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Bio-Herbicidal Potential of the Essential Oils from Different *Rosmarinus officinalis* L. Chemotypes in Laboratory Assays

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Abstract: The current study aimed to assess the allelopathic effect of *Rosmarinus officinalis* L. essential oils (EOs) to define the potent effect against weed species, by exploring distinct chemotypes and their main compounds. The EOs from eight accessions were characterized. Their components were identified by gas chromatography, and four chemotypes were defined; C1 (*α*-pinene), C2 (camphor), C3 (*α*-pinene/1,8-cineole), and C4 (*α*-pinene/1,8-cineole/camphor). Four concentrations of the EOs (400, 800, 1200, and 2400 µL/L) and the main compounds of each chemotype were tested in a laboratory assay against *Amaranthus retroflexus* L. and *Lolium perenne* L. in pre- and post-germination. The results showed that the EOs significantly affected all the tested parameters (germination, early growth, and physiological and histological parameters of the weeds under study) in a dose, chemotype, and species dependent manner. *A. retroflexus* was more sensitive than *L. perenne* at germination level being significantly inhibited at the lowest dose of all the chemotypes. The latter all exhibited significant effects but with a higher potency of C2 (camphor chemotype) and C3 (*α*-pinene/1,8-cineole chemotype), as well qualitative differences in the induced damage. Our results thus increase knowledge about the role of the monoterpene composition in bio-herbicidal effect, which can help in the development of EO based bio-herbicides.

Keywords: sustainable weed management; monoterpenes; *Rosmarinus officinalis* L.; chemotypes; bio-herbicides; germination; post-germination

1. Introduction

In all farming systems, weeds account for the most expensive pest to manage causing up to 30% of total potential losses from all pests [1]. In organic farming, they are the number one constraint organic farmers face in the field and are even their first preoccupation when considering conversion to organic farming [2]. Indeed, as weed management can reach up to 50% of the total production cost, notably in intensive organic horticulture [3]. This higher complexity is mainly related to the restriction of the use of synthetic herbicides and the lack of potent alternatives under allowed natural herbicides [4]. The latter class is available to organic farmers currently in very few options and at a high cost for the necessary rates [5,6].

In this context, many studies have documented an urgency to explore new options for more effective and viable direct tools for weed management under organic farming. Much attention is being given, in that sense, to the utilization of the allelopathic potential in plants as an ecologically based

weed control tool. Allelopathy can be defined as a form of interaction that occurs between plants by means of chemical substances (allelochemicals) released in their surroundings from living or decaying tissues [1]. Many plant species can release allelopathic compounds with a highly phytotoxic effect, which can negatively interfere with the growth of other plants [7,8].

A large number of the widely known allelopathic species, such as *Calamintha nepeta* L. *Origanum vulgare* L., *Satureja hortensis* L., and *Rosmarinus officinalis* L., belong to the Lamiaceae family, which were extensively explored for bio-herbicidal potential [9–20]. The phytotoxic effect of their extracts, particularly essential oils (EOs), was linked to the presence of volatile bioactive compounds, such as α-pinene, limonene, 1,8-cineole, carvacrol, camphor, and thymol, which possess varying phytotoxic levels [7,21–26]. Nevertheless, considering the critics regarding the high costs of the EOs in question, care should be taken with the production cost and EO yield when choosing the source plant species.

R. officinalis (Rosemary) is one EO bearing species belonging to the Lamiaceae family, which is native to the Mediterranean area and now cultivated worldwide. It is a low-demand plant with an important amount of EO yield, reaching up to 2.5% [27]. According to many authors, its EO is rich in monoterpenes, such as 1,8-cineole, α-pinene, and camphor, which are known to be involved in allelopathic interactions and to have a bio-herbicidal effect against various weed species [12,13,16].

However, the use of *R. officinalis* EO as a bio-herbicide is still facing many limitations. First, this species has been reported to have intraspecific chemical variations, resulting in different chemotypes [28–33]. This could be a problem in the sense that the described bio-herbicidal effect in the literature is not specific, as it is not clear whether the chemical variation affects the level of phytotoxicity of the EO. In addition, the previously reported effect is unclearly selective [12,14,16], and the mechanism of action is not well understood.

Thus, the present work aimed at defining the potent effect of *R. officinalis* EO against weed species by exploring distinct chemotypes and their main compounds.

2. Materials and Methods

2.1. Plant Material

Eight *R. officinalis* (R1–R8) accessions were collected and identified, according to Good Agricultural and Collection Practices (GACP), from different areas of southern Italy and maintained ex situ (field collection) under the same conditions 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"). At the flowering stage in April 2019, the aerial parts of the plants were cut, dried in an oven at 35 °C for two days, and then stored into paper bags at room temperature in the dark until the time of extraction.

The experimental weed species were *Amaranthus retroflexus* L. and *Lolium perenne* L., which were chosen due to their wide distribution, their easy germination, and considering the two main groups (monocots and dicots); they were purchased from Herbiseed Company and stored at 4 °C for use during the experiment.

2.2. Essential Oil Extraction and Analyses

Leaves of the eight *R. officinalis* accessions separated from stems were subjected to hydrodistillation following the guidelines of European Pharmacopoeia, 2016, Ph. Eur. 9 [34]. The distillation was held for 3 h in a Clevenger-type apparatus. The oil was dried over anhydrous sodium sulphate and then recovered in amber glass bottles sealed with parafilm and stored at 4 °C for further analysis and use. Three sets of extractions were completed and served as analytical replicates.

The chemical characterization was carried out by a gas chromatography—mass spectrometry (GC/MS); gas chromatograph (Agilent 6890N) coupled to an Agilent mass spectrometer 5973N (Agilent Technologies, Cernusco sul Naviglio, MI, Italy). The latter is equipped with a data processor (Agilent enhanced Chemstation MSD G1701DA D.03.00.611 version). The injection of 1 μL of EO diluted in

dichloromethane (v:v, 1:300) was carried out at split ratio of 1:25 and under 1.1 mL min^{-1} flow of Helium. The separation was assured with a capillary column HP-5MS (5%-phenyl-methypolysiloxane, 0.25 mm × 30 m × 0.25 μm film thickness). The injector and the transfer line temperatures were set to 250 and 300, respectively. The heating of the column was carried out in three steps: first 60 °C, then programmed to 240 °C at 3 °C min^{-1}, then increased to 280 °C at 8 °C min^{-1}, and held for 5 min. The total run time was set at 60 min for each sample.

The chromatograms obtained were integrated, and then the retention index (Kovats index) was calculated, based on direct injection of a mixture of aliphatic hydrocarbons (C8–C30; Sigma, IT—Milan) in n-hexane into the GC according to the above mentioned temperature program, for all the integrated compounds. This index, together with the indications of the Wiley (1995) library, served to identify the compounds. In the end, a library of compounds was elaborated containing three replicates for each accession.

2.3. Classification and Chemotype Definition

In order to define distinct chemotypes from the accessions that were analyzed, the hierarchical clustering method was used. Among the volatile constituents, those which showed an average percentage equal to or higher than 1% in all accessions were selected, which were to be included in the cluster analysis, 11 out of 20 (55%). After conducting the statistical analyses, the optimal number of clusters was determined by using a statistical approach but also considering the objective of the study (testing the differences in bio-herbicidal effect between variable combinations of volatile compounds). Finally, to establish the chemotype for the number of the resulting clusters, a percentage criterion was defined to suit the data that was obtained:

- single main compound chemotype: one compound representing more than 33% of the total composition and all the rest was less than 20%
- two main compounds chemotype: two compounds together representing more than 50% of the total composition and all the rest was less than 15%
- three main compounds chemotype: three compounds together representing more than 60% and all rest was less than 5%.

2.4. Germination Test

A germination bioassay was carried out on the two experimental weed species using the EOs from the defined *R. officinalis* chemotypes. To comparatively examine their inhibitory effect and relate it to the presence of the main compounds, a range of concentrations were tested. For the EOs: 400, 800, and 1200 μL/L, while for the main compounds their solutions were prepared in a way to simulate their concentrations in the EOs (60% camphor, 40% α-pinene, and 25% 1,8-cineole), and were tested at a concentration of 1200 μL/L.

The main standard compounds were purchased from Sigma Company at a grade of purity of: 98, 96, and 99% for α-pinene, camphor, and 1,8-cineole, respectively.

All the solutions were prepared using Tween 20 (T20) (1%) diluted in distilled water. The distilled water alone and added to T20 were used as two negative controls, while a registered commercial bio-herbicide (C.B) based on pelargonic acid served as a positive control (Finalsan 18.9% w/w pelargonic acid). To apply the treatments, ten seeds of each weed species were sterilized using 1% and 5% NaOCl for *A. retroflexus* and *L. perenne*, respectively, and then were placed on the filter paper in 9 cm diameter Petri dishes, and 2 mL of each treatment (EOs, standard solutions, controls) were added. Immediately after, the Petri dishes were sealed with parafilm and placed in a controlled growth chamber in the dark at 25 °C. All the treatments and controls were replicated three times following a completely randomized design.

The germination was monitored every 24 h, and seeds showing radicles of more than 1 mm were considered as germinated. After seven days (when no further germination was observed in the water

control), the percentage of the total number of germinated seeds (GP), the seedlings length, and the weight were measured per Petri dish, and served to calculate the germination inhibition (GI) and the seedling vigor index (SVI).

Germination percent (GP) [19]

$$GP = ((\text{Number of germinated seeds at final count})/(\text{Total number of seeds for bioassay})) \times 100. \quad (1)$$

Germination inhibition (GI) [12]

$$GI = ((GP\ (\text{Water}) - GP)/(GP\ (\text{Water}))) \times 100. \quad (2)$$

GI is germination inhibition percent (%); GP (Water) is the germination percent of water treatment; GP is the germination percent for each treatment.

Seedling vigor index (SVI) [19]

$$SVI = ((\text{Seedling length (mm)} \times GP)/100). \quad (3)$$

In order to study the role of the main compounds, their individual induced GI and then their additive one in respect to their combinations in the chemotypes were calculated and compared to the GI of the respective chemotypes in order to understand the possible synergies. The additive effect was calculated by using the simple addition of individual effects in accordance with the method adopted in studying the effect of a drugs' synergistic combination [35].

2.5. Post Germination Test

To simulate the action of the EOs and the main compound solutions on post-emergence, a test was conducted on seven-day-old seedlings using the three different controls and the same treatments as in the germination trial, plus one more, represented by a double concentration (2400 μl/L) in respect to a lower sensitivity observed in preliminary tests.

All the treatments and controls were replicated three times following a completely randomized design.

The weed seeds (50 for each species) were sterilized in the same manner as the germination test, put in Petri dishes with 2 mL of distilled water, and placed in growth chamber at the seeds optimal growth conditions (25 °C dark) for seven days. On the seventh day, samples were taken for weight measurement (W0) before applying all the treatments. Immediately after the application, the Petri dishes were sealed with parafilm and placed in a growth chamber in 16 h/8 h light/dark at 25 °C for 48 h. In order to investigate the eventual damage, different samples of fresh seedlings were taken for the range of the analysis at the end of the 48 h: (i) Weight increase measurement after treatment (Wt), (ii) Electrolyte Leakage (EL), (iii) Membrane peroxidation, and (iv) Plasma membrane integrity.

i.: Weight increase measurement

Total fresh and dry weight (Wt) of three seedlings for each replicate were taken to evaluate the relative growth rate (RGR) using the method proposed by Hoffmann and Poorter [36]. The drying was done in an oven at 50 °C for 72 h. The formula used was the following:

$$RGR = \left(\text{In}\ (Wt) - \text{In}^{f0}(W0)\right)/(t1 - t0) \quad (4)$$

where W0 and Wt are weight before and 48 h after treatment, respectively, and t0 and t1 are time of the (W0) and Wt measurements, respectively.

ii: Electrolyte leakage

To measure the electrolyte leakage (EL), 200 mg of seedlings at 48 h after treatment were cut and placed in test tubes containing 10 mL of distilled deionized water. The tubes were incubated at 25 °C on a rotary shaker for 24 h to allow the tissues to leak solutes into the solution, and, subsequently,

the electrical conductivity of the solution (L0) was measured. Samples were then autoclaved at 120 °C for 20 min and the final electrical conductivity (Lt) was measured after equilibrium at 25 °C. The measurements of electrical conductivity were made by using a conductimeter WTW (LF330 model). The EL was then determined by using the following formula [37]:

$$EL\ (\%) = (Lt/L0) \times 100. \quad (5)$$

iii.: In situ detection of membrane peroxidation

A histochemical detection of lipid peroxidation was performed by using Schiff's reagent (Carlo Erba Reagents s.r.l.). The latter 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) $K_2S_2O_5$ or $Na_2S_2O_5$ in 0.05 M HCl). Later three roots were taken from different replicates for treatment. The terminal apex of the roots was cut and photographed under a microscope (Leica DMLB100, Milan, Italy) at 100x magnification equipped with an imaging system program. The color intensity was quantified by measuring the image pixel intensity using X-PRO Analysis Image X-Pro analytical software (Alexasoft, Florence, Italy) designed to optimize image capture with Nikon DXM 1200 Digital Camera (Tokyo, Japan). A linear distance of 1000 μm from the root apex was considered, and the pixels reported were an average of 10 representative measurement points of the area. The values are inversely proportional to the intensity considering a scale from 0 (black) to 255 (white) pixel. A lower intensity of red subpixels indicated a more intensive red staining and a higher lipid peroxidation

iv.: Plasma membrane integrity

Another assessment for the weeds' cell membrane integrity was performed using Trypan blue (Carlo Erba reagents s.r.l.). Three roots for each replicate were placed in test tubes with Trypan blue solution 10 mg/mL for 15 min. After this the colorant was removed from the tubes and the roots were rinsed three times with distilled water [38]. Directly afterwards, the choice of representative roots and the processing under the microscope were handled in the same way as described in the membrane peroxidation test (see Section 3). In this case, a lower intensity of blue subpixels indicated a more intensive blue staining and a greater effect on the loss of membrane integrity.

2.6. Statistical Analyses

The EOs content data was analyzed using one way analysis of variance (ANOVA) after checking for normality and homoscedasticity assumptions. Tukey's mean comparison test was used. For the chemotype definition hierarchical cluster analysis was adopted using the linkage between groups and simple Euclidian distance. After conducting cluster analysis, the optimal number of clusters was determined using the elbow method (minimizes within groups differences). Regarding the phytotoxicity tests parameters, the parametric test assumptions (Normality/Variance equality) were verified for the original data and for the transformed ones with a range of arithmetic transformations (Log, sqrt, arcsin). According to this verification, the KRUSKAL WALLIS test, followed by the Dunn test to compare the means, were conducted for GI, SVI, and RGR, while, for EL and pixel intensity, as indirect measurements of the EL, two-way ANOVA (chemotype and concentration factors) was conducted, followed by Tukey's test to compare the means. All tests were conducted by using SPSS 20.0 software (SPSS, Chicago, IL, USA).

3. Results

3.1. EOs Content and Chemical Composition

The average yield resulting from four extractions per accession is reported in the Table 1. It showed that the EO content differed significantly between the accessions. It ranged from 1.35 to 4.12% (v/w) for R5 and R4, respectively. Regarding the chemical composition, in Table 2 the identified compounds based on GC/MS analysis are reported, supported by the Kovats retention index (RI) calculation.

In all the accessions, they represented more than 90% of the total composition. The results showed a considerable variation in the proportions of compounds between the accessions.

Table 1. Essential oils (EOs) content of the eight accessions.

Accession	R1	R2	R3	R4	R5	R6	R7	R8
EO * (V/W%)	1.76 ± 0.04 ^{cd}	1.94 ± 0.08 ^{cd}	1.43 ± 0.09 ^{cd}	4.12 ± 0.32 ^{a}	1.35 ± 0.18 ^{d}	1.76 ± 0.15 ^{cd}	2.15 ± 0.88 ^{c}	2.95 ± 0.80 ^{b}

* Values represent mean ± standard deviation. Means with different letters are significantly different ($p < 0.05$) Tukey's test.

Table 2. The chemical composition (%) of the EO from the *R. officinalis* accessions.

Component	Composition (%)									
	RI *	R1	R2	R3	R4	R5	R6	R7	R8	Average
α-Pinene	933	41.85	38.38	36.58	9.54	33.80	34.96	38.89	21.60	31.94
Camphene	948	7.70	8.21	7.72	5.68	5.87	7.56	8.32	7.28	7.27
Verbenene	953	0.15								0.15
β-Pinene	977	0.63								0.63
3-Octanone	984	0.72						0.15		0.43
β-Myrcene	988	3.26	2.76	3.83		1.11	3.13	5.00	1.22	2.89
α-Phellandrene	1001								2.24	0.28
α-Terpinene	1014								3.76	0.47
ρ-Cymene	1023			1.44		1.00			1.65	0.51
1,8-Cineole	1029	13.70	12.09	12.85	9.53	26.24	11.66	24.54	24.89	16.93
γ-Terpinene	1058	0.80	1.27		1.35	1.58	1.52	2.74	2.04	1.60
Linalool	1096	1.40	1.56	2.53		3.04	1.98	0.92		1.89
Camphor	1142	7.85	8.93	9.50	62.0		10.16	6.16	17.25	17.39
Borneol	1164	3.15	4.26	5.28		7.82	4.67	3.44	3.80	4.63
ρ-Cymen-8-ol	1187	1.49	1.75	0.93	1.79	2.18	1.85	1.53	2.16	1.70
Verbenone	1205	2.96	2.79	2.38		1.88	2.86			2.55
Bornyl acetate	1283	8.93	9.34	7.77		10.89	9.85	4.95	4.09	7.97
Total		94.58	91.36	90.81	90.0	95.29	90.02	96.63	91.96	

* RI: Retention Index.

According to the cluster analyses results illustrated in the dendrogram (Figure 1), they could be clustered into different number of groups depending on the distance. The most discriminating compounds between these groups were mainly α-pinene, camphor, and 1,8-cineole.

Using the elbow method, the optimal number of groups was four, which were: (i) R1; R2; R3; R6, (ii) R4, (iii) R5, and iv) R8 (Table 3). Groups, that according to the percentage criterion established, permitted the identification of four chemotypes: α-pinene (C1), camphor (C2), α-pinene/1,8-cineole (C3), and α-pinene/camphor/1,8-cineole (C4).

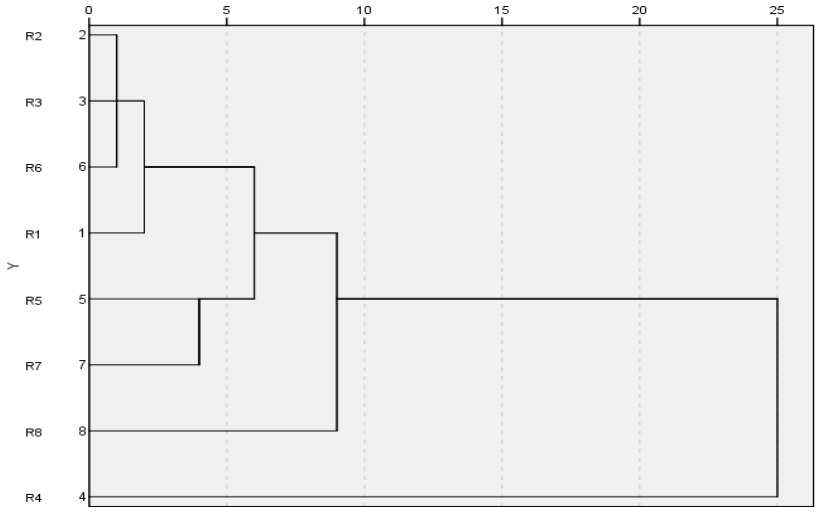

Figure 1. Dendrogram of the distance between the eight accessions of *Rosmarinus officinalis* L., using hierarchical clustering method.

Table 3. Grouping of eight *Rosmarinus officinalis* L. accessions into chemotypes based on the major component of essential oils.

Code	Accessions	Chemotypes
C1	R1, R2, R3, R6	α-pinene
C2	R4	camphor
C3	R5	α-pinene/1,8-cineole
C4	R8	α-pinene/1,8-cineole/camphor

3.2. Germination Test

The final GI calculation showed that the EOs significantly inhibited the seed germination of both *L. perenne* and *A. retroflexus*, as shown in Table 4. The observed response depended significantly on the concentration, while the chemotype showed less difference. In the case of *L. perenne*, only the highest applied concentration (1200 μL/L) of the EOs obtained from all the chemotypes caused significant germination inhibition. The values recorded ranged from 81% (C1 and C4) to 96% (C2).

As for *A. retroflexus*, the application of the EOs from the four chemotypes at all concentrations significantly reduced the germination compared to the water control, except for C4 at the lowest rate (400 μL/L), where only 32% of the seeds were not able to germinate. The T20 treatment did not affect the germination of both species, confirming the hypothesis that its addition to the treatment with essential oils is irrelevant.

On the contrary, the commercial bio-herbicide (C.B) totally inhibited the germination of both species in the same way of all the chemotypes at 800 and 1200 μL/L rate applied on *A. retroflexus*, while, in the case of *L. perenne*, it was comparable only to the C2 and C3 EOs at 1200 μL/L.

EOs from the C2 and C3 caused the highest germination inhibition for both species, notably for *A. retroflexus*, for which C2 completely inhibited the germination at both 800 and 1200 μL/L rates. Regarding the comparison with the main compounds, the calculated GI showed that C1 EO containing 40% of α-pinene was significantly more effective in inhibiting the germination than the same percentage of pure α-pinene in both weed species (Figure 2). Moreover, the germination inhibition effect of C3 EO (1,8-cineole/α-pinene chemotype) was significantly higher than that of 25% 1,8-cineole and 40% α-pinene in either their individually or combined effects. However, when comparing the effect of C2 EO containing 60% camphor with the individual effect of the same amount of the pure compound, it showed that they were statistically comparable as they both expressed the highest effects.

Nevertheless, camphor EO (C 2) had a slightly reduced effect than the individual compound for *L. perenne*. Furthermore, although the C3 and C4 EOs (1,8-cineole/α-pinene/camphor chemotype) had statistically comparable effects, a trend of lower one for the latter was visible for both species.

Table 4. Effect of the different EOs on seed germination inhibition and on seedling vigor index of *A. retroflexus* and *L. perenne*.

Treatments		*L. perenne*		*A. retroflexus*	
		GI (%)	**SVI (mm)**	**GI (%)**	**SVI (mm)**
Controls	**Concentrations** (μL/L)				
Water			12.80 a		4.54 a
T20		7 e	9.33 ab	2 d	2.57 a
C.B		100 a	0.00 f	100 a	0.00 c
	Chemotypes				
C1	400	44 bcde	1.27 bcd	73 abc	0.23 bc
	800	74 bcde	0.08 def	86 abc	0.12 bc
	1200	81 bcd	0.03 def	82 abc	0.16 bc
C2	400	30 cde	1.97 bcd	86 abc	0.10 bc
	800	56 bcde	0.54 cde	100 a	0.00 bc
	1200	96 ab	0.01 ef	100 a	0.00 bc
C3	400	26 de	3.03 bc	64 bc	0.32 bc
	800	74 bcde	0.55 cde	91 abc	0.07 bc
	1200	89 abc	0.16 cde	91 abc	0.07 bc
C4	400	22 cde	2.54 bc	32 c	0.67 b
	800	48 bcde	1.40 bcd	95 abc	0.03 bc
	1200	81 bcde	0.09 def	86 abc	0.10 bc

Means with the same letter are not significantly different at $p \leq 0.05$ significant level (Dunn test). T20: Tween 20, C.B: Commercial bio-herbicide, C1: α-pinene, C2: camphor, C3: α-pinene/1,8-cineole, C4: α-pinene/camphor/1,8-cineole, GI: germination inhibition, SVI: Seedling vigor index.

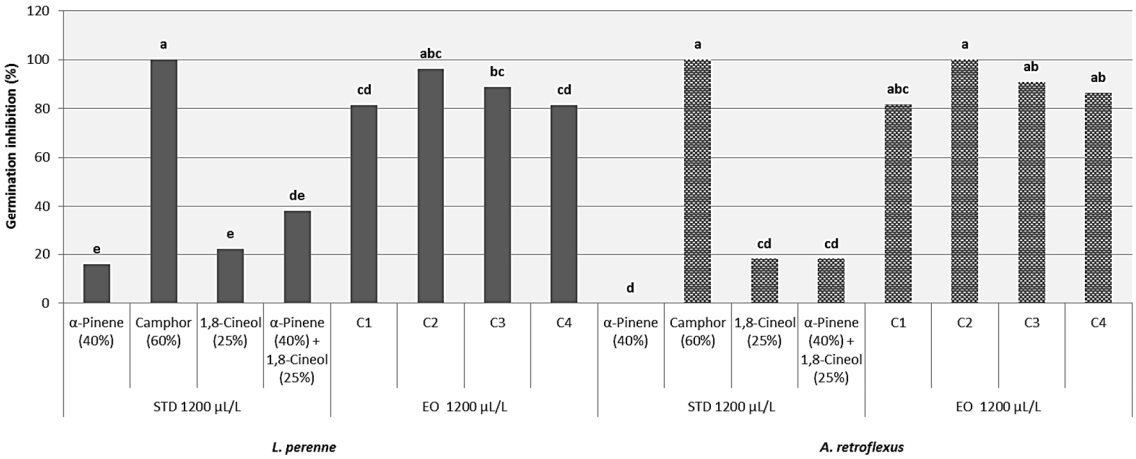

Figure 2. Comparative effect of the EOs and their corresponding main compounds at 1200 μL/L on the germination inhibition of *A. retroflexus* and *L. perenne*. C1: α-pinene, C2: camphor, C3: α-pinene/1,8-cineole, C4: α-pinene/camphor/1,8-cineole, STD: standard. Means with the same letter are not significantly different at $p \leq 0.05$ significant level (Dunn test).

When considering the SVI (Table 4), the effect of the EOs was more pronounced. On the seventh day, all the EO treatments induced a significantly lower vigor index than the controls for both species studied, as shown in Table 4. Moreover, the inhibition level was concentration dependent in the case of *L. perenne*, notably with C2 and C4 EOs where significantly higher effect was detected at the higher applied rate (1200 μL/L) than the lower one. In the case of *A. retroflexus*, a high sensitivity was already noted at 400 μL/L with significant difference compared to the non-treated control, which also highlights the higher sensitivity of this species. The chemotype variation did not show significant differences on seedling vigor inhibition, confirming what was also detected in the GP response.

3.3. Post-Germination Test

3.3.1. Relative Growth Rate

The evaluation of the relative growth rate of the young seedlings at 48 h after treatment showed a varying response between chemotypes, concentrations, and species (Table 5).

Table 5. Effect of the EOs on the relative growth rate of *A. retroflexus* and *L. perenne* seedlings 48 h after treatment.

Treatments		**Relative Growth Rate (mg/h)**	
		L. perenne	***A. retroflexus***
Controls	**Concentrations** (μL/L)	NS	
Water		1.93	8.61 a
T20		2.92	8.72 ab
C.B		0.00	0.00 c
Chemotypes			
C1	800	0.00	0.73 abc
	1200	1.89	4.00 abc
	2400	6.74	6.14 abc
C2	800	0.00	0.00 c
	1200	0.00	0.00 c
	2400	1.82	0.00 c
C3	800	3.43	0.00 c
	1200	1.31	0.00 c
	2400	1.32	0.00 c
C4	800	2.42	5.07 abc
	1200	4.36	2.00 abc
	2400	0.66	0.00 bc

Means with the same letter are not significantly different at $p \leq 0.05$ significant level (Dunn test). T 20: Tween 20, C.B: Commercial bio-herbicide, C1: α-pinene, C2: camphor, C3: α-pinene/1,8-cineole, C4: α-pinene/camphor/1,8-cineole, NS: Not Significant.

Only the RGR of *A. retroflexus*, which was in general higher than that of *L. perenne*, was significantly affected by the treatments when compared with the non-treated control. Moreover, the C2 and C3 were particularly inhibitory at all concentrations where no increase in RGR was recorded 48 h after treatment, and C4 induced significantly lower growth rate than the control only at the highest concentration. However, C1 induced the lowest RGR at 800 μL/L but with no significance compared with the non-treated control.

All the treatments were not significantly effective in the case of *L. perenne*, which can probably be explained by the generally low growth rate exhibited by this species at this young stage.

3.3.2. Electrolyte Leakage and Histochemical Analysis

At the end of 48 h, no visible injury or wilting was detectable in the EO treatments in contrast to the C.B that caused visible damage. However, the measurement of the relative electrolyte leakage (EL) and the evaluation of the roots staining showed an evident effect of the EOs on membrane permeability and integrity of the seven-day-old seedlings. The observed effect was dose, chemotype, and species dependent.

L. perenne seedlings expressed no excessive EL when measured at 48 h following the application of the different EOs at 400, 800 and 1200 μL/L rates compared to the non-treated control, and only at the rate of 2400 μL/L was the EL significantly higher (Figure 3).

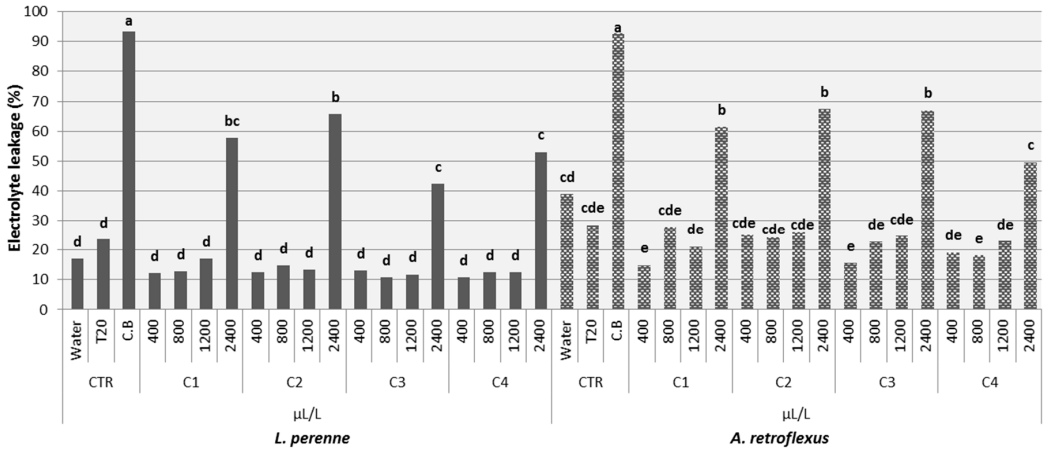

Figure 3. Effect of the different EOs at different doses on the relative electrolyte leakage of *A. retroflexus* and *L. perenne* seedlings, measured 48 h after treatment. CTR: Controls, T20: Tween 20 and C.B: Commercial bio-herbicide; C1: α-pinene, C2: camphor, C3: α-pinene/1,8-cineole, C4: α-pinene/camphor/1,8-cineole. Means with the same letter are not significantly different at $p \leq 0.05$ significant level (Tukey's test).

However, the EO-induced damage even at the highest concentration was not comparable to the C.B induced damage (92%), which is known to cause electrolyte leakage. Furthermore, the C 2 chemotype (65.74% EL) showed an EL effect closer to the C.B and significantly different from the farthest C 3 (42.36% EL).

The membrane damage was also visible with the Trypan blue staining (Figure 4), a negatively charged molecule that can penetrate and blue stain only the cells which had lost their membrane integrity [39].

Therefore, the results showed an increase in the induced damage following the dose increase, as visible visually and expressed by the measured coloration intensity (expressed in white to blue pixels; Figure 4). This, however, was not observed for all the chemotypes, as C3 EO was clearly less damaging to the root cell membrane than all the other tested EOs. The results described in this staining test confirmed the data recorded with the EL test. Indeed, in the case of the C3 EO, the cells showed a lower blue color intensity testifying to less damage to the integrity of the membrane. On the contrary, with C.B, the greater color intensity was associated with the highest value of EL. In addition, the color intensity trend for the C1 and C2 EOs showed an increase at 1200 μL/L, which is in line with their significantly faster growth curve as compared with the others.

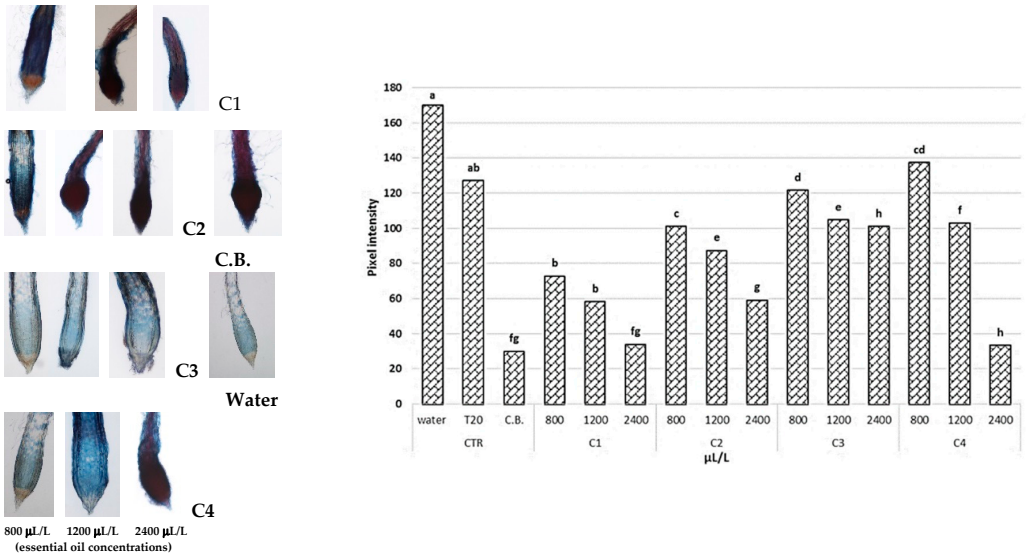

Figure 4. Effect of different chemotype EO concentrations on the intensity of loss of membrane integrity in roots of *L. perenne* stained with Trypan blue (**left**) and relative pixel intensity (lower intensity indicates a higher staining blue intensity and a higher loss of membrane integrity) (**right**). **C1**: α-pinene, **C2**: camphor, **C3**: α-pinene/1,8-cineole, **C4**: α-pinene/camphor/1,8-cineole. CTR: water, T20: Tween 20 and C.B: Commercial bio-herbicide. Means with the same letter are not significantly different at $p \leq 0.05$ significant level (Tukey's test).

Concerning Shiff's staining, it is a reagent which reacts with aldehydes from the lipid peroxidation resulting in a red coloration of the tissues. Hence, the results illustrated in Figure 5 showed the same dose-dependent effect observed with the Trypan blue test, as seen in the increased red color intensity, and confirmed by the decreased red subpixel measurement. However, a different chemotype-dependent trend from Trypan blue and EL can be noticed; as the roots treated with C3 EO were the most intensely stained, those treated with C1 were the least intense. Nevertheless, this difference between chemotypes was not as wide as in the EL and Trypan blue tests. In fact, Shiff's reagent test suggested the ability of all the EOs to induce membrane peroxidation at especially high doses, such as 2400 μL/L, at which they performed better than the C.B. Nevertheless, this mode of action was unlikely to be responsible for the EL, since it could not explain the higher one in the C.B, the C2, and C1 EOs treated seedlings, nor the lower one applying the C3 EO.

The response of *A. retroflexus* seedlings to the application of the EOs expressed a significantly high EL compared to the non-treated control only at the rate of 2400 μL/L (Figure 3).

However, the EOs were not able to induce comparable EL to the C.B (93%), even at the highest concentration. Values not dissimilar to each other and much closer to treatment with CB occurred with the chemotypes C1, C2, and C3 at the highest dose (61.47, 67.39, and 66.98% EL, respectively). In general, the response of *A. retroflexus* seedlings to the application of the EOs was similar to *L. perenne* in terms of cell membrane damage.

As for root staining with Trypan blue, the results showed a difference in sensitivity between the species, since all the treatments had comparable staining also expressed by similar pixel intensity (data not shown). Furthermore, no clear trend was visible following an increase in the dose. These results suggested a lower sensitivity of *A. retroflexus* root membrane integrity to the EOs, or indicate the sensitivity of its root tissue to the Trypan blue stain, which is reported to be cytotoxic and can induce damage to cells [40]. And since a significant increase in electrolyte leakage following the application of the EOs at only 2400 μL/L was demonstrated, the latter hypothesis seems to be more likely.

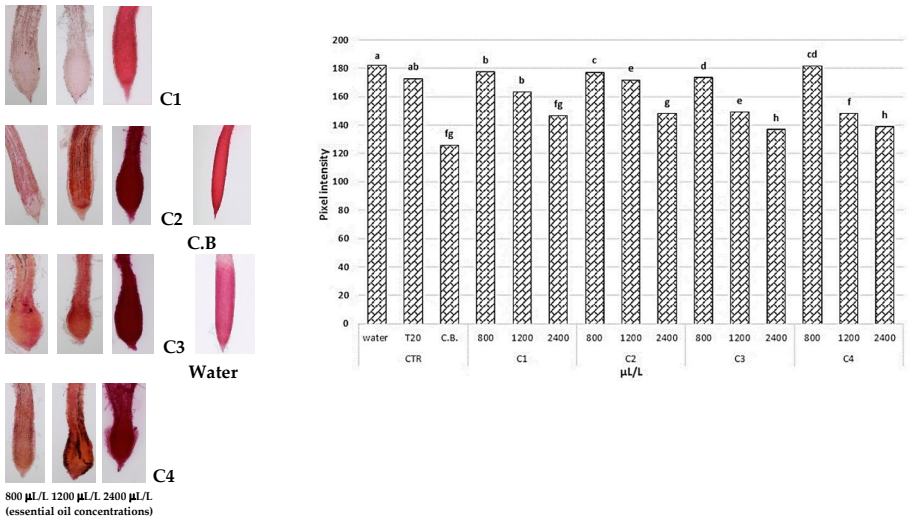

Figure 5. Effect of different chemotype EO concentrations on the intensity of lipid peroxidation in roots of *L. perenne* stained with Shiff's (**left**) and relative pixel intensity (lower intensity indicates a higher staining red intensity and a higher lipid peroxidation) (**right**). **C1**: α-pinene, **C2**: camphor, **C3**: α-pinene/1,8-cineole, **C4**: α-pinene/camphor/1,8-cineole. CTR: water, T20: Tween 20 and **C.B**: Commercial bio-herbicide. Means with the same letter are not significantly different at $p \leq 0.05$ significant level (Tukey's test).

The visual assessment (Figure 6) of Shiff's reagent staining, as well as the color intensity measurement on the pixel scale, showed an almost equal value for all the treatments, which was comparable to one of the controls. Only the C.B induced color intensity was slightly higher than the non-treated control, although no significant difference was found for any treatment. These results might indicate a lower sensitivity of *A. retroflexus* roots compared to those of *L. perenne*, as well as an unlikely ability of EOs to induce the membrane peroxidase on the roots of both species. Therefore, the induction of excessive EL by the EOs at high concentration could be related to a different action mechanism.

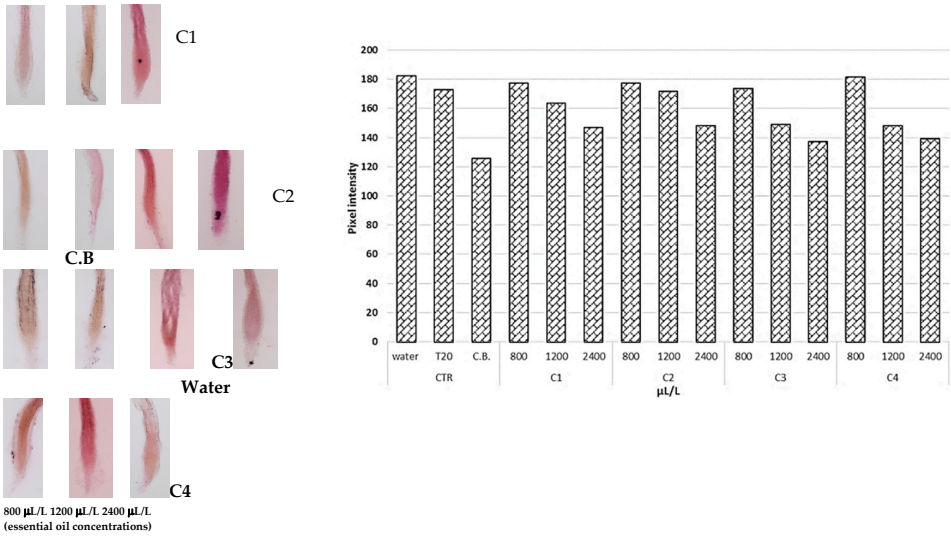

Figure 6. Effect of different chemotype EO concentrations on the intensity of lipid peroxidation in roots of *A. retroflexus* stained with Shiff's (**left**) and relative pixel intensity (lower intensity indicates a higher staining red intensity and a higher lipid peroxidation) (**right**). **C1**: α-pinene, **C2**: camphor, **C3**: α-pinene/1,8-cineole, **C4**: α-pinene/camphor/1,8-cineole. CTR: water, T20: Tween 20 and **C.B**: Commercial bio-herbicide.

4. Discussion

The resulting quantitative and qualitative characteristics of the *R. officinalis* EOs were generally comparable to those reported in previous studies [11,16,27,41,42]. What is more, the existence of different chemotypes was also highlighted in the literature and similar ones to those defined in the present study have already been described by other authors [27,43].

When testing their bio-herbicidal potential, the EOs of *R. officinalis* at the range of studied chemotypes under laboratory conditions were found generally more effective when applied on the seed stage rather than to young seedlings for both studied weed species.

For the germination test, the observed effect was more dose dependent than chemotype dependent since no significant differences were detected between the chemotypes. The germination of *A. retroflexus* was found to be more affected than *L. perenne*, as it was significantly inhibited by the lowest applied concentration (400 μL/L). Similar studies regarding the germination inhibition of *R. officinalis* EO to *A. retroflexus* also report its sensitivity at low concentrations, such as 300 or 400 μL/L [13,16]. The germination of *L. perenne* on the other hand was significantly reduced only at 1200 μL/L regardless the chemotype (at least 87% reduction). This finding is in line with what has been reported in previous studies about the higher sensitivity of dicots compared to monocots [12,14,16].

However, as the seedling vigor of *L. perenne* was also affected by the lower applied concentration, the tegument permeability and size of the seeds of this species could be behind the limitation of the effectiveness on germination. This effect of the tegument type on the germination inhibition efficacy with EOs has also been reported by previous studies [44–46].

As for the early growth test, the application of *R. officinalis* EOs at the range of studied chemotypes was found to cause varying levels and types of phytotoxicity depending on the species, chemotype and dose. The latter was generally the most determining for all the studied parameters, notably for the ones with membrane damage, which were affected at only the highest applied dose regardless of the chemotype. However, the chemotypes were also determining for the damage level and type, differently from the germination test. Actually, significant differences were detectable between the chemotypes, as C2 EO had significantly higher effect than C3 on EL and plasma membrane integrity, while C3 induced the highest membrane peroxidation. Hence, the chemotypes were found to affect the mode of action. Moreover, the damage type to the membrane differed between the two studied weed species, as *L. perenne* seedlings were more likely to undergo membrane peroxidation than *A. retroflexus*. This can be relatable to the findings of Mutlu et al. [47], who assessed membrane peroxidation induced by catmint EO on seven different weed species and found that only *A. retroflexus* was not susceptible to this mechanism of action. However, from our findings, no direct relationship could be concluded between this difference in sensibility to membrane peroxidation and the membrane functioning, which was affected in the same dose-dependent manner for both species. Membrane dysfunction, mainly the increased permeability expressed in EL, could be more related to the results of the Trypan blue test in the case of these study results, which can suggest the creation of lyses or pores in the cell membrane as a possible mechanism of action of the EOs since no signs for cell death were detectable [48].

In other studies, EOs can induce weed tissues EL in a dose and time dependent manner when tested under laboratory conditions [37,49–51]. However, the significantly effective doses reported are generally lower than those found in our study. This could be related to the different EOs and terpenes tested in those investigations, as they could be significantly determining for the effect as reported in the present study. The membrane peroxidation results can also comply with previous studies, as EOs were reported to cause peroxidation of polyunsaturated fatty acids in the weeds' bio membranes [52,53]. However, differently from our findings, the detected membrane peroxidation was in many cases correlated to the EL [54]. This can also be explained by the different EOs used, as shown in the presented results, even varying chemotypes in the same species resulted in varying damage types.

An attempt to relate the expressed membrane damage to the growth rate reduction showed that the latter is unlikely to be caused by the observed contact damage. This is because no growth reduction

was detectable within the 48 h in the case of *L. perenne*, and the described membrane damage was not relatable to RGR decrease in the case of *A. retroflexus*. In fact, the seedlings in some treatments expressed a decrease in RGR but no EL increase (C2 and C3 at 800 and 1200 μL/L), while C1 at 2400 μL/L expressed the opposite trend. Therefore, the EO treatments that showed effectiveness in reducing the seedlings early growth may act by a different mechanism than direct damage to the membrane. Previous investigations found that allelochemicals, including EOs, can inhibit the growth of weeds by the alteration of cells division and cycles [54–56] or by reducing their elongation through action on hormones, such as auxin, cytokinin, and ethylene [57]. However, the effect found on the membrane can be considered to be an early sign of phytotoxicity that can result later in a significant effect on the weeds growth or cause programmed cells death.

Regarding the investigation on the main monoterpenes' role in the effect of the EOs, α-pinene and 1,8-cineole applied individually were unable to express significant phytotoxicity at the tested doses, but they showed high toxicity when present in EO complexes (mainly C3 EO). Moreover, the addition of these two compounds' effects was still significantly lower than that of the C3 EO, which eliminates the action of their simple additive effect. Therefore, the high phytotoxicity observed with the C3 EO could be related to a synergic effect of α-pinene and 1,8-cineole, or to the effect of minor compounds, such as bornyl acetate (10%). The first hypotheses can be supported by previous studies proving the existence of synergic effects between different monoterpenes [57]. For camphor however, it was found to be able to cause high phytotoxicity individually, which was relatable to the effect of the C2 EO, meaning that it enhanced its effect. This is in agreement with the results of Sekine et al. [58], who demonstrated that camphor was the major inhibitory compound in rosemary. However, this claim is probably only valid if this compound is present in a dominant way. When applied alone, camphor was slightly more effective than the EO containing it at the same proportion. Furthermore, the C4 EO, containing camphor in combination with the other two compounds, was found slightly lower than that of C3 EO and was, in some cases, only non-significantly effective. Hence, we can hypothesize that camphor could only have a high effect at certain high concentrations or that it could cause an antagonist effect to the other compounds when present with them in the EO complexes. Other studies confirm the high toxicity of camphor as a ketonic monoterpene as compared to other compounds, like α-pinene or 1,8-cineole [22,59]. Nevertheless, no evidence was previously reported about an antagonistic effect in EOs related to this compound.

5. Conclusions

The EO of *R. officinalis* at the range of studied chemotypes was phytotoxic to the experimental weed species, but with a visible higher potency of C2 (camphor chemotype) and C3 chemotypes (α-pinene/1,8-cineole chemotype), and some differences in the type of induced damage. Moreover, as the high phytotoxicity of C2 EO could be related to its camphor content, we can suggest camphorous *R. officinalis* EO (when camphor is dominant: >60%) as a particularly good bio-herbicide candidate. As for the C3 EO, further investigations (effect of the minor compounds) are needed to chemically specify and standardize its effect. Interestingly, the camphor chemotype had a considerably higher EO yield, which also supports its higher cost efficient potential. The dose was the greatest determining factor in all the studied effects; dicots were more susceptible than monocots, and the seed stage was more susceptible than young seedlings when the damage was measured 48 h after treatment. The mode of action of the observed effect in both stages could probably be related to cell division and elongation inhibition rather than direct damage to the membrane by contact, which can probably explain the higher sensitivity during the first days of germination expressed by low SVI. However, the detected damage on the membrane may be an early sign of additional phytotoxicity, which can be confirmed by monitoring weeds in the periods after the application. Our results thus increased knowledge about the role of the monoterpene composition as natural products that provide new and important biological agents at the forefront of integrated weed management strategies. In order to confirm the utility of

these results, the next step of our studies will be testing the EO's interaction with soil under greenhouse and field conditions.

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