

Proceeding Paper

The Effects of Fertilization Regime on Growth Parameters and Bioactive Properties of Pot Grown *Cichorium spinosum* L. Plants [†]

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Abstract: *Cichorium spinosum* L. is a wild edible species found mostly in coastal areas of the Mediterranean basin. In this experiment, seven fertilization treatments varying in the amounts of N:P:K were applied via nutrient solution feeding in pot-grown *C. spinosum* plants, namely 100:100:100 (C111), 200:100:100 (C211), 200:200:200 (C222), 300:100:100 (C311), 300:200:200 (C322) and 300:300:300 (C333) ppm ratio of N:P:K and control where no fertilizers were added (C0). The growth parameters tested included the number and the fresh and dry weight of leaves, SPAD index, leaf area index (LAI) and specific leaf area (SLA). Bioactive parameters, including the antioxidant (OxHLIA and TBARS assays), anti-inflammatory (RAW 264.7 cell line) and cytotoxic (PLP2, AGS, CaCo2, VERO and MCF7 cell lines) were examined in aqueous and hydroethanolic extracts of leaves. Fertilizer treatments benefited the growth parameters of spiny chicory, while the highest antioxidant activity was recorded for the C222 and C311 treatments. The application of fertilizers on *C. spinosum* plants had positive effects on plant growth, chemical composition and bioactive properties.

Keywords: spiny chicory; wild edible plants; chemical fertilizers; healthy food; bioactive compounds

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1. Introduction

Cichorium spinosum L. is a wild edible species, commonly known as stamnagkathi found in many parts of Greece, especially in Crete island, as well as in other Mediterranean countries. It is a plant that presents a wide adaptability and it can be grown even at coastal areas with low soil fertility and exhibits considerable tolerance to salt stress [1]. Nowadays, the ever-growing interest for the consumption of healthy foods and the ongoing climate crisis has created a market niche for high added value products that ensure high use efficiency or natural resources [2]. Wild edible plants could be a promising prospect for the production of functional healthy foods such as spiny chicory which presents a high content in vitamins E and K1, antioxidants compounds, ω -3 fatty acids and several mineral elements [3,4]. Fertilizers have been an important factor for the farmers to increase yield and farmer's income by restraining yield losses and enhancing the quality of the final product. Despite the minimum agronomic inputs of wild edible plants when cultivated under commercial cultivation conditions, growing and environmental conditions

and plant nutrition could significantly affect the growth parameters, yield, chemical composition and bioactive properties of plants [5]. As previously reported in the study of Petropoulos et al. [4], the antioxidant properties of commercially grown *Cichorium spinosum* were significantly affected by the different fertilizer treatments. Therefore, the ongoing climate crisis and land degradation enforce the necessity for the design and establishment of novel farming applications in terms of sustainability. In this context, the Mediterranean basin is an admirable hotspot of wild edible plants with a remarkable variation, which according to several authors they show a great potential to be integrated in cropping systems as complementary crops. Considering the above, the aim of the current study was to evaluate the effects of fertilization regime on growth parameters and bioactive properties of pot grown *C. spinosum* plants and further explore the potential of commercially grow these plants.

2. Material and Methods

2.1. Plant Material, Experimental Treatments and Growing Condition

The present research was carried out at the experimental farm of the University of Thessaly in Velestino, Greece. Seeds of *Cichorium spinosum* were sown in seed trays filled with peat and after emergence young seedlings were transplanted in 2 L plastic pots containing peat (Klassman-Deilmann KTS2, Geeste, Germany) and perlite (1:1, v/v) when they reached the stage of 3–4 true leaves on 12 March 2021. Firstly, cultivation took place in an unheated glasshouse at the experimental farm of University and later on the pots were transferred outside due to the rising temperatures within the glasshouse. For the conduction of the experiment, seven treatments were used which varied in the amounts of N:P:K (Atlas 20-20-20 + TE; Gavriel; S.A., Volos, Greece), namely 100:100:100 (C111), 200:100:100 (C211), 200:200:200 (C222), 300:100:100 (C311), 300:200:200 (C322) and 300:300:300 (C333) ppm ratio of N:P:K) and the control treatment where no fertilizers were added (C0). All treatments were applied via nutrient solution in the *C. spinosum* plants. Each treatment included fifteen pots (n = 15) and there were used 105 pots in total, according to the Completely Randomized Design (CRD). All the treatments received the same amount of nutrient solution in which the plants were fertigated manually once a week comprising of 150 mL of N-P-K per plastic plot. Before harvest, it was recorded the chlorophyll content of leaves (SPAD values). Harvest took place on 26th of April when the rosette reached the marketable size in order to examine the effect of different fertilization regimes on crop performance. The recorded traits included the number of leaves/plant, weight of leaves/plant (g), dry matter of leaves (%), leaf area (cm²) and specific leaf area (m²/kg). For dry weight evaluation, samples of fresh leaves were oven dried at 72 °C to a constant weight. Fresh samples of leaves were lyophilized (Sublimator model EKS, Christian Zirbus Co., Germany), reduced to a fine powder (~20 mesh) using a domestic grinder, and stored in an air-sealed bag under protection from the light in a deep freezer (−80 °C) until further analysis.

2.2. Antioxidant Activity

The antioxidant activity was determined by applying two-cell based assays: the oxidative haemolysis (OxHLIA) assay evaluated for Δt for 60 min and the thiobarbituric acid reactive substances (TBARS) formation inhibition assays as previously mentioned by Mandim et al. [6,7]. Trolox was the positive control.

2.3. Anti-Inflammatory Activity

The anti-inflammatory activity was evaluated through the determination of the extracts capacity to inhibit the lipopolysaccharide (LPS)-induced nitric oxide (NO) production by a murine macrophage cell line (RAW 264.7) and using the Griess Reagent System Kit (Promega, Madison, WI, USA) as previously described by Silva et al. [8]. Results were

expressed as the extract concentration responsible for 50% of NO production inhibition (IC_{50} , $\mu\text{g/mL}$).

2.4. Hepatotoxicity and Cytotoxicity Assays

Leaf extracts were dissolved in water and diluted by successive dilutions to obtain the range of concentrations (0.125–8 mg/mL) to be tested. The different extract concentrations were incubated with the tested cell lines (190 μL , 10,000 cells/mL), being the range of final concentrations tested between 6.25 and 400 $\mu\text{g/mL}$. Four tumour cell lines were used: cervical carcinoma—HeLa, breast carcinoma—MCF-7, hepatocellular carcinoma—HepG2, and non-small cell lung cancer (NCI-H460). The hepatotoxic potential was evaluated using a non-tumour porcine liver primary culture (PLP2) [6]. The cytotoxic and hepatotoxic potentials were evaluated using the sulforhodamine B assay [9]. Results were expressed as the extract concentration responsible for 50% of cell growth inhibition (GI_{50} , $\mu\text{g/mL}$).

2.5. Statistical Analysis

The growth parameters were determined in 15 plants per treatment ($n = 15$), whereas the harvested material from each treatment was used to prepare three batch samples ($n = 3$) for the described chemical analyses. Chemical composition and bioactive properties analyses were performed in three different samples for each treatment and all the analyses were performed in triplicate ($n = 3$). Statistical analysis was carried out with JMP v. 16.1 (SAS Institute Inc.). Before the conduction of the statistical analysis, all data were examined for normal distribution according to the Shapiro-Wilk test. The results of the study are expressed as mean values and standard deviations (SD). Data were analyzed using the one-way analysis of variance (ANOVA), while means were compared using the Tukey HSD-test at $p = 0.05$.

3. Results and Discussion

The results regarding the effect of fertilization regime on growth parameters of *C. spinosum* plants are presented in Table 1. The application of fertilizers on spiny chicory plants had a varied effect on the number of leaves/plant. Particularly, the highest number of leaves/plant (30.36) was recorded for the C322 treatment, while the lowest number of leaves/plant (24.21) was achieved by C211 treatment. Equally, there were recorded significant differences for the weight of leaves/plant where the highest fresh weight of leaves (12.90 g) was recorded for C211 fertilizer treatment and the lowest one (9.91 g) for the C222 treatment. On the other hand, the application of fertilizers had a significant impact on dry matter of leaves, with control showing the highest (8.27%) and C311 the lowest (5.56%) dry matter content. Based on the SPAD index results, C0 (94.81) and C222 (98.14) treatments presented the highest chlorophyll content, whereas the C311 (62.10) treatment recorded the lowest content respectively. Moreover, the tested fertilization regimes had a significant effect on the leaf area index and specific leaf area index on the plants of *C. spinosum*. Briefly, the C211 fertilization regime was noted for the highest leaf area index (324.75 cm^2) and C311 achieved the lowest index (250.42 cm^2), while C311 (39.20 m^2/kg) and C0 (27.09 m^2/kg) treatments presented the highest and lowest specific leaf area, respectively.

The results of the study are in accordance with the findings of Petropoulos et al. [10] who reported similar values regarding the number of leaves and dry matter of leaves, whereas he recorded a slightly increased fresh weight in comparison to our study which could be attributed to the different growing and harvesting period and cultivation practices. According to the findings of the study, the C211 treatment affected positively the fresh weight by achieving the highest yield, while the increased ratio of N:P:K didn't present any significant impact on fresh weight of *C. spinosum* indicating that spiny chicory is a wild edible species with relatively low nutrient demands. Similar trends have also been

confirmed by Petropoulos et al. [4] who reported that higher contents of ammonium nitrogen in the nutrient solution had no beneficial effect related to the fresh weight. Equally, Chatzigianni et al. [5] observed that the increasing amounts of ammonium nitrogen in nutrient solution had no differential responded to *C. spinosum* implying a tolerance of the species in high amounts of ammonium. Therefore, based on the literature reports, the low requirements for nutrition required by spiny chicory could make it a noteworthy prospect for the commercial cultivation of the species.

Table 1. The effect of fertilization regimes on number of leaves/plant, weight of leaves/plant (g), dry matter of leaves (%), chlorophyll content of leaves (SPAD Index), leaf area index (m²) and specific leaf area index (m²/kg) of *C. spinosum*.

Treatments	Traits					
	Number of Leaves/Plant	Weight of Leaves/Plant (g)	Dry Matter of Leaves (%)	Chlorophyll Content (SPAD Index)	Leaf Area Index (cm ²)	Specific Leaf Area (m ² /kg)
C0	29.54 ± 1.35 a	11.49 ± 0.97 c	8.27 ± 2.16 a	94.81 ± 12.21 (a)	297.12 ± 8.50 (b)	27.09 ± 1.69 (e)
C111	29.14 ± 1.13 a	11.59 ± 1.22 c	6.55 ± 1.02 e	82.82 ± 7.79 (bc)	282.82 ± 7.86 (c)	31.17 ± 1.71 (bc)
C211	24.21 ± 1.38 c	12.90 ± 1.35 a	6.69 ± 0.09 d	74.19 ± 6.61 (c)	324.75 ± 8.57 (a)	28.20 ± 1.73 (e)
C222	27.33 ± 0.73 b	9.91 ± 1.12 e	6.08 ± 1.24 e	98.14 ± 13.10 (a)	260.23 ± 11.39 (d)	37.61 ± 1.98 (a)
C311	27.29 ± 1.27 b	12.02 ± 1.69 b	5.56 ± 2.55 f	62.10 ± 7.00 (d)	250.42 ± 6.76 (d0)	39.20 ± 1.45 (a)
C322	30.36 ± 1.73 a	11.54 ± 1.26 c	7.93 ± 1.05 b	87.89 ± 7.24 (ab)	278.37 ± 8.28 (c)	30.82 ± 1.64 (cd)
C333	29.73 ± 1.30 a	10.85 ± 0.77 d	6.92 ± 2.57 c	76.08 ± 7.79 (c)	255.81 ± 7.99 (d)	29.78 ± 1.46 (d)

* Means in the same column followed by different Latin letters are significantly different at $p < 0.05$, according to Tukey's HSD test.

The results of antioxidant and anti-inflammatory activity, hepatotoxicity and cytotoxicity are presented in Table 2. Regarding the OxHLIA assay results of hydroethanolic extracts, the highest antioxidant activity was recorded by C111 (53 µg/mL), followed by C222 (61 µg/mL) and C311 (65 µg/mL) treatments, whereas for the TBARS assay the treatment C311 (151 µg/mL) had the highest antioxidant activity. Similarly, regarding the aqueous extract, the highest antioxidant activity was recorded by C222 (20 µg/mL), followed by C221 (25 µg/mL) treatment in the case of OxHLIA assay, while for the TBARS assay the C222 (116 µg/mL) treatment was noted for the highest antioxidant activity. These results are in coincidence with the findings of previous studies [3,10,11] which reported varied antioxidant efficacy based on the EC₅₀ values of TBARS and OxHLIA assays. The high antioxidant activity recorded on the current research could be attributed to high total tocopherols and phenolic acids content due to the fact that the bioactive compounds content of the wild edible species is positively correlated with antioxidant activity [12]. Moreover, the application of different fertilizations regimes showed diversified results regarding the antioxidant activity; however, these discrepancies are in agreement with previous studies [5,13,14] where the effect of production cycle, genotype, fertilization treatments and growing systems was highlighted. Based on the current findings of this study, we could conclude that the fertilizer treatments with an intermediate ratio of N:P:K such as C111, C222 and C311 for both extracts had the highest antioxidant activity in comparison to the control treatment and the maximum ratio of N:P:K namely C322 and C333. Similar results have also been recorded by Cruz et al. [15] who reported that the lowest nitrogen application of 200 ppm produced the highest antioxidant activity of Green and Red-Colored Basil cultivars in comparison to the increased nitrogen application of 400 and 600 ppm. Finally, at the maximum studied concentration (400 mg/mL) the *C. spinosum* samples did not present cytotoxic, hepatotoxic and anti-inflammatory activity (data not shown) in relation to the different fertilization regimes studied, results which are in full agreement with the findings of Petropoulos et al. [14] who also reported that the nitrogen source had no effect on the cytotoxicity and hepatotoxicity activity of *C. spinosum* leaves.

Likewise, Petropoulos et al. [16] have reported that none of *Centaurea raphanina* samples showed toxicity against non-tumor cell lines (porcine liver primary cultures; PLP2) and tumor cell lines (breast carcinoma; MC7) with an exception of the 600 ppm nitrogen application rate who recorded a mild toxicity effect. On the contrary, Petropoulos et al. [17] observed a mild toxicity against tumor cell-lines (HeLa, HepG2, MCF-7 and NCI-H460) and no toxicity against non-tumor cell lines apart from the control treatment. These discrepancies recorded between the wild edible plants, according to the literature reports could be attributed to the growing conditions and cultivations practices, while many authors have highlighted that extraction protocol could affect the phytochemicals composition of the obtained extracts [17–20]. To best of our knowledge, further studies are required in order to find out the optimum balance between fertilization regime and bioactive properties of *C. spinosum*.

Table 2. Antioxidant (IC₅₀ values µg/mL) activity of the hydroethanolic extracts (HE) and aqueous extracts (AE) of *C. spinosum* (Mean ± SD).

Bioactive Properties	Extracts	Traits							
		C0	C111	C211	C222	C311	C322	C333	
Antioxidant activity ^A	OxHLIA	HE	322 ± 20 b	53 ± 3 g	339 ± 18 a	61 ± 2 f	65 ± 2 e	103 ± 4 d	123 ± 7 c
		AE	131 ± 5 c	97 ± 5 d	25 ± 2 e	20 ± 1 f	207 ± 12 b	278 ± 9 a	207 ± 13 b
	TBARS	HE	479 ± 9 b	411 ± 15 c	408 ± 6 c	363 ± 16 d	151 ± 6 e	465 ± 15 b	547 ± 27 a
		AE	357 ± 11 a	143 ± 2 e	167 ± 6 c	116 ± 5 f	225 ± 8 b	163 ± 8 cd	159 ± 7 d
Anti-inflammatory activity ^B	RAW 264,7	HE	>400	>400	>400	>400	>400	>400	>400
		AE	>400	>400	>400	>400	>400	>400	>400
Hepatotoxicity ^C	PLP2	HE	>400	>400	>400	>400	>400	>400	>400
		AE	>400	>400	>400	>400	>400	>400	>400
Cytotoxicity Activity ^C	AGS	HE	>400	>400	>400	>400	>400	>400	>400
		AE	>400	>400	>400	>400	>400	>400	>400
	CaCo2	HE	>400	>400	>400	>400	>400	>400	>400
		AE	>400	>400	>400	>400	>400	>400	>400
	VERO	HE	>400	>400	>400	>400	>400	>400	>400
		AE	>400	>400	>400	>400	>400	>400	>400
	MCF7	HE	>400	>400	>400	>400	>400	>400	>400
		AE	>400	>400	>400	>400	>400	>400	>400

^A Trolox IC₅₀ values: 5.8 ± 0.6 µg/mL (TBARS), 21.8 ± 0.3 µg/mL (OxHLIA 60 min); ^B Dexametaxone IC₅₀ value: 6.3 ± 0.4 µg/mL; ^C Ellipticine GI₅₀ values: 1.4 ± 0.1 µg/mL (PLP2), 1.23 ± 0.03 µg/mL (AGS), 1.21 ± 0.02 µg/mL (CaCo2), 1.41 ± 0.06 µg/mL (VERO) and 1.02 ± 0.02 µg/mL (MCF-7).

4. Conclusions

The results of the current study provide valuable information related to the response of *C. spinosum* plants to the different fertilization regimes and bioactive properties, which could be further exploited by farmers within the context of integrating the commercial cultivation of spine chicory as a complementary/alternative crop. Fertilization regime affected the growth parameters of spiny chicory, especially the C211 treatment which significantly increased plant fresh weight, while the highest anti-oxidant activity was recorded for the C222 and C311 treatments. In contrast, cytotoxicity, hepatotoxicity and anti-inflammatory properties were not affected by the fertilization regime. In conclusion, commercial cultivation of wild edible species is a promising cropping alternative under the climate change conditions; however, further research is demanded in terms of evaluating and standardizing the cultivation protocols in order to establish the commercial cultivation of such species.

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data curation, N.P., B.P. and M.I.D.; writing—original draft preparation, N.P., B.P., M.C. and M.I.D.; writing—review and editing, L.B. and S.A.P.; visualization, S.A.P.; supervision, L.B. and S.A.P.; project administration, L.B. and S.A.P.; funding acquisition, S.A.P. All authors have read and agreed to the published version of the manuscript.

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