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# Impacts of Alexandrian Clover Living Mulch on the Yield, Phenolic Content, and Antioxidant Capacity of Leek and Shallot

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**Abstract:** The use of living mulches (LM) grown in-season together with the cash crop is a potentially important method of organic vegetable production. There are only a few reports on the influence of LM on the biological value of vegetable crops. The impacts of LM of Alexandrian clover on the yields and levels of phenolic compounds in leeks and shallots were investigated. There were three sowing dates for the clover plants: 3 weeks before planting the leeks and shallot, at the planting date, and three weeks from planting the leeks and shallots. The yields of leeks and shallots with LM were higher than without the clover LM; the plants grown with LM accumulated more total phenolic (TP) compounds, and the plant extracts showed significantly higher antioxidant activity (AA). In shallot bulbs, the LM of Alexandrian clover increased the ferulic acid and chlorogenic acid levels, while in leek pseudo-stems the quercetin level was increased. The level of bioactive compounds depended on the date of clover planting. The most favorable sowing dates for clover planting were at the time of planting the leeks and shallots and three weeks after planting the plants. The LM of Alexandrian clover can be considered as a tool that can influence the nutritional value of leeks and shallots.

**Keywords:** *A. ampeloprasum* var. *porrum* L.; *A. ascalonicum* L.; *Trifolium alexandrinum*; total phenolics; living mulch; caffeic acid; quercetin

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## 1. Introduction

Allium species include vegetable, spice, and medicinal plants, including leek (*Allium ampeloprasum* var. *porrum*) and shallot (*Allium cepa* L. var. *ascalonicum* Backer), with a wide range of uses. The largest share of leek production occurs in Europe (80%), including in France (3200 ha), Belgium (2429 ha), the Netherlands (2156 ha), Germany (2050 ha), and Poland (1200 ha) [1]. Among the onion vegetables, the cultivation of shallots is less widespread [2].

These vegetables are abundant in polyphenols, including phenolic acids (and their derivatives) and flavonoids (flavan, flavanone, flavones, flavonol, dihydroflavonol, flavan-3-ol, flavan-4-ol, and flavan-3,4-diol). They contain organic sulfur compounds such as cysteine, methionine, ajoene, glutathione, alliin, and allicin, as well as vitamins such as riboflavin, thiamine, nicotinic acid, and vitamin C [3]. The various bioactive substances have antioxidant properties; therefore, consuming vegetables rich in these compounds can reduce the occurrence of many diseases, such as ischemic heart disease, some forms of cancer, and atherosclerosis [4–6].

In agrotechnics, bulbous vegetables are grown in wide inter-rows (usually every 30–40 cm). This cultivation system promotes soil erosion and humus mineralization. A good way to counteract these processes is via tillage with living mulch (LM). A living mulch is a one-year or perennial cover crop grown during the main crop cultivation period [7]. Numerous studies have documented the beneficial effects of LMs; they can be sources of nitrogen for subsequent crops [8] and can reduce soil erosion [9], enrich the soil with organic matter [10], and reduce runoff and potential water contamination [11]. In addition, living mulches can reduce the amounts of leached nitrogen [12], can have a positive effect on soil water management [13], can improve the physical and biological properties of the soil [14], and can reduce the occurrence of nematodes [15]. Numerous studies have demonstrated the ability of live mulches to suppress weeds in various growing systems [15–17]. Pfeiffer et al. [18] found that intercropping with a LM can reduce the weed biomass as compared to homogeneous cultivation. The weed suppression is often due to the allopathic effect [7], nutrient depletion [19], or both. Vigorous LM growth more effectively suppresses weeds but also limits crop yields [20]. Adamczewska-Sowińska and Kołota [21] found a reduction in the yield of sweet peppers grown with white clover (by 27.6%) and perennial ryegrass (by 12.4%) compared to the cultivation without catch crops. Carruthers et al. [22] and Bhaskar [23] noted the lack of economic benefits in using LM. In contrast, Båth et al. [24] found that a living mulch farming system can achieve higher or comparable yields when there is little interspecific competition. Higher yields achieved by reducing weed infestations were found in the cultivation of cabbage, leek, zucchini, tomato, and potato crops [18,25–27]. Higher yields were found in cultivation with LM in a temperate climate in *Allium* species such as onions [28], garlic [29], and leeks [30].

The effect of using LM with vegetable plants depends on several factors, including the soil and water conditions, type of cover plant, sowing date, and varietal characteristics of the vegetable [31–33]. In practice, it is crucial to control the amount and date of LM sowing concerning the plant growth phase of the main crop, so as to avoid problems related to plant competition [32,33].

Robačer et al. [9], regarding the principles of plant selection they formulated, emphasize several features of LM, namely its high tolerance to drought, the durability of its sodding, and its low agrotechnical requirements, including its fertilization needs. In this context, the Alexandrian clover (*Trifolium alexandrinum* L.; Egyptian clover, beersem clover) is considered a promising annual species for intercropping. Alexandrian clover plants produce abundant biomass within a short time and bloom late [34]. The biomass consisting of legumes, after the re-incorporation of soil nitrogen into the plant molecules, may satisfy the additional nitrogen demands of the other intercrop plants. In this way, it can continue biomass production and increase soil fertility [10].

From an economic standpoint, cultivation with LM involves additional work and the purchase of appropriate machines and resources. However, the long-term benefits of using residual LM organic matter make it more of an investment than a cost. The use of LM in combination with other species as main crops is a new topic in horticultural practice. During cultivation with LM, the key seems to be to recognize the phenology of the plants as a determinant of the expected competition period, so that there is no competition for nutrients and water. To minimize this risk, the cultivated vegetable species should be adapted to intercropping. The effects of intercropping on the levels of bioactive substances in the edible parts of vegetables remain completely unknown. In this study, it was hypothesized that the Alexandrian clover as an LM in cultivation with leeks and shallots would not reduce the yield or content of bioactive substances in pseudo-leek stalks and shallot bulbs.

## 2. Materials and Methods

### 2.1. Plant Material and Growth Conditions

The agronomic experiments were carried out at the Felin Research Station of the University of Life Sciences in Lublin, Poland (51.23°N, 22.56°E).

Two species of vegetable plants of the genus *Allium*, leek (*Allium ampeloprasum* var. *porrum* L.) and shallot (*A. ascalonicum* L.), were cultivated with living mulch (LM) of the Alexandrian clover (*Trifolium alexandrinum* L.). The experiment was set up as a two-factor split-plot design with four replications. The first factor had two levels: no living mulch (control, without living mulch) and without moving the LM throughout the cultivation cycle (LM). The second factor was the date of living mulch establishment: 3 weeks before planting the leek and shallot plants, at the planting date, and three weeks from planting the leek and shallot plants. In the experiments, the clover was sown in an amount of 8 kg ha$^{-1}$ at three dates: (1) 3 weeks before leek and shallot planting; (2) at the planting date; (3) 3 weeks from the planting date. The fresh weights of plants in an area measuring 1 m$^2$ ranged from 3.7 to 5.2 kg. The LM covered the soil surface until the shallots and leeks were harvested. The clover LM initially covered 35% of the soil surface and covered 65% at the end of cultivation.

The soils in the area were classified as Luvisols [35] and were developed in a plain and with a climate in which the dry and wet seasons are markedly defined. The main characteristic in Luvisols is the clay content leaching from upper to lower horizons, where it accumulates. The notable clay content (39%) compared to the rest of the textural fractions (sand (35.2%) and silt (25.8%)) gives rise to soils with a clay loam texture, a slightly acidic character (pH = 6.7), and a low content of organic matter (1.6%). The total N (0.68%), Ca 4.5 (%); P 1.2 (%), K 1.8 (%), and Mg 0.9 (%) contents revealed the mineral content of the topsoil in the field.

The shallot and leek cultivation was carried out following local practice rules. In each of the two experiments, ploughing (30 cm depth), harrowing, and fertilization were carried out before planting. The differences between the crops mainly related to the mineral fertilization, carried out using fertilizers in the doses described below. No plant protection products or irrigation procedures were used to cultivate the shallot or leek plants.

### 2.2. Shallot

The research carried out in 2018 and 2019 included the evaluation of the yields and contents of dry matter and total phenolic compounds (TP) in bulbs of the cultivar 'Toto' (PlantiCo Zielonki Sp. z o.o., Zielonki near Warsaw, Poland), with a dry straw-yellow husk and white-cream flesh scales.

The size of a single plot was 3 m$^2$ (2.0 × 1.5 m). The seedlings prepared from the sowing of seeds in boxes, during the second week of March, were sown in the field on 26 April 2018 and 29 April 2019 in rows spaced apart every 20 cm, with a row distance between every seedling of 10 cm (plant density, 50 plants m$^{-2}$). On the planting day, the plants were about 12–15 cm high with 2–3 leaves.

Carrots were grown before the shallots. Before sowing the clover plants and planting shallot seedlings, the following fertilization was applied: 10 kg ha$^{-1}$ N; 40 kg ha$^{-1}$ $P_2O_5$, and 60 kg ha$^{-1}$ $K_2O$ in mineral fertilizers (ammonium nitrate, triple superphosphate, potassium sulfate).

The harvesting was carried out once on 25 July (2018 and 2019), during the breaking and drying phase of the chives.

### 2.3. Leek

The test plant was the leek of the 'Jolant' cultivar (bred by Bejo Poland, Ożarów Mazowiecki, Poland), grown for summer harvest. The average length of the bleached pseudo-stem part (8–9 cm) and the poorly marked onion characterize plants of this cultivar.

The leek seedlings were planted on 25 April 2018 and 28 April 2019 in plots with an area of 3.0 m$^2$ (2.0 × 1.5 m). The seedlings prepared in the same way as in the previous case (shallot), during the second week of February, were transplanted on the flat in rows spaced apart every 35 cm. In the rows, the seedlings were placed every 7 cm (plant density, 40 plants m$^{-2}$). On the planting day, the plants were about 15–20 cm high with 3–5 leaves.

Before sowing the clover plants and planting the leeks, mineral fertilization was applied: 10 kg ha$^{-1}$ N, 50 kg ha$^{-1}$ $P_2O_5$ and 70 kg ha$^{-1}$ $K_2O$ (the same type of fertilizer for the shallots). No weed control treatments were carried out during the growing season. The leek harvest was carried out once each on August 23 (2018) and August 28 (2019), and after shortening the roots, the marketable yield (above-ground part) was assessed.

### 2.4. Weather Conditions

From April to September 2018, the total rainfall was 425 mm, 57 mm higher than the average multi-year total (1955–2010) for this period (Table 1). In 2018, the dry month was April (40 mm) and the wettest month was July (124 mm). In 2019, the lowest rainfall rates were recorded in June and July (37 and 38 mm, respectively) and the highest in August (102 mm). The average air temperatures for this period, both in 2018 and 2019, were higher by 2.7 and 2.4 °C, respectively, than the long-term average (1955–2010). The average air temperatures in 2018 in the summer months (July, 20.6 °C; August, 20.8 °C) were higher than the multi-year average by 2.8 and 3.7 °C, respectively. In 2019, the average temperature in July was 19.4 °C and in August 20.3 °C, which were higher by 1.6 and 3.2 °C, respectively, than the average long-term air temperature for these months.

**Table 1.** Average air temperatures and rainfall during the experimental trials (2018–2019)—data from the Meteorological Station in Felin, Poland (51, 13° N; 22, 37° N).

| Years | Month | | | | | | |
|---|---|---|---|---|---|---|---|
| | **April** | **May** | **June** | **July** | **August** | **September** | **Average/Sum** |
| | Temperature (°C) | | | | | | |
| 2018 | 7.5 | 16.7 | 18.8 | 20.6 | 20.8 | 15.5 | 16.7 |
| 2019 | 9.5 | 13.4 | 21.5 | 19.4 | 20.3 | 14.5 | 16.4 |
| 1951–2010 | 7.4 | 13.0 | 16.2 | 17.8 | 17.1 | 12.6 | 14.0 |
| | Rainfall (mm) | | | | | | |
| 2018 | 40 | 56 | 65 | 124 | 72 | 68 | 425 |
| 2019 | 49 | 93 | 37 | 38 | 102 | 52 | 371 |
| 1951–2010 | 39 | 58 | 66 | 84 | 69 | 54 | 368 |

### 2.5. Yield and Morphological Parameter Evaluation

In the case of shallots, after the bulbs were harvested and cleaned from the dried chives, the marketable yield of the bulbs was assessed from 50 plants $m^{-2}$ in each plot. The bulbs were dried in natural conditions (drying room) at a temperature of 20 °C during the day and 15 °C at night. During harvest, the weight of 100 bulbs was assessed, and samples (100 bulbs) were taken for the chemical analysis from each plot.

In the case of leek, harvests of the mature plants were performed when the pseudo-stems had reached their maximum growth, and the leaf blades were trimmed to 15.0 cm in length to obtain a marketable product. In each plot, determinations were made of the weight of the commercial product (pseudo-stems with 15-cm-long leaf blades) from 40 plant samples (from 1 $m^2$). Further, the roots were cut from the collected plant samples, gently washed with water to remove surface contaminants, and dried with filter paper. The pseudo-stems and leaves were separated, cut with a plastic knife, dried to a constant weight, and homogenized. During the harvest, measurements of the length (cm) and diameter (cm) of the pseudo-stems were carried out on 40 randomly selected plants.

### 2.6. Sample Preparation and Analyses

The leek (pseudo-stem) and shallot samples were cleaned, cut into 1 cm pieces, and stored at T = −80 °C before lyophilization. The phytochemical analyses of the leek samples were carried out successively for three weeks. The phytochemical analyses of the shallots (onions) were performed six weeks after harvest. Immediately before the chemical analyses were performed, the plant material was ground. In shallot bulbs and pseudo-leek stems, the dry weight was determined by drying to a constant weight at 75 °C over 3 days [36].

### 2.7. Extraction

After grinding the sample (3 g), it was extracted with 80% ($v/v$) methanol at a solid material-to-solvent ratio of 1:10 ($w/v$) [37]. The extractions were carried out in an RVO

400SD rotary vacuum evaporator (Ingos, Prague, Czech Republic) at a temperature of 65 °C for 3 h. After filtration through Whatman filter paper (gradation 42; Merck, Warsaw, Poland), the material was retreated with 80% methanol and extracted twice for 2 h at room temperature. The methanol extracts were joined, and after solvent evaporation the residues were eluted with hot water (50 mL). The water solutions were left in the refrigerator for 24 h. All samples were stored at a temperature of T = −22 °C until further analyses. All extractions were performed in triplicate.

*2.8. Total Phenolics Measurement*

The total phenolic (TP) content was assessed using the Folin–Ciocalteu test [38] as a modification of Yen and Chen's method [39]. The sample extracts (0.2 mL) were mixed with sodium carbonate (64 mL, 6% in distilled water), and after 1 min 0.2 mL of freshly diluted Folin–Ciocalteu reagent mixed 1:1 with water ($v/v$) was added. The reaction mixture was incubated for 2 hours at room temperature, and the absorbance of the mixture was read at 750 nm with a UV–Vis spectrophotometer (Model UV-1800, Shimadzu Corp., Kyoto, Japan). The TP content was standardized against gallic acid and expressed as mg gallic acid equivalents (GAE) per 100 g dry weight sample (DW).

*2.9. HPLC Analysis*

The HPLC separation was performed on a Shimadzu UFLC series apparatus (Shimadzu Corp., Tokyo, Japan) coupled to a diode array detector (DAD). The separation was performed on a Phenomenex Synergi Fusion-RP column (4 µm, 250 × 4.6 mm i.d.; Phenomenex, Santa Clara, USA) with a sample injection volume of 20 µL. The compounds were eluted with a linear gradient system of solvents A (acetonitrile-water-trifluoroacetic acid, 5:95:0.1, $v/v/v$) and B (acetonitrile-trifluoroacetic acid, 100:0.1, $v/v$) [40]: 0–18 min, 0–60% B. The flow rate was 1 mL $min^{-1}$, and the column temperature was 30 °C. The detection was performed by scanning the wavelength range of 190 to 400 nm. The contents of individual phenolic compounds were expressed based on the calibration curves of the appropriate standards or structurally related substances.

*2.10. Determination of Total Antioxidant Capacity*

The total antioxidant capacity levels of *A. ampeloprasum* and *A. ascalonicum* were assessed spectrophotometrically (UV-1800 model, Shimadzu Corp., Kyoto, Japan) via 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging tests [41]. The DPPH radical scavenging activity was determined by analyzing a mixture of 1 mL of sample with 2 mL of 0.1 mM DPPH radical at 517 nm after 30 min in the dark. The results are expressed as mM Trolox equivalents per 1 g of sample in dry weight (DW).

Consistent with the Ferric-reducing antioxidant power (FRAP), the total reduction capacity was determined according to earlier reports by Gouveia and Castilho [42] with modifications. At low pH, the orange iron–tripyridyltriazine complex ($Fe^{3+}$-TPTZ) was reduced to the blue iron complex ($Fe^{2+}$-TPTZ) by the action of electron-donating antioxidants. After this reaction, an increase in absorbance was observed at 593 nm. The reagent working solution was prepared with 100 mL of 300 mM acetate buffer at pH 3.6, 10 mL of 10 mM 2,4,6-tris (2-pyridyl)-s-triazine (TPTZ) in 40 mM hydrochloric acid, and 10 mL of 20 mM iron(III) chloride hexahydrate. The absorbance of the variance of the reaction mixture was recorded at 593 nm with a spectrophotometer (Shimadzu UV-1800 spectrophotometer). Methanol solutions with known Fe(II) concentrations were used to prepare the calibration curve. The results are expressed as µM $Fe^{2+}$ per 1 g of dry weight (DW).

*2.11. Chemicals and Standards*

Pure caffeoylquinic acids and flavonoids determined or used for calibration were purchased as certified materials from Merck (Darmstadt, Germany). All solvents and reagents used for preparing standard solutions and extracting phenolic acids (methanol, ethanol, ethyl acetate, toluene, acetic acid, and formic acid) were of analytical purity, while the methanol used for the chromatographic analysis was of HPLC quality. All solvents were obtained from Sigma-Aldrich (Poznań, Poland).

### 2.12. Statistical Analysis

The analysis of the morphological parameters, yields, contents of phenols and phenolic compounds, and antioxidant activity levels of the leeks and shallots was carried out in three repetitions. The data were analyzed via an analysis of variance (ANOVA) using Statistica PL ver. 13.0 (StatSof Inc., Tulsa, OK, USA). The compliance of the distribution of the determined features with the normal distribution was verified with the Shapiro–Wilk test, and through Levene's test the homogeneity of the variance was checked. The results were considered statistically significant at $\alpha = 0.05$, and Tukey's test identified homogeneous groups.

## 3. Results

### 3.1. Leek

For the yield level and selected morphological traits of the edible part (pseudo-stem) of the leek samples, a significant effect of the living mulch (LM) of clover as the main effect was demonstrated (Tables 2 and 3). In the cultivation plot with clover, the average leek yield was higher by 36% compared to the plants cultivated without LM. With clover, the leek stalks were characterized by a greater weight and smaller diameter. A higher yield of leeks with a higher unit weight for the pseudo-stems was obtained when clover plants were sown three weeks from planting the seedlings as compared to earlier dates. The pseudo-stems had a larger diameter and length in the simultaneous planting of leek seedlings with clover sowing. In 2019, the leek yield was higher by an average of 12 Mg ha$^{-1}$, with a higher average pseudo-stem weight than in 2018. For the leek yield, the significance of the interaction 'sowing date × cultivation season' (F = 7.972) was confirmed (Table 2). In 2019, the leek yield was higher by 5.7 Mg ha$^{-1}$ than in 2018, with a late sowing term for clover plants (3 weeks after planting seedlings) (Figure 1).

**Table 2.** The *F*-statistics for the leek parameters (ANOVA).

| Parameter | LM [a] | ST [b] | GS [c] | (LM) × (ST) | (LM) × (GS) | (ST) × (GS) | (LM) × (ST) × (GS) |
|---|---|---|---|---|---|---|---|
| Yield | 39.428 * | 9.328 * | 29.633 * | 1.326 NS | 1.502 NS | 7.972 * | 2.152 NS |
| Mean pseudo-stem weight | 3.613 * | 3.496 * | 5.185 * | 0.202 NS | 0.932 NS | 0.283 NS | 2.308 NS |
| Length of pseudo-stems | 0.00 NS | 4.99 * | 1.56 NS | 6.22 * | 0.00 NS | 0.01 NS | 0.69 NS |
| Diameter of pseudo-stems | 11.64 * | 25.95 * | 4.55 * | 7.14 * | 2.91 NS | 4.41 * | 3.59 * |
| Dry weight | 124.023 * | 12.235 * | 11.273 * | 7.156 * | 0.028 NS | 3.018 NS | 2.514 NS |
| Total phenolics | 58.257 * | 4.104 * | 16.541 * | 0.048 NS | 0.048 NS | 0.808 NS | 0.048 NS |
| Ferulic acid | 152.197 * | 0.452 NS | 65.724 * | 0.124 NS | 0.124 NS | 1.615 NS | 0.124 NS |
| Chlorogenic acid | 17.091 * | 30.158 * | 238.793 * | 0.047 NS | 0.426 NS | 0.047 NS | 0.426 NS |
| Caffeic acid | 2.02 NS | 111.44 * | 212.58 * | 9.45 * | 9.78 * | 3.51 * | 3.91 * |
| FRAP | 60.446 * | 0.638 NS | 3.021 NS | 2.418 NS | 0.269 NS | 0.486 NS | 0.269 NS |
| DPPH | 35.121 * | 1.830 NS | 0.173 NS | 0.103 NS | 0.026 NS | 3.978 * | 0.103 NS |

[a] LM: living mulch; [b] ST: sowing term; [c] GS: growing season; * indicates significance at $p < 0.05$; NS, not significant.

**Table 3.** The marketable yield (Mg ha$^{-1}$), pseudo-stem weight (g), pseudo-stem length, and pseudo-stem diameter (cm); (±standard deviation) in leeks as affected by the main experimental factors; means followed by different letters (a,b) are significantly different for each parameter at $p < 0.05$.

| Main Factor | Marketable Yield | Mean Pseudo-Stem Weight | Length of Pseudo-Stems | Diameter of Pseudo-Stems |
|---|---|---|---|---|
| | | Living mulch | | |
| Clover | 38.32 ± 1.4 a | 249 ± 12 a | 9.07 ± 0.54 a | 3.21 ± 0.09 b |
| Without mulch | 24.38 ± 0.7 b | 190 ± 70 b | 9.07 ± 0.23 a | 3.30 ± 0.17 a |

**Table 3.** *Cont.*

| Main Factor | Marketable Yield | Mean Pseudo-Stem Weight | Length of Pseudo-Stems | Diameter of Pseudo-Stems |
|---|---|---|---|---|
| | | Sowing term | | |
| 3 weeks before leek planting | 26.26 ± 0.9 b | 170 ± 11 b | 9.16 ± 0.47 ab | 3.17 ± 0.09 b |
| At time of leek planting | 30.01 ± 1.2 b | 219 ± 60 ab | 9.23 ± 0.15 a | 3.39 ± 0.11 a |
| 3 weeks after leek planting | 37.78 ± 1.5 a | 270 ± 11 a | 8.81 ± 0.43 b | 3.21 ± 0.13 b |
| | | Growing season | | |
| 2018 | 25.31 ± 0.09 b | 185 ± 10 b | 9.14 ± 0.36 a | 3.28 ± 0.17 a |
| 2019 | 37.40 ± 0.14 a | 255 ± 10 a | 9.00 ± 0.45 a | 3.23 ± 0.11 a |

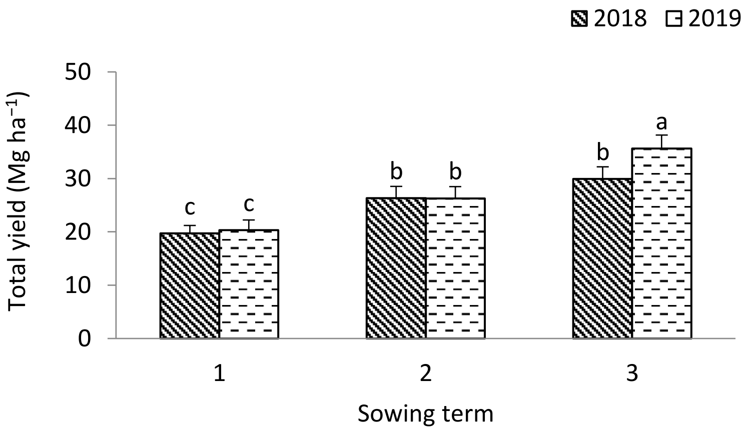


**Figure 1.** The total yield of the leeks affected by the 'sowing term–growing season' interaction. Sowing terms: 1: 3 weeks before leek planting; 2: at time of leek planting; 3: 3 weeks after leek planting. According to Tukey's test, means with the same letters do not differ significantly at $p \leq 0.05$. Bars indicate standard deviations.

Table 4 shows that the applied LM and sowing date affected the dry weight and total phenolic (TP) compound content. The dry weight was higher, the plants contained more TP compounds, and the ferulic acid and chlorogenic acid concentrations were higher in the cultivated plants than in the control. The LM of clover caused higher antioxidant activity (AA) in the extracts when the FRAP tests were used. However, the plants cultivated without clover showed a lower ability to scavenge DPPH radicals.

**Table 4.** Dry weight, total phenolics (mg GAE 100 $g^{-1}$ dw), ferulic acid, chlorogenic acid, caffeic acid (mg $g^{-1}$ dw), and antioxidant capacity contents (expressed by FRAP assay, µmol $Fe^{2+}$ $g^{-1}$; DPPH assay, µmol Trolox $g^{-1}$); (±standard deviation) in leeks as affected by the main experimental factors; means followed by different letters (a,b) are significantly different for each parameter at $p < 0.05$.

| Main Factor | DW * | TP | FER | CHL | CAF | FRAP | DPPH |
|---|---|---|---|---|---|---|---|
| | | | | Living mulch | | | |
| Clover | 20.96 ± 3.13 a | 655 ± 1.87 a | 62.74 ± 0.08 a | 8.65 ± 0.23 a | 93.42 ± 1.51 a | 8.01 ± 0.11 a | 1.01 ± 0.11 a |
| Without mulch | 13.52 ± 3.21 b | 616 ± 1.85 b | 43.30 ± 0.07 b | 7.59 ± 0.24 b | 90.92 ± 2.43 a | 5.51 ± 0.08 b | 0.80 ± 0.08 b |
| | | | | Sowing term | | | |
| 3 weeks before leek planting | 15.33 ± 5.23 b | 626 ± 2.50 b | 52.13 ± 0.12 a | 6.98 ± 0.21 c | 81.31 ± 1.23 b | 7.00 ± 0.20 a | 0.87 ± 0.20 a |
| At time of leek planting | 19.36 ± 2.87 a | 644 ± 2.45 a | 53.96 ± 0.12 a | 9.40 ± 0.23 a | 84.56 ± 1.41 b | 6.70 ± 0.14 a | 0.89 ± 0.14 a |
| 3 weeks after leek planting | 17.03 ± 5.64 a | 636 ± 3.01 ab | 52.96 ± 0.14 a | 7.98 ± 0.21 b | 110.65 ± 1.89 a | 6.57 ± 0.12 a | 0.95 ± 0.12 a |
| | | | | Growing season | | | |
| 2018 | 16.12 ± 5.18 b | 625 ± 2.63 b | 46.63 ± 0.10 b | 10.10 ± 0.12 a | 79.35 ± 1.29 b | 6.48 ± 0.16 a | 0.90 ± 0.16 a |
| 2019 | 18.36 ± 4.46 a | 645 ± 2.40 a | 59.41 ± 0.11 a | 6.15 ± 0.13 b | 105.00 ± 1.76 a | 7.04 ± 0.15 a | 0.91 ± 0.15 a |

* DW: dry weight; TP: total phenolics; FER: ferulic acid; CHL: chlorogenic acid; CAF: caffeic acid.

When the clover plants were sown and the leeks were planted simultaneously or three weeks after planting, the plants were characterized by a higher dry weight and contained more TP compounds, chlorogenic acid, or caffeic acid. There was no significant effect of the clover sowing date on the content of ferulic acid and the AA values. In 2019, as compared to 2018, the plants were characterized by a higher dry weight content and they had more TP compounds, ferulic acid, and caffeic acid and less chlorogenic acid. No effect of the growing season was found for AA.

Statistically, significant differences in caffeic acid content were found depending on the clover sowing date and the growing season (Table 2). In the cultivation with clover, the caffeic acid content was higher at each sowing date—three weeks before planting, at the time of planting, and three weeks after planting the leek plants (no significant differences) (Figure 2). In 2019, as compared to 2018, the caffeic acid content was higher at each clover sowing date (Figure 3).

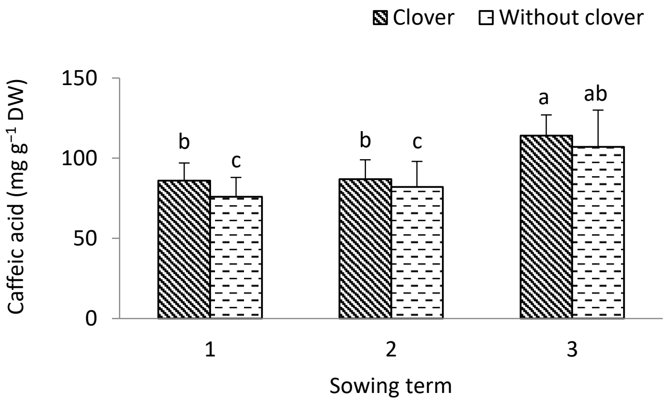


**Figure 2.** The caffeic acid in the leek plants was affected by the 'living mulch–sowing term' interaction. Sowing terms: 1: 3 weeks before leek planting; 2: at time of leek planting; 3: 3 weeks after leek planting. According to Tukey's test, means with the same letters do not differ significantly at $p \leq 0.05$. Bars indicate standard deviations.

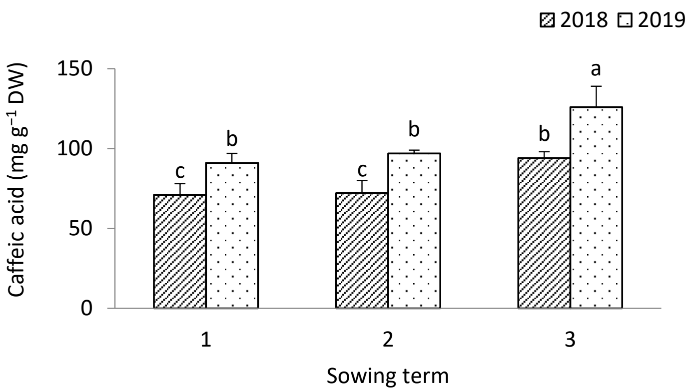


**Figure 3.** The caffeic acid in the leek plants was affected by the 'sowing term–growing season' interaction. Sowing term: 1: 3 weeks before leek planting; 2: at time of leek planting; 3: 3 weeks after leek planting. According to Tukey's test, means with the same letters do not differ significantly at $p \leq 0.05$. Bars indicate standard deviations.

*3.2. Shallot*

In the cultivation of shallots with the LM of clover, the bulb yield was higher by 23%, the weight of 100 bulbs was higher by 20%, and the bulbs contained more dry weight in comparison to plants cultivated without living mulch (Tables 5 and 6). The sowing date of the clover plants also significantly impacted the yield. When the clover plants were sown on the same planting date as the shallot seedlings and later (three weeks after planting the bulbs), the yield averages were 29–31% higher and the bulbs contained more dry weight than three weeks before planting.

**Table 5.** The *F*-statistics for the shallot parameters (ANOVA).

| Parameter | LM [a] | ST [b] | GS [c] | (LM) × (ST) | (LM) × (GS) | (ST) × (GS) | (LM) × (ST) × (GS) |
|---|---|---|---|---|---|---|---|
| Yield | 17.12 * | 12.58 * | 0.79 NS | 1.21 NS | 3.23 NS | 2.42 NS | 0.2 NS |
| Weight of 100 bulbs | 8.33 * | 7.58 * | 1.44 NS | 1.24 NS | 3.53 NS | 0.20 NS | 0.43 NS |
| Dry weight | 31.60 * | 14.63 * | 44.83 * | 0.48 NS | 0.40 NS | 1.06 NS | 0.05 NS |
| Total phenolics | 5.96 ** | 9.36 ** | 46.43 ** | 1.98 NS | 5.08 ** | 8.59 ** | 0.39 NS |
| *p*-coumaric acid | 4.43 ** | 1.72 NS | 72.01 ** | 0.11 NS | 0.03 NS | 1.94 NS | 0.61 NS |
| Quercetin | 7.86 ** | 3.58 ** | 6.87 ** | 1.11 NS | 3.98 NS | 0.65 NS | 2.57 NS |
| Chlorogenic acid | 1.30 NS | 0.37 NS | 92.26 ** | 0.22 NS | 0.00 NS | 0.31 NS | 0.01 NS |
| FRAP | 0.27 NS | 16.82 ** | 68.98 ** | 1.75 NS | 7.47 ** | 0.35 NS | 2.13 NS |
| DPPH | 0.33 NS | 0.48 NS | 121.11 ** | 0.89 NS | 23.25 ** | 12.78 ** | 6.75 ** |

[a] LM: living mulch; [b] ST: sowing term; [c] GS: growing season; * and ** indicate significance at $p < 0.05$ and $p < 0.01$, respectively; NS, not significant.

**Table 6.** Marketable yield (Mg ha$^{-1}$), weight of 100 bulbs (kg), and dry weight of bulbs; (±standard deviation) in shallots as affected by the main experimental factors; means followed by different letters (a,b) are significantly different for each parameter at $p < 0.05$.

| Main Factor | Yield | Weight of 100 Bulbs | Dry Weight |
|---|---|---|---|
| Living mulch | | | |
| Clover | 26.24 ± 0.64 a | 8.04 ± 0.23 a | 13.41 ± 1.08 a |
| Without mulch | 20.20 ± 0.64 b | 6.44 ± 0.20 b | 12.42 ± 0.88 b |
| Sowing term | | | |
| 3 weeks before shallot planting | 18.05 ± 0.69 b | 5.71 ± 0.21 b | 12.31 ± 0.89 b |
| At time of shallot planting | 25.55 ± 0.61 a | 7.98 ± 0.21 a | 13.47 ± 1.06 a |
| Three weeks after shallot planting | 26.05 ± 0.52 a | 8.03 ± 0.19 a | 12.99 ± 1.10 a |
| Growing season | | | |
| 2018 | 23.02 ± 0.76 a | 6.91 ± 0.25 a | 12.33 ± 0.93 b |
| 2019 | 23.41 ± 0.65 a | 7.57 ± 0.21 a | 13.51 ± 0.92 a |

The cultivation of shallots with clover plants as compared to the cultivation without living mulch did not significantly affect the TP, *p*-coumaric acid, chlorogenic acid, and AA values in the bulbs; the differences were insignificant (Tables 5 and 7). The bulbs harvested from plots with clover contained more quercetin than in the control, but the statistical value was low ($F = 7.86$) (Table 5).

**Table 7.** Total phenolics (mg GAE 100 g$^{-1}$ dw), *p*-coumaric acid, chlorogenic acid, quercetin (mg g$^{-1}$ dw), and antioxidant capacity contents (expressed by FRAP assay, µmol $Fe^{2+}$ g$^{-1}$ and DPPH assay, µmol Trolox g$^{-1}$); (±standard deviation) in shallots as affected by the main experimental factors; means followed by different letters (a,b) are significantly different for each parameter (level of significance *p*-value, is shown in the Table 5).

| Main Factor | TP * | COU | CHL | QUE | FRAP | DPPH |
|---|---|---|---|---|---|---|
| Living mulch | | | | | | |
| Clover | 137 ± 22.90 a | 5323 ± 53.48 a | 2.35 ± 0.67 a | 116 ± 6.12 a | 2.70 ± 0.76 a | 1.17 ± 0.06 a |
| Without mulch | 129 ± 18.84 a | 5122 ± 51.66 a | 2.22 ± 0.63 a | 96 ± 4.56 b | 2.65 ± 0.50 a | 1.13 ± 0.04 a |
| Sowing term | | | | | | |
| 3 weeks before shallot planting | 125 ± 23.10 b | 5102 ± 56.43 a | 2.24 ± 0.71 a | 94 ± 3.56 b | 2.26 ± 0.51 b | 1.14 ± 0.04 a |
| In time of shallot planting | 130 ± 22.80 b | 5311 ± 58.17 a | 2.35 ± 0.65 a | 107 ± 7.01 ab | 2.84 ± 0.61 a | 1.12 ± 0.06 a |
| 3 weeks after shallot planting | 144 ± 12.67 a | 5254 ± 44.52 a | 2.26 ± 0.61 a | 117 ± 6.23 a | 2.93 ± 0.61 a | 1.20 ± 0.04 a |
| Growing season | | | | | | |
| 2018 | 121 ± 14.81 b | 4818 ± 42.44 b | 1.74 ± 0.49 b | 97 ± 5.04 b | 2.25 ± 0.49 b | 0.78 ± 0.03 b |
| 2019 | 145 ± 19.56 a | 5627 ± 22.85 a | 2.82 ± 0.11 a | 115 ± 6.34 a | 3.10 ± 0.48 a | 1.53 ± 0.03 a |

* TP: total phenolics; COU: *p*-coumaric acid; CHL: chlorogenic acid; QUE: quercetin.

Higher TP levels were found in bulbs when the living mulch was sown three weeks after planting the bulbs than on the earlier dates. Clover sowing three weeks before planting lowered the quercetin content in the bulbs, which was accompanied by a low AA value as determined by the FRAP. The LM sowing date had no significant effect on the contents of *p*-coumaric acid, chlorogenic acid, and AA as determined by the DPPH method.

The total phenolic compounds, *p*-coumaric acid, chlorogenic acid, quercetin, and AA values were higher in 2019 than in 2018.

In 2019, in the cultivation with clover, the TP content in the bulbs was higher than in the cultivation without LM, while in 2018 these differences were insignificant (Figure 4). In the cultivation with LM, for each clover sowing term, the TP level was higher than without clover (Figure 5).

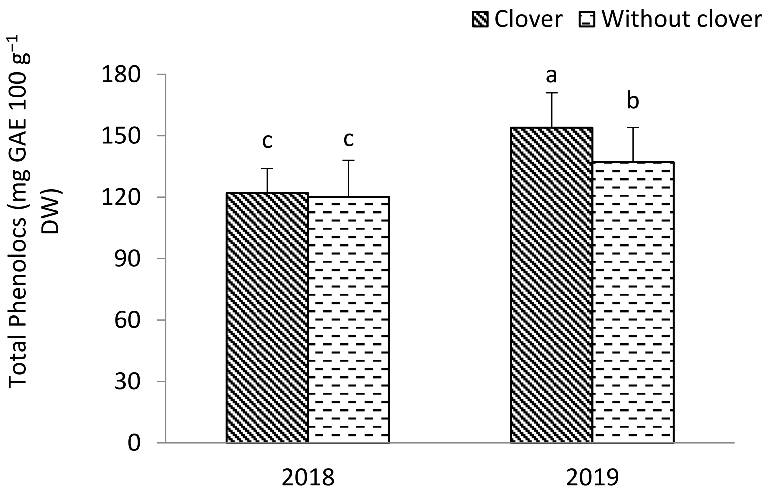


**Figure 4.** The total phenolics in shallots as affected by the 'living mulch–growing season' interaction. Sowing terms: 1: 3 weeks before leek planting; 2: in term leek planting; 3: 3 weeks after leek planting. According to Tukey's test, means with the same letters do not differ significantly at $p \leq 0.05$. Bars indicate standard deviations.

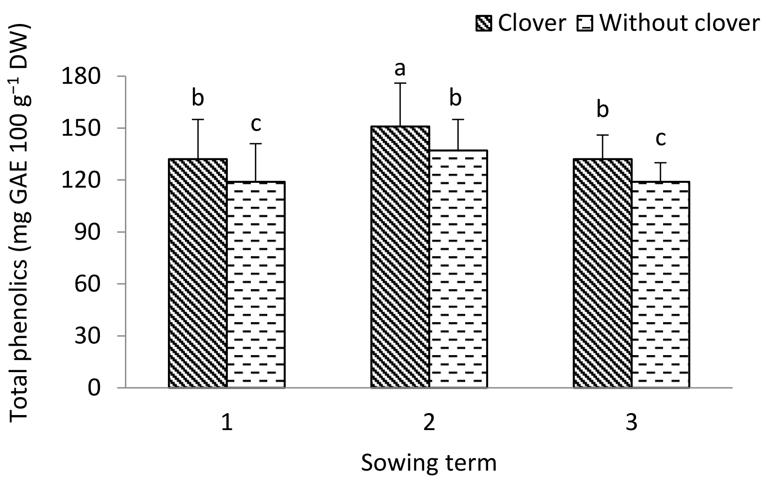


**Figure 5.** The total phenolics in shallots as affected by the 'living mulch–sowing term' interaction. Sowing term: 1: 3 weeks before leek planting; 2: in time of leek planting; 3: 3 weeks after leek planting. According to Tukey's test, means with the same letters do not differ significantly at $p \leq 0.05$. Bars indicate standard deviations.

## 4. Discussion

### *4.1. The Yield of Leek Above-Ground Mass and the Yield of Shallot Bulbs*

The influence of the weather conditions on the yielding of two species, *Allium ampeloprasum* var. *porrum* and *Allium ascalonicum*, was varied. The weather conditions had an effect on the leek yield but not on the shallot yield. This was related to the different cultivation periods. The ripe 'Toto' shallots were harvested in July, while the 'Jolant' leeks were harvested in August. In Poland, the period of intensive plant growth covers July and August (the period of acute sensitivity to water shortage). In 2018, the amount of rainfall in July was higher than in 2019. On the contrary, in August, the amount of rainfall in 2019 was higher than in 2018. In the cultivation of shallots in the short cultivation period, the amount of rainfall was of less importance compared to the season.

In this study, the average yield of the leek cultivar 'Jolant' was 31.4 Mg ha$^{-1}$, and the yield of the shallot bulbs of the 'Toto' cultivar was 23.2 Mg ha$^{-1}$. In the study by Golubkina et al. [43], the yields of the fresh leek mass ranged from 23.8 to 40.2 Mg ha$^{-1}$. The total yield of the shallot bulbs of the cultivar 'Toto' was similar to that described for this cultivar by Tendaj et al. [2], at 22.7 Mg ha$^{-1}$.

These studies show that the co-cultivation of leeks and shallots with Alexandrian clover does not reduce the marketable yield. The average yield of leeks with clover was 38.32 Mg ha$^{-1}$ ha, which was higher by 36% compared to the cultivation without LM. The yield of shallots was 26.24 Mg ha$^{-1}$, which was higher by 23%. The unit weight of the leek pseudo-stems in the cultivation with clover was 249 g and was higher by 24%, while the weight of 100 shallot bulbs was 8.04 kg and was 20% higher compared to the cultivation without mulch. Thus, the use of living mulch led to many positive responses in the plants, as shown above. The leek and shallot species of the genus *Allium* are cultivated in wide inter-row spaces and are perfect for growing with LM. Thus, it was confirmed that one of the factors determining the success of the cultivation is the choice of the sown species [8]. According to Campanelli and Canali [34], plants intended for use as living mulch should have a low demand for nitrogen, so that they do not compete with the crop. Several previous studies have shown that *Trifolium repens*, *Ornithopus sativus*, and *Tagetes patula* plants can work well in co-cultivation with leeks [31,32,44]. Some results indicate that living mulch from *Isatis tinctora* plants placed in a belt system can increase the leek yield by 112% compared to cultivation without a living mulch [27]. Trinchera et al. [45] explained that clover plants in co-cultivation with other species positively affect the activity, while mycorrhiza colonization guarantees the plant an adequate supply of nutrients.

In our experiment, the most competitive approach for leeks and shallots was the use of *Trifolium aleksandrinum* sowed three weeks before planting, which resulted in yield reductions for both the leeks and shallots. At this clover sowing time, a smaller diameter and unit weight of the leek pseudo-stems and a lower average weight for 100 shallots were recorded. The interaction between the sowing date and the cultivation season for the yield of the above-ground part of the season is noteworthy. With low rainfall in June and July of 2019, a higher yield was harvested in the clover crop, but only when sown in the third term (3 weeks after planting).

### *4.2. The Biological Value of Leek Pseudostems and Shallot Bulbs*

Sowing clover in the 2nd and 3rd terms (at the time of vegetable crop planting and three weeks after planting, respectively) ensured more dry weight in the leeks and shallots than in the 1st term. Similarly, in the cultivation of eggplants, tomatoes, and peppers, changing the LM sowing date from perennial ryegrass caused an increase in dry weight [31–33]. It can be suggested that the appropriate selection of the LM species and sowing date is crucial in influencing the dry weight of the edible parts of plants.

Several studies have showed that the cultivation of vegetables with LM may cause an increase in TP content values in the edible parts of onion [28], cauliflower [46], pumpkin [47], cabbage [48] plants. In this study, the TP content in the edible parts of the leeks in LM cultivation was 655 mg GAE 100 g$^{-1}$ DW, and without mulch it was 616 mg GAE

100 g$^{-1}$ DW. On the other hand, in comparison to leeks, the TP content in shallot onions was lower in cultivation with live mulch, amounting to 137 mg GAE 100 g$^{-1}$ DW, while it amounted to 129 mg GAE 100 g$^{-1}$ DW in the monoculture (no significant differences). *A. ampeloprasum* is considered a potential source of TP and was compared favorably with the amount reported by Golubkina et al. [43] (284–555 mg GAE 100 g$^{-1}$ DW). A two-fold higher level of TP (on average, 14 mg GAE g$^{-1}$ DW) was found in 30 varieties of *A. ampeloprasum* [49]. In the fresh leek plants, the TP content reported by García-Herrera et al. [50] was 5.70 mg GAE g$^{-1}$ FW, while Proteggente et al. [51] reported 22 mg GAE 100 g$^{-1}$ FW. The content of polyphenols in shallots in the fleshy scales of the bulbs is 40.8–43.2 mg 100 g$^{-1}$ FW, and in dry scales it is higher, amounting to 1670–1840 mg 100 g$^{-1}$ FW [2]. The higher TP content is characteristic of the leaves more than the bulb extracts [52].

The results of these studies show that there was a more significant total level of phenolic compounds in the leeks than in the shallots. The level of phenolic compounds in plants is influenced by many factors [30], so it is difficult to interpret the results. It should be assumed that the more extended period of leek cultivation than for the shallots (by one month) favored the accumulation of more phenols in the leek plants. Lisiewska and Kmiecik [52] reported that the phenolic content is determined, to a high degree, by environmental conditions, while the temperature remains the main stress factor. Variations in the phytochemical levels of *Allium* can be caused by many factors, such as genotypic differences, growing conditions, and agricultural practices [30].

In our research, shortening the vegetation period of Alexandrian clover by 3 and 6 weeks, thereby reducing the produced biomass at the beginning of leek and shallot vegetation, increased the TP content. These results, in line with the previous results, indicate that when LM is sown too early, it excessively competes with the main crops for nutrients, water, space, and sunlight, and consequently may reduce the TP level and the biological value of the crop [31,32,53]. Several studies have shown a correlation between the increase in TP levels and exposure to UV-B radiation in barley [54] and *Arabidopsis* [55]. Plants produce UV-B-absorbing phenolic compounds that accumulate on the leaf surfaces [56]. Additionally, PAR may increase the content of polyphenols [57]. In the field cultivation of *A. cepa*, it was observed that plants accumulate more TP compounds in years with higher solar radiation [58]. In our studies, delaying the LM sowing time in leek and shallot plants limited the intensity of the competition for light, favoring the biosynthesis of phenols.

Studies that are more recent indicate that delaying the sowing or planting of living mulch is a reliable method to limit competition with the main crop [20,23]. Alternatively, competition between the living mulch and the main crop can be reduced by interrupting the companion plant's growth. For example, trimming barley to a height of 18 cm in the cultivation of onions reduces the competition from the live barley mulch [59].

The role of environmental factors is emphasized in shaping the level of secondary metabolites; according to Bibi et al. [60], the level of TP increases or decreases in response to environmental stimuli. In this context, the results of our research allowed us to establish that the applied LM had a positive effect, with increases in TP, ferulic acid, and chlorogenic acid in leek plants. The living mulch may have limited the heating of the soil in various ways, thereby reducing evaporation and promoting water absorption and infiltration. It was noticed that in the cultivation of leeks and shallots, the level of TP was highly influenced by the weather factor. In 2019, with a lower amount of rainfall, more ferulic acid and caffeic acid was found in the leeks, and more *p*-coumaric acid, chlorogenic acid, and quercetin was found in the shallots. In general, the differences in the chemical compositions of the leeks and shallots, as in other plant tissues, are influenced by many factors, such as the temperature, rainfall, sun exposure, soil type, growth phase, and interactions of different plants or animals in the ecosystem [50].

The effectiveness of polyphenolic compounds depends mainly on their molecular weight, structure, and degree of oxidation. The activity of phenolic acids increases significantly if they contain two ortho hydroxyl groups in the molecule. An example of a compound with high antioxidant activity is caffeic acid. In our study, growing leeks with clover sown three weeks after planting increased the level of caffeic acid and decreased the chlorogenic acid. It is worth noting that during leek cultivation, at each clover sowing time, the content of caffeic acid was higher than in the cultivation without living mulch. Contrary to the cultivation of shallots, the date of clover planting did not affect the level of caffeic acid in the bulbs of this species.

### *4.3. Antioxidant Value*

The direct mechanisms of phenolic compounds' antioxidant activity mainly consist of capturing or scavenging free oxygen radicals and inhibiting lipid peroxidation. The multidirectional action of phenolic compounds as antioxidants makes it challenging to estimate the antioxidant potential of the plant extracts. In our research, we chose to extinguish DPPH and FRAP free radicals. This approach relies on electron transfer to determine the antioxidant capacity [61].

In the leek cultivation, more TP compounds were determined with clover cultivation, which was accompanied by higher antioxidant activity (AA) as determined only by the FRAP test. The LM sowing date and growing season did not affect the AA. This difference between the DPPH and FRAP values is in line with previously published data from Bernaert et al. [49]. It is related to the fact that the antioxidant activity of phenolic compounds also depends on their chemical structure and the different evaluation methods used.

Moreover, our results showed that in the cultivation of shallots, clover plants sown at the time of planting seedlings and three weeks after planting increased the quercetin level in the bulbs and the AA (FRAP) value. It is known that the activity levels of TP and the AA levels in plants vary with growth conditions [62] and with the methods of extracting compounds [63]. The higher TP values in the *Allium* methanol extracts were correlated with higher in vitro radical scavenging capacity and the more robust inhibition of tumor cell proliferation [5]. Similarly, in our study, the antioxidant effect was more substantial as the quercetin level in shallot increased. There was no difference between the clover sowing dates in terms of the DPPH scavenging activity of the shallot bulb extracts.

## 5. Conclusions

Growing leeks and shallots in wide rows creates the risk of leaching nutrients from the soil and erosion. Using clover living mulch (LM) for coordinated cultivation positively affected the yield and quality of leek and shallot crops. During cultivation with LM, the leeks and shallots were characterized by higher dry weights and higher total phenolic (TP) compound contents. The extracts showed more significant antioxidant activity (AA) than those cultivated without mulch. Crop growth with Alexandrian clover mulch contributed to higher levels of ferulic acid and chlorogenic acid in the leek pseudo-stems and higher levels of quercetin in the shallot bulbs. In the cultivation of leeks with clover, higher AA in the extracts was also found using the FRAP test and the DPPH test. The cultivation of shallots with clover did not significantly affect the AA value of the bulbs. The levels of bioactive compounds depended on the date of clover sowing. The most advantageous sowing dates for the clover plants were when the leeks were planted three weeks after planting the seedlings and at the time of shallot planting or three weeks after planting.

To sum up, the LM from Alexandrian clover used in cultivation with leeks and shallots can optimize their growth, positively influencing the yield and biological value of the plants. Such a cultivation system also increases the agri-biodiversity of the field. These studies complement the existing knowledge and suggest that cultivation in an LM system is very demanding, because sometimes the benefits are not directly visible and difficult to quantify. Therefore, there is a need for further research; however, in our opinion, living mulches may increase the yield and enhance the biological value of vegetable crops.

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H.M.-R.; supervision, A.S.; project administration, R.N.-W.; funding acquisition, A.S. and A.K. All authors have read and agreed to the published version of the manuscript.

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