

Article

Chamomile and Anise Cultivation in Olive Agroforestry Systems

Georgios I. Katsoulis ¹, Athanassios C. Kimbaris ², Eirini Anastasaki ³, Christos A. Damalas ²
and Apostolos P. Kyriazopoulos ^{1,*}

¹ Department of Forestry and Management of the Environment and Natural Resources, Democritus University of Thrace, 193 Pantazidou Str., 68200 Orestiada, Greece; geokatsoulis@hotmail.gr

² Department of Agricultural Development, Democritus University of Thrace, 193 Pantazidou Str., 68200 Orestiada, Greece; kimparis@agro.duth.gr (A.C.K.); cdamalas@agro.duth.gr (C.A.D.)

³ Scientific Directorate of Entomology and Agricultural Zoology, Benaki Phytopathological Institute, 8 Stefanou Delta Str., Kifissia, 14561 Attica, Greece; e.anastasaki@bpi.gr

* Correspondence: apkyriaz@fmenr.duth.gr; Tel.: +30-25520-41109

Abstract: Agroforestry systems have been practiced for hundreds of years with multiple benefits both environmentally and economically in terms of productivity. Olive cultivation is widespread in the countries of the Mediterranean basin, including Greece. Agroforestry practices are common in olive groves, but little research has been conducted on the productivity of such systems, especially with medicinal–aromatic plants (MAPs) as understory crops. Natural populations of MAPs can be found in various ecosystems, while some of them are cultivated. The purpose of this research was to study the effects of fertilization and shading both on yield and chemical composition of essential oils derived from chamomile (*Matricaria recutita* L.) and anise (*Pimpinella anisum* L.), grown in olive silvoarable systems. Fertilization and shading increased the plant height of chamomile and delayed the flowering. In addition, fertilization increased the concentration of α -bisabolol oxide A and (Z)-spiroether, and reduced the α -bisabolone oxide A and hamazulen. Shade also reduced α -bisabolone oxide A and hamazulen but increased the α -bisabolol oxide B. In the case of anise, fertilization increased plant height, decreased the concentration of limonene, and increased the concentration of E-anethole. Shading reduced plant height. Intercropping of olive trees with chamomile and anise yielded essential oils rich in the substances defined by the commercial specifications.

Keywords: silvoarable systems; shade; fertilization; *Matricaria recutita*; *Pimpinella anisum*; essential oil



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1. Introduction

The interest in silvoarable agroforestry systems has significantly increased in recent years because these systems are sustainable in terms of productivity and more environmentally friendly. They produce a variety of goods, thus improving the overall productivity of agricultural holdings, contribute to the reduction of rural migration by providing jobs, and bring profit and prosperity to rural and disadvantaged communities [1–3]. It is also well documented that silvoarable systems can reduce soil erosion and enrich the soil with organic matter [4], play a crucial role in carbon sequestration [5], improve microclimatic conditions and air quality, and enhance biodiversity [6].

Silvoarable agroforestry practices are applied to 358,000 hectares, accounting for merely 0.39% of the total agricultural land in Europe [7]. The most common silvoarable systems in the Mediterranean region are co-cultivation of broadleaf trees with cereals and co-cultivation of olive trees with vegetables and cereals [8]. In olive agroforestry systems, the trees are usually planted in lines, but they can also be in a scattered pattern, after thinning [9]. Common agroforestry systems are applied in olive groves or in other cultivated tree species such as oak (*Quercus* spp.), carob (*Ceratonia siliqua* L.), walnut (*Juglans regia* L.), and almond (*Prunus dulcis* (Mill.) D.A. Webb) [9,10] with the cultivation

of cereals, vegetables or legumes. Such systems can also combine grazing of the understory by small ruminants usually following the sowing of forage crops. Olive agroforestry systems produce a variety of products including olive oil and edible olives, firewood, timber, derivatives from co-cultivation, and grazing [10]. Agroforestry practices in olive groves used to be a traditional practice in the Mediterranean countries [9,11]; however, presently they are not as widespread in most countries. In Italy, only about 20,000 hectares of olive groves are co-cultivated or grazed, mainly in Umbria and Lazio; in Spain they are very rare, participating with very small numbers in the total land of olive groves, while in France they are completely demised [9]. In contrast, 124,300 hectares of the total area covered of olive groves estimated to 690,000 hectares [12] are co-cultivated or grazed in Greece [10].

From ancient times medicinal–aromatic plants (MAPs) have been widely used for their properties in cooking, medicine, cosmetics and elsewhere [13]. About 2500 species of MAPs are traded worldwide [14], with the biggest portion deriving from native vegetation [15]. Essential oils are widely used for their properties: antimicrobial, antiviral–antiepileptic, antifungal, antioxidant, anti-cancer, anti-inflammatory, analgesic, sedative, expectorant, antispasmodic, anesthetic, insecticidal [16,17]. The collection of self-seeded material from nature led to the significant reduction of natural populations, their genetic degradation [18], and even their extinction [18,19]. Only a few species of MAPs are cultivated presently [18] and for the above reasons it is essential to expand the number of species cultivated.

Chamomile (*Matricaria recutita* L.) is an annual species naturally distributed in the Mediterranean basin, but it also grows naturally in several areas of Europe, Asia, India, Algeria, Siberia, Australia, and the Americas [20,21]. It is cultivated in temperate countries such as Germany, Hungary, Russia, Kashmir, Lebanon, Argentina, Colombia, and elsewhere. It thrives in all types of soil acidity, but performs better in soils with high pH values. It can also be grown in clayey, shallow, and moist soils, while it grows better in soils rich in organic matter and temperatures from 7 to 26 °C [21]. The acreage yield of chamomile, both in dry plant material and in essential oil, but also its quality, is influenced by the climatic factors of the area [22]. Chamomile is categorized mainly into five chemotypes, based on its content of α -bisabolol, chamazulen, bisabolol oxide A and B, and bisabolone oxide in its essential oil. The components of the essential oil of each chamomile variety depend on the chemotype to which it belongs. The main ones are β -farnesene, germacrene D, bicyclogermacrene, α -farnesene, bisabolol oxide A, bisabolol oxide B, bisabolone oxide, α -bisabolol, chamazulene, and *cis-trans*- ϵ -dicycloether [23–27]. For the marketing of chamomile essential oil there are some limits for certain substances, categorizing the essential oil into two categories—oil rich in bisabolol oxides (total of bisabolol oxides from 29% to 81% and chamazulen > 1%), and oil rich in bisabolol (bisabolol from 10% to 65%, chamazulen $\geq$ 1% and bisabolol with its oxides $\geq$ 20%) [25,28].

Anise (*Pimpinella anisum* L.) is an annual species mainly distributed in the Eastern Mediterranean and Western Asian countries [29], and it is cultivated in Spain, Mexico, France, Turkey, Lebanon, Russia, Bulgaria, Cyprus, India, China, Japan, Argentina and Greece. Anise consumption in European countries is higher than production, with Germany being the largest consumer [30]. Anise prefers open warm areas, with ideal growth temperatures from 18 to 25 °C. It is cultivated in plains and semi-mountainous areas and prefers medium composition, mainly calcareous, fertile and well-drained soils, with pH 6–8. The yields of anise in terms of seed and essential oil production are influenced mainly by climatic conditions [31], the period of sowing and harvesting, and cultivation treatments, such as irrigation and applied fertilization [30,32,33]. The categorization of anise derives from the percentage of the seed content in essential oil. The most important components of the oil are linalool with values ranging from 0.1% to 1.5%, estragol (0.5%–6.0%), α -terpineol (0.1%–1.5%), *cis*-anethole (less than 0.5%), *trans*-anethole (84%–93%) and anisaldehyde (0.1%–3.5%). Other components of the essential oil are pinene, limonene, *p*-methoxy acetophenone, and γ -himchalen (~2.0%). In the Greek varieties of anise, essential oil has a yield of up to 2.5% for whole seeds and up to 6% for fragmented seeds. It consists mainly of

anethole (up to 95%), estragole (2.0%–3.0%), γ -himchalen (0.5%–2.5%), and *p*-anisaldehyde (0.3%–0.6%) [27].

Although MAPs have low requirements for nutrients, their productivity is affected by nutrients [34,35], so it is necessary that the absorbed nutrients are replenished on an annual basis, depending on the species, and especially during spring [19]. Plant growth seems to be affected using nitrogen (N) fertilizers [36]. The overall productivity of chamomile is affected by various macronutrients and micronutrients [37]. The productivity of chamomile in essential oil does not seem to be particularly affected by fertilization [38], with the phosphorous (P) fertilization to increase the essential oil content [39]. It has been observed that N fertilization rate of 75 kg/ha yields the largest amount of essential oil [36], but additional use of N fertilizer delays flowering, which negatively affects the production of essential oil [36,38]. Fertilization greatly affects the growth and productivity (seeds and essential oil) of anise [39]. More specifically, N [35] and P fertilization affect plant growth and the quantitative yield of anise essential oil [34]. Reduced N intake affects anise, as with various other MAPs, in terms of growth and productivity [40]. P affects metabolic processes, improves fertility, stimulates flowering, helps seed growth, and accelerates ripening [36].

The cultivation of MAPs in agroforestry silvoarable systems is possible [41], with high-quality products, equal to that of the native ones [18]. The cultivation of MAPs has multiple advantages over various other crops. MAPs offer higher yield per unit area and are less likely to be attacked by insects and diseases. For example, anise has been reported to act as an insecticide in the biological control of *Bactrocera oleae* (Rossi) [29]. MAPs can upgrade poor and unprofitable soils, yield long-term products, and ensure profit for farmers [42]. The most suitable MAPs for intercropping with trees are those that develop a deep root system and are shade-resistant [18], because of the competition that will be developed between the trees and the understory crop for sunlight and soil moisture. The impact of this competition can be negative or positive depending mainly on the combination of species selected [43]. It has been observed that some MAPs produce better yields when grown in agroforestry systems, such as species of the genus *Mentha*, *Cymbopogon martinii* (Roxb.) W. Watson, *Cymbopogon flexuosus* (Nees ex Steud) Wats. and *Piper longum* L. in eucalyptus, poplar, and leuca (*Leucaena leucocephala* (Lam.) de Wit) plantations [42]. Tree density affects the growth and chemical composition of MAPs [18], the total amount of biomass produced, and the composition and quality of their essential oils [18,19], but not to the same degree for all the MAP species [18]. Singh et al. [44] found that light shading provided by poplar trees did not affect the essential oil components of *Cymbopogon winterianus* Jowitt ex Bor and *Cymbopogon martinii*, but the oil of *Mentha arvensis* L. had lower amounts of menthone and higher amounts of menthol when grown under shading. Thus, the appropriate choice of the understory MAP species will have a significant impact on the success of the agroforestry system.

The purpose of this research was to study the effects of fertilization and shading on the productivity of anise and chamomile essential oils grown in olive silvoarable systems in terms of both yield and essential oil chemical composition. Our test hypothesis was that fertilization would benefit the plant growth, essential oil yield and chemical composition of anise and chamomile either alone or together with shading by olive trees. The research question of this study was: can anise and chamomile grow in olive agroforestry systems, and what are the effects of this co-cultivation on the productivity of those MAPs in terms of biomass and essential oil yields?

2. Materials and Methods

2.1. Study Area

The study area is in Tolofonas, Fokida region, central Greece (38°21' N, 22°12' E) at 10 m a.s.l. The climate of the area is classified as Mediterranean, with a mean air temperature of 19.0 °C and an annual rainfall of 392 mm. In 2018, the year the experiment was conducted, the mean annual precipitation was 483 mm, which was considerably higher than the annual average, and the mean temperature was 19.2 °C.

An olive grove with tree spacing of 8×8 m and an adjacent open field were selected in the study area to establish the experiment. The soil of the research area was analyzed (Soil Survey Laboratory “The Union” 2013) before the onset of the experiment. The soil texture in the olive grove is clay loam, while in the open field is silty clay (Table 1).

Table 1. Soil characteristics of the experimental fields.

Experimental Surface	Olive Meadow	Open Field
Characterization	Clay Loam	Silty Clay
Sand	20.8%	16.8%
Silt	45.2%	51.2%
Clay	34.0%	32.0%
pH	7.7	7.6
Organic matter	1.3%	2.0%
CaCO ₃	9.7%	9.7%
Electrical conductivity	1.5 mS cm ⁻¹	1.4 mS cm ⁻¹
Nutrients		
NO ₃ -N	2.6 mg kg ⁻¹	197 mg kg ⁻¹
P	13 mg kg ⁻¹	44.5 mg kg ⁻¹
K	107.6 mg kg ⁻¹	143.4 mg kg ⁻¹
Mg	522 mg kg ⁻¹	422 mg kg ⁻¹
Fe	8.1 mg kg ⁻¹	11.6 mg kg ⁻¹
Mn	4.8 mg kg ⁻¹	5.5 mg kg ⁻¹
Zn	1.2 mg kg ⁻¹	0.5 mg kg ⁻¹
Cu	0.6 mg kg ⁻¹	2.4 mg kg ⁻¹
B	0.82 mg kg ⁻¹	0.78 mg kg ⁻¹

2.2. Experimental Design, Plant Material and Sampling Methods

The olive grove (shading) and the adjacent open field (control) served as shading treatments in the experiments. In the understory of the olive grove average light intensity was approximately $900 \mu\text{E m}^{-2} \text{s}^{-1}$ (75% of the total radiation), while light intensity in the open field was $1200 \mu\text{E m}^{-2} \text{s}^{-1}$ (100% of the total radiation). Light intensity was measured by a Licor quantum sensor (Li 190 SB). Chamomile and anise were cultivated in plots of 8 m^2 ($2 \times 4 \text{ m}$) in each shading treatment. Eight plots per species were established in each shading treatment. The plots were separated by a bare earth strip of 2 m to avoid edge effects and to allow easy movement during the sampling periods. The minimum distance from the olive trees was 1 m.

The seeds of chamomile (*Matricaria recutita* L.) were of a B chemotype and diploid variety. They were sown in early January 2018 in boxes placed in a greenhouse. Then the seedlings were transplanted in the plots in late March 2018. The distances used for the transplanting were 33.3 cm between the rows and 20 cm between the plants. Anise (*Pimpinella anisum* L.) was directly shown in the plots at distances of 40 cm between the lines and 0.3–0.5 cm between the plants, in late March 2018. The seeds of anise were from a local cultivation on the island of Evia, central Greece.

Half of the plots with chamomile in both the olive grove and the open field were fertilized with triple hyperphosphate (0-46-0) with 34.90 kg ha^{-1} , potassium sulfate (0-0-50) with 16.86 kg ha^{-1} and ammonia sulfate (21-0-0) with 23.80 kg ha^{-1} . Half of the plots with anise in shaded and unshaded treatments were also fertilized with 39.88 kg ha^{-1} of triple hyperphosphate, 19.27 kg ha^{-1} of potassium sulfate and with 23.80 kg ha^{-1} of ammonia sulfate. The fertilizers were applied twice, in early and late May 2018. The main macronutrients of crop growth (N-P-K) were considered, while no special consideration was given to sulfur, which was assumed adequate in the fertilized plots due to the sulfate form

of the applied fertilizers. Fertilization rates were defined based on the literature [16]. All plots were irrigated to field capacity once a week. Hand weeding was done when required.

Plant height was measured for both species prior to the plant material collection. The collection of chamomile flower heads was done manually in late June 2018, when the lingual flowers were in a horizontal position. The percentage of full bloom flower heads was estimated. Only those that were in full bloom condition (premium quality) were used for chemical analysis. The flower heads were dried naturally in a ventilated room on a cloth, shaded, with shredding, for a period of 7 days and then weighted. Anise was collected in mid-August 2018 manually. Only the ripe fruit were selected. They dried in an atrium, under shading, by shoveling in containers for a period of 2 days, stored in airtight containers, and then weighed. The plant material from both species was stored in airtight containers in cool and dry place away from light.

2.3. Isolation of Essential Oils

The dry stored plant material (see Section 2.2) was combined, per species and cultivation treatment, and grinded in a blender until fine particles formed. Then, each sample species that derived under different treatment was weighed, divided into three equal amounts, placed into a round bottom flask with deionized distilled water and submitted to hydrodistillation for 3 h using a Clevenger apparatus. The obtained essential oils, were dried over anhydrous magnesium sulfate, filtrated and finally stored in labeled sterile screw capped bottle at $-22\text{ }^{\circ}\text{C}$ until analysis. Overall, four types of essential oils (according to the treatment applied) per species isolated and known as SnFCEO (shade without Fertilizer Chamomile Essential Oil), SFCEO (shade with Fertilizer Chamomile Essential Oil), nSnFCEO (without Shade without Fertilizer Chamomile Essential Oil), nSFCEO (without Shade with Fertilizer Chamomile Essential Oil), SnFAEO (shade without Fertilizer Anise Essential Oil), SFAEO (shade with Fertilizer Anise Essential Oil), nSnFAEO (without Shade without Fertilizer Anise Essential Oil), and nSFAEO (without Shade with Fertilizer Anise Essential Oil). Yield of the essential oils was determined on average of three replicates (per species and treatment) and expressed as mL of essential oil/100 g of dry material.

2.4. Gas Chromatography–Mass Spectrometry (GC–MS) Analysis

Identification of essential oil was performed using gas chromatography–mass spectrometry (GC–MS) on a Varian CP-3800 GC, with a 1079 injector coupled with a 1200 L quadrupole mass spectrometer. One microliter of the essential oil (1:10 dilution) was used for the analysis. Separation of the analytes was performed with a TG-5MS capillary column (5% diphenyl/95% dimethyl polysiloxane) with dimensions 30-m length, 0.25-mm i.d., 0.25- μm film thickness (Thermo Scientific, Waltham, MA, USA). Splitless mode was set for 1 min. The flow rate of the carrier gas helium was 1 mL min^{-1} . The oven temperature was set at $60\text{ }^{\circ}\text{C}$ and increased with a rate of $3\text{ }^{\circ}\text{C min}^{-1}$ to the final temperature of $250\text{ }^{\circ}\text{C}$, with a total run of 63.33 min. Mass spectrometer was operated in Electron ionization mode (EI) with ion energy of -70 eV , filament current $50\text{ }\mu\text{A}$ and source temperature $200\text{ }^{\circ}\text{C}$. Data acquisition was performed in full scan (MS) with scanning range 40–300 amu. Tentative identification was achieved by comparing their elution order, the mass spectra with those from mass spectra libraries [45] (NIST 2005, Wiley 275) and literature data [45] (NIST-WebBook site). Retention indices (RI) of a series of n-alkane ($\text{C}_8\text{--C}_{20}$) were also used. Wherever possible, retention time and mass spectra were compared with commercial standards. The total ion chromatogram was processed by Varian MS Workstation software (version 6.9) based on the retention time and mass spectrum. Relative percentages (%) of the compounds were obtained electronically from the area percent data.

2.5. Data Analysis

The experimental design for each species consisted of two factors (two shading treatments and two fertilization levels). Within each of the two shading treatments (olive grove and open field), fertilization treatments were arranged in a randomized complete

block design with four replications. The replicate measurements were not true replication (pseudoreplication), since only one contiguous area represented each shading treatment. Although this is a common case when working with existing field studies, it does introduce additional uncertainty into interpretation of the analysis. In this case, it is important to acknowledge that responses associated with either the main effect of shading, or interactions with shading, could be influenced by factors in addition to shading (such as soil variation) which are unmeasured but confounded with the unreplicated shade blocks. General linear models' procedure (SPSS 18 for Windows) was used for two-way ANOVA. Prior to ANOVA all data sets were tested for normality using the Kolmogorov Smirnov test. The least significant difference (LSD) test [46] at $p \leq 0.05$ was used to detect differences among means.

3. Results

3.1. Cultivation of Chamomile

Shading and fertilization application significantly affected the plant height of chamomile and the percentage concentrations of α -bisabolol oxide B, α -bisabolone oxide A, α -bisabolol oxide A, chamazulene and Z-spiroether (Table 2). Additionally, significant differences for full bloom flower heads percentage and dry flower head yield were recorded between fertilization treatments. The interaction of shading and fertilization was significant for essential oil yield as well as for all the main components of the oil besides chamazulene (Table 2).

Table 2. Statistical significance of F ratios for growth parameters and essential oil main components of chamomile.

Parameter	Shade	Fertilization	S $\times$ F
Plant height	14.10 **	113.71 ***	0.02
Flower heads	0.81	23.17 ***	0.26
Dry flower head yield	0.37	5.01 *	3.74
Essential oil yield	0.09	6.65	12.93 *
α -bisabolol oxide B	38.42 ***	21.97 **	21.97 **
α -bisabolone oxide A	64.16 **	25.20 **	25.20 **
α -bisabolol oxide A	9.99 *	18.01 **	50.88 **
Chamazulene	11.45 **	103.05 ***	3.53
Z-spiroether	246.43 ***	52.34 **	141.04 **

* Significant at $p \leq 0.05$; ** Significant at $p \leq 0.01$; *** Significant at $p \leq 0.001$.

The plant height of chamomile across fertilization treatments was reduced in the olive grove (under shading). Shading did not affect the percentage of full bloom flower heads and their dry yield, as well as the essential oil yield (Table 3). The percentage concentrations of α -bisabolol oxide B, α -bisabolol oxide A, and Z-spiroether were significantly higher under shading, while those of α -bisabolone oxide A and chamazulene were lower (Table 3).

Table 3. Effects of shading (across fertilization application treatments) on growth parameters and essential oil main components (mean $\pm$ SD) of chamomile.

Parameter	Shading	Control
Plant height (cm)	27.25 $\pm$ 10.26 b	33.50 $\pm$ 9.68 a
Flower heads (%)	52.22 $\pm$ 8.25 a	49.80 $\pm$ 8.90 a
Dry flower head yield (g m ⁻²)	34.36 $\pm$ 4.26 a	41.23 $\pm$ 18.33 a
Essential oil yield (mL·100 g ⁻¹)	0.61 $\pm$ 0.10 a	0.63 $\pm$ 0.22 a
α -bisabolol oxide B (%)	34.00 $\pm$ 3.48 a	29.90 $\pm$ 1.22 b
α -bisabolone oxide A (%)	13.55 $\pm$ 2.74 b	17.30 $\pm$ 0.40 a
α -bisabolol oxide A (%)	19.15 $\pm$ 3.61 a	17.40 $\pm$ 1.06 b
Chamazulene (%)	10.25 $\pm$ 1.95 b	11.60 $\pm$ 2.67 a
Z-spiroether (%)	11.80 $\pm$ 3.89 a	6.05 $\pm$ 1.02 b

Means within each row followed by the same letter are not significantly different ($p \leq 0.05$).

The plant height and the dry flower head yield of chamomile increased under fertilization, while the essential oil yield was not affected by shading. The percentage of full bloom flower heads was reduced by fertilization. The concentrations of α -bisabolol oxide B, α -bisabolone oxide A, and Z-spiroether were significantly higher in the control treatment, while those of α -bisabolol oxide A and chamazulene were higher under fertilization (Table 4).

Table 4. Effects of fertilization (across shading treatments) on growth parameters and essential oil main components (mean $\pm$ SD) of chamomile.

Parameter	Fertilization	Control
Plant height (cm)	39.25 $\pm$ 4.43 a	21.50 $\pm$ 4.66 b
Flower heads (%)	44.52 $\pm$ 2.79 b	57.50 $\pm$ 6.82 a
Dry flower head yield (g m ⁻²)	50.44 $\pm$ 12.77 a	25.14 $\pm$ 8.68 b
Essential oil yield (mL·100 g ⁻¹)	0.54 $\pm$ 0.12 a	0.70 $\pm$ 0.15 a
α -bisabolol oxide B (%)	30.40 $\pm$ 1.07 b	33.50 $\pm$ 4.10 a
α -bisabolone oxide A (%)	14.25 $\pm$ 3.15 b	16.60 $\pm$ 0.94 a
α -bisabolol oxide A (%)	19.45 $\pm$ 3.28 a	17.10 $\pm$ 1.38 b
Chamazulene (%)	8.90 $\pm$ 0.65 b	12.95 $\pm$ 1.33 a
Z-spiroether (%)	10.25 $\pm$ 5.54 a	7.60 $\pm$ 1.04 b

Means within each row followed by the same letter are not significantly different ($p \leq 0.05$).

Fertilization significantly reduced the essential oil yield of chamomile in the open field, while no significant effect was observed under shade condition. The essential oil yield of chamomile was higher under shade for fertilized plants, and it was lower for the unfertilized ones (Figure 1).

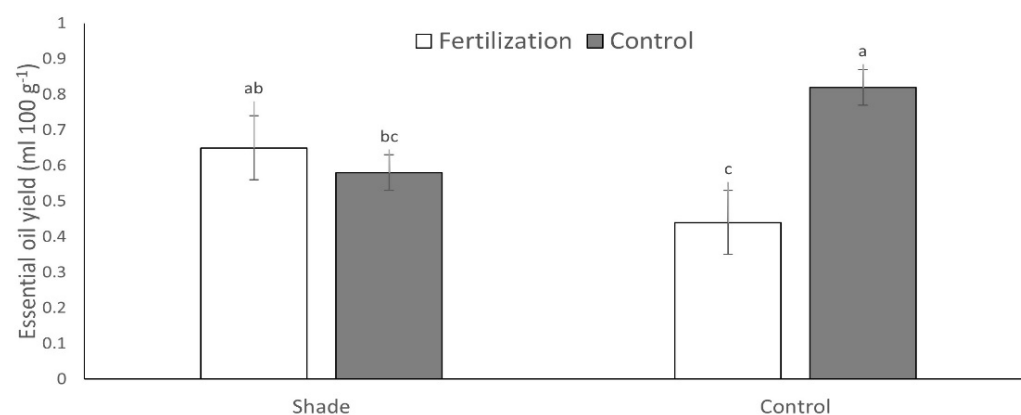


Figure 1. Effects of shading and fertilizer application on the essential oil yield of chamomile. Columns followed by the same letter are not significantly different ($p \leq 0.05$).

The essential oil main components of chamomile grown in the open field were not affected by fertilization, except for Z-spiroether, which decreased with fertilizer application. Fertilization of shaded plants increased the percentage concentrations of α -bisabolol oxide B, α -bisabolol A, oxide, and Z-spiroether, but reduced the concentration of α -bisabolone oxide A. A tendency for increased concentrations under shading was detected especially in the fertilized plots for all the essential oil main components of chamomile, apart from α -bisabolone oxide A, for which the opposite trend was observed (Table 5).

The identification rate of the components of the chamomile essential oil in all four combinations of treatments ranged from 97.10% to 98.10% with a total of 28 ingredients being identified. Specifically, the highest number of substances (28) was recorded for samples grown in fertilized plots without shading, while the lowest number (16) was recorded in plants grown under shading without fertilization application (Table A1).

Table 5. Effects of shading and fertilizer application on the essential oil major components (mean $\pm$ SD) of chamomile.

Treatment	α -Bisabolol Oxide B (%)	α -Bisabolone Oxide A (%)	α -Bisabolol Oxide A (%)	Z-Spiroether (%)
Olive grove				
Fertilized	37.10 $\pm$ 0.82 a	11.20 $\pm$ 1.25 b	22.30 $\pm$ 1.42 a	15.30 $\pm$ 0.56 a
Control	30.90 $\pm$ 0.92 b	15.90 $\pm$ 0.82 a	16.00 $\pm$ 0.89 c	8.30 $\pm$ 0.92 b
Open field				
Fertilized	29.90 $\pm$ 1.13 b	17.30 $\pm$ 0.56 a	16.60 $\pm$ 0.72 bc	5.20 $\pm$ 0.26 d
Control	29.90 $\pm$ 1.57 b	17.30 $\pm$ 0.30 a	18.20 $\pm$ 0.60 b	6.90 $\pm$ 0.62 c
LSD _{0.05}	2.18	1.53	1.81	1.19

Means within each column followed by the same letter are not significantly different ($p \leq 0.05$).

3.2. Cultivation of Anise

Shading and fertilization application significantly affected only the plant height of anise (Table 6). Additionally, significant differences for the percentage concentrations of *E*-anethole and limonene were recorded between fertilization treatments. No significant interactions were observed between shading and fertilization treatments for all the measured parameters (Table 6).

Table 6. Statistical significance of F ratios from the analysis of variance for growth parameters and essential oil main components of anise.

Parameter	Shade	Fertilization	S $\times$ F
Plant height	5.03 *	288.00 ***	2.00
Dry seeds yield	0.77	0.01	0.41
Essential oil yield	2.94	0.01	1.19
<i>E</i> -anethole	0.33	39.63 ***	0.12
Limonene	0.18	22.22 **	0.51

* Significant at $p \leq 0.05$; ** Significant at $p \leq 0.01$; *** Significant at $p \leq 0.001$.

The plant height (across fertilization treatments) of anise was reduced in the olive grove (under shading). Shading did not affect dry seed and essential oil yield or the concentrations of *E*-anethole and limonene (Table 7).

Table 7. Effects of shading (across fertilization application treatments) on growth parameters and essential oil main components (mean $\pm$ SD) of anise.

Parameter	Shading	Control
Plant height (cm)	26.87 $\pm$ 7.49 b	27.12 $\pm$ 8.87 a
Dry seeds yield (g m ⁻²)	19.67 $\pm$ 13.21 a	26.34 $\pm$ 15.29 a
Essential oil yield (mL·100 g ⁻¹)	5.19 $\pm$ 0.74 a	4.16 $\pm$ 0.83 a
<i>E</i> -anethole (%)	88.65 $\pm$ 1.71 a	88.90 $\pm$ 1.60 a
Limonene (%)	5.55 $\pm$ 1.13 a	5.40 $\pm$ 0.99 a

Means within each row followed by the same letter are not significantly different ($p \leq 0.05$).

The plant height (across shading treatments) of anise increased under fertilization, while dry seed and essential oil yield did not differentiate. The percentage concentration of *E*-anethole increased with fertilization, while this of limonene was reduced (Table 8).

The identification rate of the components of the anise essential oil in all the combinations of treatments ranged from 98.9% to 99.9% with 17 ingredients being identified. Specifically, the highest number of substances (16) was recorded for samples grown under shade without fertilization, and the lowest (13) was recorded in plants grown under shading with fertilization application (Table A2).

Table 8. Effects of fertilization (across shading treatments) on growth parameters and essential oil main components (mean $\pm$ SD) of anise.

Parameter	Fertilization	Control
Plant height (cm)	34.50 $\pm$ 1.41 a	19.50 $\pm$ 2.07 b
Dry seeds yield (g m ⁻²)	23.05 $\pm$ 13.27 a	22.96 $\pm$ 16.04 a
Essential oil yield (mL·100 g ⁻¹)	4.66 $\pm$ 1.06 a	4.69 $\pm$ 0.97 a
<i>E</i> -anethole (%)	90.15 $\pm$ 0.48 a	87.40 $\pm$ 0.86 b
Limonene (%)	4.65 $\pm$ 0.71 b	6.30 $\pm$ 0.37 a

Means within each row followed by the same letter are not significantly different ($p \leq 0.05$).

4. Discussion

The effects of fertilization and shading on the production of essential oils in terms of both yield and chemical composition were not the same for anise and chamomile. There were significant effects of shading and fertilization interactions for essential oil yield and almost all the main components of the oil in chamomile. The effects of fertilization on oil yield and components depended on shading. Contrary to chamomile, there were no significant effects of shading and fertilization interactions for any of the studied characteristics. The major effects were verified for fertilization treatment, while the shade factor was significant only for height.

4.1. Chamomile

The height of chamomile plants increased with fertilizer application in the open field, while under shading the plants had lower height at harvest. The increased plant height with fertilizer application in chamomile plants could be attributed to the nitrogen fertilization applied in the form of ammonia, which has been reported to result in better plant growth in terms of height [36,47], while potassium and sulfur may have played a positive role through promoting photosynthesis and plant metabolism. On the other hand, the negative effect of shading on plant height is most likely related to reduced photosynthesis rates of plants [48]. Nevertheless, this point requires further clarification because interactions between shade and yields are possible in agroforestry systems due to differences in precipitation and soil nutrient status [49]. It is noteworthy that much higher heights of chamomile have been reported under fertilization in Iran [24,50]. This difference can be explained by the different climatic conditions and the different soil texture in the study areas. It has been documented that geographical and climatic differences affect the morphology of chamomile [51].

The percentage of the bloomed flower heads was significantly lower in the fertilized plots. This effect is probably associated with the excessive N uptake, which contributed to delayed flowering of plants [36,38]. Shading did not affect the opening of the flower heads of the plants, probably because of the wide olive tree spacing. Fertilizer application increased the dry matter productivity of flower heads, while shading did not have a significant effect. Previous studies have reported the positive effects of ammonia fertilization [21,50,52], vermicompost and zeolite application [53] on the flower head production of chamomile.

The production of essential oil was not affected by fertilization or by shading. However, the interaction of those treatments produced significant results. Plants in the fertilized plots grown under shade produced significantly higher amounts of essential oil than those grown in the open field. This finding can probably be attributed to the better development of the root system of the plants under shading due to the increased soil moisture [54,55], and therefore the greater capacity for nutrient binding, especially N [36] and P, from the soil to the flowers, which ultimately improved oil yield [37]. It has been reported that under shading conditions for the fertilized plants the percentage of essential oil yield was higher, as with ammonium fertilization enhanced the formation of essential oil cells [56], while in the absence of shading, the highest percentage was observed in non-fertilized plants. A recent study [57] suggested that the essential oil yield in lemon balm plants under partial shade was higher than that under full sunlight, probably because assimilates might

have been directed to repair mechanisms necessary to support plant growth under high irradiance conditions.

The essential oil obtained from all the combinations of shading and fertilization meets the eligibility requirements defined by the European Pharmacopoeia [28]. Fertilization reduced the α -bisabolol oxide B percentage concentration, while shading increased it. The biosynthesis of this substance, as well as of the α -bisabolol oxide A, is controlled by gene interactions [56]. The percentages of these terpenes were expected to be increased until the final stage of flowering [53], but as excessive ammonia fertilization affects the duration of flowering, it is likely that it also negatively affected the biosynthesis of the components discussed. On the other hand, the increase in the concentration of α -bisabolol oxide B in plants grown under the olive tree shade is most likely explained by the balancing of the rhythms of the photosynthetic process, which were reduced due to the prolonged duration of flowering. According to a previous study in Iran, fertilization led to a slight increase of α -bisabolol oxide B [53], possibly because of the different fertilizer used and the differences in soil texture. The effects of fertilization were stronger under shading probably because of the competition between hydrocarbon terpenes and oxygenated terpenes, which tends to inversely relate the substances of one group with their concentration rates [56].

Fertilization and shading reduced the concentration of α -bisabolone oxide A. This reduction can be related to the delayed maturity of the flowers caused by ammonia fertilization [53] and the reduction of the photosynthetic activity due to shading. The effects of fertilization were stronger under shading. The concentration of α -bisabolone oxide A was generally higher than that reported in previous studies [23,24,50].

Shading and fertilizer application resulted in an increase of α -bisabolol oxide A concentration. This result contradicts previous studies, which reported that fertilization, and in particular ammonia, reduced the concentration of this substance in chamomile essential oil [52,58], but it is in accordance with the results of Salehi et al. [53], who reported a positive effect of various fertilizers in the α -bisabolol oxide A concentration. This increase was significant in the olive grove, but not in the open field. Plants grown under shading and fertilization develop an extensive and more active root system due to increased soil moisture and ammonia fertilization [53], which in turn result in better uptake of N and P [36], which play an important role in the biosynthesis of the substance [53]. In contrast, non-fertilized plants in the open field had a relative higher concentration of this substance, although not statistically significant. Nutrient deficiencies, high evaporation, and low soil moisture are quite stressful conditions for plants, and play an important role in the composition of the secondary metabolites [59].

Fertilization as well as shading reduced the percentage concentration of chamazulene in chamomile. Fertilization effects on this substance depend on fertilization type and quantity. Increased concentration of chamazulene with vermicompost, zeolite and ammonia fertilization has been recorded [52,53], while Emongor et al. [56] stated that excessive fertilization reduce it. Although N fertilization increases photosynthetic rates [53], this effect is weak under shading conditions and consequently the concentration of chamazulene in the essential oil remained low.

Fertilization significantly increased the percentage concentration of Z-spiroether, which is probably associated with the competition between hydrocarbons and oxygenated terpenes. The opposite relationship between the two groups of terpenes means that the concentrations of hydrocarbons increase as those of oxygenated ones decrease and vice versa [56]. Generally, it has been observed that N fertilization has a positive effect on the component percentages of Z-spiroether [56]. In contrast, shading reduced the concentration of this compound, albeit slightly, by reducing the photosynthetic capacity of plants, which significantly affects the production of secondary metabolites through the synthesis of primary metabolites [60].

Under shading conditions, the fertilized plants had higher concentration of Z-spiroether, as they had developed a more extensive root system [53] that enabled better nutrient uptake.

The opposite trend was detected in non-shaded plots related to higher evaporation and the lower soil moisture [59].

4.2. Anise

The height of anise plants grown in fertilized plots was higher as a result of N and P application [34,35]. Shading had a negative impact on the plant height of anise, probably due to the reduced photosynthesis caused by the decreased intake of sunlight [48]. Similar findings regarding anise height grown under artificial shading have been reported by Ullah et al. [32].

Dry anise seed productivity was not affected by either the fertilizer application or shading. In contrast to the results of previous studies conducted in Turkey [34] and Spain [31] that reported higher seed yields of anise under fertilization, this was not confirmed in the current study. This result is probably related to the significantly lower amount of P and N applied to the experimental plots compared to those used in the other studies. The crucial role of tree density on the seed production of MAPs has been well documented [18,19]. The relatively low density of olive trees in the present study was the main reason for the stable seed production in the olive grove.

The essential oil productivity of anise was not affected by shading and fertilization. The yields of some MAPs do not appear to be affected by the uptake of sunlight, but are mainly affected by their growth cycle and nutrient yields [18], with the use of chemical fertilizers significantly affecting essential oil yields [39]. The use of limited amounts of P and N fertilization in the present study is the main reason behind the differentiated result regarding the yield of essential oil in the fertilized plots. It must be noted that the essential oil yield of anise found in this study ranged from 107.4 to 198.8 mL ha⁻¹, and exceeded the average yields reported by Orav et al. [61] for various European countries, as well as those reported by Kara [31] in Turkey and by Ullah et al. [32] in Germany.

The essential oil obtained from all the combinations of shading and fertilization meets the eligibility requirements as defined by European Pharmacopoeia [28]. Both *E*-anethole and limonene percentage concentrations were not affected by shading, indicating that the shading produced by the olive trees had minimal effects on the essential oil components of anise. Similar to this result, Degani et al. [62] reported minimal effects of moderate shading on the essential oil composition of *Eucalyptus citriodora* Hook. The significant increase of the concentration of *E*-anethole after fertilization is related to the increase of available nutrients [63]. Anethole concentration in the present study was higher than the one reported by Ullah et al. [32] and similar to the findings of Fitsiou et al. [64]. In contrast, fertilization significantly reduced the concentration of limonene. The composition of secondary metabolites in MAPs depends mainly on the floral state and the stressful conditions [59]. Although generally, fertilization, particularly with N, increases the content of the components of the essential oils of MAPs, a similar reduction has been observed for limonene for fennel (*Foeniculum vulgare* Mill.) [65], while Buntain and Chung [66] reported that the fennel oil components were not affected by N fertilization.

5. Conclusions

The use of both chamomile and anise as understory crops in olive agroforestry systems in the Mediterranean area is a very promising practice, supplementing the annual income of the farmers. The essential oil yield of both species was not affected by shading, and it was of high quality, meeting the criteria of commercial specifications. Fertilizer application seems to guarantee a high production in terms quantity and quality of the essential oils. Nevertheless, the productivity and the chemical composition of MAPs essential oils are influenced by various factors, such as soil and climatic conditions, cultivation practices and post-harvest handling and cultivated variety. Thus, further research is required to explore the effects of those factors on both species in olive silvoarable systems of various tree spacings. The study provides a useful first evaluation of the effects of fertilization and shading on the productivity of anise and chamomile grown in olive silvoarable systems

in terms of both yield and essential oil chemical composition, for which limited data exist in the literature. However, conclusions on those effects, albeit promising, are restricted by only one year of data, which is a limitation of the current study.

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Appendix A

Table A1. Chamomile’s essential oils chemical composition under different treatments. SnFCEO: Shade without Fertilizer Chamomile Essential Oil, SFCEO: Shade with Fertilizer Chamomile Essential Oil, nSnFCEO: without Shade without Fertilizer Chamomile Essential Oil, nSFCEO: without Shade with Fertilizer Chamomile Essential Oil. Components are listed in order of elution from an TG-5MS column. Retention indices (RI), relative to *n*-alkanes, are listed alongside published values (Pub. RI from Adams 2007). Mean $\pm$ SD ($n = 3$).

	RI	Components	Relative Percentage Concentration			
			SnFCEO	SFCEO	nSnFCEO	nSFCEO
1	999	yomogi alcohol	-	-	-	0.2 $\pm$ 0.1
2	1058	artemisia ketone	-	-	-	0.5 $\pm$ 0.2
3	1179	naphthalene	-	-	-	0.2 $\pm$ 0.1
4	1235	(3Z)-hexenyl 3-methyl butanoate	-	-	-	0.2 $\pm$ 0.1
5	1272	4,8-dimethyl-nona-3,8-dien-2-one	0.2 $\pm$ 0.1	0.2 $\pm$ 0.1	0.1 $\pm$ 0.0	0.2 $\pm$ 0.0
6	1284	<i>trans</i> anethole	1.1 $\pm$ 0.3	0.8 $\pm$ 0.2	0.8 $\pm$ 0.2	1.0 $\pm$ 0.2
7	1336	δ -elemene	-	0.2 $\pm$ 0.1	0.1 $\pm$ 0.0	0.2 $\pm$ 0.1
8	1356	eugenol	-	-	-	0.1 $\pm$ 0.0
9	1391	β -elemene	-	0.2 $\pm$ 0.1	0.2 $\pm$ 0.1	0.2 $\pm$ 0.1
10	1447	methyl naphthol	0.1 $\pm$ 0.0	0.3 $\pm$ 0.1	0.2 $\pm$ 0.1	0.1 $\pm$ 0.0
11	1458	(E)- β -farnesene	1.7 $\pm$ 0.4	2.2 $\pm$ 0.3	3.1 $\pm$ 0.3	7.6 $\pm$ 1.2
12	1479	γ -muurolene	-	0.2 $\pm$ 0.1	0.3 $\pm$ 0.1	0.6 $\pm$ 0.2
13	1495	bicyclogermacrene	-	0.2 $\pm$ 0.1	0.3 $\pm$ 0.1	0.5 $\pm$ 0.1
14	1507	(E, E)- α -farnesene	-	-	-	0.3 $\pm$ 0.2
15	1522	δ -cadinene	-	-	-	0.2 $\pm$ 0.1
16	1565	(E)-nerolidol	-	0.7 $\pm$ 0.2	1.1 $\pm$ 0.2	1.1 $\pm$ 0.3
17	1578	spathulenol	3.8 $\pm$ 0.4	3.4 $\pm$ 0.5	3.6 $\pm$ 0.3	4.4 $\pm$ 0.6

Table A1. Cont.

RI		Components	Relative Percentage Concentration			
			SnFCEO	SFCEO	nSnFCEO	nSFCEO
18	1591	salvial-4(14)-en-1-one	-	-	-	0.2 ± 0.1
19	1630	nerolidol oxide	-	-	-	0.6 ± 0.2
20	1639	<i>epi</i> - α -cadinol	0.3 ± 0.1	0.5 ± 0.2	0.3 ± 0.2	0.6 ± 0.1
21	1660	α -bisabolol oxide B	37.1 ± 0.8	30.9 ± 0.9	29.9 ± 1.6	29.9 ± 1.1
22	1686	α -bisabolone oxide A	15.9 ± 0.8	11.2 ± 1.2	17.3 ± 0.3	17.3 ± 0.6
23	1730	chamazulene	11.9 ± 0.8	8.6 ± 0.8	14.0 ± 0.7	9.2 ± 0.4
24	1751	α -bisabolol oxide A	16.0 ± 0.9	22.3 ± 1.4	18.2 ± 0.6	16.6 ± 0.7
25	1773	dimethyl biphenyl	0.2 ± 0.1	0.3 ± 0.1	0.2 ± 0.1	0.2 ± 0.1
26	1880	(Z)-spiroether	8.3 ± 0.9	15.3 ± 0.6	6.9 ± 0.6	5.2 ± 0.3
27	1891	(E)-spiroether	0.6 ± 0.2	0.2 ± 0.1	0.3 ± 0.1	0.5 ± 0.1
28	1948	1,4-dimethyl-7-(1-methylethyl)-azulene-2-ol	0.3 ± 0.1	0.4 ± 0.1	0.2 ± 0.1	0.2 ± 0.1
Total			97.5	98.1	97.1	98.1

Table A2. Anise's essential oils chemical composition under different treatments. % (mean ± SD). SnFAEO: Shade without Fertilizer Anise Essential Oil, SFAEO: Shade with Fertilizer Anise Essential Oil, nSnFAEO: without Shade without Fertilizer Anise Essential Oil, nSFAEO: without Shade with Fertilizer Anise Essential Oil. Components are listed in order of elution from an TG-5MS column. Retention indices (RI), relative to *n*-alkanes, are listed alongside published values (Pub. RI from Adams 2007). Mean ± SD (*n* = 3).

RI		Components	Relative Percentage Concentration			
			SnFAEO	SFAEO	nSnFAEO	nSFAEO
1	932	α -pinene	0.4 ± 0.1	0.2 ± 0.1	0.2 ± 0.2	0.2 ± 0.1
2	971	sabinene	0.2 ± 0.1	0.2 ± 0.1	0.2 ± 0.1	0.2 ± 0.1
3	990	myrcene	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0
4	1004	α -phelladrene	0.1 ± 0.0	-	0.1 ± 0.0	-
5	1022	<i>p</i> -cymene	0.2 ± 0.1	-	-	0.1 ± 0.0
6	1029	limonene	6.5 ± 0.4	4.6 ± 0.6	6.1 ± 0.2	4.7 ± 1.0
7	1036	(Z)- β -ocimene	1.1 ± 0.2	0.9 ± 0.1	1.2 ± 0.2	0.9 ± 0.1
8	1057	γ -terpinene	0.6 ± 0.1	0.6 ± 0.2	0.6 ± 0.2	0.6 ± 0.1
9	1087	fenchone	0.2 ± 0.1	0.2 ± 0.0	0.1 ± 0.0	-
10	1129	<i>allo</i> -ocimene	0.2 ± 0.1	0.3 ± 0.0	0.2 ± 0.1	0.1 ± 0.0
11	1199	methyl chavicol	2.2 ± 0.3	2.1 ± 0.2	2.9 ± 0.2	2.1 ± 0.2
12	1253	(Z)-anethole	0.3 ± 0.1	0.2 ± 0.0	0.2 ± 0.1	0.2 ± 0.1
13	1288	(E)-anethole	87.2 ± 0.8	90.1 ± 0.6	87.6 ± 1.0	90.2 ± 0.5
14	1376	α -copaene	0.1 ± 0.0	0.2 ± 0.1	0.1 ± 0.0	-
15	1458	(E)- β -farnesene	0.2 ± 0.1	-	-	0.1 ± 0.0
16	1480	germacrene D	-	0.2 ± 0.1	0.2 ± 0.0	0.3 ± 0.1
17	1522	δ -cadinene	0.2 ± 0.1	-	-	0.1 ± 0.0
Total			99.8	99.9	99.9	99.8

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