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Effect of Different Parameters (Treatment Administration Mode, Concentration and Phenological Weed Stage) on *Thymbra capitata* L. Essential Oil Herbicidal Activity

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Abstract: The essential oil (EO) of *Thymbra capitata* has been demonstrated to possess herbicidal activity and could be used as an alternative to synthetic herbicides with reduced persistence in soil and new mode of action. Nevertheless, it is necessary to determine the adequate doses for its use, the proper way for its application and the best phenological stage of weeds and crops in which the EO should be applied to obtain maximum efficacy against weeds without compromising crop production. In this work, *T. capitata* EO was tested at three different concentrations against weeds grown from a citrus orchard soil seedbank untreated with herbicides and against three important weed species grown in substrate to determine the efficacy of the concentrations on different weed species. All experiments were carried out under greenhouse conditions. To find out the best way for applying the EO, it was applied by irrigation and by spraying on the targeted weeds, and to verify the influence of timing, it was tested on *Lolium rigidum* at two different phenological stages and on wheat at a later phenological stage than weeds. The highest concentration tested (12 $\mu L \cdot mL^{-1}$) showed the best performance to control weeds. The more effective mode of application was by spraying on dicotyledons and by irrigation on monocotyledons at the earliest phenological stage. *T. capitata* EO was phytotoxic for wheat. More trials in different crops are needed to determine the best conditions for its use.

Keywords: essential oils; *Thymbra capitata*; natural herbicides; abiotic stress; weed control; integrated weed management; herbicidal activity; irrigation; spraying; phenological stage

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1. Introduction

The world's population will increase from 7 billion in 2010 to 9.8 billion in 2050, which means that global demand for food will increase by more than 50%. A set of intertwined challenges aim to generate a sustainable food system [1]. The biotic stress factors that cause the most losses in crops are weeds (34%) followed by pathogens and insects (18–16%, respectively) [2]. Depending on different factors, like weed emergence and density, weed type and crop, weeds can cause 100% of yield losses if they are not controlled [3]. Losses in the period 2001–2003 caused by weeds in maize worldwide averaged 10%, but varied between continents, ranging from 5% in Western Europe to 19% in West Africa. At the same time, losses in wheat, maize and cotton were 7.7, 10.5 and 8.6, respectively [4]. In India, rice crops have the highest losses due to weeds, with a loss of 14% which economically means a

loss of USD 4420 million, followed by wheat USD 3376 million and soybeans USD 1559 million [5].

Weeds are difficult to eradicate because they have several characteristics that make them more competitive with crops, like competitiveness in terms of resource utilization to complete their cycle; the ability to spread rapidly, and to adapt to environmental plasticity; the ability to grow under a wide climatic range and to remain dormant when climatic conditions are unfavorable, thus increasing their viability; and the similarity between weed seeds and the seeds of the crop that they infest make it difficult to separate them. It is very important to understand weed biology and ecology before planning and developing any weed control approach [6–8].

With the discovery of synthetic (systemic or hormonal) herbicides in the 1940s, a revolution in chemical weed control in agriculture began. The first herbicide developed was 2,4-dichlorophenoxy-acetic acid (2,4-D) [9]. Herbicides interfere with the growth of weeds. They are classified according to different factors, including their mode of action, application and selectivity [10]. Repeated application of herbicides with the same mode of action produces a selection of herbicide-resistant weed biotypes. This is one of the challenges for herbicides use maintenance [7,11]. In the European Union (EU), through Directive 2009/128/EC of the European Parliament and of the Council establishing a framework for Community action to achieve the sustainable use of pesticides, it is mandatory to implement the principles of integrated pest management (IPM), which is an ecosystem-based strategy that focuses on long-term prevention of pests or their damage through a combination of techniques and different pest management and control methods, prioritizing the use of cultural, physical, biological, and other non-chemical control methods to the use of phytosanitary products. The number of authorized herbicide active ingredients in the EU was 78 in 2017; however, this number is anticipated to decline since several herbicide active ingredients have been discontinued in previous years but are still in use in the USA. Currently, glyphosate is the most used herbicide in the EU, which has increased its utilization in the latest years, at the same time that concerns about its effects on human health and the environment have grown [12]. Further work is required for the development of non-chemical alternative techniques to control weeds, tools and procedures, as well as strategies that support farmer communication and the adoption of integrated weed management techniques [13].

Bioherbicides, which are weed control substances of natural origin, could be utilized as a substitute to lessen the abiotic stressors caused by synthetic herbicides. Bioherbicides can be living organisms and micro-organisms and their derived products, including the natural metabolites that plants and microorganisms naturally produce during their growth and development [14]. The use of bioherbicides can be another tool and a sustainable method for weed control in the future, contributing to solving the necessities of agriculture, in combination with other innovative techniques and technology [15,16]. Many studies reported the inhibitory effects on weed seed germination and the phytotoxic effects on weed plants of essential oils (EOs) and the allelochemicals they contain, as the terpenes present in the majority of EOs. The interest in these compounds by the industry is growing. The challenges in the knowledge of bioherbicides today are to determine the mechanisms of action of the compounds, the formulation of bioherbicides combining different secondary metabolites and to reduce the costs required for their production [17].

Thymbra capitata (L.) Cav. belongs to the Lamiaceae family. It is found across the Mediterranean region and grows between 0 and 600 m above sea level [18,19]. The biological, nutritional and industrial uses and applications of *T. capitata* EO are well recognized. In the composition of *T. capitata* EO, there is a variety of secondary metabolites, where terpenoids predominate [20]. By studying the EOs of *T. capitata* from both wild and domesticated plants in Spain, carvacrol was determined as the major constituent in all of them [18]. As an oxygenated monoterpene, carvacrol is thought to act by expanding and rupturing membranes as these metabolites gradually accumulate in the cells. In addition, these sub-

stances slow down cellular respiration by dissociating oxidative photophosphorylation [21]. *T. capitata* EO has antimicrobial, antifungal and antioxidant effects [18,22–26].

The herbicidal effects of *T. capitata* EO have been previously demonstrated [27–29]. The germination of various weed species treated in vitro conditions with *T. capitata* EO was reduced or blocked completely: *Lolium rigidum* decreased its germination by 76.6% with the concentrations 1 $\mu L \cdot mL^{-1}$ [26]. The species for which germination was 100% inhibited by *T. capitata* EO were *Erigeron canadensis*, *Sonchus oleraceus* and *Chenopodium album*, with a concentration of 0.125 $\mu L \cdot mL^{-1}$; *Setaria verticillata*, *Avena fatua* and *Solanum nigrum* at 0.5 $\mu L \cdot mL^{-1}$; *Amaranthus retroflexus*, *Portulaca oleracea* and *Echinochloa crus-galli* at 2 $\mu L \cdot mL^{-1}$ [27]; *Araujia sericifera* at 0.5 $\mu L\ ml^{-1}$ and *Erigeron bonariensis* at 0.125 $\mu L \cdot mL^{-1}$ [28]. The phytotoxic effects of *T. capitata* EO have been tested in vivo under greenhouse conditions on numerous weed species, like *E. bonariensis*, *P. oleracea*, *A. fatua*, *E. crus-galli*, *A. retroflexus* and *A. sericifera* in previous works of our research group [27–29]. Nevertheless, it is necessary to determine the optimal concentrations of *T. capitata* EO that would be effective to control a great number of weeds in field conditions and the optimal phenological stages of the weeds and crops in which the EO should be applied to cause maximum damage in weeds without compromising crop production.

In this article, the herbicidal effect of *T. capitata* EO applied against the weeds developed from an untreated agricultural soil seedbank was studied in order to determine the best concentration to control a large number of different weeds. Also, the effect of *T. capitata* EO applied in two different ways, by irrigation and by spraying, was tested against two important dicotyledonous weeds in Mediterranean crops (*Sonchus oleraceus* and *Chenopodium album*) and *Lolium rigidum*, a monocotyledonous weed, very competitive in cereals and other crops, to determine the best way of applying the EO. On *L. rigidum*, *T. capitata* EO was applied at two different phenological stages to understand the importance of timing in *T. capitata* EO herbicidal efficacy. We also studied the effect of the EO on *Triticum aestivum* in order to verify its possible use on this crop for weed control. The EO was applied on the crop in a more advanced phenological stage than it was applied on weeds, to cause less phytotoxicity.

2. Materials and Methods

2.1. Essential Oil Tested

Thymbra capitata (L.) Cav. EO was purchased from Bordas S.A. (Sevilla, Spain). The composition of the EO used in this work was determined by gas chromatography (GC) and gas chromatography coupled with mass spectrometry (GC-MS) analyses in our previous studies [27]. Carvacrol was the main compound of the EO, representing 72.30% of the composition, followed by *p*-cymene 8.93% and γ-Terpinene 7.77%.

For the assays, the EO was mixed with water using Fitoil® (Xeda Italia S.r.l., Forlí, Italy), which is a natural adjuvant made from soya oil (400 g/L), as emulsifier. It was added at 0.05%. In previous assays, it was verified that Fitoil® at the used concentration did not show any effect on different weed species. A Fitoil® control was added in the experiments also to corroborate that it did not have any effect on weeds.

2.2. In Vivo Herbicidal Activity Assays on Spontaneous Weeds Grown from the Soil Seedbank

A postemergence trial was carried out under greenhouse conditions against the spontaneous flora developed from the soil seedbank of an abandoned orange orchard non-treated with herbicides located at 39°37′24.8″ N, 0°17′25.6″ W in Puzol, Valencia province, Spain. The soil was collected from the first 10 cm in a stand, removing the weed species grown in the vicinity. The species present were *Portulaca oleracea* L., *Araujia sericifera* L., *Setaria* spp., *Amaranthus retroflexus* L., *Amaranthus blitoides* L., *Portulaca oleracea* L. and *Sonchus oleraceus* L.

To perform the experiment, 3 trays of 32 × 23 × 7 cm (3 repetitions) were prepared for each treatment. The trays were filled with 200 g of perlite at the bottom as drainage and 1500 g of soil, previously homogenized, on top. The trays were irrigated with 250 mL of water three times a week to germinate the seeds present in the soil seedbank and then

to maintain the growing plantlets. The weeds were treated when monocotyledonous had 3–4 leaves and dicotyledonous had 4–8 leaves, phenological stages *Biologische Bundesanstalt*, *Bundessortenamt und Chemische Industrie* (BBCH) [30] 13–14 and 14–18, respectively.

The treatments (Table 1) were assigned by random to the different trays, and were applied by spraying with a glass sprayer VFOC.712/10 (VidraFOC, Barcelona, Spain) equipped with balloon and liquid recovery hood, which provided a uniform spray mist. The volume applied was 250 mL per tray.

Table 1. Treatments tested on spontaneous weeds from the soil seedbank.

	Treatments	Codes
T1	Water control	WATER CONTROL
T2	Water plus Fitoil® (0.05%) control	FITOIL CONTROL
T3	*Thymbra capitata* EO 4 $\mu L \cdot mL^{-1}$	TC4
T4	*Thymbra capitata* EO 8 $\mu L \cdot mL^{-1}$	TC8
T5	*Thymbra capitata* EO 12 $\mu L \cdot mL^{-1}$	TC12

The duration of the experiment and the temperature and humidity conditions of the greenhouse during the experiments are shown in Table 2. Data were recorded with a HOBO U23 Pro v2 data logger (Onset Computer Corporation, Bourner, MA, USA).

Table 2. Greenhouse conditions during the herbicidal tests against spontaneous weeds developed from the soil seedbank.

Starting–End Date	Temperature (°C)			Relative Humidity (%)		
	Mean	Max.	Min.	Mean	Max.	Min.
30 August 2018–14 September 2018	27.45	38.03	22.13	71.32	88.88	38.86

To evaluate the experiment, images from the trays with the plantlets were taken with a compact digital camera Canon PowerShot SX730 HS (Canon, Tokyo, Japan) during the trial on days 0, 1, 3, 7, 10 and 15 after the application of the treatments. The images were processed by UTHSCSA Image Tool 3.0 (University of Texas Health Science Center, San Antonio, TX, USA), to count the number of plants present on each tray. The number of plants counted on day 0, before spraying, in each tray, was considered as 100% of weeds and the next days, the percentage of plants present on each tray was calculated related to the number of plants that were present in the tray when the experiment started. The number of plantlets grown in the different trays at the beginning of the experiment was very similar (Table S1). There were no significative differences in the number of plantlets grown between trays on day 0. The number of monocotyledonous and dicotyledonous plants in each tray was also counted separately.

2.3. In Vivo Herbicidal Activity Assays on Targeted Plants

2.3.1. Plant Material

Seeds of *Chenopodium album* L. were collected from an organic persimmon orchard situated in L'Alcùdia (Valencia province, Spain) in 2018. Seeds of *Sonchus oleraceus* L. were collected from horticultural crop fields located in Puzol (Valencia province, Spain) in 2020. Seeds of *Lolium rigidum* Gaudin were purchased from Herbiseed (Reading, UK) (year of collection 2019). *Triticum aestivum* L. seeds were purchased from the company of Antonio Banegas in Abarán (Murcia province, Spain) in 2020.

Plants were germinated under greenhouse conditions in nurseries in trays of dimensions 36 × 27.3 × 9 cm. A seedbed was prepared for each species and when the plants had one leaf, the plants were transplanted individually to pots (8 × 8 × 7 cm) filled with peat and perlite in 3:1 proportion. The plants were irrigated by bottom watering during the assays.

2.3.2. Treatments on Targeted Plants

Ten pots were tested for each treatment (Figure 1). The treatments (Table 3) were applied by irrigation and spraying when the plants reached the phenological stage of 2–3 leaves (12–13 BBCH) for the monocotyledonous weeds and 3–4 leaves (13–14 BBCH) in the case of dicotyledonous weeds (Figure 1). To produce the minimum damage to the crop, *T. aestivum* was treated in a phenological stage more developed than monocotyledonous weeds, when it had 4–5 leaves (14–15 BBCH). To determine the effect of the phenological stage on the herbicidal activity of the EO, two phenological stages of application were tested in *L. rigidum*, 1) the same as for all monocotyledonous weeds, 3–4 leaves (13–14 BBCH) and 2) the stage tested for *T. aestivum*, 4–5 leaves (14–15 BBCH). The volume of treatment was 20 mL for each plant.

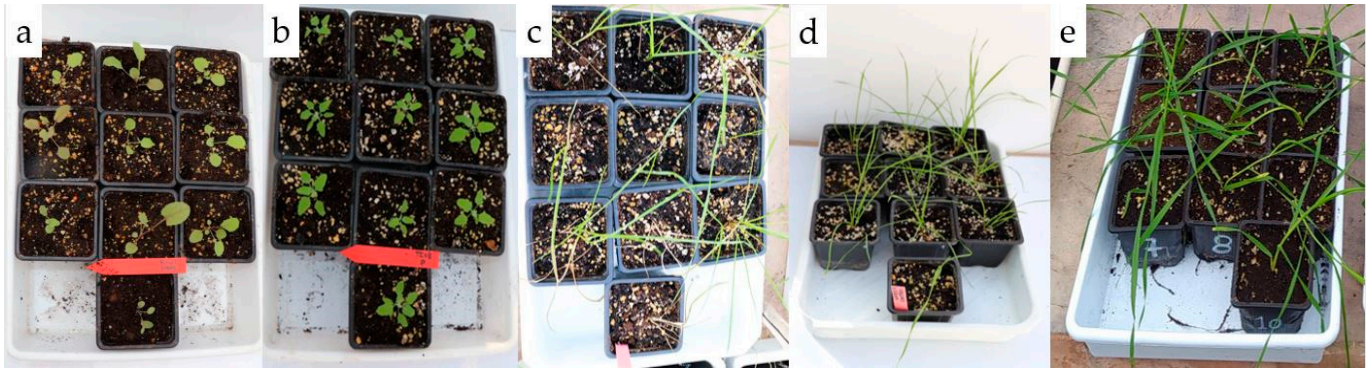

Figure 1. Species tested before applying the treatments: (**a**) *Sonchus oleraceus*, (**b**) *Chenopodium album*, (**c**) *Lolium rigidum* (BBCH 12–13), (**d**) *Lolium rigidum* (BBCH 14–15), (**e**) *Triticum aestivum*.

Table 3. Treatments tested on targeted plants.

	Treatments	Codes
T1	Water irrigation control	WATER IRRIGATION
T2	Water spray control	WATER SPRAYING
T3	Water plus Fitoil® (0.05%) irrigation control	FITOIL IRRIGATION
T4	Water plus Fitoil® (0.05%) spray control	FITOIL SPRAYING
T5	*Thymbra capitata* 4 µL ml^{-1} irrigation	TC4 IRRIGATION
T6	*Thymbra capitata* 4 µL ml^{-1} spray	TC4 SPRAYING
T7	*Thymbra capitata* 8 µL ml^{-1} irrigation	TC8 IRRIGATION
T8	*Thymbra capitata* 8 µL ml^{-1} spray	TC8 SPRAYING
T9	*Thymbra capitata* 12 µL ml^{-1} irrigation	TC12 IRRIGATION
T10	*Thymbra capitata* 12 µL ml^{-1} spray	TC12 SPRAYING

The species tested, the dates of the experiments and the greenhouse conditions during the experiments are reported in Table 4. Data were registered by a HOBO U23 Pro v2 data logger (Onset Computer Corporation, Bourner, MA, USA).

Table 4. Greenhouse conditions during the herbicidal tests on targeted plants.

Species	Starting–End Date	Temperature (°C)			Relative Humidity (%)		
		Mean	Max.	Min.	Mean	Max.	Min.
L. rigidum (15–16 BBCH)	14 October–14 November 2020	21.43	36.07	12.44	66.31	90.29	24.79
L. rigidum (13–14 BBCH)	19 October–19 November 2020	21.25	36.07	12.44	67.76	89.59	24.79
Chenopodium album	26 October–26 November 2020	21.02	36.07	12.44	65.44	86.78	24.79
Sonchus oleraceus	13 November–13 December 2020	20.13	33.24	14.89	64.53	86.78	31.22
Triticum aestivum	9 April–9 May 2021	21.42	36.04	14.58	59.74	93.34	17.46

2.3.3. Evaluation of the Herbicidal Activity Assays on Targeted Plants

To evaluate all parameters, images from all the pots were taken with a single lens reflex (SLR) camera Canon EOS 77D mounted on a tripod during the trial, 0, 1, 3, 7, 15 and 30 days after treatment application. The images were analyzed by Digimizer v.4.6.1 software (MedCalc Software, Ostend, Belgium, 2005–2016) to measure the height of the plants during the experiment and the total, aerial and root parts length of plants at the end of the experiment. To evaluate the herbicidal activity, two variables were measured: the efficacy and the damage level. The efficacy was defined as a variable that was assessed 100 if the plant was dead and 0 if the plant was alive. The level of damage was evaluated for each plant according to a damage scale that was defined as described in Table 5 and Figure 2. At the end of the experiment, the fresh and dry weight of the plants were also obtained.

Table 5. Damage level assessment.

Level of Damage	
0	Undamaged plant
1	Plant with slight damage
2	Plant with severe damage
3	Dead plant

2.4. Statistical Analyses

The results obtained from the experiments were processed with Statgraphics® Centurion XVIII software (StatPoint Technologies Inc., Warrenton, VA, USA).

In the experiment 1, on spontaneous weeds grown from the soil seedbank, a one-way ANOVA was performed for the % of plants grown and the % of monocotyledons and dicotyledons grown by treatment for each counting day. The means were compared using Fisher's least significant difference (LSD) test ($p < 0.05$).

In the experiment 2, on targeted weeds, a multifactorial analysis of variance (ANOVA) was performed for the efficacy and damage level, including as effects the species, treatments, time after application of the treatments and their double significant interactions (all double interactions except for damage level, *Lolium rigidum* BBCH stage and day interaction effect) (Tables S2–S5) (Figures S1–S11). In addition, one-way ANOVA was performed at the end of the trial to evaluate the effect of *T. capitata* applied against *L. rigidum* at 13–14 and at 14–15 BBCH, *C. album*, *S. oleraceus* and *T. aestivum* for the following variables: efficacy, damage level and plant biometric variables (aerial part, root and total length, fresh and dry weight). Fisher's multiple comparison test (LSD intervals, least significant difference, at $p < 0.05$) was used to separate the means.

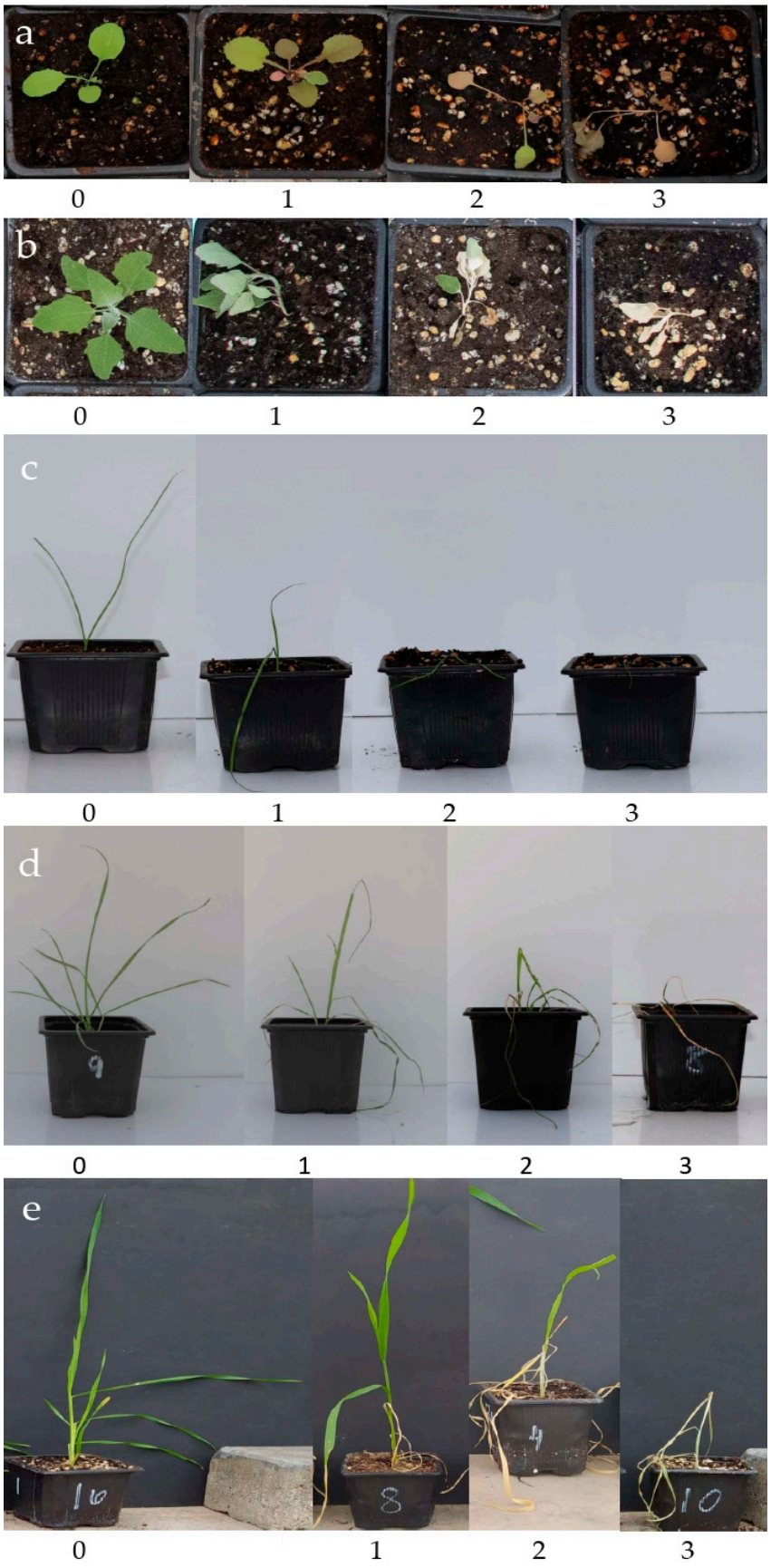

Figure 2. Damage scale for each species: (**a**) *Sonchus oleraceus*, (**b**) *Chenopodium album*, (**c**) *Lolium rigidum* (13–14 BBCH), (**d**) *Lolium rigidum* (14–15 BBCH), (**e**) *Triticum aestivum*.

3. Results and Discussion

3.1. Herbicidal Activity of T. capitata EO against Spontaneous Weeds Developed from the Soil Weed Seedbank

T. capitata EO showed greater herbicidal activity with increasing concentrations (Figure 3) and it was more effective against dicotyledonous (Figure 3A) than monocotyledonous (Figure 3B) weeds. The lowest concentration tested of the EO did not control the growth of monocotyledonous species (Figure 3B). The dicotyledonous species developed were *P. oleracea*, *A. sericifera*, *A. retroflexus*, *A. blitoides* and *S. oleraceus.* Treatment TC4 reduced 45% of the dicotyledonous weeds present in the trays at the end of the trial, while TC8 controlled 84% of the developed dicotyledonous weeds and TC12 controlled 100% of the dicotyledonous weeds from day 1 after treatment application until the end of the assay. Previous studies have demonstrated the efficacy of *T. capitata* EO to control spontaneous dicotyledonous weeds from the soil seedbank (*A. blitoides*, *Amaranthus albus*, *E. bonariensis* and *Euphorbia prostata*) with an efficacy of 63.1% and 82.4%, respectively, when applied at a concentration of 4 $\mu L \cdot mL^{-1}$ (the same of TC4) with a volume of 1.83 L/m^2 and 2.775 L/m^2 [27]. Previous studies with *T. capitata* EO tested against targeted plants had shown 100% of efficacy on *A. retroflexus* when applied at concentrations of 8 and 12 $\mu L \cdot mL^{-1}$ (same as TC8 and TC12) and 90% of efficacy on *P. oleracea* at 12 $\mu L \cdot mL^{-1}$ (TC12) [29].

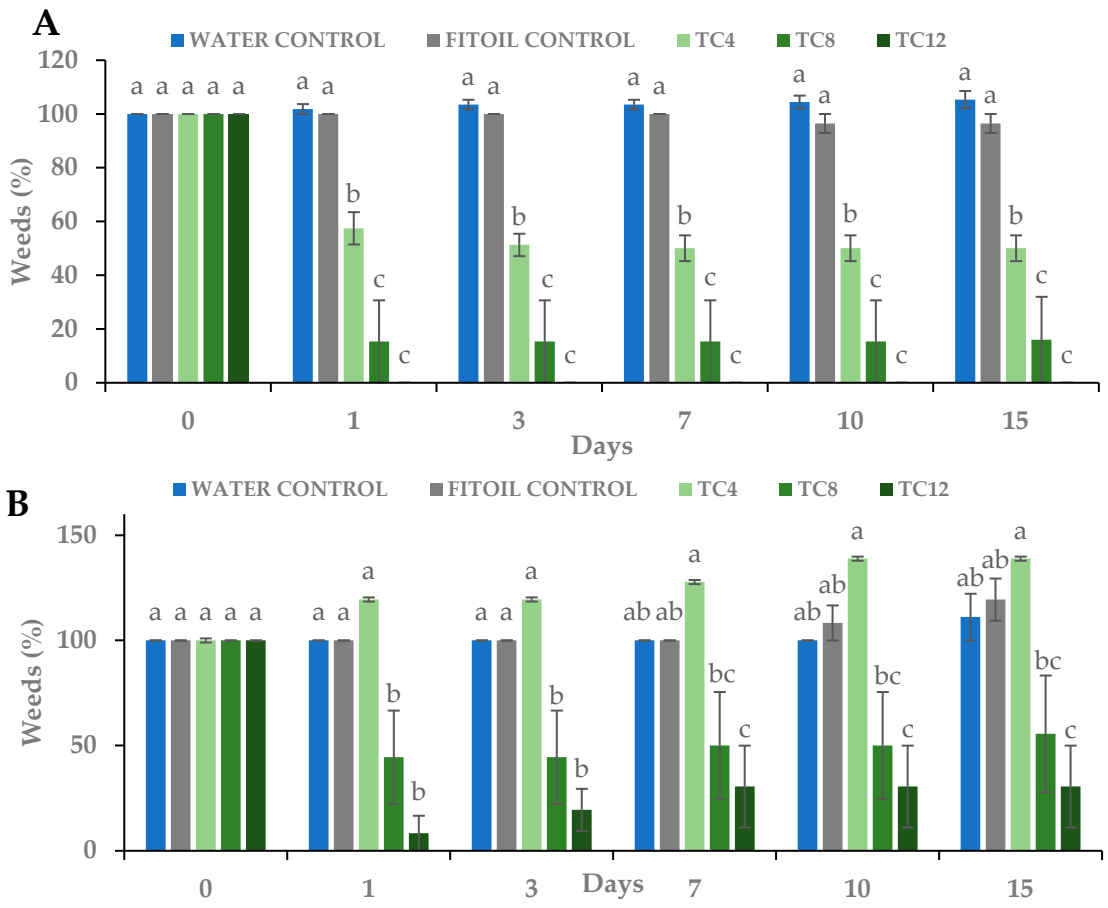

Figure 3. Effect of the treatments applied in post-emergence on the % of weeds (mean ± standard error) in the trays where they were applied on dicotyledonous (**A**) and monocotyledonous (**B**) weed species. Different letters in the same day group indicate significative differences between treatments (one-way ANOVA using Fisher's LSD test, $p < 0.05$).

As indicated above, *T. capitata* EO needed higher concentrations to control monocotyledonous weeds (Figure 3B). One day after spraying, the treatments TC8 and TC12

(Figure 3B) decreased the coverage of monocotyledons (*Setaria verticillata* and *Sorghum halepensis* among other species) by 55.56% and 91.67%, respectively. At the end of the experiment, new monocotyledonous plants grew, being reduced the weed coverage of the trays treated with TC8 and TC12 by 44.44% and 69.44%, respectively. In previous studies, *T. capitata* EO was tested against monocotyledonous weed species (*Echinochloa crus galli* and *Avena fatua*) applied at 4, 8 and 12 $\mu L \cdot mL^{-1}$, *E. crus-galli* (plants were not totally controlled by the concentrations tested) being more resistant than *A. fatua* (100% of plants controlled at 12 $\mu L \cdot mL^{-1}$) [27].

3.2. Herbicidal Activity of *T. capitata* EO on Target Plants

According to Table 6, regarding the results of the multifactorial ANOVA for the efficacy of *T. capitata* EO on weed species applied at the ideal application time (BBCH 13–14), it can be concluded that plants from all treatments with *T. capitata* EO showed statistically significative differences with control plants, all the treatments being effective. Spray treatments at the same concentrations were more effective and caused higher damage levels than irrigation treatments. Dicotyledonous species were more vulnerable to the EO treatments, showing significative differences in their response to them, *S. oleraceus* being the most susceptible species and *L. rigidum* the most resistant (Table 6). These findings—that dicotyledonous plants were more vulnerable, and irrigation was the most effective application technique—have been confirmed by several previous assays [27]. In terms of timing, it was observed that efficacy increased until 15 days after treatments application, without significative statistical differences between days 7, 15 and 30. The damage level shown by plants was significantly higher from day 1 onwards (Table 6).

Table 6. Efficacy and damage level according to treatment, species (in the phenological stage BBCH 13–14) and time.

Treatment	Efficacy	Damage Level
WATER IRRIGATION	0.00 ± 0.00 f	0.00 ± 0.00 e
WATER SPRAYING	0.00 ± 0.00 f	0.00 ± 0.00 e
FITOIL IRRIGATION	0.00 ± 0.00 f	0.00 ± 0.00 e
FITOIL SPRAYING	0.00 ± 0.00 f	0.00 ± 0.00 e
TC4 IRRIGATION	25.33 ± 2.68 e	1.17 ± 0.06 d
TC4 SPRAYING	57.33 ± 2.68 c	2.32 ± 0.06 b
TC8 IRRIGATION	42.00 ± 2.68 d	1.77 ± 0.06 c
TC8 SPRAYING	68.00 ± 2.68 ab	2.52 ± 0.06 a
TC12 IRRIGATION	60.67 ± 2.68 bc	2.32 ± 0.06 b
TC12 SPRAYING	72.00 ± 2.68 a	2.49 ± 0.06 ab
Species	**Efficacy**	**Damage Level**
S. oleraceus	45.20 ± 1.47 a	1.56 ± 0.03 a
C. album	35.00 ± 1.47 b	1.27 ± 0.03 b
L. rigidum (13–14 BBCH)	17.40 ± 1.47 c	0.95 ± 0.03 c
Time	**Efficacy**	**Damage Level**
1	11.00 ± 1.89 c	1.05 ± 0.05 b
3	29.00 ± 1.89 b	1.28 ± 0.05 a
7	38.00 ± 1.89 a	1.36 ± 0.05 a
15	42.67 ± 1.89 a	1.33 ± 0.05 a
30	42.00 ± 1.89 a	1.28 ± 0.05 a

Values are mean ± standard error. Means followed by different letters in the same column indicate significative differences between treatments (multifactorial ANOVA using Fisher's LSD test, $p < 0.05$).

The efficacy of *L. rigidum* showed a similar pattern to the *S. oleraceus* in all treatments except for the TC12 spraying when *S. oleraceus* had similar efficacy to *L. rigidum*. However, a similar damage level was observed in *C. album* and in *S. oleraceus* spraying treatments, whereas the latter showed a higher damage in irrigation treatments. Water and Fitoil treatments showed no efficacy throughout the experiment. Irrigation treatments showed

similar patterns among them along the experiment and a different pattern from spraying treatments. Similar behavior was obtained for damage levels. *L. rigidum* and *S. oleraceus* showed a similar pattern during the experiment, whereas *C. album* showed a high increment in efficacy from 1 to 3 days (Figures S1–S6).

After analyzing the results of *L. rigidum* treated at the different phenological stages BBCH 13–14 and 14–15 (Table 7), it can be concluded that irrigation treatments were more successful than spray treatments at the same concentrations, except for the higher concentrations applied, which showed the same efficacy. The timing was crucial for the efficacy of the treatments with *T. capitata* EO, since even one more leaf or phenological phase could significantly diminish the effectiveness by more than 50%.

Table 7. Efficacy and level of damage according to treatment, species phenology and time on *Lolium rigidum*.

Treatment	Efficacy	Damage Level
WATER IRRIGATION	0.00 ± 0.00 d	0.00 ± 0.00 e
WATER SPRAYING	0.00 ± 0.00 d	0.00 ± 0.00 e
FITOIL IRRIGATION	0.00 ± 0.00 d	0.00 ± 0.00 e
FITOIL SPRAYING	0.00 ± 0.00 d	0.00 ± 0.00 e
TC4 IRRIGATION	14.00 ± 2.80 c	0.62 ± 0.08 d
TC4 SPRAYING	0.00 ± 2.80 d	0.81 ± 0.08 d
TC8 IRRIGATION	22.00 ± 2.80 b	1.40 ± 0.08 b
TC8 SPRAYING	12.00 ± 2.80 c	1.17 ± 0.08 c
TC12 IRRIGATION	36.00 ± 2.80 a	1.70 ± 0.08 a
TC12 SPRAYING	32.00 ± 2.80 a	1.85 ± 0.08 a
Species	**Efficacy**	**Damage Level**
L. rigidum (14–15 BBCH)	5.80 ± 1.25 b	0.56 ± 0.04 b
L. rigidum (13–14 BBCH)	17.40 ± 1.25 a	0.95 ± 0.04 a
Time	**Efficacy**	**Damage Level**
1	1.00 ± 1.98 c	0.73 ± 0.05 abc
3	8.00 ± 1.98 b	0.85 ± 0.05 ab
7	14.50 ± 1.98 a	0.88 ± 0.05 a
15	17.00 ± 1.98 a	0.71 ± 0.05 bc
30	17.50 ± 1.98 a	0.61 ± 0.05 c

Values are means ± standard error. Means followed by different letters in the same column indicate significative differences between treatments ($p < 0.05$).

The efficacy of *Lolium rigidum* treated at 13–14 BBCH with TC12 spraying showed similar efficacy and damage level than at 14–15 BBCH whereas in most of the treatments *Lolium rigidum* treated at 13–14 BBCH had a higher efficacy and damage. A similar pattern of efficacy was obtained using irrigations treatments. Regarding the damage level, no patter was established taking into account the treatments. Finally, differences in efficacy were increasing along the assay (Figures S7–S11).

Considering the results at the end of the experiment, 30 days after treatment application, on *L. rigidum* at 13–14 BBCH (Table 8) *T. capitata* EO was more effective applied at higher dosages. The most effective treatment was irrigation at the maximum dosage (TC12). The same outcomes had been verified in previous assays; for monocotyledonous plants, the irrigation mode of administration produced the highest efficacies [27].

The results of *T. capitata* EO treatments applied on *L. rigidum* at stage BBCH 14–15 at the end of the experiment (Table 9) showed that only the highest concentrations applied were able to control *L. rigidum* at some extent. Although the highest efficacy (50) was achieved with the spray application method for TC12, it did not show significant statistical differences with respect to the irrigation method (30 efficacy) (Table 9). The level of damage in all EO treatments was not higher than 2, so there was a slight damage to the plants. Only the plants treated with TC12 applied by spraying showed significant differences in length with control plants.

Table 8. Effects of *T. capitata* EO (TC4, TC8 and TC12 are 4, 8 and 12 µL mL^{-1}) applied by irrigation and spraying against *L. rigidum* at 13–14 BBCH on efficacy, damage level and plant biometric variables (aerial part, root and total length, fresh and dry weight) at the end of the trial.

	Mean ± St. Error						
Treatments	**Efficacy**	**Damage Level**	**Aerial Part Length (cm)**	**Root Length (cm)**	**Total Length (cm)**	**Fresh Weight (g)**	**Dry Weight (g)**
WATER IRRIGATION	0.00 ± 0.00 c	0.00 ± 0.00 c	28.58 ± 1.23 a	23.85 ± 1.26 ab	52.42 ± 2.05 a	0.84 ± 0.10 a	0.12 ± 0.01 b
WATER SPRAYING	0.00 ± 0.00 c	0.00 ± 0.00 c	20.56 ± 1.31 b	23.21 ± 1.50 ab	43.77 ± 1.62 a	0.64 ± 0.09 ab	0.10 ± 0.01 bc
FITOIL IRRIGATION	0.00 ± 0.00 c	0.00 ± 0.00 c	23.61 ± 1.73 ab	24.98 ± 1.56 a	48.59 ± 2.38 a	0.36 ± 0.00 cd	0.27 ± 0.00 a
FITOIL SPRAYING	0.00 ± 0.00 c	0.00 ± 0.00 c	20.17 ± 1.06 b	26.75 ± 1.95 a	46.91 ± 2.21 a	0.59 ± 0.12 bc	0.08 ± 0.01 c
TC4 IRRIGATION	40.00 ± 16.33 b	1.20 ± 0.49 b	11.87 ± 3.33 c	17.09 ± 5.12 bc	28.95 ± 8.10 b	0.33 ± 0.10 de	0.05 ± 0.01 d
TC4 SPRAYING	0.00 ± 0.00 c	0.00 ± 0.00 c	23.87 ± 2.23 ab	20.33 ± 1.49 ab	44.21 ± 2.64 a	0.39 ± 0.08 cd	0.05 ± 0.01 d
TC8 IRRIGATION	50.00 ± 16.67 ab	1.50 ± 0.50 ab	10.58 ± 3.62 c	10.14 ± 3.47 cd	20.72 ± 6.96 bc	0.27 ± 0.12 def	0.03 ± 0.01 de
TC8 SPRAYING	40.00 ± 16.33 b	1.20 ± 0.49 b	6.55 ± 2.08 cd	8.30 ± 2.69 d	14.85 ± 4.47 cd	0.09 ± 0.04 fg	0.01 ± 0.00 e
TC12 IRRIGATION	80.00 ± 13.33 a	2.40 ± 0.40 a	3.43 ± 2.30 cd	3.70 ± 2.51 d	7.13 ± 4.75 d	0.10 ± 0.07 efg	0.00 ± 0.00 e
TC12 SPRAYING	40.00 ± 16.33 b	1.20 ± 0.49 b	7.23 ± 1.78 cd	6.17 ± 1.46 d	13.41 ± 3.14 cd	0.03 ± 0.01 g	0.01 ± 0.00 e

Values are mean of ten replicates ± standard error. Different letters in the same column indicate statistical differences between treatments ($p < 0.05$) using Fisher's least significant difference (LSD) test.

The results of the treatments with *T. capitata* EO on *C. album* at the end of the trial, 30 days after treatment application (Table 10), showed a complete control of this species by T. capitata EO applied by spraying at all concentrations tested. When the EO was applied by irrigation, an increase in efficacy was observed with increasing concentrations, reaching the value of 80 efficacy for concentration TC12. In previous studies, the same EO was used to treat *E. bonariensis* and *P. oleracea* plants at the same dosages, and the results were identical to the ones observed here: the spray application method was more effective than irrigation for dicotyledonous species control [27]. All treatments caused a significant level of damage, and significant reductions in total length and fresh and dry weight on treated plants compared to the controls.

The results for *S. oleraceus* at the end of the experiment (Table 11) indicated total efficacy (100) for all the concentrations of *T. capitata* EO administered by spraying. For irrigation application, more efficacy was observed as the concentrations rose, reaching 100 of efficacy at the highest concentration. All the EO treatments tested caused very high levels of damage and all the measured parameters were reduced significantly for the plants treated with *T. capitata* EO compared to the control plants. Based on the results obtained, this species was more sensitive to the treatments with *T. capitata* EO than *C. album* and *L. rigidum*. Once again, it can be affirmed that *T. capitata* EO was more effective in dicotyledonous plants administered by spraying than in monocotyledonous, with the particularity that very high efficacies were obtained at the highest concentration TC12 although these results depended, among other factors, on the weed species in which the EO was tested. In the case of *P. oleracea*, 90 efficacy was obtained with the same concentration of EO tested in previous works [27].

Table 9. Effects of *T. capitata* EO (TC4, TC8 and TC12 are 4, 8 and 12 μL·mL^{-1}) applied by irrigation and spraying against *L. rigidum* at 14–15 BBCH on efficacy, damage level and plant biometric variables (aerial part, root and total length, fresh and dry weight) at the end of the trial.

	Mean ± St. Error						
Treatments	**Efficacy**	**Damage Level**	**Aerial Part Length (cm)**	**Root Length (cm)**	**Total Length (cm)**	**Fresh Weight (g)**	**Dry Weight (g)**
WATER IRRIGATION	0.00 ± 0.00 c	0.00 ± 0.00 b	20.46 ± 0.79 ab	21.93 ± 1.19 ab	42.39 ± 1.06 abc	18.84 ± 2.37 ab	0.34 ± 0.04 a
WATER SPRAYING	0.00 ± 0.00 c	0.00 ± 0.00 b	23.29 ± 1.36 a	20.14 ± 1.41 bc	43.43 ± 2.26 abc	3.71 ± 0.41 bc	0.38 ± 0.02 a
FITOIL IRRIGATION	0.00 ± 0.00 c	0.00 ± 0.00 b	23.32 ± 0.98 a	26.00 ± 1.89 ab	49.32 ± 1.59 ab	3.62 ± 0.28 bc	0.30 ± 0.01 ab
FITOIL SPRAYING	0.00 ± 0.00 c	0.00 ± 0.00 b	21.08 ± 1.00 ab	24.81 ± 2.07 ab	45.90 ± 2.05 abc	3.45 ± 0.38 bc	0.32 ± 0.04 a
TC4 IRRIGATION	0.00 ± 0.00 c	0.00 ± 0.00 b	20.53 ± 1.28 ab	21.54 ± 1.25 ab	42.08 ± 1.73 abc	4.90 ± 0.57 bc	0.37 ± 0.04 a
TC4 SPRAYING	0.00 ± 0.00 c	0.00 ± 0.00 b	22.00 ± 3.12 ab	28.57 ± 2.04 a	50.58 ± 3.91 a	3.45 ± 0.39 a	0.29 ± 0.04 ab
TC8 IRRIGATION	20.00 ± 13.33 bc	1.30 ± 0.37 a	16.76 ± 2.04 bc	19.04 ± 3.40 bc	33.81 ± 5.86 cd	1.88 ± 0.54 c	0.15 ± 0.05 cd
TC8 SPRAYING	0.00 ± 0.00 c	0.00 ± 0.00 b	18.17 ± 0.95 ab	21.85 ± 2.03 ab	40.03 ± 2.79 abc	2.23 ± 0.28 bc	0.21 ± 0.03 bc
TC12 IRRIGATION	30.00 ± 15.28 ab	1.60 ± 0.37 a	17.42 ± 3.91 abc	19.23 ± 4.39 bc	36.65 ± 8.23 bcd	1.58 ± 0.51 c	0.15 ± 0.05 cd
TC12 SPRAYING	50.00 ± 16.67 a	1.80 ± 0.42 a	11.36 ± 3.92 c	13.32 ± 5.04 c	24.68 ± 8.89 d	1.53 ± 0.59 c	0.09 ± 0.04 d

Values are mean of ten replicates ± standard error. Different letters in the same column indicate statistical differences ($p < 0.05$) using Fisher's least significant difference (LSD) test.

The results for *T. aestivum* at the end of the trial, 30 days after treatment application (Table 12), showed 100 efficacy for spray treatment TC12, indicating that spray application could cause damage to the crop not being adequate to control weeds on this crop. Instead, TC12 administered by irrigation could be recommended for its use, since it did not cause damage to wheat plants when applied at the stage of four or five true leaves, unlike *L. rigidum*, which showed damage at the two phenological stages tested.

Table 10. Effects of *T. capitata* EO (TC4, TC8 and TC12 are 4, 8 and 12 μL·mL^{-1}) applied by irrigation and spraying against *C. album* on efficacy, damage level and plant biometric variables (aerial part, root and total length, fresh and dry weight) at the end of the trial.

	Mean ± St. Error						
Treatments	**Efficacy**	**Damage Level**	**Aerial Part Length (cm)**	**Root Length (cm)**	**Total Length (cm)**	**Fresh Weight (g)**	**Dry Weight (g)**
WATER IRRIGATION	0.00 ± 0.00 d	0.00 ± 0.00 d	24.71 ± 1.77 a	27.33 ± 2.63 a	52.05 ± 3.34 a	3.67 ± 0.24 a	0.49 ± 0.04 bc
WATER SPRAYING	0.00 ± 0.00 d	0.00 ± 0.00 d	24.17 ± 1.94 ab	24.08 ± 2.00 a	48.25 ± 2.38 a	3.43 ± 0.25 a	0.59 ± 0.04 ab
FITOIL IRRIGATION	0.00 ± 0.00 d	0.00 ± 0.00 d	25.69 ± 1.73 a	21.85 ± 1.61 a	47.55 ± 2.67 a	4.06 ± 0.30 a	0.66 ± 0.05 a
FITOIL SPRAYING	0.00 ± 0.00 d	0.00 ± 0.00 d	29.96 ± 2.32 a	24.94 ± 1.81 a	54.90 ± 3.07 a	2.12 ± 0.12 b	0.44 ± 0.03 cd
TC4 IRRIGATION	30.00 ± 15.28 c	0.90 ± 0.46 c	17.04 ± 4.16 bc	14.44 ± 3.30 b	31.49 ± 7.22 b	1.85 ± 0.42 bc	0.35 ± 0.08 d
TC4 SPRAYING	100.00 ± 0.00 a	3.00 ± 0.00 a	0.00 ± 0.00 e	0.00 ± 0.00 d	0.00 ± 0.00 d	0.00 ± 0.00 e	0.00 ± 0.00 f

Table 10. *Cont.*

	Mean ± St. Error						
Treatments	**Efficacy**	**Damage Level**	**Aerial Part Length (cm)**	**Root Length (cm)**	**Total Length (cm)**	**Fresh Weight (g)**	**Dry Weight (g)**
TC8 IRRIGATION	60.00 ± 16.33 b	1.80 ± 0.49 b	9.80 ± 4.27 cd	8.15 ± 3.45 bc	17.95 ± 7.67 c	1.12 ± 0.50 cd	0.18 ± 0.08 f
TC8 SPRAYING	100.00 ± 0.00 a	3.00 ± 0.00 a	0.00 ± 0.00 e	0.00 ± 0.00 d	0.00 ± 0.00 d	0.00 ± 0.00 e	0.00 ± 0.00 f
TC12 IRRIGATION	80.00 ± 13.33 ab	2.40 ± 0.40 ab	6.57 ± 4.43 de	5.71 ± 3.86 cd	12.28 ± 8.28 cd	0.73 ± 0.49 de	0.11 ± 0.08 ef
TC12 SPRAYING	100.00 ± 0.00 a	3.00 ± 0.00 a	0.00 ± 0.00 e	0.00 ± 0.00 d	0.00 ± 0.00 d	0.00 ± 0.00 e	0.00 ± 0.00 f

Values are mean of ten replicates ± standard error. Different letters in the same column indicate statistical differences between treatments ($p < 0.05$) using Fisher's least significant difference (LSD) test.

Table 11. Effects of *T. capitata* EO (TC4, TC8 and TC12 are 4, 8 and 12 µL mL^{-1}) applied by irrigation and spraying against *S. oleraceus* on efficacy, damage level and plant biometric variables (aerial part, root and total length, fresh and dry weight) at the end of the experiment.

	Mean ± St. Error						
Treatments	**Efficacy**	**Damage Level**	**Aerial Part Length (cm)**	**Root Length (cm)**	**Total Length (cm)**	**Fresh Weight (g)**	**Dry Weight (g)**
WATER IRRIGATION	0.00 ± 0.00 d	0.00 ± 0.00 c	7.37 ± 1.26 a	22.33 ± 1.14 a	29.70 ± 1.96 a	0.45 ± 0.07 b	0.08 ± 0.01 b
WATER SPRAYING	0.00 ± 0.00 d	0.00 ± 0.00 c	6.86 ± 0.56 a	21.54 ± 2.21 a	28.41 ± 2.59 a	0.37 ± 0.04 b	0.05 ± 0.01 bc
FITOIL IRRIGATION	0.00 ± 0.00 d	0.00 ± 0.00 c	5.90 ± 0.38 a	20.78 ± 1.95 a	26.68 ± 1.88 a	0.26 ± 0.04 b	0.04 ± 0.01 bc
FITOIL SPRAYING	0.00 ± 0.00 d	0.00 ± 0.00 c	6.04 ± 0.43 a	20.34 ± 2.06 a	26.38 ± 2.20 a	0.07 ± 0.04 b	0.01 ± 0.01 c
TC4 IRRIGATION	60.00 ± 16.33 c	2.30 ± 0.30 b	1.80 ± 0.95 b	4.82 ± 2.63 b	6.62 ± 3.51 b	0.00 ± 0.00 b	0.00 ± 0.00 c
TC4 SPRAYING	100.00 ± 0.00 a	3.00 ± 0.00 a	0.00 ± 0.00 b	0.00 ± 0.00 b	0.00 ± 0.00 c	0.16 ± 0.10 b	0.02 ± 0.01 c
TC8 IRRIGATION	80.00 ± 13.33 b	2.50 ± 0.34 b	1.57 ± 1.05 b	4.49 ± 3.00 b	6.06 ± 4.04 bc	0.00 ± 0.00 b	0.00 ± 0.00 c
TC8 SPRAYING	100.00 ± 0.00 a	3.00 ± 0.00 a	0.00 ± 0.00 b	0.00 ± 0.00 b	0.00 ± 0.00 c	0.00 ± 0.00 b	0.00 ± 0.00 c
TC12 IRRIGATION	100.00 ± 0.00 a	3.00 ± 0.00 a	0.00 ± 0.00 b	0.00 ± 0.00 b	0.00 ± 0.00 c	0.00 ± 0.00 b	0.00 ± 0.00 c
TC12 SPRAYING	100.00 ± 0.00 a	3.00 ± 0.00 a	0.00 ± 0.00 b	0.00 ± 0.00 b	0.00 ± 0.00 c	0.45 ± 0.07 b	0.08 ± 0.01 b

Values are mean of ten replicates ± standard error. Different letters in the same column indicate statistical differences ($p < 0.05$) using Fisher's least significant difference (LSD) test.

In the future, the application of TC4 in spray to wheat could be studied, since at these concentrations *C. album* and *S. oleraceus* weeds were controlled. The effect on the crop should be evaluated because, as can be observed in Table 12, the weights of the spray-applied plants showed a difference in weight with respect to the control plants due to the damage that occurred after EO application. Monocotyledonous plants have the ability to regenerate more quickly than dicotyledonous plants and are therefore more resistant to bioherbicides. The fact that monocotyledons are more tolerant to the stress of bioherbicides was proven with *Acacia nilotica* and *Eucalyptus rostrata* in *Zea mays* and *Phasoleus vulgaris* [31]. While certain dicotyledonous plant species may be more tolerant to various forms of stress, such as drought, other monocotyledonous plant species may be

more tolerant to specific types of stress, such as salt. Due to elements like genetic diversity and environmental variables, resistance can also differ among plant species [32].

Table 12. Effects of *T. capitata* EO (TC4, TC8 and TC12 are 4, 8 and 12 $\mu L \cdot mL^{-1}$) applied by irrigation and spraying against *Triticum aestivum* on efficacy, damage level and plant biometric variables (aerial part, root and total length, fresh and dry weight) at the end of the experiment.

	Mean ± St. Error						
Treatments	**Efficacy**	**Damage Level**	**Aerial Part Length (cm)**	**Root Length (cm)**	**Total Length (cm)**	**Fresh Weight (g)**	**Dry Weight (g)**
WATER IRRIGATION	0.00 ± 0.00 c	0.00 ± 0.00 c	62.20 ± 0.94 a	19.13 ± 1.15 ab	81.32 ± 1.74 a	4.77 ± 0.24 b	1.55 ± 40.08 ab
WATER SPRAYING	0.00 ± 0.00 c	0.00 ± 0.00 c	60.69 ± 0.68 a	17.26 ± 1.18 b	77.95 ± 1.31 a	5.03 ± 0.13 ab	1.45 ± 0.02 ab
FITOIL IRRIGATION	0.00 ± 0.00 c	0.00 ± 0.00 c	60.18 ± 0.92 a	18.11 ± 0.85 b	78.29 ± 1.18 a	5.58 ± 0.25 a	1.41 ± 0.07 ab
FITOIL SPRAYING	0.00 ± 0.00 c	0.00 ± 0.00 c	61.23 ± 1.40 a	16.71 ± 1.16 b	77.94 ± 2.30 a	5.07 ± 0.24 ab	1.46 ± 0.08 ab
TC4 IRRIGATION	0.00 ± 0.00 c	0.00 ± 0.00 c	57.14 ± 1.15 a	17.44 ± 0.82 b	74.59 ± 0.91 ab	4.55 ± 0.26 b	2.28 ± 1.02 a
TC4 SPRAYING	10.00 ± 10.00 c	0.30 ± 0.30 c	45.60 ± 5.24 b	16.07 ± 2.20 b	61.66 ± 7.26 b	2.78 ± 0.42 c	0.80 ± 0.13 bcd
TC8 IRRIGATION	0.00 ± 0.00 c	0.00 ± 0.00 c	57.95 ± 1.23 a	30.46 ± 12.29 b	88.41 ± 12.51 a	4.41 ± 0.20 b	1.26 ± 0.10 bc
TC8 SPRAYING	30.00 ± 15.28 b	0.90 ± 0.46 b	31.62 ± 7.46 c	11.79 ± 2.92 b	43.41 ± 9.99 c	1.87 ± 0.51 d	0.40 ± 0.12 cd
TC12 IRRIGATION	0.00 ± 0.00 c	0.00 ± 0.00 c	58.02 ± 1.75 a	17.57 ± 0.86 b	75.59 ± 2.06 ab	4.39 ± 0.22 b	1.22 ± 0.08 bc
TC12 SPRAYING	100.00 ± 0.00 a	3.00 ± 0.00 a	0.00 ± 0.00 d	0.00 ± 0.00 c	0.00 ± 0.00 d	0.00 ± 0.00 e	0.00 ± 0.00 d

Values are means of ten replicates ± standard error. Different letters in the same column indicate statistical differences between treatments ($p < 0.05$) using Fisher's least significant difference (LSD) test.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/agronomy13122938/s1, Table S1. Number of plantlets grown on each tray during the experiment. Table S2. Multifactorial ANOVA analysis for Efficacy including treatment, species, and time as main factors as well as the double interaction effects. Table S3. Multifactorial ANOVA analysis for Damage level including treatment, species, and time as main factors as well as the double interaction effects. Table S4. Multifactorial ANOVA analysis for Efficacy including treatment, species, and time as main factors as well as the double interaction effects. Table S5. Multifactorial ANOVA analysis for Damage level including treatment, species, and time as main factors as well as the double interaction effects. Figure S1. Interaction effect of species and treatment for efficacy. Figure S2. Interaction effect of day and treatment for efficacy. Figure S3. Interaction effect of species and day for efficacy. Figure S4. Interaction effect of species and treatment for damage level. Figure S5. Interaction effect of day and treatment for damage level. Figure S6. Interaction effect of species and day for damage level. Figure S7. Interaction effect of *Lolium rigidum* BBCH stage and treatment for efficacy. Figure S8. Interaction effect of treatment and day for efficacy. Figure S9. Interaction effect of *Lolium rigidum* BBCH stage and day for efficacy. Figure S10. Interaction effect of *Lolium rigidum* BBCH stage and treatment for damage level. Figure S11. Interaction effect of treatment and day for damage level.

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