractable C more than AEO and such an increase was proportionally to the amount of aqueous extract added (Figure 2). MBC was affected by aqueous extracts only at the end of the incubation when it was the highest in AEC treatment and the lowest in the control. On the contrary, MBN was affected only by AEC and AEO treatments at day 7 showing lower values than in the control. Microbial respiration rate, proportionally to the applied dose, was stimulated more by AEC than AEO. The biological available C and the total C mineralized followed the same pattern, being the highest in AEC treatment compared to AEO. The turnover rate constant decreased according to the following order: AEO1=AEO2>AEC1>AEC2>control (Figure 2). Bacteria were increased by AEC and AEO only at day 35 being such increase the highest with AEC1. Except AEO1, also fungi were increased by both treatments at day 35, whereas at day 7 only by the two AEO doses. Among bacteria, both bacG+ and G- were stimulated by AEC and AEO although with not a univocal trend: bacG+ were affected mainly by AEO at day 7, whereas bacG- were not affected only by AEO at day 7. AEC was the treatment, on average, which more stimulated bacG- The fungi/bacteria ratio was increased only by AEO treatments, except AEO2 at day 35, whereas the bacG+/G- ratio was lowered by all the treatments with no clear trend among them (Table 4).

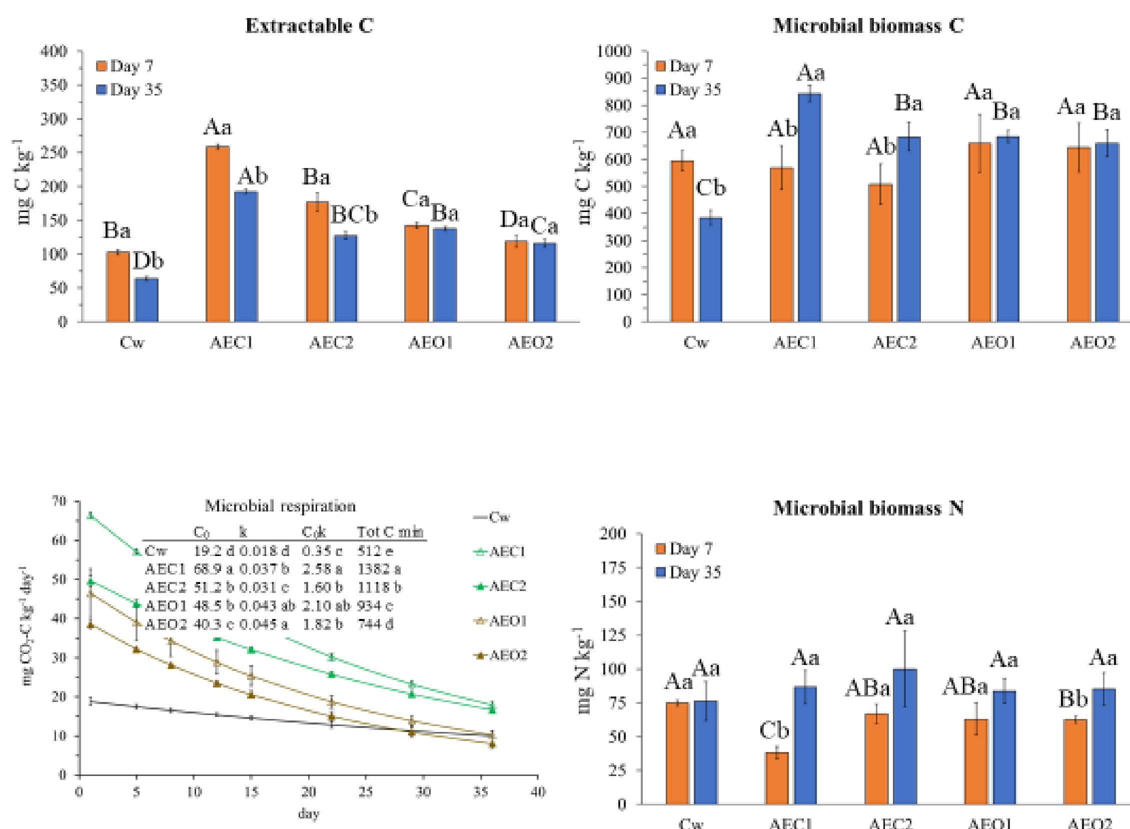

Figure 2. Biochemical soil variables determined at 7 and 35 incubation days after moistening it up to 50% of its water holding capacity with aqueous extracts of *E. camaldulensis* (EC) and *E. occidentalis* (EO) at two different doses: as they are (AEC1 and AEO1) or diluted with water at 1:1 ratio (AEC2 and AEO2). Microbial respiration rate fitted to the exponential first order decay function (Mineralized C = C_0 e^{-kt}) and derived parameters (C_0, biological available C; k, turnover constant rate; C_0k, initial potential rate of C mineralization). Reported results are means ±standard deviations (n=4). Capital letters indicate significant differences among doses within the same incubation day. Lower case letters indicate significant differences between the two-incubation day at the same treatment. Numbers in bold indicate significant differences with the control.

Table 4. Main microbial groups in soil moistened up to 50% of its water holding capacity with of *E. camaldulensis* (EC) and *E. occidentalis* (EO) aqueous extracts at two different doses: as they were obtained (AEC1 and AEO1) or diluted with water at 1:1 ratio (AEC2 and AEO2). Capital letters indicate significant differences among treatments within the same incubation day. Lower case letters indicate significant differences between the two incubation days at the same treatment. Numbers in bold indicate significant differences with the control.

Treatment	day	Microbial biomass	Bacteria	Fungi	BacGp	BacGn	F_B	Bp_Bn
Cw	7	401 a	120 a	16 a	53 a	50 a	0.14 a	1.05 a
Cw	35	541 a	119 a	14 a	61 a	50 a	0.13 a	1.27 a
AEC1	7	486 Ab	148 Ab	24 Aa	49 Aa	**90** Aa	0.16 Aa	**0.55** Ba
AEC2	7	464 Ab	142 Ab	23 ABa	58 Aa	**70** ABa	0.16 Aa	**0.84** Aa
AEO1	7	334 Bb	84 Bb	**14** Ca	**26** Ba	54 Ca	**0.17** Aa	**0.49** Ba
AEO2	7	373 Bb	94 Bb	**17** BCa	**31** Ba	57 BCa	**0.18** Ab	**0.56** Ba
AEC1	35	**816** Aa	**244** Aa	**38** Ab	**87** Ab	**139** Ab	0.16 Ba	**0.63** ABa
AEC2	35	680 Ba	**187** Ba	**32** Ba	73 Aba	**99** Bb	0.17 Ba	**0.74** Aa
AEO1	35	**655** Ba	**178** Ba	26 ABa	59 Bb	**104** Bb	0.14 Ba	**0.57** Ba
AEO2	35	705 Ba	**168** Ba	**38** Ab	66 Bb	**96** Bb	**0.23** Aa	**0.68** Aa

3.4 Effects of hydrolates on soil biochemical properties

After 7 days since the application of hydrolates both extractable and microbial biomass C were increased compared to the control with no differences among the treatments (Figure 3). At day 35 extractable C and MBC decreased compared to day 7, but the former was still higher in all the treatments compared to the control and the latter only in HEC1. On the contrary to MBC, MBN was inhibited by HEC2, HEO1 and HEO2 treatments at day 7, whereas at day 15 no differences were observed between treatments and control. Microbial respiration rate, as well as the biological available C and total C mineralized, was stimulated at the beginning of the incubation only by HEC and HEO (Figure 3). Bacteria were stimulated by HEC treatments only at day 35, whereas fungi by all of the treatments (except for HEC2 at day 7) with slight differences among them. Among bacteria only the bacG- were affected being increased by HEO treatments at day 7 and by HEC at day 35. The fungi/bacteria were generally increased by treatments but with not clear trend among them. On the other hand, the bacG+/G- ratio was decreased mainly by HEO treatments at both days of incubation (Table 5).

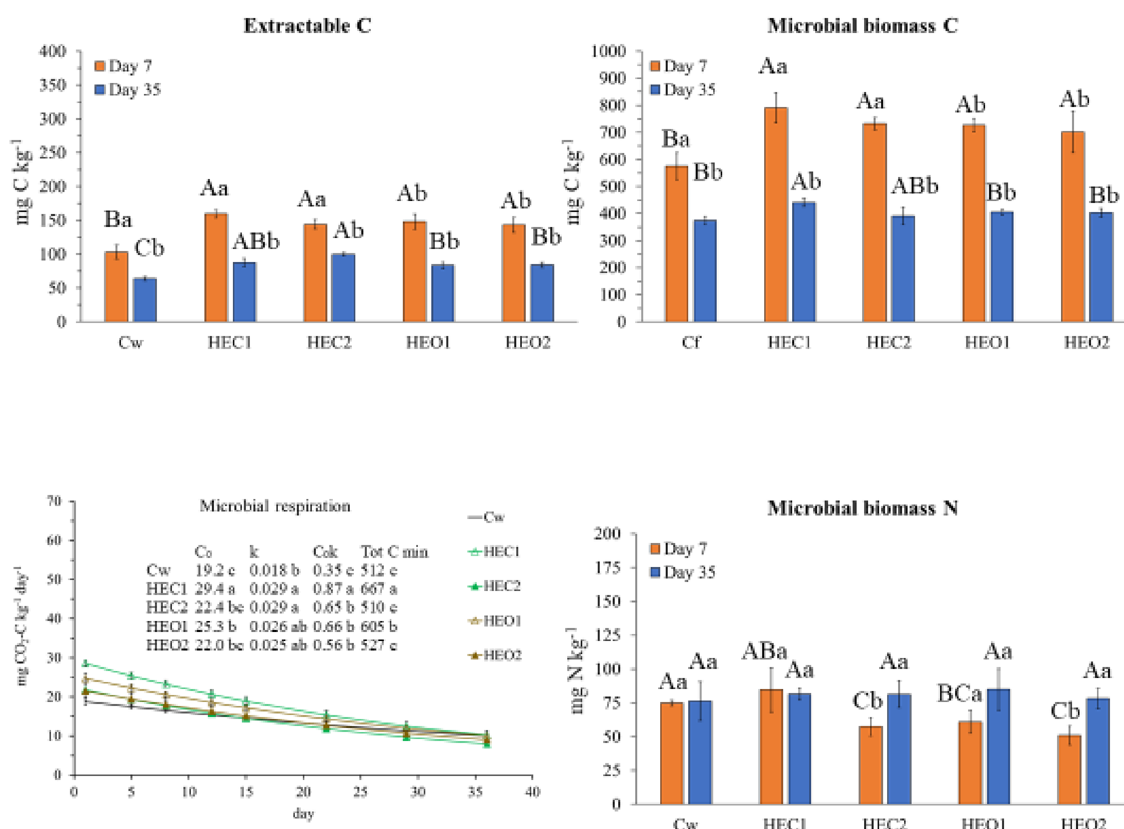

Figure 3. Biochemical soil variables determined at 7 and 35 incubation days after moistening it up to 50% of its water holding capacity with hydrosol obtained during the hydrodistillation process of *E. camaldulensis* (EC) and *E. occidentalis* (EO) leaves at two different doses: as they are (HEC1 and HEO1) or diluted with water at 1:1 ratio (HEC2 and HEO2). Microbial respiration rate fitted to the exponential first order decay function (Mineralized C = C_0 e^{-kt}) and derived parameters (C_0, biological available C; k, turnover constant rate; C_0k, initial potential rate of C mineralization). Reported results are means ±standard deviations (n=4). Capital letters indicate significant differences among doses within the same incubation day. Lower case letters indicate significant differences between the two-incubation day at the same treatment. Numbers in bold indicate significant differences with the control.

Table 5. Main microbial groups in soil moistened up to 50% of its water holding capacity with hydrosol obtained during the hydrodistillation process of *E. camaldulensis* (EC) and *E. occidentalis* (EO) leaves at two different doses: as they were obtained (HEC1 and HEO1) or diluted with water at 1:1 ratio (HEC2 and HEO2). Capital letters indicate significant differences among treatments within the same incubation day. Lower case letters indicate significant differences between the two incubation days at the same treatment. Numbers in bold indicate significant differences with the control.

Treat	day	Microbial biomass	Bacteria	Fungi	BacGp	BacGn	F_B	Bp_Bn
Cw	7	401 a	120 a	16 a	53 a	50 a	0.14 a	1.05 a
Cw	35	541 a	119 a	14 a	61 a	50 a	0.13 a	1.27 a
HEC1	7	491 Ab	131 Ab	**46** Aa	45 Aa	54 Ba	**0.36** Aa	0.84 Aa
HEC2	7	422 Ab	109 Ab	21 Bb	46 Aa	60 ABa	0.20 ABa	**0.81** Aa
HEO1	7	513 Aa	151 Aa	**23** Ba	59 Aa	**83** Aa	0.16 Ba	**0.70** Aa
HEO2	7	500 Aa	126 Aa	**26** Ba	47 Aa	**72** ABa	**0.21** Ba	**0.66** Aa
HEC1	35	**751** Aa	**230** Aa	**28** ABb	105 Ab	**120** Aa	0.13 Bb	0.88 Aa
HEC2	35	**710** Aa	**179** Aa	**37** Aa	77 Ab	**98** Aa	**0.21** Aa	0.79 ABa
HEO1	35	450 Ba	118 Cb	**23** Ba	50 Aa	65 Ba	**0.19** Aa	**0.76** ABa
HEO2	35	**489** Ba	142 Bb	**25** Ba	54 Aa	**77** Ba	0.18 Aa	**0.70** Ba

3.5 Effects of essential oils on soil biochemical properties

At day 7, extractable C increased following the addition of the EOs and, at a given dose, *E. occidentalis* EOs raised it more than *E. camaldulensis* EOs. At day 35, extractable C was greater than the control only with EO treatments and in particular with EO2 (Figure 4). Microbial biomass C was generally decreased by EOs. At day 7, the lowest MBC values occurred at the two highest doses of EOs, whereas EC1 treatment did not affected MBC. At day 35, all the treatments decreased MBC according to the following order: EO2>EC2>EO1=EO1. It is to note that except for EC2 treatment, MBC was lower at day 35 compared to that at day 7. MBN resembled MBC pattern at day 7. At day 35, all treatments decreased MBN occurring the greatest decrease with the highest EOs dose. On the contrary to what observed for MBC, MBN decreased during the incubation only with EC treatments. During the first 10 days of incubation, microbial respiration rate was stimulated proportionally to the applied EOs dose. Then, EO2 treatment still increased microbial respiration rate compared to EO1, whereas the opposite occurred with EC treatments being microbial respiration rate greater with EC1 compared to EC2. The biological available C was the highest with EC2 and EO2, followed by EC1 and then by EO1. The turnover constant rate did not show a univocal trend among treatment since it was lower with EC1 compared to EC2 treatment, whereas did not differ between the other two treatments. Finally, the total C mineralized was higher with EC treatments, that did not differ between them, compared to EO treatments, which total C mineralized was higher in EO2 compared to EO1 (Figure 4). Bacteria were decreased

by EOs at the highest dose at both days of incubation, except for EC2, that did not affect them at day 35. At day 7, mainly bacG+ were decreased by treatments, whereas bacG- were reduced only by EO2. At day 35, bacG+ were decreased only by EC2 whereas bacG- by EC1 and EO2. Fungi were also decreased by all the treatments except for EC2 and EO1 at day 35. Notably, the highest decreased of fungi was observed at day 35 with EO2. The fungi/bacteria ratio was reduced by all the treatments although such a reduction was significant only at day 7 with EC1. The bacG+/G- ratio was negatively affected by EC treatments with the highest decreased observed at day 35 with EC2 (Table 6).

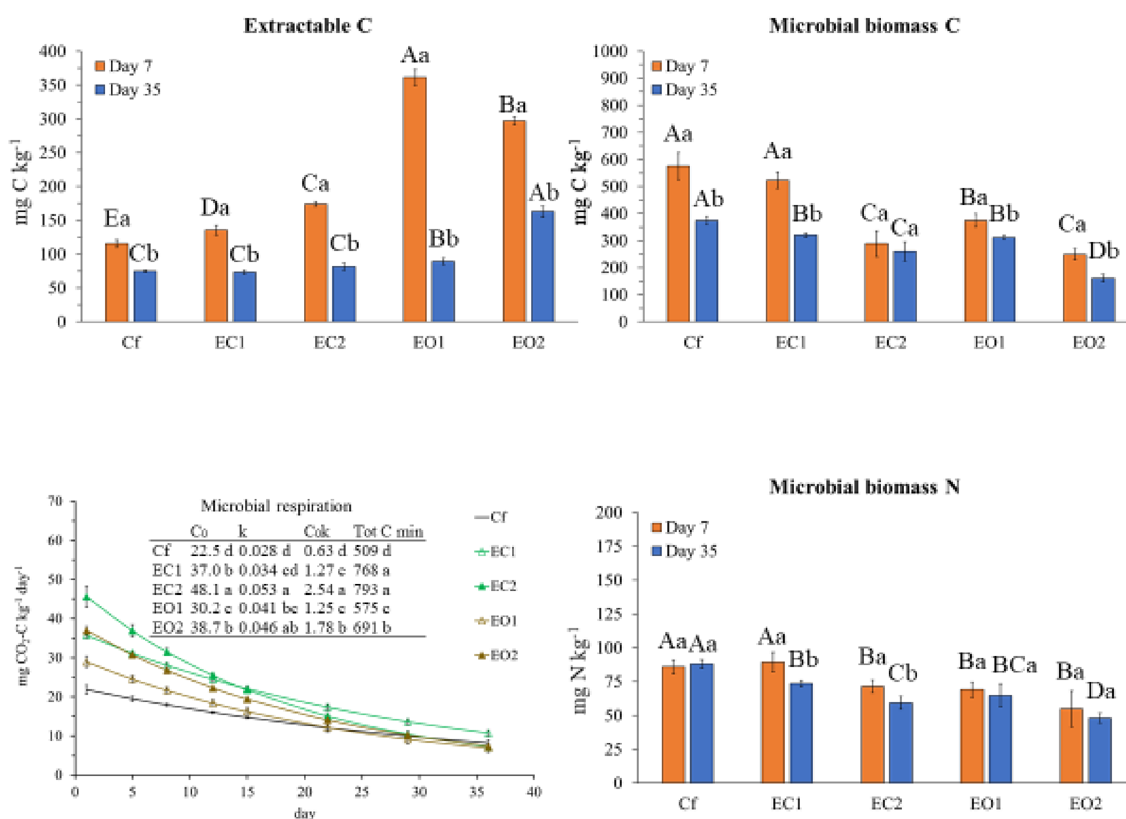

Figure 3. Biochemical soil variables determined at 7 and 35 incubation days after moistening it up to 50% of its water holding capacity with an emulsion containing essential oils extracted by hydrodistillation of *E. camaldulensis* (EC) and *E. occidentalis* (EO) leaves. Treatments are: EC1 and EC2, soil moistened up to 50% of its WHC with *E. camaldulensis* EO emulsion to have 2.64 µL and 3.52 mL of EO g^{-1} of soil, respectively; EO1 and EO2, soil moistened up to 50% of its WHC with *E. occidentalis* EO emulsion to have 2.64 µL and 3.52 mL of EO g^{-1} of soil, respectively. Microbial respiration rate fitted to the exponential first order decay function (Mineralized C = $C_0 e^{-kt}$) and derived parameters (C_0, biological available C; k, turnover constant rate; C_0k, initial potential rate of C mineralization). Reported results are means ±standard deviations (n=4). Capital letters indicate significant differences among doses within the same incubation day. Lower case letters indicate significant differences between the two-incubation day at the same treatment. Numbers in bold indicate significant differences with the control.

Table 6. Main microbial groups in soil moistened up to 50% of its water holding capacity (WHC) with an emulsion containing *E. camaldulensis* (EC) and *E. occidentalis* essential oils. Treatments are: EC1 and EC2, soil moistened up to 50% of its WHC with *E. camaldulensis* EO emulsion to have 2.64 µL and 3.52 mL of EO g^{-1} of soil, respectively; EO1 and EO2, soil moistened up to 50% of its WHC with *E. occidentalis* EO emulsion to have 2.64 µL and 3.52 mL of EO g^{-1} of soil, respectively. Capital letters indicate significant differences among treatments within the same incubation day. Lower case letters indicate significant differences between the two incubation days at the same treatment. Numbers in bold indicate significant differences with the control.

Treat	day	Microbial biomass	Bacteria	Fungi	BacGp	BacGn	F_B	Bp_Bn
Cf	7	467 a	147 a	30 a	59 a	63 a	0.21 a	1.03 a
Cf	35	363 b	91 b	15 b	42 b	44 a	0.16 a	0.99 a
EC1	7	506 Aa	141 Aa	**13** Ab	54 Aa	69 Aa	**0.09** Bb	**0.77** ABa
EC2	7	417 Aa	**108** ACa	**16** Aa	**44** Aa	56 Aa	0.14 Aa	**0.78** ABa
EO1	7	431 Ba	117 ABa	**16** Aa	**46** Aba	63 Aa	0.14 Aa	**0.72** Ba
EO2	7	**338** Ca	**88** Ca	**12** Aa	**36** Ba	**38** Ba	0.14 Aa	0.98 Aa
EC1	35	325 Ab	125 Aa	**25** Aa	46 Aa	**70** Aa	0.20 Aa	**0.66** Ba
EC2	35	**279** Bb	78 Bb	10 Bb	**26** Cb	47 Ba	0.13 Ba	**0.56** Bb
EO1	35	306 Ab	92 ABCa	10 BCb	38 Aba	50 ABa	0.12 ABa	0.82 ABa
EO2	35	**215** Cb	**54** Cb	**6** Cb	26 BCb	**26** Ca	0.11 Ba	0.99 Aa

4 Discussion

4.1 Yield and chemical composition of EOs

The yield of *E. camaldulensis* (0.21%) was similar to that reported by Barra *et al.* (2010) for the same species grown in Sardinia (Italy) and lower than that reported in other studies (Moudachirou *et al.*, 1999; Verdeguer *et al.*, 2009; Jemâa *et al.*, 2011) in which it is reported to be 0.6% in *E. camaldulensis* from Bénin, 0.71% from Spain and 0.85% from Tunisia, respectively. On the other hand, the yield of *E. occidentalis* (0.43) was lower than that reported for the same species grown in Australia (1.34%) and in Tunisia (1.1%) (Bignell *et al.*, 1996; Elaissi *et al.*, 2010). Concerning the chemical composition, the high content (39%) of *p*-cymene found in *E. camaldulensis* EO agreed with Nadiye *et al.* (2016) that reported 32.6%, but lower than that found by Basak and Candan (2010) (68.43%). Other compositions, with different main compounds have been reported for *E. camaldulensis* EOs from different origins (Verdeguer *et al.* 2009; Maaloul *et al.*, 2019). Regarding *E. occidentalis* EO, the determined composition, with high amounts of 1,8-cineol (57%) and α-pinene (24%) was different from that reported by Bande-Borujeni *et al.* (2018). However, such differences in chemical composition among EOs extracted from the same species from different origins have been

already reported and could be due to genetic differences as well as to abiotic factors (Ndiaye *et al*., 2016).

4.2 Changes in soil biochemical properties and in main microbial groups following the addition of eucalyptus leaves

The addition of eucalyptus leaves stimulated soil microorganisms so leading to an increase of MBC and of microbial respiration rate. Such an increase can be due to the enhanced availability of organic C substrates in treated soils as confirmed by the increase of extractable C. Similar stimulatory effect has been reported by Chander *et al*. (1995) that found an increase of MBC after 10 days since the addition of *E. tereticornis* leaves in a similar laboratory incubation experiment. The decline of MBC and respiration rate observed during the incubation suggested that the easily biodegradable pool of organic C present in leaves was progressively consumed. Similar behaviour has been reported in field experiments and in laboratory experiments, when soils were amended with different types of organic substrates (Ocio *et al*., 1991). However, after 35 days of incubation, the stimulatory effect on MBC was still evident.

Considering that the extractable C content was similar between the two treatments, the higher values of MBC at day 35 and those of microbial respiration rate during the first 8 days of incubation with LEC compared to LEO suggested a greater palatability of the organic C substrates supplied by *E. camaldulensis* leaves. Such a hypothesis was also confirmed by the higher values of the biological available C (C_0) and of the turnover constant rate. The mismatch stimulating effect of eucalyptus leaves on MBC and microbial respiration during incubation indicated that the organic substrate supplied to microorganisms by the addition of eucalyptus leaves was firstly respired and then, towards the end of the incubation, preferentially immobilized. The greater palatability of LEC compared to LEO was also evident on microbial groups since bacteria were more stimulated by the former. Indeed, bacteria are more adapted at exploiting easily degradable resources (Swift *et al*., 1979; Paul, 2015) and hence experience higher growth in the presence of more easily decomposable organic C substrates. This finding agreed with Fanin *et al*. (2019), who reported that gram negative bacteria were associated with simple C compounds whereas gram positive bacteria were more strongly associated with more complex C forms.

4.3 Changes in soil biochemical properties and in main microbial groups following the addition of eucalyptus aqueous extracts

The higher amount of extractable C in AEC treatments compared to AEO ones reflected the C content of the aqueous extracts. The increase of labile C, in turn, stimulated microbial activity as suggested by the higher respiration rate. Such results agreed with Di Bene *et al*. (2013) and Gamba *et al*. (2005) following the addition of available C by olive mill wastewaters and have been ascribed to the rapid mineralization of added organic matter (Di Serio *et al*., 2008; Mechri *et al*., 2007). In general, the addition of organic substrates enhances microbial respiration, because they are potential energetic sources consumed through the oxidative metabolism of the heterotrophic soil microbiota (Bhattacharyya *et al*., 2001). However, to such higher amount of labile C at day 7 did not correspond an increase of MBC at the same day of observation. Such results were in contrast with Adrover *et al*. (2012) and Chen *et al*. (2008) who suggested that the positive effects of greater amount of labile C supplied by treated wastewater irrigation on soil microbial biomass can be attributed to the addition of easily decomposable organic matter and nutrients. However, MBC increased at day 35. Overall, the promptly increase of microbial respiration and the delayed increase of MBC following the addition of aqueous extracts indicated that the C supplied by aqueous extract was firstly mineralized and then immobilized by soil microorganisms. The absence of a concurrently increase of MBN suggested that changes of main microbial groups occurred. Furthermore, results indicated that, as already observed for the leaves treatment, AEC treatments provided more easily decomposable substrates as confirmed by the higher biological available C and total C mineralized in AEC treatments. Regarding the stimulation of microbial biomass as suggested by the MBC and MBN patterns, fungi were stimulated more than bacteria. On the other hand, among bacteria, the bacG- more than bacG+ took advantages by the addition of aqueous extracts so decreasing the bacG+/G- ratio and indicating a greater C availability for bacteria (Fanin *et al*., 2019).

4.4 Changes in soil biochemical properties and in main microbial groups following the addition of eucalyptus hydrolates

The C added by hydrolates was promptly immobilized by soil microorganisms and only partially respired as confirmed by the high increase of MBC at day 7 and the low microbial respiration rate at the beginning of the incubation. On the other hand, the reduction of MBN registered at day 7, i.e. when MBC increased, suggested changes of main microbial groups. Such a hypothesis was confirmed by the increase of fungi. Among bacteria, the gram-negative

ones were favored over gram positive so leading to, on average, a decrease of the bacG+/bacG- ratio as already reported for aqueous extract and leaves.

4.5 Changes in soil biochemical properties and in main microbial groups following the addition of eucalyptus essential oils

The increase in extractable C following the addition of EOs and the concomitant decrease in MBC, as well as MBN, suggested that EOs had a biocidal effect on soil microorganisms and that the cytoplasmic material released following their death was not completely used by the surviving microorganisms. Indeed, although soil respiration increased following the addition of EOs, such an increase was not proportional to the observed increase of labile C. Furthermore, it is to note that at the highest dose of EOs, the greatest reduction of MBC, MBN and microbial respiration rate was observed, whereas with regard to EO type, Eucalyptus *occidentalis* showed the highest deleterious effects on soil microorganisms. Fungi were the most negatively affected main groups since they were halved by EOs with no differences among treatments, whereas bacteria were reduced only at the highest doses. Notably EO2 at day 35 still reduced both bacteria and fungi.

5 Conclusions

The results of this study provide evidence that *Eucalyptus* leaves and their extracts (EOs, hydrolates and aqueous extracts), at contrasting way, affect soil microbial community. Furthermore, those effects were dependent on the *Eucalyptus* species. *Eucalyptus* leaves, aqueous extracts and hydrolates, by providing labile C, stimulated both microbial biomass and respiration, although in the case of *Eucalyptus* leaves and aqueous extracts a mismatch between microbial immobilization and respiration occurred. On the other hand, *Eucalyptus* EOs killed soil microorganisms and, consequently, the microbial cytoplasmic material released following their death was used by the surviving ones. Finally, also the relative abundance of the main microbial groups was affected. The addition of labile C by eucalyptus leaves, aqueous extracts and hydrolates increased bacteria, mainly the gram-negative bacteria, whereas EOs strongly reduced fungi.

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