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Herbicidal Potential of the Natural Compounds Carvacrol, Thymol, Eugenol, *p*-Cymene, Citral and Pelargonic Acid in Field Conditions: Indications for Better Performance

Natalia Torres-Pagán [1,2], Marta Muñoz [1,3], Sara Barbero [1], Roberta Mamone [2], Rosa Peiró [4], Alessandra Carrubba [2], Adela M. Sánchez-Moreiras [5,6], Diego Gómez de Barreda [7,*] and Mercedes Verdeguer [1,*]

1 Instituto Agroforestal Mediterráneo (IAM), Universitat Politècnica de València, Camino de Vera s/n, 46022 Valencia, Spain; natorpa@upv.es (N.T.-P.); marmuous@upv.es (M.M.); sbarper@upv.es (S.B.)
2 Department of Agricultural, Food and Forestry Sciences, Università degli Studi di Palermo, Viale delle Scienze, Building 4, 90128 Palermo, Italy; roberta.mamone@unipa.it (R.M.); alessandra.carrubba@unipa.it (A.C.)
3 Seipasa S.A. C/Ciudad Darío, Polígono Industrial La Creu Naves 1-3-5, 46250 L'Alcudia, Spain
4 Centro de Conservación y Mejora de la Agrodiversidad Valenciana (COMAV), Universitat Politècnica de València, Camino de Vera s/n, 46022 Valencia, Spain; ropeibar@btc.upv.es
5 Departamento de Bioloxía Vexetal e Ciencia do Solo, Facultade de Bioloxía, Universidade de Vigo, 36310 Vigo, Spain; adela@uvigo.es
6 Instituto de Agroecoloxía e Alimentación (IAA), Universidade de Vigo, Campus Auga, 32004 Ourense, Spain
7 Departamento de Producción Vegetal, Universitat Politècnica de València, Camino de Vera s/n, 46022 Valencia, Spain
* Correspondence: diegode@btc.upv.es (D.G.d.B.); merversa@eaf.upv.es (M.V.)

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Abstract: In recent years, interest in natural products with herbicidal activity as new tools for integrated weed management has increased. The European Union is demanding a reduction in the number of herbicides used, forbidding use of the most toxic ones, despite the problem of weed resistance increasing. Pelargonic acid (PA) is the only natural herbicide available in Spain. In this work, two field assays were performed with the natural compounds carvacrol (CAR), citral (CIT), eugenol (EUG), thymol (THY), *p*-cymene (P-CYM), (PA), and the combination of PA with CIT—all except P-CYM formulated by Seipasa—to test their herbicidal efficacy in real conditions. They were compared with commercial PA, glyphosate (GLY) and oxyfluorfen (OXY). In both experiments, GLY achieved the best weed control. Considering the natural herbicides, PA formulated by Seipasa and PA plus CIT were the most effective. From both experiments, some conclusions can be extracted for better herbicidal performance of natural products: (1) use products on sensitive weed species, (2) treat weeds at earlier phenological stages, (3) find the active doses in field conditions, (4) cover weeds well when treating, (5) ensure adequate formulation of products, and (6) develop a strategy for correct application.

Keywords: weed control; bioherbicides; field trials; natural products; integrated weed management; plant secondary metabolites

1. Introduction

One of the challenges facing humanity is meeting the projected food needs of the population in the future, as current levels of agricultural production are insufficient [1,2] to feed the expected world population, projected to be approximately 10 billion by 2050 [3]. Crop losses due to weeds, pests, pathogens and viruses reduce food production. Approximately 34% of the world's agricultural losses are due to weeds, while 18–16% are losses caused by diseases and pests [4]. The characteristics of weeds allow them to compete with crops, leading to increased food production costs and reduced crop quality [5]. Crop losses due to weeds vary depending on the crop, how it competes with weeds and weed management. Globally, weed losses in 2006 were 7.7% for wheat, 10.5% for maize and 8.6% for cotton [4]. In the United States, winter wheat production loss due to weeds was predicted to be from 2.9% to 34.4%, with an average of 25.6% [6]. To ensure good weed control, the following are needed: more investment in research, education and regulation; more sustainable weed management practices; encouraging innovation [1]. There are major challenges in crop and weed management in the European Union (EU) to avoid climate change, herbicide resistance, the withdrawal of effective active substances, invasive plants, and restrictions on chemical inputs. The EU requires the application of integrated pest management (IPM) practices through Directive 2009/128/EC, which establishes a framework to achieve the sustainable use of pesticides. IPM is an ecosystem-based strategy that prioritizes the use of cultural, physical, biological, and other non-chemical control methods over the use of phytosanitary products. By 2030, the EU27 countries are projected to have drastically reduced their herbicide use. Implementation of the proposed comprehensive framework for optimizing weed management in the EU Green Deal era would ensure a reduction in herbicide use in the medium and long terms. Providing desirable agro-ecosystem services and integrating and optimizing non-chemical alternatives for weed management are two of the most promising ways to minimize herbicide use, protect crops, increase biodiversity and safeguard farmers' incomes in the EU Green Deal era [7]. A single control strategy will not be sufficient to manage weeds in the long term [2]. The use of herbicides is the most effective practice for weed management in fruit orchards, although there are other sustainable alternatives for weed management such as the use of shallow tillage systems and mulching [8]. Bioherbicides include products derived from living organisms or microorganisms and the substances that they produce in their life cycle, like secondary metabolites, used for weed control [9]. Bioherbicides are tools that offer great potential in integrated weed management (IWM); but for these products to be commercially viable on the market, they must have high performance in the field [10]. There are thirteen bioherbicides registered in the EU; nine of them are based on bacterial microorganisms, three on fungal microorganisms and only one contains a natural plant extract as an active ingredient. Beloukha® is a bioherbicide registered in Europe since 2015, and the active ingredient is pelargonic acid. The development of natural herbicides based on organic acids or essential oils (EOs) could reduce negative effects on the environment due to their low persistence and herbicide resistance, as natural products have different modes of action [11,12]. Many plant species belonging to the *Lamiaceae* family, mainly distributed in the Mediterranean area, contain bioactive secondary metabolites rich in terpenes with bioherbicidal potential [13]. These products of natural origin are not 100% miscible with water, so they need to be formulated. One solution to increase the efficacy of bioherbicides in the field is formulation and nanoencapsulation [14–16]. In this work, the herbicidal potential of different natural products, with known herbicidal properties—carvacrol, thymol, eugenol, citral, *p*-cymene and pelargonic acid—were formulated by the company Seipasa and tested in field conditions. Carvacrol and thymol are phenolic monoterpenes that can be found in the EOs of plants in the *Lamiaceae* family, including *Thymbra capitata* (L.) Cav., *Thymus vulgaris* L. and *Origanum* spp. These compounds have demonstrated the ability to inhibit the germination of weed seeds and cause phytotoxicity in plants [12,17]. Solutions of carvacrol and thymol were applied to *Lactuca sativa* L. at concentrations of 0.015–0.060% *w*/*v*. Carvacrol solutions decreased the mitotic index (MI), which can be used to evaluate the cytotoxicity of allelopathic compounds, and thymol solutions increased the mitotic index significantly compared to control. The effect of carvacrol could be attributed to the alteration of growth and development, while thymol affected cell division, increasing it, and leading to cell proliferation and abnormalities in chromosomes [18,19]. Eugenol is a phenolic compound found in the EOs of Lamiaceae, Lauraceae, Myrtaceae and Myristicaceae. Eugenol is the major compound in the EO of *Syzygium aromaticum* (L.) Merr. & L.M. Perry [20]. Eugenol inhibited germination and reduced plant growth of *Echinochloa crus-galli* (L.) Beauv., *Phalaris minor* Retz., *Sorghum halepense* (L.) Pers. and *Leptochloa chi-*

nensis (L.) Nees, as it caused phytotoxicity due to low cell respiration and chlorophyll concentration. The effect of this monoterpene was greater on monocotyledons [14]. The compound *p*-cymene is a monoterpene, precursor of carvacrol. *p*-cymene is found in many EOs, such as *Artemisia* spp., *Origanum* spp., *Protium* spp., and *T. capitata*, and in fruits and vegetables (orange juice, raspberries, carrots) [21]. The effects of thymol, carvacrol and *p*-cymene were tested in vitro and in vivo on *Amaranthus retroflexus* L., *Chenopodium album* L. and *Rumex crispus* L. species. The results showed that thymol and carvacrol inhibited germination in vitro in contrast to *p*-cymene; but under greenhouse conditions at doses of 1 mg mL^{-1}, carvacrol was more effective than thymol, and *p*-cymene showed no phytotoxic effects [22]. *P*-cymene inhibited 28.1% of the germination of *E. crus-galli* at 8 mM in in vitro conditions and reduced its root growth (EC50 = 0.22 mM), while thymol and eugenol at 4 mM completely prevented barnyard grass germination [23]. Thymol at 160 nL cm^{-3} prevented seed germination of *Lolium rigidum* L. Gaudin, while *p*-cymene was scarcely phytotoxic at the doses tested (0–640 nL cm^{-3}) [24]. Citral (3,7-dimethyl-2,6-octadienal) is a monoterpene that is extensively utilized in the pharmaceutical and cosmetic industries. Citral is a blend of geranial and neral aldehydes, with geranial making up 1.5–3-fold the amount of geranial in EOs compared to neral [25]. Citral was 100% effective for weed control under greenhouse conditions at a concentration of 100 kg ha^{-1} for five dicots: *Aeschynomene indica* L., *Abutilon theophrasti* Medik., *Gossypium hirsutum* Cav., *Glycine max* (L.) Merr. and *Ipomoea autrobasiliensis* J.R.I. Wood & Scotland, as well as five monocots: *Echinochloa crus-galli* (L.) P.Beauv, *Digitaria ciliaris* (Retz.) Koeler, *Setaria viridis* (L.) P.Beauv., *Poa annua* L. and *Zea mays* L. At a concentration of 50 kg ha^{-1}, citral completely controlled all weeds, except *P. annua*. Eugenol was not as effective as citral in terms of phytotoxicity. At a concentration of 100 kg ha^{-1}, eugenol was not able to completely control the species [26]. Citral has the potential to be developed into acceptable and environmentally friendly weed control solutions [27]. Pelargonic acid is a saturated, nine-carbon fatty acid that was firstly isolated from the leaves of *Pelargoniun roseum* Willd, and can also be found in vegetables and fruits (orange, grape, apple, and potato), milk, cheese, and beef. Pelargonic acid is used to control weeds in gardens, parks, lawns, golf courses, roads, pavements, and industrial areas [28]. As stated previously, it is the only plant-based bioherbicide registered in the EU. The herbicidal activity of pelargonic acid has been widely studied, specially in recent years [11,12,29–34]. Pelargonic acid can be a useful alternative to synthetic herbicides in IWM; for example, an environmentally beneficial method of managing annual weeds in soybean fields is to combine pelargonic acid herbicide with fake or stale seedbed technology to achieve a reduction of 95% compared to a normal seedbed [35]. Few studies have been carried out to assess the effectiveness of bioherbicide weed management in field conditions and identify which crops may benefit from the use of such weed control techniques [12]. The aim of this work was to study the herbicidal activity of the natural products carvacrol, thymol, eugenol, *p*-cymene, citral, and pelargonic acid in field conditions. This is an important step in the investigation of natural herbicides for the control of spontaneous weeds as there are very few field trial studies.

2. Materials and Methods

2.1. Experimental Sites

Two similar experiments were conducted in two different places of the Valencia province agricultural area (Eastern Spain) during 2021. The first one was conducted in the lanes of a young citrus field located in Guadassuar (GUA) village (39°16′6″ N, 0°31′10″ W; 40 m asl) during July to August; and the second one in a vegetable field, previous to plantation, at the Polytechnic University of Valencia (UPV) experimental farm (39°29′3″ N, 0°20′11″ W; 5 m asl) during October to November. Climatic conditions during the experimentation period and soil characteristics are shown in Table 1.

Table 1. Main soil characteristics of the experimental fields and climatic conditions during the experimental period.

Soil Characteristics		
	Guadassuar (GUA)	**University (UPV)**
Texture (sand-silt-clay, %)	53.3–16.0–30.7	76.7–13.3–10.0
pH	8.30	8.52
Electrical conductivity (dS·m^{-1})	0.20	0.17
Organic matter (%)	1.06	0.25
Carbonate content (%)	21.4	29.6
Climatic Conditions [1]		
	Guadassuar (GUA)	**University (UPV)**
Mean Maximum temperature (°C)	32.33	23.46
Mean temperature (°C)	26.34	19.19
Mean Maximum temperature (°C)	20.69	15.21
Cumulated precipitation (mm)	15.90	13.00

[1] Source: Agricultural Cooperative "Sant Bernat" Coop.V., municipality of Carlet, located 15 km from the GUA experimental field, and own climatic station located 200 m from the UPV experimental field.

2.2. Treatments and Experimental Design

Six natural products were evaluated: carvacrol, thymol, *p*-cymene, eugenol, citral and pelargonic acid. Carvacrol, thymol, eugenol and *p*-cymene were purchased from Sigma Aldrich® (Merck KGaA, Darmstadt, Germany); citral and pelargonic acid were provided by Seipasa. All natural products except *p*-cymene were formulated as emulsifiable concentrates (EC) by Seipasa, including the mixture of pelargonic acid plus citral. The herbicidal activity of these compounds was compared with a natural herbicide based on pelargonic acid (68%), already in the market (Beloukha®, Belchim Crop Protection NV/SA, Londerzeel, Belgium), and two synthetic herbicides, which are the most used in the area, glyphosate (Karda®, Lainco S.A., Barcelona, Spain) and oxyfluorfen (Fenfen®, Lainco S.A., Barcelona, Spain) (Table 2). The products already marketed were tested at the label doses. The natural products carvacrol, thymol, *p*-cymene, eugenol and citral were tested in field conditions for the first time, and the doses were selected based on previous greenhouse assays. Due to the results obtained in the GUA assay, additionally, at the UPV location, the natural products carvacrol, thymol and eugenol were applied at the same dose to GUA and at a triple dose. The doses of SEITHOR® were selected according to the results of previous field trials. Ten herbicidal treatments were evaluated at GUA and thirteen at UPV in a randomized complete-block design (RCBD) with four replications. The elemental plot size was 3 m^2 (3 × 1 m) (Figure 1), and each herbicide was applied on four different plots. Applications were made with a CO_2-pressured sprayer (R&D Sprayers, Opelousas, LA, USA) calibrated to deliver 500 L ha^{-1} with a single 9504 E flat-fan nozzle (Tee Jet Spraying Systems Co., Roswell, GA, USA) at 300 kPa.

At GUA, weed species had an average elemental plot ground coverage of 28.4%, and comprised *Amaranthus retroflexus* L., *Amaranthus blitoides* S. Wats., *Convolvulus arvensis* L., *Cyperus rotundus* L., *Diplotaxis erucoides* (L.) DC., *Heliotropium europaeum* L., *Poa annua* L., *Portulaca oleracea* L., and *Tribulus terrestris* L. The phenological stage of the weeds when the treatments were applied was 4–8 leaves, equivalent to stage 14–18 Biologische Bundesanstalt, Bundessortenamt und Chemische Industrie (BBCH) scale [36] (Figure 1A). At UPV, weed species had an average elemental plot ground coverage of 48.0%, and the species present were mainly *P. oleracea*, *Sisymbrium officinale* (L.) Scop. and *Chenopodium album* L. At treatment application, the phenological stage of weeds was 2–4 leaves (12–14 BBCH scale) (Figure 1B).

Table 2. List of treatments tested, with their acronyms and application rates.

	Acronym	Application Rate	
		L ha^{-1}	Kg a.i. * ha^{-1}
Pelargonic acid (55%) SEITHOR®	PA-55	15.0	8.3
Citral (30%)	CIT	15.0	4.5
Pelargonic acid (38%) + citral (30%)	PA-38+CIT	15.0	5.7 + 4.5
Carvacrol (32%)	CAR	15.0	4.8
Carvacrol (32%) × 3	CARX3	45.0	14.4
Eugenol (15%)	EUG	80.0	12.0
Eugenol (15%) × 3	EUGX3	240.0	36.0
Thymol (15%)	THY	80.0	12.0
Thymol (15%) × 3	THYX3	240.0	36.0
P-cymene (18%)	P-CYM	42.7	7.7
Pelargonic acid (68%) (Beloukha®)	PA-68	16.0	10.9
Glyphosate (36%) (Karda®)	GLY	1.5	0.54
Oxyfluorfen (24%) (Fenfen®)	OXY	0.6	0.14
Untreated control	UNT	-	-

* a.i.: active ingredient.

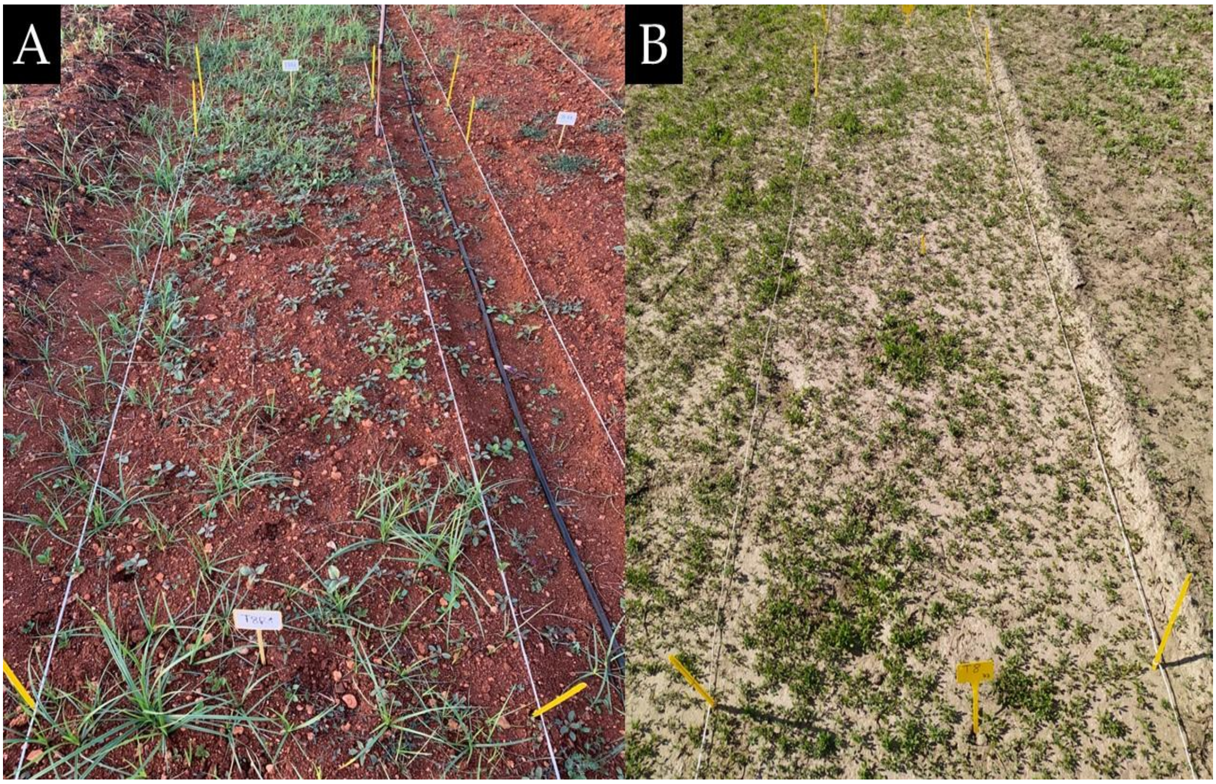

Figure 1. Control plots at the beginning of the experiments at: (**A**) Guadassuar (GUA); (**B**) the Polytechnic University of Valencia (UPV).

2.3. Determinations and Statistical Analyses

The following determinations were conducted in both locations at 0, 1, 2, and 4 weeks after treatment application (WAT): (i) weed ground coverage as a subjective visual percentage of the plot; (ii) weed ground coverage processing images of the whole plot with the software Digimizer v.4.6.1 (MedCalc Software, Ostend, Belgium, 2005–2016) (Figure S1); (iii) Normalized Difference Vegetation Index (NDVI) values of the whole plot using a GreenSeeker Handheld Crop Sensor (Trimble, Folsom, CA, USA); (iv) weed damage using a subjective visual scale from 1 (no damage) to 9 (completely dead plant); and (v) fresh weed biomass removed from one sample per plot using a 42 cm of diameter circular frame.

Weed coverage using the Digimizer image analysis software is an objective weed coverage assessment method, although it depends on the software analyzer. Weed coverage was also visually determined and plotted against the Digimizer output for both locations and each sampling date to validate the method (Figure S2).

Statistical analysis was carried out with the software Statgraphics Centurion XIX version 18.1.13 (StatPoint Technologies, Warrenton, VA, USA). A one-way ANOVA was performed for each variable determined (weed coverage, NVDI, weed damage and weed fresh weight) in each measurement day. Fisher's least significant difference (LSD) test ($p < 0.05$) was used to compare the means.

3. Results

3.1. Effect on Weed Coverage

One week after treatment (WAT) in Guadassuar (GUA), there was an increase in weed coverage in the untreated control (UNT) plots (48%), and plots treated with natural products (5.2 to 46.4% range); however, the weed coverage increase in natural product plots was different depending on the product. Plots treated with pelargonic acid (55%) SEITHOR® (PA-55), pelargonic acid + citral (PA-38+CIT), and carvacrol (CAR) showed at least a slight herbicidal effect as they had less weed coverage than UNT plots—25.6, 34.0, and 50.2%, respectively (Table 3)—while the other natural products citral (CIT), eugenol (EUG), *p*-cymene (P-CYM), thymol (THY) and the reference bioherbicide Beloukha® (PA-68) had the same weed coverage as the UNT plots. In contrast, the plots treated with the synthetic herbicides glyphosate (GLY) and oxyflurofen (OXY) showed a decrease in weed coverage from 30.7 to 8.0% and from 24.5 to 19.9%, respectively, at one WAT. On the following sampling date (2 WAT), plots treated with PA-55 and PA-38+CIT still exhibited less weed coverage than UNT plots, but weed control was not as satisfactory as that achieved with GLY (6.7%) and OXY (32.7%). At four WAT, only the plots treated with GLY (8.7% weed coverage) and OXY (58.4% weed coverage) presented reduced weed coverage compared with UNT plots, although OXY displayed a poorer performance than GLY, while the natural products exhibited a weed coverage in the range of 90.2–100%.

In the second experiment, located at the Polytechnic University of Valencia (UPV), the initial weed coverage was larger than at the first experiment (GUA), although the weed BBCH stage was earlier (Figure 1). In addition, at UPV, three of the natural products (CAR, EUG, and THY) were sprayed at two different doses, the same one as at GUA and at a triple dose, because of the weak herbicidal effect observed at GUA. At one WAT (Table 3), UNT plot weed coverage evolved from 48.2 to 78.4%. Contrary to what happened at GUA when all natural products induced a weed coverage increase, at UPV, only the CAR, EUG, THY, and P-CYM plots increased the weed coverage. All natural products containing pelargonic acid and CIT reduced the weed coverage, some of them (PA-55, and PA-38+CIT) at the same level as GLY and OXY. At two WAT, the UNT plot had a weed coverage of 87.5%, the same weed coverage achieved by CAR, EUG, THY, and P-CYM again, showing no weed control, while the rest of natural products, GLY and OXY presented less weed coverage. The natural products PA-55 and PA-38+CIT exerted weed control similar to GLY, which produced a total weed control, while weeds in the OXY-treated plots continued growing and covered 41.3% of the plot. At the end of the evaluation period (4 WAT), herbicide treatments could be classified into three groups according to the weed coverage, those producing no weed control (CAR, EUG, P-CYM, and THY), those producing deficient weed control (CIT, OXY, and PA-68), and herbicides with good to excellent weed control (PA-55, PA-38+CIT, and GLY). All plots treated with natural products at a triple dose exhibited less weed coverage at 4 WAT than plots treated at the normal rate. CAR, EUG, and THY at the triple-rate plots showed 44.6, 25.9, and 24.2%, respectively, less weed coverage that normal dose-treated plots, but this was still unsatisfactory in terms of weed control efficacy.

Table 3. Total weed coverage (%) obtained with Digimizer during the two experiments (Guadassuar and the Polytechnic University of Valencia). PA-55 (pelargonic acid 55%), CIT (citral), PA-38+CIT (pelargonic acid 38% + citral), CAR (carvacrol), CAR ×3 (carvacrol—triple dose), EUG (eugenol), EUG ×3 (eugenol—triple dose), THY (thymol), THY ×3 (thymol—triple dose), P-CYM (*p*-cymene), PA-68 (pelargonic acid 68%), GLY (glyphosate), OXY (oxyfluorfen), and UNT (untreated control). Different letters in the same column indicate significant differences according to Fisher's least significant difference (LSD) test ($p < 0.05$).

	Total Weed Coverage (%)							
	Guadassuar (GUA)				University (UPV)			
	Weeks after Initial Treatment				Weeks after Initial Treatment			
	0	1	2	4	0	1	2	4
PA-55	20.4 a	25.6 de	58.5 c	90.2 a	38.4 a	3.4 f	5.3 f	10.1 ef
CIT	22.7 a	54.6 abc	85.6 a	96.5 a	49.1 a	36.5 c	58.6 bc	75.2 bc
PA-38+CIT	26.0 a	34.0 cde	65.2 bc	93.8 a	55.1 a	11.9 ef	13.9 f	24.4 e
CAR	29.7 a	50.2 bcd	80.7 ab	97.8 a	52.4 a	70.3 ab	83.5 a	99.6 a
CAR ×3	-	-	-	-	52.3 a	16.2 def	31.2 e	55.2 d
EUG	29.5 a	60.9 abc	87.5 a	99.7 a	41.8 a	73.1 ab	83.9 a	99.2 a
EUG ×3	-	-	-	-	53.4 a	30.6 cd	51.0 cd	75.2 bc
THY	22.0 a	68.4 ab	95.0 a	100 a	41.8 a	64.7 b	74.0 ab	91.9 ab
THY ×3	-	-	-	-	53.0 a	22.9 cde	43.1 cde	68.1 cd
P-CYM	36.2 a	74.7 ab	93.5 a	99.7 a	50.7 a	79.9 a	89.9 a	99.9 a
PA-68	41.3 a	62.0 ab	87.5 a	92.5 a	37.4 a	31.1 c	52.7 cd	69.2 cd
GLY	30.7 a	8.0 e	6.7 e	8.7 c	47.7 a	8.3 ef	0.0 f	2.3 f
OXY	24.5 a	19.9 e	32.7 d	58.4 b	50.0 a	11.3 ef	41.3 de	73.2 c
UNT	29.6 a	77.6 a	91.8 a	98.9 a	48.2 a	78.4 ab	87.5 a	100.0 a

The "-" symbol within a table cell means no available data as triple doses were only used in the university experiment.

3.2. Effect on the Normalized Difference Vegetation Index (NDVI)

The NDVI is a useful quantitative assessment method for the determination of healthy vegetal soil coverage. NDVI values at GUA on the treatment date ranged between 0.19 and 0.24, with no statistical differences among treatments (Table 4). At one WAT, UNT plots' NDVI average value increased to 0.48, and the NDVI values of the plots treated with PA-68, EUG, P-CYM, and THY were equal to that of the UNT plots. Plots treated with CIT, CAR, PA-55, and PA-38+CIT showed increased NDVI values, although by less than the plots treated with the previous reported natural products and the UNT plots. The plots treated with both synthetic herbicides, GLY and OXY, did not show increased NDVI values. At two WAT, all natural product-treated plots' NDVI, but that of PA-55 and PA-38+CIT, did not differ from the NDVI of UNT plots, and again, GLY- and OXY-treated plots' NDVI values were lower than those of the rest of the treatments. Finally, on the four WAT date, apart from the synthetic herbicide-treated plots, which showed lower NDVI values than at 0 WAT, the only NDVI value different to that of the UNT plot (0.58) was the PA-55-treated plot NDVI (0.42), although the difference was not large and the reduction in weed coverage (42%) represented poor weed control.

Results from the UPV experimental field were different to those from GUA from the beginning. On the treatment date, the NDVI value ranged from 0.21 (CIT) to 0.28 (GLY), a little higher than at GUA, as weed coverage was 28.4% at GUA, vs. 48.0% at UPV. NDVI values showed significant differences between the CIT- and GLY-treated plots. At one WAT, there was a NDVI decrease in plots treated with CIT, CAR, PA-55, PA-68, PA-38+CIT, and both synthetic herbicides, while EUG-, P-CYM- and THY-treated plots´ NDVI increased and were equal to that of the UNT plots. At four WAT, only EUG- and P-CYM-treated plots´ NDVI were equal to that of UNT plots; however, the NDVI value of plots treated with CAR (0.43), THY (0.43), and PA-68 (0.34) indicated that weed control was not satisfactory. On the other hand, PA-55- and PA-38+CIT-treated plots´ NDVI stayed low (0.08 and 0.11, respectively), at the same level as the plots treated with GLY (0.08). Plots treated with CAR, EUG and THY at a triple dose obtained at four WAT lower NDVI values than plots treated at the normal rates, specifically, showed a reduction of 58.1, 60.4, and 32.0% of the NDVI compared with at the normal rates. Plots treated with the triple dose of CAR did not show significant statistical differences to plots treated with GLY.

Table 4. Normalized Difference Vegetation Index (NDVI) during the during the two experiments (Guadassuar and the Polytechnic University of Valencia). PA-55 (pelargonic acid 55%), CIT (citral), PA-38+CIT (pelargonic acid 38% + citral), CAR (carvacrol), CAR ×3 (carvacrol—triple dose), EUG (eugenol), EUG ×3 (eugenol—triple dose), THY (thymol), THY ×3 (thymol—triple dose), P-CYM (*p*-cymene), PA-68 (pelargonic acid 68%), GLY (glyphosate), OXY (oxyfluorfen), and UNT (untreated control). Different letters in the same column indicate significant differences according to Fisher's least significant difference (LSD) test ($p < 0.05$).

	NDVI							
	Guadassuar (GUA)				University (UPV)			
	Weeks after Initial Treatment				Weeks after Initial Treatment			
	0	1	2	4	0	1	2	4
PA-55	0.19 a	0.22 de	0.33 cd	0.42 c	0.23 ab	0.09 f	0.10 f	0.08 f
CIT	0.20 a	0.33 bcd	0.53 ab	0.52 abc	0,21 b	0.14 def	0.18 ef	0.28 de
PA-38+CIT	0.20 a	0.27 cde	0.41 bc	0.47 bc	0.26 ab	0.10 ef	0.12 f	0.11 f
CAR	0.20 a	0.31 bcde	0.53 ab	0.61 a	0.28 a	0.26 bc	0.33 bcd	0.43 bc
CAR ×3	-	-	-	-	0.28 ab	0.12 def	0.14 f	0.18 ef
EUG	0.20 a	0.37 abc	0.55 ab	0.62 a	0.23 ab	0.28 abc	0.39 abc	0.50 ab
EUG ×3	-	-	-	-	0.22 ab	0.20 cd	0.26 de	0.34 cd
THY	0.19 a	0.38 abc	0.57 a	0.58 ab	0.25 ab	0.27 abc	0.38 abc	0.43 bc
THY ×3	-	-	-	-	0.22 ab	0.13 def	0.17 ef	0.24 de
P-CYM	0.24 a	0.42 ab	0.57 a	0.58 ab	0.25 ab	0.34 ab	0.43 ab	0.57 a
PA-68	0.22 a	0.37 abc	0.59 a	0.61 a	0.23 ab	0.19 cde	0.28 cde	0.34 cd
GLY	0.22 a	0.18 e	0.22 d	0.14 d	0.28 a	0.16 def	0.10 f	0.08 f
OXY	0.19 a	0.19 de	0.20 d	0.17 d	0.27 ab	0.16 def	0.17 ef	0.27 de
UNT	0.20 a	0.48 a	0.61 a	0.58 ab	0.24 ab	0.36 a	0.45 a	0.56 a

The "-" symbol within a table cell means no available data as triple doses were only used in the university experiment.

3.3. Effect on Weed Damage

In both experiments, weeds at 0 WAT were in good condition, with no damage due to external abiotic or biotic factors. Weeds at GUA were in a later BBCH stage (14–18) than at UPV (12–14). On the first WAT, there were damage differences in weeds among treatments (Table 5). At GUA, weeds of the UNT-, EUG-, P-CYM-, THY-, and PA-68-treated plots did not show any significant injury, while weeds of the GLY-treated plot were almost dead (7.8). The only product showing the same level of weed damage as in the GLY plot was OXY (6.5); however, the PA-55- and PA-38+CIT-treated plots showed a good weed damage level (5.8), followed by CIT alone and CAR (3.5). Nevertheless, at four WAT, the only herbicides producing weed damage were GLY (7.8) and OXY (4.0).

In the second experiment (UPV), the EUG-, P-CYM-, and THY-treated plots were, as at GUA, the only products showing the same low weed damage level as UNT plots, while the PA-55- and PA-38+CIT-treated plots showed a very good weed damage level (8.8 and 8.3, respectively), overcoming the weed damage of both synthetic herbicides, GLY (7.3) and OXY (7.8), but without statistically significant differences among them. The rest of the natural products (CAR, CIT, and PA-68) caused an intermediate weed damage level, in the range of 3.5–5.0. This weed damage level remained stable at two WAT, with the only exception of OXY-treated plots, whose weeds did not seem so damaged (6.3) like the weeds in the PA-55- and PA-38+CIT-, and GLY-treated plots (8.6, 7.9, and 8.8, respectively). These three latter products were the only ones producing excellent weed damage on the last evaluation date (4 WAT), while the rest did not achieve satisfactory weed control. When CAR and EUG were applied at a triple dose, the weed damage effect was higher than at the normal dose, but the only product achieving good weed damage at the end of the evaluation period (4 WAT) was CAR (5.8), although not as satisfactory as in the PA-55-, PA-38+CIT-, and GLY-treated plots.

Table 5. Weed damage (1 to 9 visual scale, being 1 no damage and 9 dead plant) during the two experiments (Guadassuar and the Polytechnic University of Valencia). PA-55 (pelargonic acid 55%), CIT (citral), PA-38+CIT (pelargonic acid 38% + citral), CAR (carvacrol), CAR ×3 (carvacrol—triple dose), EUG (eugenol), EUG ×3 (eugenol—triple dose), THY (thymol), THY ×3 (thymol—triple dose), P-CYM (*p*-cymene), PA-68 (pelargonic acid 68%), GLY (glyphosate), OXY (oxyfluorfen), and UNT (untreated control). Different letters in the same column indicate significant differences according to Fisher's least significant difference (LSD) test ($p < 0.05$).

	Weed Damage						
	Guadassuar (GUA)			University (UPV)			
	Weeks after Treatment (WAT)			Weeks after Treatment (WAT)			
	0	1	4	0	1	2	4
PA-55	1.0 a	5.8 b	1.8 c	1.0 a	8.8 a	8.6 a	8.0 a
CIT	1.0 a	3.5 c	2.0 c	1.0 a	4.8 cd	5.3 bc	3.5 cd
PA-38+CIT	1.0 a	5.8 b	1.8 c	1.0 a	8.3 a	7.9 a	7.5 a
CAR	1.0 a	3.5 c	1.3 c	1.0 a	3.5 de	5.0 bcd	2.8 de
CAR ×3	-	-	-	1.0 a	7.5 a	6.3 b	5.8 b
EUG	1.0 a	1 d	1.8 c	1.0 a	2.0 ef	2.5 ef	1.8 ef
EUG ×3	-	-	-	1.0 a	5.5 bc	4.3 cd	3.5 cd
THY	1.0 a	1 d	1.5 c	1.0 a	2.5 ef	2.5 ef	2.5 de
THY ×3	-	-	-	1.0 a	5.5 bc	3.5 de	3.3 cd
P-CYM	1.0 a	1.5 d	1.5 c	1.0 a	2.3 ef	1.3 f	1.5 ef
PA-68	1.0 a	2.5 cd	1.5 c	1.0 a	5.0 cd	4.5 cd	3.8 cd
GLY	1.0 a	7.8 a	7.8 a	1.0 a	7.3 ab	8.8 a	8.5 a
OXY	1.0 a	6.5 ab	4.0 b	1.0 a	7.8 a	6.3 b	4.3 c
UNT	1.0 a	1 d	1.0 c	1.0 a	1.0 f	1.0 f	1.0 f

The "-" symbol within a table cell means no available data as triple doses were only used in the university experiment.

3.4. Effect on Weed Biomass

At the end of the evaluation period (4 WAT), weeds from a uniform 0.14 m^2 plot portion (selected by a circular frame of 42 cm of diameter) of each elemental plot were removed and weighed. At the GUA experimental field (Figure 2A), all products, except PA-68, caused a weed fresh weight reduction in comparison with the UNT plots. There were no significant statistical differences in fresh weight among the plots treated with the rest of products, with the only exception being that the THY-treated plots produced more weed biomass than plots treated with GLY and OXY. At the UPV experimental field (Figure 2B), the removed weed biomass was lower than at GUA. The CAR-, EUG-, P-CYM-, THY-, and PA-68-treated plots presented the same weed biomass as UNT plots, while the CIT-, GLY-, OXY-, PA-55-, and PA-38+CIT-treated plots produced less weed biomass than the UNT plots. The three natural products that were also applied at a triple dose (CAR, EUG, and THY) produced less weed biomass than when they were treated with at normal dose, but these differences were not statistically significant.

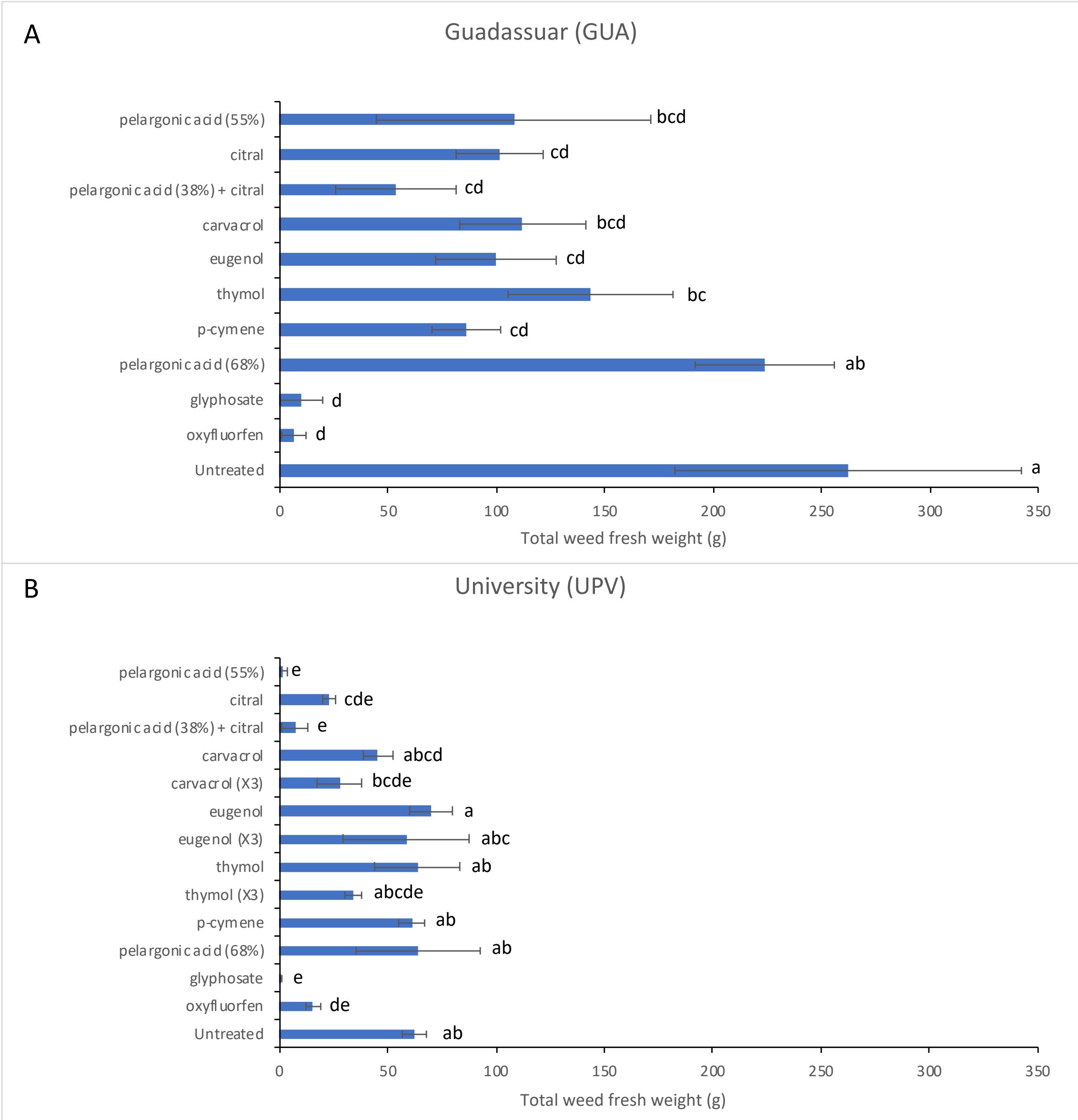

Figure 2. Weed fresh weight (g) (media of the four repetitions per treatment ± standard error) at the end of the trial at both experimental fields: (**A**) Guadassuar (GUA) and (**B**) the university (UPV). In each panel, different letters in the bars indicate significant differences, according to Fisher's least significant difference (LSD) test ($p < 0.05$).

4. Discussion

Little field research has been conducted on the efficacy of natural herbicides for weed control, as the majority have studied pelargonic acid (PA) [11,29–33]. To our knowledge, there are no reports of field research on the herbicidal activity of the natural products carvacrol (CAR), thymol (THY), eugenol (EUG), *p*-cymene (P-CYM), and citral (CIT). It is believed that the success of natural herbicides for post-emergence weed control is highly dependent on the following factors: weed species, weed phenological stage, product dose, concentration and formulation, water volume rate, treatment strategy, and weather conditions. In the present research, some of the above-mentioned factors have been tested.

Webber and Shrefler [30], reported that PA weed control was greater for broadleaf than for grass weeds. Ahuja et al. [14], in an in vitro experiment, concluded that eugenol caused retardation in seed germination and suppressed seedling growth. They observed that grassy plants were more sensitive than broad-leaved ones. This could be the explanation for the results obtained with EUG. In the experiment at UPV, there were only dicotyledonous species present. EUG at the normal dose did not achieve any weed control, while at GUA, with some monocotyledonous weeds present, the EUG-treated plots had less weed biomass than the UNT plots. The same effect was described by Grul'ová et al. [37], reporting that the essential oil (EO) from *Origanum vulgare* L. thymol chemotype (76% thymol content) exerted phytotoxic effects against the monocotyledonous species *Hordeum vulgare* L. and *Triticum aestivum* L. and stimulatory effects on the dicotyledonous species *Sinapis alba* L. and *Lepidium sativum* L. Other authors reported good monocotyledonous weeds control using different natural compounds, the mixture of lemmon grass oil and pelargonic acid resulted in 77% lower dry weight for *L. rigidum* Gaud. In comparison to untreated control, while *Avena sterilis* L. resulted in 31–33% lower dry weight [11]. In our case, we obtained a fresh weight reduction of 44.2% at GUA and 62.7% at UPV with mainly broadleaf weeds. In addition, at the GUA location, two perennial weed species were present, *C. rotundus* and *C. arvensis*, both with an undeniable power of recuperation that was only controlled with glyphosate.

In addition, Webber and Shrefler [30] verified that PA weed control was significantly better when applied to younger weeds. The UPV experiment was conducted at an earlier BBCH broadleaf weed stage than in GUA (12–14 vs. 14–18, respectively), resulting in excellent weed control with PA-55 SEITHOR® in terms of weed coverage, NDVI, weed damage, and weed biomass. Also, the mixture PA-38+CIT showed good weed control performance in terms of NDVI, weed damage, and weed biomass. In addition, other natural products achieved at least some level of weed control when they were treated at UPV, over less developed weeds: CIT and PA-68 in terms of weed coverage; CIT, CAR, THY, and PA-68 in terms of NDVI; CAR, THY, and PA-68 for weed damage; and CIT for weed biomass. Other researchers have reported the same conclusions on weed phenological stage sensitiveness. Ogbanwor and Söchting [32] concluded that the efficacy of PA against important weeds was less outstanding in BBCH 25 in comparison to BBCH 12. Covarelli and Contemori [29] found that PA applied at 30 kg ha^{-1} was able to control common purslane (*P. oleracea*) at cotyledon growth stage, while the efficacy was reduced 30%when applied at late growth stages (18–25).

The weed recovery capacity Is another important effect regarding weed stage when spraying a contact post-emergent herbicide like the tested natural compounds. Kanatas et al. [31] reported that based on NDVI measurements conducted at one day after treatment with several products including PA, they obtained low NDVI values compared to untreated plots; however, in the final measurement conducted ten days after treatment, weeds treated with PA seemed to recover since increased NDVI values were recorded. We obtained the same results for some of the PA formulations at the GUA location (higher initial weed BBCH stages than at UPV): PA-38+CIT and PA-55 showed good herbicidal effect in the first weeks but treated weeds recovered, while PA-68-treated weed NDVI values increased since the beginning. At UPV, as weeds were in a lower BBCH stage, they did not recover at the end of the experiment and NDVI values of PA-38+CIT- and PA-55-treated weeds remained low. This weed recovery effect could also be due to the tested volume rate. In the present research, we used 500 L ha^{-1}, a higher water volume rate than Panacci et al. [33], who obtained a PA response curve for several weeds using 300 L ha^{-1}, but lower than other authors, like Webber and Shrefler [30], who applied 935 L ha^{-1} obtaining good weed control.

Another important factor when experimenting with new natural herbicides is the application rate. Panacci et al. [33] determined the dose–response curve of PA for several broadleaf weeds using the following rate range: 1.4–21.8 kg ha^{-1}. They reported ED50 values for *A. retroflexus*, *Heliotropium europaeum* L., and *P. oleracea*, which were 11.7, 3.0, and 18.7 kg ha^{-1}, respectively, while our average control in terms of ground coverage at GUA and UPV was 48.0 and 81.3% at one WAT, 23.0 and 72.6% at two WAT, and 6.8 and 65.4% at four WAT using 5.7, 8.3, and 10.9 kg ha^{-1} of PA different formulations. In fact, the way the active ingredient is formulated is also paramount—not only the active ingredient concentration, but also its mixture with other compounds, which can potentiate its herbicidal activity. The active ingredient of herbicides must be formulated with other ingredients to allow mixing, dilution, application, and stability. The emulsifier system is probably the most important adjuvant in the formulation [38]. In order to store, market, and easily handle and apply bioherbicides, they must be formulated with co-formulants, the compositions of which are not normally made public. The compositions ensure the effectiveness of the product [10]. In our research, we obtained better weed control with formulations containing lower PA concentration (PA-38+CIT and PA-55) than with a more concentrated commercial formulation (PA-68). This could be explained by the different formulations of the active ingredient (PA), which contained different co-formulants that obtained different results in the performance of the active ingredient. The importance of the application rate was demonstrated at the UPV location when CAR, EUG and THY were also treated at a triple dose. All three natural compounds applied at a triple dose improved weed control compared to the normal dose at least for one of the determined parameters. CARX3 and EUGX3 achieved better weed coverage, NDVI, and damage than CAR and EUG, respectively, and THYX3 was better than THY for weed coverage and NDVI. However, this improvement in weed control was not always satisfactory and still higher doses should be explored. We also reported poor weed control when applying oxyfluorfen, which was in part explained by the fact that the early post-emergent effect of this herbicide is limited and perhaps the lower application rate in the present experiment (0.14 kg ha^{-1}) compared to that used by Bhowmik and Mc Glew [39] (0.43 kg ha^{-1}) for an acceptable post-emergence weed control was definitive.

A final factor to be considered when interpreting the obtained results is climate. Hot and dry conditions can promote leaf traits that decrease weed sensitivity by reducing herbicide penetration inside leaves [34]. In the present research, the mean temperature of both experiments differed by 7.2 °C. At the GUA location, the experiment was conducted during July to August, with a mean temperature of 26.3 °C (Table 1), which is perhaps high enough to prevent good herbicide performance. However, at UPV, the experiment was conducted in October, with a more suitable mean temperature (19.2 °C) for herbicide action on the plant. Rainfall was low and very similar during both experimental periods, 15.9 mm at GUA and 13.0 mm at UPV (Table 1). The only difference was that at GUA, the rainfall was concentrated over just 5 days after treatment and at UPV 14 days after treatment. This, together with the higher mean temperature and higher phenological stage, could explain why there was a generally large weed coverage increment at GUA—because the rainfall provoked new weed germination processes. At UPV, since the main rainfall event was later, the new germination processes did not affect general weed coverage as much. Perhaps a two-application strategy with a 15-day interval rather than one would have been decisive in obtaining a good weed control.

5. Conclusions

In summary, the natural compounds PA-55 and PA-38+CIT showed excellent weed control when used to treat annual broadleaf weeds at early phenological stages, with results comparable to those of the effective herbicide glyphosate, and superior to those of the also well-known commercial formulation of pelargonic acid (PA-68). The findings of the present field study revealed that, in general, the tested natural products have at least some herbicidal effect, which could be improved when factors such as water volume, product rate and formulation, application strategy, weed type and phenological stage at treatment time, and climatic conditions are considered. Future field research is needed to test these above-mentioned factors.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/agronomy14030537/s1, Figure S1: Image of a plot processed with Digimizer to measure the weed coverage; Figure S2: Correlation between weed coverage assessment methods (Digital image analysis software vs visual score).

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