

Article

Effect of Plant Density on *Artemisia annua* L. Biomass and Essential Oil Yield and Its Weed Seed Germination Suppression

Flavio Polito ^{1,*}, Michele Denora ^{2,*}, Donato Casiello ², Pierluigi Casiero ², Lorian Cardone ²,
Vincenzo Candido ², Michele Perniola ², Vincenzo De Feo ¹, Valentino Palombo ³ and Sebastiano Delfine ³

¹ Department of Pharmacy, University of Salerno, Via Giovanni Paolo II, 132, 84084 Fisciano, Italy; defeo@unisa.it

² Department of Agriculture, Forest, Food and Environmental Sciences, University of Basilicata, Viale dell'Ateneo Lucano, 10, 85100 Potenza, Italy; docasiello@gmail.com (D.C.); pierluigi.casiero@unibas.it (P.C.); lorian.cardone@unibas.it (L.C.); vincenzo.candido@unibas.it (V.C.); michele.perniola@unibas.it (M.P.)

³ Department of Agriculture, Environment and Foods, University of Molise, Via De Sanctis, 86100 Campobasso, Italy; abg@animol.it (V.P.); delfine@animol.it (S.D.)

* Correspondence: fpolito@unisa.it (F.P.); michele.denora@unibas.it (M.D.)

Abstract

During spring–summer 2023 and 2024, an *Artemisia annua* crop was carried out, using two planting densities (20D = 5.0 plants m⁻²; 40D = 2.5 plants m⁻²). Morphological traits were measured, including height, stem number, diameter, weight, and dry above-ground biomass. The aerial parts were hydro-distilled, and the essential oil (EO) yield increased from the 1st to 2nd year, from 0.117 to 0.439% for 20D and from 0.157 to 0.550% for 40D. There were significant variations in chemical composition between the years, with an increase in the presence of oxygenated monoterpenes in the 2nd year and the disappearance of oxygenated sesquiterpenes. In the 1st year, sesquiterpene hydrocarbons were the main class, while in the 2nd, oxygenated monoterpenes predominated. The main components were artemisia ketone (8.05–65.77%), eucalyptol (4.70–13.14%), and β-selinene (5.38–37.53%), present in all the EOs, and *trans*-caryophyllene (11.65%), present only in the 1st year EOs. The possible phytotoxicity of the EOs on seeds of plants found in the *A. annua* crops was evaluated. The most susceptible seeds were *Sinapis alba*, *Papaver rhoeas*, and *Portulaca oleracea*. The phytotoxicity was greater in the 2nd year, with more marked effects on the germination of *P. rhoeas* and *P. oleracea* (up to 100%). The inhibition of root elongation reached 100% for those at the higher concentrations tested.

Keywords: *Artemisia annua* L.; plant density; morphological traits; essential oil; phytotoxicity



Academic Editor: William A. Payne

Received: 4 May 2025

Revised: 13 June 2025

Accepted: 18 June 2025

Published: 20 June 2025

Citation: Polito, F.; Denora, M.; Casiello, D.; Casiero, P.; Cardone, L.; Candido, V.; Perniola, M.; De Feo, V.; Palombo, V.; Delfine, S. Effect of Plant Density on *Artemisia annua* L. Biomass and Essential Oil Yield and Its Weed Seed Germination Suppression.

Agriculture **2025**, *15*, 1330.

<https://doi.org/10.3390/agriculture15131330>

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1. Introduction

Essential oils (EOs) are widely used in food [1], herbal [2], and pharmaceutical [3] industries. Recently, their use is also common in agriculture [4] and post-harvest [5] to defend plants and food from pests. The scientific research behind the spread of the EOs in these productive areas, however, often produces contradictory results. These inconsistencies can be attributed to differences related to genetics [6,7], the environment [8,9], and the cultivation practice adopted [10]. Specifically, an important role of EO yield and composition is played by plant density, which has the potential to induce natural variations in the development of the plant and, therefore, in its primary and secondary metabolism [11–13]. *Artemisia* L. is one of the largest and most important aromatic and medicinal genera of the Asteraceae family and comprises more than 400 species [14]. The annual wormwood, *Artemisia annua* L., native to

temperate Asia, has become endemic in a higher number of countries [15]. *Artemisia annua* is widely studied for its bioactive compounds, and its essential oils are considered promising candidates for sustainable weed control due to their allelopathic activity. EOs are known for their several modes of action against pests [16,17]. The main compounds detected in EOs of *A. annua* in previous investigations include camphor, 1,8-cineole, and artemisia ketones, but the chemical composition varied in accordance with the geographic area, time of harvest, method of extraction, and part of the plant [18]. Furthermore, the scientific importance of this plant comes from its biologically active substances, such as sesquiterpenes and terpenoids (mainly monoterpenes in EOs) [19]. These phytochemical classes are recognized for a wide variety of biological activities [20]. Cultivated annual wormwood, not only for EO production, is certainly an interesting and promising product, given the various uses including agricultural ones. Despite their promising bioherbicidal potential, *A. annua* essential oils may pose toxicity risks to mammals if not properly formulated or dosed, which limits their direct use and requires further toxicological assessment [21].

This cultivation, with defined cultural management, could represent a valuable opportunity to increase agro-environmental sustainability and diversification of typical Mediterranean crops, as well as to help reduce the use of synthetic molecules for pest management. Considering the potential economic importance of this crop, this study aimed at evaluating the possibility of implementing targeted crop management (plant density) to standardize the quality of annual wormwood EOs in order to better enhance them for agricultural uses from a sustainable perspective. The influence of the plant density, at the flowering stage, on the main agronomic and phytotoxic traits (plant biomass production and weed seed germination suppression performance) and on EO yield and composition have been evaluated.

2. Materials and Methods

2.1. Study Area and Plant Material

The study was conducted during the spring–summer seasons of 2023 and 2024 at an experimental field site in the Basilicata region (South of Italy). The Basilicata region is positioned on the eastern side of the Apennines watershed and has a typical Mediterranean climate of interior lands in southern Italy. The field was located in a hilly environment, close to Genzano di Lucania (40° 82' N, 16°08' E; 252 m a.s.l.). The experimental site presents a high fertility considering the texture of the soil, organic matter, and nutrient content (Table 1).

Seeds of *Artemisia annua* used in the experiment were obtained from a certified commercial supplier (Sativa Biosaatgut; Jestetten, Germany). The seed lot was selected for genetic uniformity and health quality and tested for purity and germination rate according to standard protocols for medicinal plant propagation. Seedlings were initially sown in nursery trays on 23 April 2023 and 2 May 2024, respectively, and transplanted into the field after 30 days of growth under controlled conditions. The experimental design was structured as a randomized complete block design (RCBD) to evaluate the effects of different planting densities on growth, EO yield and composition, and phytotoxic activity. This design was chosen to minimize the effects of variability in environmental conditions across the experimental site. The primary treatment factor was the planting density, which included two levels: 20D (High Density), with 5.0 plants per m² and with seedlings spaced 0.2 m apart within rows, and 40D (Low Density), with 2.5 plants per m² and with seedlings spaced 0.4 m apart within rows. The two densities (5.0 and 2.5 plants m⁻²) were selected to allow for mechanical weed control operations and are supported by previous studies showing their agronomic and economic feasibility, as well as their influence on biomass and secondary metabolite production [22].

Table 1. The soil properties (particle distribution and physical–chemical properties) and weather data of the experimental field site.

Parameters	Values
Sand (%)	37.1
Silt (%)	26.0
Clay (%)	36.9
pH (in water)	8.2
Total CaCO ₃ (%)	5.0
Organic matter (%)	2.28
Cation exchange capacity (meq/100 g)	25.16
Active CaCO ₃ (%)	2.5
Total N (‰)	0.138
Electrical conductivity (mS/cm)	0.188
Exchangeable K (ppm)	862
Assimilable P (ppm)	7

Each treatment was replicated three times, resulting in a total of six experimental plots. The plots were arranged to ensure that each treatment was exposed to similar environmental conditions, such as sunlight, soil type, and moisture levels. Each plot measured 10 m², containing 25 plants at the lower density (2.5 plants m⁻²) and 50 plants at the higher density (5.0 plants m⁻²). For each treatment and replicate ($n = 3$), ten representative plants were randomly selected and harvested for morphological, yield, and chemical analyses. In each experimental plot, sufficient space was allowed for plant growth, and competition between plants within the same plot was minimized. The plots were separated by buffer zones of 1 m to reduce the potential for cross-contamination of treatments and to facilitate maintenance activities.

Before fertilizer applications, soil samples (0–30 cm) were taken from each block and analyzed according to standard procedures [23].

The soil texture was characterized as average and as sand–clay (Table 1). In general, organic matter and total N contents were good. The soil profile was overall uniform, containing good amounts of available P and exchangeable K. Soils had very low active CaCO₃, and pH was average alkaline; salinity was low.

Following transplanting, the field was irrigated using drip lines positioned along each row. The initial irrigation brought the soil to Field Water Capacity (FWC). During the crop cycle, irrigation was performed periodically when climatic conditions indicated a significant decrease in soil moisture, with the aim of maintaining levels near FWC and avoiding drought stress. This water management strategy followed standard recommendations for crop evapotranspiration and irrigation scheduling [24].

Durum wheat was the previous crop. After plowing (35 cm depth), 90 kg P ha⁻¹, 90 kg K₂O ha⁻¹, and 100 kg N ha⁻¹ were applied. The same fertilization protocol was applied to both planting densities, as no significant nutrient competition was observed in the field or reported in the literature under comparable conditions [22,25].

Harvesting was conducted at full flowering (balsamic stage) on 31 July 2023 and 12 August 2024, respectively, to ensure maximum accumulation of bioactive compounds in the aerial parts.

The agronomic traits analyzed were plant height, stems per plant, fresh biomass, dry matter content, and dry biomass. The crop was harvested manually in both years. The

herbal-yield biomass was weighed, after which the plant samples were heated at 105 °C for one hour and then dried at 75 °C to constant weight.

2.2. Climatic Parameters

The area of study is characterized by a warm temperate climate with dry summers, and precipitation is concentrated in the winter and spring seasons.

A conventional meteorological station placed in a field site recorded climatic data. The meteorological data for the periods 1 April–30 September 2023 and 1 April–30 September 2024 are illustrated in Figures 1 and 2. Total precipitation in the 1st growing season (2023) was 332 mm, whereas in the 2nd season (2024) it decreased to 161 mm. The average temperature increased from 21 °C in 2023 to 22.7 °C in 2024.

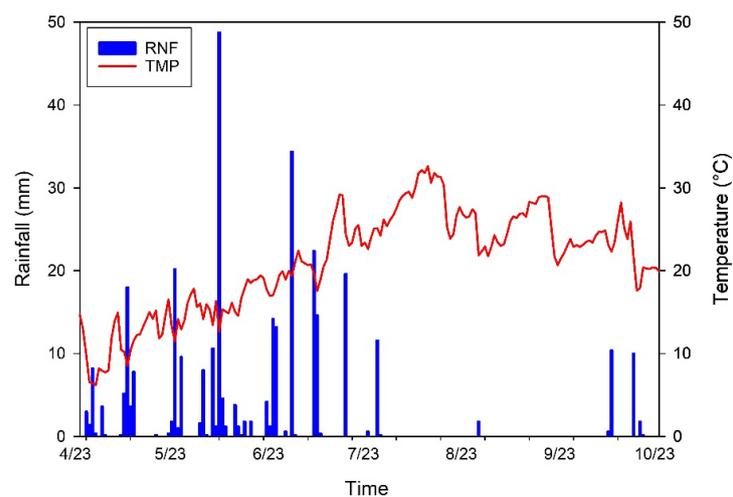


Figure 1. Daily rainfall (RNF, mm) and average temperature (TMP, °C) recorded during the 2023 growing season. The full flowering stage (BBCH 65) of *Artemisia annua* occurred between 25 July and 5 August.

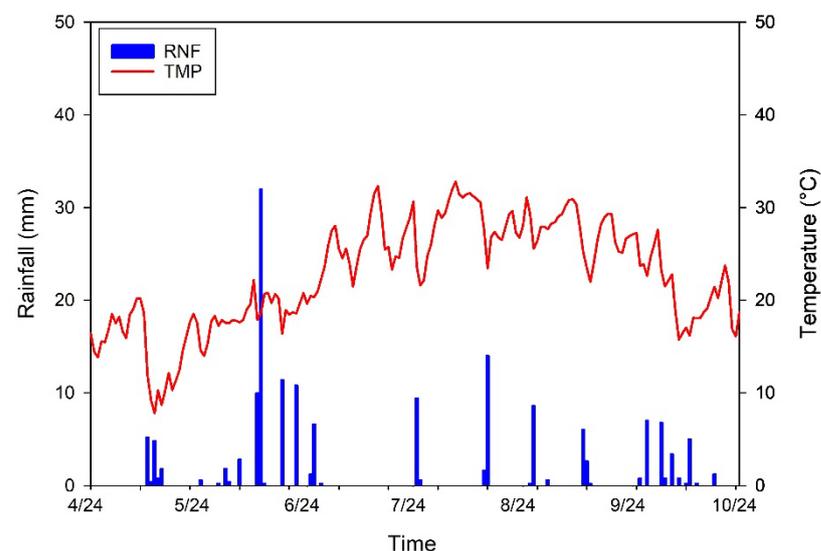


Figure 2. Daily rainfall (RNF, mm) and average temperature (TMP, °C) recorded during the 2024 growing season. The full flowering stage (BBCH 65) of *Artemisia annua* occurred between 05 August and 15 August.

2.3. Isolation of Essential Oils

In both years, leaves and young stems of *A. annua* were subjected to oil extraction. Plants were harvested for oil isolation at full flowering (BBCH 65), corresponding to

the balsamic stage when the concentration of essential oils and bioactive compounds is expected to peak. To isolate the EOs, each sample (1000 g in three replications) was subject to hydro-distillation for 3 h, using a Clevenger-type apparatus based on the method already described [9]. The EOs were obtained with different yields in the years, 20D 0.117 ± 0.011 –40D $0.157 \pm 0.01\%$ (2023) and 20D 0.430 ± 0.021 –40D $0.550 \pm 0.015\%$ (2024), on a fresh mass and were yellowish (20D intense and 40D light). The EOs were dried over anhydrous sodium sulphate and stored under N_2 at $+4^\circ C$ in the dark until tested and analyzed.

2.4. GC and GC-MS Analysis

GC analysis was performed using a Perkin-Elmer Sigma 115 gas chromatograph (Waltham, MA, USA) equipped with a flame ionization detector (FID) on a non-polar HP-5 MS capillary column of fused silica (30 m \times 0.25 mm; 0.25 μm film thickness). The operating conditions were as follows: injector and detector temperatures, 250 and 290 $^\circ C$, respectively. The analysis was conducted on a scheduled basis, 5 min isothermally at 40 $^\circ C$; subsequently, the temperature was increased by 2 $^\circ C/min$ until 270 $^\circ C$, and finally it was kept in isotherm for 20 min. The analysis was also performed on an HP Innowax column (50 m \times 0.20 mm; 0.25 μm film thickness). In both cases, He was used as a carrier gas (1.0 mL/min). GC-MS analysis was performed using an Agilent 6850 Ser. II Apparatus (Santa Clara, CA, USA) equipped with a DB-5 fused silica capillary column (30 m \times 0.25 mm; 0.25 μm film thickness) and connected to an Agilent Mass Selective Detector (MSD 5973) (Santa Clara, CA, USA) with an ionization voltage of 70 V and an ion multiplier energy of 2000 V. The mass spectra were scanned in the range of 40–500 amu, with five scans per second. The chromatographic conditions were as reported above, and the transfer line temperature was 295 $^\circ C$. Most of the components were identified by comparing their Kovats indices (Ki) with those of the literature [26–29] and by a careful analysis of the mass spectra compared to those of the pure compounds or to those present in the NIST 14 and Wiley 257 mass libraries [30]. The Kovats indices were determined in relation to a homologous series of *n*-alkanes (C10–C35) under the same operating conditions. The identification of some compounds was confirmed by co-injection with standard samples. The components' relative concentrations were calculated by peak area normalization. Response factors were not considered.

2.5. Phytotoxic Activity

The phytotoxic activity was evaluated against the seed germination and radicle emergence/elongation of the weeds *Lolium multiflorum* Lam., *Sinapis alba* L., *Papaver rhoeas* L., and *Portulaca oleracea* L., as well as the horticultural crops, *Vicia lens* (L.) Coss. & Germ. and *Triticum durum* L. The weed species used for phytotoxicity testing were selected based on their prevalence in Mediterranean cereal-based cropping systems, particularly in durum wheat fields, to ensure the relevance and applicability of the results under field conditions. The seeds were surface sterilized in 96% ethanol for 15 s and sown in Petri dishes ($\varnothing = 90$ mm) on three layers of Whatman filter paper. They were impregnated with 7 mL of deionized water, and then 7 mL of a water–acetone mixture (99.5:0.5, *v/v*) was used as the control since EOs were dissolved in this mixture due to their lipophilicity. Finally, 7 mL of the EO solution at different concentrations (1000, 500, 250, and 125 $\mu g/mL$) was tested. The controls carried out with the water–acetone mixture alone showed no differences in comparison to the controls in water alone. The germination conditions were $20 \pm 1^\circ C$, with a natural photoperiod. Seed germination was checked in Petri dishes every 24 h. A seed was considered germinated when the protrusion of the root became evident [31]. On the fifth day (after 120 h) for *S. alba*, the fifteenth day for *P. rhoeas* (after 360 h), and the seventh

day (after 168 h) for the other seeds, the effects on radicle elongation were determined by measuring the root length in cm. Each evaluation was replicated three times, using Petri dishes containing 10 seeds each. The data were expressed as the mean \pm standard deviation for both germination and radicle elongation.

2.6. Statistical Analysis

Agronomic traits were analyzed with analysis of variance (ANOVA), and mean values were separated using the Student Newman–Keuls' (SNK) test with $p \leq 0.05$.

3. Results and Discussion

3.1. Effects of Density on Morpho-Productive Traits of *A. annua*

Table 2 summarizes the effect of plant density on several morpho-productive traits of *A. annua* over two years (2023 and 2024). Significant differences in the morphological traits between the two densities have been found. In 2023, the average height of plants in the higher density (20D) was 176.0 ± 1.2 cm, compared to 156.8 ± 1.7 cm in the lower density (40D). Additionally, the number of stems per plant was higher in 40D (3.5 ± 0.1) than in 20D (2.5 ± 0.2). The fresh biomass was also greater in 20D (3683.5 ± 185.2 g/m²) compared to 40D (3071.3 ± 199.0 g/m²), although the dry biomass content was comparable between 40D ($40.6 \pm 0.2\%$) and 20D ($40.4 \pm 0.7\%$).

Table 2. Effect of plant density on morpho-productive traits of *A. annua* crop in two years.

Year	Plant Densities	Plant Height (cm)	Stems Per Plant (n.)	Fresh Biomass (g/m ²)	Dry Matter Content (%)	Dry Biomass (g/m ²)
2023	20D	176.0 ± 1.2	2.5 ± 0.2	3683.5 ± 185.2	40.4 ± 0.7	1489.1 ± 99.1
2023	40D	156.8 ± 1.7	3.5 ± 0.1	3071.3 ± 199.0	40.6 ± 0.2	1248.5 ± 36.4
2024	20D	95.6 ± 4.0	3.1 ± 0.4	1840.1 ± 99.1	44.0 ± 0.1	810.3 ± 28.4
2024	40D	95.5 ± 1.0	4.9 ± 0.4	1211.1 ± 98.3	47.1 ± 0.6	571.6 ± 19.1
Significance		**	*	**	**	**

Mean values followed by a different letter are significantly different at $p \leq 0.05$, according to the SNK test. *, significance at $p \leq 0.05$; **, significance at $p \leq 0.01$.

In 2024, the 20D plants exhibited reduced height but maintained a relatively higher number of stems per plant and higher dry matter content. Conversely, the 40D plants showed shorter heights but produced the highest number of stems, albeit with lower total biomass and reduced dry weight. This suggests that while denser plantings may promote more vigorous growth in terms of height and stem proliferation, they may also face limitations in biomass production and dry matter content in subsequent years, possibly due to increased competition for resources. The climatic differences observed in the two years of research had a direct impact on soil water availability and plant stress levels during critical development stages. The higher precipitation recorded in 2023 ensured better water availability, promoting vegetative growth. Conversely, the reduction in precipitation in 2024 intensified environmental stress, limiting biomass production. The markedly higher dry biomass recorded for the 40D treatment in 2024 (571.6 ± 19.1 g/m²) may be attributed to favorable climatic conditions during early plant development, including above-average rainfall in June, coupled with reduced interplant competition at lower density, which likely enhanced vegetative growth at the single-plant level. This aligns with the findings by Scarcella et al. [32], who observed that adequate water availability significantly enhances biomass yield in *Artemisia annua*, whereas water stress conditions lead to reduced plant growth and productivity in semi-arid environments.

3.2. Essential Oil Yield and Composition

Table 3 reports the yield and composition of the EOs. The EO yield increases from the 1st to 2nd year, from $0.117 \pm 0.011\%$ to $0.430 \pm 0.021\%$ for 20D (about 3.7 times) and from $0.157 \pm 0.01\%$ to $0.550 \pm 0.015\%$ for 40D (about 3.5 times). This trend has been reported for the EO yield in the *Artemisia* genus [33]. The analyses showed 68 compounds in total: 61 in the 20D 1st year (93.35% of the total), 17 in the 20D 2nd year (98.57%), 28 in the 40D 1st year (95.33%), and 14 in the 40D 2nd year (98.71%). The EO from the 20D 1st year has a different composition compared to that of the 2nd year. In the 1st year, the most representative class is that of sesquiterpene hydrocarbons (53.52%), followed by oxygenated sesquiterpenes and oxygenated monoterpenes in similar percentages (18.78% and 17.60%, respectively). Monoterpene hydrocarbons were poorly represented (1.16%), while 2.29% were metabolites belonging to other classes. In the 2nd year, the predominant class is that of oxygenated monoterpenes (78.19%), followed by sesquiterpene hydrocarbons (11.32%) and monoterpene hydrocarbons (6.84%). Only 2.22% is represented by molecules belonging to other classes. Oxygenated sesquiterpenes were absent. Even the EO of the 40D 1st year presented differences with the composition of the 2nd year. The predominant class is that of sesquiterpene hydrocarbons (48.92%), followed by oxygenated monoterpenes (35.83%), oxygenated sesquiterpenes (6.99%), and monoterpene hydrocarbons (2.90%); 0.69% belonged to other chemical classes. In the 2nd year, the predominant class was instead that of oxygenated monoterpenes (84.59%), while the other classes did not reach 10% (7.73% for sesquiterpene hydrocarbons, 4.38% for monoterpene hydrocarbons, and 2.01% for others); oxygenated sesquiterpenes were absent. Comparing the compositions of 20D and 40D, a similar trend can be noted. In the 1st year, all classes of components were present, with a predominance of sesquiterpene hydrocarbons, while in the 2nd year, in both EOs, oxygenated monoterpenes were the main class, and oxygenated sesquiterpenes disappeared completely. The main components were the same in all EOs (artemisia ketone, eucalyptol, and β -selinene); however, *trans*-caryophyllene was among the main components only in the EO 20D 1st year. Also, their amount between the 1st and 2nd year and between the two EOs follow the same trend. In the 1st year, 20D had β -selinene (34.35%), *trans*-caryophyllene (11.65%), and artemisia ketone (8.05%) as the main components, while 40D showed β -selinene (37.53%), artemisia ketone (14.01%), and eucalyptol (11.19%) as the main components. In the 2nd year, artemisia ketone resulted in the main component (50.32% in 20D and 65.77% in 40D, respectively), followed by eucalyptol and β -selinene in both EOs (13.14% and 8.20% in 20D and 8.97% and 5.38% in 40D). The compositions were probably influenced by the climatic differences between the two harvest years. In fact, environmental conditions, including temperature and water availability, are among the critical factors that influence the characteristics of EOs [34–36]. The 1st year (2023) was characterized by greater water availability and less warm temperatures, leading to less stress on the plants that grew abundantly and with a more balanced composition between sesquiterpene hydrocarbons and oxygenated monoterpenes. On the contrary, the reduction in rainfall and the increase in temperature in 2024 caused more stress on the plants, resulting in a greater production of more volatile compounds, such as oxygenated monoterpenes [37,38]. The literature reports numerous components in *A. annua* EO, with components varying by geographic and environmental factors [39]. Variations in climatic conditions and planting density are known to influence the biosynthesis of secondary metabolites in *Artemisia annua*. Environmental stresses, such as drought and temperature increase, can modulate the expression of genes involved in terpenoid pathways, including those encoding terpene synthases, ultimately affecting essential oil composition [40,41] (Xie et al., 2016; Arsenault et al., 2010). Regarding essential oils, a higher yield was obtained, on average, for the annual wormwood collected in 2024. Instead, despite the season, the plant density

D20 has produced lower oil than D40. The different oil and plant biomass yields in the two seasons can be related to the climatic differences found. The detected climatic differences may have caused a reduction in soil water availability for the annual wormwood crop, resulting in stressful conditions for the plant during critical developmental stages [42] (Yadav et al., 2014). Water availability is inversely related to dry plant biomass and oil production, as previously reported by Delfine et al. [9] for mallow under moderate water stress conditions. In addition, the higher temperatures recorded in 2024 intensified stress conditions, limiting biomass production while affecting essential oil yield [43] (Zehra et al., 2019). The most frequently found compounds are artemisia ketone, borneol, camphene, camphor, β -caryophyllene, carvone, 1,8-cineole, limonene, myrtenol, α - and β -pinene, and α -terpinene [44–47]. Except for carvone and limonene, all of them were present in the studied EOs, and in some cases (as happens for artemisia ketone and β -caryophyllene), they were among the main components. Comparing the composition with that of other Italian *A. annua* EOs, it is possible to note a certain heterogeneity in the compositions, which are not always in agreement with that of this work. In 2024, an EO of *A. annua* from the Campania region (Southern Italy) resulted rich in β -pinene (14.4%) and *trans*-chrysantenyl acetate (6.3%) [48]. An *A. annua* EO from the Marche region, central Italy, was found to be rich in camphor (14.63%), cubenol (2.15%), and spathulenol (2.05%). In our samples, camphor was present in lower amounts (1.95–6.26%), while spathulenol and cubenol were present only in the 1st year EOs (0.74–2.12%) [49]. Artemisia ketone (24.0%), camphor (17.7%), and 1,8-cineole (16.1%) are the main components of a Tuscan EO, which, therefore, presents similarities with that of this work, being that all three are present in the studied EOs as main components [50].

Table 3. Essential oil yield and composition.

	%				Ki ^a	Ki ^b	Identification ^c
	20D 1st Year	20D 2nd Year	40D 1st Year	40D 2nd Year			
Yield	0.117	0.430	0.157	0.550			
1 α -Pinene	0.20	-	0.67	-	862	1025	1,2,3
2 Camphene	0.23	1.10	0.83	0.77	874	1068	1,2,3
3 β -Pinene	0.38	2.07	0.89	1.34	899	1110	1,2,3
4 β -Myrcene	-	3.67	-	2.27	920	1145	1,2
5 Yomogi alcohol	0.65	1.18	0.84	1.38	928	1395	1,2
6 <i>p</i> -Cymene	0.35	-	0.51	-	946	1270	1,2,3
7 Eucalyptol	4.70	13.14	11.19	8.97	951	1211	1,2,3
8 Artemisia ketone	8.05	50.32	14.01	65.77	985	1344	1,2
9 Artemisia alcohol	0.27	2.77	1.08	2.73	1002	1510	1,2
10 <i>p</i> -Mentha- <i>trans</i> -2,8-dien-1-ol	-	2.37	-	1.42	1025	1639	1,2
11 Butanoic acid, 2-methyl-, 3-methyl-3-butenyl ester	0.10	-	-	-	1032		1,2
12 <i>trans</i> -Pinocarveol	0.12	-	-	-	1049	1661	1,2
13 Camphor	1.95	6.26	6.25	4.32	1054	1515	1,2,3
14 4,8-dimethyl-1,3,7-Nonatriene	-	1.67	-	1.04	1060	1309	1,2
15 2,6-dimethyl-1,5,7-Octatrien-3-ol	-	-	-	0.97	1064		1,2
16 Borneol	0.11	1.61	-	-	1076	1700	1,2,3
17 Terpinen-4-ol	0.76	0.54	1.30	-	1088	1601	1,2
18 α -Terpineol	0.32	-	-	-	1094	1694	1,2,3
19 Myrtenol	0.21	-	0.38	-	1100	1790	1,2

Table 3. Cont.

		%				Ki ^a	Ki ^b	Identification ^c
		20D 1st Year	20D 2nd Year	40D 1st Year	40D 2nd Year			
20	<i>cis</i> -3-Hexenyl isovalerate	0.12	-	-	-	1141		1,2
21	Carvacrol,methyl ether	0.14	-	-	-	1197	1599	1,2
22	<i>p</i> -Menth-en-3,8-diol	0.16	-	0.39	-	1198		1,2
23	α -Copaene	0.56	-	0.53	-	1269	1491	1,2,3
24	Butanoic acid, 3-methyl-, phenylmethyl ester	0.95	-	1.08	-	1283	1902	1,2
25	Benzyl isovalerate	-	0.55	-	-	1286	1851	1,2
26	<i>cis</i> -Jasmone	0.18	-	-	-	1293	1933	1,2
27	<i>trans</i> -Caryophyllene	11.65	1.52	5.97	1.48	1303	1588	1,2
28	α -Humulene	0.77	-	0.46	-	1337	1667	1,2
29	Amorpha-4,11-diene	0.76	-	0.45	-	1345		1,2
30	<i>trans</i> - β -Farnesene	0.17	0.54	-	-	1347	1665	1,2
31	α -Acoradiene	0.41	-	0.55	-	1360	1690	1,2
32	Germacrene D	-	1.06	3.43	0.87	1364	1708	1,2
33	γ -Muurolene	3.84	-	-	-	1365	1690	1,2
34	β -Selinene	34.35	8.2	37.53	5.38	1369	1717	1,2
35	δ -Selinene	0.15	-	-	-	1376	1756	1,2
36	Bicyclogermacrene	0.26	-	-	-	1379	1734	1,2
37	Indipone	0.25	-	-	-	1381		1,2
38	γ -Patchoulene	0.42	-	-	-	1397	1664	1,2
39	δ -Cadinene	0.18	-	-	-	1402	1749	1,2
40	<i>cis</i> -Nerolidol	0.12	-	-	-	1407	2007	1,2
41	<i>trans</i> -Nerolidol	0.22	-	-	-	1445	2036	1,2
42	Palustrol	0.25	-	-	-	1449	1953	1,2
43	Spathulenol	0.76	-	0.74	-	1452	2127	1,2
44	Caryophyllene oxide	2.16	-	1.04	-	1457	1986	1,2
45	Isoaromadendrene epoxide	2.31	-	1.8	-	1460	1807	1,2
46	Globulol	0.18	-	-	-	1484	2082	1,2
47	Longiborneol	1.11	-	0.49	-	1493		1,2
48	Junenol	0.12	-	-	-	1496		1,2
49	10- <i>epi</i> - γ -Eudesmol	0.35	-	-	-	1498	1624	1,2
50	<i>allo</i> -Aromadendrene epoxide	0.25	-	-	-	1497	2095	1,2
51	Cubenol	2.12	-	1.29	-	1500	2068	1,2
52	Cedr-8(15)-en-9- α -ol	0.35	-	-	-	1519	-	1,2
53	7- <i>epi</i> - α -Eudesmol	0.24	-	-	-	1526	2224	1,2
54	Ylangenal	0.25	-	-	-	1529		1,2
55	Aromadendrene oxide-(2)	1.43	-	0.56	-	1532		1,2
56	Alloaromadendrene oxide-(1)	0.24	-	-	-	1545		1,2
57	(1R,7S)-Germacra-4(15),5,10(14)-trien-1 β -ol	1.11	-	0.46	-	1550		1,2
58	Eudesm-7(11)-en-4-ol	0.14	-	-	-	1558	2271	1,2
59	8- α -11-Elmodiol	0.2	-	-	-	1579		1,2
60	Aristolone	1.13	-	0.61	-	1586		1,2
61	α -Costol	0.12	-	-	-	1599	2604	1,2
62	γ -Eudesmol acetate	0.17	-	-	-	1600	2174	1,2

Table 3. Cont.

		%				Ki ^a	Ki ^b	Identification ^c
		20D 1st Year	20D 2nd Year	40D 1st Year	40D 2nd Year			
63	8-Cedren-13-ol acetate	0.8	-	-	-	1603		1,2
64	Isovalencenol	1.89	-	-	-	1626		1,2
65	Acid <i>cis</i> -thujopsenic	0.16	-	-	-	1693		1,2
66	8S,13-Cedranediol	0.16	-	-	-	1694		1,2
67	11,12-dihydroxy-Valencene	0.44	-	-	-	1731		1,2
68	Phytol	0.85	-	-	-	1951	2622	1,2
Total		93.35	98.57	95.33	98.71			
	Monoterpene hydrocarbons	1.16	6.84	2.90	4.38			
	Oxygenated monoterpenes	17.60	78.19	35.83	84.59			
	Sesquiterpene hydrocarbons	53.52	11.32	48.92	7.73			
	Oxygenated sesquiterpenes	18.78	-	6.99	-			
	Others	2.29	2.22	0.69	2.01			

^{a,b} The Kovats retention indices are relative to a series of *n*-alkanes (C10–C35) on the apolar DB-5 and the polar HP Innowax capillary columns, respectively. ^c Identification method: 1 = comparison of the Kovats retention indices with published data, 2 = comparison of mass spectra with those listed in the NIST 17 and Wiley 275 libraries and with published data, and 3 = co-injection with authentic compounds; - = absent.

3.3. Phytotoxic Activity

Tables 4–7 show the phytotoxic activity of the EOs. The results were reported as percentage inhibition (%), with reference to the treatment carried out with the control only (water–acetone mixture (99.5:0.5, *v/v*)) to which an inhibition of 0.0% was attributed. A heat map was generated for all data, using the green color for positive inhibition values and the red color for negative inhibitions (which indicate a stimulating rather than inhibitory effect). The white color was used for the control and when there was no action (0.0%). The more intense the color, the greater the activity. The complete data are available in the Supplementary Material section. They include all measurements performed on the number of germinated seeds and on the radical elongation processes (in cm) expressed as a mean of three experiments \pm SD, with attached statistical treatment (*p*-value).

3.3.1. Effect on Germination

- The EO 20D 1st year showed significant inhibitory effects at all concentrations on *P. oleracea* (from 28.6 to 57.1%) and on *A. fatua* (97.0% at 1000 μ g/mL). On *P. rhoeas*, it showed contrasting effects, with significant stimulations at low concentrations. On the other seeds, it showed weak inhibitory activities (up to 20.0%).
- The EO 20D 2nd year increased its inhibitory activity on *P. rhoeas* (up to 100%), *P. oleracea* (up to 61.3%), and *S. alba* (up to 73.6%) at all concentrations and on *A. fatua* (87.0% at 1000 μ g/mL). *L. multiflorum* instead underwent stimulating effects. On the other seeds, it showed weak inhibitory activities (up to 20.0%).
- In the EO 40D 1st year, the greatest inhibitory effects were present at the highest concentrations on *P. rhoeas* (up to 46.0%) and *P. oleracea* (up to 100%). On the other seeds, it showed weak inhibitory activities (up to 20.0%), and in some cases, at low concentrations, stimulating activities.
- The EO 40D 2nd year's inhibitory activity increased, especially at the highest concentrations, on *P. rhoeas* (up to 83.7%), *P. oleracea* (up to 100%), and *S. alba* (up to 70.0%). On the other seeds, it showed weak inhibitory activities (up to 22.2%), and only in rare cases, low concentrations, stimulating activities.

Table 4. Phytotoxic activity of the EO 20D 1st year. Green: positive inhibition values; red: negative inhibition values (stimulating rather than inhibitory effect); white: used for control and when there was no action (0.0%). The more intense the color, the greater the activity.

Number of Germinated Seeds							
	<i>L. multiflorum</i>	<i>S. alba</i>	<i>A. fatua</i>	<i>P. rhoeas</i>	<i>P. oleracea</i>	<i>V. lens</i>	<i>T. durum</i>
Control	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Treatment (µg/mL)							
125	3.8	7.0	0.0	−42.6	28.6	0.0	0.0
250	0.0	10.0	0.0	−12.8	38.6	0.0	10.0
500	−16.3	20.0	7.0	21.3	42.8	20.0	20.0
1000	0.0	20.0	97.0	29.8	57.1	20.0	20.0
Radical Length (cm)							
	<i>L. multiflorum</i>	<i>S. alba</i>	<i>A. fatua</i>	<i>P. rhoeas</i>	<i>P. oleracea</i>	<i>V. lens</i>	<i>T. durum</i>
Control	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Treatment (µg/mL)							
125	16.7	12.0	3.7	−125.0	15.6	0.0	17.0
250	5.6	32.0	18.5	−125.0	28.1	0.0	18.9
500	11.1	36.0	25.9	−25.0	40.6	8.7	26.4
1000	61.1	52.0	25.9	0.0	59.4	17.4	34.0

Table 5. Phytotoxic activity of the EO 20D 2nd year. Green: positive inhibition values; red: negative inhibition values (stimulating rather than inhibitory effect); white: used for control and when there was no action (0.0%). The more intense the color, the greater the activity.

Number of Germinated Seeds							
	<i>L. multiflorum</i>	<i>S. alba</i>	<i>A. fatua</i>	<i>P. rhoeas</i>	<i>P. oleracea</i>	<i>V. lens</i>	<i>T. durum</i>
Control	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Treatment (µg/mL)							
125	−13.0	8.0	0.0	−9.3	50.0	10.0	0.0
250	−7.8	31.1	10.0	60.5	47.5	10.0	0.0
500	−13.0	69.0	20.0	69.8	58.8	10.0	10.0
1000	−7.8	73.6	87.0	100.0	61.3	20.0	20.0
Radical Length (cm)							
	<i>L. multiflorum</i>	<i>S. alba</i>	<i>A. fatua</i>	<i>P. rhoeas</i>	<i>P. oleracea</i>	<i>V. lens</i>	<i>T. durum</i>
Control	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Treatment (µg/mL)							
125	4.4	42.9	3.2	−71.4	7.14	8.5	39.0
250	55.6	57.1	12.9	71.4	16.7	16.9	65.9
500	46.7	57.1	19.4	42.9	35.7	30.5	73.2
1000	66.7	64.3	25.8	100.0	57.1	37.3	80.5

3.3.2. Effect on Radical Elongation

In general, the inhibition of root elongation was more marked than germination.

- The EO 20D 1st year significantly inhibited all seeds (up to 61.1%), except *P. rhoeas*, for which it showed very intense stimulating activities (−125%).
- The EO 20D 2nd year maintained the strong inhibitory activity of the first year, showing strong inhibitions also on *P. rhoeas* (up to 100%) and significant increases on *T. durum* (up to 80.5%).

- The EO 40D 1st year showed high inhibitory activities on all seeds, with inhibitions reaching 100.0%. Its activities were variable instead towards *L. multiflorum* and *P. rhoeas*, where for some concentrations there was a mild inhibition (up to 33.3%), and for others, there were strong stimulating effects (up to −133.3%).
- The EO 40D 2nd year expanded its inhibitory activity on all seeds, and it was particularly high on *P. rhoeas* and *P. oleracea*, with percentages of 100% at 500 and 1000 µg/mL. The very strong stimulating activities of the first year have disappeared and are now confined only to two cases with extremely low values (−2.0 and −14.3%).

Table 6. Phytotoxic activity of the EO 40D 1st year. Green: positive inhibition values; red: negative inhibition values (stimulating rather than inhibitory effect); white: used for control and when there was no action (0.0%). The more intense the color, the greater the activity.

Number of Germinated Seeds							
	<i>L. multiflorum</i>	<i>S. alba</i>	<i>A. fatua</i>	<i>P. rhoeas</i>	<i>P. oleracea</i>	<i>V. lens</i>	<i>T. durum</i>
Control	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Treatment (µg/mL)							
125	−3.8	−4.3	−12.5	−35.1	6.98	0.0	10.0
250	−12.5	0.0	−12.5	0.0	16.28	10.0	20.0
500	0.0	3.23	0.0	46.0	69.8	20.0	20.0
1000	8.8	10.8	0.0	46.0	100	20.0	30.0
Radical Length (cm)							
	<i>L. multiflorum</i>	<i>S. alba</i>	<i>A. fatua</i>	<i>P. rhoeas</i>	<i>P. oleracea</i>	<i>V. lens</i>	<i>T. durum</i>
Control	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Treatment (µg/mL)							
125	7.7	40.0	13.0	−100.0	19.1	18.2	47.4
250	38.5	48.0	26.1	33.3	42.9	24.2	56.1
500	−107.7	60.0	30.4	−33.3	61.9	23.8	77.2
1000	30.8	68.0	39.1	−133.0	100.0	28.6	86.0

Table 7. Phytotoxic activity of the EO 40D 2nd year. Green: positive inhibition values; red: negative inhibition values (stimulating rather than inhibitory effect); white: used for control and when there was no action (0.0%). The more intense the color, the greater the activity.

Number of Germinated Seeds							
	<i>L. multiflorum</i>	<i>S. alba</i>	<i>A. fatua</i>	<i>P. rhoeas</i>	<i>P. oleracea</i>	<i>V. lens</i>	<i>T. durum</i>
Control	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Treatment (µg/mL)							
125	0.0	18.9	0.0	−16.3	11.3	0.0	0.0
250	27.6	41.1	0.0	30.2	28.3	10.0	10.0
500	4.6.0	55.6	7.8	83.7	43.4	10.0	10.0
1000	−3.4.0	70.0	22.2	83.7	100.0	10.0	20.0
Radical Length (cm)							
	<i>L. multiflorum</i>	<i>S. alba</i>	<i>A. fatua</i>	<i>P. rhoeas</i>	<i>P. oleracea</i>	<i>V. lens</i>	<i>T. durum</i>
Control	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Treatment (µg/mL)							
125	4.0	50.0	25.0	−14.3	23.8	13.7	50.0
250	24.0	58.3	33.3	14.3	38.1	23.5	67.3
500	−2.0	50.0	58.3	100.0	66.7	39.2	71.2
1000	12.0	33.3	66.7	100.0	100.0	47.1	76.9

Table 8 shows a comparison of the phytotoxic activity of EOs for each process (germination, radical elongation). It shows the most sensitive species to each EO, the maximum inhibition observed (%), and the most effective concentration.

Table 8. Comparison of the phytotoxic activity of EOs.

EO	Process	Most Sensitive Species	Maximum Inhibition Observed (%)	Most Effective Concentration ($\mu\text{g}/\text{ml}$)
EO 20D 1st year	Germination	<i>A. fatua</i> , <i>P. oleracea</i>	97.0 (<i>A. fatua</i>)	1000
	Radical elongation	<i>P. rhoeas</i> , <i>T. durum</i>	61.1 (<i>L. multiflorum</i>)	1000
EO 20D 2nd year	Germination	<i>P. rhoeas</i> , <i>S. alba</i> , <i>A. fatua</i>	100.0 (<i>P. rhoeas</i>)	1000
	Radical elongation	<i>P. rhoeas</i> , <i>T. durum</i>	100.0 (<i>P. rhoeas</i>)	1000
EO 40D 1st year	Germination	<i>P. rhoeas</i> , <i>P. oleracea</i>	100.0 (<i>P. oleracea</i>)	1000
	Radical elongation	<i>T. durum</i> , <i>P. oleracea</i>	100.0 (<i>P. oleracea</i>)	1000
EO 40D 2nd year	Germination	<i>P. rhoeas</i> , <i>P. oleracea</i>	100.0 (<i>P. oleracea</i> , <i>P. rhoeas</i>)	1000
	Radical elongation	<i>P. rhoeas</i> , <i>P. oleracea</i> , <i>T. durum</i>	100.0 (<i>P. rhoeas</i> , <i>P. oleracea</i>)	1000

3.3.3. Comparison with the Literature

The phytotoxic activity of EOs was highly heterogeneous. It was high both when dealing with different seeds and within the same seed with different concentrations of EOs. In fact, the phenomenon of hormesis was very marked, an event by which at certain concentrations (high or low) EOs can stimulate some physiological events in the seed, while at other concentrations, they can inhibit them [51].

There are not a lot of studies on the possible phytotoxic activity of the EO of *A. annua*. The inhibitory activity of an EO was reported against *Amaranthus retroflexus* and *Setaria viridis*, with a total inhibition of germination at 10 and 100 $\mu\text{g}/\text{mL}$, respectively [52,53], and on *Cydonon dactylon* at 250–1000 ppm [54].

The phytotoxicity found in the present work is obviously related to the composition. In fact, some of the main components of the EOs have been recognized as having phytotoxic properties: artemisia ketone [55,56], borneol [52,57], camphor [58,59], *p*-cymene, terpinen-4-ol, and α - and β -pinene [52,56]. The observed phytotoxicity of *A. annua* essential oils against common weed species reinforces their potential use in bioherbicide formulations. As supported by recent findings, the integration of plant-derived allelochemicals into weed management strategies may reduce the dependence on synthetic herbicides and support more sustainable cropping systems.

4. Conclusions

The findings obtained in this study highlighted that the phytochemical composition of annual wormwood EOs can be modified by crop management (plant density), becoming a specific product to be used in specific organic supply chains (e.g., weeds management). Future studies should explore the possibility of implementing specific crop management strategies to successfully modulate the EO bioactive molecules composition that enhances weed management. This threshold in annual wormwood must be clearly identified to optimize oil yields, particularly if this aromatic species is indicated as being a good alternative crop for pest management, from an agroecological perspective, for marginal lands of Mediterranean-type agro-ecosystems.

The EOs were phytotoxic on both germination and radical elongation, depending on the target seed and the dose. This dual action emphasizes the potential of *A. annua* EOs in both preventing germination and stunting root development. The variability in effectiveness among different weed species suggests that tailored applications could be

developed for specific weed management scenarios. Additionally, the findings advocate for the use of *A. annua* EOs as a sustainable alternative to synthetic herbicides, aligning with eco-friendly agricultural practices that promote plant health without introducing toxic residues. Future research should focus on identifying the specific chemical compounds responsible for these phytotoxic effects and exploring their mechanisms of action, alongside conducting field trials to verify the actual weed control effect of EOs and to assess practical applications in real-world agricultural settings. A further important consideration may concern the potential for mixing EOs with other biopesticides to develop synergistic formulations, increase treatment efficacy, and reduce the risk of resistance. Overall, *Artemisia annua* EOs represent a promising avenue for sustainable weed management in agriculture.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/agriculture15131330/s1>, Table S1. Phytotoxic activity of EO 20D 1st year. Table S2. Phytotoxic activity of EO 20D 2nd year. Table S3. Phytotoxic activity of EO 40D 1st year. Table S4. Phytotoxic activity of EO 40D 2nd year.

Author Contributions: Conceptualization, V.C., F.P., M.D., M.P., S.D. and V.D.F.; methodology, V.C., F.P., M.D., M.P., S.D. and V.D.F.; formal analysis, V.C., F.P., M.D., S.D., V.P. and V.D.F.; data curation, V.C., D.C., F.P., M.D., S.D., V.D.F. and V.P.; software, D.C., F.P., L.C., M.D. and P.C.; investigation, V.C., F.P., M.D., M.P., V.P., S.D. and V.D.F.; supervision, V.C., M.P. and V.D.F.; writing—original draft preparation, V.C., L.C., F.P., M.D., V.D.F. and S.D.; writing—review and editing, V.C., D.C., M.D., P.C., S.D. and V.D.F.; project administration, M.P. and V.C.; funding acquisition, M.P. All authors have read and agreed to the published version of the manuscript.

Funding: This work was supported by the project AgrEcoMed—New Agroecological Approach for Soil Fertility and Biodiversity Restoration to Improve Economic and Social Resilience of Mediterranean Farming Systems, funded under PRIMA Section 2—Multi-topic 2021 (CUP H93C21000130005), as part of the PRIMA Programme supported by the European Union.

Data Availability Statement: Data will be made available by the corresponding authors upon request.

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

Abbreviations

EO Essential oil

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