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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 the combined climate and biodiversity crisis, the sustainable utilization of phytogenetic resources stands as a one-way alternative, while nutrient management strategies are gaining 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 soil application). Foliar application comprised inorganic fertilization (conventional) or integrated nutrient management (INM). Soil application consisted of conventional inorganic fertilizers, biostimulants, or INM with biostimulants. Above-ground biomass content of nutrients, leaf chlorophyll fluorescence, and color parameters (SPAD meter, DA meter, Chroma Meter) were estimated. The leaf chlorophyll content, three key antioxidant compounds, and nutrient titers were also determined. 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 organs 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; MAPs; phenols; biostimulant

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 the human population relies on wild plants for remedies [1,2]. Furthermore, an increasing demand for a wide variety of natural products is also noted in the perfumery, cosmetics, and food industries, coupled with intense global quests for new ornamental plants originating from rich biodiversity hotspots [3].

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 [4]. To satisfy the multisectoral growing demand for novel products without further exhausting natural resources, recruiting neglected and underutilized species of economic interest and introducing them into cultivation systems stands out as a promising alternative and sustainable strategy for the development of new crops [3]. Well-documented initial plant material should be employed coupled with extant propagation protocols and cultivation guidelines applied in well-managed cultivation settings to be operative in such an endeavor. From this perspective, fertilization schemes tailor-made to the species in concern ought to be established and applied [5,6].

The key task in agriculture to date is to increase crop yield and quality in a sustainable way for the present and future. Nutrients are conventionally added to the soil of any crop using inorganic conventional fertilizers to support optimal plant growth. However, their over-application or insufficient use has been associated with a range of negative effects on the environment [7]. Major offsets include soil quality degradation and nutrient leaching [8]. These nutrients, which are lost from agricultural lands, pollute water bodies globally and, in this way, adversely affect aquatic systems and biodiversity [7,9]. Accumulative evidence suggests that the respective environmental impact is comparatively limited when alternative fertilization schemes are employed instead [8], such as biostimulants, which may trigger root nutrient uptake [10], or integrated nutrient management (INM), potentially promoting plant growth and productivity with a low environmental footprint [11]. Generally, it is well established that fertilization schemes determine plant growth, productivity, and quality aspects of the cultivated plant material [8]. An increasing number of studies indicate that applying organic fertilizers and INM strategies may upgrade the antioxidant level of medicinal plant material compared to conventional inorganic fertilizers [10,12–14]. INM schemes and organic fertilizers have also been previously related to increased chlorophyll content [15] or to the intensity and homogeneity of leaf greenness [16] or plants of medicinal interest [5,6,17]. Antioxidants are important compounds that deactivate free radicals and reactive oxygen species within cells [18]. The plant antioxidant profiles represent one of the most 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 a strong defense against injuries triggered by these free radicals [18].

Species-wise, this investigation is focused on *Carlina diae* (Rech. f.) Meusel and A. Kástner (Asteraceae) due to its ornamental value [3] and medicinal interest [17], its uniqueness as an 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 (included in Appendix I of Bern Convention and covered by the Greek Presidential Decree 67/1981) [17]. Context-wise, this investigation builds on previous studies exploring its sustainable exploitation potential in the ornamental [3] and the medicinal sectors and on developing effective species-specific propagation protocols for integrated conservation actions [17]. Using different schemes of conventional inorganic fertilizers or integrated nutrient management (INM), this study aimed to develop a species-specific fertilization protocol promoting optimal plant growth and key herbal quality aspects, such as antioxidant metabolite levels.

2. Materials and Methods

2.1. Plant Material

The collection data regarding the focal *C. diae* GR-1-BBGK-19,006 (Figure 1A,B) and the species-specific propagation protocols used to produce ex situ enough plant individuals for field experimentation are reported in our previous study [17]. The plants used in the experimental procedure were transferred to Crete in 2 L pots prepared by AFI GLAVAKI KE SIA OE Tree & Plant Nurseries Company, Aridea, PELLAS, GR-58400, Greece.

(**A**) (**B**) (**C**)

(**D**)

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**,**B**): M. Avramakis, Natural History Museum of Crete (reproduced with permission).

2.2. Field Experiment Establishment and Soil Properties

The general description of the established field experiment (Figure 1C) employing completely randomized blocks (CRB) of 10 individuals per block and three randomly arranged blocks in three rows per treatment at the Hellenic Mediterranean University Campus, Heraklion, Crete (Greece), as well as the soil analyses and properties of the research field are reported in previous publications [5,6] and are provided briefly in Supplementary Materials Methods S1.

2.3. Fertilization Schemes

Details on the fertilization schemes used in this experimentation are given briefly in Supplementary Material Methods S1 and are provided in our previous studies [5,6]. The fertilization management of the pilot cultivation of *C. diae* included:

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

B. Conventional inorganic fertilization as foliar application (ChF-fa): The nutrient solution contained NH_4NO_3 (34.4-0-0, Neofert®, Neochim PLC, Dimitrovgrad, Bulgaria) 2.7 g L^{-1}, $Ca(NO_3)_2$ (NITROCAL, Agrohimiki, Patras, Greece) 1.7 g L^{-1}, 10-47-10 3.2 g L^{-1}, K_2SO_4 (0-0-52) 2.27 g L^{-1}, $MgSO_4$ (Mg 25.6%) at 0.6 g L^{-1} and micronutrients Plex Mix at 1.5 mL L^{-1}.

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

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

E. Conventional inorganic fertilization as soil application (ChF-sa): The nutrient solution contained NH_4NO_3 (34.4-0-0) 2.7 g L^{-1}, $Ca(NO_3)_2$ (NITROCAL) 1.7 g L^{-1}, 10-47-10 3.2 g L^{-1}, K_2SO_4 (0-0-52) 2.3 g L^{-1}, $MgSO_4$ (Mg 25.6%) 0.3 g L^{-1} and micronutrients Plex Mix 1.5 mL L^{-1}.

F. Mixture of plant extracts (biostimulant) as soil application (MPE-sa): The nutrient solution contained THEOMASS 10 mL L^{-1}.

2.4. Plant Measurements

Several plant and leaf measurements were regularly performed till 25 May 2021, ending with the completion of the flowering of *C. diae*. All sampled leaves were fully expanded, grown under direct light, and lacked visual symptoms of pathogen infections or insect injuries. In all instances, the time from sampling to the initiation of measurements was shorter than 15 min. Whenever this was not feasible, aliquots were put in vials, frozen in liquid nitrogen, and transported to a freezer (−80 °C) for storage. Replicate leaves were taken from different plant individuals.

Details on each of the following plant measurements are given briefly in Supplementary Material Methods S2 and provided in our previous studies [5,6].

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

In this study, leaf coloration was assessed using three different methods (for the instruments used, see Supplementary Materials Methods S2 and [5,6]): (i) Leaf SPAD value, approximating chlorophyll content; (ii) the index of absorbance difference (IAD) computed as the variance between the absorbance values at 670 and 720 nm, close to the chlorophyll absorbance peak; (iii) quantified leaf color using a Chroma Meter. These measurements were conducted in situ on attached leaves of intact plant individuals at the vegetative, early flowering, and full flowering stages. Three spots were noted per each replicate leaf and were averaged. Three aliquot leaves were estimated per treatment.

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

As a valid display of leaf photosynthetic performance, the variable to maximum chlorophyll fluorescence (Fv/Fm) ratio was assessed (for instruments and conditions, see Supplementary Materials Methods S2 and [5,6]). These measurements were conducted in situ in dark-adapted attached leaves of intact plant individuals at vegetative, early flowering, and full flowering stages. Three spots were noted per each replicate leaf and were averaged. Three aliquot leaves were estimated per treatment.

2.4.3. Leaf Shape Indicators

A morphometric examination was performed by evaluating leaf form in terms of the aspect ratio, circularity, roundness, and solidity (for instruments, metrics, and values, see Supplementary Materials Methods S2 and [5,6]). Thirty leaves (5 per plant individual $\times$ 6 plant individuals) were examined per treatment.

2.4.4. Plant Growth and Biomass Partitioning to Generative Organs

Above-ground plant and inflorescence (fresh and dry) masses were determined ($\pm$0.01 g; MXX-412; Denver Instruments, Bohemia, NY, USA). Aliquots were put in an air-drying oven 72 h at 80 °C to measure dry weight. Samples from six replicate plants were evaluated per treatment.

2.4.5. Leaf Chlorophyll and Carotenoid Contents

The chlorophyll content is important for leaf coloration and photosynthetic performance. Carotenoids are key non-enzymatic antioxidants. Therefore, the effect of growth conditions on chlorophyll and carotenoid contents was assessed (see details in Supplementary Materials Methods S2 and [5,6]). Three leaves were estimated per treatment. Four aliquots taken from different plant individuals were assembled for individual replicates, and the quantitation assay was implemented twice.

2.4.6. Leaf Total Phenolics and Total Flavonoids

As phenols and flavonoids represent critical non-enzymatic antioxidants, their total contents were estimated using the Folin–Ciocalteu assay and aluminum chloride colorimetric assay, respectively (see details in Supplementary Materials Methods S2 and [5,6]). Three distinct leaves were evaluated per treatment. Four aliquots from different plant individuals were assembled for every replicate, and the assay was performed twice.

2.4.7. Leaf-Soluble Sugar Contents

Since carbohydrate status is related to photosynthetic activity, the effect of the fertilization scheme on carbohydrate status was assessed (see details in Supplementary Materials Methods S2 and [5,6]). These assays were guided on three replicates per treatment. Four aliquots from different plant individuals were joined for every replicate, and the quantitation assay was performed twice.

2.4.8. Leaf and Inflorescence Nutrient Analyses

To evaluate the role of the fertilization scheme on nutrient uptake, leaf and inflorescence mineral analysis was conducted (see details in Supplementary Materials Methods S2 and [5,6]). Three replicates were assayed per treatment. Four aliquots from distinct plant individuals were assembled for every replicate, and the quantitation assay was performed twice.

2.5. Statistical Analyses

Data analyses were performed using the SPSS statistical software Version 22 (IBM Corp., Armonk, NY, USA). Data were examined for homogeneity of variances (Duncan's test), and a one-way analysis of variance (ANOVA) was conducted. Afterward, the least significant differences (LSD) test was used for mean comparisons among treatments (INM-fa, INM-sa, ChF-fa, ChF-sa, MPE-sa) (p = 0.05).

3. Results

3.1. The Fertilization Scheme Exerted Minor Effects on Leaf Colour

At the vegetative stage, foliar fertilization (INM-fa or ChF-fa) was associated with a higher leaf SPAD value compared to INM-sa (Figure 2A). At the early and full flowering stages, the fertilization scheme did not affect the leaf SPAD value (Figure 2B,C). At the vegetative stage, conventional inorganic fertilization, regardless of application method (ChF-fa or ChF-sa), was related to lower leaf IAD compared to the rest of the treatments (Supplementary Figure S1A). At the early and full flowering stages, the fertilization scheme did not affect leaf IAD (Supplementary Figure S1B,C).

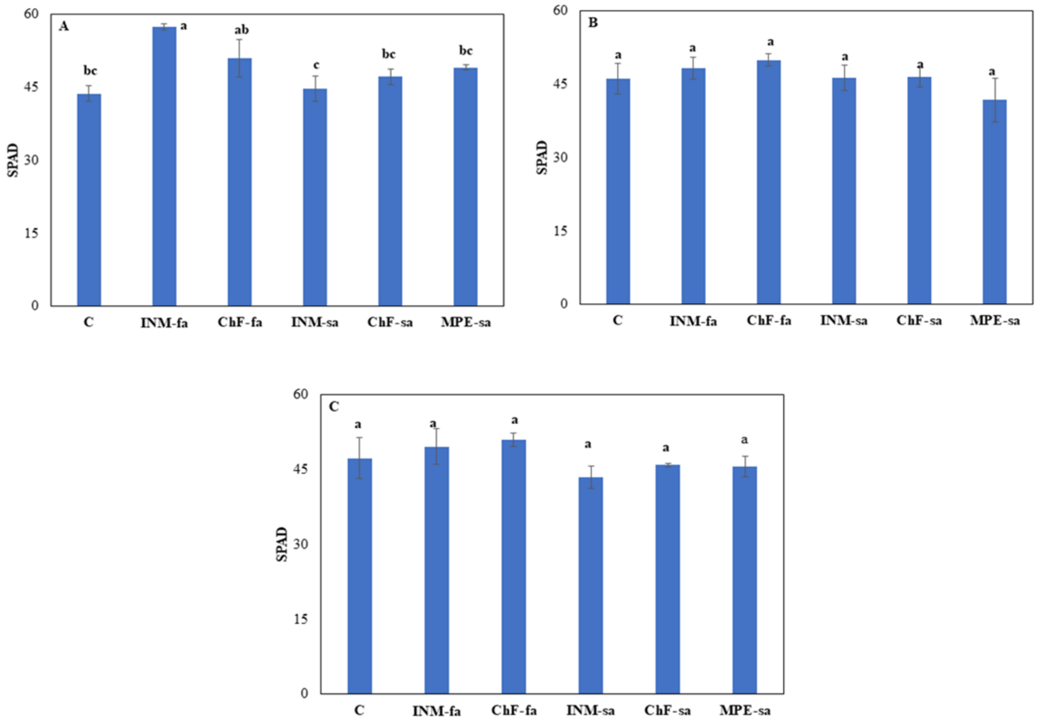

Figure 2. Effects of the fertilization scheme and application method (soil/foliar) on leaf SPAD value of *Carlina diae* at the vegetative (**A**), early flowering (**B**), and full flowering (**C**) stages. C: Control (water); INM-fa: integrated nutrient management (INM) by foliar application; ChF-fa: conventional inorganic fertilization by foliar application; INM-sa: INM by soil application; ChF-sa: conventional inorganic fertilization by soil application; MPE-sa: mixture of plant extracts as biostimulant by soil application (THEOMASS). Columns symbolize the means of three replicates ± SEM. Within every plot, different letters indicate statistically significant differences.

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

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

At the vegetative stage, ChF-sa and MPE-sa resulted in higher leaf chlorophyll fluorescence values compared to the control plants and plants treated with foliar fertilization (INM-fa or ChF-fa; Figure 3A). At the early flowering stage, these two fertilization schemes (ChF-sa and MPE-sa) showed a significant difference when compared to ChF-fa (Figure 3B). The fertilization scheme did not affect leaf chlorophyll fluorescence value at the full flowering stage (Figure 3C).

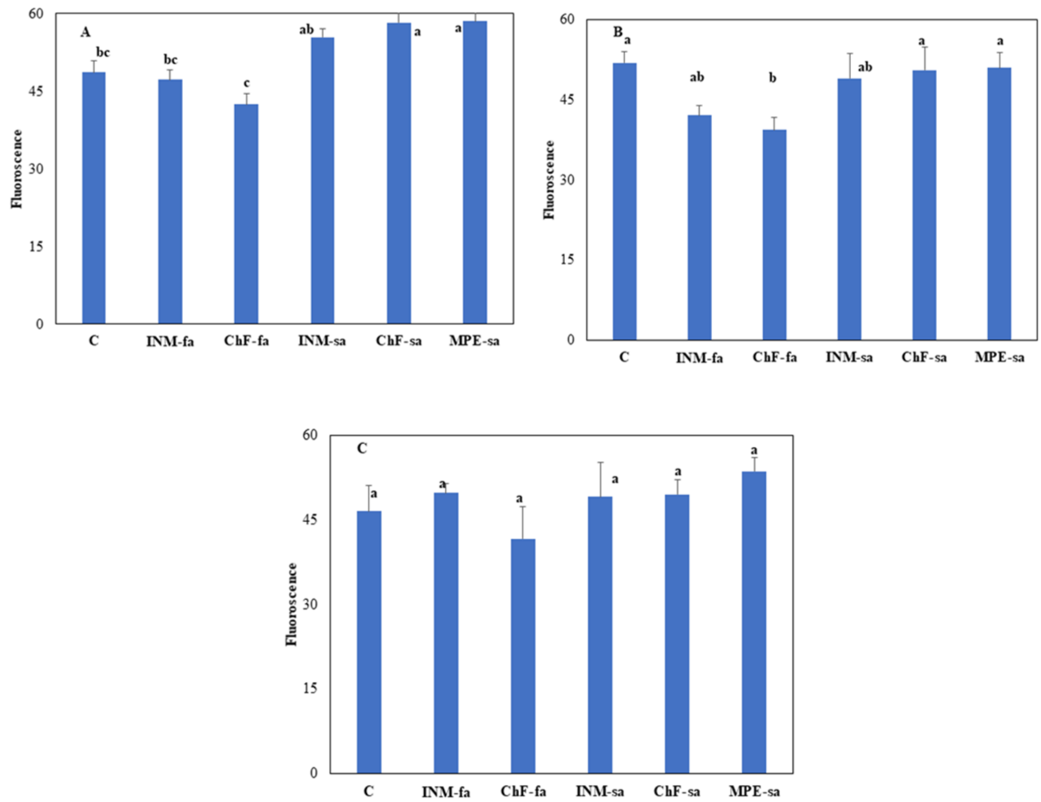

Figure 3. Effects of the fertilization scheme and application method (soil/foliar) on the chlorophyll fluorescence value of *Carlina diae* at the vegetative (**A**), early flowering (**B**), and full flowering (**C**) stages. C: control (water); INM-fa: integrated nutrient management (INM) by foliar application; ChF-fa: conventional inorganic fertilization by foliar application; INM-sa: INM by soil application; ChF-sa: conventional inorganic fertilization by soil application; MPE-sa: mixture of plant extracts as biostimulant by soil application (THEOMASS). Columns symbolize the means of three replicates ± SEM. Within every plot, different letters indicate statistically significant differences.

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

The morphometric analysis was conducted by determining four leaf shape factors (aspect ratio, circularity, roundness, and solidity). The fertilization scheme did not affect the aspect ratio, roundness, and solidity (Figure 4A,C,D). Plants cultivated under ChF-sa and MPE-sa presented higher leaf circularity than plants treated with foliar fertilization (INM-fa or ChF-fa; Figure 4B).

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 or INM-sa) was associated with lower water content compared to the controls (Figure 5B). Plants treated with foliar fertilization (INM-fa or ChF-fa) had increased biomass partitioning to the inflorescences compared to the controls, while plants treated with INM-fa had the highest partitioning to the inflorescences compared to the remaining schemes (Figure 5C).

3.5. Fertilization Generally Improved Leaf Chlorophyll Content

The control plants and the plants treated with conventional inorganic fertilization through foliar application (ChF-fa) showed the lowest leaf chlorophyll content (Figure 6A). Plants under MPE-sa showed the highest leaf chlorophyll content compared to the rest of the schemes besides ChF-sa (Figure 6A).

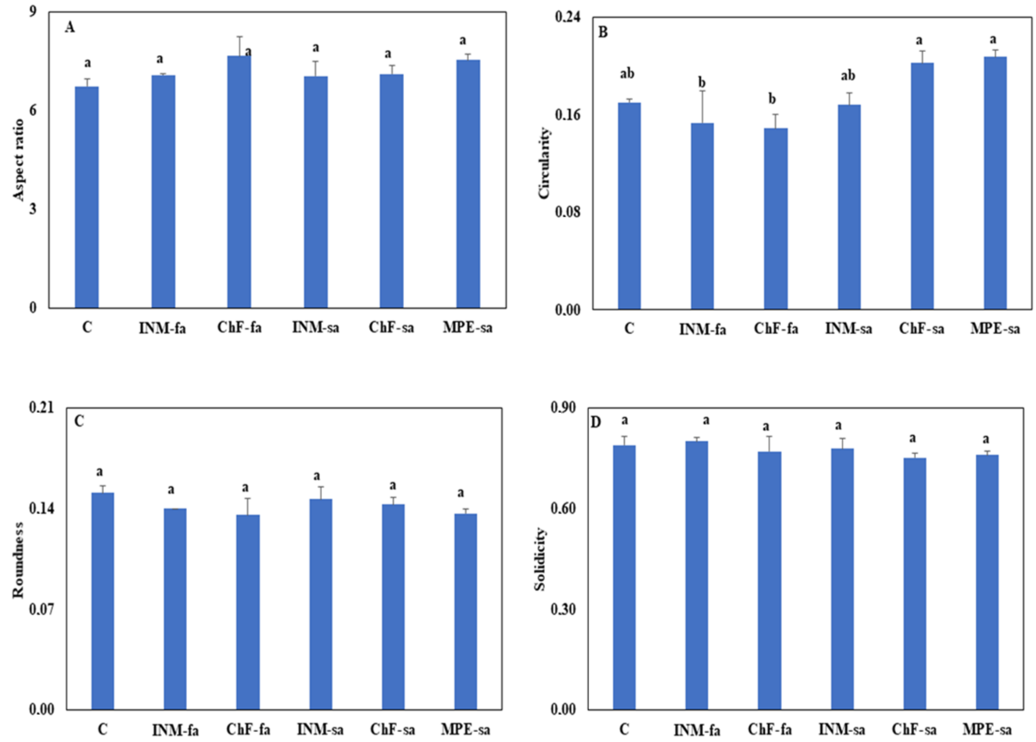

Figure 4. Effects of the fertilization scheme and application method (soil/foliar) on four leaf shape factors of *Carlina diae* namely aspect ratio (**A**), circularity (**B**), roundness (**C**), and solidicity (**D**). C: control (water); INM-fa: integrated nutrient management (INM) by foliar application; ChF-fa: conventional inorganic fertilization by foliar application; INM-sa: INM by soil application; ChF-sa: conventional inorganic fertilization by soil application; MPE-sa: mixture of plant extracts as biostimulant by soil application (THEOMASS). Columns symbolize the means of six replicates ± SEM. Within every plot, different letters indicate statistically significant differences.

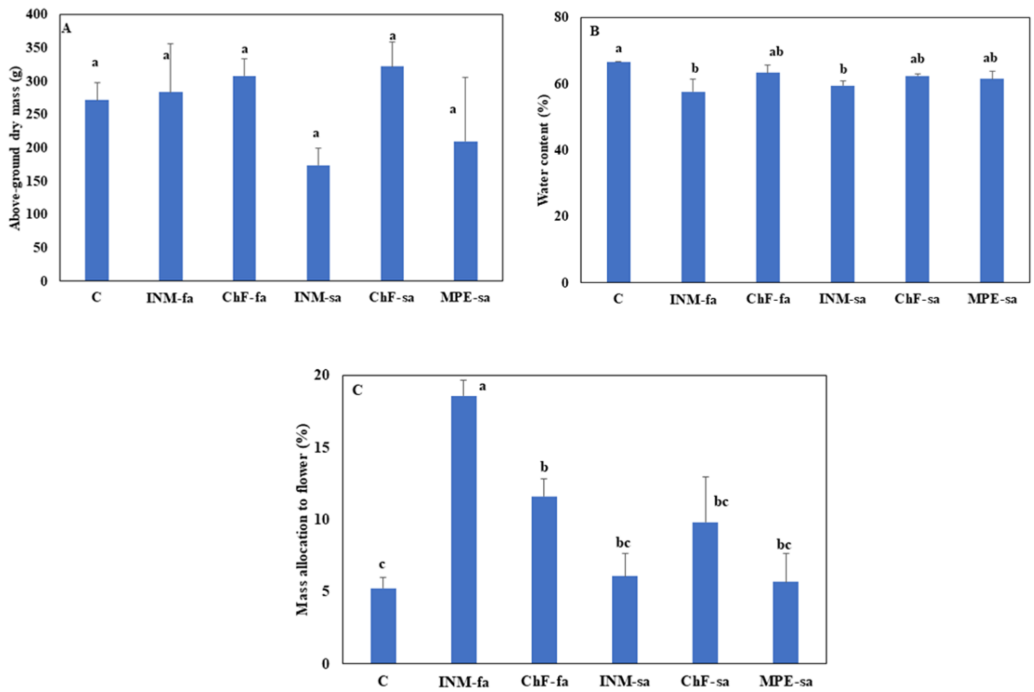

Figure 5. Effects of the fertilization scheme and application method (soil/foliar) on above-ground dry weight (**A**), water content (**B**), and dry weight partitioning to inflorescences (**C**) of *Carlina diae*.

C: control (water); INM-fa: integrated nutrient management (INM) by foliar application; ChF-fa: conventional inorganic fertilization by foliar application; INM-sa: INM by soil application; ChF-sa: conventional inorganic fertilization by soil application; MPE-sa: mixture of plant extracts as biostimulant by soil application (THEOMASS). Columns symbolize the means of six replicates ± SEM. Within every plot, different letters indicate statistically significant differences.

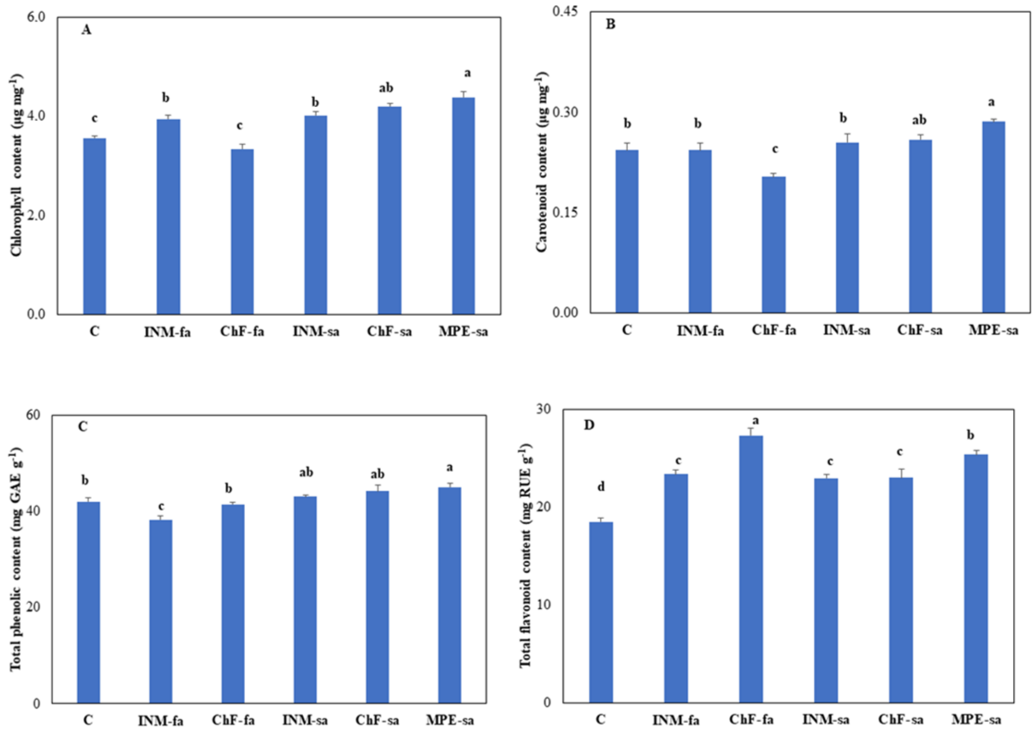

Figure 6. Effects of the fertilization scheme and application method (soil/foliar) on the leaf chlorophyll (**A**), carotenoid (**B**), total phenol (**C**), and total flavonoid (**D**) contents of *Carlina diae*. C: control (water); INM-fa: integrated nutrient management (INM) by foliar application; ChF-fa: conventional inorganic fertilization by foliar application; INM-sa: INM by soil application; ChF-sa: conventional inorganic fertilization by soil application; MPE-sa: mixture of plant extracts as biostimulant by soil application (THEOMASS). Columns symbolize the means of three replicates ± SEM. Within every plot, different letters indicate statistically significant differences.

3.6. Leaf Antioxidant Compound Content

Carotenoids, flavonoids, and phenols are critical non-enzymatic antioxidants. Plants under MPE-sa exhibited the highest leaf carotenoid content compared to all remaining schemes besides ChF-sa (Figure 6B). The lowest leaf carotenoid contents were detected in plants treated with ChF-fa (Figure 6B).

Plants under MPE-sa exhibited the highest leaf phenolic content compared to other schemes besides ChF-sa and INM-sa (Figure 6C). The lowest leaf phenolic contents were detected in plants treated with 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 contents were noted under the MPE-sa and ChF-fa treatments (Figure 7).

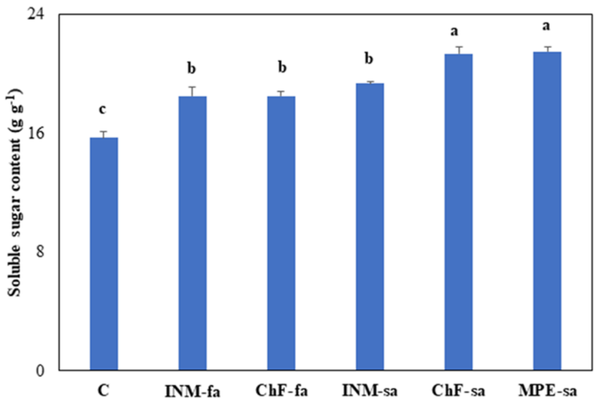

Figure 7. Effects of the fertilization scheme and application method (soil/foliar) on leaf soluble sugar contents of *Carlina diae*. C: control (water); INM-fa: integrated nutrient management (INM) by foliar application; ChF-fa: conventional inorganic fertilization by foliar application; INM-sa: INM by soil application; ChF-sa: conventional inorganic fertilization by soil application; MPE-sa: mixture of plant extracts as biostimulant by soil application (THEOMASS). Columns symbolize the means of three replicates ± SEM. Different letters indicate statistically significant differences.

3.8. Leaf and Floral Nutrient Analysis

The fertilization scheme and application method significantly affected the nutrient leaf content of plants in certain cases (Tables 1 and 2). Specifically, the 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 in the control. No significant differences were observed among treatments regarding Ca and Mg (Table 1).

Regarding micronutrient leaf content, ChF-fa treatment showed the highest Cu, Zn, Fe and Mn concentrations compared 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 the control. Boron concentrations in the INM-fa, INM-sa, ChF-sa, and MPE-sa treatments were higher than the control, with the MPE-sa treatment showing the highest value (Table 2).

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

Table 1. Effects of the fertilization scheme and application method (soil/foliar) on the leaf essential macronutrient content of *Carlina diae*. C: control (water); INM-fa: integrated nutrient management (INM) by foliar application; ChF-fa: conventional inorganic fertilization by foliar application; INM-sa: INM by soil application; ChF-sa: conventional inorganic fertilization by soil application; MPE-sa: mixture of plant extracts as biostimulant by soil application (THEOMASS). Nutrient content is expressed on a dry weight basis. Values denote the means of three replicates ± SEM.

Treatment	**N**	**P**	**K**	**Ca**	**Mg**
			(g kg^{-1})		
C	9.8 ± 0.5 a *	1.1 ± 0.0 c	21.9 ± 0.2 b	18.2 ± 0.8 a	2.3 ± 0.1 a
INM-fa	12.3 ± 1.1 a	2.7 ± 0.1 b	35.1 ± 1.7 a	26.4 ± 1.5 a	2.6 ± 0.1 a
ChF-fa	11.4 ± 0.5 a	3.3 ± 0.1 a	33.7 ± 1.0 a	27.8 ± 1.4 a	2.6 ± 0.1 a
INM-sa	5.9 ± 0.2 b	1.2 ± 0.0 c	30.1 ± 1.1 a	27.0 ± 1.5 a	2.6 ± 0.1 a
ChF-sa	6.0 ± 0.1 b	1.3 ± 0.0 c	32.1 ± 0.1 a	24.8 ± 0.9 a	2.8 ± 0.1 a
MPE-sa	5.5 ± 0.2 b	1.5 ± 0.1 c	30.3 ± 1.6 a	24.8 ± 1.9 a	2.5 ± 0.1 a
p F-test	<0.001	<0.001	0.014	NS #	NS

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

Table 2. Effects of the fertilization scheme and application method (soil/foliar) on the leaf essential micronutrient contents of *Carlina diae*. C: control (water); INM-fa: Integrated nutrient management (INM) by foliar application; ChF-fa: conventional inorganic fertilization by foliar application; INM-sa: INM by soil application; ChF-sa: conventional inorganic fertilization by soil application; MPE-sa: mixture of plant extracts as biostimulant by soil application (THEOMASS). Nutrient content is expressed on a dry weight basis. Values denote the means of three replicates ±SEM.

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

* Means followed by different letters within the same column statistically differ at $p \leq 0.05$.

Table 3. Effects of the fertilization scheme and application method (soil/foliar) on the inflorescence essential macronutrient contents of *Carlina diae*. C: control (water); INM-fa: integrated nutrient management (INM) by foliar application; ChF-fa: conventional inorganic fertilization by foliar application; INM-sa: INM by soil application; ChF-sa: conventional inorganic fertilization by soil application; MPE-sa: mixture of plant extracts as biostimulant by soil application (THEOMASS). Nutrient content is expressed on a dry weight basis. Values denote the means of three replicates ± SEM.

Treatment	N	P	K	Ca	Mg
			(mg kg^{-1})		
C	10.46 ± 0.49 a *	2.11 ± 0.35 a	15.08 ± 0.05 a	3.88 ± 0.05 a	1.59 ± 0.05 a
INM-fa	9.42 ± 0.03 a	1.32 ± 0.11 b	12.70 ± 1.03 a	3.04 ± 0.70 a	1.02 ± 0.10 b
ChF-fa	8.66 ± 0.32 a	1.75 ± 0.07 b	14.22 ± 0.77 a	3.97 ± 0.32 a	1.38 ± 0.19 b
INM-sa	9.62 ± 1.60 a	1.08 ± 0.27 b	12.99 ± 0.64 a	2.94 ± 0.42 a	1.32 ± 0.15 b
ChF-sa	9.29 ± 1.02 a	1.23 ± 0.22 b	13.82 ± 0.57 a	3.33 ± 0.07 a	1.30 ± 0.13 b
MPE-sa	4.71 ± 0.27 b	1.65 ± 0.27 b	12.89 ± 0.05 a	2.83 ± 0.14 a	1.08 ± 0.00 b
p F-test	0.007	0.076	NS #	NS	0.06

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

Table 4. Effects of the fertilization scheme and application method (soil/foliar) on the floral essential micronutrient contents of *Carlina diae*. C: control (water); INM-fa: integrated nutrient management (INM) by foliar application; ChF-fa: conventional inorganic fertilization by foliar application; INM-sa: INM by soil application; ChF-sa: conventional inorganic fertilization by soil application; MPE-sa: mixture of plant extracts as biostimulant by soil application (THEOMASS). Nutrient content is on a dry weight basis. Values denote the means of three replicates ± SEM.

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

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

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

4. Discussion

This pivotal field study presents the first steps in introducing *Carlina diae*—an Endangered local endemic of Dia Island and locally in Crete, Greece, with ornamental [3] and medicinal interest [17]—into sustainable cultivation. It assesses the fertilization scheme and application method suitable for stimulating plant growth and herbal quality in terms of ornamental and/or medicinal interest. In this regard, the potential of employing INM instead of conventional inorganic fertilizers was explored owing to the reduced environmental footprint and the benefit of respective certification, which is greatly valued in the markets [19–22].

The 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 than the controls (Figure 5B). Foliar fertilization (INM-fa or ChF-fa) was associated with increased biomass partitioning to the inflorescences compared to the controls, while plants under INM-fa had the highest inflorescence partitioning compared to all treatments (Figure 5C). Since inflorescence tissue represents the harvestable organ for ornamental purposes, foliar fertilization appears advantageous, especially when employing INM in *C. diae*, since it can increase yield.

Macroscopic grading of herbal materials is based on visual inspection of leaf shape and color. The intensity and homogeneity of greenness are commonly employed as a quality criterion across the supply chain [16]. In *C. diae*, the fertilization scheme and application method did not generally affect leaf coloration, as determined by three different instruments (SPAD meter, DA meter, Chroma Meter; Figure 2 and Figures S1–S4). In other taxa, organic fertilizers have been associated with enhanced leaf greenness [15]. Analysis of four diverse metrics showed that the fertilization scheme also exerted a rather limited influence on leaf shape (Figure 4). Considering both leaf color and shape, it is concluded that the fertilization scheme and application method did not alter the visual herbal quality aspects in *C. diae*.

Since consumers are currently emphasizing the positive aspects of antioxidant intake, the consumer-perceived value of the antioxidant content of the herbal medicinal material is continuously rising [9,18]. This study determined three key non-enzymatic antioxidants (carotenoids, flavonoids, phenols) in *C. diae*, which also has medicinal interest. The optimum fertilization scheme varied depending on the metabolite (Figure 6B–D). Considering all three antioxidants together, MPE-sa appears to be 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 enhancing effect of INM on the antioxidant compound contents has similarly been previously shown in other taxa [13,23]. Previous research lines employ other local endemic plants of Crete, such as *Verbascum arcturus* L. [5] and *Origanum microphyllum* (Benth.) Vegel [6] offer first-time reports regarding the evaluation of the total phenolic and flavonoid contents, which may further inspire the usage of fertilizers in stimulating the herbal antioxidant content deprived of optical quality and/or yield in medicinal crops. Such investigations supplement 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 similarly to fertilization. Furthermore, the fertilization strategies employed in such studies incorporate a multidisciplinary approach dealing with the biological, physiological, phytochemical, and agronomical aspects of different species with promising potential in different economic sectors [3], all being useful for the establishment of distinct and species-specific value chains [24–26].

With the exception of K, which increased in plant leaves upon application of both fertilization schemes and methods of application, only P among macronutrients responded to the foliar application of INM or conventional inorganic fertilizers. The picture was quite different regarding the micronutrients, which responded to both fertilization schemes and application methods in most cases. Regarding micronutrient leaf content, the ChF-fa treatment showed the highest Cu, Zn, Fe, and Mn concentrations compared 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 an 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 the INM-fa and MPE-sa treatments for Zn. Considering the lack of effect of fertilization treatment on above-ground dry biomass as shown here, these findings may suggest the effect of the initial macro and micro-nutrient availabilities in the soil [5,6], which have probably masked or reduced any potential effect on inflorescence nutrient contents.

Since the results reported in the study were obtained from only one season of field experimentation, a longer period of research is needed to shed light on the optimum fertilization schemes and application methods for *C. diae* with respect to the plant's quantitative and qualitative characteristics. However, the current investigation provided baseline information for several herbal quality aspects in *C. diae* for the first time. 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 and locally in Crete (Greece) with high ornamental and medicinal potential. This pilot field study highlights the ameliorative effects of fertilization strategies on crop productivity and the antioxidant profile of *C. diae*. Foliar fertilization employing INM increased biomass partitioning to inflorescences (harvestable organs) and decreased the tissue water content (facilitating processing). The optimum fertilization scheme varied depending on the metabolite. Considering all three antioxidants together (carotenoids, flavonoids, and phenols), INM with biostimulant appears to be the optimum scheme since it was associated with the highest (carotenoids and 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 toward its use in the ornamental sector and/or in medicine.

Supplementary Materials: The supporting information shown below can be downloaded at: https://www.mdpi.com/article/10.3390/agriculture14020259/s1, Methods S1. Experimental design, climate, soil analyses, and fertilization treatments; Methods S2. Plant measurements performed; Figure S1. Effects of the fertilization scheme and application method (soil/foliar) on leaf index of the absorbance difference of *Carlina diae*; Figure S2. Effects of the fertilization scheme and application method (soil/foliar) on leaf L values of *Carlina diae*; Figure S3. Effects of the fertilization scheme and application method (soil/foliar) on leaf "a" values of *Carlina diae*; Figure S4. Effects of the fertilization scheme and application method (soil/foliar) on leaf "b" values of *Carlina diae*; Figures S1–S4 fertilization schemes. C: control (water); INM-fa: integrated nutrient management (INM) by foliar application; ChF-fa: conventional inorganic fertilization by foliar application; INM-sa: INM by soil application; ChF-sa: conventional inorganic fertilization by soil application; MPE-sa: mixture of phytoextracts as bioreactor by soil application (THEOMASS). Columns symbolize the means of three replicates ± SEM. Within every plot, different letters indicate statistically significant differences.

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Abbreviations

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

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