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Integrated Nutrient Management Boosts Inflorescence Biomass and Antioxidant Profile of *Carlina diae* (Asteraceae)—An Endangered Local Endemic Plant of Crete with Medicinal and Ornamental Value

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Abstract: Due to combined climate and biodiversity crisis, the sustainable utilization of phytogenetic resources stands as one-way alternative while nutrient management strategies gain an increasing role in agriculture. Building on previous studies regarding the Endangered local endemic of Crete (Greece) *Carlina diae* (Asteraceae) with medicinal and ornamental value, this investigation focused on its pilot cultivation and fertilization (foliar or root application). Foliar application included chemical fertilizer (conventional) or integrated nutrient management (INM). Root application consisted of chemical fertilizer, biostimulant, or INM with biostimulant. Above-ground biomass content of nutrients, together with leaf chlorophyll fluorescence and colour parameters (SPAD meter, DA meter, Chroma Meter) were determined. The leaf content of chlorophyll, three key antioxidant compounds and minerals' titers were also assessed. The fertilization scheme did not influence plant growth and visually perceived quality (leaf color and shape). Notably, foliar INM fertilization increased biomass partitioning to inflorescences (harvestable organ for either medicinal or ornamental purposes), and decreased tissue water content (facilitating processing). Considering all three antioxidants together, INM with biostimulant appeared the optimum scheme, being associated with the highest (carotenoids, phenolics) or the second highest (flavonoid) content. In *C. diae*, therefore, INM fertilization was optimal for upgrading yield (foliar) and herbal quality in terms of antioxidant profile (INM with biostimulant), which might be embraced as an eco-friendly approach for high quality yields.

Keywords: INM; enhanced efficiency fertilizers; carotenoids; flavonoids; Greece; herbal quality; ornamental value; medicinal plant; phenols; biostimulant

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1. Introduction

In traditional and complementary (alternative) medicine, plant materials are extensively employed for healing purposes while a wide range of conventional (pharmaceutical) drugs derive from wild plants and a large part of human population rely on wild plants for remedies [1,2]. Furthermore, an increasing demand for a broad variety of natural products is also noted in perfumery, cosmetics, and food industry [3,4] coupled with intense quests globally for new ornamental plants originating from rich biodiversity hotspots [5].

Due to limited public awareness and ineffective control mechanisms combined with ever-increasing commercial demand and modern consumerism trends, a substantial segment of the traded plant material globally still originates from wild phytogenetic resources, thus depleting wild-growing populations [6]. To satisfy the multi-sectorial growing demand for novel products without further exhausting natural resources, recruiting focal neglected and underutilized species of economic interest to introduce them into cultivation systems, stands out as promising alternative and sustainable strategy for the development of new crops [5]. To be operative such an endeavor, a well-documented initial plant material should be employed coupled with extant propagation protocols and cultivation guidelines applied in well-managed cultivation settings. From this perspective, fertilization schemes tailor-made to the species in concern ought to be established and applied [7,8].

The key challenge for agriculture is to increase crop yield and quality in a sustainable way for present and future. To support optimal plant growth, nutrients are conventionally added to the soil of any crop by means of chemical (inorganic) fertilizers. However, their over-application or insufficient use has been associated with a range of negative effects on the environment [9]. Major offsets include soil quality degradation, and nutrient leaching [10]. These nutrients, which are lost from agricultural lands, contaminate water bodies globally, and, in this way, adversely affect aquatic systems and biodiversity [9,11]. Accumulative evidence suggests that the respective environmental impact is comparatively limited when alternative fertilization schemes are employed instead [10], such as biostimulants which may trigger root nutrient uptake [12], or integrated nutrient management (INM) potentially promoting plant growth and productivity with a low environmental footprint [13]. Generally, it is well established that fertilization schemes determine not only plant growth and productivity, but also quality aspects of the cultivated plant material [10,14,15]. An increasing number of studies indicates that application of organic fertilizers and INM strategies may upgrade the antioxidant level of medicinal plant material, as compared to chemical fertilizers [12,16–18]. Organic fertilizers and INM schemes have also been previously related to increased chlorophyll content [19], or to the intensity and homogeneity of leaf greenness [20] or plants of medicinal interest [7,8,21]. Antioxidants are important sets of compounds that deactivate free radicals and reactive oxygen species within cells [23]. The plant antioxidant profiles represent one of the utmost attention-grabbing parameters in agriculture. As free radicals play a central role in the development of various chronic diseases, among others, cardiovascular syndromes, aging, heart diseases, anemias, cancers and inflammations, the plant antioxidants used in nutrition/pharmacology offer strong defense against injuries triggered by these free radicals [24].

Species-wise, this study is focused on *Carlina diae* (Rech. f.) Meusel and A. Kástner (Asteraceae) due to its ornamental value [5] and medicinal interest [21], its uniqueness as ancient, Tertiary relict, range-restricted species (local endemic of Dia Island off-shore from Crete Island) coupled with contemporary rarity and Endangered IUCN (International Union for the Conservation of Nature) status due to overgrazing of its small-sized wild-growing populations under protection (covered by Appendix I of the Bern Convention and the Greek Presidential Decree 67/1981) [22]. Context-wise, this investigation builds on previous studies exploring its sustainable exploitation potential in the ornamental sector [5] and the medicinal sector, as well as on the development of effective species-specific propagation protocols for integrated conservation actions [22]. Herein, this combined field and laboratory study deals with the establishment of a pilot cultivation of *C. diae* in Crete and aimed to the development of a species-specific fertilization protocol promoting optimally, not only plant growth, but also key herbal quality aspects, such as antioxidant metabolite levels.

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2. Materials and Methods

2.1. Origin of Plant Material

The authorized collection data regarding the focal *C. diae* GR-1-BBGK-19,006 (Figure 1A,B) as well as the species-specific propagation protocols used to produce ex-situ enough plant individuals for field experimentation are reported in previous own study [22]. The plants used in the experimental procedure were transplanted in 2 L plastic pots by the company of AFI GLAVAKI KE SIA OE Tree & Plant Nurseries, Aridea, PELLAS, GR-58400, Greece.

Figure 1. Wild-growing individual of *Carlina diae* as a rock-dweller in Dia Island offshore from Crete, Greece (A), harvested inflorescences from the wild utilized as domestic dried ornamental (B) and cultivated plant individual of *C. diae* (C) in the pilot field experiment at the campus of the Hellenic Mediterranean University, Heraklion, Crete (D). Photos A and B: M. Avramakis, Natural History Museum of Crete (reproduced with permission).

2.2. Establishment of Field Experiment and Soil Analysis

The general description of the established field experiment (Figure 1C) in the campus of the Hellenic Mediterranean University, Heraklion, Crete (Greece) and the soil properties of the research field are reported in previous publications [7,8] and are provided briefly in Supplementary Materials Method S1.

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2.3. Fertilization Schemes

Details on the fertilization regime used in this experimentation are given briefly in Supplementary Materials Method S1 and are provided by previous own studies [7,8]. The fertilization treatments of the pilot cultivation of *C. diae* involved:

A. INM by foliar application (INM-fa): The nutrient solution consisted of THEORUN at 7 ml L^{-1}, THEOCAL at 1.5 g L^{-1}, THEOFAST at 5 ml L^{-1}, 10-47-10 (AGRI.FE.M. LTD Fertilizers, Greece) at 3.2 g L^{-1}, K_2SO_4 (0-0-52, AGRI.FE.M. LTD Fertilizers, Greece) at 2.07 g L^{-1}, micronutrients (Plex Mix, AGRI.FE.M. LTD Fertilizers, Greece) at 1.5 ml L^{-1} and $MgSO_4$ (Mg 25.6 %, AGRI.FE.M. LTD Fertilizers, Greece) at 0.6 g L^{-1}.

B. Chemical (inorganic) fertilization by foliar application (ChF-fa): The nutrient solution consisted of NH4NO3 (34,4-0-0, Neofert®, Neochim PLC, Bulgaria) at 2.7 g L^{-1}, Ca(NO3)2 (NITROCAL, Agrohimiki, Greece) at 1.7 g L^{-1}, 10-47-10 at 3.2 g L^{-1}, K_2SO_4 (0-0-52) at 2.27 g L^{-1}, micronutrients Plex Mix at 1.5 ml L^{-1} and $MgSO_4$ (Mg 25.6 %) at 0.6 g L^{-1}.

C. Control, with foliar and soil applications with tap water.

D. INM by soil application (INM-sa): The nutrient solution consisted of THEORUN at 7 ml L^{-1}, THEOCAL at 1.5 g L^{-1}, THEOMASS at 10 ml L^{-1}, 10-47-10 at 3.2 g L^{-1}, K_2SO_4 (0-0-52) at 2.1 g L^{-1}, micronutrients Plex Mix at 1.5 ml L^{-1} and $MgSO_4$ (Mg 25.6 %) at 0.3 g L^{-1}.

E. Chemical (inorganic) fertilization by soil application (ChF-sa): The nutrient solution was consisted of NH4NO3 (34,4-0-0) at 2.7 g L^{-1}, $Ca(NO_3)_2$ (NITROCAL) at 1.7 g L^{-1}, 10-47-10 at 3.2 g L^{-1}, K_2SO_4 (0-0-52) at 2.3 g L^{-1}, micronutrients Plex Mix at 1.5 ml L^{-1} and $MgSO_4$ (Mg 25.6 %) at 0.3 g L^{-1}.

F. Mixture of plant extracts as bioreactor by soil application (MPE-sa): The nutrient solution was consisted of THEOMASS at 10 ml L^{-1}.

2.4. Plant Measurements

Several plant and leaf level measurements were regularly conducted until May 25, 2021, ending with completion of flowering of *C. diae*. All sampled leaves were fully expanded, grown under direct light and devoid of visual symptoms of either pathogen infection or insect damage. In all instances, the time between sampling and the initiation of the evaluation was shorter than 15 min. When this was not feasible, samples were placed in vials, flash-frozen in liquid nitrogen and were transferred to a freezer (-80°C) for storage. Replicate leaves were sampled from separate plant individuals.

Details on the each of the following plant measurements are given briefly in Supplementary MaterialS Method S2 and are provided by previous own studies [7,8].

2.4.1. Non-Invasive Evaluation of Leaf Coloration in Growth Stages

In this study, leaf coloration was assessed by three different methods (for instruments used see Supplementary Materials Method S2 and [7,8]): (i) Leaf SPAD value, approximating chlorophyll content; (ii) The index of absorbance difference (IAD) computed as the difference between the absorbance values at 670 and 720 nm, near the chlorophyll absorbance peak; (iii) Quantified leaf colour by using a Chroma Meter. These measurements were in situ conducted in attached leaves of intact plant individuals at vegetative, early flowering, and full flowering stages. Three points were recorded per replicate leaf and were further averaged. Three replicate leaves were assessed per treatment.

2.4.2. Non-Invasive Evaluation of Photosynthetic Performance in Growth Stages

As a valid indicator of leaf photosynthetic performance [25], the ratio of variable to maximum chlorophyll fluorescence (Fv/Fm) was assessed (for instruments and conditions see Supplementary Materials Method S2 and [7,8]). These measurements were in situ conducted in dark-adapted attached leaves of intact plant individuals at vegetative, early flowering, and full flowering stages. Three points were recorded per replicate leaf and were further averaged. Three replicate leaves were assessed per treatment.

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2.4.3. Leaf Shape Indicators

A morphometric analysis was performed by analyzing leaf form in terms of aspect ratio, circularity, roundness, and solidity (for instruments, metrics and values see Supplementary Materials Method S2 and [7,8]). Thirty leaves (5 per plant individual × 6 plant individuals) were analyzed per treatment.

2.4.4. Plant Growth and Biomass Partitioning to Generative Organs

Above-ground plant and inflorescence (fresh and dry) masses were determined (± 0.01 g; MXX-412; Denver Instruments, Bohemia, NY, USA). For measuring dry weight, samples were placed in a forced-air drying oven for 72 h at 80°C. Six replicate plants were evaluated per treatment.

2.4.5. Leaf Chlorophyll and Carotenoid Contents

Chlorophyll content is important for leaf coloration and photosynthetic performance. Carotenoids are key non-enzymatic antioxidants [26]. The effect of growth conditions on chlorophyll and carotenoid contents was therefore assessed (see details in Supplementary Materials Method S2 and [7,8]). Three leaves were assessed per treatment. For each replicate, four samples collected from different plant individuals were pooled, and the assay was performed twice.

2.4.6. Leaf Total Phenolic and Total Flavonoid Contents

Phenols and flavonoids are critical non-enzymatic antioxidants [26,27]. The contents of total phenolic and total flavonoid were determined by using the Folin-Ciocalteu assay and aluminum chloride colorimetric assay, respectively (see details in Supplementary Materials Method S2 and [7,8]), following [27]. Three leaves were assessed per treatment. For each replicate, four samples from different plant individuals were pooled and the assay was performed twice.

2.4.7. Leaf Soluble Sugar Content

Since carbohydrate status is related to photosynthetic activity, the effect of fertilization scheme on carbohydrate status was assessed (see details in Supplementary Materials Method S2 and [7,8]). These measurements were conducted on three replicates per treatment. For each replicate, four samples from different plant individuals were pooled and the assay was performed twice.

2.4.8. Leaf and Inflorescence Mineral Analysis

To assess the role of fertilization regime on mineral uptake, leaf and inflorescence mineral analysis was conducted (see details in Supplementary Materials Method S2 and [7,8]). Three replicates were evaluated per treatment. For each replicate, four samples from different plant individuals were pooled and the assay was performed twice.

2.5. Statistical Analysis

Data analyses were carried out using the SAS statistical software (SAS Institute, Cary, NC, USA). Data were tested for homogeneity of variances (Duncan's test) and one way analysis of variance (ANOVA) was conducted. Subsequently, estimated least significant differences (LSD) of treatment effects were determined ($P = 0.05$).

3. Results

3.1. The Fertilization Scheme Exerted Minor Effects on Leaf Colour

At vegetative stage, foliar fertilization (INM-fa or ChF-fa) was associated with higher leaf SPAD value, as compared to INM-sa (Figure 2A). At early and full flowering stages, the fertilization scheme did not affect the leaf SPAD value (Figure 2B,C). At vegetative stage, chemical fertilization regardless of application method (ChF-fa or ChF-sa) was related to lower leaf IAD, as compared to the rest of

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the treatments (Supplementary Figure S1A). At early and full flowering stages, the fertilization scheme did not affect leaf IAD (Supplementary Figure S1B,C).

INM-fa resulted to higher leaf L value as compared to the control plants at vegetative stage, while it led to higher leaf L value as compared to INM-sa at full flowering stage (Supplementary Figure S2A,C). With these two minor exceptions, leaf L value was not affected by the fertilization scheme (Supplementary Figure S2). The fertilization scheme did not also affect leaf a and b values at all three growth stages (Supplementary Figures S3 and S4).

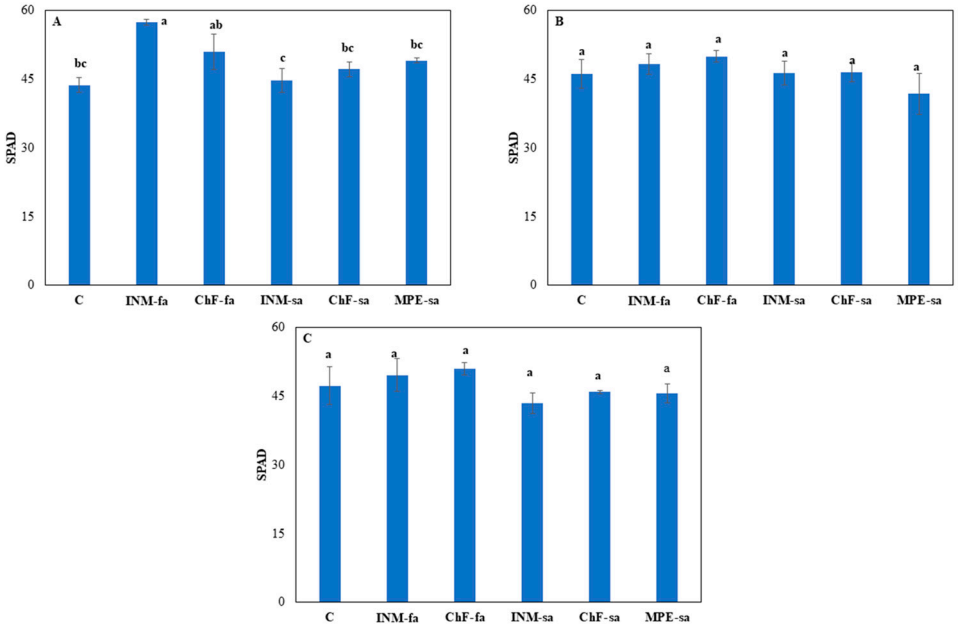
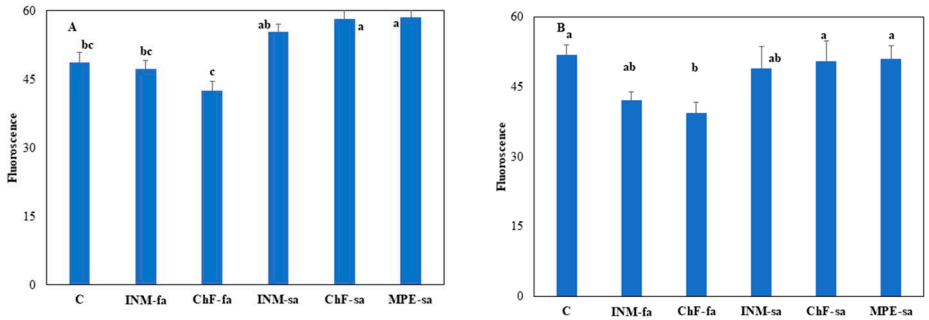


Figure 2. Effect of fertilization regime through different (root/foliar) application methods on leaf SPAD value of *Carlina diae* at vegetative (A), early flowering (B), and full flowering (C) stage. C: Control (water); INM-fa: Integrated nutrient management (INM) by foliar application; ChF-fa: Chemical fertilization by foliar application; INM-sa: INM by soil application; ChF-sa: Chemical fertilization by soil application; MPE-sa: Mixture of plant extracts as biostimulant by soil application (THEOMASS). Values represent the mean of three replicates ± SEM. Within each plot, different letters indicate significant differences.

3.2. *The Fertilization Scheme Induced Limited Effects on Leaf Photosynthetic Performance*

At vegetative stage, ChF-sa and MPE-sa resulted to higher leaf chlorophyll fluorescence value as compared to controls plants and plants receiving foliar fertilization (INM-fa or ChF-fa; Figure 3A). At early flowering stage, these two regimes (ChF-sa and MPE-sa) showed a significant difference when compared to ChF-fa (Figure 3B). Fertilization scheme did not affect leaf chlorophyll fluorescence value at full flowering stage (Figure 3C).



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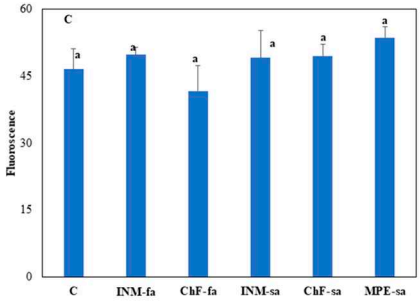

Figure 3. Effect of fertilization regime through different (root/foliar) application methods on chlorophyll fluorescence value of *Carlina diae* at vegetative (A), early flowering (B), and full flowering (C) stage. C: Control (water); INM-fa: Integrated nutrient management (INM) by foliar application; ChF-fa: Chemical fertilization by foliar application; INM-sa: INM by soil application; ChF-sa: Chemical fertilization by soil application; MPE-sa: Mixture of plant extracts as biostimulant by soil application (THEOMASS). Values represent the mean of three replicates ± SEM. Within each plot, different letters indicate significant differences.

3.3. Leaf Shape Was Generally Not Affected by the Fertilization Scheme

A morphometric analysis was conducted by determining four leaf shape factors (aspect ratio, circularity, roundness, solidity). Aspect ratio, roundness, and solidity were not affected by the fertilization scheme (Figure 4A,C,D). Plant cultivated under ChF-sa and MPE-sa had higher leaf circularity as compared to plants receiving foliar fertilization (INM-fa or ChF-fa; Figure 4B).

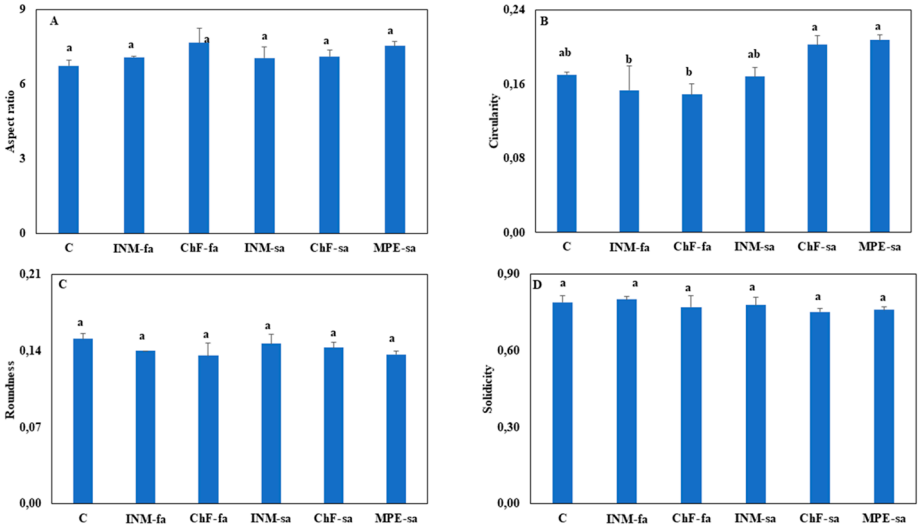

Figure 4. Effect of fertilization regime through different (root/foliar) application methods on four leaf shape factors of *Carlina diae*. C: Control (water); INM-fa: Integrated nutrient management (INM) by foliar application; ChF-fa: Chemical fertilization by foliar application; INM-sa: INM by soil application; ChF-sa: Chemical fertilization by soil application; MPE-sa: Mixture of plant extracts as biostimulant by soil application (THEOMASS). Values represent the mean of six replicates ± SEM. Within each plot, different letters indicate significant differences.

3.4. Fertilization Scheme Did Not Affect Plant Growth, though Foliar Fertilization Stimulated Biomass Allocation to Generative Organs

Above-ground plant biomass was not affected by the fertilization scheme (Figure 5A). INM by either application method (INM-fa, INM-sa) was associated with lower water content, as compared to controls (Figure 5B). Plants receiving foliar fertilization (INM-fa or ChF-fa) had increased biomass partitioning to the inflorescences as compared to controls, while plants cultivated under INM-fa had the highest partitioning to the inflorescences as compared to the remaining regimes (Figure 5C).

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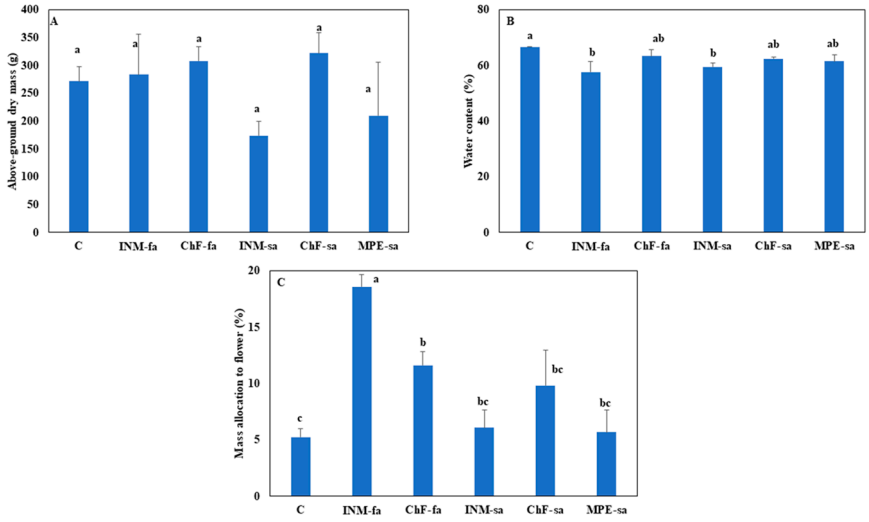

Figure 5. Effect of fertilization regime through different (root/foliar) application methods on above-ground dry weight (A), water content (B) and dry weight partitioning to inflorescences (C) of *Carlina diae*. C: Control (water); INM-fa: Integrated nutrient management (INM) by foliar application; ChF-fa: Chemical fertilization by foliar application; INM-sa: INM by soil application; ChF-sa: Chemical fertilization by soil application; MPE-sa: Mixture of plant extracts as biostimulant by soil application (THEOMASS). Values represent the mean of six replicates ± SEM. Within each plot, different letters indicate significant differences.

3.5. Fertilization Generally Improved Leaf Chlorophyll Content

Control plants and plants receiving chemical fertilization through foliar application (ChF-fa) had the lowest leaf chlorophyll content (Figure 6A). Plants under MPE-sa had the highest leaf chlorophyll content as compared to all remaining regimes, besides ChF-sa (Figure 6A).

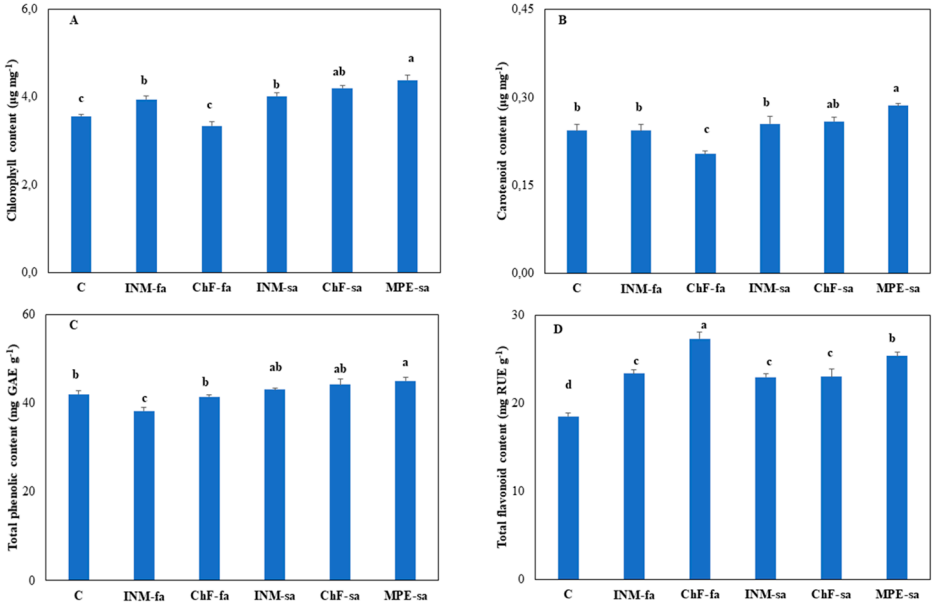

Figure 6. Effect of fertilization regime through different (root/foliar) application methods on leaf chlorophyll (A), carotenoid (B), total phenol (C) and total flavonoid (D) content of *Carlina diae*. C: Control (water); INM-fa: Integrated nutrient management (INM) by foliar application; ChF-fa: Chemical fertilization by foliar application; INM-sa: INM by soil application; ChF-sa: Chemical fertilization by soil application; MPE-sa: Mixture of plant extracts as biostimulant by soil application (THEOMASS). Values represent the mean of three replicates ± SEM. Within each plot, different letters indicate significant differences.

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3.6. Leaf Antioxidant Compound Content

Carotenoids, flavonoids, and phenols are critical non enzymatic antioxidants. Plants under MPE-sa had the highest leaf carotenoid content as compared to all remaining regimes, besides ChF-sa (Figure 6B). The lowest leaf carotenoid content was noted in plants receiving ChF-fa (Figure 6B).

Plants under MPE-sa had the highest leaf phenolic content as compared to the remaining regimes, besides ChF-sa and INM-sa (Figure 6C). The lowest leaf phenolic content was noted in plants receiving INM-fa (Figure 6C).

In all cases, fertilization stimulated leaf flavonoid content (Figure 6D). The highest leaf flavonoid content was noted under ChF-fa, followed by MPE-sa (Figure 6D).

3.7. Fertilization Stimulated Leaf Soluble Sugar Content

In all cases, fertilization was associated with enhanced leaf soluble sugar content (Figure 7). The highest leaf soluble sugar content was noted under the MPE-sa and ChF-fa schemes (Figure 7).

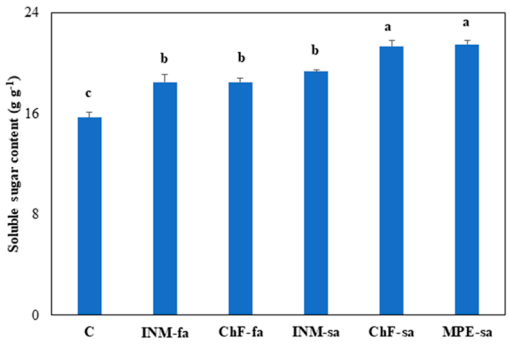

Figure 7. Effect of fertilization regime through different (root/foliar) application methods on leaf soluble sugar content of *Carlina diae*. C: Control (water); INM-fa: Integrated nutrient management (INM) by foliar application; ChF-fa: Chemical fertilization by foliar application; INM-sa: INM by soil application; ChF-sa: Chemical fertilization by soil application; MPE-sa: Mixture of plant extracts as biostimulant by soil application (THEOMASS). Values represent the mean of three replicates ± SEM. Within each plot, different letters indicate significant differences.

3.8. Leaf and Floral Mineral Analysis

Fertilization scheme and mode of application affected significantly the nutrients' leaf content of plants in certain cases (Tables 1 and 2). Specifically, INM-fa and ChF-fa treatments showed the highest N and P content compared to all other treatments, besides C (control) in the case of N. Furthermore, K concentrations in all fertilization treatments were higher than control. No significant differences were observed among treatments regarding Ca and Mg (Table 1).

Table 1. Effect of fertilization scheme through different (root/foliar) application methods on leaf essential macronutrient content of *Carlina diae*. C: Control (water); INM-fa: Integrated nutrient management (INM) by foliar application; ChF-fa: Chemical fertilization by foliar application; INM-sa: INM by soil application; ChF-sa: Chemical fertilization by soil application; MPE-sa: Mixture of plant extracts as biostimulant by soil application (THEOMASS). Mineral content was expressed per dry weight basis. Values represent the mean of three replicates ±SEM.

Treatment	N	P	K	Ca	Mg
			(g kg^{-1})		
C	9.8±0.5a*	1.1±0.0c	21.9±0.2b	18.2±0.8a	2.3±0.1a
INM-fa	12.3±1.1a	2.7±0.1b	35.1±1.7a	26.4±1.5a	2.6±0.1a
ChF-fa	11.4±0.5a	3.3±0.1a	33.7±1.0a	27.8±1.4a	2.6±0.1a

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INM-sa	5.9±0.2b	1.2±0.0c	30.1±1.1a	27.0±1.5a	2.6±0.1a
ChF-sa	6.0±0.1b	1.3±0.0c	32.1±0.1a	24.8±0.9a	2.8±0.1a
MPE-sa	5.5±0.2b	1.5±0.1c	30.3±1.6a	24.8±1.9a	2.5±0.1a
***p* F-test**	< 0.001	< 0.001	0.014	NS#	NS

* Means followed by different letters within the same column are statistically different at p ≤ 0.05. #NS Non-significant.

Table 2. Effect of fertilization scheme through different (root/foliar) application methods on leaf essential micronutrient content of *Carlina diae*. C: Control (water); INM-fa: Integrated nutrient management (INM) by foliar application; ChF-fa: Chemical fertilization by foliar application; INM-sa: INM by soil application; ChF-sa: Chemical fertilization by soil application; MPE-sa: Mixture of plant extracts as biostimulant by soil application (THEOMASS). Mineral content was expressed per dry weight basis. Values represent the mean of three replicates ±SEM.

Treatment	Cu	Zn	Fe	Mn	B
			(mg kg^{-1})		
C	10.5±0.2c*	36.3±0.3b	439±18b	114±2bc	29.5±0.5c
INM-fa	12.3±0.4abc	41.3±2.0ab	721±2ab	136±8ab	41.8±1.9ab
ChF-fa	13.8±0.6a	48.4±0.4a	932±64a	156±3a	36.4±1.0bc
INM-sa	11.3±0.2bc	36.2±0.8b	886±74a	113±4bc	39.7±1.3ab
ChF-sa	11.4±0.3bc	42.1±1.9ab	865±43a	115±1bc	38.9±1.2ab
MPE-sa	13.3±0.3ab	44.0±1.5ab	778±81a	107±2c	46.9±1.8a
***p* F-test**	0.028	0.037	0.059	0.005	0.007

As far as micronutrients' leaf content is concerned, ChF-fa treatment showed the highest Cu, Zn, Fe and Mn concentrations comparing to all other treatments (including control). Moreover, concentrations of Cu in MPE-sa and Fe in INM-sa, ChF-sa and MPE-sa treatments were higher than C (control). Boron concentrations in the INM-fa, INM-sa, ChF-sa and MPE-sa treatments were higher than C (control), with the MPE-sa treatment showing the highest value (Table 2).

Inflorescence mineral analysis showed significant differences among treatments in certain cases of nutrients (Tables 3 and 4). MPE-sa showed the lowest N content and C (control) the highest P and Mg content. No significant differences were observed among treatments regarding K and Ca content (Table 3).

Regarding inflorescence micronutrient content of *C. diae* (Table 4), the significantly highest Zn content was observed in C (control) and ChF-sa treatments and the lowest value in MPE-sa. No significant differences were observed among treatments in terms of Fe and B content (Table 4).

Table 3. Effect of fertilization regime through different (root/foliar) application methods on inflorescence essential macronutrient content of *Carlina diae*. C: Control (water); INM-fa: Integrated nutrient management (INM) by foliar application; ChF-fa: Chemical fertilization by foliar application; INM-sa: INM by soil application; ChF-sa: Chemical fertilization by soil application; MPE-sa: Mixture of plant extracts as biostimulant by soil application (THEOMASS). Values represent the mean of three replicates ± SEM.

Treatment	N	P	K	Ca	Mg
			(mg kg^{-1})		
C	10.46±0.49a*	2.11±0.35a	15.08±0.05a	3.88±0.05a	1.59±0.05a
INM-fa	9.42±0.03a	1.32±0.11b	12.70±1.03a	3.04±0.70a	1.02±0.10b
ChF-fa	8.66±0.32a	1.75±0.07b	14.22±0.77a	3.97±0.32a	1.38±0.19b
INM-sa	9.62±1.60a	1.08±0.27b	12.99±0.64a	2.94±0.42a	1.32±0.15b

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ChF-sa	9.29±1.02a	1.23±0.22b	13.82±0.57a	3.33±0.07a	1.30±0.13b
MPE-sa	4.71±0.27b	1.65±0.27b	12.89±0.05a	2.83±0.14a	1.08±0.00b
***p* F-test**	0.007	0.076	NS	NS	0.06

* Means followed by different letters within the same column are statistically different at $p \leq 0.05$. #NS Non-significant.

Table 4. Effect of fertilization regime through different (root/foliar) application methods on floral essential micronutrient content of *Carlina diae*. C: Control (water); INM-fa: Integrated nutrient management (INM) by foliar application; ChF-fa: Chemical fertilization by foliar application; INM-sa: INM by soil application; ChF-sa: Chemical fertilization by soil application; MPE-sa: Mixture of plant extracts as biostimulant by soil application (THEOMASS). Values represent the mean of three replicates ± SEM.

Treatment	**Zn**	**Fe**	**B**
		mg kg^{-1}	
C	19.65±1.29a*	72.54±15.21a	13.05±0.14a
INM-fa	15.86±0.14b	52.70±0.45a	12.86±0.16a
ChF-fa	17.78±0.89ab	58.74±6.52a	12.85±0.54a
INM-sa	17.67±1.42ab	45.95±8.20a	25.51±12.47a
ChF-sa	20.58±1.46a	34.19±6.92a	19.52±5.27a
MPE-sa	16.11±0.50b	51.30±12.79a	16.73±1.58a
***p* F-test**	0.051	NS	NS

* Means followed by different letters within the same column are statistically different at $p \leq 0.05$. #NS Non-significant.

4. Discussion

This pivotal field study presents the first steps of introducing *Carlina diae* – an Endangered local endemic to Dia Island offshore from Crete, Greece with ornamental [5] and medicinal interest [21] - into sustainable cultivation, and assesses the fertilization regime which is suitable for stimulating both plant growth and herbal quality in terms of ornamental and/or medicinal interest. In this regard, the potential of employing INM instead conventional (inorganic) fertilizers was explored owing not only to the reduced environmental footprint, but also to the benefit of respective certification, which is greatly valued in the markets [28].

Fertilization scheme did not affect plant biomass production in *C. diae* (Figure 5A). INM by either application method (INM-fa, INM-sa) was associated with lower water content, as compared to controls (Figure 5B). This reduced water content owing to INM schemes is expected to facilitate processing, which reduces moisture content (through drying) to establish a shelf-stable herb product [3]. Foliar fertilization (INM-fa or ChF-fa) was associated with increased biomass partitioning to the inflorescences as compared to controls, while plants under INM-fa had the highest inflorescence partitioning as compared to all treatments (Figure 5C). Since inflorescence tissue represents the harvestable organ for ornamental purposes, foliar fertilization appears to be advantageous especially when employing INM in *C. diae*, since it increases yield.

Macroscopic grading of herbal materials is based on visual inspection of leaf shape, and colour. The intensity and homogeneity of greenness is commonly employed as a quality criterion across the supply chain [20]. In *C. diae*, the fertilization scheme did not generally affect leaf coloration, as determined by three different instruments (SPAD meter, DA meter, Chroma Meter; Figure 2, and Supplementary Figures S1–S4). In other taxa, instead, organic fertilizers have been associated with enhanced leaf greenness [19]. Through analysis of four diverse metrics, it became apparent that the fertilization scheme also exerted a rather limited influence on leaf shape (Figure 4). Considering both

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leaf color and shape, it is concluded that the fertilization scheme did not alter the visual herbal quality aspects in *C. diae*.

Since consumers are currently placing more emphasis on the positive aspects of antioxidant intake, the value of antioxidant content of the herbal medicinal material is continuously rising [11,27]. In this study, three key non enzymatic antioxidants (carotenoids, flavonoids, phenols) were determined in *C. diae* which has also medicinal interest. The optimum fertilization scheme varied depending on the metabolite (Figure 6B,C,D). Considering all three antioxidants together, MPE-sa appears the more stimulatory scheme, since it gives the highest [carotenoids (along with ChF-sa; Figure 6B), phenolics (along with ChF-sa, INM-sa; Figure 6C)] or the second highest [flavonoid (after ChF-fa); Figure 6D] content. The promotive effect of INM on the antioxidant compound content has also been earlier shown in other taxa [17,29]. Previous own research lines employing other local endemic plants of Crete such as *Verbascum arcturus* L. [7] and *Origanum microphyllum* (Benth.) Vegel [8] offer first-time reports regarding the evaluation of the total phenolic and flavonoid contents which may further inspire the usage of fertilization in stimulating the herbal antioxidant content without compromised optical quality or yield in medicinal crops. Such investigations are supplementary to the current study dealing with *C. diae* as they offer insight into several cases of exemplary neglected and underutilized local endemic species of Crete responding in similar ways to fertilization. Furthermore, the fertilization strategies employed in such studies incorporate a multidisciplinary approach dealing with biological, physiological, phytochemical, and agronomical aspects of different species with promising potential in different economic sectors, all being useful for the establishment of distinct species-specific value chains [5].

With the exception of K, which increased in plant leaves upon application of both fertilization schemes and modes of application, only P among macronutrients responded to the foliar application of the integrated nutrient management or conventional fertilizers. The picture was quite differerent regarding the micronutrients, which responded to both fertilization schemes and modes of application in most cases. As far as micronutrients' leaf content is concerned, ChF-fa treatment showed the highest Cu, Zn, Fe and Mn concentrations comparing to all other treatments (including control). Despite the variations among treatments, it is concluded that the conventional or integrated nutrient management fertilization did not cause increase in any of the macro- or micro-nutrient concentrations in the inflorescences of C. diae. By contrast, lower values were observed in all treatments for P and Mg concentrations compared to the control (C). Moreover, lower content compared to the control was found in INM-fa, INM-sa and MPE-sa with regards to K, and lower INM-fa, and MPE-sa for Zn. Considering, also, the lack of effect of fertilization management on above-ground dry biomass as shown here, these findings may suggest the effect of the initial macro- and micro-nutrients availabilities in the soil [7,8], which have probably masked or reduced any potential effect on inflorescence nutrients contents. Besides the above-mentioned, interestingly, the MPE-sa treatment showed lower Ca values compared to control and other treatments, which also needs further research given the significance of Ca on physiology of plant species; Ca is important for the stability of cell wall and membrane, being also a second messenger for specific developmental and physiological processes [30].

Since the results reported in the study were obtained from only one season of field experimentation, longer period of research is needed to shed light into the optimum fertilization schemes and practices for *C. diae* in respect to both quantitative and qualitative characteristics of the plant. However, the current investigation provided for the first time baseline information for several herbal quality aspects in *C. diae*. Considering yield foliar fertilization, employing INM appears optimal, whereas MPE-sa is the best option when antioxidant compound content is the primary standard.

5. Conclusions

Carlina diae is an Endangered local endemic of Dia Island offshore from Crete (Greece) with high ornamental and medicinal potential. This pilot field study highlights the ameliorative effects of fertilization strategies on crop productivity and antioxidant profile of *C. diae*. Foliar fertilization

employing INM increased biomass partitioning to inflorescences (harvestable organ), and decreased tissue water content (facilitating processing). The optimum fertilization scheme varied depending on the metabolite. Considering all three antioxidants together (carotenoids, flavonoids, phenols), INM with biostimulant appears the optimum scheme, since it was associated with the highest (carotenoids, phenolics) or the second highest (flavonoid) content. The present results in *C. diae* indicate that INM fertilization was optimal for upgrading yield (foliar application) or herbal quality in terms of antioxidant profiles (INM with biostimulant), thus facilitating the way towards its use in ornamental sector and/or medicine.

Supplementary Materials: The following supporting information can be downloaded at the website of this paper posted on Preprints.org, Method S1. Experimental design, soil analysis and fertilization treatments; Method S2. Plant measurements performed; Figure S1. Effect of fertilization regime through different (root/foliar) application methods on leaf index of absorbance difference of *Carlina diae*; Figure S2. Effect of fertilization regime through different (root/foliar) application methods on leaf L value of *Carlina diae*; Figure S3. Effect of fertilization regime through different (root/foliar) application methods on leaf "a" value of *Carlina diae*; Figure S4. Effect of fertilization regime through different (root/foliar) application methods on leaf "b" value of *Carlina diae*; Figures S1-S4 fertilization regimes. C: Control (water); INM-fa: Integrated nutrient management (INM) by foliar application; ChF-fa: Chemical fertilization by foliar application; INM-sa: INM by soil application; ChF-sa: Chemical fertilization by soil application; MPE-sa: Mixture of plant extracts as bioreactor by soil application (THEOMASS). Values represent the mean of three replicates ± SEM. Within each plot, different letters indicate significant differences.

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Data Availability Statement: All data supporting the results of this study are included in the manuscript and the datasets are available upon request.

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Abbreviations

a*, green to red range intensity; b*, blue to yellow range intensity; Fv/Fm, ratio of variable to maximum chlorophyll fluorescence; GAE, gallic acid equivalent; IAD, index of absorbance difference; INM, integrated nutrient management; L*, lightness; RUE, rutin equivalent; SAR, sodium absorption ratio.

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