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Article

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

Rym Boukhalfa ¹, Claudia Ruta ^{1,*}, Saida Messgo-Moumene ², Generosa J. Calabrese ³, Maria Pia Argentieri ⁴ and Giuseppe De Mastro ¹

¹ Department of Soil, Plant and Food Sciences (DiSSPA), University of Bari Aldo Moro, 70126 Bari, Italy

² Department of Biotechnologies, University Saad Dahleb, Blida 09101, Algeria

³ International Centre for Advanced Mediterranean Agronomic Studies Mediterranean Agronomic Institute of Bari (CIHEAM-IAMB), 70010 Bari, Italy

⁴ Department of Pharmacy-Drug Sciences, University of Bari Aldo Moro, 70125 Bari, Italy

* Correspondence: claudia.ruta@uniba.it; Tel.: +39-080-5442975

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 (500, 750, 1000 and 2000 µl/100 mL) were applied. All the EOs expressed an important bioherbicidal activity against the target species. A total inhibition of seed germination was displayed at 2000 µl whereas a potent effectiveness was achieved even at 500 µl. On the contrary, for post-emergence experiments, *L. perenne* showed resistance to the EOs while *A. retroflexus* was highly sensitive, arguing a selectivity of the EOs. Bioassays were also 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.

Keywords: Thyme; bioherbicidal activity; pre and post emergence; *A. thaliana* (L) Heynh; structural modification

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 [1,2]. 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 [3].

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 [4,5]. 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 [6].

In that regard and in the perspective of promoting integrated weed management (IWM), research on bio-herbicides has greatly increased [7]. 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 [7,8]. Thus, bioherbicides are developed, especially those of plant origin [9,10]. 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 [11,12]; the mode of action of these secondary compounds seems to be similar to synthetic herbicides. In this perspective, allelochemicals can act by altering seed germination or interfering with seedlings' establishment [13]. Given their ecological role in natural environments, these secondary metabolites could be considered promising molecules to be employed for the production of new classes of plant-based herbicides [14,15].

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 [16–18]. 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) [19]. [20] 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 [21–24]. 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.

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 [25]. For this species, the biological effectiveness of its EOs has been demonstrated, especially thanks to their large chemotype diversity.

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. Results

2.1. Herbicidal Activity of the EOs against Target Weeds

2.1.1. 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 Figure 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 2000 μL an effective herbicidal potential for *L. perenne* was expressed by all the tested EOs, inducing a total inhibition of the germination. At 1000, no statistical differences were found between the EOs; all of them significantly inhibited seed germination, reducing it by over 90%. A similar results was obtained using the concentration of 750 μL for T1, T2 and T4, while T3 seemed less effective, even if, also at the lowest concentration (500 μL) the reduction of seed germination was between 83% (T1) and 77,5% (T3) (Figure 1a).

T2 expressed the strongest herbicidal effect on *A. retroflexus*, where a total inhibition of seed germination was induced at 2000 and 1000 μL . At 750 and 500 μL , 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 a total inhibition at 2000 μL 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 a less significant effectiveness in comparison with T2 and T3. An important reduction of seed germination was noticed for the other concentrations (Figure 1b). However, it is still important that a total inhibition was achieved at 2000 μL 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*.

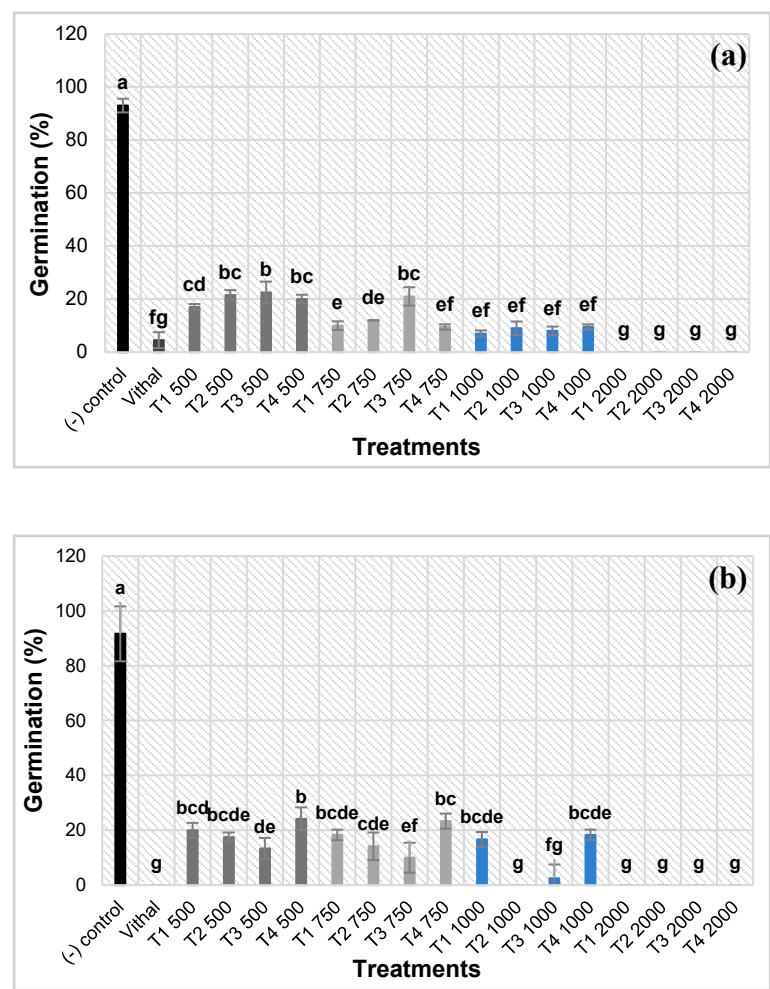


Figure 1. Effect of the different treatments and doses on seed germination of *L. perenne* (a) and *A. retroflexus* (b) after 15 days exposure to a 500- 2000 µl/100 mL range of concentrations of the four tested EOs. T1: *T. algeriensis*, T2: *T. ciliatus* subsp. *coloratus*, T3: *T. vulgaris* cultivar Varico 3, T4: *T. vulgaris* ecotype Fasano. Data are means of three replicates. At each dose, column with different letters are significantly different ($p \leq 0.05$, Tukey's test).

2.1.2. 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 (2000 µl), where the mortality of all the plantlets was observed. For the other concentrations, all the seedlings were able to survive after the exposure to the treatments. On the contrary, *A. retroflexus* strongly reacted to the treatments from the first application. It displayed wilting symptoms few hours after the exposure to the EOs, inducing to the mortality 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 demonstrate the high sensitivity of *A. retroflexus* plantlets to the treatments (Figure 2).

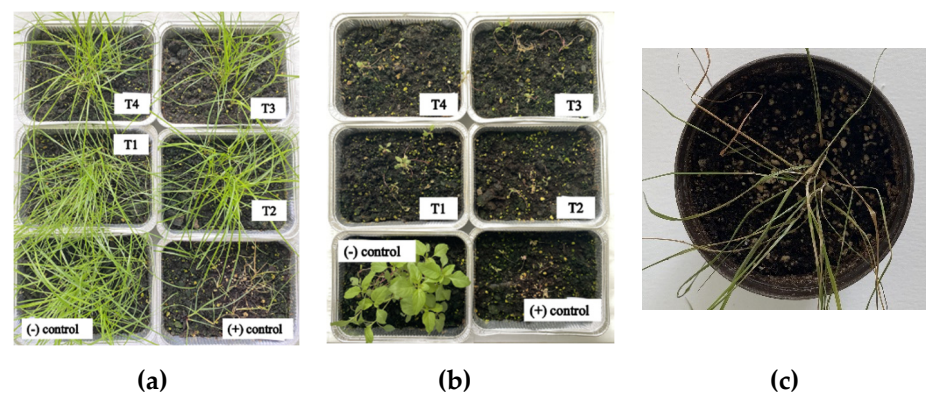
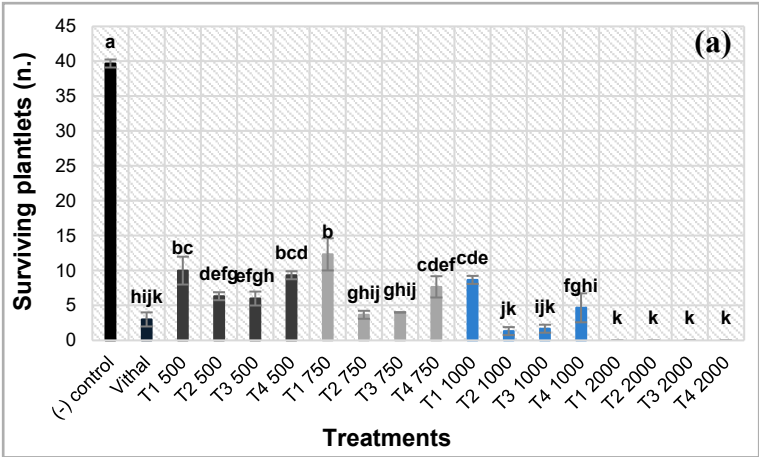


Figure 2. Effect of the treatments on target weed species plantlets. (a): *L. perenne* seedling treated with EOs at 1000 µl, (b): *A. retroflexus* seedling treated with EOs at 1000 µl, (c): *L. perenne* seedling treated with EOs at 2000 µl.

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 (2000 µl), all the seedlings died, while the number of surviving ones was considerably reduced at 1000 µl. The same effectiveness was observed at 750 and 500 µl where the number of surviving plantlets was importantly reduced. T4 provoked the mortality of all the plantlets at the concentration of 2000 µl. 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 (500 µl). 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 mortality of the seedlings at 2000 µl, and drastically decreased it to less than 13 plantlets for 750 µl, which was the less effective dose (Figure 3a). The results of the fresh biomass of *A. retroflexus* are correlated with those of the number of surviving plants (Figure 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 (2000 µl) exhibited an effective effect of the treatments on the seedlings. For all the treatments, the biomass was null at 2000 µl and insignificant at 1000, 750 and 500 µl.



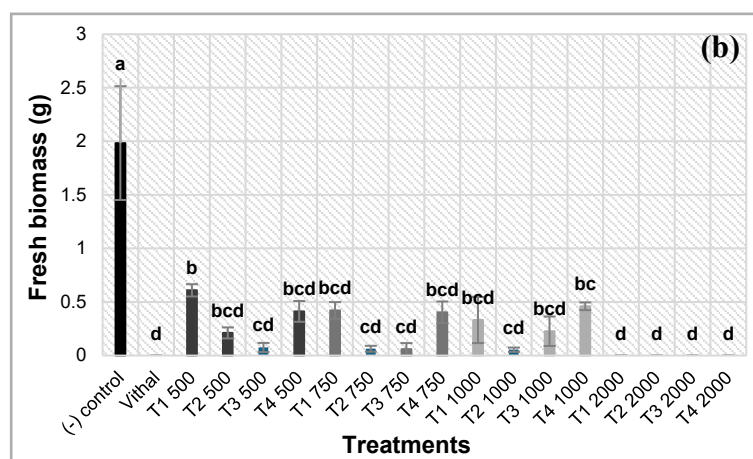


Figure 3. Number of surviving (a) and fresh biomass (b) of controls and treated *A. retroflexus* plants, 24 hours after exposure to the different treatments and doses (500- 2000 μ l/100 mL range of concentrations) of the four tested EOs. T1: *T. algeriensis*, T2: *T. ciliatus* subsp. *coloratus*, T3: *T. vulgaris* cultivar Varico 3, T4: *T. vulgaris* ecotype Fasano. Data are means of three replicates. At each dose, column with different letters are significantly different ($p \leq 0.05$, Tukey's test).

2.3. Phytotoxic activity of EOs on *Arabidopsis thaliana*

The mode of action of the studied EOs was evaluated on the model plant *A. thaliana*. The EOs treatments did not significantly affected *A. thaliana* germination; however, the root growth and development of the EO-treated seedlings were significantly affected. After 14 days of growth, EO-treated seedlings were visualized under a stereomicroscope to evaluate the morphological changes induced by the EOs on the seedlings. Several anomalies were observed depending on the EO. Roots were either shorter, thinner, with visible proliferation of adventitious roots, with important condensation or without root hair in comparison to that of control (Figure 4). Furthermore, EOs reduced the number and length of the root hairs. Some *A. thaliana* seedlings even presented a loss of gravitropism.

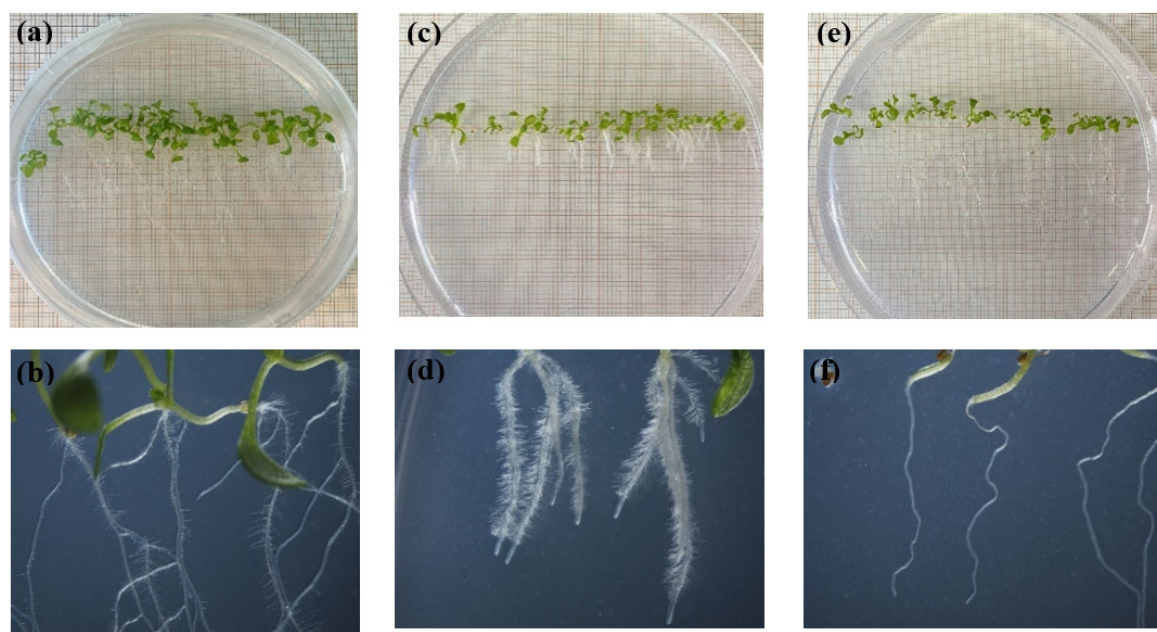


Figure 4. Images of *Arabidopsis thaliana* seedlings control and treated with thyme EOs. (a, b): control; (c, d): short roots and condensate root hair belonging to seedlings treated with T1; (e, f): absence of lateral and root hair of seedlings treated with T3.

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 [26,29], 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 (Figure 5).

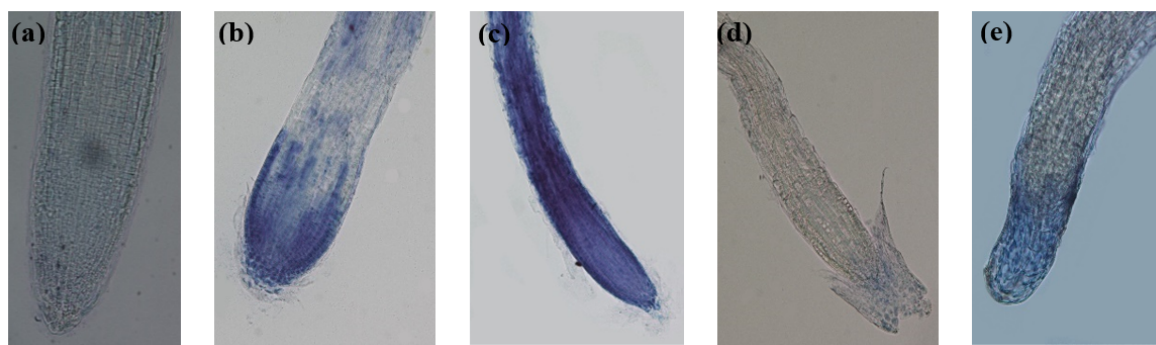


Figure 5. Effect of thyme EOs on the loss of membrane integrity of apical root longitudinal sections of *A. thaliana* stained with Evans Blue reagent visualized under light microscopy. (a): control, (b): EO-treated roots with T1; (c): EO-treated roots with T2. (d): EO-treated roots with T3 and (e): EO-treated roots with T4. Images taken at 10 and 20X magnification.

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 (Figure 6).

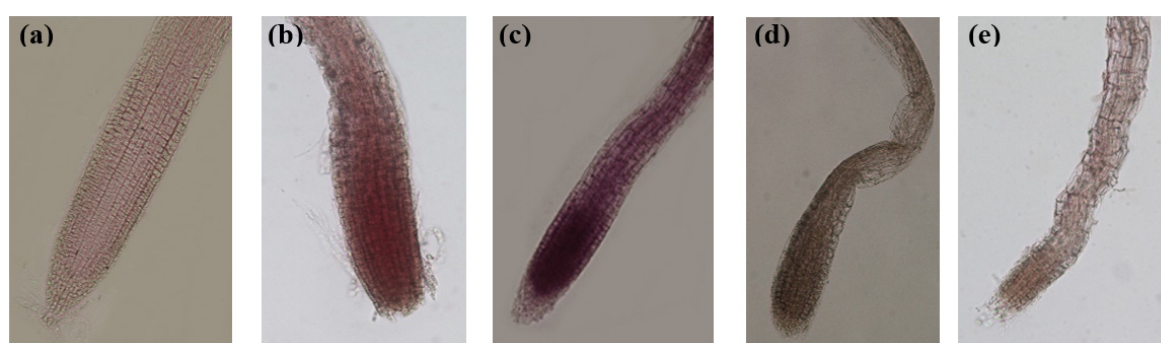


Figure 6. Histochemical determination of lipid peroxidation in *A. thaliana* roots under the effect of thyme EOs by Schiff's reagent (longitudinal sections of root apices). (a): control, (b): EO-treated roots with T1; (c): EO-treated roots with T2. (d): EO-treated roots with T3 and (e): EO-treated roots with T4. Images taken at 10 and 20X magnification.

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, while EO-treated roots presented

an evident and significant tissue disruption. The calyptra was thicker and the cell rows were disorganized and hardly distinguishable. Torsions with zig-zag development were also observed in the elongation zone; and in some samples the growth patterns were completely altered, showing toxic effects, such as contraction phenomena. In some EO-treated seedlings, the columella, including the statocytes, was destructed; this could explain the loss of the gravity perception of the roots.

This section may be divided by subheadings. It should provide a concise and precise description of the experimental results, their interpretation, as well as the experimental conclusions that can be drawn.

3. Discussion

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. Indeed, the herbicidal potential of *Thymus* sp. pl. EOs were investigated by several researchers who demonstrated significant efficacy in inhibiting seed germination and seedling growth of indicator test and weed species [24,27–33].

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 [34,35], which according to [36], 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 [36].

The inhibition and/or the slowdown of seedling growth by thyme EOs, in turn, could be associated with the disturbance of photosynthesis inducing to an oxidative stress [23,37]. [38] 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 [39–41] 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 (H₂O₂), can accumulate and cause irreversible damage to plasma membranes when defense mechanisms are inefficient. The ROS causes damage on proteins, DNA, lipids, cell membranes and photosynthetic pigments, and finally leads to plant mortality [41].

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 [42] 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 [43–45]. [46] suggested that the seedlings release bioactive molecules in the medium that might contrast the effect of the applied treatments. [47] 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 [48]. According to [49], this is due to the high genetic diversity of *Lolium* spp., an obligate outcrossing mating system and a recurrent selection with a herbicide might lead to metabolic cross-resistance to other herbicides even from different MOA

[49,50]. 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 [16,51], which supports our findings. The tested thyme EOs used in this study contain an important percentage of monoterpenes, particularly, α -pinene, thymol, carvacrol, o-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 [52,53]. 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 [54–56]. 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 [57]. In addition, the molecular structure of each component can be responsible for the specific MOA [58].

The *in vitro* experiments on the model plant *A. thaliana* were performed to better understand the alterations caused by thyme EOs. Morphological and structural damages were observed, mainly 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 widely reported in plants exposed to allelochemicals. [59], 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 [19,60], 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; [61] 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 [62]. 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) [61]. 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 [63]. [64] 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 [65,66]. 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 [67]. 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. Materials and Methods

4.1. Plant material and EOs Extraction and Characterization

Aerial part of 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 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) [68]. EOs were extracted by hydro-distillation and identified by gas chromatography coupled with mass spectrometry in a previous study [68]. The chemical composition of the EOs is presented in Table 1. EOs were stored at 4°C until their use in the biological activity.

Table 1. 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	tricyclene	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-3ylacetate	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	p-mentha-1,5 dien-8-ol	1170	1174	1.72	--	1.76	1.65

N°	Compound	KI ¹	KI ²	T1	T2	T3	T4
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 : literature; ² : calculated							

4.2. 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. 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 [69]. Four concentrations (500, 750, 1000 and 2000 µl/100 ml of 2% of Tween® 20 solution) were applied. Water 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 21x16 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.

4.2.1. Pre-emergence assay

Fifty (50) 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 [70]:

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

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 to 60% of humidity and were monitored daily for irrigation.

4.2.2. 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, fifty (50) 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 to 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.

4.3. Phytotoxic Activity of EOs on *Arabidopsis thaliana* (L.) Heynh

To evaluate the physiological damages caused by the EOs on the target weed species, assays on *Arabidopsis thaliana* were conducted. 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 [26,59] 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 Petri 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 [59].

Each treatment and control was prepared in triplicate. The morphology was analyzed at the 14th day using a magnifier (Nikon SMZ 1500) and light microscope (Leica DMLB100, Milan, Italy). Root structure, thickness, morphology and abundance of root hairs of EOs-treated and control radicles of *A. thaliana* were 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 [71]. 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; K₂S₂O₅ or Na₂S₂O₅ 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 replicate 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).

4.4. 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 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 ($p < 0.05$) [72].

5. 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 results revealed a likely hormonal balance alteration.

Taken together, these results demonstrated that thyme EOs altered the hormonal balance of the recipient weed species, inducing a possible increase of both auxin and ethylene content, and stimulated oxidative damage. Both hormones and ROS could alter the normal plant growth interfering with cell division and cytokinesis. The discovery of natural molecules that present in thyme EOs, characterized by a potent herbicidal potential is important for the development of a selective new bio-chemicals able to overcome the use of synthetic inputs. However, due to their chemical instability, EOs can be easily degraded, a way to protect them could be the nano-encapsulation as it might protect them and enhance their stability against environmental conditions; future studies on the preparation of bio-nanostructured systems to encapsulate thyme EOs would allow not only to investigate and better understand thyme EOs' effects on recipient plant metabolism but could be interesting to enhance their stability while preserving their efficacy as bio-herbicide.

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