UNIVERSITÁ DEGLI STUDI DI BARI ALDO MORO

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Mediterranean Aromatic Plants and Eco-friendly Techniques for Agricultural Weed Management: Exploration of the Diversity of Secondary Metabolites of Algerian and Italian *Thymu*s Species.

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Dedication

To my mother and father, with all my love and gratitude.

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Table of Contents

List of tables

List of figures

Summary

The significant growth of the world population combined with accelerating climate change has caused unprecedented pressure on the agricultural systems to produce sufficient food and feed. While the Green Revolution of the 1960s and 1970s demonstrated human's capability for agricultural innovation through high-yielding crops and modern farming techniques, today's challenges require new sustainable solutions that balance high productivity with ecological preservation. In this perspective, weeds are considered one of the major threats to crop production as they compete with cash crops and act as a reservoir of pests, which make their control at the center of agricultural practices. Nevertheless, weed control is mainly based on the intensive use of herbicides that poses human and environmental risks. In addition, the continuous usage of the same active molecules with the same modes of action (MOA) led to the emergence of herbicide-resistant weeds. These concerns created an urgent need to find sustainable and efficient alternatives to synthetic agrochemicals to control weeds. Natural compounds with herbicidal potential could provide suitable solutions and the Mediterranean basin is a partially unexplored source of plants producing bioactive compounds.

In this context, this PhD research project aimed to perform a study of some *Thymus* sp. pl. collected from natural environments in Algeria and cultivated in Italy to identify bioactive compounds that could express a potential herbicidal activity and be used as active ingredients in the formulation of a nano-herbicide for innovative weed control. To achieve this purpose, the study was divided into three main parts represented as chapters.

The first chapter was dedicated to the investigation and the identification of the plant species that may express a potential herbicidal activity. After a deep literature review, it was highlighted that *Thymus algeriensis* Boiss. and Reut., *T. ciliatus* Desf subspecies *coloratus* (Boiss. et Reut.) Batt., *T. vulgaris* ecotype Fasano, and *T. vulgaris* var Varico 3, known for the biological activities of their essential oils (EOs), deserved further attention. EOs of these species were extracted, chemically characterized, and their phytotoxicity was screened on seed germination and early growth of two weed species (*Lolium perenne* L. and *Aramanthus retroflexus* L.) under *in vitro* conditions. Five concentrations (1.0, 2.5, 5.0, 7.5, and 10 µL/mL) were considered to determine the minimum inhibitory concentration (MIC). The phytochemical analysis revealed a high diversity of compounds. The Algerian species EOs (*T. algeriensis* and *T. ciliatus*) were characterized by the absence of carvacrol and low content of thymol in *T. ciliatus* while the Italian ones (*T. vulgaris* ecotype Fasano and *T. vulgaris* var Varico 3) were characterized by a *p*-cymene chemotype and important contents of thymol and carvacrol. Biologically, all the EOs displayed a significant phytotoxic activity against the target weed species. Total inhibition of seed germination and seedling growth were demonstrated starting from 5 µL/mL, suggesting that this concentration is the MIC. Moreover, *T. vulgaris* ecotype Fasano expressed the most phytotoxic potential.

The second chapter was developed in continuity with the experiments initiated during the first chapter. The results obtained under *in vitro* conditions were a preliminary screening of the phytotoxic potential of thyme EOs. During this second part of the study, the experiments were conducted under *in vivo* conditions. The purpose of the bioassays was to confirm the results previously obtained under conditions close to the field ones. The trials assessed the effect of thyme EOs on the pre and post-emergence of the same target weed species. Four concentrations of EO emulsions (5.0, 7.5, 10, and 20 µl/mL) were applied. The achieved results confirmed the *in vitro* experiments findings. All the concentrations effectively inhibited seed germination of both weed species. Contrariwise, for post-emergence experiments, the effect on seedling growth was only significant on *A. retroflexus*, arguing a selectivity of thyme EOs. Furthermore, the results confirmed the greater herbicidal potential of *T. vulgaris* ecotype Fasano EO. For a better understanding of the mechanisms of action of thyme EOs, cytological analyses were performed on the model plant *Arabidopsis thaliana* (L.) Heynh. The root system of EO-treated *A. thaliana* seedlings displayed significant structural damages indicating a possible alteration of the hormonal balance of the seedlings and oxidative stress.

The last chapter of this PhD research project focused on assays of fabrication of a prototype of a nano-herbicide with the aim of coating the EO to protect it from environmental conditions and preserve its herbicidal efficacy. After establishing the effectiveness of thyme EOs as a possible bioherbicide, the encapsulation tests of thyme EO were performed. *T. vulgaris* cultivar Varico 3 was selected because of its botanical and agronomic characteristics. This species is a hybrid developed for a higher EO content, stability of active compounds, and constant production of fresh biomass. Three volumes (500 µL, 750 µL, and 1000 µL) of EOs were encapsulated in silica nanoparticles (SiNPs). The efficacies of these nanomaterials were compared to that of pristine EO. Transmission Electron Microscopy (TEM) revealed spherical and regular EO-SiNPs with a size range of 220-300 nm. FT-IR analysis confirmed EO loading by the presence of characteristic peaks of isoprenoids and isomeric compounds with loading efficiencies of 26%, 42%, and 64% according to the EO concentration. Biological assays with pristine thyme EO in post-emergence treatments on *A. retroflexus* seedlings demonstrated a significant concentration-dependent herbicidal activity. Encapsulation with SiNPs increased the herbicidal efficacy of thyme EO. Compared to the pristine EO, the encapsulated form increased shoot and root malondialdehyde (MDA) content and antioxidant enzyme activities (ascorbate peroxidase, catalase, and superoxide dismutase, respectively, APX, CAT, and SOD). These results demonstrated a systemic effect of EO-SiNPs, inducing a significant reactive oxygen species (ROS) production leading to cell membrane damage and an imbalanced antioxidant system.

The findings achieved during this PhD thesis are promising and open the way for further research for developing a nano-bioherbicide using thyme EO encapsulated in SiNPs, for a novel and sustainable control of weeds.

Key words: *Thyme, essential oil, bioherbicidal activity, nanotechnology, biosystem delivery.*

Riassunto

La crescita significativa della popolazione mondiale, combinata con l'accelerazione del cambiamento climatico, ha causato una pressione senza precedenti sui sistemi agricoli finalizzati alla produzione di cibo e mangimi sufficienti. Mentre la Rivoluzione Verde degli anni '60 e '70 ha dimostrato la capacità di innovazione dell'uomo in campo agricolo attraverso colture ad alta resa e tecniche agricole moderne, le sfide di oggi richiedono nuove soluzioni sostenibili che bilancino l'alta produttività preservando l'ecologia e la biodiversità. In questa prospettiva, le erbe infestanti sono considerate una delle principali minacce alla produzione agricola poiché competono con le colture da reddito e fungono da serbatoio per i parassiti, il che rende il loro controllo al centro delle pratiche agricole. Tuttavia, il controllo delle erbe infestanti si basa principalmente sull'uso intensivo di erbicidi che pongono rischi per l'uomo e l'ambiente. Inoltre, l'uso continuo delle stesse molecole attive con gli stessi modi d'azione (MOA) ha portato alla selezione di erbe infestanti resistenti agli erbicidi. Queste preoccupazioni hanno creato un bisogno urgente di trovare alternative sostenibili ed efficienti agli agrofarmaci sintetici per il controllo delle erbe infestanti. I composti naturali con potenziale erbicida potrebbero fornire soluzioni adeguate e il bacino del Mediterraneo è una fonte parzialmente inesplorata di piante che producono composti bioattivi.

In questo contesto, questa progetto di ricerca di dottorato ha mirato a condurre uno studio di alcuni *Thymus* sp. pl. raccolti da ambienti naturali in Algeria o selezionati dal campo collezione presso l'azienda sperimentale "Enrico Pantanelli" dell'Università di Bari Aldo Moro (Policoro, MT), per identificare composti bioattivi che potrebbero esprimere una potenziale attività erbicida ed essere utilizzati come principi attivi nella formulazione di un nano-erbicida per il controllo innovativo delle erbe infestanti. Per raggiungere questo scopo, lo studio è stato suddiviso in tre parti principali rappresentate come capitoli.

Il primo capitolo è stato dedicato all'indagine e all'identificazione delle specie vegetali che potrebbero esprimere una potenziale attività erbicida. Dopo un'approfondita revisione della letteratura, è stato evidenziato che *Thymus algeriensis* Boiss. e Reut., *T. ciliatus* Desf sottospecie *coloratus* (Boiss. et Reut.) Batt., *T. vulgaris* ecotipo Fasano e *T. vulgaris* var Varico 3, noti per le attività biologiche dei loro oli essenziali (OE), meritavano ulteriore attenzione. Gli OE di queste specie sono stati estratti, caratterizzati chimicamente e la loro fitotossicità è stata valutata sulla germinazione dei semi e sulla crescita precoce di due specie infestanti (*Lolium perenne* L. rappresentativa delle specie monocotiledoni e *Aramanthus retroflexus* L. per le dicotiledoni) in condizioni *in vitro*. Sono state considerate cinque concentrazioni (1, 2,5, 5, 7,5 e 10 µL/mL) per determinare la concentrazione minima inibitoria (MIC). L'analisi fitochimica ha rivelato un'elevata diversità di composti. Gli OE delle specie algerine (*T. algeriensis* e *T. ciliatus*) erano caratterizzati dall'assenza di carvacrolo e dal basso contenuto di timolo nel *T. ciliatus,* mentre quelli italiani (*T. vulgaris* ecotipo Fasano e *T. vulgaris* var Varico 3) erano caratterizzati da un chemotipo *p*-cimene

e importanti contenuti di timolo e carvacrolo. Biologicamente, tutti gli OE hanno mostrato una significativa attività fitotossica nei confronti delle specie infestanti bersaglio. L'inibizione totale della germinazione dei semi e della crescita delle plantule è stata dimostrata a partire da 5 µL/mL, suggerendo che questa concentrazione è la MIC. Inoltre, *T. vulgaris* ecotipo Fasano ha espresso il maggior potenziale fitotossico.

Il secondo capitolo è stato sviluppato in continuità con gli esperimenti iniziati durante il primo capitolo. I risultati ottenuti in condizioni *in vitro* sono stati uno screening preliminare del potenziale fitotossico degli OE di timo. Durante questa seconda parte dello studio, gli esperimenti sono stati condotti *in vivo*. Lo scopo dei biosaggi era di confermare i risultati precedentemente ottenuti in condizioni simili a quelle di campo. Le prove hanno valutato l'effetto degli OE di timo sulla pre e post-emergenza delle stesse specie infestanti bersaglio. Sono state applicate quattro concentrazioni di emulsioni di OE (5, 7,5, 10 e 20 µl/mL). I risultati ottenuti hanno confermato i risultati degli esperimenti *in vitro*. Tutte le concentrazioni hanno efficacemente inibito la germinazione dei semi di entrambe le specie infestanti. Al contrario, per gli esperimenti in post-emergenza, l'effetto sulla crescita delle plantule è stato significativo solo su *A. retroflexus*, evidenziando una selettività degli OE di timo. Inoltre, i risultati hanno confermato il maggiore potenziale erbicida di *T. vulgaris* ecotipo Fasano. Per una migliore comprensione dei meccanismi d'azione degli OE di timo, sono state effettuate analisi citologiche sulla pianta modello *Arabidopsis thaliana* (L.) Heynh. Il sistema radicale delle plantule di *A. thaliana* trattato con OE ha mostrato significativi danni strutturali indicando una possibile alterazione dell'equilibrio ormonale delle plantule e stress ossidativo.

L'ultimo capitolo di questo progetto di ricerca di dottorato si è concentrato su prove di fabbricazione di un prototipo di nano-erbicida con l'obiettivo di rivestire l'OE per proteggerlo dalle condizioni ambientali e preservarne l'efficacia erbicida. Dopo aver stabilito l'efficacia degli OE di timo come possibile bioerbicida, sono stati effettuati i test di incapsulamento dell'OE di timo. *T. vulgaris* cultivar Varico 3 è stato selezionato per le sue caratteristiche botaniche e agronomiche. Questa specie è un ibrido caratterizzato da un maggiore contenuto di OE, stabilità dei composti attivi e produzione costante di biomassa fresca. Tre volumi (500 µL, 750 µL e 1000 µL) di OE sono stati incapsulati in nanoparticelle di silice (SiNPs). Le efficacie di questi nanomateriali sono state confrontate con quella dell'OE puro. La Microscopia Elettronica a Trasmissione (TEM) ha rivelato OE-SiNPs sferiche e regolari con una dimensione compresa tra 220-300 nm. L'analisi FT-IR ha confermato il caricamento dell'OE attraverso la presenza di picchi caratteristici di isoprenoidi e composti isomerici con efficienze di caricamento del 26%, 42% e 64% in base alla concentrazione di OE. I saggi biologici con OE di timo puro in trattamenti post-emergenza su plantule di *A. retroflexus* hanno dimostrato una significativa attività erbicida dipendente dalla concentrazione. L'incapsulamento con SiNPs ha aumentato l'efficacia erbicida dell'OE di timo. Rispetto all'OE puro, la forma incapsulata ha aumentato il contenuto di malondialdeide (MDA) nei germogli e nelle radici e le attività degli enzimi antiossidanti (rispettivamente ascorbato perossidasi, catalasi e superossido dismutasi, APX, CAT e SOD). Questi risultati hanno dimostrato un effetto sistemico degli OE-SiNPs, inducendo una significativa produzione di specie reattive dell'ossigeno (ROS) che porta a danni alla membrana cellulare e a uno squilibrio del sistema antiossidante.

I risultati ottenuti durante questa tesi di dottorato sono promettenti e aprono la strada a ulteriori ricerche per lo sviluppo di un nano-bioerbicida che utilizzi l'olio essenziale di timo incapsulato in SiNP, per un controllo innovativo e sostenibile delle erbe infestanti.

Parole chiave: *Timo, olio essenziale, attività bioerbicida, nanotecnologia, sistema di distribuzione biologico*.

GENERAL INTRODUCTION

General introduction

By 2050, the world population is projected to reach 9.7 billion (UN, 2024). The growing global population has created a need to produce more food, causing, consequently, greater pressure on agricultural production. In addition, problems related to climate change, water scarcity, or decreasing amounts of arable land are widely considered major threats to food security (Monteiro & Santos, 2022). Although the precise consequences of climate change are impossible to forecast, the broad view is that global crop production will be negatively affected (Zhao *et al*., 2017). Responding to these challenges, crop production worldwide is heavily based on the massive use of chemical inputs.

1. Overview on weeds

Weeds are considered one of the major threats to crop production because they decrease product quality and productivity through the competition for resources such as light, water, and nutrients and act as reservoirs of plant pathogens, pests, and insect vectors (Monteiro & Santos, 2022). However, before to go further in the topic, some definitions are necessary. The word "weed" can be define in several ways. The Oxford English Dictionary describes the word weed as "*a wild plant growing where it is not wanted*"; the Weed Science Society of America (WSSA) describes it as "*any plant that is objectionable or interferes with the activities or welfare of man*"; while for the European Weed Research Society (EWRS), a weed "*is any plant or vegetation, excluding fungi, which interferes with human objectives*". Some researchers define weeds as plants persistent and damaging that disrupt the growth of other crop plants and affect human agricultural activities and national economies (Vats, 2015). Whereas, others qualify weeds as organisms able to adapt and survive even in extreme conditions, particularly in cropped fields, where weed populations undergo frequent and intensive selection imposed by human agricultural practices (Liu *et al*., 2020). All these definitions revolve around the same concept, thus weeds could be defined as a highly diverse wild plant or vegetation which grows on fields and interferes with human agricultural activities.

The global cost of weed control is important, with evaluations often exceeding billions of dollars annually. Agricultural economic reports and research studies have provided insights into these costs, estimating that weed infestation causes economic losses of over $32 billion per year, while the potential crop yield loss is about 31 to 34% of crop production, 23% for wheat, 37% for rice, 40% for corn, 30% for potato, 37% for soybean and 36% for cotton. This prompts an intensive use of herbicides (Oerke, 2006; Jabran *et al*., 2015; Qu *et al*., 2021; Kubiak *et al*., 2022). Indeed, although several control methods are available, including physical, mechanical, and biological approaches, the most common is still the chemical one, based on the use of herbicides (Müller-Schärer and Collins, 2020).

2. Weed control approaches

2.1. Chemical weed control approach

By definition, herbicides are a subcategory of pesticides (Zimdahl, 2018). They are chemical molecules used to control weeds. Besides killing plants, they have also the particularity to modify the chemical environment of plants. Herbicides have different properties, which allow them to be classified according to chemical families, activities, methods of application, sites of action, or period of application (Vats, 2015). From a chemical point of view, herbicides are classified according to two systems: one belongs to the Weed Science Society of America (WSSA) developed in 2007, the other one has been introduced in 2010 by the Herbicide Resistance Action Committee (HRAC). The WSSA system currently includes 200 active ingredients and 145 chemical families while the HRAC one covers 291 active ingredients and 58 chemical families.

Depending on their period or time of application, herbicides are divided into three groups: (i) pre-plant herbicides, which are applied to the soil before planting; (ii) pre-emergence herbicides, which are applied before weed seedling emerge and (iii) post-emergence herbicides, which are applied after the weeds have emerged (Vats, 2015). Furthermore, they can be selective or non-selective, which means, respectively, they control target species or suppress all plants with which they come into contact. However, the most common classification of herbicides is based on their site or mode of action (MOA) because within the same site of action class, herbicides produce similar symptoms on susceptible plants (Zimdahl, 2018).

Being the most efficient weed control tool, herbicides have revolutionized agriculture over the last decades and contributed to the significant increase of worldwide crop yields. Consequently, the use of herbicides is constantly rising. Developed countries have rapidly adopted the use of herbicides in the 1950s-1970s as a consequence of rural exodus, which leads to a lack of labor for hand weeding and soil tillage (Gianessi, 2013). Today, the same phenomenon is shown in many developing countries such as India, China, and Bangladesh. In those countries, two main factors lead to the overuse of herbicides: the shortage of laborers as millions of rural populations are moving to urban areas and the affordability and availability of herbicides (Gianessi, 2013).

In 2022, 1.94 million tons of herbicides were used worldwide, representing, thereby, 52.53% of the total pesticide use. Herbicides were mainly used in developed and developing countries, representing 92% of the total use of herbicides, while in Africa the value was very low (3%) (figure 1). Concerning the herbicides use by countries, Brazil used more than 492.400 tons, followed by the United States of America (405.497 tons) and Argentina (249.796 tons). Unfortunately, the data available for China and India are not representative (FAO, 2022).

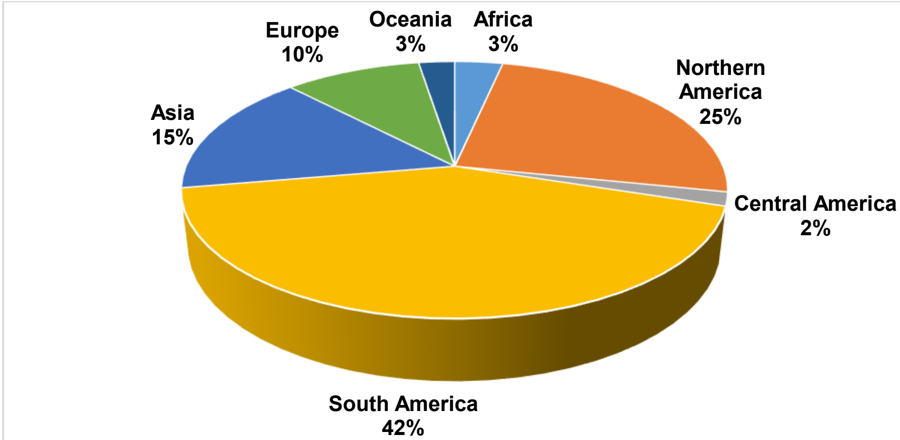


Figure 1. World herbicide use for 2022 (FAO, 2022).

- ***Herbicide weed resistance***

Between the early 1950s and the mid-1980s, herbicides achieved an important success thanks to the discovery of one distinct mechanism of action (MOA) every two years. The introduction of glyphosate-resistant crops in the last 30 years dramatically changed this situation, leading to the emergence of herbicide-resistant weeds (Dayan *et al*., 2019). By definition, herbicide weed resistance is the ability of a given plant's biotype to survive and reproduce after contact with a supposed lethal dose of herbicide to plants of the same species (Vats, 2015). Shaner and Beckie (2014) explained this resistance by the use of the same herbicides with the same modes of action. So far, two types of resistance mechanisms are reported: target-site and non-target-site both caused by structural or regulatory mutations (Délye *et al*., 2015). In 2024, 273 herbicide-resistant weed species (156 dicots and 117 monocots) in 101 different crops have been reported, affecting the efficacy of 168 different herbicides from 21 of the 31 known modes of action (MOAs), adding to this, no herbicides with actual new molecular targets have been commercialized in the last 30 years (He *et al*., 2022; Heap, 2024). The United States of America, Australia, South America, and the European Union have registered the highest cases of herbicide-resistant weeds, nevertheless, differences in crop management, herbicide availability, and weed composition among countries can generate variation rates of herbicide resistance evolution (Délye *et al*., 2015; Owen, 2016; Mascanzoni *et al*, 2018). Countries vary in their use of agronomic strategies such as boosting crop competitiveness, employing effective crop rotations, using herbicide mixtures, strategic tillage, and precision weed management to mitigate herbicide resistance. Additionally, different weed species may not all develop resistance to the same herbicide modes of action or exhibit similar mechanisms of resistance, making their response to external factors heterogeneous. Considering that management interventions and resistance mechanisms affect selection pressures on herbicide resistance on a field level, it may be challenging to distinguish their overall impact when comparing various countries on a global scale (Beckie *et al*., 2019; Hulme, 2023).

- ***Impacts of herbicides on human health and environment***

The excessive and often inappropriate use of synthetic active ingredients, in particular those available in the past, has also caused considerable injuries to human health, environment and natural habitat. Indeed, even if the use of synthetic molecules, like herbicides or pesticides in general, has undoubtedly contributed to increasing agricultural production, their intensive application has considerably harmed the global health. As a consequence of this widespread application of these agrochemicals, human health and environmental concerns also have been raised worldwide (Mubeen *et al*., 2023). In fact, herbicides negatively affect human health either directly through active ingredients or commercial formulations; often containing other chemicals as co-formulants. Studies revealed that they could provoke neurological disorders, alter metabolic and reproductive functions, endocrine disruption, or even mutation and cancer (Defarge *et al*., 2016; Mostafalou and Abdollahi, 2017). The general population, is exposed to chemical inputs through ingestion of food products that might be contaminated with residues. Indeed, some dangerous pesticide residues can persist for a long time in the ecosystems and enter the food chain reaching the consumer's body at higher strata, through the bioaccumulation and biomagnification (Chawla *et al*., 2018). For these reasons, several countries have restricted their legislation of the use of chemical inputs, and the most harmful ones are banned. The European Commission, for example, has restricted the use of glyphosate and banned one of its co-formulants: polyethoxylated tallow amine (Tarazona *et al*., 2017; Nath *et al*., 2024). It is important to point out that glyphosate is by far the most applied active compound and is used in more than 750 different herbicide formulations; accounting for almost one-third of all herbicide usage, an order of magnitude greater amount used than 2,4-D or atrazine, the next two most used active ingredients. This large use is intensified by the spread of glyphosate-tolerant transgenic plants (Maggi *et al*., 2019; Nagy *et al*., 2019).

From the ecological point of view, herbicides have considerably harmed the environment and wildlife, through animal and microorganisms poisoning, disturbance of the abundance, the species composition, and the activities of beneficial insects and in most cases kill them, potential leaching and runoff into groundwater, or contamination of water (Ndakidemi *et al*., 2016; Sondhia *et al*., 2018; Poudyal and Cregg, 2019).

2.2. Sustainable weed management approach

This is why today there is increasing recognition of the harmful impacts of the use of chemicals on human health and the environment. Governments, policymakers, producers, and consumers are aware that agriculture must produce more sustainably and aspire to more eco-friendly cropping systems. Under these adverse conditions and projections, novel and sustainable approaches to increase agricultural productivity and keep pests and pathogens under the damaging threshold, so that the global food supply chain may be maintained to ensure food security, are more than necessary. Indeed, even if weeds are an important threat to crop production, they are imperative for plant diversity in agricultural landscapes (Colbach *et al*., 2018). Thus, weed management must both control weed harmfulness for crop production and foster their essential role in enhancing biodiversity and designing agricultural landscapes. In addition, cropping systems should not be focused only on the limitation of weed-related yield loss but must take particular attention to their potential contribution to biodiversity (Mézière *et al*., 2015). These two opposite tasks could be reconciled by maintaining weed diversity and preventing the dominance of a few competitive weed species. Storkey and Neve (2018) and Adeux *et al*. (2019) argued that simplification of agroecosystems and weed management strategies are the main cause of the emergence of herbicides resistance and the loss of weed diversity, and thereby their related ecosystem services, which are essential for sustainable crop production. Moreover, weed injuries depend on the biological characteristics of the species present in the agroecosystems and their abundance (Storkey and Neve, 2018). From this perspective, weed flora should not be excluded from the agricultural landscape but to be kept under the injury threshold. This hypothesis was demonstrated thanks to the ecological-niche-based hypothesis (niche breadth) (Rocha *et al*., 2020).

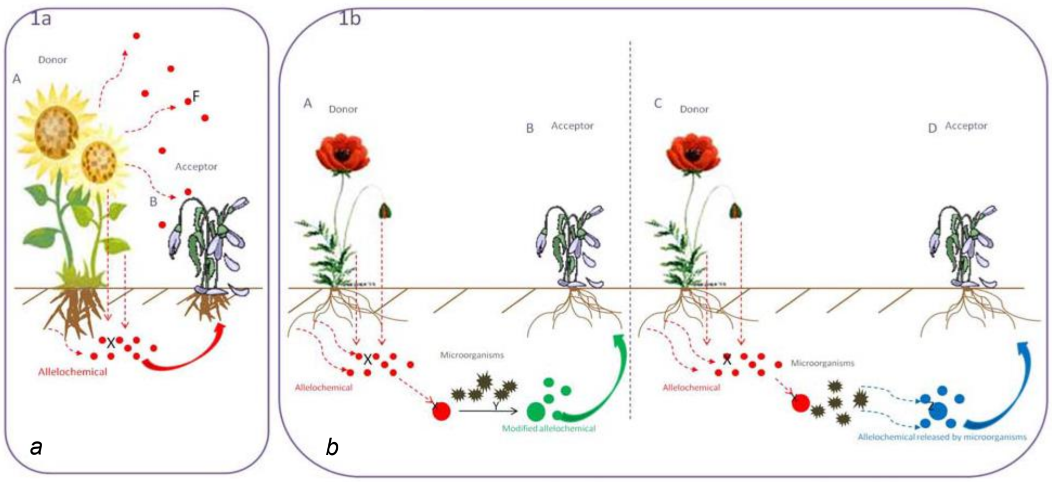
For all the reasons mentioned previously, eco-friendly practices for weed management are the sought alternative. Based, from the agronomic point of view, on agroecological practices such as minimum soil disturbance, permanent soil cover, crop rotation, diversity at farm level…etc. can contribute to a better weed control (Wezel *et al*., 2014; Tuck *et al*., 2014; Nichols *et al*., 2015). Indeed, several studies demonstrated for example the positive effects of cover crops as a preventive method in weed management. They can suppress 70–95% of weeds in the fall-to-spring period, then cover crop residues can reduce weed emergence during early development of the following cash crop acting as a physical barrier and releasing allelopathic compounds into the soil solution (Gerhards and Schappert, 2020). Furthermore, thanks to their allelopathic properties, some plant extracts/essential oils (EOs) can be used as bio-herbicides for weed management.

3. Allelopathy: mechanisms of plant-plant phytotoxicity

Allelopathy, thanks to its important role in regulating ecosystem services, is a promising and serious pathway to consider for discovering next-generation herbicides with high biomolecules diversity. By definition, allelopathy is a biochemical interaction between plants, in which a donor plant releases secondary metabolites called allelochemicals, which interfere negatively with the germination, growth, and development of its neighboring plants, such as the inhibition of photosynthesis, enzymatic activity, and respiration, free radical production, decline of chlorophyll content, disturbance of water, and hormonal balance…etc. (Schandry & Becker, 2020). In scientific jargon, the plant that secretes allelochemicals is called “donor” while plants that are affected by these allelochemicals are referred to as “target plants”. The allelochemicals, are released into the surrounding environment by almost all the plant organs, but most commonly by roots, rhizomes, leaves, and seeds (figure 2.a) (Soltys *et al*., 2013).

The underlying cause of the action of allelochemicals is mainly the inhibition of enzyme activity. The ability of an allelochemical to inhibit or delay seed germination and/or plant growth is usually defined as its "allelopathic potential". Furthermore, the allelopathic interaction and the accumulation of secondary metabolites can cause soil depletion. The intensity and the allelopathic effect are manifold due to the modifications that they generate in the soil.

Most of these secondary metabolites penetrate the soil in an active compound form, such as phenolic acids, cyanamide, momilactones, heliannuols...etc. while others must undergo the action of microorganisms or specific environmental conditions (pH, humidity, temperature, light, oxygen, etc.), to be transformed into an active compound (juglone, benzoxazolin-2-one (BOA), 2-amino-3-H-phenoxazin-3-one (APO)) (figure 2.b) (Hussain and Reigosa, 2011; Iqbal and Fry, 2012; Soltys *et al*., 2013).



(a) Plant A releases allelochemicals X and F which directly affect the growth of plant B. (b) left side; Plant A releases allelochemical X which is modified or activated by microorganisms to allelochemical Y that affects the growth of plant B. (b) right side; Plant C releases allelochemical X which stimulates microorganisms to produce allelochemical Z that affects the growth of plant D.

Figure 2. Multi-dimensional nature of allelopathic interactions (Soltys *et al*., 2013).

3.1. Chemical classes of allelochemicals

From the chemical point of view, secondary metabolites or allelochemicals can be classified into three main groups: terpenes, phenolic compounds, and nitrogen-containing compounds (alkaloids), according to their biosynthetic pathways (Rea *et al*., 2010; Maurya *et al*., 2022). Terpenes are synthesized via the mevalonic pathway from precursor acetyl-CoA, phenolic compounds are produced via the shikimic acid pathway or the mevalonic pathway, and alkaloids are formed first from aliphatic amino acids derived from the tricarboxilic acid pathway or aromatic acids derived from the shikimic acid pathway, via acetyl-CoA and mevalonic acid (Jimenez-Garcia *et al*., 2013).

- ***Terpenes***

Terpenes/isoterpenoids are the major group constituting the EOs. Terpenes are characterized by their molecular structures containing carbon backbones of 2-methylbuta-1,3-diene (isoprene units) which can be rearranged into cyclic structures. The number of isoprene units is responsible for the structural diversity of terpenes; considering the number of isoprene subunits they contain (condensation of two or more isoprene units), terpenes are classified in different subgroups, such as hemi- (C5), mono-(C10), sesqui- (C15), di-(C20), sester- (C25), tri- (C30), tetra- (C40) and polyterpenes (C5)n (isoprene units > 8).

Monoterpenes are the major components of EOs (90%), followed by sesquiterpenes. Diterpenes, triterpenes, and tetraterpenes with their oxygenated derivatives are also detected in small amounts (Falleh *et al*., 2020; Stephane & Jules, 2020; Masyita *et al*., 2022). Terpenoids are another type of terpenes containing oxygen molecules that are constructed via biochemical modifications (removal or addition of methyl groups). Terpenoids can be divided into aldehydes, alcohols, esters, ether, ketones, phenols...etc. Terpenes and terpenoids are synthesized by the mevalonic acid (MVA) pathway in the cytosol and the 2C-methyl-D-erythritol-4- phosphate (MEP) pathway in the plastid for the formation of precursors: isopentenyl pyrophosphate (IPP) and dimethylallyl pyrophosphate (DMAPP) (Stephane & Jules, 2020; Masyita *et al*., 2022). This allows to form a large diversity of compounds that is also enhanced by the modification of a common scaffold structure and since the differential modification of common backbone structures can change and/or modify the biological activity, the new compounds can demonstrate a potential new biological activity (Araniti *et al*., 2016; Kong *et al*., 2021).

- ***Phenolic compounds***

Phenolic compounds consist of hydroxyl groups, which are attached to an aromatic hydrocarbon group (Jimenez-Garcia *et al*., 2013). They are divided into non-soluble compounds such as condensed tannins, lignins, cell-wall bound hydroxycinammic acids, and soluble phenolics such as phenolic acids, flavonoids, and quinones (Krzyzanowska *et al*., 2010). They include many classes of structurally diverse molecules biogenetically arising from the shikimate-phenylpropanoids-flavonoids pathways (Krzyzanowska *et al*., 2010). The most abundant polyphenolics are flavonoids, which are categorized into 13 classes (Rea *et al*., 2010). The most important flavonoids include flavonols, flavones, flavanols, flavanones, anthocyanidins, and isoflavones (Rea *et al*., 2010; Jimenez-Garcia *et al*., 2013). In addition, polyphenols include other chemical compounds such as tannins and coumarins, which are, respectively, complex polyphenols that can be degraded to sugars and phenolic acids by both enzymatic and non-enzymatic hydrolytic processes and lactones derived by cyclization of cis-ortho-hydroxycinnamic acid (Rea *et al*., 2010).

- ***Nitrogen-containing compounds***

Nitrogen-containing compounds or alkaloids are a large group of bioactive molecules containing usually basic nitrogen atoms, derived from an amino acid. Their classification is based on the nitrogen-containing ring system (pyrrolidine, piperidine, etc.) and their biosynthetic origin, amino acids, amines, alcamides, cyanogenic glycosides, and glucosinolates (Khadem and Marles, 2012). Furthermore, because of their toxicity, most alkaloids express chemical defense properties against herbivores and pathogens (Jimenez-Garcia *et al*., 2013).

3.2. Mode of actions of allelochemicals

The mode of action of some allelochemicals can be similar to synthetic herbicides. This characteristic allows them to be considered as potential candidates for the synthesis of a new generation of herbicides, commonly known as botanical herbicides (Schandry and Becker, 2020). In general, secondary metabolites produced by plants can inhibit recipient plant physiological and biochemical activities and restricting weed potential, mainly by an induction of changes to enzymes, genetics, hormones, and metabolic processes, leading to important several disturbances and gradual death (Maurya *et al*., 2022). Several plant sources have been identified with the potential to be formulated as bioherbicides, including *Cynara cardunculus* L., *Dittrichia viscosa* Greuter., *Myrtus communis* L., *Ocimum basilicum* L., *Ruta graveolens* L. and *Sorghum bicolor* L. Studies have shown that higher concentrations of *Datura stramonium* L. treatment in soybeans hinder primary and lateral root development and interfere with chromosome number and structure. Ethyl acetate fraction of *Aglaia odorata* leaves inhibits cell division in *Allium cepa* roots by destroying chromatin and microtubule organization (Teerarak *et al*., 2012; Ben Kaab *et al*., 2020; Grauso *et al*. 2020; Boari *et al*., 2021; Reyes-Vaquero *et al*., 2021, Bruxel *et al*., 2022).

EOs, particularly, have also shown success in controlling weeds, causing severe damage to DNA, biochemical processes, and cellular functions (Ben Kaab *et al*., 2020; Travlos *et al*., 2020; Hasan *et al*., 2021; Anwar *et al*., 2021; Duke *et al*., 2022). Monoterpenes, the primary components of EOs, can cause morphological and physiological alterations in plant seedlings, accretion of lipoid globules, and decline in organelles. Understanding the mechanism and action of allelochemicals requires exploring their herbicidal activities. Citral, a key component of lemongrass EO, have been found to have a strong allelopathic potential in various crops, interacting with microtubules in wheat and *Arabidopsis thaliana*, and interfering with cell ultrastructure in seedlings (Table 1) (Maurya *et al*., 2022).

Table 1. Allelopathic effect of some medicinal and aromatic plants on weeds (Maurya *et al*., 2022).

Family	***Plant species***	***Extract/EO***	***Test weed***
Apiaceae	*Carum carvi*	EO	*Amaranthus retroflexus* *Centaurea salsotitialis* *Raphanus raphanistrum* *Rumex nepalensis* *Sinapis arvensis*
	Pituranthos chloranthus	Extract	*Avena fatua*
	Coriandrum sativum	EO Extract	*Vicia villosa* *Lathyrus annus* *Chenopodium album*
	Foeniculum vulgare	EO	*Vicia villosa* *Lathyrus annus*
	Cuminum cyminum	EO	*Vicia villosa* *Lathyrus annus*
	Foeniculum vulgare	EO	*Echinochloa crus-galli*
Asteraceae	*Artemisia scorpia*	EO	*Echinochloa crus-galli* *Vicia villosa* *Lathyrus annus*
	Artemisia herba-alba	Extract EO	*Avena fatua* *Mantisalea salmentica* *Sinapis arvensis* *Hordeum murimum*
	Artemisia annua	Extract	*Chenopodium album* *Amaranthus retroflexus*
	Artemisia dubia	Extract	*Ageratum conyzoides* *Galinsoga parviflora* *Cyperus rotundus*
	Matricarria chamomilla	Extract/compound	*Avena fatua* *Lolium multiflorum*
	Tagetes minuta	EO Dried powder	*Chenopodium miracle* *Phalaris minor* *Amaranthus tricolor* *Chenopodium muracle* *Bidens pilosa* *Amaranthus viridis* *Echinochloa crus-galli* *Cyperus rotundus*
	Sunflower	Extract	*Coronopus didhymus* *Chenopodium album* *Avena fatua*
Capparaceae	*Capparis spinosa*	Extract	*Chenopodium muracle* *Amaranthus retroflexus*
Cucurbitaceae	*Citrullus colocynthis*	Extract	*Chenopodium muracle* *Amaranthus retroflexus*

Family	Plant species	Extract/EO	Test weed
Euphorbiaceae	Ricinus communis	Extract EO	Chenopodium muracle Chenopodium album
Lamiaceae	Origanum onites	EO	Amaranthus retroflexus Centaurea salsotitialis Raphanus raphanistrum Rumex nepalensis Sinapis arvensis
	Thymbra spicata	EO	Amaranthus retroflexus Centaurea salsotitialis Raphanus raphanistrum Rumex nepalensis Sinapis arvensis
	Mentha arvensis	EO	Angallis arvensis Cynodon dactyalon Cyperus rotundus
	Mentha spicata	EO Extract	Amaranthus retroflexus Centaurea salsotitialis Raphanus raphanistrum Rumex nepalensis Sinapis arvensis Sonchus oleraceus Sorghum halepense Cynodon dactylon Rumex crispus
	Mentha longifolia	EO	Vicia villosa Lathyrus annus
	Mentha pipperita	Extract EO	Chenopodium album Sorghum halepense Convolvulus arvensis Echinochloa colona Echinochloa colona Amaranthus retroflexus Portulaca oleracea Vicia sativa Microencapsulated Chenopodium album Echinochloa crus-galli
	Salvia officinalis	Extract	Amaranthus retroflexus Chenopodium album
	Rosmarinus officianalis	Extract	Chenopodium muracle Amaranthus retroflexus Chenopodium album
	Thymus vulgaris Thymus capitatus Origanum syriacum	Extract Extract Extract	Chenopodium album Medicago polymorpha Chenopodium muracle Amaranthus retroflexus

Allelochemicals also affect plant growth and development by influencing phytohormone production, photosynthesis, and antioxidant enzyme activity. They also produce reactive oxygen species (ROS) and increase free radical levels, creating an imbalance in the antioxidant system. Lemongrass essential oil has been found to damage barnyardgrass membrane systems, damage cells, and increase cell membrane permeability. Allelochemicals adversely affect various stages of respiration, including electron transfer in mitochondria, oxidative phosphorylation, CO2 generation, and ATP enzyme activity (figure 3) (Wang *et al*., 2014; Maurya *et al*., 2022).

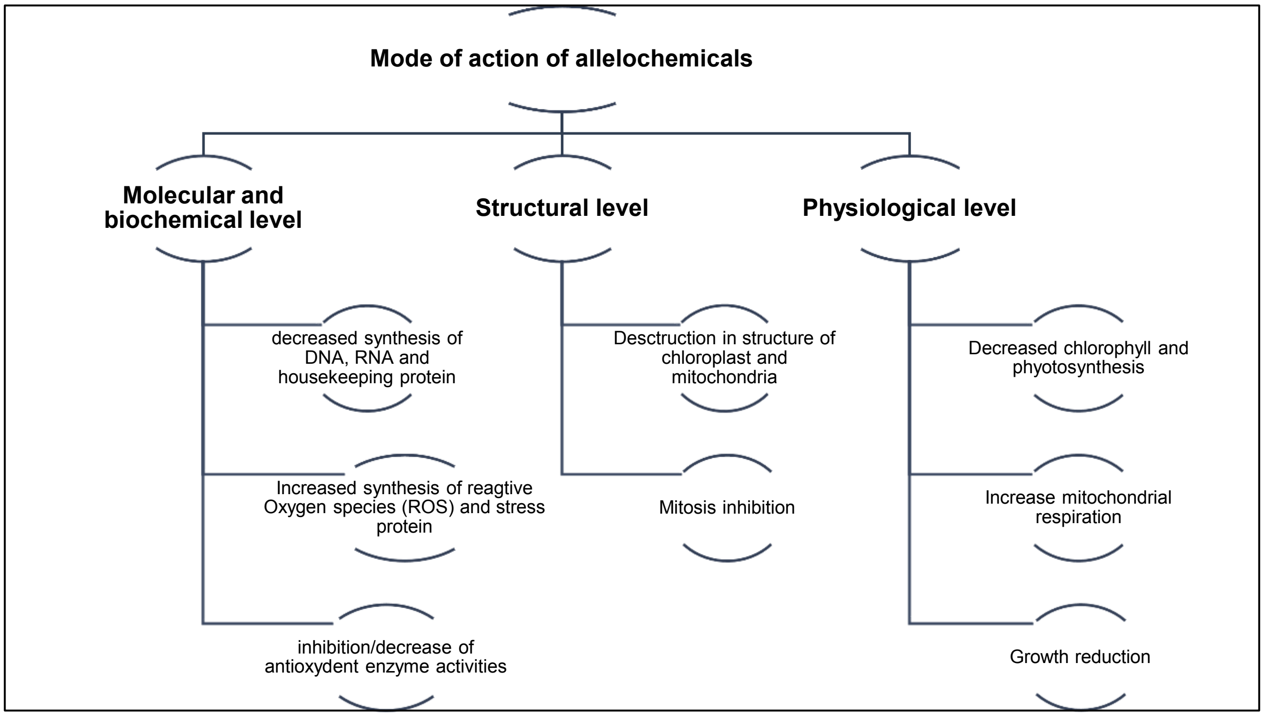


Figure 3. Modes of action of allelochemicals (Maurya *et al*., 2022).

3.3. Herbicidal potential of EOs and their application limitations

In general, the herbicidal activity of EOs has been demonstrated through cell injury (hinder cell division and differentiation, gene expression and signal transduction, and truncate cell membrane permeability), oxidative stress damage, DNA and RNA damage, photosynthesis and respiration inhibition, ultimately leading to cell death (Arora *et al*., 2024). Though their mechanisms are complex, diverse, and insufficiently elucidated, EOs are currently being considered for commercial herbicide products formulations. As of 2020, seven commercial bioherbicides — Matratec, GreenMatch, GreenMatchEX, WeedZap, Weed Slayer, Avenger Weed Killer, BioWeed, and WeedLock — were registered and available in the USA, Australia, and Malaysia. For example, BioWeed (Barmac, Lidcombe, Australia), Avenger Weed Killer (Avenger Products, LLC, Buford, Georgia), and Weed Slayer (Agresearch International, LLC, McKinney, United States) have been used to effectively control *Ochna serrulata* Walp., *Digitaria sanguinalis* (L.) Scop., and *Echinochloa crus-galli* (L.) P. Beauv., respectively.

However, it is not economically sustainable for large-scale weed control and requires further scaling up of its formulation to allow the application on a large scale (Travlos *et al*., 2020; Verdeguer *et al*., 2020; Arora *et al*., 2024). Furthermore, EOs are highly hydrophobic, chemically unstable, volatile, and easily degraded in nature. Indeed, EOs are easily dissipated through evaporation, degraded (oxidised, isomerized, dehydrogenised, or polymerised) by environmental factors such as light, heat, or air unless they are protected. Another limitation factor for their application is their insolubility in the aqueous phase may also restrict their applications; thus, their encapsulation represents an important alternative to preserve their properties (Mun and Townley, 2021; Santos *et al*., 2023).

3.4. Novel approaches for targeted active ingredients delivery

In this regard, nano-biotechnology provides innovative and novel methods to manipulate and enhance crop production and protection. In this regard, nano-enable materials offer a new and smart platform to deliver active compounds for plants to increase yield, optimize water and nutrient uptake, and protect them against pests and pathogens. In addition to these advantages, they ensure a controlled release and site-directed delivery of agrochemicals. So far, various types of nanomaterials have been tested and the results are promising in terms of productivity and quality enhancement of crop production (Mali *et al*., 2020; Periakaruppan *et al*., 2023). Indeed, they have given controlled release formulations (CRF) a new dimension thanks to their optimal active ingredient delivery, leaf surface adhesion, and longer contact time on plant foliage, without washing into a runoff or leached out immediately after application (Itodo *et al*., 2017; Sousa *et al*., 2018; Grillo *et al*., 2021; Pontes *et al*., 2021). It is worth precise that material generated through nanotechnology techniques is in nanometer scale ranging from 1 to 100 nm and thanks to their small size, the ratio between surface area and volume is increased in the nanomaterials in comparison with the bulk forms. In this way, the biochemical reactivity and physical properties of the fabricated nanomaterial are improved (Ali *et al*., 2014).

Given the physical and chemical properties of EOs, strategic design using nano-enable materials to ensure the optimal efficacy on target organisms can be consider. Several nano-enable materials can be used for this purpose. Indeed, the process of encapsulating bioactive compounds consists in enclosing droplets of the compounds with coatings or submerging them in heterogeneous or homogeneous matrices (Mun and Townley, 2021). In this sense, several types of nanoencapsulation materials have been used to increase the availability of EOs, including polymer-based, inorganic nanoparticle systems…etc. From a chemical point of view, EOs are able to create hydrophobic interactions with the hydrophobic cavities found in nanocapsules or within the polymers of nanocapsules and nanospheres. They might also engage with the surfactants found in nanoliposomes or the lipid phases of nanoemulsions, solid lipid nanoparticles, and nanostructured lipid carriers, while they may interact with inorganic nanoparticle systems through van der Waals forces, or electrostatic interactions for metal nanoparticles. In this sense, a large number of nano-enable material as coating material can be found in the literature (figure 4).

- **Organic nano-enabled material**

Organic nanomaterials are outstanding materials based on organic polymers such as lignocellulosic materials, proteins, complex macromolecules as dendrimers, etc. these types of polymers are widely used in nano-enabled delivery of active compounds due to their biodegradability and biocompatibility (Shakiba *et al*., 2020; Forini *et al*., 2022). Furthermore, synthesis of biopolymer-based nanocarriers such as chitosan was reported using chemical crosslinking agents as tripolyphosphate (TPP) or functionalization with molecules such as 11-mercapto undecanoic acid (MUA) or N-octyl derivatives may offer a good nano-enabled product (Mohammadi *et al*., 2021). Pectin and gums/gelatin can also be consider to encapsulate EOs (Hazrati *et al*., 2017; Taban *et al*., 2020; Taban *et al.,* 2021).

- **Inorganic nano-enabled material**

Given their potential benefits in agriculture, inorganic nano-enabled materials have a particular attention from the scientific community. Indeed, they can enhance crop yield and herbicidal effectiveness, leading to decreases in waste and runoff (Cartwright *et al*., 2020). Inorganic enable-materials are based on silica, silver metal and mesoporous silica nanoparticles (MSNs). This later (MSNs) has a honeycomb structure with a large number of pores, ranging from 2 to 50 nm. They have a high specific surface area, adjustable particle size, and pore size, as well as the possibility of surface functionalization (Weisany *et al*., 2024). MSNs were successfully used for the encapsulation of *Thymus eriocalyx* and *Thymus kotschyanus* EOs for insecticidal purposes (Ebadollahi *et al*., 2017). Inorganic nano-enabled materials are able to release ions effectively, whereas other metal-based nano-material can trap organic molecules and release ions efficiently in a controlled manner. This type of nanoformulations have been developed as nanoherbicides with zinc/aluminum hydroxides or magnesium-aluminum in conjunction with sepiolite clay (Sharif *et al*. 2020; Forini *et al*. 2022). These systems are capable of effectively trapping hydrophobic molecules within their layers like EOs (Sutthanont *et al*., 2019; Veisi *et al*., 2019).

- **Hybrid nano-enabled herbicides**

Hybrid materials can blend the benefits of different materials, like organic and inorganic, within a unified structure (Gao *et al*., 2020). These versatile nanomaterials can exhibit diverse attributes, dimensions, shapes, and chemical properties (Ananikov, 2019). In addition, hybrid nano-material can enhance targetability, traceability, and stimuli-responsiveness features (Zhao *et al*., 2020; Li *et al*., 2021). Research has been conducted on biomass-based hybrids made up of lignin, xylan, starch, and cellulose for enclosing active ingredients and employing in targeted active compounds delivery, owing to their biocompatibility, biodegradability, natural abundance, and ease of functionalization. In addition, inorganic materials can be used also in hybrid nano-enable material combined to organic or other inorganic materials (Granetto *et al*., 2022). Chemical interactions among different compounds can transform materials into effective carriers for nano-enabled active compounds. As an example, hydrophilic materials can be modified to coat hydrophobic molecules (Hao *et al*., 2020). Therefore, hybrid nanocarriers are becoming increasingly efficient in coating active molecules thanks to a better spreadability, better leaf adhesion, and less UV irradiation degradation.

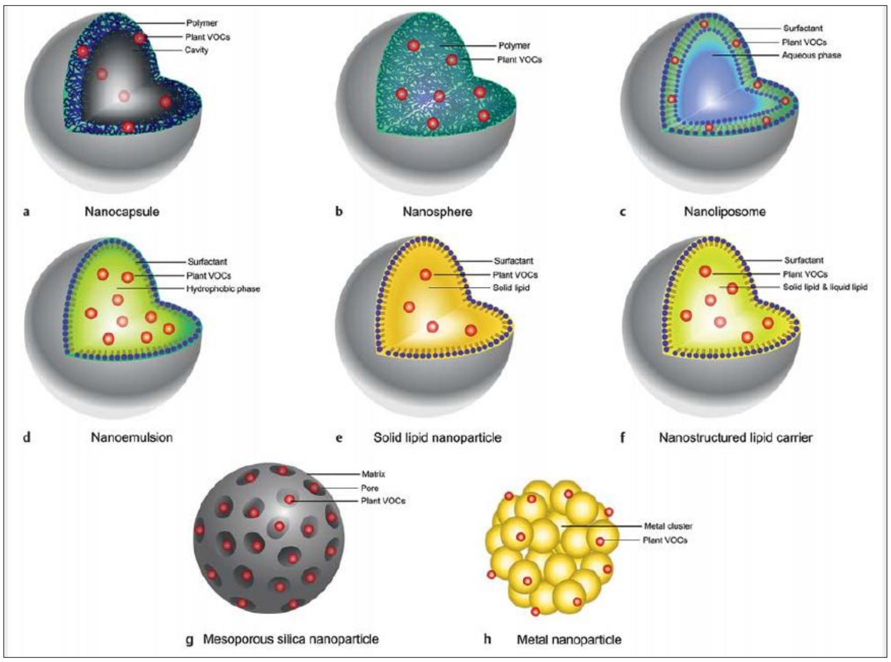


Figure 4. Structures of nanoparticle platforms carrying plant volatile compounds. Nanocapsule (a) and nanosphere (b): polymer-based nanoparticles; nanoliposome (c), nanoemulsion (d), solid lipid nanoparticle (e), and nanostructured lipid carrier (f): fabricated from lipid to form the lipid-based nanoparticle system. MNSs (g) and metal nanoparticles (h): inorganic nanoparticle system (Mun and Townley, 2021).

In conventional agriculture, nanoherbicides have demonstrated significant potential for weed control, with various types of nanoherbicides being developed utilizing both organic and inorganic nanocarriers (Dong *et al*., 2021; Pontes *et al*., 2021; Lima *et al*., 2021; Takeshita *et al*., 2025). Studies demonstrated that nano-enabled herbicides may offer greater efficiency and environmental advantages than conventional ones (Aguirre-Becerra *et al*., 2022), which allows their potential use in weed control in sustainable farming systems. Despite their increased effectiveness, studies regarding human and environmental risk assessments of nano-herbicides are still incipient; moreover, it is important to do not lose sight of the fact that active ingredients in many nanoherbicides are often synthetic molecules that may pose human and ecological hazards. Hence, there is growing interest in using natural products as active ingredients (Taban *et al*., 2020; Forini *et al*., 2022).

4. Valorization of the Mediterranean flora

The Mediterranean area is one of the richest hotspots of the world with 24.000 species distributed in more than 2.3 million km^2. Almost 2000 species have been recorded classified into 200 families. Plant assemblages consequently result in a high number of habitats and vegetation types, arranged in five vegetation belts: thermo-Mediterranean (typically from sea level to 300 m), meso-Mediterranean (300–800 m), supra-Mediterranean (800–1700 m), oro-Mediterranean (1700–2100 m) and cryoro-Mediterranean (from 2100 over 3000 m) (Vargas, 2020). Biodiversity in this area is formed by more complex patterns (Blondel *et al*., 2010). Moreover, the actual Mediterranean flora has been molded by three different factors: (i) geological history and climate change, (ii) environmental conditions, and (iii) ancient human activity (Nieto Feliner, 2011).

Some of these Mediterranean plant species are very common and widespread and proved to have an allelopathic potential. They were studied for several purposes and in some cases, they were also considered for their herbicidal potential. The *Lamiaceae* family is among the most important families considered for their allelopathic properties. Indeed, it is an important medicinal and aromatic plants family which contains about 236 genera and more than 7.000 species (Raja, 2012). *Lamiaceae* species are rich in essential oils which are used in natural medicine, pharmacology, cosmetology, and aromatherapy and flavoring (Karpiński, 2020). In addition, extracts of some *Lamiaceae* species showed an inhibitory effect of the germination and growth of many weed species; and EOs from species such as oregano, thyme, rosemary, sage and mint exhibit a strong bioherbicidal activity (Maccioni *et al*., 2020). The phytotoxic effect of these extracts, specifically EOs, is mainly attributed to the presence of volatile bioactive compounds such as carvacrol, camphor, thymol, α-pinene, limonene and 1,8-cineole (Abd-Elgawad *et al*., 2020).

Taking into account what has been mentioned above and thanks to their phytochemical diversity and biological activities, the genus *Thymus* deserve a particular attention.

4.1. *Thymus* genus

The genus *Thymus* belongs to the *Nepetoideae* (subfamily of *Lamiaceae* family). It consists of around 250 species (comprising 214 species and 36 subspecies) of herbaceous or sub-shrubs aromatic perennial plants and small shrubs. The classification of thyme species attributes to the chromosomal information, which is crucial for taxonomy. However, identifying the chromosomes of *Thymus* species is challenging due to their small size and similar morphology. The Mediterranean region is considered the center of the genus, particularly the Iberian Peninsula and North-west Africa. Nevertheless, it has spread to Europe, Greenland, Northern America, Abyssinia, and Asia (Stahl-Biskup *et al*., 2012; Ghasemi *et al*., 2015; Dall'Acqua *et al*., 2017). *Thymus vulgaris* L., commonly named "common thyme", is the most important species on the genus; other species such as *T. zygis* L. (Spain thyme), *T. serpyllum* L. (wild thyme), and *T. pulegioides* L. (large thyme) are largely commercialized around the world (Stahl-Biskup *et al*., 2012; Nabavi *et al*., 2015). Thanks to their aromatic and medicinal properties, *Thymus* genus species are largely utilized as culinary herb, in cosmetics, perfumery, and pharmaceutical industries. Thyme plants are commonly added to infusions as traditional remedies for digestive and respiratory issues (Alarcón *et al*., 2015; Salehi *et al*., 2019a). From the botanical, genetic and phytochemical points of view, thyme species can show different particularities.

The genus *Thymus* is a widely distributed in the Mediterranean region with several endemic species.

4.1.1. *Thymus* genus in Algeria

Thymus species are endemic and widespread in Algeria and are growing wild. They are used as an aromatic herb for culinary purposes and in traditional medicine against many health problems (Hazzit *et al*., 2009; Nouasri *et al*., 2015). The genus is represented by twelve species and three subspecies, among them nine are endemic: *Thymus algeriensis* Boiss. & Reut, *T. capitatus* Hoffmanns. & Link., *T. ciliatus* Desf., *T. commutatus* Batt., *T. dreatensis* Batt., *T. glandulosus* Reg., *T. guyonii* de Noé., *T. hirtus* Banks. & Sol., *T. lanceolatus* Desf., *T. numidicus* Poir., *T. pallidus* Coss. ex Batt., *T. fontanesii* Boiss. & Reut and *T. pallescens* (Bekhechi *et al*., 2007).

T. algeriensis Boiss. & Reut. is the most widespread species but *T. ciliatus* Desf. and *T. pallescens* are also common in the northern Algeria, as well as *T. numidicus* Poiret, which grows mainly in Constantine (north-eastern of Algeria) and Kabylie region (north-central of Algeria) (Quezel & Santa, 1963). *T. ciliatus* Desf. subsp. *eu-ciliatus* Mair, is widespread in all the regions of Algeria, subsp. *coloratus* (Boiss. et Reut.) Batt. is common in Tell of Algeria while subps. *munbyanus* (Bois. et Reut.) Batt. is relatively rare and localized only in Western Algeria (the Oranais and the Hautes Plaines) (Quezel & Santa, 1963). *T. dreatensis* and *T. lanceolatus* Desf. are rare and are present, respectively, in Aures mountains (Batna region) and Djurdjura mountains (Kabylie region) of eastern Algeria for the first species and in mountains of Tlemcen, Tiaret, Ben chicaou (province Medea) and Sour el Ghozlene (province Bouira) for the second one (Quezel & Santa, 1963).

Table 2. Geographical localization of *Thymus* species in Algeria (Quezel & Santa, 1963).

Species	**Localization**
Thymus capitatus Hoffmanns. & Link	Rare, present in Tlemcen region
Thymus fontanesii Boiss. & Reut.	Common in the Tell- Endemic to Eastern part of Algeria
Thymus commutatus Batt.	Endemic to Oran Region – Western part of Algeria
Thymus numidicus Poir.	Relatively common – present in The Tell Atlas sub-sector, Constantine and Kabylie region and From Skikda to the Tunisian borders.
Thymus guyonii de Noé	Rare- Present in Algiers and Oran region but also Constantine and Hautes Plaines sub-sector
Thymus lanceolatus Desf.	Rare – Present from Terni to Benchicao (Medea) and the sub-sector of the Hautes Plaines (Tiaret et Constantine)
Thymus pallidus Coss. ex Batt	Very rare- Present in the sub-sector of the Saharian Atlas and Constantine region
Thymus ciliatus Desf.	Common in all the regions except the coastal one
Thymus glandulosus Reg.	Very rare- Present in the sub-sector of the Hautes Plaines
Thymus algeriensis Boiss & Reut.	Very common- Present in the sub-sector of the Hautes Plaines of the center and Western part of Algeria
Thymus munbyanus Boiss & Reuter	Western of Algeria

Concerning *T. ciliatus* Desf. and *T. munbyanus*, some doubts are expressed. According to the classification reported by Quezel & Santa (1963), *T. ciliatus* (Desf.) Benth. includes three subspecies: ssp. *eu-ciliatus* Maire, ssp. *coloratus* (Boiss. et Reut.) Batt. and ssp. *munbyanus* (Boiss. et Reut.) Batt while Stahl-Biskup and Sáez (2002) considered these three subspecies and established that each one could be considered as a species in its own right. Thus, they were named *Thymus ciliatus*, *Thymus munbyanus* and *Thymus coloratus*, indicating that these three species could easily hybridize to each other. The authors have also indicated that the botanical differentiation between them would be difficult and thus the chemical composition could help to make this classification. Boudiaf *et al*. (2017) have reported a third classification. The authors indicated, according to Plant List Database – 2013, that *T. munbyanus* includes three subspecies, namely subsp. *abylaeus* Front (Quer & Maire), subsp. *ciliatus* (Desf.) Greuter & Burdet and subsp. *coloratus* (Boiss. & Reut.) Greuter & Burdet. The same Database reported the most recent classification of *T. ciliatus* and indicated that *T. ciliatus* (Desf.) Benth. is a synonym of *T. munbyanus* subsp. ciliatus (Desf.) Greuter & Burdet (= *T. ciliatus* subsp. *eu-ciliatus* Maire); *T. coloratus* Boiss. & Reut. is a synonym of *T. munbyanus* subsp. *coloratus* (Boiss. & Reut.) Greuter & Burdet (= *T. ciliatus* subsp. *coloratus* (Boiss. et Reut.) Batt.); and *T. munbyanus* Boiss. & Reut. is a synonym of *T. munbyanus* subsp. *munbyanus* (= *T. munbyanus* subsp. *abylaeus* (Font Quer & Maire), = *T. ciliatus* subsp. *munbyanus* (Bois. et Reut.) Batt.) (Boudiaf *et al*., 2017).

Unfortunately, there is a lack of literature review concerning the botanical description of the Algerian thyme species.

4.1.2. *Thymus vulgaris*

T. vulgaris is an erect and sometimes decumbent sub-shrub, about 10–40 cm and with a woody base and herbaceous characteristics in the upper zone. The stems are reddish, pubescent with short hairs. The leaves of the plant are small, about 3.5 to 6.5 mm long and 0.8 to 3.0 mm wide, linear to ovate-lanceolate, pointed, with the margins rolled under, greenish-gray in color and with spheroidal glands. The inflorescence is 10–15 mm in diameter, with a capituliform or spiciform structure and more or less separated verticillasters. *T. vulgaris* has small purple to white flowers that appear in late spring to early summer. The flower consists of five petals fused into a cup or tube, forming a corolla smaller than 5 mm and with the upper lip low cut and the lower one with a central lobe greater than the lateral ones. The fruit of the plant is a smooth, dry and dark colored nutlet but does not open when ripe (figure 5). The species has a fragrant and aromatic smell when crushed, and the flavor is also aromatic, warm and spicy (Christopher, 2008; Silva *et al*., 2021).

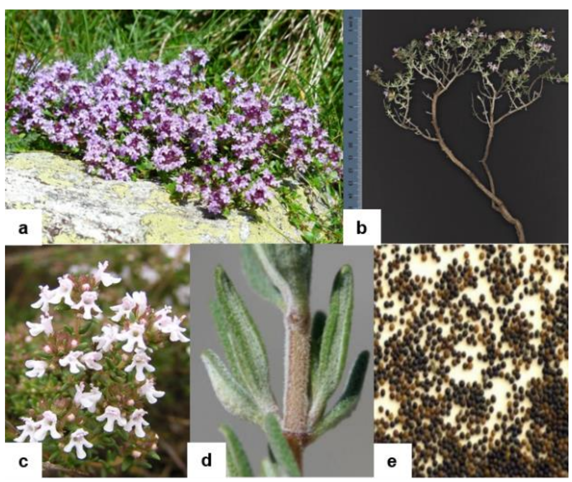


Figure 5. Morphology of *Thymus vulgaris* L.; (a): Plant; (b): branch; (c): flower; (d): leaves; (e): seeds of thyme (Patil *et al*., 2021).

T. vulgaris, as other thyme species can be grown is temperate to warm and sunny climates, with rough, coarse and well-drained calcareous soils (Kuete, 2017). It can grow from sea level to heights above 800 m but it also takes up deep freezes and can be found on mountain highlands (Hosseinzadeh *et al*., 2015). The plant can be propagated by seeds, cuttings, or by dividing rooted sections. *T. vulgaris* seeds generally germinate 1–2 weeks after sowing at 10–30 °C and sufficient sunlight (Iapichino *et al*., 2018).

T. vulgaris L. has a diverse genetic constitution. It shows aneuploidy where the number of chromosomes differs between plants within the same species, where 2n=28, 30, 56 and 60 are considered as the most frequent numbers for other plants belonging to the genus *Thymus*. Aneuploidy has played an important role during evolution of the species and may also contribute to its morphological variation. Several sub-species are also present that can further add to the complexities of genetic research. The variation in *T. vulgaris* essential oil yield and composition depends also on environmental and ecological conditions (Nabavi *et al*., 2015; Satyal *et al*., 2016; Lemos *et al*., 2017).

In view of his economic importance as medicinal plant and as condiment (Salehi *et al*., 2018), *T. vulgaris* has been the largely studied in terms of phytochemistry. These studies revealed that essential oil of *T. vulgaris* is produced in higher amounts during the flowering stage (Golmakani, 2008). Furthermore, plant growth stage, environmental factors, harvesting time, geographical location and climatic conditions can influence the content of volatile compounds qualitatively and quantitatively which leads the occurrence of different chemotypes. In this sense, several chemotypes of *T. vulgaris* exist and are named according to its dominant monoterpene (György *et al*., 2020). Presently, more than 13 different chemotypes have been identified, including geraniol (G), α-terpineol (A), thuyanol-4 (U), linalool (L), carvacrol (C), 1,8-cineole and thymol (T) (Satyal *et al*., 2016).

The phytochemical analysis of *T. vulgaris* L. revealed the presence of a myriad of chemical compounds as phenolic compounds, terpenes/terpenoids, flavonoids, steroids, alkaloids, tannins, and saponins. Most of them are volatile compounds extracted from plant EO. The characterization of the phytochemical composition of *T. vulgaris* was performed mostly by using gas chromatography-mass spectroscopy (GC-MS) and high-performance liquid chromatography/thin layer chromatography (HPLC/HPTLC) techniques (Abdelli *et al*., 2017; Stefanis *et al*., 2019). The results depict the prevalence of essential oil components over the other metabolites.

T. vulgaris L. is one of the principal sources of phenolic compounds. Heidari *et al.* (2018) identified new phenolic compounds using GC-MS technique, including creosol 2-methoxy-4-methylphenol), thiophenol (benzenethiol), quininic acid (6-methoxyquinoline-4-carboxylic acid), loliolide [(6S,7aR)-6-hydroxy-4,4,7a-trimethyl-6,7-dihydro-5H-1-benzofuran-2-one], phenol 4-(3- hydroxy-1- propenyl-2- methoxy), and 3-methoxy-5- methylphenol. Also, previous studies have identified the presence of rosmaric acid, caffeic acid, p-coumaric acid, geranic acid, p-hydroxybenzoic acid, gentisic acid, syringic acid, and ferulic acid, rosmarinic and salvianolic acid, danshensu-hexoside, danshensu, caffeic acid-hexoside, eriodictyol, eriodictyol-diglucoside, eriodictyol-hexoside, eriodictyol-glucuronide, p-cumaric acid-hexoside, apigenin, apigenin-di-C-glucoside, apigenin-glucuronide, hydroxybenzoic acid, hydroxyrosmarinic acid, kynurenic acid, caffeic acid, hydroxyluteolin-hexoside, eriodictyol-rutinoside, quercetin-glucuronide, dihydroquercetin, naringenin, naringenin-glucoside, naringenin-rutinoside, luteolin-glucoside, luteolin-glucuronide, luteolin-glucuronide-hexoside, luteolin-acetyl-glucoside, rosmarinic acid, salvianolic acid A, salvianolic acid K, salvianolic acid I, cirsimaritin and cirsilineol (Kuete, 2017; Schött *et al.*, 2017; Patil *et al*., 2021).

The characterization of the EOs of *T. vulgaris* L. reveals the presence of hydrocarbons, oxides, alcohols/esters, and aldehydes/ketones with different concentrations. Among all the reported volatile compounds, thymol, carvacrol, geraniol, linalool, α- and β-pinene, p-cymene, and γ-terpinene have been reported (Salehi *et al*., 2019b). In addition, hydrocarbons like 2,6-Octadienal, cis-Sabinene hydrate, germacrene D, limonene, β-ocimene, myrcene, β-caryophyllene, α-thujene, α-phellandrene and α-humulene, oxides like 1,8-cineol, caryophyllene oxide; alcohol/esters including α-terpineol, borneol, 1-octen-3-ol, 3-octanol, p-cymen-8-ol, terpinen-4-ol, thymol methyl ether, carvacrol methyl ether; aldehydes/ketones like 3-octanone, camphor, thymoquinone, and geranial are present. The GC-MS analysis also showed the presence of esters including butanoic acid, 2-methyl-, methyl ester, bornyl acetate, and geranyl propanoate (Nabavi *et al*., 2015; Schött *et al*., 2017; Patil *et al*., 2021).

Furthermore, the phytochemical analysis of *T. vulgaris* L. reveals the presence of flavones like 6-hydroxyluteolin, apigenin, luteolin, methyl-flavones including cirsimaritin or genkwanin, cirsilineol, 5-desmethylnobiletin, 8-methoxycirsilineol, 7-methoxyluteolin, gardenin B, salvigenin, thymonin, sideritoflavone, xanthomicrol and thymusin (Kuete, 2017; Patil *et al*., 2021). In their study using HPTLC technique, Asha *et al*. (2017) assessed the methanolic extract of *T. vulgaris* L. The results showed the presence of 9 alkaloids, 14 saponins, 12 steroids, and 8 tannins. The study revealed a good resolution with 10 bands of EOs, along with bands corresponding to tannins (9.2%) and saponins (23.1%). As most of the chemical components belong to EOs and phenolic compounds, metabolites like steroids, tannins, alkaloids, and saponins have not been evaluated in detail.

The alcohol insoluble residue prepared from *T. vulgaris* leaf were analyzed in terms of polysaccharides. The results revealed that the leaf extract was reported to contain 37% total sugars of which about 8% were uronic acids. Xylose, arabinose, glucose, galactose and rhamnose were reported to be the main sugars, while mannose and fucose units were in small amount. Alcohol insoluble residue also contained galacturonic acid and glucuronic acid. Besides polysaccharides, the residue was indicated to have 7% protein content (Banerjee *et al*., 2019).

The highly diversified phytochemical composition conferred to *T. vulgaris* its important biological activities, hence its use in traditional medicine. The plant has an antiseptic, astringent, carminative, tonic, anthelminthic, antibacterial and antifungal properties. It is used for respiratory diseases including whooping cough, bronchitis, and catarrh, intestinal infections and infestations caused by ascarids, hookworms, fungi, yeast, and bacteria, dermatological problems like acne, oily skin, dermatitis, bug bites as well as sciatica and rheumatic aches. It is also reported that *T. vulgaris* contributes in the amelioration of oxidative stress and cell mediated immune response (Benameur *et al*., 2019 ; Cabarkapa *et al*., 2019 ; Tian *et al*., 2019; Arrais *et al*., 2023; Al-Rimawi *et al*., 2024).

4.1.3. Herbicidal activity of some *Thymus* species

Thymus genus species have been considered for their allelopathic potential. The herbicidal activity of *Thymus* sp. EO and their constituents has been reported, especially monoterpenes, on seed germination and plant growth of the test species and thus influence plant community's composition and dynamic (Linhart *et al*., 2015). The following table (table 3) summarizes some of the studies performed on thyme species for their herbicidal potential.

Table 3. Summary of some research studies on the herbicidal potential of thyme species.

Species	***Test weed***	***Target***	***Reference***
T. satureioides	*Amaranthus retroflexus*	seed germination post-emergence growth	Miloudi *et al*. (2024)
T. pulegioides α-terpinyl acetate chemotype	*Poa pratensis* L.	seed germination and radicle growth	Vaiciulyte *et al.* (2021)
T. proximus	*Amaranthus retroflexus* *Poa anuua* *Amaranthus* sp. *Rottboellia cochinchinensis*	seed germination seedling growth	Zhou *et al.* (2021) Portuguez-García *et al.* (2021)
T. vulgaris	*Chenopodium album* *Echinochloa crus-galli* *Amaranthus retroflexus* *Poa anuua* *Amaranthus sp.* *Rottboellia cochinchinensis*	seed germination seedling growth	Elghobashy *et al*. (2024) Zhou *et al.* (2021) Portuguez-García *et al.* (2021)
T. decussatus	*Lactuca sativa*	seed germination shoot/root growth	Saleh *et al*., (2020)
T. fontanesii	*Sinapis arvensis, Avena fatua, Sonchus oleraceus and Cyperus rotundus*	seed germination	Sara *et al*. (2019)
T. eigii	*Lactuca sativa* *Lepidium sativum* *Portulaca oleracea*	seed germination	Zeynep *et al*., (2018)
T. algeriensis	*Medicago sativa* *Triticum astivum*	shoot and root growth	Ali *et al.* (2015)
T. daenensis Celak	*A. retroflexus*	shoot and root growth	Ali *et al*. (2015)

➢ Aims and objectives

In the light of these considerations, the Mediterranean area is one of the richest hotspots of biodiversity in the world. Plant assemblages consequently result in a high number of habitats and vegetation types. Some of these Mediterranean plant species are very common and widespread and proved to have strong allelopathic activity and phytotoxic bioactive substances, though the potential use of their extracts for controlling weeds is still widely unexplored. Preliminary investigations showed that EOs for *Thymus* sp. pl. could have the potential for controlling weeds (Ghasemi *et al*., 2020; Saleh *et al*., 2020; Vaiciulyte *et al*., 2021; Zhou *et al*., 2021). However, to the best of our knowledge, few studies considered large screenings of the herbicidal potential of thyme species and even less the use of plant active compounds for a nano-fabrication of bioherbicides. This supports the viability of exploiting natural compounds from thyme species to novel sustainable weed control. From this, some research questions arise:

1. What is the variability of bioactive compounds with a potential herbicidal activity within the genus *Thymus*?
2. What could be the most effective mode of application of such active compounds?
3. Could bioherbicidal efficacy be enhanced using nanoparticles?
4. What could be the modes of action of thyme bioactive compounds on target weed species?

In this context, this PhD research project performed a study, focusing on four *Thymus* sp. pl. common and widespread throughout the Mediterranean Basin, to identify allelopathic or bioactive compounds that may be the starting point for developing novel and innovative tools for weed control in sustainable farming systems.

The specific objectives of this research were:

1. Identification of some thyme species able to provide EOs with a potential herbicidal activity.
2. Chemical characterization of the thyme EOs and identification of bioactive compounds responsible for the potential herbicidal activity.
3. Identification of the most effective modes of application of the thyme EOs.
4. Determination of the modes of action of thyme EOs.
5. Formulation of a prototype of a nano-herbicide based on thyme EOs.

To achieve these objectives the following research activities were performed:

- Identification and harvesting: four *Thymus* sp. pl. species (*Thymus algeriensis* Boiss. and Reut., *T. ciliatus* Desf. subspecies *coloratus* (Boiss. et Reut.) Batt., *T. vulgaris* ecotype Fasano and *T. vulgaris* var Varico 3) were selected and harvested from Algeria and Italy.
- Extraction: EOs were extracted and chemically characterized to evaluate the possible allelopathic potential or identify bioactive compounds potentially able to control weeds.
- Bioassays: several *in vitro* and *in vivo* bioassays were performed to evaluate the potential herbicidal activity of the EOs and identify the most effective application methods.

- Nano-formulation: the EO that revealed the most important herbicidal potential was used to formulate a prototype of nano-herbicide.
- Cytological and biochemical assays: some plant defense mechanisms were evaluated to determine the modes of action of the nano-formulated bioherbicide.

It is worth mentioning that this PhD thesis is written in the form of a collection of scientific publications classified according to the progression of the research work and findings.

CHAPTER I

Valorization of Mediterranean Species of Thyme for the Formulation of Bio-Herbicides

agronomy

Article

Valorization of Mediterranean Species of Thyme for the Formulation of Bio-Herbicides

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Abstract: This study focused on the evaluation of the phytotoxic activity of four essential oils (EOs) from the Mediterranean species of *Thymus* sp. pl., namely *Thymus algeriensis* Boiss. et Reut., *T. ciliatus* Desf. subspecies *coloratus* (Boiss. et Reut.) Batt., *T. vulgaris* L. ecotype Fasano and *T. vulgaris* cultivar L. Varico 3, to identify new biomolecules with herbicide potential. The chemical characterization of EOs was performed by GC-MS. The evaluation of the phytotoxicity of the EOs was conducted under *in vitro* conditions, and the inhibition of germination and seedling growth of *Lolium perenne* L. and *Amaranthus retroflexus* L. were assessed. Five concentrations (100, 250, 500, 750 and 1000 µL/100 mL) were considered. Phytochemical analysis revealed a great diversity of compounds. *T. algeriensis* and *T. ciliatus* EOs were characterized by the absence of carvacrol and a low content of thymol in *T. ciliatus*. On the contrary, *T. vulgaris* ecotype Fasano and *T. vulgaris* cultivar Varico 3 were characterized by an important content of *p*-cymene, thymol and carvacrol. All the EOs expressed a potent phytotoxic activity against the tested species. The total inhibition of seed germination and seedling growth were recorded for the highest concentrations of all the EOs. *T. vulgaris* ecotype Fasano expressed the most effective activity.

Keywords: thyme; essential oils; GC-MS; phytotoxicity; germination; early growth

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1. Introduction

Today, food security, undermined by a fast-growing human population, is the foremost challenge for governments, policymakers and producers. To increase yields and improve food quality, crop production worldwide is consequently still based on massive chemical inputs. In 2021, 3.53 million tons of pesticides were used, and herbicide consumption reached 1.73 million tons, representing almost half of the total pesticide use [1]. Indeed, weeds are the most damaging category for crop production. They compete for resources such as light, water and nutrients and act as a reservoir of plant pathogens, pests and disease vectors. Therefore, they are considered a major abiotic and biotic cause of yield losses and crop quality depreciation [2–4].

Although several control methods are available, including physical, mechanical and biological approaches, the most common is still the chemical approach, based on the use of herbicides [5]. The overuse of these herbicides has caused negative impacts on human health and the environment [6–8]. Moreover, synthetic chemicals affect both soil organic matter and microbial community composition by decreasing soil bacterial diversity and abundance, especially for rapidly growing species because they can be immobilized in the soil by adsorption or binding to colloids [9,10].

Furthermore, the continuous use of the same molecules, combined with transgenic crops tolerant to herbicides, has contributed to developing increasing herbicidal resistances in many weeds and farmland biodiversity depletion [6,7]. In 2024, 272 herbicide-resistant weed species had been reported in 100 different crops, affecting the efficacy of 168 different herbicides and 21 of the 31 known modes of action (MOAs) [11].

To overcome this issue, several studies have explored new sources of active molecules, which can be used in weed control. These studies have demonstrated that plants, through the release of volatile organic compounds, can alter the physiological processes of neighboring species and affect plant community composition [12]. In this context, plant-based products with herbicidal activity, such as essential oils (EOs), could be an alternative to chemical herbicides due to their allelopathic properties and easy degradation in the environment [8,13,14]. Among bio-herbicides, EOs could be promising candidates due to their high content of monoterpenes, sesquiterpenes and diterpenes, which have important phytotoxic effects, volatility and biodegradability [15].

Within the *Lamiaceae* family, several genera showed interesting potential in terms of molecules with biocidal action. Among these genera, *Thymus* is certainly one of the most important with more than 250 taxa, for which the effectiveness of their EOs and terpenes have been demonstrated to have biological activity, especially antimicrobial properties. Understanding the relationship between the structures of these compounds and their various biological activities is crucial. In an agro-ecological context, it is of great interest to study the potential bioherbicidal activity of certain *Thymus* species to analyze and valorize their biodiversity as an alternative to chemical herbicides with a view to sustainable agriculture. Some thyme species have already been considered for their herbicidal properties in previous research. However, few aspects have been studied, and none of the previous studies have performed large-scale screening [16,17]. Therefore, this genus deserves renewed consideration and further investigations.

Based on the important chemotype variability of *Thymus* species detected in different environments in Algeria and Italy, four species were identified as deserving further attention: two Algerian endemic species, *T. algeriensis* Boiss. et Reut., *T. ciliatus* Desf. subsp. *coloratus* (Boiss. et Reut.) Batt.; an Italian landrace, *T. vulgaris* L. ecotype Fasano; and a Swiss commercial hybrid, *T. vulgaris* L. cultivar Varico 3, cultivated in Italy. In this context, our study aims to perform a preliminary analysis of several thyme species to investigate potential allelopathic activity that could be exploited for sustainable management of weeds. The experiments were conducted under in vitro conditions using two common weed test species.

2. Materials and Methods

2.1. Plant Material and EOs Extraction and Characterization

Aerial part of plant samples of *Thymus* species, namely *T. algeriensis* Boiss. et Reut.; *T. ciliatus* Desf. subsp. *coloratus* (Boiss. et Reut.) Batt.; *T. vulgaris* L. cultivar Varico 3 and *T. vulgaris* L. ecotype Fasano, were collected at the full flowering stage during May and June 2022 (Table 1). *T. algeriensis* Boiss. et Reut. and *T. ciliatus* Desf. subsp. *coloratus* (Boiss. et Reut.) Batt were identified in the Hamma Botanical Garden (Algiers, Algeria) based on the Quezel and Santa (1963) classification. Botanical vouchers were deposited at the herbarium of the garden. *T. vulgaris* L. ecotype Fasano was identified in the Botanical Garden Museum University of Bari Aldo Moro (Italy) and maintained ex situ (field collection) at the experimental farm "Enrico Pantanelli" of the University of Bari A. Moro, located in Policoro (southern Italy, 40°10′20″ N, 16°39′04″). Furthermore, the *T. vulgaris* cultivar Varico 3 is a hybrid that was obtained in 2000 by crossing two accessions from the Agroscope Changins-Wädenswil Research Station (Agroscope ACW—Switzerland) breeding material.

Dried samples were submitted to hydrodistillation for three hours, using a Clevenger-type apparatus, according to the European Pharmacopeia 2022 (https://www.edqm.eu/en/european-pharmacopoeia-ph.-eur.-11th-edition#-, accessed on 24 May 2022). The essential oils (EOs) obtained were yielded and stored at 4 °C until their use.

Table 1. Data on the plant species used in the study.

Species	Habitat	Code	Collecting Area	Geographical Localization	Altitude
T. algeriensis Boiss. et Reut.	Endemic wild species	T1	Chrea National Park, Blida (Algeria)	N 36°27′08.5″ E 002°54′47.2″	1465 m
T. ciliatus Desf. subsp. *coloratus* (Boiss. et Reut.) Batt.	Endemic wild species	T2	Hammam Melouane-Chrea National Park, Blida (Algeria)	N 36°28′12″ E 003°00′36″	211 m
T. vulgaris L. cultivar Varico 3	Cultivated hybrid	T3	Policoro, Basilicata (Italy)	40°10′28″ N 16°39′26″ E	15 m
T. vulgaris L. ecotype Fasano	Cultivated local species	T4			

Samples of the *Thymus* EOs were analyzed with a trace GC-FID Ultra Thermo Finnigan gas chromatograph equipped with an Agilent DB-5 (J&W Scientific, Milan, Italy) fused silica capillary column (30 m × 0.25 mm; 0.25 µm film thickness). Adopted analytical conditions were as follows: detector temperature was 300 °C; the column temperature was programmed from 60 °C (5 min isothermal) to 280 °C (30 min isothermal) at 4 °C/min. Hydrogen was the carrier gas (35 kP; 2.0 mL/min). Data were processed using a Chrom-Card 32-bit version 2.0 computing software. Analyses were run in the cold on-column mode.

GC-MS analyses were performed with a Hewlett Packard 6890 (MSD)-5973 (GC) GC-MS System interfaced with an HP Chemstation (Agilent, Scientific Instruments, Milan, Italy). The following analytical parameters were used: column oven program of 60 °C (5 min iso-thermal) to 240 °C (15 min isothermal) at 3 °C/min; injector, 280 °C. Helium was the carrier gas (flow rate, 1 mL·min^{-1}). Chromatographic separation was performed with an HP-5 MS capillary column (30 m × 0.25 mm; 0.25 µm film thickness). MS operating conditions were as follows: ion source, 70 eV; ion source temperature, 200 °C; mass spectra acquisition, over 40–800 amu range at 1 scan·s^{-1}. The ion source was operating in electron impact mode. Samples (1 µL) were injected using the splitless sampling technique.

The chemical composition of the analyzed EOs was achieved by a comparison of GC retention times of their constituents with authentic reference compounds in combinations with the Kovats index (KI) and by means of reference mass spectra from standard compounds and/or from library files [18,19].

KI values were calculated using an n-alkane series (C6–C32) under the same GC conditions as that for the samples. The relative amounts of individual components of the oil were expressed as percent peak area relative to the total peak area from the GC-FID analysis of the whole extracts without the use of correction factors. A linear proportion between the areas was used, assuming an equal response factor for all detected compounds.

2.2. Phytotoxicity of the EOs against Target Weeds

Two weed species, *Amaranthus retroflexus* L. and *Lolium perenne* L., were used to test in vitro the phytotoxic effect of the EOs on germination and early growth conditions. Seeds were purchased from Weberseeds Company and stored at 4 °C for use during the experiment.

Seeds were surface-sterilized with sodium hypochlorite (NaClO, 2%—*v*/*v*) for 2 min and rinsed three times with sterile distilled water [20]. The viability of the seeds was assessed by the germination test of 100 seeds in Petri dishes fitted with two layers of Whatman filter paper wetted with distilled water. Petri dishes were sealed immediately with parafilm and placed in a controlled growth chamber (24 ± 1 °C with adjusted light conditions of 16/8 h light/dark cycle).

Subsequently, the phytotoxicity of *Thymus* EOs against the weed test species was estimated in dose–response laboratory bioassays on seed germination and seedling early growth. Suspensions of T1, T2, T3 and T4 were prepared using 2% of Tween® 20 (Sigma-Aldrich, Milan, Italy) as an emulsifying agent, according to Abd-El Gawad et al. [21]. Briefly, five concentrations (100, 250, 500, 750 and 1000 µL/100 mL) of EOs were prepared by diluting the EOs in 2% of Tween 20, and then the volumes were adjusted to 100 mL with sterilized distilled water. A total of 2% of Tween® 20 solution was considered as negative control, while two pelargonic acid-based bio-herbicides (Finalsan Ultima 18.9% *w*/*w* pelargonic acid and Vithal 51.9% *w*/*w* pelargonic acid) were considered as positive controls. The concentrations of the positive controls were prepared as indicated on the labels of the products (1 L of Finalsan Ultima per 5 L of water and 2.2 L of Vithal per 5 L of water).

2.2.1. Germination Test

Ten seeds of *A. retroflexus* and *L. perenne* were placed in three Petri dishes for each EO and concentration, previously fitted with two layers of Whatman filter paper wetted with 3 mL of each treatment. Petri dishes were immediately sealed with parafilm to prevent loss of moisture and oil volatilization and placed in a controlled growth chamber at 24 ± 1 °C with adjusted light conditions of 16/8 h light/dark cycle and light: LEDs with a broad spectrum between 400 and 700 nm, a white/pink ratio of 2:1 and a light intensity of 50 µm s^{-1} m^{-2}. Trials were conducted in a completely randomized design in triplicate. After 7 days, the number of germinated seeds was counted. Seeds showing radicles of more than 2 mm were considered germinated [22,23]. The results are expressed in a percentage of germinated seeds, determined as follows:

$$GP\ (\%) = (n)/(N) \times 100$$

where GP: Germination percent; n: Number of germinated seeds at final count; N: Total number of seeds [24].

2.2.2. Seedling Early Growth Test

Seeds of both target species were germinated in three Petri dishes (150 mm in diameter) for each EO and concentration. All the Petri dishes were fitted with two layers of filter paper wetted with distilled water and placed in a controlled growth chamber at the seeds' optimal growth conditions for four days. Afterward, ten *A. retroflexus* and *L. perenne* seedlings were placed in 90 mm Petri dishes previously fitted with two layers of Whatman filter paper wetted with 3 mL of EO suspension at different concentrations. The Petri dishes were immediately sealed with parafilm to prevent loss of moisture and oil volatilization and placed in a controlled growth chamber at 24 ± 1 °C with adjusted light conditions of 16/8 h light/dark cycle and light: LEDs with a broad spectrum between 400 and 700 nm, a white/pink ratio of 2:1 and a light intensity of 50 µm s^{-1} m^{-2}. Trials were conducted in a completely randomized design in triplicate. After 7 days, shoot and radical lengths were measured using a ruler and caliper.

2.3. Statistical Analysis

Data obtained were statistically analyzed using Minitab® version 19.2020.1 (Minitab software, State College, PA, USA). Analysis of the variance (one-way ANOVA) of the seed germination percentage and root and shoot growth was performed to assess the phytotoxicity of the treatments. Differences among means was run using Tukey's test. Statistical significance was accepted when the probability of the result, assuming the null hypothesis, (p) was less than 0.05 ($p < 0.05$) [25].

3. Results and Discussion

3.1. Chemical Characterization of the EOs

The hydrodistillation of the aerial part of the samples resulted in different essential oil (EO) yields of the studied *Thymus* species, which ranged from 0.43% to 1.10% (*v*/*w*). The highest yield was observed in *T. ciliatus* subsp. *coloratus* (1.05%) and *T. vulgaris* cv Varico 3 (1.10%), while the lowest ones were found in *T. algeriensis* (0.43%) and *T. vulgaris* ecotype Fasano (0.60%) samples.

Gas chromatography analysis allowed for the identification of 43 components, representing 91.2–98.9% of the oils, with the remaining components present only in trace amounts (<0.1%). The percentage of the composition and indexes of the components are listed in Table 2. Based on the dominant compound of each EO, three different types were identified: α-pinene for *T. algeriensis*, linalool for *T. ciliatus* subsp. *coloratus* and *p*-cymene for *T. vulgaris* cv Varico 3 and *T. vulgaris* ecotype Fasano. The chemical analysis of the *T. algeriensis* EO revealed the dominance mainly of α-pinene (19.73%), α-terpenyl acetate (13.21%), borneol (11.31%), camphene (8.64%), isobornyl acetate (5.28%), β-pinene (4.37%), camphor (4.32%) and linalool (3.09%), while the *T. ciliatus* EO was characterized by an important content of linalool (93.06%), β-cedrene (1.98%) and limonene (1.36%). The chemical analysis of *T. algeriensis* (T1) and *T. ciliatus* subsp. *coloratus* (T2) EOs revealed the absence of carvacrol in both samples and a low content of thymol in *T. ciliatus* (0.55%).

Table 2. Chemical composition of the studied *Thymus* EOs.

N°	Compound	KI [1]	KI [2]	T1	T2	T3	T4
01	santolina triene	908	910	0.36	--	--	--
02	trycyclene	926	924	0.47	--	--	--
03	α-thujene	926	924	--	0.20	0.48	0.66
04	α-pinene	936	940	19.73	0.21	1.03	1.15
05	camphene	954	950	8.64	0.29	0.88	0.93
06	verbenene	967	968	0.34	--	0.05	--
07	sabinene	975	975	0.87	0.05	--	--
08	β-pinene	979	980	4.37	0.14	0.21	--
09	myrcene	991	992	2.77	0.36	0.52	1.35
10	α-terpinene	1017	1012	0.25	0.05	0.13	0.24
11	*p*-cymene	1022	1021	0.30	0.13	35.63	23.85
12	limonene	1029	1026	1.95	1.36	0.79	1.33
13	1,8 cineole	1031	1028	2.99	0.16	3.53	3.30
14	β(E)-ocymene	1050	1047	1.17	0.07	--	--
15	γ-terpinene	1059	1058	0.54	0.98	2.35	10.36
16	*cis*-sabinene hydrate	1070	1067	0.45	0.10	--	--
17	terpinolene	1088	1088	0.47	--	0.48	0.17
18	linalool	1096	1103	3.09	93.06	2.57	2.54
19	1-octen-3γlacetate	1110	1116	0.43	--	--	--
20	α-campholenal	1125	1125	0.99	--	--	--
21	camphor	1143	1142	4.32	0.38	1.66	1.20
22	trans-verbenol	1144	1145	2.61	--	--	--
23	pinocarvone	1162	1158	0.56	--	--	--
24	borneol	1165	1166	11.31	--	1.47	1.22
25	ρ-mentha-1,5 dien-8-ol	1170	1174	1.72	--	1.76	1.65
26	terpin-4-ol	1177	1182	0.76	--	--	--
27	α-terpineol	1189	1187	0.96	--	--	--
28	myrtenal	1193	1190	0.90	--	--	--
29	verbenone	1204	1215	0.73	--	--	--
30	isobonyl formate	1233	1243	0.33	--	--	--
31	thymol methyl ester	1235	1234	--	--	1.97	1.48
32	linalool acetate	1257	1262	3.96	--	--	--
33	isobornyl acetate	1285	1289	5.28	--	--	--
34	thymol	1290	1293	--	0.55	20.35	21.77
35	carvacrol	1298	1300	--	--	11.76	18.15
36	trans-carvyl acetate	1328	1337	0.11	--	--	--
37	α-terpenyl acetate	1350	1353	13.21	--	--	--
38	α-copaene	1376	1370	0.49	--	--	--
39	β-bourbonene	1384	1376	0.22	--	--	--
40	β-cedrene	1418	1404	0.51	1.98	7.69	6.56
41	germacrene	1480	1475	1.58	--	--	--
42	Δ-cadinene	1524	1530	0.26	--	--	--
43	caryophyllene oxide	1581	1573	--	--	5.57	2.09
Monoterpene hydrocarbons				42.23	3.77	42.85	40.04
Oxygen-containing monoterpenes				54.71	94.18	43.89	51.31
Sesquiterpene hydrocarbons				3.06	1.98	7.69	6.56
Oxygen-containing sesquiterpenes				-	-	5.57	2.09
Others				-	-	-	-

KI: Kovats index; [1]: the literature; [2]: calculated.

Overall, these results are in agreement with other studies on *Thymus* species. Similar results for *T. algeriensis* were reported by [26–28]. They pointed out that the phytochemical profiles of *T. algeriensis* harvested from Chrea National Park at the same altitude and Medea (north-central Algeria) were characterized by the predominance of terpinyl acetate (18.0%), nerolidol (12.6%), geranyl acetate (16.4%), α-pinene (11.1–27.1%), borneol (9.0%), bornyl acetate (7.7%) and in some samples, the absence of thymol. In contrast, [26] observed that a sample collected in Chrea National Park at 800 m altitude was characterized by thymol as the predominant component. Great variability was also found in *T. algeriensis* populations from the central-northern, central-southern and eastern regions of the country [29–32]. These results confirm the important role that environmental conditions play in the synthesis of different essential oil compounds.

Concerning *T. ciliatus* subsp. *coloratus*, our results indicated that the chemical profile of the EOs of the species growing in Hammam Melouane differed significantly from what has been reported in the literature. Indeed, our findings identified linalool as the major compound (93.06%) for this species for the first time. Generally, the North Algerian *T. ciliatus* EO is characterized by the predominance of thymol, carvacrol, *p*-cymene and γ-terpinene [33–35].

T. vulgaris cv Varico 3 and *T. vulgaris* ecotype Fasano (T3 and T4) have similar chemical compositions, characterized by *p*-cymene (35.63% and 23.85%, respectively), thymol (20.35% and 21.77%, respectively), carvacrol (11.76% and 18.15%, respectively), α-cedrene (7.69% and 6.56%, respectively), caryophyllene oxide (5.57 and 2.09%, respectively), γ-terpinene (2.65% and 10.36%, respectively) and 1,8 cineol (2.35% and 3.30%, respectively) as major components.

T. vulgaris is the most studied *Thymus* species in terms of phytochemistry. Since 2000, more than one hundred articles have been published, but as far as we know and according to the literature, few studies have focused on the *T. vulgaris* cultivar Varico 3 and none on *T. vulgaris* ecotype Fasano. Regarding the *T. vulgaris* cultivar Varico 3, [36] obtained different results than ours. In their study on the chemical composition of the volatile oil and genetic fingerprint of ten *T. vulgaris* clones, they found that *T. vulgaris* Varico 3, cultivated in Switzerland, was rich in thymol (75.44%), with *p*-cymene as the second most abundant compound (8.14%). Previously, [37] revealed that thymol is the main compound (60.2%), followed by *p*-cymene (19.9%) and carvacrol (10.3%) for this species cultivated in the Czech Republic. Generally, the EOs of *T. vulgaris* can have different chemotypes depending on the main compounds. Our results showed that the main compounds of both EOs are *p*-cymene, thymol and carvacrol, suggesting that both belong to the *p*-cymene chemotype. These results are in accordance with those found by [38] in their cluster analysis on the compositions of 85 *T. vulgaris* EOs. In addition to the already-established seven chemotypes (thymol, carvacrol, linalool, geraniol, thujanol-4, terpineol and 1,8-cineole) [39], they recorded *p*-cymene/thymol as the second most common chemotype represented by 18 samples, even though the most dominant chemotype reported by the literature remains the "thymol chemotype".

It is important to note that, apart from the main component that defines the chemotype of the species, some compounds are predictable, as they are metabolically related. For thymol and carvacrol chemotypes, *p*-cymene and γ-terpinene are expected to be present in considerable amounts, as they are their precursors [40]. Indeed, the metabolic pathway for the formation of thymol and carvacrol starts with the autoxidation of γ-terpinene to *p*-cymene, followed by hydroxylation to thymol [41].

The great variability and diversity of compounds observed in the chemical composition of *Thymus* species EOs could be attributed to several factors, such as response mechanisms to abiotic variations. In essence, environmental conditions can affect biosynthetic pathways [29,35,42,43].

3.2. Phytotoxicity of the EOs against Target Weeds

3.2.1. Phytotoxic Effect on Seed Germination

The phytotoxic effect of the different EOs extracted from aerial parts of thyme species on seed germination of *L. perenne* and *A. retroflexus* is summarized in Figure 1. All the treatments prevented seed germination of both species where their influence varied significantly depending on the EO and concentration. Seven days after treatment, T1 and T4 completely inhibited seed germination at 500, 750 and 1000 µL in a similar way to the positive controls. T1 and T4 also reduced seed germination at 100 µL; however, this reduction was not significant compared to the negative control. T3 completely suppressed seed germination at 750 and 1000 µL and reduced it considerably at 500 µL (83% less than negative control). In contrast, at 100 and 250 µL, the seed germination percentage was close to that of the negative control. T2 showed the least significant effect, suppressing seed germination only at 1000 µL. Germination percentages were almost similar to the negative control at 100, 250, and 500 µL. T4 exerted the most potent activity against the treated species (Figure 1a).

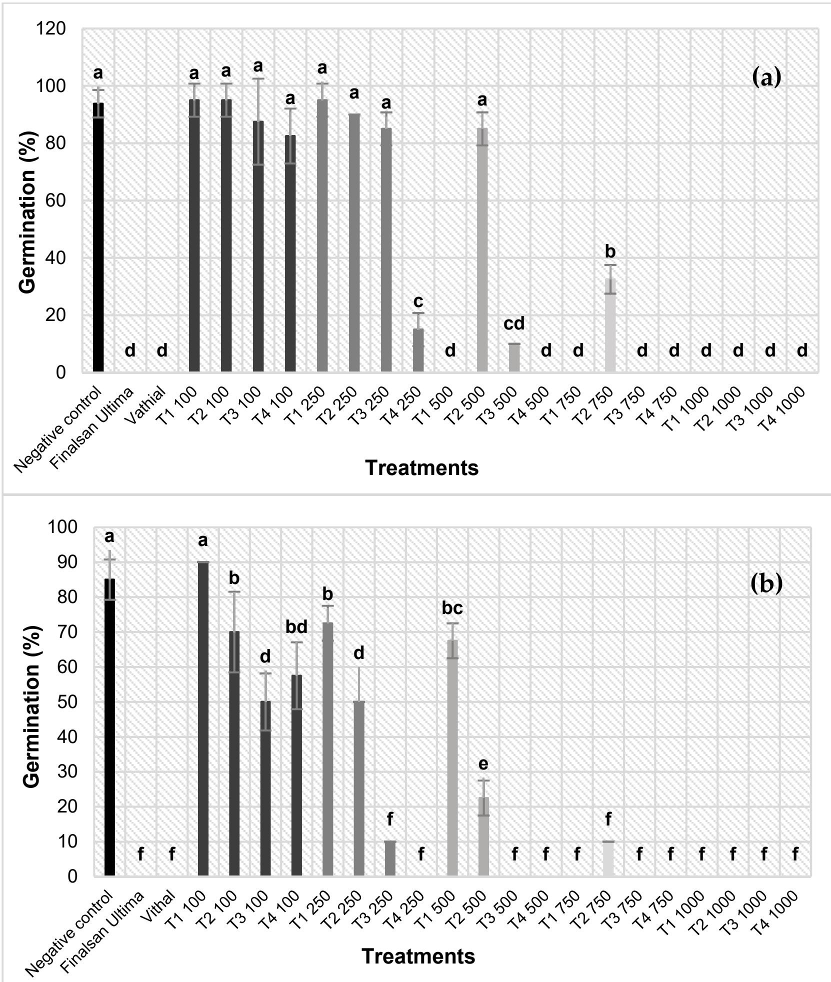

Figure 1. Effect of the different treatments and doses on seed germination of *L. perenne* (**a**) and *A. retrofelxus* (**b**) after 7 days of exposure to a 100–1000 µL/100 mL range of concentrations of the four tested EOs. T1: *T. algeriensis*, T2: *T. ciliatus*, T3: *T. vulgaris* cultivar Varico 3, T4: *T. vulgaris* ecotype Fasano. Data are means of three replicates. At each dose, data followed by different letters are significantly different ($p \leq 0.05$, Tukey's test).

A similar pattern was observed for *A. retroflexus*. T4 completely inhibited seed germination at 250, 500, 750 and 1000 µL and reduced it by almost half at 100 µL. T3 showed strong effectiveness at 500, 750 and 1000 µL, extremely reduced seed germination at 250 µL and reduced it by half at 100 µL. T1 completely inhibited seed germination at 750 and 1000 µL and reduced it by almost 20% and 13% at 250 and 500 µL, respectively. T2 was the least effective treatment, inhibiting seed germination only at the highest concentration (1000 µL) and slowing it down at the other concentrations. As with *L. perenne*, it was noticeable that T4 exerted the strongest phytotoxic effect against *A. retroflexus* by totally inhibiting seed germination at four concentrations (Figure 1b).

Overall, all the EOs exhibited significant phytotoxic activity against the tested weed species. T4 was the most phytotoxic treatment, while T2 was the least effective.

3.2.2. Phytotoxic Effect on Seedlings' Early Growth

The allelopathic effect of the EOs extracted from aerial parts of thyme species on *L. perenne* L. and *A. retroflexus* L. root and shoot growth is summarized in Figure 2. The results demonstrated a strong inhibitory effect of the EOs on the growth of the target plants. Root and shoot elongation was strongly inhibited in a dose-dependent manner. For *L. perenne*, T1, T2, T3 and T4 showed potent toxicity on root growth at all four concentrations (250, 500, 750 and 1000 µL), with T4 having a particularly strong effect, reducing root elongation by about 30 mm and 45 mm compared to the mean root elongation of the other treatments and the negative control, respectively, even at 100 µL (Figure 2a).

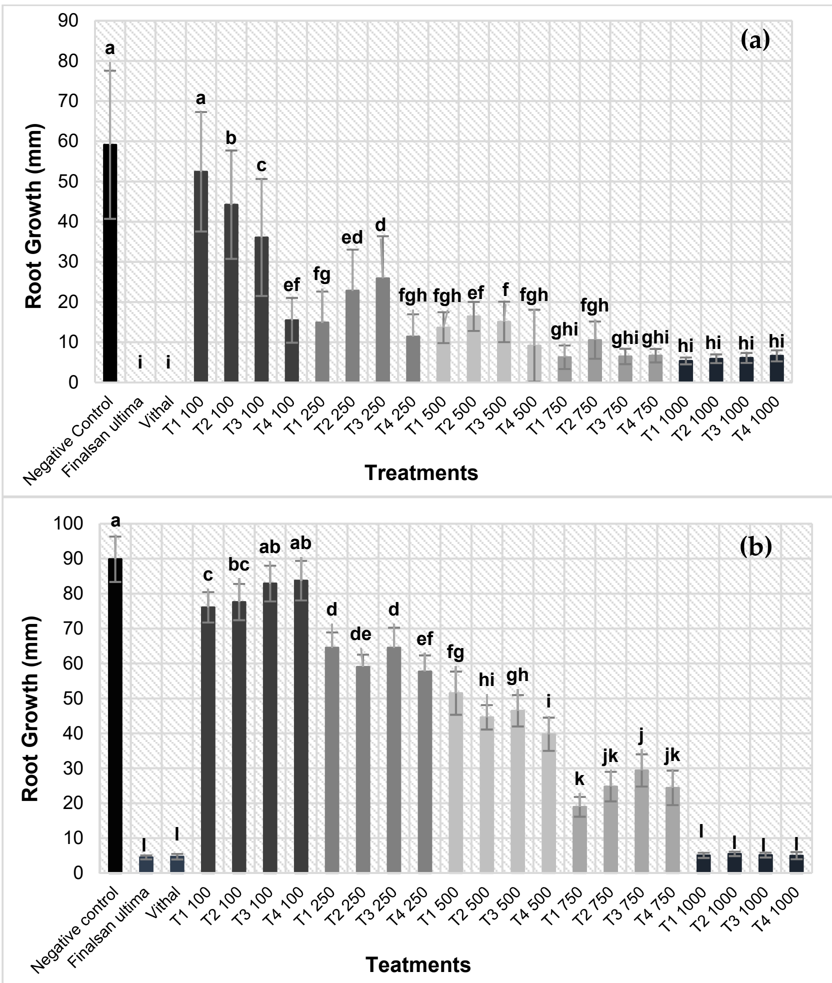

Figure 2. Effect of the different treatments and doses on root elongation of *L. perenne* (**a**) and *A. retrofelxus* (**b**) seedlings after 7 days of exposure to a 100–1000 µL/100 mL range of concentrations of the four tested EOs and active compounds. T1: *T. algeriensis*, T2: *T. ciliatus*, T3: *T. vulgaris* cultivar Varico 3, T4: *T. vulgaris* ecotype Fasano. Data are means of three replicates. At each dose, data followed by different letters are significantly different ($p \leq 0.05$, Tukey's test).

The same effect was recorded for *A. retroflexus*. All the EOs negatively affected root growth. They significantly reduced it at 750 and 1000 µL and decreased it by half at 250 and 500 µL. At 100 µL, no major effect was recorded. At the lower concentration, the effect was almost the same as that of the negative controls (Figure 2b).

For *L. perenne*, shoot growth responded to the application of EOs in a quite similar manner to root growth. T4 completely inhibited shoot growth at 750 and 1000 µL comparable to the positive controls and greatly reduced it at 500, 250 and 100 µL. In particular, the reduction in shoot growth was 25 mm less than the average of the other treatments at 250 µL and 30 mm for 100 µL. T1 also inhibited shoot elongation at 750 and 1000 µL and considerably reduced it at 500 and 250 µL, while at 100 µL, the effect was almost similar to that of the negative control. T3 prevented shoot growth only at 1000 µL but significantly slowed it down at 250, 500 and 750 µL. T2 was the least effective treatment compared to the other three. It did not prevent shoot growth; however, it significantly decreased it at 500, 750 and 1000 µL in comparison to the negative control with decreases of about 36, 39 and 47 mm, respectively (Figure 3).

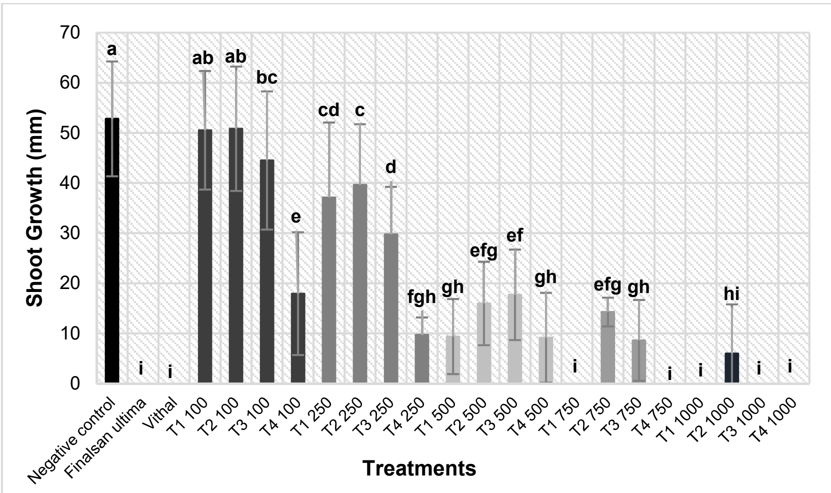

Figure 3. Effect of the different treatments and doses on shoot elongation of *L. perenne* seedlings after 7 days of exposure to a 100–1000 µL/100 mL range of concentrations of the four tested EOs and active compounds. T1: *T. algeriensis*, T2: *T. ciliatus*, T3: *T. vulgaris* cultivar Varico 3, T4: *T. vulgaris* ecotype Fasano. Data are means of three replicates. At each dose, data followed by different letters are significantly different ($p \leq 0.05$, Tukey's test).

It is noteworthy that all the EO-based treatments completely prevented the shoot growth of *A. retroflexus*, and T4 was the most effective in inhibiting or slowing down root and shoot growth of both target species in a dose-dependent manner.

The results obtained from the in vitro trials confirmed the phytotoxic effect of the studied *Thymus* EOs. All treatments inhibited seed germination and seedlings' growth of the target weed species at higher concentrations, with T4 being effective even at lower concentrations. These results are highly consistent with those reported in the literature. Ref. [44] established that the *T. vulgaris* EO possessed potent activity against seven tested plants, negatively influencing their root growth and completely inhibiting their seed germination. Ref. [45] also revealed that the EO of *T. algeriensis* inhibited both shoot and root growth of *Medicago sativa* L. and of *Triticum aestivum* L. seedlings. To the best of our knowledge, no studies have been reported on the phytotoxic effect of *T. vulgaris* cultivar Varico 3, *T. vulgaris* ecotype Fasano and *T. ciliatus* subsp. *coloratus*. Nevertheless, the allelopathic activity of various *Thymus* sp. pl. EOs and their constituents has been reported to affect seed germination and plant growth of several weed species, thereby influencing plant community composition and dynamics [46]. Studies on EOs of *T. pulegioides* L., *T. proximus* Serg., *T. vulgaris*, *T. kotschyanus* Boiss. & Hohen., *T. decussatus* Benth., *T. fontanesii* Boiss. et

Reut. *T. eigii* and *T. daenensis* Celak. revealed significant and strong phytotoxic potential against a wide range of weed species, such as *Amaranthus retroflexus* L., *Poa anuua* L., *Sinapis arvensis* L. and *Avena fatua* L. [16,17,43,47–50].

To explain this biological activity, research confirmed that this potential is due to the presence of terpenic compounds, most of which are volatile [51,52]. In fact, [21] established a structure–activity relationship between phytotoxicity and EO chemical composition based on chemometric analysis. Data analysis revealed that mono- and sesquiterpenes play major roles in the phytotoxicity of EOs, concluding that α- and β-pinene, 1,8 cineole, linalool and carvacrol are the most effective monoterpenes exhibiting an allelopathic effect on many plants. Additionally, caryophyllene, a sesquiterpene compound, and its metabolites, such as germacrene, spathulenol and hexahydrofarnesyl acetone, play an important role in the phytotoxic potential of EOs. This was also reported by [21], where monoterpenes like linalool and 1,8 cineole delayed weed germination time and reduced their development. This is in accordance with our results, where *T. algeriensis, T. vulgaris* cultivar Varico 3 and *T. vulgaris* ecotype Fasano, which are rich in α-pinene, *p*-cymene, thymol and carvacrol, respectively, exhibited the most significant activity.

In addition, it was assumed that the biological properties of EOs are generally induced mainly by their major components. However, recent research has demonstrated that compounds present in minor and trace amounts can also influence plant defense strategies and could act synergistically to enhance the biological potential of EOs [53]. *T. ciliatus* was the least effective treatment, even though it contained more than 90% of linalool. Furthermore, our EOs were generally rich in certain compounds, such as α-pinene, borneol, linalool, thymol, carvacrol and caryophyllene, but their chemical compositions were particularly different. *T. algeriensis* and *T. ciliatus* did not contain thymol and carvacrol, two compounds commonly well known for their strong allelopathic activity, or contained these only in trace amounts. This supports the view that there was a synergy between the major and minor compounds of the examined EOs that affected the germination and early growth of *L. perenne* and *A. retroflexus*.

4. Conclusions

The object of this study was to exploit plant sources to obtain active ingredients for use in crop protection, particularly for weed control. With this aim, a preliminary assessment of the phytotoxic potential of some Mediterranean *Thymus* sp. pl. EOs was performed, and the presence of bioactive compounds, potentially useful for weed management, was evaluated. The chemical analysis demonstrated the high variability of secondary metabolites in *Thymus* sp. pl essential oils (EOs). Forty-three chemical compounds were identified. The Algerian species were characterized by the absence of carvacrol and traces of thymol in *T. ciliatus* subsp. *coloratus* and the predominance of linalool, α and β-pinene, α-terpenyl acetate, borneol and camphene, while *p*-cymene, thymol, carvacrol and γ-terpinene were found as the main constituents of *T. vulgaris* species. Biologically, all the EOs demonstrated a significant phytotoxic effect on *L. perenne* and *A. retroflexus*. Thyme EOs strongly inhibited the seed germination and seedling growth of both weed species. *T. algeriensis* Boiss. et Reut., *T. vulgaris* cultivar Varico 3 and *T. vulgaris* ecotype Fasano completely inhibited the seed germination of both test species at 500, 750 and 1000 µL/100 mL, while *T. ciliatus* subsp. *coloratus* considerably reduced it at 500 and 750 µL/100 mL and completely suppressed it at 1000 µL/100 mL. The seedling growth of *L. perenne* and *A. retroflexus* was drastically slowed down under the effect of thyme EOs in a dose-dependent manner; at 1000 µL/100 mL, all the EOs almost inhibited the seedling growth. Overall, *T. vulgaris* ecotype Fasano exhibited the most potent phytotoxic activity, while *T. ciliatus* subsp. *coloratus* was the least effective.

This initial screening of species of genus *Thymus* endowed with biologically active compounds represents the first stage in a process of introducing into cultivation certain species that are currently under-utilized but have the potential to become industrial crops. The high cost of the natural product could thus be reduced in favor of a more efficient and less costly method of obtaining the raw material. All of this is perfectly in line with sustainability goals (Agenda 2030), including the replacement of synthetic compounds with plant-based compounds in agriculture, as in other contexts.

Further studies and applicative investigations on the effectiveness, selectivity, modes of action and application of thyme EOs under in vivo conditions, as well as their effects on crops and soil, are necessary to develop ongoing, tailored strategies for more sustainable weed control.

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Conflicts of Interest: The authors declare no conflicts of interest.

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CHAPTER II

Assessment of the Bioherbicidal Potential of *Thymus* sp. pl. Essential Oils in Weed Control

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Assessment of the bioherbicidal potential of *Thymus* sp. pl. essential oils in weed control

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ABSTRACT

The bioherbicidal potential of four essential oils (EOs) from Mediterranean species of *Thymus* sp. pl.: *Thymus algeriensis* Boiss. et Reut., *T. ciliatus* Desf. subspecies *coloratus* (Boiss. et Reut.) Batt., *T. vulgaris* L., ecotype Fasano and *T. vulgaris* L. var. Varico was investigated to identify new sources of biomolecules. *In vivo* assays were carried out to assess the effect of the EOs on pre and post-emergence of two weed species: *Lolium perenne* L. and *Amaranthus retroflexus* L. Four concentrations of EO emulsions (5, 7.5, 10 and 20 μL/mL) were applied. All essential oils (EOs) exhibited the strongest herbicidal activity at the highest tested concentration (20 μL/mL), completely inhibiting seed germination (100 %) in the Vithal control, whereas lower concentrations were less effective. On the contrary, for post-emergence experiments and compared to the positive control, *L. perenne* showed resistance to the EOs while *A. retroflexus* was highly sensitive, arguing a selectivity of the EOs. Additionally, cytological analysis were performed on the model plant *Arabidopsis thaliana* (L.) Heynh, for a better understanding of the structural modifications induced by the EOs. The root system of EO-treated *A. thaliana* seedlings was highly damaged.

1. Introduction

Chemical inputs, especially pesticides, are among the most applied agrochemicals for pest control. Nevertheless, the repeated use of the same synthetic products led to an increased risk of the emergence of pest resistance and ecological hazards, which raises a new problem for global environment preservation (Mostafalou and Abdollahi, 2017; Mubeen et al., 2023). This potential risk effect on global health, associated with the overuse of agrochemicals, has encouraged researchers to search for eco-friendly alternatives for pest control (Oliveira et al., 2017).

Among the long list of threats to crop production, weeds are the most damaging because they compete for resources such as light, water, and nutrients and act as reservoirs of pests and pathogens (Byron et al., 2019; Monteiro and Santos, 2022). In addition, in these last decades, weeds have developed resistance mechanisms to herbicides: target-site resistance, which involve gene mutations that affect herbicide protein targets, and non-target-site resistance, involving complex interactions of genes from large gene families responsible for reduced herbicide absorption, translocation, degradation, and sequestration. These resistance mechanisms can combine at the individual level, demonstrating the important adaptability of weeds to selection pressures (Gaines et al., 2020).

In that regard and in the perspective of promoting integrated weed management (IWM), research on bio-herbicides has greatly increased (Cordeau et al., 2016). The use of bioherbicides is presented as one of the most advantageous tools for IWM, demonstrating an increased target specificity, rapid degradation with maximum residue limits, and less restrictiveness (Olson, 2015; Cordeau et al., 2016). Thus, bioherbicides are developed, especially those of plant origin (De Souza Barros et al., 2021; Hasan et al., 2021). The search for potential bioherbicides is typically based on the chemical ecology approach, in other words, on the allelopathic potential of plants. Plants can produce phytotoxic metabolites, called allelochemicals, to compete with other species (Vurro et al., 2018; Palanivel et al., 2021); the mode of action of these secondary

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compounds seems to be similar to synthetic herbicides. In this perspective, allelochemicals can act by altering seed germination or interfering with seedlings' establishment (Hierro and Callaway, 2021). Given their ecological role in natural environments, these secondary metabolites could be considered promising molecules to be employed to produce new classes of plant-based herbicides (Araniti et al., 2015; Berestetskiy, 2023).

In this context, essential oils (EOs) are widely studied as potential candidates for new-generation and eco-friendly herbicides for weed management, due to their important phytotoxicity. EOs demonstrated their potential as promising bioherbicides, thanks to their ability to inhibit seed germination and seedling growth, causing oxidative stress and disrupting photosynthesis and respiration mechanisms (Pouresmaeil et al., 2020; Verdeguer et al., 2020a; Werrie et al., 2020). EOs are chemically composed of terpenes, which are derived from the universal C5 precursor isopentenyl di-phosphate (IPP) and its allylic isomer dimethylallyl diphosphate, which form a large diversity of compounds derived from the branched C5 skeleton of isoprene, including volatile monoterpenes (C10) and sesquiterpenes (C15) (Araniti et al., 2016). Trindade et al. (2018) explained that this diversity is generated by the modification of a common scaffold structure, to which diversity is added. Since the differential modification of common backbone structures can change and/or modify the biological activity, the new compounds can demonstrate a potential new biological activity (Kong et al., 2021; Casella et al., 2023; Li et al., 2023; Miloudi et al., 2024). In this sense, some of these secondary metabolites demonstrated a strong herbicidal potential against a large range of weed species and also microbial and insecticidal activities. Nevertheless, it is worthy to highlight that frequently the effects of these compounds, observed in experimental studies on seedlings, do not coincide with those obtained on adult plants of the same species or crops (Silva et al., 2016). Indeed, secondary metabolites are also able to act as priming molecules both in biotic and abiotic plant stress, inducing better plant performances and resistance to pests. This "biostimulant" effect can be expressed by an increase of phenolic content, antioxidant enzyme activities (SOD, POD, and CAT), a promotion of histone modifications of defense genes and trigger a multilayered immune system (Sellamuthu et al., 2013; Sukegawa et al., 2018; Rienth et al., 2019; Soudani et al., 2022).

The Mediterranean area is one of the richest hotspots of biodiversity in the world with 24.000 species. Almost 2000 plants have been recorded, among them *Thymus* genus (Vargas, 2020). For this genus, the biological effectiveness of its EOs has been demonstrated, especially thanks to their large chemotype diversity. Preliminary investigations showed that EOs for *Thymus* sp. pl. could have the potential for controlling weeds (Ghasemi et al., 2020; Saleh et al., 2020; Vaiciulyte et al., 2021; Zhou et al., 2021), nevertheless, to the best of our knowledge, few studies considered the herbicidal activity of thyme EOs and even fewer a large screening of this genus and their modes of action.

A large diversity of species belonging to this genus is present in different environments in Algeria and Italy, therefore this study focuses on the evaluation of the herbicidal activity of thyme EOs of two Algerian endemic species, harvested from natural environments, an Italian local variety and a Swiss commercial hybrid, cultivated in Italy.

2. Materials and methods

2.1. Plant material and EOs extraction and characterization

Aerial part of dried plant samples of four *Thymus* species: *T. algeriensis* Boiss. et Reut., *T. ciliatus* Desf. subsp. *coloratus* (Boiss. et Reut.) Batt., *T. vulgaris* L. cultivar Varico 3 and *T. vulgaris* L. ecotype Fasano, referred hereafter in the document, respectively, as T1, T2, T3 and T4, were collected at full flowering stage during May and June 2022 from the Chrea National Park and Hammam Melouane (Blida, Algeria) and Policoro (Basilicata, Italy) (Boukhalfa et al., 2024a). EOs were extracted by hydrodistillation and identified by gas chromatography coupled with mass spectrometry in a previous study. The yields of the EOs were 0.43, 0.60, 1.05 and 1.10 %, respectively, for *T. algeriensis*, *T. vulgaris* ecotype Fasano, *T. ciliatus* subsp. *coloratus* and *T. vulgaris* cv Varico 3 (Boukhalfa et al., 2024a). The chemical composition of the EOs is presented in Table 1. EOs were stored at 4°C until their use in the biological activity.

2.2. Bioherbicidal activity of the EOs against target weeds

Two weed species *Lolium perenne* L. and *Amaranthus retroflexus* L. were used to assess the herbicidal activity of the EOs on germination and early growth under *in vivo* conditions. These species were selected because of their availability, high germination rate, fast and uniform growth and belonging to the two main groups: monocots and dicots.

Table 1
Chemical composition of the studied *Thymus* EOs.

N°	Compound	KI[a]	KI[b]	T1	T2	T3	T4
01	santolina triene	908	910	0.36	–	–	–
02	trycyclene	926	924	0.47	–	–	–
03	α-thujene	926	924	–	0.20	0.48	0.66
04	α-pinene	936	940	19.73	0.21	1.03	1.15
05	camphene	954	950	8.64	0.29	0.88	0.93
06	verbenene	967	968	0.34	–	0.05	–
07	sabinene	975	975	0.87	0.05	–	–
08	β-pinene	979	980	4.37	0.14	0.21	–
09	myrcene	991	992	2.77	0.36	0.52	1.35
10	α-terpinene	1017	1012	0.25	0.05	0.13	0.24
11	*p*-cymene	1022	1021	0.30	0.13	35.63	23.85
12	limonene	1029	1026	1.95	1.36	0.79	1.33
13	1,8 cineole	1031	1028	2.99	0.16	3.53	3.30
14	β(*E*)- ocymene	1050	1047	1.17	0.07	–	–
15	γ-terpinene	1059	1058	0.54	0.98	2.35	10.36
16	*cis*-sabinene hydrate	1070	1067	0.45	0.10	–	–
17	terpinolene	1088	1088	0.47	–	0.48	0.17
18	linalool	1096	1103	3.09	93.06	2.57	2.54
19	1-octen−3γlacetate	1110	1116	0.43	–	–	–
20	α-campholenal	1125	1125	0.99	–	–	–
21	camphor	1143	1142	4.32	0.38	1.66	1.20
22	trans-verbenol	1144	1145	2.61	–	–	–
23	pinocarvone	1162	1158	0.56	–	–	–
24	borneol	1165	1166	11.31	–	1.47	1.22
25	ρ-mentha−1,5 dien−8-ol	1170	1174	1.72	–	1.76	1.65
26	terpin−4-ol	1177	1182	0.76	–	–	–
27	α-terpineol	1189	1187	0.96	–	–	–
28	myrtenal	1193	1190	0.90	–	–	–
29	verbenone	1204	1215	0.73	–	–	–
30	isobonyl formate	1233	1243	0.33	–	–	–
31	thymol methyl ester	1235	1234	–	–	1.97	1.48
32	linalool acetate	1257	1262	3.96	–	–	–
33	isobornyl acetate	1285	1289	5.28	–	–	–
34	thymol	1290	1293	–	0.55	20.35	21.77
35	carvacrol	1298	1300	–	–	11,76	18.15
36	trans-carvyl acetate	1328	1337	0.11	–	–	–
37	α-terpenyl acetate	1350	1353	13.21	–	–	–
38	α-copaene	1376	1370	0.49	–	–	–
39	β-bourbonene	1384	1376	0.22	–	–	–
40	β-cedrene	1418	1404	0.51	1.98	7.69	6.56
41	germacrene	1480	1475	1.58	–	–	–
42	Δ-cadinene	1524	1530	0.26	–	–	–
43	caryophyllene oxide	1581	1573	–	–	5.57	2.09
Monoterpene hydrocarbons				42.23	3.77	42.85	40.04
Oxygen-containing monoterpenes				54.71	94.18	43.89	51.31
Sesquiterpene hydrocarbons				3.06	1.98	7.69	6.56
Oxygen-containing sesquiterpenes				-	-	5.57	2.09
Others				-	-	-	-
Total identified				70.01	85.00	41.17	44.68

KI: Kovats index.
[a] : literature.
[b] : calculated.

Seeds were purchased from Weberseeds Company and stored at 4 °C for use during the experiments.

The herbicidal potential of thyme EOs against the test weed species was estimated in dose–response bioassays on seed germination (pre-emergence) and seedling early growth (post-emergence), under greenhouse conditions. Emulsions of T1, T2, T3 and T4 were prepared using 2 % Tween® 20 (Sigma- Aldrich, Milan, Italy) as an emulsifying agent, according to Abd-El Gawad et al. (2021). Four concentrations (5.0, 7.5, 10 and 20 μL/mL of 2 % of Tween® 20 water solution) were applied. The 2 % of Tween® 20 water solution and a pelargonic acid based bio-herbicide (Vithal, 51.9 % w/w pelargonic acid) were considered, respectively, as negative and positive controls. The concentration of the positive control was prepared as indicated in the label of the product.

Peat was used as substrate in these experiments with the purpose to evaluate the behavior of the test species in response to the treatments in controlled conditions. Trays of 21 × 16 cm were fitted with 2 cm drainage layer of perlite and 700 g of peat. All trays were irrigated and brought to their optimal water holding capacity and left to leach the excess of water.

2.2.1. Pre-emergence assay

Hundred (100) seeds of *L. perenne* L. and *A. retroflexus* L. were sown in each tray, then 10 mL of each treatment was spread using a micro-sprayer to guarantee its homogeneity. The number of germinated seeds was counted after fifteen (15) days and seed germination rate was calculated according to the following formula (Islam et al., 2014):

$$GP\ (\%) = (n)/(N) \times 100$$

Where: GP: germination percent; n: number of germinated seeds at final count; N: total number of seeds.

Trials were conducted in a complete randomized design with three replicates. The trays were placed in the greenhouse at 22–25°C and 50–60 % of humidity and were monitored daily for irrigation.

2.2.2. Post-emergence assay

For this test, pre-cultivation of the weed species was required. Seeds were allowed to germinate in nursery trays (72 holes) until the emergence of two true leaves. Then, hundred (100) seedlings of *L. perenne* and *A. retroflexus* were transplanted in trays already prepared as described previously. The seedlings received 10 mL of each treatment once they reached the phenological stage “three true leaves”, corresponding to 12–13 BBCH (Biologische Bundesanstalt, Bundessortenamt and Chemical industry) scale for *L. perenne* and “three to four true leaves”, corresponding to 13–14 BBCH scale for *A. retroflexus*. The EOs emulsions were applied using a micro-sprayer as contact treatments. The treatments were applied until the appearance of wilting symptoms on the plantlets.

The trays were placed in the greenhouse at 22–25°C and 50–60 % of humidity and were monitored daily for irrigation. All the treatments and controls were replicated three times in a complete randomized design.

At the end of the experiment, the number of surviving plantlets and fresh weight were determined. The fresh biomass was measured using a weighing scale on the same day.

2.3. Phytotoxic activity of EOs on Arabidopsis thaliana (L.) Heynh

To evaluate the physiological damage caused by the EOs on the target weed species, cytological assays on *Arabidopsis thaliana* were conducted. Additionally, the percentage of seed germination and root length of *A. thaliana* were also assessed. Seeds of *A. thaliana* (L.) Heynh Columbia ecotype (Col-0) were surface sterilized with ethanol (50 %) and NaOCl (0.5 %) for 3 min each and washed three times with sterilized distilled water, then stored in agar (0.1 %) at 4 °C for 72 h to favor vernalization. Meanwhile, Petri dishes (90 mm) with agar medium (0.8 % w/v) enriched with 0.44 % Murashige-Skoog growth medium and supplemented with 1 % sucrose (Díaz-Tielas et al., 2012; Verdeguer et al., 2020b) were prepared.

In order to determine the phytotoxic effect of the studied EOs on the growth of *A. thaliana*, emulsions were prepared with EOs at the concentration of 1000 μM using 0.1 % EtOH (v/v) as solvent and added to the growth medium. Control plates included 0.1 % EtOH. Afterward, twenty seeds were sown in each Petri dish under sterile conditions. All the Perti dishes were kept vertically in growth chamber at 22°C, with a photoperiod of 8 h light/16 h darkness, and a relative humidity of 55 % for 14 days (Verdeguer et al., 2020b).

Each treatment and control were prepared in triplicate. The percentage of seed germination and root growth were determined at the 7th and 14th day as previously described for the target weed species while the morphology of the seedlings was analyzed at the 14th day using a magnifier (Nikon SMZ 1500) and light microscope (Leica DMLB100, Milan, Italy). Root structure, morphology and abundance of root hairs of EOs-treated and control radicles of *A. thaliana* were visually evaluated using a magnifier (Nikon SMZ 1500) equipped with Nikon DXM 1200 Digital Camera (Tokyo, Japan). At the same time, Schiff’s staining method was used as histochemical indicator of membrane damage due to membrane peroxidation, while Evans blue staining method was followed for the determination of cell viability and root plasma membrane integrity (El Mahdi et al., 2020). Schiff’s reagent (Carlo Erba Reagents s. r.l.) was prepared and applied to three roots from each replicate in test tubes and left to react for 20 min. After this, the reagent was removed from the tubes and root samples were rinsed with a sulfite solution to retain the staining color (0.5 % w/v; K_2S2O_5 or Na_2S2O_5 in 0.05 M HCl). The solution of Evan blue (Carlo Erba reagents s.r.l.) (10 mg/mL) was placed in test tubes containing three roots for each replication for 15 min. After this, the stain was removed from the tubes and the roots were rinsed three times with distilled water. Afterward, three roots were taken from different replicates per treatment. The terminal apex of the roots was cut and photographed under a microscope (Leica DMLB100, Milan, Italy) at 200x magnification equipped with Nikon DXM 1200 Digital Camera (Tokyo, Japan).

2.4. Statistical analysis

Data obtained were statistically analyzed using Minitab® version 19.2020.1 (Minitab software, State College, Pennsylvania, USA). Analysis of the variance (one-way ANOVA) of the seed germination percentage, number of surviving plantlets and fresh biomass of the two target weed species as well as the seed germination percentage and root length of *A. thaliana* was performed to assess the herbicidal activity of the treatments. Difference among means was run using Tukey’s test. Statistical significance was accepted when the probability of the result assuming the null hypothesis (p) was less than 0.05 ($p < 0.05$) (Alonso et al., 2003; Shedden, 2015).

3. Results and discussion

3.1. Herbicidal activity of the EOs against target weeds

3.1.1. Pre-emergence assay

The herbicidal effect of the different EOs of thyme species on seed germination of *L. perenne* and *A. retroflexus* is presented in Fig. 1. All the treatments inhibited or reduced seed germination of both target weed species in a dose-response manner and depending on the EO. At the concentration of 20 μL/mL an effective herbicidal potential for *L. perenne* was expressed by all the tested EOs, inducing a total inhibition of the germination. At 10 μL/mL, no statistical differences were found between the EOs; all of them significantly inhibited seed germination, reducing it by over 90 % in comparison to the negative control. Similar results were obtained using the concentration of 7.5 μL/mL for T1, T2 and T4. T3 seemed less effective, even if, also at the lowest concentration (5.0 μL/mL) the reduction of seed germination was between 83 % (T1) and 77,5 % (T3) (Fig. 1a).

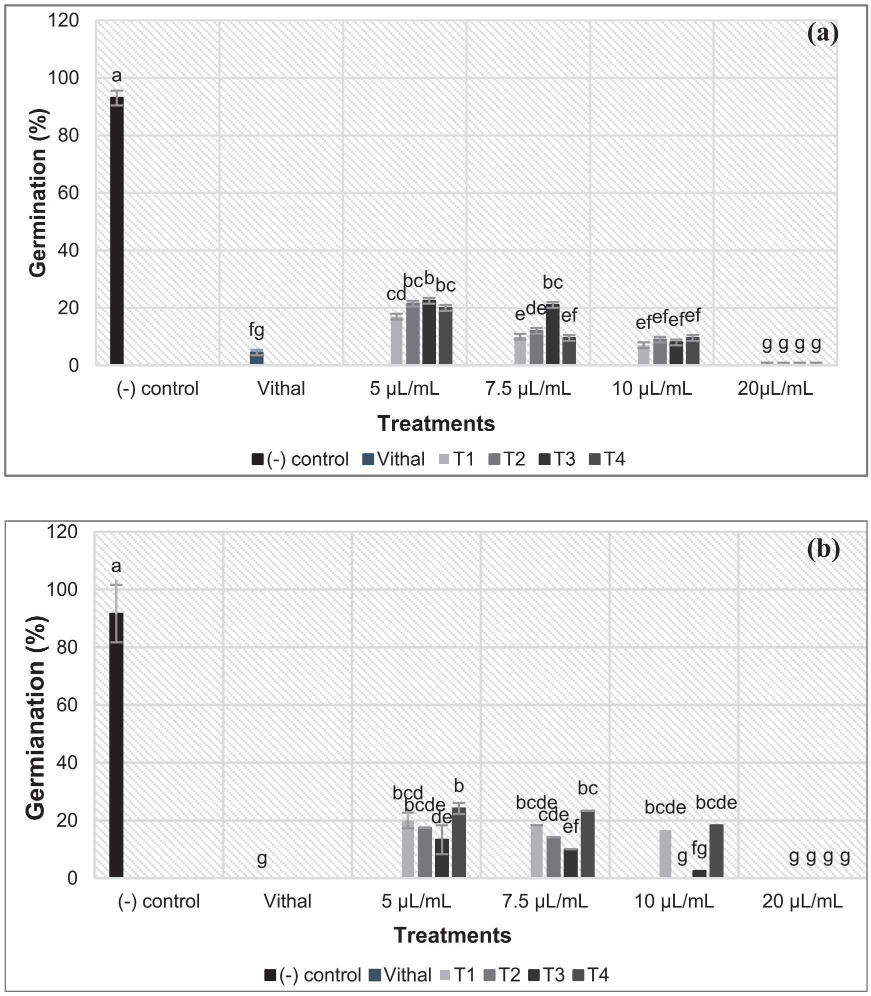

Fig. 1. Effect of the different treatments and doses on seed germination of *L. perenne* **(a)** and *A. retroflexus* **(b)** after 14 days exposure to a 5.0–20 µL/mL range of concentrations of the four tested EOs. T1: *T. algeriensis*, T2: *T. ciliatus* subsp. *coloratus*, T3: *T. vulgaris* cultivar Varico 3, T4: *T. vulgaris* ecotype Fasano. Values are given as mean ± SD (n = 3). At each dose, column with different letters are significantly different ($p \leq 0.05$, Tukey's multiple comparison test).

T2 expressed the strongest herbicidal effect on *A. retroflexus*, where a total inhibition of seed germination was induced at 20 and 10 µL/mL. At 7.5 and 5.0 µL/mL, it was extremely reduced at a percentage of 14.2 and 17.5, respectively, while the germination of the negative control was 91.7 %. T3 demonstrated total inhibition at 20 µL/mL and significantly slowed it down at the other concentrations (2.5, 10.0 and 13.3 % respectively). Contrary to what was observed for *L. perenne*, T1 and T4 expressed less significant effectiveness in comparison with T2 and T3. An important reduction in seed germination was noticed for the other concentrations (Fig. 1b). However, it is still important that a total inhibition was achieved at 20 µL/mL and a strong reduction in seed germination of about 80 % was noted for the other concentration.

In summary, T1 and T4 expressed the higher herbicidal effect against *L. perenne* while T2 and T3 were more effective against *A. retroflexus*.

3.1.2. Post-emergence assay

Regarding the herbicidal effect on target species seedlings, *A. retroflexus* was more sensitive than *L. perenne* to thyme EOs treatments. *L. perenne* seedlings were affected by the treatments only at the highest concentration (20 µL/mL), where the mortality of all the plantlets was observed. For the other concentrations, all the seedlings were able to survive after exposure to the treatments. On the contrary, *A. retroflexus* strongly reacted to the treatments already from the first application. It displayed wilting symptoms few hours after the exposure to the EOs, inducing the death of the plantlets in less than 24 hours. The fresh weight of *A. retroflexus* biomass subjected to the treatments was consistent with the number of surviving seedlings. This demonstrates the high sensitivity of *A. retroflexus* plantlets to the treatments (Fig. 2).

The potent herbicidal effect of all the EOs on *A. retroflexus*, as compared to the negative control, was well demonstrated by T2 and T3. At the highest concentration (20 µL/mL), no seedlings survived, while the number of surviving ones was considerably reduced at 10 µL/mL. The same effectiveness was observed at 7.5 and 5.0 µL/mL where the number of surviving plantlets was importantly reduced. T4 provoked the mortality of all the plantlets at the concentration of 20 µL/mL. The other concentrations induced an important mortality of the seedlings, where the number of the surviving ones did not exceed 10 plantlets for the lowest concentration (5 µL/mL). T1, even if it expressed the less important herbicidal effect in comparison to the other EOs, reduced considerably the number of surviving plantlets. As for the other treatments, it induced a total death of the seedlings at 20 µL/mL, and drastically decreased it to less than 13 plantlets for 7.5 µL/mL, which was the less effective dose (Fig. 3a). The results of the fresh biomass of *A. retroflexus* are correlated with those of the number of surviving plants (Fig. 3b).

Furthermore, it is important to note that as for the surviving plantlets, there were no significant differences in the herbicidal effect of the treatments on *L. perenne* plant biomass in comparison to the negative control; only the highest concentration (20 µL/mL) exhibited an effective effect of the treatments on the seedlings. Over all, for all the

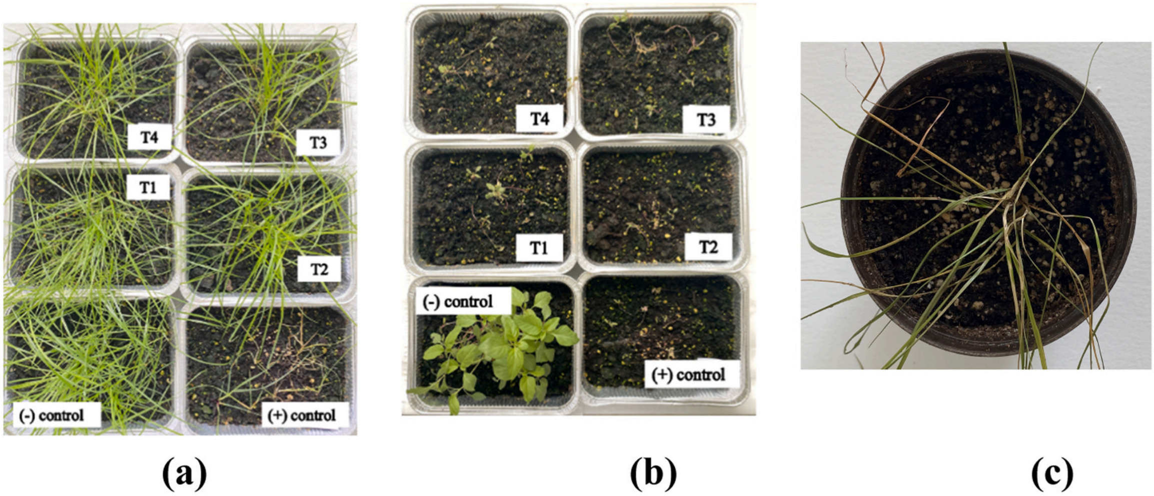

Fig. 2. Effect of the treatments on target weed species plantlets. (a): *L. perenne* seedling treated with EOs at 10 µL/mL, (b): *A. retroflexus* seedling treated with EOs at 10 µL/mL, (c): *L. perenne* seedling treated with EOs at 20 µL/mL. T1: *T. algeriensis*, T2: *T. ciliatus* subsp. *coloratus*, T3: *T. vulgaris* cultivar Varico 3, T4: *T. vulgaris* ecotype Fasano. Pictures were taken one (01) day after the application of the treatments.

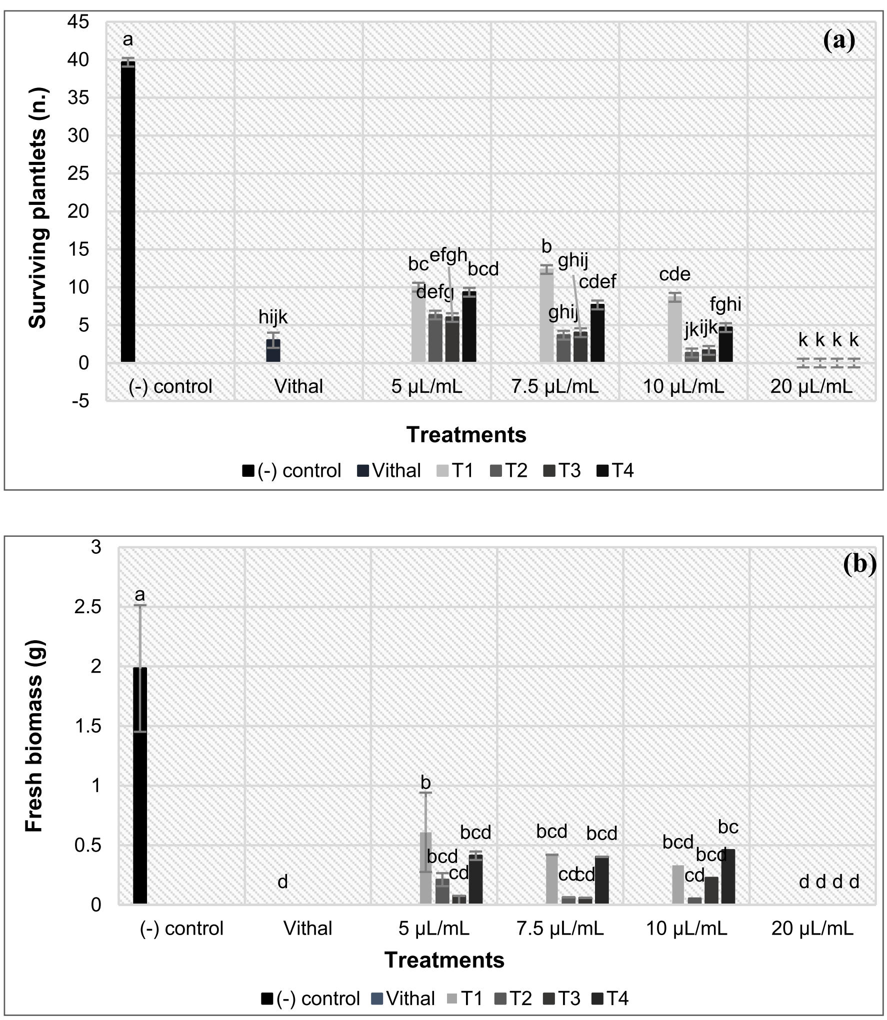

Fig. 3. Number of surviving (a) and fresh biomass (b) of controls and treated *A. retroflexus* plants, 24 hours after exposure to the different treatments and doses (5–20 µL/mL range of concentrations) of the four tested EOs. T1: *T. algeriensis*, T2: *T. ciliatus* subsp. *coloratus*, T3: *T. vulgaris* cultivar Varico 3, T4: *T. vulgaris* ecotype Fasano. Values are given as mean ± SD (n = 3). At each dose, column with different letters are significantly different ($p \leq 0.05$, Tukey's multiple comparison test).

treatments, the biomass was null at 20 μL/mL and insignificant at 10, 7.5 and 5 μL/mL.

The results obtained from the greenhouse experiments demonstrated an important herbicidal activity of the studied thyme EOs. All the EOs showed efficacy in negatively affecting seed germination of both weed test species at the highest concentration and drastically reducing it at lower concentrations. However, this effectiveness was selective on seedlings growth. *L. perenne* demonstrated a resistance where treatments were effective only at the highest concentration. On the contrary, *A. retroflexus* was highly sensitive to the application of the EOs at all concentrations. Globally, these results are highly consistent with those reported by the literature considering other species of thyme. Indeed, the herbicidal potential of some *Thymus* sp. pl. EOs, such as *T. fontanesii*, *T. kotschyanus, T. decussatus, T. pulegioides*, were investigated by researchers who demonstrated the significant efficacy in inhibiting seed germination and seedling growth of indicator test and weed species (Benchaa et al., 2019; Ghasemi et al., 2020; Saleh et al., 2020; Portuguez-García et al., 2021; Vaiciulyte et al., 2021; Zhou et al., 2021; Elghobashy et al., 2024; Miloudi et al., 2024). Our results, investigating EOs of different species, broaden the perspective of using new and widespread thyme species for their herbicidal potential.

The negative impact of EOs on seed germination and seedling growth could be associated with physiological disturbances of the treated plant species. Secondary metabolites affect the vigor of the seeds by acting directly in the processes of cell and membrane degradation (Qian et al., 2009; Silva et al., 2011), which according to De Oliveira Roberto et al. (2023), disturb the leakage of electrolytes and provoke the loss of selectivity of the membrane. This disturbance can inhibit the synthesis of ATP and increase the production of reactive oxygen species, causing the rupture of membranes and leading to cell necrosis and germination reduction. The effect of these thyme EOs can also be responsible for the decrease of the weed seeds' vigor. Indeed, the seed vigor is related to the integrity of the cell membrane system; and when the seeds are immersed in the treatments, cytoplasmic solutes are released in the medium, proportionally to the state of disorganization in which the membranes are found (De Oliveira Roberto et al. 2023).

The inhibition and/or the slowdown of seedling growth by thyme EOs, in turn, could be associated with the disturbance of photosynthesis inducing oxidative stress (Hasan et al., 2022; Li et al., 2023). Amri et al. (2013) argue that the inhibition of plant growth by EOs may occur via the generation of reactive oxygen species (ROS), subsequent oxidative stress, characterized primarily by markers specifically, and increase of malondialdehyde and proline concentrations. For several authors (Romero-Puertas et al., 2004; Silva et al., 2017; Bruxel et al., 2022), the exposure to EO causes oxidative stress in recipient species by activating several signaling pathways, causing metabolic damage. This could explain the results obtained in the present study. Plant tissues can become oxidized and reactive oxygen species (ROS), primarily hydrogen peroxide (H_2O_2), can accumulate and cause irreversible damage to plasma membranes when defense mechanisms are inefficient. The ROS causes damage to proteins, DNA, lipids, cell membranes and photosynthetic pigments, and finally leads to plant mortality (Bruxel et al., 2022).

In this sense and addressing plant defense mechanisms, *L. perenne* seedlings displayed a significant resistance while *A. retrofluxus* expressed a potent sensitivity to the EO treatments. These results coincide with those of Omezzine et al. (2011) on *D. viscosa*. It is suggested that there is a selectivity in the action of bioactive molecules regarding the target plant species and their types, whether monocots or dicots (Tuyen et al., 2018; Lebedev et al., 2019; Jmii et al., 2020). Rial et al. (2018) suggested that the seedlings release bioactive molecules in the medium that might contrast the effect of the applied treatments. Jouini et al. (2020) abounded in this sense and demonstrated that the effectiveness of the treatments depended on the targeted weed species and the type of EOs. In this regard, *L. perenne* is a notable resistant weed species to herbicides where resistance to 14 mechanisms of action (MOA) has been confirmed in this species (Heap, 2024). According to Matzrafi et al. (2021), this is due to the high genetic diversity of *Lolium* spp., an obligate outcrossing mating system and a recurring-rent selection with a herbicide might lead to metabolic cross-resistance to other herbicides even from different MOA (Neve and Powles, 2005; Matzrafi et al., 2021). The results of this study revealed the high selectivity of thyme EOs and this could be interesting for the development of selective bio-herbicides.

In that regard, it has been reported that monoterpene-rich EOs significantly affect weed germination and growth rather than sesquiterpene-rich EOs (Verdeguer et al., 2020a; Amri et al., 2023), which supports our findings. The tested thyme EOs used in this study contain an important percentage of monoterpenes, particularly, α-pinene, thymol, carvacrol, p-cymene and linalool. Oxygenated monoterpenes (essentially thymol, carvacrol, and p-cymene) are known to have potent phytotoxic effects, resulting in the inhibition of α-amylase, suppression of seed water uptake, disturbance of mitochondrial respiration, as well as interference with cell division processes (Somala et al., 2022; Amato et al., 2023). In fact, thymol can exhibit clastogenic and aneugenic mechanisms of action, causing DNA damages and the mitotic spindle. Carvacrol displays also clastogenic mechanisms of action and causes damage to the DNA (De Assis Alves et al., 2018). Presenting a similar structure with the synthetic herbicide cinmethylin, 1,4 cineole is of particular interest because it can alter the mitotic process affecting the prophase, induced growth abnormalities in shoots. Similarly, 1,8-cineole can inhibit the plant growth affecting mitochondrial respiration, inducing the formation of swollen root tips (in onions) and the inhibiting all the mitosis stages (Verdeguer et al., 2020a).

Furthermore, others identified compounds in the thyme EOs, such as p-cymene, γ-terpinene, and linalool have been confirmed to possess phytotoxic effects against many weed species (De Assis Alves et al., 2018; Bozhuyuk, 2020; Khammassi et al., 2023). However, it is important to highlight that the phytotoxic potential of EOs can be attributed to antagonistic effects due to interactions among EO constituents, even those present at low concentrations (Xianfei et al., 2007). In addition, the molecular structure of each component can be responsible for the specific MOA (Lins et al., 2019).

3.2. Phytotoxic activity of EOs on *Arabidopsis thaliana*

The phytotoxic effect and the mode of action of the studied EOs were evaluated on the model plant *A. thaliana*. The treatments did not significantly affect *A. thaliana* germination. However, the root growth of EO-treated seedlings was strongly influenced by the treatments (Table 2). At day 7, T1 drastically reduced the root growth of treated seedling followed by T3 and T4 while T2 was the less effective one. After 14 days of exposure to the different EOs, the roots of the treated seedlings were unable to resume their development, except those treated with T2. On the contrary, seedlings treated with T1, T3 and T4 displayed a very slow growth in comparison to the control; where T1 was the most effective treatment in slowing down the root elongation of *A. thaliana,* closely followed by T3.

In addition, a reduction of the root hairs was detected in the treated plants: condensate root hairs were detected in seedlings treated with T1

Table 2
Effect of thyme EOs on *A. thaliana* germination and root growth.

	Control	T1	T2	T3	T4
Germination (%)	93.33 ± 2.89a	80 ± 13.23a	86.67 ± 10.41a	53.33 ± 12.58a	61.70 ± 28.4a
Root length at day 7 (mm)	24.39 ± 3.19a	1.74 ± 0.27c	16.28 ± 3.37b	4.21 ± 1.28c	7.62 ± 0.97c
Root length at day 14 (mm)	47.28 ± 7.46a	5.01 ± 3.12b	44.26 ± 11.35a	5.66 ± 2.09b	18.18 ± 10.62b

Values are given as mean ± SD (n = 3). For each treatment, different letters indicate statistically significant differences ($p \leq 0.05$, Tukey's multiple comparison test).

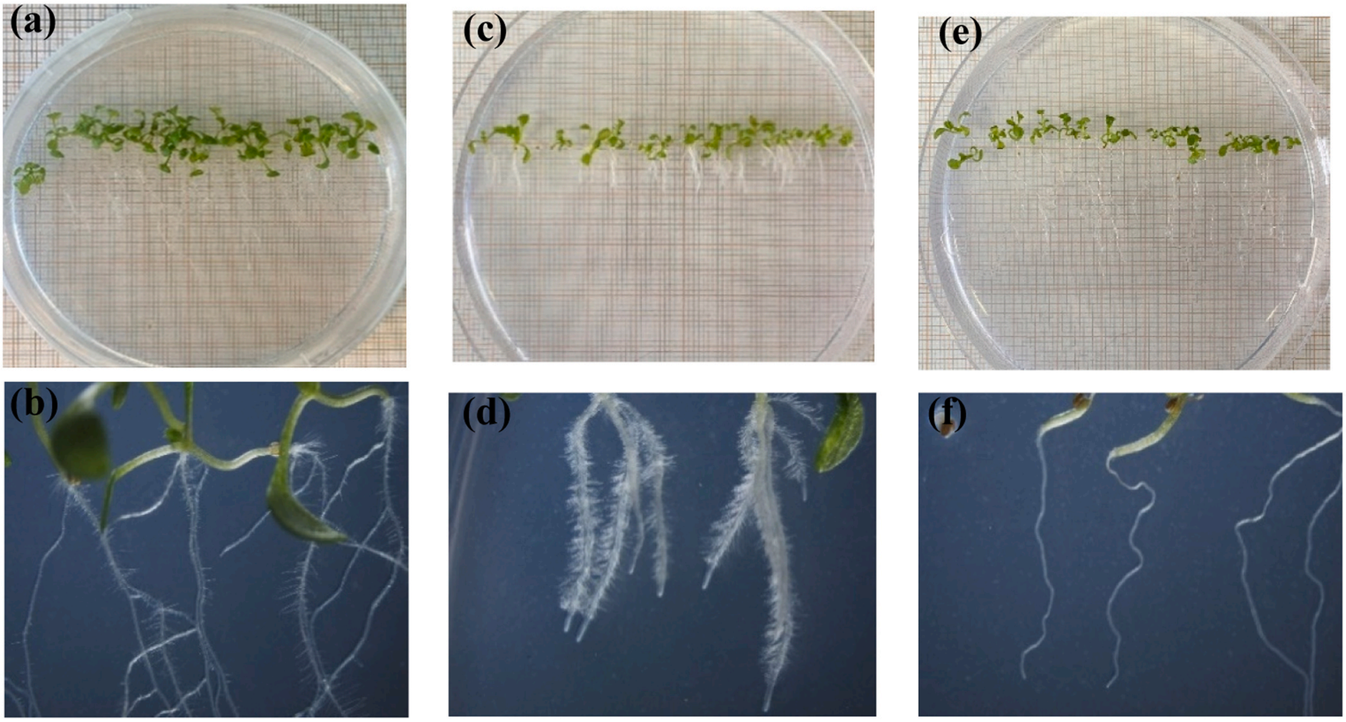

Fig. 4. Images of 14 days *A. thaliana* seedlings control and treated with thyme EOs. (a, b): control; (c, d): short roots and condensate root hair belonging to seedlings treated with T1 (*T. algeriensis*); (e, f): absence of lateral and root hair of seedlings treated with T3 (*T. vulgaris* L. cultivar Varico 3).

while the total absence of them was clear in T3 (Fig. 4). The visualization under a stereomicroscope to evaluate the morphological changes induced by the EOs on the seedlings after 14 days of growth showed also several anomalies depending on the EO. Furthermore, EOs reduced production and length of the root hairs. Some *A. thaliana* seedlings even presented a loss of gravitropism.

The microscopic observation of roots with Evans Blue staining showed a clear membrane damage induced by the treatments which is in accordance with what was reported by previous studies (Díaz-Tielas et al., 2012; Zhou et al., 2021), who established that Evans Blue only penetrate and color cells that have lost their membrane integrity. In this sense, all the EO-treated roots were blue stained with different intensity. This means that the effect was also correlated with the EO. T2 showed the most intense blue color arguing an important damage to the cell membrane integrity, while T1, T3 and T4 displayed low to moderate blue stained roots mostly in the root cap (Fig. 5).

The histochemical detection of lipid peroxidation of the roots induced by the EOs was performed using Schiff's reagent. This reagent reacts with aldehydes from the lipid peroxidation inducing to a magenta coloration of the tissues. The microscopic observations showed, in this sense, magenta stained EO-treated roots, demonstrating a membrane degradation. However, as for Evans Blue staining, the intensity of the red color differed according to the EO applied; EO-treated roots with T2 displayed the most intense coloration, followed by T1, which indicates an important lipid peroxidation. T3 and T4 treated roots showed low coloration but with evident torsions and structural modifications (Fig. 6).

It is worthy to mention also that structural modifications have been observed on EO-treated roots. *A. thaliana* control roots demonstrated a normal growth with homogenous and symmetric cell rows and layers corresponding to the different root tissues in the division, similar thickness along their entire length and growing straight according to gravitropism (Fig. 6a), while EO-treated roots presented an evident and significant tissue disruption. The calyptra was thicker and the cell rows

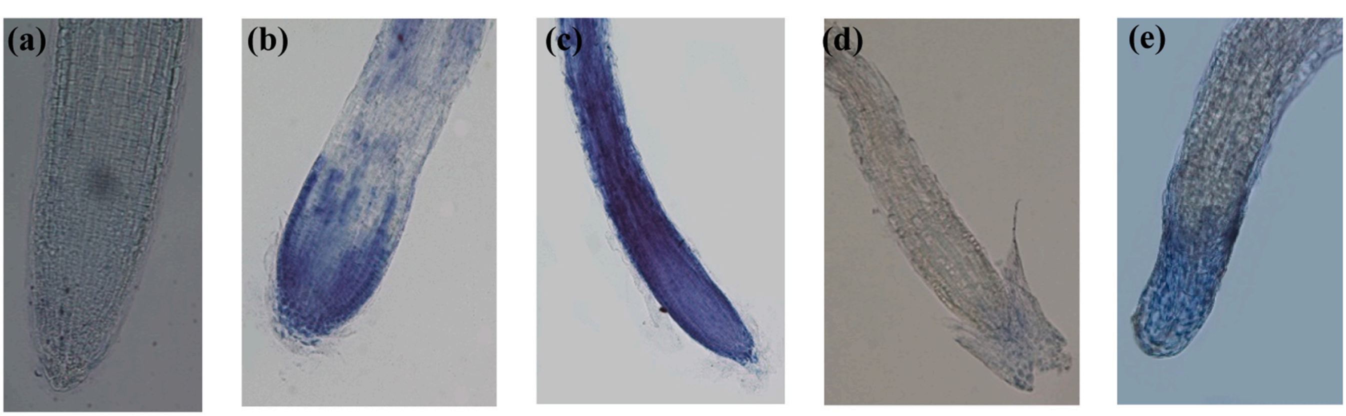

Fig. 5. Effect of thyme EOs on the loss of membrane integrity of apical root longitudinal sections of 14 days *A. thaliana* roots stained with Evans Blue reagent visualized under light microscopy. (a): control, (b): EO-treated roots with T1 (*T. algeriensis*); (c): EO-treated roots with T2 (*T. ciliatus*). (d): EO-treated roots with T3 (*T. vulgaris* L. cultivar Varico 3) and (e): EO-treated roots with T4 (*T. vulgaris* ecotype Fasano). Images taken at 10 (c, d, e) and 20X magnification (a, b).

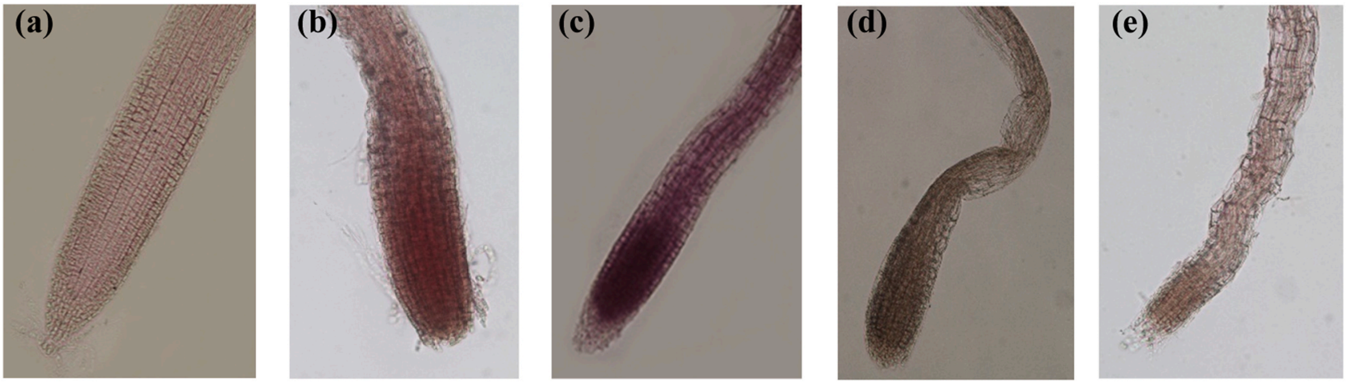

Fig. 6. Histochemical determination of lipid peroxidation in 14 days *A. thaliana* roots under the effect of thyme EOs by Schiff's reagent (longitudinal sections of root apices). (a): control, (b): EO-treated roots with T1 (*T. algeriensis*); (c): EO-treated roots with T2 (*T. ciliatus*). (d): EO-treated roots with T3 (*T. vulgaris* cultivar Varico 3) and (e): EO-treated roots with T4 (*T. vulgaris* ecotype Fasano). Images taken at 10 (c, d, e) and 20X magnification (a, b).

were disorganized and hardly distinguishable (Fig. 6b,c,d,e). Torsions with zig-zag development were also observed in the elongation zone (Fig. 6c); and in some samples the growth patterns were completely altered, showing toxic effects, such as contraction phenomena.

The *in vitro* experiments on the model plant *A. thaliana* were mainly performed to better understand the alterations caused by thyme EOs. Morphological and structural damages were observed, mostly on the root part of the seedlings. The EO-treated seedlings presented, among others, a zig-zag and torsion growth of the roots and a destruction of the calyptra. These observations of the alterations of *A. thaliana* exposed to different EOs have been wildly reported in plants exposed to allelochemicals. Verdeguer et al. (2020b), in their study of the effect of carvacrol on *A. thaliana*, established that this secondary metabolite caused shorter and thinner roots of the treated seedlings. The roots were also characterized by torsion and zig-zag development, reduction of the number and length of root hairs at the transition zone between hypocotyl and epicotyl, an increased number of ectopic roots close to the root tip and roots growing in different directions related to the loss of gravitropism. According to other authors (Blume et al., 2012; Araniti et al., 2016), these morphological and structural modifications and alterations could be attributed to the related to microtubule condensation and disorganization, the strong increment in ethylene production and unbalanced auxin content; López-González et al. (2020) reached the same results. Treated seedlings with norharmane, an indol alkaloid, presented an inhibition of the growth with the formation of adventitious roots. This phenomenon is a phytohormone-driven process, particularly auxin, responsible for the formation of adventitious roots (Lakehal and Bellini, 2019). Therefore, the morphology of seedlings exposed to, for example, *T. algeriensis* EO (T1), which have short roots with more adventitious roots and root hairs than the control, suggests that the EO could be responsible for an alteration in auxin synthesis or polar auxin transport (PAT) (López-González et al., 2020). This latter suggests that EO affects polar auxin transport (PAT) by reducing the number of PIN2, PIN3 and PIN7 proteins. This could provoke the accumulation of auxin in several root areas, without reaching the root cap, resulting in the adventitious root formation. This alteration in transport also has a negative effect on microtubules, causing inhibitory processes linked to cell elongation. Moreover, ethylene stimulates acropetal auxin transport in the plant roots, which is associated with a reduction of lateral root formation in a PIN3 and PIN7-dependent way (Lewis et al., 2011). Ivanchenko et al. (2008) established the synergistic role played by auxin and ethylene in inhibiting lateral root initiation. At high concentrations, ethylene inhibits the ability of pericycle cells to initiate new lateral root primordia. These results are in accordance with those obtained in this study. EOs might increase endogenous ethylene concentration in the EO-treated seedlings roots leading to the inhibition of the emergence of lateral roots, as per *T. vulgaris* cultivar Varico (T3) EO-treated seedlings.

Furthermore, the root morphology in *A. thaliana* is regulated by ethylene and auxin, agonistically or antagonistically (Swarup et al., 2002; Alonso et al., 2003). Our results suggest that the ultrastructure alteration, probably due to auxin and/or ethylene accumulation, and the macroscopic effects observed on root structure are linked. Moreover, thyme EOs, probably through the stimulation of auxin production, could interfere with microtubule dynamics by acting as a stabilizer of the tubulin, modifying the proper orientation of microtubules, which are essential components of cell cytoskeleton. Indeed, they are involved in vital processes, including cell division, growth and differentiation. These activities are highly dependent on the dynamic balance between the different subunits that form the microtubules (Ellis et al., 1994). When this balance is altered, the orientation of microtubules in the cell is affected, as well as all the cellular processes depending on them. The observations made in this study stipulate the clear evidence of altered microtubule organization, including the disruption of mitotic and cortical microtubules and by inducing ultra-structural cell malformations, thus modifications on root morphology through altering the cross-talk between auxin and ethylene. The results obtained from the experiments on *A. thaliana* point out that the mode of action of thyme EOs could be probably related to the alteration of the hormonal balance and bio-chemical reactions, inducing morphological damages and generating the accumulation of ROS as a side product in recipient plants.

4. Conclusions

The present study demonstrated that all *Thymus* sp. pl. EOs tested exhibited a significant herbicidal potential against *L. perenne* and *A. retroflexus*. Thyme EOs were strongly effective as pre-emergence treatments by either totally inhibiting or drastically slowing down seed germination. This effectiveness was also obtained as a post-emergence treatment against *A. retroflexus*. On the contrary, *L. perenne* expressed a resistance reaction. This achievement argues a selectivity of the herbicidal potential of thyme EOs. As for the *A. thaliana* experiment, the tested EOs showed a clear phytotoxic effect on the roots.

The discovery of natural molecules present in thyme EOs, characterized by a potent herbicidal potential, is important for the development of a new biobased products able to overcome the use of synthetic inputs. However, due to their chemical instability, EOs can be easily degraded, which currently limits their widespread use as bioherbicides. Several studies have been conducted on the possibility of adopting innovative techniques such as nanotechnology to overcome this problem and improve their stability while preserving their efficacy as bioherbicides (Boukhalfa et al., 2024b). To this end, further studies assessing the effect of thyme EOs on target (other weeds species) and non-target organisms (crops and soil microorganisms) and introducing into cultivation these species to guarantee a constant raw material production, EO yield and stable phytochemical composition are ongoing.

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CRediT authorship contribution statement

Boukhalfa Rym: Writing – review & editing, Writing – original draft, Validation, Supervision, Investigation, Data curation. **Messgo-Moumene Saida:** Writing – original draft, Validation, Supervision, Investigation, Data curation. **Ruta Claudia:** Writing – review & editing, Validation, Supervision, Investigation, Data curation, Conceptualization. **Argentieri Maria Pia:** Validation, Supervision, Investigation, Data curation. **Calabrese Generosa J.:** Writing – original draft, Supervision, Project administration. **De Mastro Giuseppe:** Writing – review & editing, Validation, Supervision, Project administration, Investigation, Data curation, Conceptualization.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Giuseppe De Mastro reports financial support was provided by University of Bari Department of Soil Plant and Food Science. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Data Availability

Data will be made available on request.

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CHAPTER III

Encapsulation in Silica Nanoparticles Increases the Phytotoxicity of Essential Oil from *Thymus vulgaris* in a Weed Species

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Encapsulation in Silica Nanoparticles Increases the Phytotoxicity of Essential Oil from *Thymus vulgaris* in a Weed Species

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ABSTRACT: Weed control poses a significant challenge to agriculture, warranting the development of effective but environmentally safe herbicides. Encapsulation of plant essential oils (EOs) with herbicidal properties in nanoscale polymers can offer high loading capacity as well as controlled and tunable agrochemical delivery. This study investigated the use of encapsulated thyme EO against redroot pigweed (*Amaranthus retroflexus* L.), a difficult-to-control weed resistant to multiple herbicides. Three volumes of thyme EO (500, 750, and 1000 μL) were encapsulated in a silica nanoparticle (SiNP) suspension to achieve 250 μL/mL (hereinafter "500"), 375 μL/mL (hereinafter "750"), and 500 μL/mL (hereinafter "1000") EO concentrations. The efficacies of these preparations were compared to that of pristine EO. The loading efficiencies were 26, 42, and 64% for the "500", "750", and "1000" EO preparations, respectively. Transmission electron microscopy (TEM) revealed spherical and regular SiNPs with a size range of 220−300 nm. Fourier transform infrared (FT-IR) spectroscopy confirmed EO loading by the presence of characteristic peaks of isoprenoids and isomeric compounds. Herbicidal bioassays with pristine thyme EO in postemergence treatments on *A. retroflexus* seedlings exhibited a significant ($p \leq 0.05$) concentration-dependent herbicidal activity, reducing shoot biomass by 85% at the highest tested concentration ("1000"), compared to the control (Tween 20). Encapsulation with SiNPs further enhanced the herbicidal efficacy compared to the control, reaching 96% at the highest concentration. Compared to the pristine EO, EO-SiNPs induced significant ROS production at the highest concentration, leading to cell membrane damage and an imbalanced antioxidant system, as demonstrated by the increased shoot malondialdehyde content (40%) and activities of the antioxidant enzymes ascorbate peroxidase (APX) (65%), catalase (CAT) (52%), and superoxide dismutase (SOD) (36%). These results suggest significant potential for developing an effective nanobioherbicide using thyme EO encapsulated in SiNPs.

KEYWORDS: *herbicidal activity, thyme essential oil, silica nanoparticles, encapsulation, enhanced agrochemical delivery*

1. INTRODUCTION

Weed species pose a significant challenge to modern agriculture and, in conjunction with nutrient and water deficiency, cause major yield depletion and crop loss.[1,2] Globally, crop loss due to weed infestation is estimated at 31.5%,[3] prompting the extensive use of herbicides.[4] Glyphosate is by far the most widely applied active compound and is used in more than 750 different herbicide formulations. This large use is intensified by the spread of glyphosate-tolerant transgenic plants.[5,6] Nevertheless, the widespread application of these agrochemicals causes adverse environmental impacts, significant human health concerns, and weed resistance.[7,8] In addition to these issues are the uncertainties associated with food insecurity and climatic variabilities and extremes.[9] Juxtaposed with the postulation that the global population is projected to reach 9.7 billion by 2050, which warrants an increase in food production of 25−70%, it has become critically necessary to respond to the grave issues affecting crop production.[10,11]

Consequently, novel and sustainable strategies for crop protection are needed under these adverse conditions and projections. In this regard, nanotechnology has had a profound influence across various research fields, including agriculture, particularly in the development of nanoscale agrochemicals, including fertilizers, pesticides, and herbicides.[12,13] Nano-herbicides have demonstrated significant potential for weed control, with various types of nanoherbicides being developed utilizing both organic and inorganic nanocarriers.[14−17] Studies have shown that nanoenabled herbicides can offer greater efficiency and environmental advantages than conventional

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herbicides.[16,18] This is due to their higher diffusion rates, improved adhesion, and longer contact times on leaf surfaces.[19] Inorganic nanomaterials, such as silica, silver, and mesoporous silica nanoparticles (MSNs), are frequently used to enhance herbicidal performance by efficiently encapsulating organic molecules and providing controlled and even tunable active ingredient release.[18,20] Despite their increased effectiveness, the active ingredients in many nanoherbicides are often synthetic molecules that have a tendency to persist in the soil and pose environmental hazards. Hence, there is growing interest in using natural products as active ingredients.[7]

Aromatic plant extracts, particularly essential oils (EOs), are emerging as green alternatives to synthetic herbicides due to their beneficial properties.[21] In recent years, EOs have gained attention owing to their diverse active compounds that have shown significant potential for the control of various weed species.[22−24] These active compounds, referred to as allelochemicals, offer several advantages: they are biodegradable, generally safe for human and environmental health, and exhibit diverse modes of action.[25] Reported mechanisms include cell injury, oxidative stress damage, DNA and RNA damage, and photosynthesis inhibition, ultimately leading to cell death.[26] The herbicidal mechanisms of EOs are complex, diverse, and have been insufficiently elucidated. However, EOs from several plant species are currently being considered for commercial herbicide product formulation. As of 2020, seven commercial bioherbicides—Matratec, GreenMatch, GreenMatchEX, WeedZap, Weed Slayer, Avenger Weed Killer, BioWeed, and WeedLock—were registered and available in the United States, Australia, and Malaysia. For example, BioWeed (Barmac, Lidcombe, Australia), Avenger Weed Killer (Avenger Products, LLC, Buford, Georgia), and Weed Slayer (Agresearch International, LLC, McKinney) have been used to effectively control *Ochna serrulata* Walp., *Digitaria sanguinalis* (L.) Scop., and *Echinochloa crus-galli* (L.) P. Beauv., respectively.[27,28] Notably, EOs are hydrophobic, chemically unstable, and easily degraded, all of which can complicate their use.[29] However, nanoparticles can be used to protect EOs and enhance their stability against environmental conditions, necessitating the development of bionanostructured systems such as nanocapsules. In this regard, several studies have demonstrated the benefits of the encapsulation of EOs for their effective delivery. In their review on the research progress of scenario-oriented nanopesticides,[30] some research studies were reported demonstrating these advantages, mainly an excellent stability and more efficacy of the EOs. Previously, several authors[26,29,31−34] noted that the encapsulation of EOs in different nanocarriers considerably enhanced EO biological activities against different pathogens.

Given the well-known herbicidal potential of EOs from *Thymus* sp. pl., in the current study, a novel bionanoherbicide was synthesized by encapsulating the EO from *Thymus vulgaris* cultivar Varico 3 within nanoscale silica (SiNPs). The SiNP-EO formulation was characterized and then assessed for herbicidal activity on redroot pigweed (*Amaranthus retroflexus* L.). The choice of *T. vulgaris* cv. Varico 3 was motivated by its botanical and agronomic characteristics; it is a hybrid developed for a higher EO content, stability of molecules, and constant production of fresh biomass. Importantly, only a few studies have explored the nanoencapsulation of EOs for herbicidal activity. The specific objectives of this study, therefore, were (i) to synthesize SiNPs and to load thyme EO into this nanocarrier; (ii) to assess the herbicidal efficacy of emulsions of pristine and SiNP-encapsulated EO against *A. retroflexus* L.; and (iii) to determine the mechanisms of action of the synthesized nanoherbicide.

2. MATERIALS AND METHODS

2.1. Plant Material and EO Extraction and Characterization. Aerial parts of *T. vulgaris* L. cultivar Varico 3, a hybrid obtained by Agroscope Changins-Wädenswil Research Station (Switzerland), were collected at the full flowering stage in May 2022 from the experimental farm of the University of Bari Aldo Moro in Policoro (southern Italy, 40°10′20″ N, 16°39′04″), Basilicata, Italy. The EO was extracted by hydrodistillation and characterized by gas chromatography coupled with mass spectrometry in a previous study.[35] The EO was stored at 4 °C until used in the present study. The chemical composition of the EO is presented in Table 1.

Table 1. Chemical Composition of *T. vulgaris* Cultivar Varico 3 EO[a]

no.	compound	KI[1]	KI[2]	content (%)
01	α-thurjene	926	924	0.48
02	α-pinene	936	940	1.03
03	camphene	954	950	0.88
04	verbenene	967	968	0.05
05	β-pinene	979	980	0.21
06	myrcene	991	992	0.52
07	α-terpinene	1017	1012	0.13
08	*p*-cymene	1022	1021	35.63
09	limonene	1029	1026	1.18
10	1,8 cineole	1031	1028	3.53
11	γ-terpinene	1059	1058	2.65
12	terpinolene	1088	1088	0.48
13	linalool	1096	1103	2.57
14	camphor	1143	1142	1.66
15	borneol	1165	1166	1.47
16	ρ-mentha-1,5 dien-8-ol	1170	1174	1.76
17	thymol methyl ester	1235	1234	1.97
18	thymol	1290	1293	20.3
19	carvacrol	1298	1300	11,76
20	β-cedrene	1418	1404	7.69
21	caryophyllene oxide	1581	1573	5.57
identified components (%)				100%
monoterpene hydrocarbons				52.39
oxygen-containing monoterpenes				38.09
sesquiterpene hydrocarbons				4.76
oxygen-containing sesquiterpenes				4.76
others				

[a]KI: Kovats index, [1]Literature, [2]Calculated.

2.2. Synthesis of Si Nanoparticles and EO-SiNPs. Silica nanoparticles (SiNPs) were prepared according to ref 36 with slight modifications. Briefly, a mixture of 515 mmol of ethanol, 33 mmol of deionized water (DI), and 32 mmol of ammonium hydroxide (25%) was prepared. The solution was stirred at 300 rpm, while 2 mmol of tetraethyl orthosilicate (TEOS, 98%) was gradually added as the silica source over a period of 6 h. The mixture was then left to stir overnight. The resulting white precipitate was separated by centrifugation for 5 cycles (2400 rpm, 5 min each at 25 °C), thoroughly washed with DI water, and then dried under vacuum at 75 °C for 24 h. The dried SiNPs were resuspended in ethanol at 50 mg/mL. Subsequently, three volumes (500, 750, and 1000 μL) of thyme EO in ethanol were added to the SiNP suspension to achieve EO concentrations of 250 μL/mL (hereinafter "500"), 375 μL/mL (hereinafter "750"), and 500 μL/mL (hereinafter "1000"), respectively, as per ref 31. The suspensions were then ultrasonicated for 20

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ACS Agric. Sci. Technol. XXXX, XXX, XXX−XXX

min, and the resulting uniform suspensions were allowed to incubate at an ambient temperature overnight to evaporate the solvent.

2.3. Characterization of EO-SiNPs. Characterization of EO-SiNPs involved several analytical techniques to determine the ζ-potential, particle size distribution, morphology, and loading efficiency (LE) of the silica nanoparticles.

2.3.1. ζ-Potential and Particle Size Distribution. The ζ-potential and particle size distribution of the pristine (blank) SiNPs and EO-SiNPs were measured by using a Malvern Zetasizer (model Nano ZS90). The electrophoretic mobility and dynamic light scattering (DLS) analyses were performed with aqueous dispersions of 0.1 g of samples in 1 mL of DI water, conducted in sextuplicate for ζ-potential and in triplicate for particle size distribution.

2.3.2. Morphology and Particle Size Analysis. The morphology and particle sizes of pristine SiNPs and EO-SiNPs were determined by transmission electron microscopy (TEM). Briefly, 0.1 g of each sample was placed in 1 mL of DI water and subjected to ultrasonic treatment for 10 min to maintain the particle dispersion. Subsequently, 2 μL of the aqueous particle dispersion was allowed to evaporate on a circular carbon-coated copper grid for 20 min. The samples were observed at an operating voltage of 100 kV using a Hitachi HT7800 RuliTEM instrument (Japan).

2.3.3. Fourier Transform Infrared (FT-IR) Spectroscopy. The loading of EO into SiNPs was further evaluated by using Fourier transform infrared (FT-IR) spectroscopy (Invenio S, Bruker Co., Germany). FT-IR spectra for blank SiNPs, pristine EO, and EO-SiNPs were recorded within the range of 500−4000 cm^{-1} to identify characteristic absorption bands corresponding to known functional groups.

2.3.4. Loading Efficiency (LE %). The loading efficiency of EO in SiNPs was determined according to ref 32. Briefly, EO-SiNPs were dispersed in 2 mL of acetonitrile, and the mixture was centrifuged at 5000 rpm for 10 min at 25 °C. The absorbance of the supernatant was measured at 240 nm using a UV−vis spectrophotometer (SpectraMax M2; Molecular Devices, San Jose, CA). The concentration of EO was estimated using a standard calibration curve for pristine thyme EO. The loading efficiency (LE %) was calculated using the following equation

$$\text{LE \%} = (\text{amount of loaded EO})/(\text{mass of loaded nanocapsules}) \times 100$$

2.4. Biological Assays. *2.4.1. Assessment of the Herbicidal Activities of Pristine EO and EO-SiNPs.* The herbicidal activity of the freshly prepared pristine thyme EO and EO-SiNPs was tested against the weed species *A. retroflexus* L. Emulsions of pristine EO were prepared according to ref 37. Briefly, preparations of "500", "750", and "1000" EO and EO-SiNPs were diluted in 2 mL of Tween 20, followed by the addition of 100 mL of DI water to the suspensions. Suspensions of Tween 20 and pristine SiNPs served as negative controls, while a pelargonic acid-based commercially available bioherbicide (Scythe herbicide, 57% w/w pelargonic acid) was used as a positive control at a concentration coinciding with the highest EO concentration in the preparations. Biological assays were conducted under greenhouse conditions to evaluate the effectiveness of pristine EO and EO-SiNPs on weed seed germination and early seedling growth as a postemergent treatment. First, seed viability was assessed through a germination test, where 100 seeds were sown in 90 mm Petri dishes fitted with two layers of Whatman filter paper wetted with 3 mL of distilled water. The Petri dishes were sealed with parafilm and placed in a controlled growth chamber maintained at 24 ± 1 °C with a 16/8 h light/dark cycle. After 7 days, the number of germinated seeds was counted and the percentage of germination was calculated. The effectiveness of the treatments was subsequently estimated in dose−response bioassays with weed seedlings. Specifically, to evaluate their impact on seedling growth, precultivation of the weed species was undertaken. Seeds were allowed to germinate in the greenhouse at 22−25 °C and 50−60% humidity, in nursery trays of 72 holes fitted with peat, until the emergence of two true leaves. Germinated seedlings were then transplanted into pots (1000 mL) filled with peat. Prior to transplantation, the pots were irrigated at optimal water holding capacity and were allowed to equilibrate and leach any excess water. All pots were placed in a greenhouse maintained at 22−25 °C with 50−60% humidity and were monitored daily for irrigation when necessary. The treatments were applied as contact treatments by spraying with a microsprayer to ensure homogeneity. Each seedling received 5 mL of the treatment once they reached the phenological stage of 13−14 (three to four true leaves) on the Biologische Bundesanstalt Bundessortenamt and Chemical Industry (BBCH) scale. Treatments were applied once for the "1000" EO, EO-SiNP, and commercial herbicide preparations (5 mL × 1 application) and twice for the "750" and "500" EO and EO-SiNP preparations and the Tween 20 and SiNP controls (5 mL × 2 applications). All treatments and controls were replicated three times. Plants were visually evaluated for potential herbicidal effects after 24 h. At the end of this period, all plantlets were harvested for biomass determination, cell injury assessment, and evaluation of the antioxidant enzyme activities.

2.4.1.1. Cell Injury Indices. Several assays were conducted to evaluate the physiological impacts induced in *A. retroflexus* as a function of the treatments. To assess cell injury, lipid peroxidation levels were estimated by measuring the malondialdehyde (MDA) content. Plant extraction was performed following the method of ref 38. Briefly, plant samples were ground into fine powders in liquid nitrogen and samples were extracted using a 0.1% trichloroacetic acid (TCA) solution. Subsequently, 160 μL of plant extract was mixed with 400 μL of 2% TCA and 0.5% thiobarbituric acid (TBA). The mixture was heated at 95 °C for 30 min and then cooled on ice. The absorbance was measured using a UV−vis spectrophotometer (SpectraMax M2; Molecular Devices, Sa Jose) at 532 and 600 nm.

2.4.2. Evaluation of Total Soluble Protein Content and Antioxidant Enzyme Activities. Biochemical analyses were conducted to evaluate the protein content and the activation of the enzymatic antioxidant defense system in the treated plants. The activities of three antioxidative enzymes were selected for this study, namely, ascorbate peroxidase (APX), catalase (CAT), and superoxide dismutase (SOD). Enzyme extraction followed the protocols of ref 39. Briefly, plant samples were ground into fine powders in liquid nitrogen, and tissues were extracted using a 25 mM potassium phosphate buffer. The protein content was determined according to the method described by ref 40. APX activity was estimated based on its ability to catalyze the conversion of ascorbic acid to ascorbate and H_2O_2.[41] For this assay, 40 μL of enzyme extract was mixed with 228 μL of ascorbic acid and 532 μL of 0.4 mM H_2O_2. The decrease in the absorbance of H_2O_2 at 290 nm was measured kinetically at 25 °C during a 2 min interval using a UV−vis spectrophotometer. CAT activity was assessed based on its ability to catalyze the decomposition of hydrogen peroxide (H_2O_2) to water.[41] For this assay, 40 μL of enzyme extract was mixed with 760 μL of a 10 mM H_2O_2 buffer. The decrease in absorbance of H_2O_2 at 240 nm was measured kinetically at 25 °C during a 3 min interval. SOD activity was determined using the photochemical reduction of nitroblue tetrazolium (NBT) method.[39,41] Briefly, 13 μL of enzyme extract was mixed with 707 μL of buffer solution containing 500 μM NBT, 78 mM methionine, 1.5 mM EDTA, and 100 mM potassium phosphate buffer. 80 μL of a 0.02 mM riboflavin solution was added in the dark. The solution was illuminated for 15 min in a light box, and the absorbance was measured at 560 nm by using a UV−vis spectrophotometer.

2.4.3. EO Profile in Treated Seedlings. To confirm EO accumulation in *A. retroflexus* leaves, a phytochemical residue analysis of the main compounds in thyme essential oil was performed. After 24 h of exposure to the pristine EO and EO-SiNP treatments, secondary metabolites were extracted from all samples (treated and negative controls) using methanol at a 1:3 (plant sample: methanol; w/v) ratio. The extraction procedure was repeated three times for each sample. The resulting extracts were pooled and filtered for chemical analysis.[42] Thymol, carvacrol, and *p*-cymene were quantified using gas chromatography-mass spectrometry (GC-MS) with an Agilent 6890N chromatography system coupled to a 5975-mass spectrometry detector (Agilent Technologies), employing an HP-5 MS capillary column (30 m × 0.25 mm, 0.25 μm film thickness). The GC column

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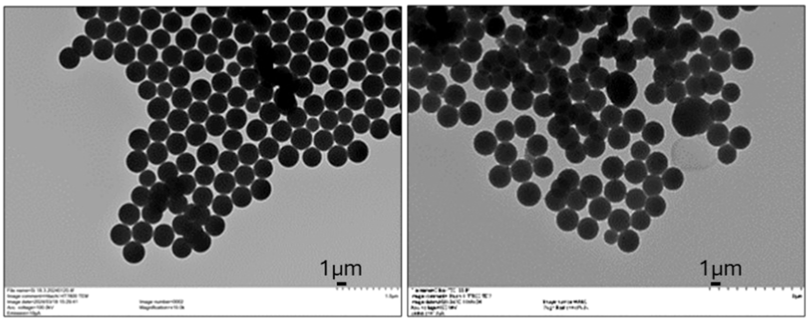

Figure 1. TEM images of SiNPs (left) and EO-SiNPs (right).

temperature program started at 50 °C for 5 min, increased to 300 °C at a rate of 15 °C/min, and then was held at 300 °C for 3 min. The injection temperature was set at 290 °C, and helium was used as the carrier gas at a flow rate of 0.8 mL/min. Mass spectra were recorded in a 70 eV electron ionization mode. The EO compounds were identified by matching their retention times and mass spectra to those available in the GC-MS WILEY database.

2.5. Statistical Analysis. Data obtained in these studies were statistically analyzed using Minitab version 19.2020.1 (Minitab Software, State College, Pennsylvania). A one-way analysis of variance (ANOVA) was performed to evaluate the effects of the treatments on root and shoot biomass, cell injuries, and antioxidant enzyme activities. Differences among means were assessed using Tukey's test. Statistical significance was accepted at a probability level of less than 0.05 ($p < 0.05$).[43]

3. RESULTS AND DISCUSSION

3.1. Characterization of EO-SiNPs. The morphology and size of SiNPs and EO-SiNPs were determined by using TEM. The SiNPs showed a regular spherical appearance and particle size ranging between 220 and 300 nm. TEM further confirmed that the EO was successfully loaded into the SiNP. The EO was distributed in both the internal and external surfaces of SiNPs, with loading appearing to affect particle size but not morphology (Figure 1). These results are consistent with those reported in refs 32,33, where SiNPs and mesoporous silica nanoparticles (MSNPs) were shown to be mostly spherical and that loading of the EOs did not affect the nanoparticle morphology. Interestingly, refs 32,33 estimated their particle sizes to be around 50−70 and 100 nm, respectively. On the other hand, ref 34 reported that cinnamon EOs encapsulated in MSNPs were approximately 500 nm. It appears that the large disparities in particle size are modulated by the effect of the molar ratio (TEOS to NH_3) as previously noted by ref 44 as well as on the type of base used in the preparation. Indeed, while refs 32,33 used sodium hydroxide, ref 34 used ammonium hydroxide, as in our study.

The hydrodynamic diameter and ζ-potential are crucial parameters in determining the availability and colloidal stability of nanosuspensions, with lower particle size often enhancing the physicochemical properties of a nanomaterial delivery system.[45] The particle size distributions of pristine SiNPs and EO-SiNPs were 236.77 ± 5.18 and 694.27 ± 20.85 nm, respectively, indicating considerable size difference between the two types of materials as also indicated by TEM. These findings can be compared with those of refs 7,45 who reported particle sizes ranging from 339.3 to 553.3 nm and 85.6 to 208.4 nm for different EOs encapsulated in organic polymers, including Gum Arabic, Persian gum/gelatin, and chitosan. The ζ-potential for the unloaded silica was −74.00 ± 0.38 mV, while for the EO-SiNPs, it was −80.43 ± 0.84 mV. According to ref 46, ζ-potential is correlated with the stability of particle dispersions, with the following scale: 0−10 mV (highly unstable), 10−20 mV (relatively stable), 20−30 mV (moderately stable), and >30 mV (highly stable). Hence, the ζ-potential analysis clearly suggested that EO-SiNPs form highly stable nanosuspensions, which is consistent with previous reports by ref 47.[47]

FT-IR analysis shows the symmetric stretching vibration of Si−O−Si (743 cm^{-1}) and the asymmetric stretching vibration of Si−O−Si (1046 cm^{-1}) and hydroxyl groups (OH) in silica (3313 cm^{-1}) in the SiNP spectrum, which agrees with refs 33,44. The absorbance bands of the EO-loaded SiNPs were different from those of the pristine SiNPs. Symmetric Si−O−Si, asymmetric Si−O−Si, and O−H band stretching vibrations shifted to 950, 1093, and 3349 cm^{-1}, respectively. The EO-SiNP spectrum clearly demonstrates the loading of the EO into SiNPs, with two new peaks at 802 and 941 cm^{-1}, characteristic of the C−H out-of-plan bending vibration from isoprenoids and isomeric compounds like thymol, carvacrol, *p*-cymene, and 1,8 cineole. In addition, for the EO-SiNP spectrum, an overlap of stretching vibrations of different groups was evident. These results are in line with previous studies (Figure 2).[48−50]

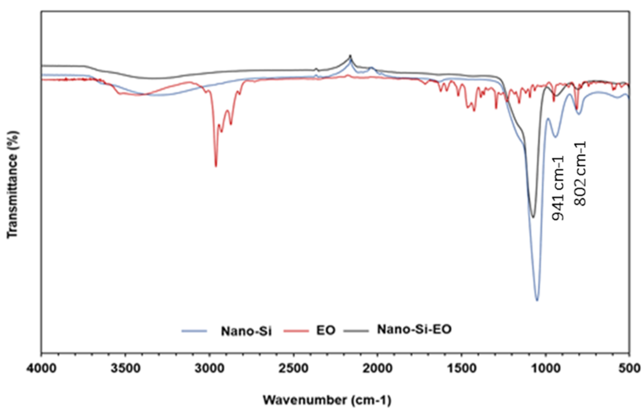

Figure 2. FT-IR spectra of pristine SiNPs (Nano-Si) and EO and EO-SiNPs (Nano-Si-EO).

The loading of SiNP with the EO was examined by UV−vis spectrophotometry. LE % was obtained using a standard curve ($y = 0.0521x + 0.0009$, $R^2 = 0.9834$). The LE analysis showed that 64% (w/w), 42%, and 26% of EO were encapsulated in the SiNPs, respectively, for the "500", "750", and "1000" EO preparations. This low LE % is mainly due to the high water solubility of *p*-cymene (23.4 mg/mL).[51] In this study, *p*-cymene is the main component of the thyme EO.

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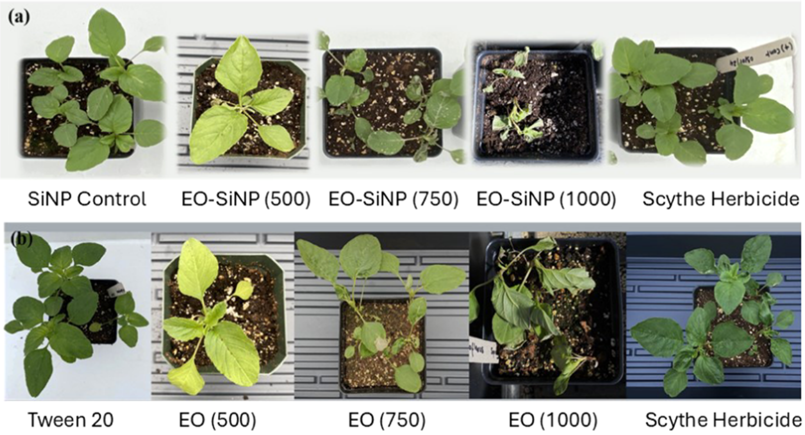

Figure 3. Photographs depicting the wilting symptoms induced by the treatments. (a) Seedlings treated with EO-SiNPs at various EO concentrations: 250 μL/mL ("500"), 375 μL/mL ("750"), and 500 μL/mL ("1000"), and a commercial herbicide (Scythe). (b) Seedlings treated with Tween 20, pristine EO at various concentrations, and the commercial herbicide.

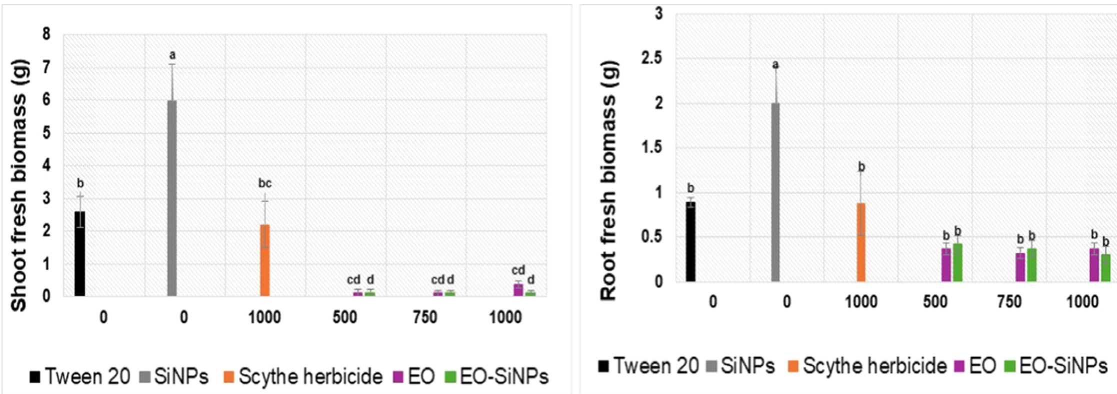

Figure 4. Effect of pristine EO and EO-SiNPs on *A. retroflexus* L. seedling fresh shoot and root biomass. Data are means and SDs of three replicates. At each dose [250 μL/mL ("500"), 375 μL/mL ("750"), and 500 μL/mL ("1000")], bars with different letters are significantly different ($p \leq 0.05$; Tukey's test).

3.2. Assessment of the Herbicidal Activities of Pristine EO and EO-SiNPs. The herbicidal effect of pristine thyme EO and EO-SiNPs against *A. retroflexus* postemergence was assessed in a dose−response assay. Significant damage to treated seedlings was observed after the first application with both treatments, with variability as a function of the treatment type and dose. The EO-SiNP treatment group caused the most severe necrosis in seedling shoots at 24 h, resulting in total wilting at the highest dose. A similar effect was observed in seedlings treated with pristine thyme EO at the same concentration, although the toxicity was less pronounced at lower concentrations of both materials, while no evident damage was noticed for seedlings treated with the commercial herbicide used as a positive control after 24 h of exposure (Figure 3).

The fresh weight of *A. retroflexus* correlated with the observed necrosis symptoms as a function of treatment; both treatment groups significantly influenced the shoot biomass. Specifically, exposure to the Tween 20 negative control resulted in an average fresh shoot biomass of 2.6 g, while the blank SiNP negative control increased the average shoot biomass to about 6 g. However, all preparations of pristine EO (500, 750, and 1000) significantly reduced shoot biomass, compared to those of negative controls. Similarly, all EO-SiNP preparations significantly reduced fresh shoot biomass, compared to the negative controls. However, the effects were not significantly different between the two EO types. The commercial herbicide (Scythe) used as a positive control resulted in a shoot biomass of 2.2 g. Notably, the reductions caused by the pristine EO doses were not statistically significant from the commercial herbicide result, whereas those caused by the EO-SiNPs were statistically different from the commercial herbicide at all doses. In contrast to the shoot observations, root biomass was increased by SiNPs, compared to other treatments, but was unaffected by all other treatments, relatively. This observation is likely a function of the mode of exposure (foliar) and the short treatment time (Figure 4).

These results align with the current literature demonstrating the ability of thyme EOs to inhibit or reduce seed germination and seedling growth.[24,52,53] However, despite the demonstrated herbicidal effect of thyme EO on *A. retroflexus*, formulating a stable EO-based bioherbicide is challenging due to its chemical characteristics, particularly high volatility. Therefore, the encapsulation of EO is proposed as a solution to preserve its efficacy and control the release of secondary metabolites.[54] The results obtained in this study regarding the use of a nanocarrier for EO delivery as a bioherbicide are promising. EO-SiNPs enhanced the efficacy of thyme EO, causing more severe wilting and necrotic symptoms in *A. retroflexus*. To the best of our knowledge, the use of polymers

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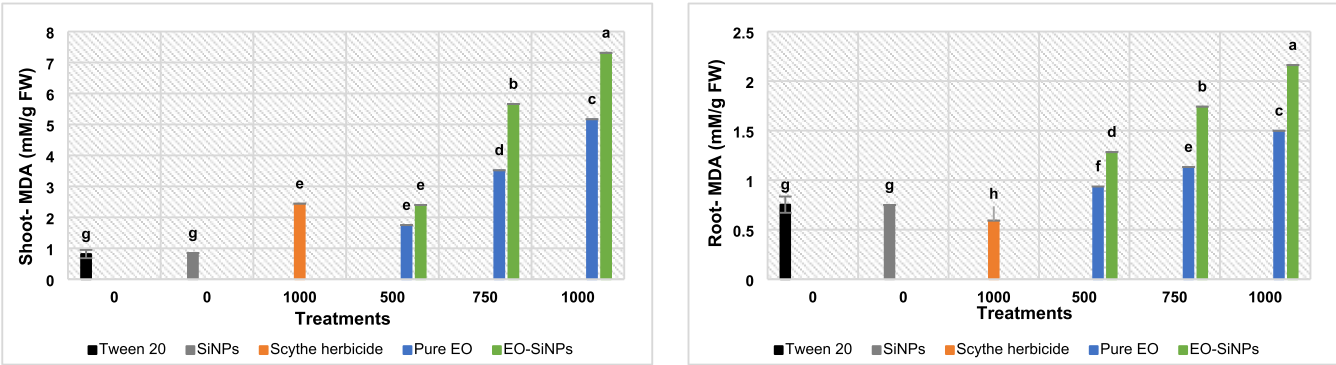

Figure 5. Effect of pristine EO and EO-Nano-Si on the malondialdehyde (MDA) content of *A. retroflexus* shoot and root tissues. Data are means and SDs of nine replicates. At each dose [250 μL/mL ("500"), 375 μL/mL ("750"), and 500 μL/mL ("1000")], bars with different letters are significantly different ($p \leq 0.05$; Tukey's test).

as nanocarriers for the delivery of essential oils for herbicidal purposes is limited to a small number of studies. Our results align with those reported by ref 7, who demonstrated that nanoencapsulated *Satureja hortensis* L. EO in Gum Arabic, Persian gum/gelatin, and Persian gum developed via cross-linking with citric acid and transglutaminase was phytotoxic to *A. retroflexus* as a postemergence treatment. When evaluated 2 days after treatment, depending on the encapsulation type, toxicity ranged between 50 and 400%, compared to the surfactant Tween 80. Unfortunately, no non-nano-encapsulated EO treatment was evaluated in that study. Similarly, refs 55,56 reported enhanced herbicidal efficiency of micro-encapsulated EOs of *Rosmarinus officinalis* L. and *Carum carvi* L. using starch and maltodextrin, respectively, as the biopolymer carriers. These treatments were incorporated into the soil as pre-emergence applications.

3.3. Cell Injury Indices. The Tween 20 and SiNP control treatments had MDA levels of 0.81 and 0.85 mM/g of FW, respectively. Both pristine EO and EO-SiNPs significantly increased the MDA content in the shoots and roots of treated *A. retroflexus* seedlings, compared to the controls (Figure 5). In shoots, the MDA content increased with dose, with encapsulated EO showing a statistically stronger effect than pristine EO across all concentrations. At the lowest EO and EO-SiNP concentration (500), the MDA content was nearly identical to the positive control (Scythe), measuring 2.4 ± 0.2, 1.7 ± 0.2, and 2.4 ± 0.3 mM/g FW for EO-SiNPs, pristine EO, and the positive control, respectively, while the MDA contents in the negative controls were lower than 1 mM/g FW. In contrast, at the highest concentration (1000), the MDA content was approximately 7.3 ± 0.25 mM/g FW for EO-SiNPs and 5.2 ± 0.25 mM/g FW for the pristine EO. Compared to the commercial herbicide (Scythe), the shoot MDA level was significantly increased by the 700 and 1000 EO and EO-SiNP preparations (Figure 5). In the roots, the Tween 20 and SiNP control treatments had MDA levels of 0.75 mM/g of FW apiece. As with the shoot, both types of EO treatments significantly increased the MDA content in a dose-dependent manner in the root. EO-SiNPs also demonstrated a superior efficacy in this case. With the 1000 preparation, the root MDA content was 2.2 ± 0.06 mM/g FW for EO-SiNPs and 1.5 ± 0.09 mM/g FW for the pristine EO. With the 500 preparation, both treatments outperformed the positive control (Scythe), thus highlighting the potentially enhanced herbicidal potential of thyme EO even at lower concentrations. This increase in the MDA content indicates that both treatments promoted lipid peroxidation, causing significant damage to the cell membrane integrity of *A. retroflexus*, with EO-SiNPs being more effective. Importantly, direct shoot exposure with the treatments resulted in root damage at the cellular level, suggesting subtle systemic effects.

The finding of MDA alterations by EO-SiNP is consistent with the results of refs 7,55,56, who observed that their treatments with different encapsulating materials increased the MDA content while decreasing the total chlorophyll, phenolic, and flavonoid contents in *A. retroflexus* and *Raphanus sativus* relative to the biopolymer free controls. According to ref 57, the molecular structure of each EO component may have its own specific mode of action. The herbicidal effect observed in our study could be attributed to the presence of oxygenated monoterpenes, such as thymol, carvacrol, and *p*-cymene, which have been shown to significantly impact weed germination and growth.[28,58,59] It is worth highlighting that the main compounds of the thyme EO used in this study are thymol, carvacrol, and *p*-cymene, which further confirms the herbicidal potential of thyme EO. Ref 22 demonstrated that the terpenic phenol thymol significantly altered the plant water status, increased the abscisic acid content, induced stomatal closure, and caused heat accumulation in the leaf lamina. These changes resulted in a significant accumulation of ROS and damage to the photosynthetic machinery. Refs 60,61 found that carvacrol increased electrolyte leakage and MDA formation in *Arabidopsis thaliana* and *Spinacia oleracea*, respectively, when studying the herbicidal effects of monoterpenes and carvacrol. Notably, the latter study involved carvacrol nanoemulsion. The elevated MDA content noted in this study reflected the leaf damage and necrosis observed during the greenhouse experiments, consistent with previous reports on allelochemicals, mainly terpenoids. These chemicals alter membrane permeability and polarization, leading to electrolyte leakage and lipid peroxidation, causing cell content leakage and resulting in slow plant growth or death.[62−65]

3.4. Evaluation of Total Soluble Protein Content and Antioxidant Enzyme Activities. The total protein content and antioxidant enzyme (APX, CAT, and SOD) activities in both the shoot and root of *A. retroflexus* were evaluated to understand the physiological mechanisms underlying the herbicidal activity of pristine thyme EO and its encapsulated form. The protein content was significantly affected by the treatments in a dose-dependent fashion, as compared to the Tween 20 (0.3 and 0.2 μg for the shoot and root, respectively) and SiNP (0.7 and 0.2 μg for the shoot and root, respectively)

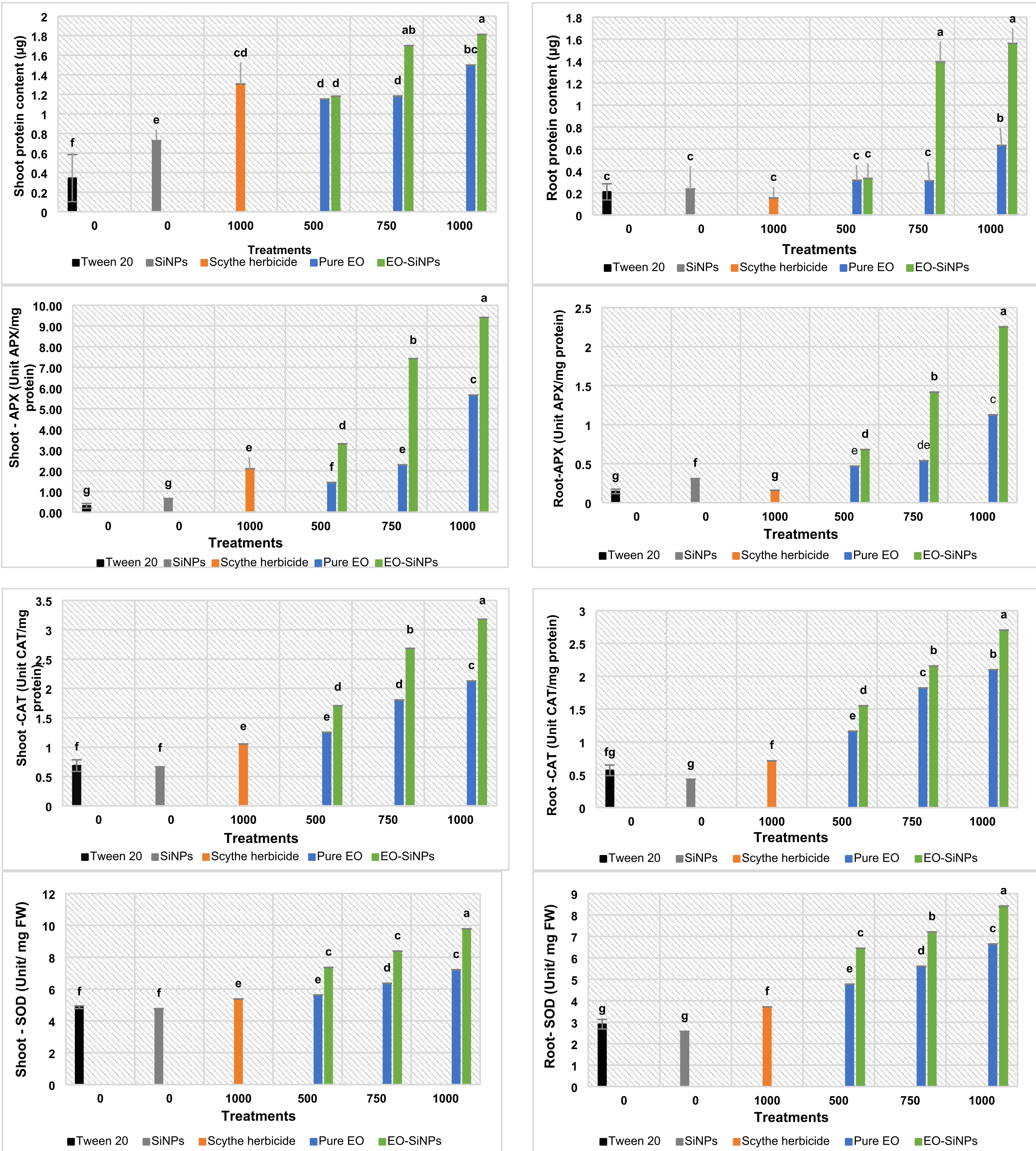

Figure 6. Effect of pristine EO and EO-SiNPs on the total protein content and antioxidant enzyme (APX, CAT, and SOD) activities in shoots and roots of *A. retroflexus* seedlings. Data are means and SDs of nine replicates. At each dose [250 μL/mL ("500"), 375 μL/mL ("750"), and 500 μL/mL ("1000")], bars with different letters are significantly different ($p \leq 0.05$, Tukey's test).

controls (Figure 6). The total soluble protein content increased significantly in the treated seedlings compared to the controls. Pristine EO, and more so EO-SiNPs, showed the highest protein content in the shoots with the 1000 preparations, where EO-SiNPs induced 1.8 ± 0.3 μg proteins in the shoots, significantly differing from 1.5 ± 0.2 μg recorded for the pristine EO. In the roots, the protein content was 1.6 ± 0.1 μg for EO-SiNPs, which also differed significantly from 0.6 ± 0.2 μg obtained for pristine EO. Notably, the shoot protein content was not affected by EO, compared to the commercial herbicide; however, the EO-SiNP 750 and 1000 preparations significantly increased the protein levels, relative to the commercial herbicide product. These outcomes were similar to the root, except in the case of EO 1000 (Figure 6).

In the shoot of *A. retroflexus*, APX, CAT, and SOD activities from the Tween 20 and SiNP control treatments were 0.3 and

0.7 units of APX/mg of protein; 0.7 units of CAT/mg of protein apiece; and 4.9 and 4.8 units of SOD/mg of FW, respectively. In the root, these values were 0.1 and 0.3 units of APX/mg protein; 0.6 and 0.4 units of CAT/mg protein; and 2.9 and 2.6 SOD units/mg FW, respectively, for Tween 20 and SiNP. Notably, APX, CAT, and SOD responded strongly to treatment with pristine thyme EO and EO-SiNPs. The enzyme activities were significantly enhanced and maintained at high levels in both shoot and root under both thyme EO and EO-SiNP exposures at all doses, relative to the above control treatments (Figure 6). Remarkably, EO-SiNPs caused a significantly greater response than EO in both plant tissues. With regard to the commercial herbicide, a shoot APX activity of 2.1 units/mg protein was recorded, which in comparison was significantly increased only by the EO 1000 preparation but more strongly so by the EO-SiNP preparation at all of the concentrations. The commercial herbicide had a shoot CAT activity of 1.1 units/mg protein, which in comparison was significantly increased by EO 750 and 1000 and by all treatments of EO-SiNPs. Similarly, the shoot SOD activity of 5.4 units/mg FW caused by the commercial herbicide was in comparison significantly increased by EO 750 and 1000 and by all treatments of EO-SiNPs. In the root, the commercial herbicide had an APX activity of 0.3 units/mg protein, a CAT activity of 0.7 units/mg protein, and an SOD activity of 3.7 units/mg FW. Notably, all EO and EO-SiNP treatments significantly increased these enzyme activities (Figure 6). Taken together, these results suggest that both pristine thyme EO and EO-SiNPs induced significant abiotic stress in *A. retroflexus* seedlings within 24 h, activating the antioxidant defense systems for reactive oxygen species scavenging. Importantly, EO-SiNPs exerted the greatest phytotoxic effect.

Together, these data demonstrated that EO-SiNPs significantly affected the defense mechanisms of *A. retroflexus*, causing more severe damage than pristine EO. Previous reports have noted similar biochemical and metabolic disturbances in weed species following the application of EO-based treatments.[21,65,66] Here, pristine thyme EO and its nanoformulation induced a generalized increase in the protein content and activated the antioxidant enzyme activities (APX, CAT, and SOD) in the shoots and roots of the seedlings. It was previously observed that treatments with different encapsulating materials increased POD enzyme activity to prevent accumulation of H_2O_2.[7] Together, these findings indicate that the treatments triggered intense ROS production in both shoots and roots, activating the plant's defense mechanisms, particularly through increased antioxidant enzyme activity for ROS scavenging and oxidative stress mitigation. Upon recognizing the stressful condition, one of the earliest plant defense responses is the production of ROS, including singlet oxygen (O), superoxide (O−), hydrogen peroxide (H_2O_2), and hydroxyl radicals (OH−).[67] Although ROS are constantly produced during aerobic metabolic reactions, their levels increase in response to stress.[68] The APX data from the current study suggest that the treatments primarily affected the membrane integrity of the plant cells, as APX significantly increased in response to the treatments. APX has a high affinity for H_2O_2 detoxification, playing a crucial role in removing H_2O_2 and maintaining ROS levels inside the cell. The results for CAT suggest that the treatments moderately impacted the photorespiration system as class I catalases, predominant in photosynthetic tissues, are involved in scavenging H_2O_2 produced during photorespiration. Overall, the inhibitory mechanisms of thyme EO are like those of EOs from other plants, though their main targets remain unclear. Commercial herbicides are generally classified into different categories based on their mode of action and active targets, such as HPPD inhibitors, ALS inhibitors, protoporphyrinogen oxidase (PPO) inhibitors, and ACCase inhibitors. Given the mode of action of the main thyme EO compounds and the evaluation results of the physiological mechanisms underlying its herbicidal activity, it appears that thyme EO is like PPO inhibitors. Protoporphyrinogen oxidase (PPO) inhibitors are primarily contact-type and postemergence herbicides that disrupt cell membranes by inhibiting the PPO enzyme located in the outer envelope of chloroplasts. This inhibition causes the colorless protoporphyrinogen (protogen) precursor to leak into the cytoplasm, where it is converted into photodynamic protoporphyrin IX (proto). In the presence of light, proto generates a burst of ROS that react with membrane lipids, leading to lipid peroxidation and subsequent cell death.[69] In their study on the role of antioxidants in protecting plants against PPO inhibitors, ref 70 noted that increases in certain antioxidants, particularly hydrophilic antioxidants such as reduced glutathione and ascorbic acid (ascorbate), were induced in response to this stress. Conversely, the addition of buthionine sulfoximine, which inhibits glutathione biosynthesis, made plants more sensitive to acifluorfen-methyl. These reducing agents protect plants by quenching ROS generated by the photoactivation of proto, with ascorbate and reduced glutathione providing superior protection against superoxide compared to hydrogen peroxide quenching by ascorbate. This aligns with our findings as we observed significant SOD activity, suggesting that the plant primarily activated both APX and SOD for ROS scavenging and preserving cell integrity. Notably, the encapsulation of EO with SiNPs heightened these enzyme activities, likely due to improved and better targeted active ingredient delivery to the plant, thereby potentiating the role of materials engineering in modulating plant biochemical responses to enhance sustainable agriculture.

3.5. Profiling of Pristine EO and EO-Nano-Si Compounds in Treated Seedlings.

GC-MS analysis of the shoots to characterize the EO profile was completed (Table 2).

Table 2. Thymol Residues Detected in *A. retroflexus* Shoot Extracts Following Treatments with EO and EO-SiNP

	residue (μg/g)		
treatment	500	750	1000
pristine EO	1.6	5.1	15.9
EO-SiNPs	1.2	5.5	

A considerable amount of thymol residue was detected in *A. retroflexus* treated with pristine EO and EO-SiNPs, whereas carvacrol and *p*-cymene were below the analytical detection limits. This indicates that the treatments were able to penetrate the cuticle of the plant leaves, likely the result of the polar surface area of each of these phytochemicals.[42] Because of a lack of shoot tissues due to severe tissue damage, it was not possible to assess the residues of the seedlings treated with EO-SiNPs 1000. Nevertheless, the results of this study, along with the proposed modes of action, were further supported by the profiling of the main active compounds of thyme EO. Thymol residues in the plant extract confirm the successful penetration of the treatments into the cells and demonstrate that SiNPs can be an effective carrier for thyme EO delivery and potentially other agrochemical cargoes.

We have demonstrated that thyme essential oil (EO) can be successfully loaded into silica nanoparticles (SiNPs) to produce a nanobioherbicide, where a combination of TEM, ζ-potential, particle size distribution, FT-IR, and UV−vis analyses confirmed the successful encapsulation of the EO into SiNPs. When used as a postemergence treatment, thyme EO demonstrated strong herbicidal activity. Encapsulating the EO in SiNPs showed a tendency to enhance its toxicity, especially at the highest concentration. Mechanistically, EO-SiNPs cause severe necrosis in seedlings, adversely affecting plant physiological processes. The treatment increased the protein and malondialdehyde content as well as APX, CAT, and SOD enzyme activities, indicating significant reactive oxygen species production and oxidative stress in the weed plant. The results suggest membrane system leakage and considerable oxidative damage to plant cells, implicating protoporphyrinogen oxidase as a potential target for thyme EO. Although Si in various forms is known to provoke plant metabolic responses under different conditions,[71,72] taken together, our data strongly indicate that all of the observed effects were not contributed to by the SiNP. Rather, the EO was responsible for the herbicidal effects that, however, were significantly accentuated by encapsulation with SiNP, due likely to the altered delivery mechanism of the EO to the plant caused by the SiNP, heightening the mechanisms that led to phytotoxicity. These findings therefore provide evidence for the potential use of EO-SiNPs as an effective bioherbicide. Though EOs on their own can control weeds, they suffer from high instability, making their long-term storage a major concern. By formulating EO encapsulated with SiNP, the chance of improving EO stability is greater alongside the enhanced active ingredient release and herbicidal efficacy. To this end, further studies to optimize loading efficiency, understand EO release kinetics under different environmental conditions (such as temperature, light, pH, and humidity) and time, evaluate product stability over time, assess the formulation against a wide range of weed species and food crops, and conduct omics-based studies to better understand the mechanisms of action are underway.

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Notes

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GENERAL CONCLUSION
&
RECOMMENDATIONS

General conclusion and recommendations

The main objective of this PhD research project was to perform a preliminary study on the following species of *Thymus*: *Thymus algeriensis* Boiss. and Reut., *T. ciliatus* Desf subspecies *coloratus* (Boiss. et Reut.) Batt. and *T. vulgaris* (ecotype Fasano and var Varico 3), collected in Algeria and Italy, in order to identify bioactive compounds that could be the starting point for the formulation of a nanoherbicide for an innovative and sustainable control of weeds. For this purpose, the essential oils (EOs) of these species were considered. The study was divided into three main parts: (i) identification of plant material, extraction and chemical characterization of EOs; (ii) *in vitro* and *in vivo* evaluation of the herbicidal activity of the EOs on some weed species; and (iii) formulation tests of a prototype of a EO based nano-herbicide.

The results positively answered the hypothesis expressed by the study. The chemical analysis of the tested *Thymus* sp. pl essential oils (EOs) displayed a high variability of secondary metabolites. Forty-three chemical compounds were identified; *Thymus algeriensis* and *T. ciliatus* subsp. *coloratus* were characterized by the absence of carvacrol and traces of thymol in *T. ciliatus* and the predominance of linalool, α and β-pinene, α-terpenyl acetate, borneol and camphene, while for *T. vulgaris* species, *p*-cymene, thymol, carvacrol were the main constituents. This finding demonstrates the high chemical variability of thyme species EOs, arguing a broad spectrum of biological activities.

Biologically, all the EOs demonstrated a significant phytotoxic potential on *Lolium perenne* and *Amaranthus retroflexus* under *in vitro* and *in vivo* conditions, confirming the presence of bioactive compounds with a potential herbicidal activity. Thyme EOs strongly inhibited seed germination of both weed species and considerably slowed down seedling growth, especially *A. retroflexus.* On the contrary, *L. perenne* seedlings expressed a resistance reaction under *in vivo* conditions. This achievement supports a selectivity of the herbicidal potential of thyme EOs. Additionally, applied as contact treatments by spraying, the minimum inhibition concentration (MIC) of seed germination for both species and *A. retroflexus* seedlings was 5 µL/mL while it was 20 µL/mL for *L. perenne* seedlings. These results suggest several modes of application of thyme EOs, e.g. pre-or post-emergence applications, as preventive or curative treatments. Furthermore, *T. vulgaris* ecotype Fasano exhibited the most potent herbicidal activity, while *T. ciliatus* subsp. *coloratus* was the least effective.

A prototype nano-herbicide based on thyme EO was successfully produced. *T. vulgaris* var Varico 3 was selected for the formulation of the nano-herbicide. This choice was motivated by the agronomical characteristics (hybrid variety) of the species and the similarity of its chemical composition with *T. vulgaris* ecotype Fasano. In that regard, *T. vulgaris* var Varico 3 EO was successfully loaded into silica nanoparticles (SiNPs). The EO-SiNPs fabricated had a regular spherical appearance, particle size ranging between 220 and 300 nm and an EO distribution in both internal and external surfaces of SiNPs. EO-SiNPs were negatively charged with a high particle dispersion stability. Through FT-IR analysis, characteristic of C–H out-of-plan bending vibration from isoprenoids and isomeric compounds such as thymol, carvacrol, *p*-cymene and eucalyptol have been identified in EO-SiNPs spectrum, confirming the loading of the EO into the SiNPs. However, the loading efficacy of thyme EO into SiNPs was relatively low with percentages ranging from 26 to 64%, according to the EO concentration.

Concerning the herbicidal potential of the fabricated nano-herbicide, applied by spraying as a post-emergence treatment, it enhanced the toxicity of thyme EO. It demonstrated a potent herbicidal activity against *A. retroflexus*. Biochemical analyses revealed a disturbance of the plant defense system through a significant increase of protein and MDA contents and antioxidant enzyme activities (APX, CAT and SOD) in both shoots and roots of the target plant. These findings suggest that nano-capsulated thyme EO induced significant abiotic stress in *A. retroflexus* seedlings, activating the antioxidant defense systems for reactive oxygen species (ROS) scavenging and oxidative stress mitigation. The increase in MDA content and APX indicates that the treatments promoted lipid peroxidation, causing significant damage to the cell membrane integrity of the target plant. Importantly, applied as contact treatment, thyme EO-SiNPs translocated to roots, causing damage at the cellular level, suggesting, in this sense, systemic effects. These results were also suggested by those obtained from the cytological trials of pristine thyme EO on *A. thaliana,* which revealed a likely hormonal balance alteration, inducing a possible increase of both auxin and ethylene contents. In summary, the biochemical and cytological results suggest membrane system leakage and considerable oxidative damage to plant cells, implicating protoporphyrinogen oxidase as a potential target for thyme EO. These findings, therefore, provide evidence for the potential use of EO-SiNPs as an effective bioherbicide. Even if pristine thyme EO expressed an important herbicidal potential, the encapsulated form insured a greater stability, enhanced the active ingredient delivery and herbicidal efficacy.

In the light of the results achieved as well as the related literature review, this study could open the way to further experiments. To the best of our knowledge, it is one of the first investigations into the use of a combination of nanomaterial and EO delivery through nanoparticles for herbicidal purposes. Given what has been achieved in this research study, some conclusions and recommendations may be drawn. Sustainable farming systems are growing fast, requiring more biological inputs for pest and disease control. In this perspective, the study offers a contribution and opens the way for further research to: a) identify pure bioactive molecules responsible for the herbicidal activity, through purification bioassays, this would allow the precise evaluation of their effectiveness, alone or in combination, to express eventual synergies. The identification of the essential oil responsible for the herbicidal activity requires a check of the efficacy of the phytocomplex and/or of the major compound, which would allow a precise evaluation of their efficacy; (b) introduce into cultivation some wild aromatic and medicinal species under-valorized and known for their herbicidal potential to guarantee a constant raw material production, EO yield and stable phytochemical composition. The herbicidal potential of some wild aromatic and medicinal species could represent an opportunity to valorize these genetic resources through a domestication process that allows a stable production in terms of quality and supports the possible creation of a production chain. This perspective has already been taken into consideration in our study by initiating bio-stimulation experiments of some thyme species using endophytic bacteria and fungi; this approach is in line with the use of biological inputs as fertilizers, aiming to address the challenges posed by climate change and meet specific soil and climate requirements. (c) extend the biological experiments to other weed species to assess the selectivity of pristine EOs and their encapsulated forms; (d) use natural soils as growing medium to evaluate the effectiveness of pristine EOs and fabricated nanomaterials under different natural conditions, with the aim to evaluate the interactions between the treatment, weed plants and soil properties; (e) understand and improve characteristics and performance of the coating nano-enable materials to optimize loading efficiency, EO/active compounds release, efficacy and stability over time; (f) in-depth understand molecular and physiological disturbances through omic studies to optimize the applied doses or modes of application; even if our results suggested protoporphyrinogen oxidase as a potential target for thyme EO, it could be interesting to better investigate this first funding; (g) study of the eco-toxicity of both forms of EOs (the pristine EO and its encapsulated forms) on human health and animals (One Health Approach), their impact on growth and yield of crops; to identify new biological molecules expressing an herbicidal activity, especially because the regulation is going stricter concerning plant protection products (PPP) allowed in agriculture; and their impact of the use of this fabricated nano-herbicide at farm level and on biodiversity and ecosystem services.

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