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Pilot Cultivation of the Vulnerable Cretan Endemic *Verbascum arcturus* L. (Scrophulariaceae): Effect of Fertilization on Growth and Quality Features

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Citation: Paschalidis, K.; Fanourakis, D.; Tsaniklidis, G.; Tzanakakis, V.A.; Bilias, F.; Samara, E.; Kalogiannakis, K.; Debouba, F.J.; Ipsilantis, I.; Tsoktouridis, G.; et al. Pilot Cultivation of the Vulnerable Cretan Endemic *Verbascum arcturus* L. (Scrophulariaceae): Effect of Fertilization on Growth and Quality Features. *Sustainability* **2021**, *13*, 14030. https://doi.org/10.3390/su132414030

Academic Editor: Bernhard Huchzermeyer

Received: 16 November 2021
Accepted: 16 December 2021
Published: 20 December 2021

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Abstract: The domestication of wild-growing plants, including cultivation and fertilization protocols, is able to alleviate the ecological risks posed by the uncontrolled harvesting of range-restricted local endemic plants. In this field study focused on *Verbascum arcturus*, a vulnerable local endemic of Crete (Greece), the effect of two kinds of fertilization applied by two methods (foliar/root) was investigated. The foliar application included conventional or integrated nutrient management (INM) fertilization. Root application included the application of conventional fertilizers, biostimulants, or INM with biostimulants. Several properties of plant growth, physiology and nutrition were determined. The results showed that fertilization treatment affected neither leaf color and shape nor plant growth, morphology, dry mass partitioning or nutrient content. However, both kinds of foliar-applied fertilization enhanced Zn and B in leaves and soil-applied biostimulant increased leaf Ca. Considering both chlorophyll and antioxidant compounds' content, foliar application of the INM fertilizers, as well as soil application of the conventional fertilizers or biostimulants, could be considered as accepted options. This study reports for the first time an assessment of the total phenolic and flavonoids content evidenced in *V. arcturus* and encourages the use of fertilization in promoting the herbal antioxidant profile without compromising visual quality or yield. The findings of this study could be considered as a documented contribution toward the sustainable exploitation of *V. arcturus*.

Keywords: antioxidant; carotenoids; flavonoids; herbal quality; medicinal plant; phenols; plant nutrition; biostimulants; Greece

1. Introduction

Although most medicinal chemical substances are presently industrially manufactured, plants continue to be a sizeable resource of pharmaceuticals and other compounds

for a wide range of sectors. For instance, over 25% of prescription medicines are derived directly or indirectly from herbal materials [1]. Notably, the respective demand for medicines, perfumery, cosmetics and food additives presently shows a rising trend [2]. Although little attention is paid to this by the denizens, a considerable portion of the herbal material still originates from wild plants, affecting their wild-growing populations [3]. Among them, medicinal and aromatic plants account for a major share. As a widely unrecognized commodity, however, communities do not safeguard whether wild-growing populations decline or remain stable, for instance, and whether an adequate time for natural reproduction and growth is allowed between harvests from the wild. This poses considerable ecological risks for specific range-restricted species, in particular, and for the whole habitat in which they thrive, in general [3].

Likewise, the ornamental plant industry is on a continuing quest for new and unique plants with impressive characteristics that are not commonplace [4]. Often, new ornamental plants are sourced initially from wild-growing populations. After successful domestication trials and propagation, cultivation and breeding strategies are effectively attempted in these new materials to allow for commercialization and the establishment of value chains. The domestication process of wild-growing plants with attractive properties, combined with rarity or endemism attributes, is extremely appreciated by the ornamental-horticultural sector as a unique source of new crops [4–6].

The targeted domestication of species of interest and their introduction into cultivation systems represents a sustainable approach in any exploitation attempt of phytogenetic resources, and this is particularly important regarding the neglected and underutilized species that are range-restricted local endemics of specific areas [4,7]. However, to tackle this challenge, the development of cultivation guidelines and fertilization protocols are required in the first place.

In general, inorganic fertilizers are conventionally applied to address the nutrient requirements of crops in agriculture. However, their use in excess has been associated with environmental concerns, such as the nutrient-induced pollution of water bodies [8]. For instance, it is estimated that more than 50% of the total N applied through conventional fertilizers is lost to the environment, leading to eutrophication problems, and is, thus, not recovered by the soil–plant system [9]. These adverse effects could be reduced when using organic instead of conventional fertilizers [8]. Biostimulants do not conventionally contain nutrients and thus cannot replace fertilizers, but they can reduce their use by facilitating nutrient uptake by plants [10]. In this perspective, the combined use of inorganic and organic fertilizers and biostimulants (Integrated Nutrient Management, INM) represents an alternative strategy that can offer enhanced productivity options for crops. In addition, INM can increase plant resistance against many types of biotic and abiotic stresses through balanced nutrient supplies matching the crop requirements, reducing at the same time the needed dose of fertilizers [11,12]. In this way, INM represents an effective fertilization and eco-friendly practice that is able to create favorable soil physicochemical conditions, safeguard the soil nutrient balance in the long run, and generate an optimum level for sustaining the desired crop productivity [11,12]. To date, INM has been associated with enhanced nutrient-use efficiency, decreased nutrient loss, minimized crop nutrient requirements, and increased cation exchange, water storage capacity, higher yields [11,13], and improved bioavailability of micronutrients in soil and their uptake by plants [11,14].

Fertilization is not only expected to optimally promote yield but also to stimulate herbal material quality features [8]. These features are very diverse and depend on the intended use [15]. The content of health-promoting compounds (e.g., antioxidants) is conventionally a key aspect in defining the quality of plant materials [9]. It has been suggested that organic fertilizers and INM strategies can stimulate the content of plant secondary metabolites, including phenols and flavonols [16]. If this hypothesis is validated, organic and INM fertilization may provide an opportunity to stimulate herbal material quality associated with health-promoting compound content. Organic fertilizers and INM schemes have also been associated with enhanced leaf chlorophyll content [17]. The

intensity and uniformity of leaf greenness is another index suggestive of quality that is commonly employed throughout the distribution chain of edible greens, medicinal–aromatic and ornamental plants [15].

In this framework, the aim of this work was to develop a pilot cultivation and fertilization protocol, optimally driving biomass production, nutrition and key herbal quality aspects (color, antioxidant compound content) in a local endemic plant of Crete (Greece), namely, *Verbascum arcturus* L. (Scrophulariaceae). This rock-dwelling species is naturally drought-tolerant and is considered as a neglected and underutilized plant of Crete with medicinal and ornamental potential [4], which is also assessed as threatened with extinction (vulnerable according to Kougioumoutzis et al., 2021 [18]). In this study, *V. arcturus* is cultivated in a pilot agricultural setting for the first time, and its fertilization protocols are comparatively assessed.

2. Materials and Methods

2.1. Origin of Plant Material

Authorized botanical expeditions were organized in 2019 to explore different areas for wild-growing *V. arcturus* populations with natural vigorous growth in the rocky wild habitats of western Crete (Figure 1a,d). The seed collections were performed under an authorized special permit from the Institute of Plant Breeding and Phytogenetic Resources, Hellenic Agricultural Organization Demeter (Permit 82336/879 of 18 May 2019 and 26895/1527, 21 April 2021). This permit is issued yearly by the Greek Ministry of Environment and Energy after detailed reporting by the applicant. The seed collections were made in the frame of the ongoing research project "Conservation and sustainable utilization of rare-threatened endemic plants of Crete for the development of new products with innovative precision fertilization" (acronym: PRECISE-M, T1EΔK-05380).

The collected herbarium samples and seeds of *V. arcturus* were taxonomically identified and, consequently, a unique IPEN (International Plant Exchange Network) accession number was given by the Institute of Plant Breeding and Genetic Resources (IPBGR) of the Hellenic Agricultural Organization, Demeter (ELGO Demeter). New plants were initially raised ex situ through seed germination trials and pilot rooting of cuttings under the IPEN accession number GR-1-BBGK-19,139 assigned to the original plant collection from the Aradaina gorge, western Crete, at an altitude of 1000 m [19]. These pilot propagation trials resulted in enough ex-situ-raised plants for further field experimentation, transplanted in 2 L plastic pots sourced from the company of AFI GLAVAKI KE SIA OE Tree & Plant Nurseries, Aridea, PELLAS, GR-58400, Greece.

2.2. Establishment of Field Experiment

The field experiment was established in the campus of the Hellenic Mediterranean University, Heraklion, Crete (Greece) in an area of 20 × 25 m (35°19′ N, 25°6′ E, 60 m). The plants were transplanted into the soil on 1 March 2021. The distance between the plants was 40 cm, the gap between the rows was 80 cm, and the rows were 20 m long. The rows had an east-west direction and included as "guard plants" other local Cretan endemics, i.e., plant individuals of *Origanum dictamnus*, *Origanum microphyllum*, *Carlina diae*, and *Sideritis syriaca* subsp. *syriaca* (Figure 1e). These buffer plants are focal species of the ongoing research project PRECISE-M and were established in equal numbers to the species under study, thus reducing heterogeneity in air velocity between the inner and outer rows.

The experimental design was of completely randomized blocks (CRB) of 10 plants of *V. arcturus* per block, with three blocks per treatment (see below), which were randomly located in three different rows per treatment. In order to achieve the same starting plant material, all plants were trimmed to 10 cm above the soil at the end of April. At the end of May (i.e., 30 d following trimming), six fertilization treatments were applied and were performed weekly until the final harvest. An automatic irrigation system was installed with 2 L/h adjustable drippers to supply water to the plants, and it was scheduled to water

them three times per week. Pest and disease control was not necessary during cultivation, but the removal of weeds was required regularly. The final harvest was carried out on 30 June 2021 (Figure 1e).

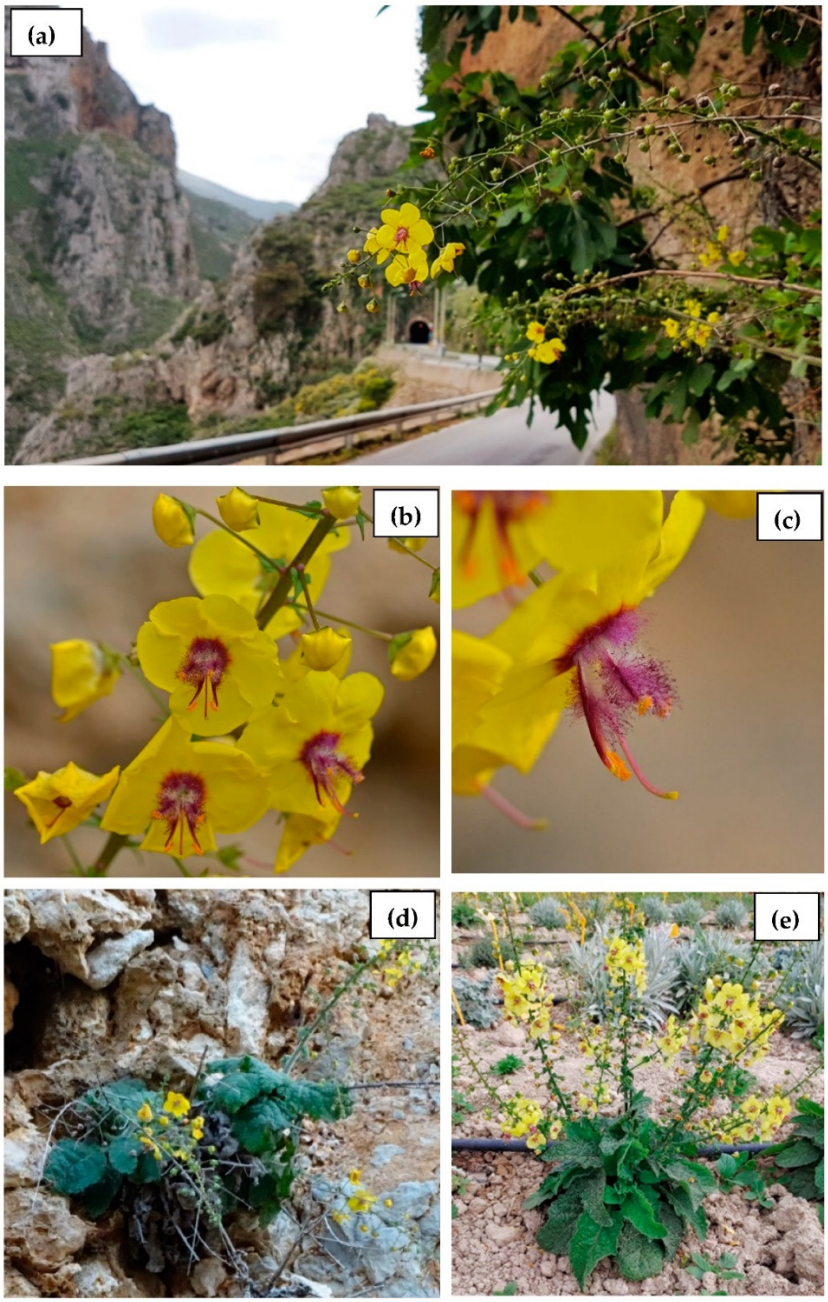

Figure 1. Aspects of the vulnerable local endemic, *Verbascum arcturus* (Scrophulariaceae) of Crete (Greece), during flowering: (**a**) vertical rocky habitat (Topolia gorge, western Crete; Photo: E. Dariotis, reproduced with permission); (**b**,**c**) part of inflorescence with densely arranged bright yellow flowers, with colorful anthers and filaments (Photos: F. Samaritakis, reproduced with permission); (**d**) growth habit of a wild-growing individual on a natural rocky substrate; (**e**) cultivated individual in the experimental field (Heraklion, Crete) before harvesting.

2.3. Soil Analysis of the Experimental Field

Soil surface (0–20 cm) sub-samples were collected from 10 different random points of the experimental field and mixed to form a composite soil sample. Then, the soil sample was passed through a 2 mm sieve and was analyzed in triplicate for the following prop-

erties, mentioned hereafter. Particle size distribution was determined by the hydrometer method [20]. Organic C was determined by the wet oxidation method [21], total N by the Kjeldahl method [22], and $CaCO_3$ by a calcimeter. The pH was measured in a 1:2 (*w*/*v*) suspension with water, the electrical conductivity was measured in the saturation extract (EC_{se}), sodium absorption ratio (SAR) was calculated by the concentrations of the water-soluble Na, Ca and Mg [23], and cation exchange capacity (CEC) was determined by employing the $(Co(NH_3)_6)Cl_3$ method (ISO 23470). Soil-available NO_3-N and NH_4-N were extracted with 1 M KCl and were determined by ultraviolet spectrometry and the sodium salicylate–sodium nitroprusside method, respectively [24]. P was extracted with 0.5 M $NaHCO_3$, pH 8.5, and was determined by the molybdenum blue–ascorbic acid method [25], whereas K, Ca and Mg were extracted with 1 M CH_3COONH_4, pH 7 [26]; K was determined by flame photometry, and Ca and Mg by atomic absorption spectrometry. Soil-available Cu, Zn, Fe and Mn were extracted with DTPA [27] and were determined by atomic absorption spectrometry, and B was extracted with hot water and was determined by the azomethine-H method [28].

2.4. Fertilization Treatments

The cultivation of *V. arcturus* followed weekly fertilization treatments using water (control), conventional and integrated nutrient management fertilizers in liquid or soluble granule form, administered with foliar and soil applications. The foliar applications were performed using a 5 L plastic handheld sprayer (low pressure) until apparent wetness, and the soil applications were performed manually per plant via 100 mL from the nutrient solution. The semi-organic fertilizers were from the THEOFRASTOS Company, Industrial Area of Korinthos, GR-20100 Korinthos, Greece. These are made from high-quality edible raw materials and limited amounts of chemical solvents. More specifically, THEORUN is mainly a N-source liquid fertilizer with: N 17% *w*/*w*; P_2O_5 0% *w*/*w*; K_2O 1.5% *w*/*w*; organic matter 3.2% *w*/*w*; C/N 0.09; pH 9.1; diurea 0.26%; electrical conductivity 86 mS/cm (liquid extract (1‰)). THEOCAL is an organic calcium powder fertilizer that mainly contains Ca substances, a pH of 7.1, Ca 30% *w*/*w* and 30% organic matter. THEOFAST includes organic matter 4.4% *w*/*w* (plant extracts), a pH of 9.5 and electrical conductivity of 78 mS/cm (liquid extract (1‰)). THEOMASS is a biostimulant made from plant extracts of edible raw materials with 5.4% organic matter *w*/*w* (plant extracts), pH 9.4 and electrical conductivity 85 mS/cm (liquid extract (1‰)). The conventional fertilizers were all in soluble powder or granule form, except the liquid fertilizer for micronutrients (Plex Mix). The fertilization treatments of the pilot cultivation of *V. arcturus* involved:

A. Integrated Nutrient Management fertilization by foliar application (INM-fa): the nutrient solution consisted of THEORUN at 7 mL/L, THEOCAL at 1.5 g/L, THEOFAST at 5 mL/L, 10-47-10 (AGRI.FE.M. LTD Fertilizers, Greece) at 3.2 g/L, K_2SO_4 (0-0-52, AGRI.FE.M. LTD Fertilizers, Greece) at 2.07 g/L, micronutrients (Plex Mix, AGRI.FE.M. LTD Fertilizers, Greece) at 1.5 mL/L and $MgSO_4$ (Mg 25.6%, AGRI.FE.M. LTD Fertilizers, Greece) at 0.6 g/L.

B. Conventional fertilization by foliar application (ChF-fa): the nutrient solution consisted of NH_4NO_3 (34,4-0-0, Neofert®, Neochim PLC, Dimitrovgrad, Bulgaria) at 2.7 g/L, $Ca(NO_3)_2$ (NITROCAL, Agrohimiki, Greece) at 1.7 g/L, 10-47-10 at 3.2 g/L, K_2SO_4 (0-0-52) at 2.27 g/L, micronutrients Plex Mix at 1.5 mL/L and $MgSO_4$ (Mg 25.6%) at 0.6 g/L.

C. Control, with foliar and soil applications only using tap water (C).

D. Integrated nutrient management fertilization by soil application (INM-sa): the nutrient solution consisted of THEORUN at 7 mL/L, THEOCAL at 1.5 g/L, THEOMASS at 10 mL/L, 10-47-10 at 3.2 g/L, K_2SO_4 (0-0-52) at 2.1 g/L, micronutrients Plex Mix at 1.5 mL/L and $MgSO_4$ (Mg 25.6%) at 0.3 g/L.

E. Conventional fertilization by soil application (ChF-sa): the nutrient solution consisted of NH_4NO_3 (34.4-0-0) at 2.7 g/L, $Ca(NO_3)_2$ (NITROCAL) at 1.7 g/L, 10-47-10 at 3.2 g/L, K_2SO_4 (0-0-52) at 2.3 g/L, micronutrients, Plex Mix at 1.5 mL/L and $MgSO_4$ (Mg 25.6%) at 0.3 g/L.

F. Mixture of plant extracts as a biostimulant by soil application (MPE-sa): the nutrient solution consisted of THEOMASS at 10 mL/L.

2.5. Plant Measurements

Multiple plant measurements and organ (leaf, stem, inflorescence) measurements (invasive and non-invasive) were conducted in *V. arcturus*. Among replicate plants, sampling was randomly conducted. Leaf coloration and chlorophyll fluorescence were assessed at vegetative, early flowering, and full flowering stages, whereas the remaining measurements were limited to the full flowering stage. For the full flowering stage, non-invasive measurements were conducted 2–5 d prior to the destructive harvest (i.e., 30 June 2021). For leaf-level measurements, leaves were selected from the upper (toward the apex) one-third of the leaf-bearing nodes of the stem. These leaves had been grown under direct natural light, were fully expanded and devoid of obvious symptoms of either pathogen infection or insect damage. In all cases, the time between sampling and the initiation of the evaluation did not exceed 15 min. When this was not possible, samples were placed in vials, flash-frozen in liquid nitrogen, and then transferred to a freezer (−80 °C) for storage. Replicate leaves, stems or inflorescences were sampled from separate plants.

2.5.1. Non-Invasive Evaluation of Leaf Coloration at Three Growth Stages

Leaf coloration is affected by fertilization and has implications for both photosynthetic capacity and visually perceived quality [29]. In this study, it was evaluated by three different methods:

(i) Leaf SPAD value, approximating chlorophyll content, was determined by using a SPAD-502 (Konica Minolta Corp., Solna, Sweden).

(ii) The index of absorbance difference (I_{AD}) accurately evaluates fruit ripeness since it is closely associated with outer mesocarp chlorophyll content [30]. I_{AD} is computed as the difference between the absorbance values at 670 and 720 nm, near the chlorophyll at the absorbance peak [30]. The potential of I_{AD} in reflecting respective differences in leaves has not been evaluated. In this study, I_{AD} was determined in leaves by using a DA meter (TR DA Meter, T.R. Turoni, Italy).

(iii) Leaf color was determined by using a Chroma Meter (Model CR-400, Minolta Corp., Japan). CIE L*a*b* coordinates were recorded using D65 illuminants and a 10° Standard Observer as a reference system. Measurements of L* (characterizing lightness and ranging from 0 (black) to 100 (white)), a* (expressing intensity in the range of green to red, where negative values refer to green and positive values to red), and b* (representing intensity in the range of blue to yellow, where negative values refer to blue and positive values to yellow) were obtained [31]. These measurements were conducted in situ at vegetative, early flowering, and full flowering stages. Three points were recorded per replicate leaf and were further averaged. These points were located midway between the leaf base and tip, as well as between the midrib and lateral margin. Three replicate leaves were assessed per treatment.

2.5.2. Non-Invasive Evaluation of Photosynthetic Performance at Three Growth Stages

As a sensitive indicator of leaf photosynthetic performance [32,33], the ratio of variable to maximum chlorophyll fluorescence (F_v/F_m) was recorded. Measurements in *V. arcturus* leaves were conducted by using a chlorophyll fluorometer (OS-30P, Opti-Sciences, Hudson, NH, USA). By employing leaf clips, leaves were dark-adapted (≥20 min) prior to evaluation. Then, F_v/F_m was determined by applying a saturated photosynthetic photon flux density of 3000 μmol m^{-2} s^{-1}. These measurements were conducted in situ in the morning (8 a.m.–10 a.m.) at vegetative, early flowering, and full flowering stages. Three points were recorded per replicate leaf and were further averaged. These points were located midway between the leaf base and tip, as well as between the midrib and lateral margin. Three replicate leaves were assessed per treatment.

2.5.3. Leaf Shape Indicators

Morphometric analysis was conducted by analyzing the leaf shape of *V. arcturus*. Leaf shape traits were derived from images acquired by a digital camera (Sony DSC-W830, Sony Corporation, Tokyo, Japan) under non-reflective glass from a distance of 0.5 m, employing a copy stand. Using specialized software (ImageJ; Wayne Rasband/NIH, Bethesda, MD, USA), leaf lamina outlines were processed to calculate the following four (dimensionless) metrics of leaf form: (a) aspect ratio {(major axis)/(minor axis); axes of the best-fitted ellipse}, (b) circularity $\{(4\pi \times \text{area})/(\text{perimeter})^2\}$, (c) roundness $\{(4 \times \text{area})/\{4\pi \times (\text{major axis})^2\}\}$, and (d) solidity {(area)/(convex area)} [34,35]. Each metric captures a distinct aspect of leaf shape. Aspect ratio and roundness are influenced by the length to width ratio, while circularity and solidity are sensitive to serration and lobing [36]. Aspect ratio ranges from 1 (circle) to a value without upper bound (infinitely narrow). Roundness ranges from 0 (infinitely narrow) to 1 (circle). Circularity ranges from 0 (infinitely narrow) to 1 (circle). Solidity ranges from 0 to 1, being inversely related to boundary irregularities. Solidity is sensitive to leaves with deep lobes or a distinct petiole and can be used to detect leaves lacking such structures [36]. Solidity, unlike circularity, is not greatly affected by serrations and minor lobing [36]. These measurements were performed on samples obtained on 30 June 2021. Thirty leaves were analyzed per treatment.

2.5.4. Plant Growth, Morphology and Biomass Allocation

Plant growth, morphology and biomass allocation were determined in cultivated *V. arcturus* and leaf, stem and inflorescence (fresh and dry) masses were recorded ($\pm$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. For leaf area assessment, leaves were imaged (Sony DSC-W830, Sony Corporation, Tokyo, Japan) and were then evaluated using specialized software (ImageJ; Wayne Rasband/NIH, Bethesda, MD, USA) [34,35]. Specific leaf area (SLA; leaf area/leaf mass), as well as above-ground biomass allocation to leaves, stem and generative organs, were calculated. The destructive harvest was carried out on 30 June 2021. Three replicate plants were evaluated per treatment.

2.5.5. Leaf Photosynthetic Pigment Content

Following fine chopping, leaf portions weighing 0.5 g were homogenized with the addition of 10 mL of 80% acetone. This primary acetone extract was then filtered, and the filtered extract was diluted by adding 2 mL of 80% acetone per mL of extract. Since chlorophyll is light-sensitive, the extraction took place in a dark room [37]. The obtained extract was subjected to reading on a spectrophotometer (Mapada UV-1800; Shanghai. Mapada Instruments Co., Ltd., Shanghai, China). Total chlorophyll and carotenoid contents were calculated [38]. These measurements were performed on samples obtained on 30 June 2021. Three leaves were assessed per treatment. For each replicate, four samples (collected from separate plants) were pooled, and the assay was performed twice.

2.5.6. Leaf Total Phenolic and Total Flavonoid Contents

Phenols and flavonoids are important non-enzymatic antioxidants [37,39]. Samples (0.1 g) were extracted with 1 mL of 80% aqueous methanol in an ultrasonic bath (10 min) and were then centrifuged (15,000 *g* for 10 min). The total phenolic and total flavonoid contents were determined using the Folin–Ciocalteu assay and aluminum chloride colorimetric assay, respectively, following the protocol of Chen et al. (2021) [39]. The absorbance against a prepared reagent blank was determined using a microplate reader (Infinite 200 PRO, TECAN, Männedorf, Switzerland). For total phenolic content, gallic acid was used as the standard reference and gallic acid equivalent (GAE) was expressed as mg per g fresh mass. For total flavonoid content, rutin was used as the standard reference and rutin equivalent (RUE) was expressed as mg per g of fresh mass. These measurements were performed on samples obtained on 30 June 2021. Three leaves were assessed per treatment. For each

replicate, four samples (collected from separate plants) were pooled, and the assay was performed twice.

2.5.7. Leaf Soluble Sugar Content

Samples (0.1 g) were incubated with 1 mL deionized water in a water bath (100 °C for 30 min). The homogenate was centrifuged (15,000× *g* for 15 min) at room temperature (25 °C). Then, 0.1 mL of the solution was mixed with anthranone ethyl acetate and H_2SO_4. Soluble sugar content was assayed in the supernatant by measuring the absorbance at 630 nm, using a spectrophotometer (Mapada UV-1800; Mapada Instruments Co., Ltd., Shanghai, China), according to the method of Dubois et al. (1956) [40]. Soluble sugar content was expressed per fresh weight basis (mg g^{-1}). These measurements were performed on samples obtained on 30 June 2021. Measurements were conducted on three replicates per treatment. For each replicate, four samples (collected from separate plants) were pooled, and the assay was performed twice.

2.5.8. Leaf, Stem and Floral Analysis

To assess the role of fertilization in nutrient absorption by plants, leaf, stem and floral mineral analysis was conducted. Samples were washed with distilled water and then dried. The biomass was dried at 70 °C, weighed, ground, and then analyzed for total N using the Kjeldahl method [22]. In addition, sub-samples were ashed at 500 °C for at least 4 h [41]; the ash was dissolved in 2 M HCl, filtered, and P, K, Ca, Mg, Cu, Zn, Fe, Mn and B were determined in the filtrate, employing the methods of analytical determinations reported previously. Mineral content was expressed per dry weight basis. These measurements were performed on samples obtained on 30 June 2021. Three replicates were evaluated per treatment. For each replicate, four samples (collected from separate plants) were pooled, and the assay was performed twice.

2.6. Statistical Analysis

Data were tested for homogeneity of variances (Duncan's test). For each parameter, an analysis of variance (ANOVA) was conducted, using the SAS statistical software (SAS Institute, Cary, NC, USA) and the LSD test, at $p = 0.05$, was used for mean comparisons.

3. Results

3.1. Soil Characteristics of the Experimental Field

The soil of the experimental field was sandy loam in texture, alkaline in reaction and calcareous, with low organic C content, EC_{se} and SAR (Table S1). Furthermore, the soil had a high content of residual NO_3-N [42] and available P and K concentrations above the sufficiency levels of 10 mg kg^{-1} [43]) and 170–250 mg kg^{-1} [44], respectively. As far as the soil-available micronutrients are concerned, they ranged at levels higher (Cu) or similar to the sufficiency levels (the rest of the micronutrients) reported by Sims and Johnson (1991) (i.e., 0.1–2.5, 0.2–2.0, 2.5–5.0, 1.0–5.0, and 0.1–2.0 mg kg^{-1}, for Cu, Zn, Fe, Mn and B, respectively) (Table S1) [45].

3.2. Leaf Colour

At three growth stages of *V. arcturus*, the effect of fertilization on leaf color (a critical herb quality feature) was assessed using three methods (SPAD meter, DA meter, Chroma Meter). In this way, the SPAD value, I_{AD} (index of absorbance difference), L* (lightness), a* (intensity in the green to red range), and b* (intensity in the blue to yellow range) were determined. The results showed that none of these color parameters was affected by the fertilization (Figures 2 and S1–S4). The only exception to this trend was the significantly higher SPAD value in the leaves of vegetative-stage plants fertilized through the root, as compared to control plants and plants fertilized through a foliar application (Figure 2A).

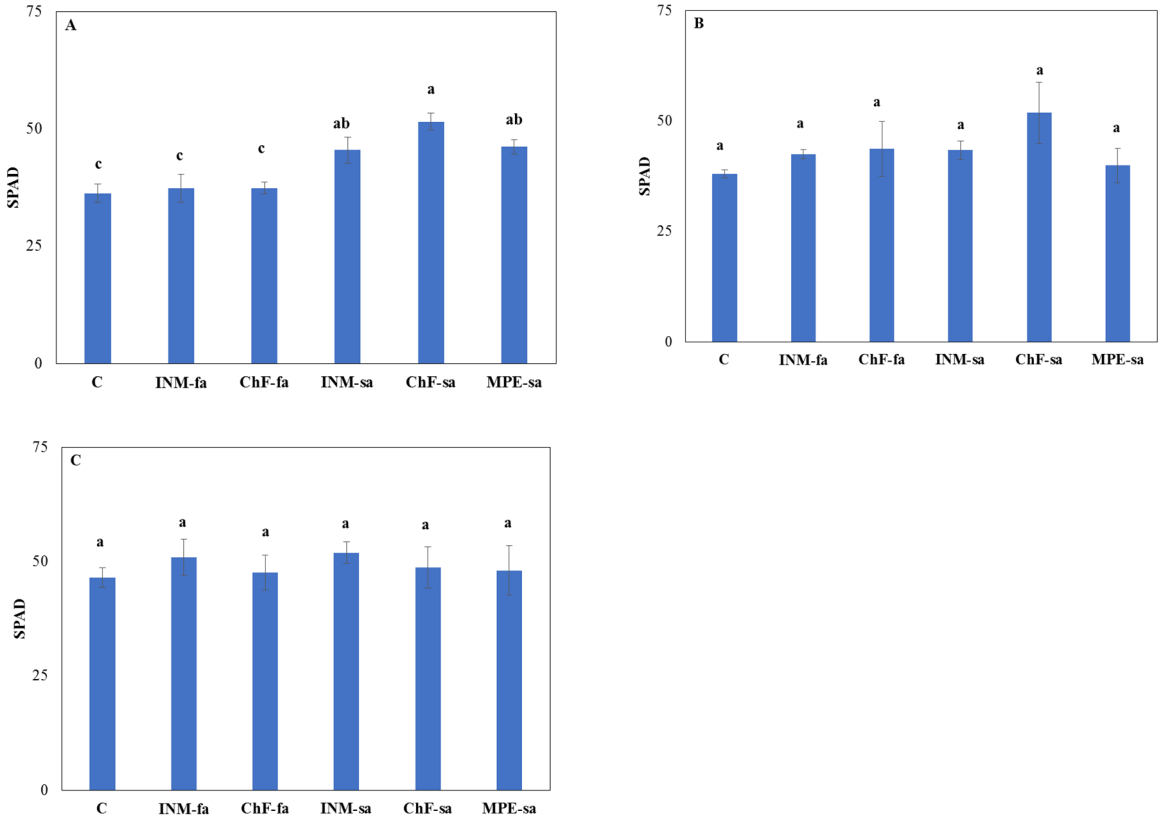

Figure 2. Effect of fertilization through different (root/foliar) application methods on the leaf SPAD value of *Verbascum arcturus* at (**A**) vegetative; (**B**) early flowering; (**C**) full flowering stages. C: Control (only water); INM-fa: integrated nutrient management fertilization by foliar application; ChF-fa: conventional fertilization by foliar application; INM-sa: integrated nutrient management fertilization by soil application; ChF-sa: conventional fertilization by soil application; MPE-sa: mixture of plant extracts as a biostimulant by soil application. Values represent the mean of three replicates ± SEM. Within each plot, different letters indicate significant differences.

3.3. Leaf Photosynthetic Performance

At three growth stages of *V. arcturus*, the effect of fertilization on overall photosynthetic functionality was assessed by determining F_v/F_m (Figure 3). At the vegetative stage, the leaves of plants receiving the integrated nutrient management (INM) fertilizers through foliar application showed the significantly highest F_v/F_m value compared to the rest of the treatments (Figure 3A). However, at the other two flowering stages, the results were rather inconclusive since no clearly significant differences were observed among treatments (Figure 3B,C).

3.4. Leaf Shape

The leaf shape response to fertilization was assessed in the cultivated plants of *V. arcturus* and four metrics (aspect ratio, circularity, roundness, solidity) were determined, each encompassing a distinct leaf-shape aspect. None of the shape metrics responded to fertilization (data not shown).

3.5. Plant Growth, Morphology and Biomass Allocation

Leaf area, leaf thickness (as approximated by SLA), above-ground dry mass, and dry mass partitioning were not affected by fertilization in *V. arcturus* (Figures 4 and 5).

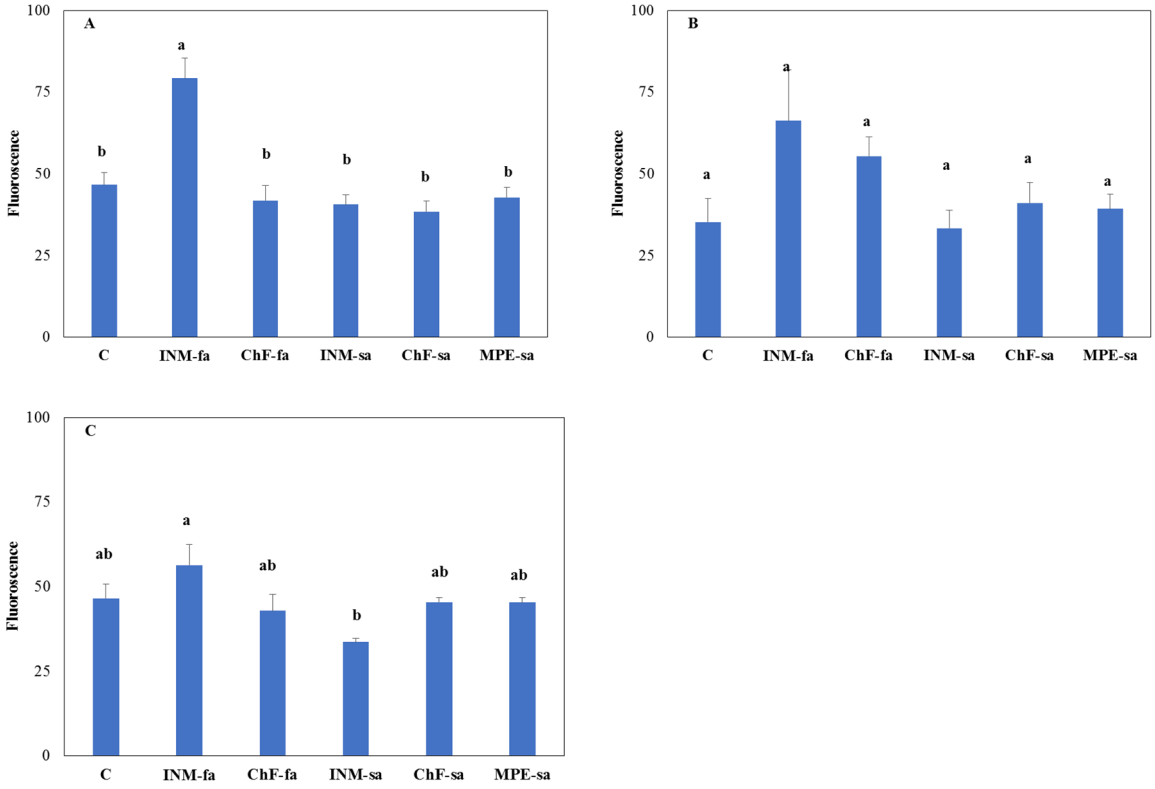

Figure 3. Effect of fertilization through different (root/foliar) application methods on the leaf chlorophyll fluorescence value of *Verbascum arcturus* at (**A**) vegetative; (**B**) early flowering; (**C**) full flowering stages. C: control (only water); INM-fa: integrated nutrient management fertilization by foliar application; ChF-fa: conventional fertilization by foliar application; INM-sa: integrated nutrient management fertilization by soil application; ChF-sa: Conventional fertilization by soil application; MPE-sa: mixture of plant extracts as a biostimulant by soil application. Values represent the mean of three replicates ± SEM. Within each plot, different letters indicate significant differences.

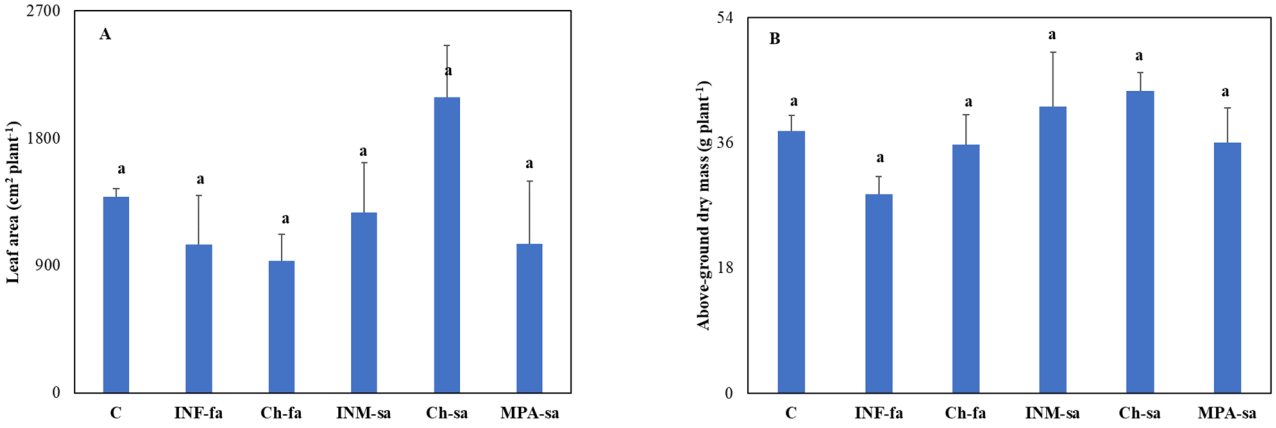

Figure 4. Effect of fertilization through different (root/foliar) application methods on (**A**) plant leaf area; (**B**) above-ground dry mass of *Verbascum arcturus*. C: control (only water); INM-fa: integrated nutrient management fertilization by foliar application; ChF-fa: conventional fertilization by foliar application; INM-sa: integrated nutrient management fertilization by soil application; ChF-sa: conventional fertilization by soil application; MPE-sa: mixture of plant extracts as a biostimulant by soil application. Values represent the mean of six replicates ± SEM. Within each plot, different letters indicate significant differences.

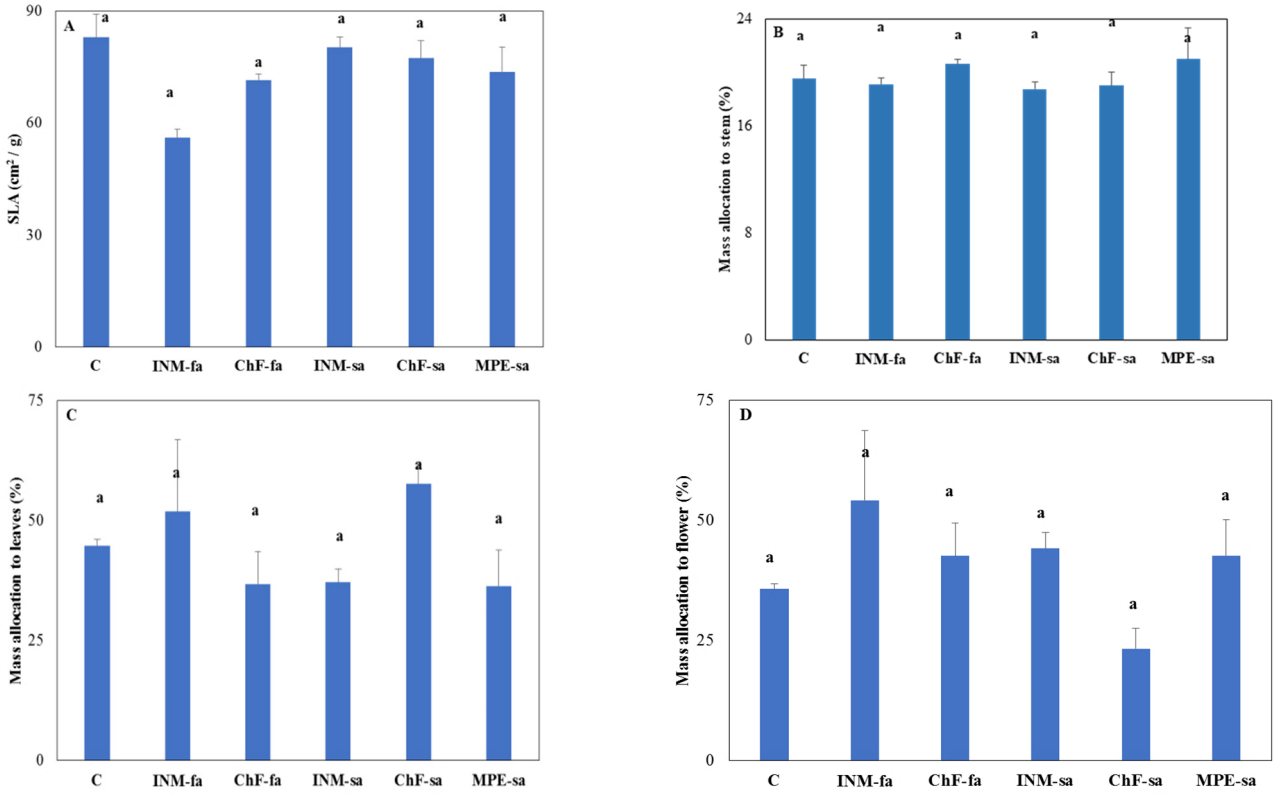

Figure 5. Effect of fertilization through different (root/foliar) application methods on (**A**) specific leaf area (SLA); (**B**) above-ground dry mass partitioning to stem; (**C**) to leaves; and (**D**) to flowers of *Verbascum arcturus*. C: control (only water); INM-fa: integrated nutrient management fertilization by foliar application; ChF-fa: conventional fertilization by foliar application; INM-sa: integrated nutrient management fertilization by soil application; ChF-sa: conventional fertilization by soil application; MPE-sa: mixture of plant extracts as a biostimulant by soil application. Values represent the mean of six replicates ± SEM. Within each plot, different letters indicate significant differences.

3.6. Leaf Chlorophyll Content

Plants receiving integrated nutrient management fertilizers by foliar application showed the highest leaf chlorophyll content, followed by those receiving conventional fertilizers or a biostimulant by root application (Figure 6A).

3.7. Leaf Antioxidant Compound Content

Plants of *V. arcturus* receiving INM fertilizers by foliar application showed higher leaf carotenoid content than the control plants and were similar to plants receiving conventional fertilizers or a biostimulant by soil application (Figure 6B).

Plants of *V. arcturus* receiving INM fertilizers in combination with a biostimulant by soil application presented the highest total phenolic content, followed by those treated with INM fertilizers by foliar application (Figure 6C).

Plants receiving INM fertilizers by foliar application or conventional fertilizers or a biostimulant by soil application showed the highest total flavonoid content (Figure 6D), while plants receiving INM fertilizers in combination with a biostimulant by soil application had the lowest flavonoid content (Figure 6D).

3.8. Leaf Soluble Sugar Content

Control plants of *V. arcturus* had the highest leaf soluble sugar content, compared to all other treatments (Figure 7).

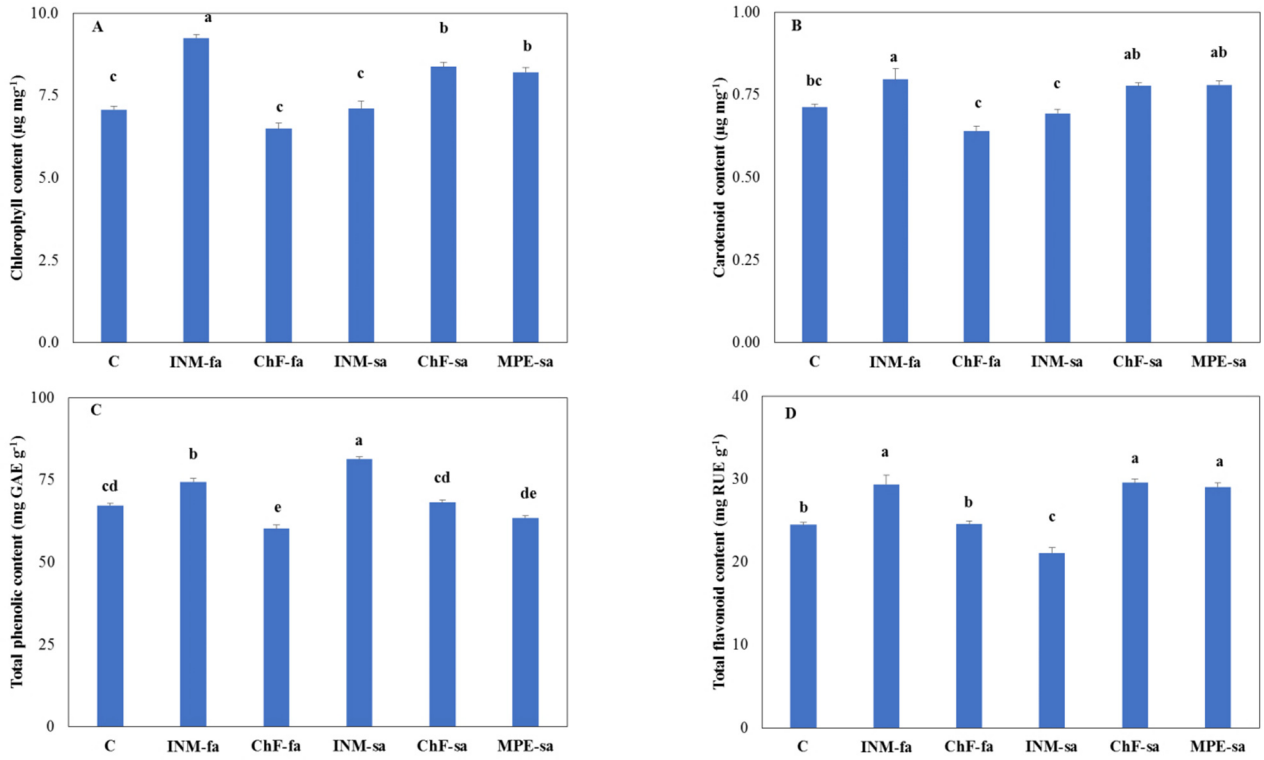

Figure 6. Effect of fertilization through different (root/foliar) application methods on (**A**) leaf chlorophyll; (**B**) carotenoids; (**C**) total phenol; (**D**) total flavonoid content of *Verbascum arcturus*. C: control (only water); INM-fa: integrated nutrient management fertilization by foliar application; ChF-fa: conventional fertilization by foliar application; INM-sa: integrated nutrient management fertilization by soil application; ChF-sa: conventional fertilization by soil application; MPE-sa: mixture of plant extracts as a biostimulant by soil application. Values represent the mean of three replicates ± SEM. Within each plot, different letters indicate significant differences.

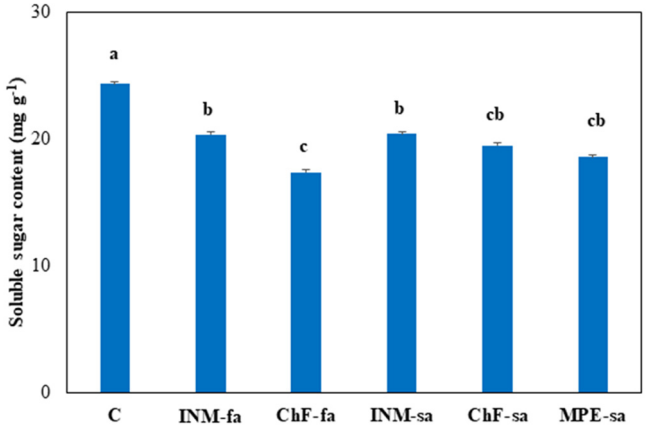

Figure 7. Effect of fertilization through different (root/foliar) application methods on leaf soluble sugar content of *Verbascum arcturus*. C: control (only water); INM-fa: integrated nutrient management fertilization by foliar application; ChF-fa: conventional fertilization by foliar application; INM-sa: integrated nutrient management fertilization by soil application; ChF-sa: conventional fertilization by soil application; MPE-sa: mixture of plant extracts as a biostimulant by soil application. Soluble protein content was expressed per fresh weight basis. Values represent the mean of three replicates ± SEM. Within each plot, different letters indicate significant differences.

3.9. Leaf, Stem and Floral Mineral Analysis

N concentrations in the leaves of plants grown under conventional or INM fertilization, in combination with a biostimulant applied in the soil, were higher than the other

treatments and similar to control. The lowest K concentration was observed with the application of INM fertilizers by foliar spray, whereas the highest Ca concentration was obtained with a biostimulant applied to the roots. Fertilization treatment did not affect leaf P and Mg contents in *V. arcturus* (Table 1).

Table 1. Effect of fertilization through two (root/foliar) application methods on leaf essential macronutrient content of *Verbascum arcturus*. C: control (only water); INM-fa: integrated nutrient management fertilization by foliar application; ChF-fa: conventional fertilization by foliar application; INM-sa: integrated nutrient management fertilization by soil application; ChF-sa: conventional fertilization by soil application; MPE-sa: mixture of plant extracts as a biostimulant by soil application. Mineral content was expressed per dry weight basis. Values represent the mean of six replicates ± SEM.

Treatment	N	P	K	Ca	Mg
			(g kg^{-1})		
C	14.3 ± 0.3 a	1.6 ± 0.0 a	14.9 ± 0.1 b	15.6 ± 0.1 b	2.5 ± 0.1 a
INM-fa	5.6 ± 0.0 d	1.7 ± 0.0 a	12.4 ± 0.2 c	17.6 ± 0.9 b	2.2 ± 0.1 a
ChF-fa	9.2 ± 0.7 cd	1.9 ± 0.0 a	18.0 ± 0.5 a	17.9 ± 0.4 b	2.3 ± 0.0 a
INM-sa	14.0 ± 0.3 a	1.7 ± 0.0 a	16.5 ± 0.2 ab	18.0 ± 0.2 b	2.4 ± 0.1 a
ChF-sa	13.5 ± 0.6 ab	1.7 ± 0.0 a	16.0 ± 0.3 ab	15.9 ± 0.4 b	2.3 ± 0.1 a
MPE-sa	9.7 ± 0.6 bc	1.7 ± 0.0 a	17.2 ± 0.3 ab	23.4 ± 0.2 a	2.5 ± 0.1 a
p F-test	0.034	NS	0.049	0.042	NS

Within each column, different letters indicate significant differences. NS: Non-significant.

Conventional or INM fertilization by foliar spray increased leaf Zn and B contents compared to control plants. The conventional fertilizers, applied by foliar spray, increased leaf Mn content compared to control plants, whereas the other two micronutrients were not affected by treatment (Table 2).

Table 2. Effect of fertilization through different (root/foliar) application methods on leaf essential micronutrients content of *Verbascum arcturus*. C: Control (only water); INM-fa: Integrated nutrient management fertilizers by foliar application; ChF-fa: Conventional fertilization by foliar application; INM-sa: Integrated nutrient management fertilizers by soil application; ChF-sa: Conventional fertilization by soil application; MPE-sa: Mixture of plant extracts as biostimulant by soil application. Mineral content was expressed per dry weight basis. Values represent the mean of six replicates ± SEM.

Treatment	Cu	Zn	Fe	Mn	B
			(mg kg^{-1})		
C	15.9 ± 0.6 a	21.2 ± 0.6 b	1085 ± 53 a	48.7 ± 2.5 bc	35.3 ± 0.5 bc
INM-fa	14.5 ± 0.9 a	33.2 ± 2.0 a	1041 ± 62 a	60.9 ± 4.0 ab	41.1 ± 0.9 a
ChF-fa	16.5 ± 0.8 a	28.9 ± 0.5 a	1158 ± 67 a	73.4 ± 3.6 a	40.4 ± 0.4 a
INM-sa	15.1 ± 0.9 a	19.2 ± 0.9 b	999 ± 30 a	45.6 ± 0.5 bc	34.1 ± 0.2 c
ChF-sa	13.7 ± 0.5 a	18.5 ± 0.6 b	1064 ± 45 a	39.2 ± 1.1 c	35.4 ± 0.6 bc
MPE-sa	14.0 ± 0.3 a	20.6 ± 0.2 b	1017 ± 41 a	50.5 ± 1.9 bc	38.6 ± 0.7 ab
p F-test	NS	<0.001	NS	0.016	0.027

Within each column, different letters indicate significant differences. NS Non-significant.

The combination of a biostimulant and INM applied through the root resulted in higher floral P content compared to INM fertilizers by foliar spray, and conventional fertilizers by root application, and were similar to control (Table 3). Floral N and K contents, as well as stem N, P, and K contents, were not affected by fertilization.

Table 3. Effect of fertilization through different (root/foliar) application methods on inflorescences and stem essential macronutrient content of *Verbascum arcturus*. C: control (only water); INM-fa: integrated nutrient management fertilization by foliar application; ChF-fa: conventional fertilization by foliar application; INM-sa: integrated nutrient management fertilization by soil application; ChF-sa: conventional fertilization by soil application; MPE-sa: mixture of plant extracts as a biostimulant by soil application. Mineral content was expressed per dry weight basis. Values represent the mean of six replicates ± SEM.

	Inflorescence			Stem		
Treatment	**N**	**P**	**K**	**N**	**P**	**K**
			(g kg^{-1})			
C	16.3 ± 1.6 a	2.4 ± 0.1 ab	11.9 ± 0.8 a	9.1 ± 0.1 a	3.7 ± 0.3 a	17.6 ± 2.0 a
INM-fa	14.8 ± 2.7 a	1.2 ± 0.5 b	9.4 ± 0.8 a	8.9 ± 0.1 a	2.2 ± 0.9 ab	20.5 ± 2.1 a
ChF-fa	13.4 ± 1.0 a	2.4 ± 0.4 ab	11.8 ± 0.7 a	8.3 ± 0.5 a	1.7 ± 0.2 b	21.5 ± 3.6 a
INM-sa	15.7 ± 0.8 a	3.0 ± 0.4 a	13.1 ± 0.4 a	10.3 ± 1.2 a	2.3 ± 0.6 ab	22.3 ± 2.2 a
ChF-sa	16.9 ± 2.2 a	1.6 ± 0.3 b	7.5 ± 3.0 a	9.3 ± 0.9 a	1.7 ± 0.3 b	19.2 ± 3.0
MPE-sa	13.6 ± 0.7 a	2.1 ± 0.1 ab	12.9 ± 0.9 a	9.0 ± 1.8 a	2.2 ± 0.5 ab	20.2 ± 4.5
p F-test	NS	0.031	NS	NS	NS	NS

Within each column, different letters indicate significant differences. NS: Non-significant.

4. Discussion

Verbascum arcturus is cultivated herein for the first time in an agricultural setting, in Heraklion, Crete (Greece), which belongs to the territory where the taxon is native and to which it is confined (local endemic). The studied species is a perennial wild-growing chasmophyte of Crete, found on calcareous cliffs and rocks, walls, ravines and boulders. Being almost exclusively a rock-dwelling plant and range-restricted, mainly in the western or western-central part of the island, from sea level up to 900 m [46], it is characterized as Vulnerable according to the criteria of the International Union for the Conservation of Nature (IUCN) and, thus, is threatened with extinction [18]. Domesticating rare and threatened wild-growing plants such as *V. arcturus* and introducing them into agriculture is a sustainable strategy to alleviate the impact of uncontrolled and excess harvesting of wild populations creating habitat alterations [3–5].

From a commercial point of view, *V. arcturus* is assessed as a potential medicinal plant [47] due to the fact that members of the genus *Verbascum*, such as *V. thapsus* L., *V. densiflorum* Bertol., and *V. phlomoides* L. have long been used for tea preparations and syrups made with the inflorescences (and/or sometimes leaves), and are currently considered as traditional herbal medicinal products with approved use by the European Medicines Agency; they are considered able to relieve the symptoms of a sore throat associated with a dry cough and cold [48]. However, it is not known whether the inflorescences of *V. arcturus* are locally collected in Crete for medicinal purposes. Although *V. arcturus* is associated with limited agro-alimentary interest [7], it is recommended as being suitable for home gardening, landscaping and xeriscaping applications, or as a pot/patio plant [4]. This is mainly due to its large leaves (8–15 × 2–5 cm) forming dense rosettes, its long flowering period (March until June, sometimes with limited blossom again in autumn) and its large flowers (25–30 mm in diameter) which are almost compactly arranged in bear-tail-like inflorescence, densely covered with glands and soft hair [46]. To date, *V. arcturus* has been associated with the satisfactory adaptation to man-made conditions, resulting in almost average potential in terms of sustainable exploitation feasibility that can only be expected in the long term [4]. However, this is mainly a consequence of scarcely applied research for this taxon to date, especially in terms of species-specific propagation methods [20], as well as cultivation and fertilization guidelines [4]. To the best of our knowledge, this is the first attempt to cultivate *V. arcturus* (a local endemic to Crete, Greece) involving the development of a fertilization protocol for optimal plant growth and herbal quality. The possibility of cultivating this species to date was pursued as an effective eco-friendly practice that bridges existing knowledge gaps effectively, thus enabling sustainable exploitation strategies to be implemented not in the long term, as previously reported [4], but much

sooner. In this way, this study fruitfully speeds up the sustainable exploitation potential of the targeted neglected and underutilized local endemic plant, through experimental proof of concept to know-how demonstrated in a relevant (agricultural) environment.

Due to the high initial concentrations of the soil-available macro- and micronutrients (higher than the sufficiency levels) (Table S1), the conventional or integrated nutrient management fertilization generally exerted no significant effects on plant growth, morphology, and dry aboveground biomass yield of *V. arcturus* (Figure 4), or on dry biomass partitioning to leaves, stems and inflorescences (Figure 5). All macro- and micronutrient concentrations in the leaves were above those measured from wild-growing samples of *V. arcturus*, except for B and Zn (unpublished data). In addition, none of the fertilizers increased the concentrations of N and P in leaves above that of the control (Table 1). The lowest leaf K concentrations were obtained with the integrated nutrient management (INM) fertilization, applied by foliar spray. Leaf Ca was only increased by the soil-applied biostimulant. Regarding the micronutrients, foliar applications of conventional or INM fertilization were the only ones that increased Zn, a nutrient that affects not only flowering [49] but also B content (Table 2). Probably, foliar applications in *V. arcturus* should be preferred to promote micronutrient absorption; however, more research is certainly needed to verify this trend over consecutive cultivation years.

The rhizosphere is a dynamic region of plants governing nutrient uptake and affecting crop yield and product quality [50]. Biostimulants are known to increase plant growth and development compared to conventional fertilizers [51]. The use of the biostimulant in *V. arcturus* as an individual treatment and in combination with INM fertilization revealed some positive effects during the field cultivation of *V. arcturus*. Leaf Ca was increased to 50% by the soil-applied biostimulant compared to control, and from 30 to 47.2% when compared to other fertilization treatments. Although there are different mechanisms involved in the mode of action of biostimulants [52], the exact mode of action of this particular biostimulant is still unknown. In general, agricultural biostimulants include diverse formulations of substances and other products that act through different pathways on the physiology of the plant and are able to improve crop robustness and increase production, quality and post-harvest shelf life [53]. The importance of Ca absorption by plants has been extensively emphasized in the literature as an essential plant nutrient for structural roles in the cell wall and membranes, as a counter-cation for inorganic and organic anions in the vacuole, and as an intracellular messenger in the cytosol [54].

The quality of medicinal herbal material and the plants' ornamental value largely depend on their intended use and include visual quality features [15]. Most often, retailers' and consumers' preferences are satisfied by dark green plants, while pale green leaf color in ornamental plants is not desirable, being perceived as a sign of environmental stress, nutrient deficiency and possibly leaf senescence, decreasing the plants' ornamental value [29,37,55]. The greenness intensity and uniformity are conventionally used as a quality measure throughout market distribution and value chains [15,56]. The results of this study indicate that, generally, the leaf coloration of *V. arcturus*, when assessed using three methods (SPAD meter, DA meter, Chroma Meter), was not affected by fertilization treatment (Figure 2 and Figures S1–S4), except for SPAD at the vegetative stage, which significantly increased upon the soil application of all kinds of fertilizers (conventional or INM or a biostimulant) (Figure 2A). In other species, leaf greenness was stimulated by using organic fertilizers [17]. Fertilization also did not affect leaf shape, as captured by four diverse metrics (Figure 3). Taken together, these results indicate that visual quality features were independent of fertilization treatment.

A key aspect of herbal material quality is the content of health-promoting compounds [9]. Carotenoids, phenolics and flavonoids are important natural antioxidants, stimulating the plant's ability to cope with stress; when these are consumed, they have beneficial properties promoting human health [36,38]. Apart from a single report derived from a general screening for antioxidant and anti-melanogenic properties [8,57–59], this study reports herein for the first time an assessment of total phenolic and flavonoid contents in

V. arcturus. Although optimal fertilization depends on the targeted secondary metabolite, when considering all three antioxidants together, INM fertilizer use by foliar application and soil application of the conventional fertilizers or biostimulants appear to be accepted options (Figure 6B–D). The content of plant secondary metabolites, including total phenols and flavonoids, was also found to be elevated via the use of organic fertilizers in other species [16]. As the current field experimentation was limited to a single growing season, further research is certainly needed to obtain solid results and conclusions. However, the findings of this study could speed up the cultivation and the sustainable exploitation of the previously non-studied, neglected and underutilized *V. arcturus*, serving as a starting point for its fertilization to produce key quality traits.

5. Conclusions

This study focused on *Verbascum arcturus*, a potentially ornamental and medicinal wild-growing plant that is exclusively confined to the island of Crete, Greece. This species has been recently domesticated and is cultivated herein for the first time when the effect of fertilization treatment on plant growth and herbal quality was initially addressed. In this pilot field experimental study, the tested conventional and integrated nutrient management fertilizers via two (foliar/root) application methods have shown that fertilization generally exerted limited effects on plant growth, morphology, biomass partitioning and nutrient contents, potentially owing to high soil fertility at the experimental site. However, both kinds of foliar-applied fertilization enhanced the Zn and B content in leaves, and a soil-applied biostimulant increased leaf Ca content. Fertilization did not alter the visually perceived herbal material quality (color, shape). Interestingly, not only foliar application of the integrated nutrient management fertilizers but also soil application of the conventional fertilizers or a biostimulant generally resulted in higher leaf antioxidant compound content than in the control. The findings of this field study may facilitate the sustainable exploitation of *V. arcturus* in the near future.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/su132414030/s1, Figure S1: Effect of fertilization through different (root/ foliar) application methods on leaf index of absorbance difference of *Verbascum arcturus* at (**a**) vegetative; (**b**) early flowering; (**c**) full flowering stages, Figure S2: Effect of fertilization through different (root/ foliar) application methods on leaf L value of *Verbascum arcturus* at (**a**) vegetative; (**b**) early flowering, (**c**) full flowering stages, Figure S3: Effect of fertilization through different (root/ foliar) application methods on leaf a value of *Verbascum arcturus* at (**a**) vegetative; (**b**) early flowering, (**c**) full flowering stages, Figure S4: Effect of fertilization through different (root/ foliar) application methods on leaf b value of *Verbascum arcturus* at (**a**) vegetative; (**b**) early flowering, (**c**) full flowering stages, Figure S5: Effect of fertilization through different (root/ foliar) application methods on (**a**) above-ground fresh mass; (**b**) water content of *Verbascum arcturus*, Table S1: Experimental field soil properties of the research garden of the Hellenic Mediterranean University, used for the fertilization trials on *Verbascum arcturus*.

Author Contributions: Conceptualization, G.T. (Georgios Tsoktouridis) and N.K.; methodology, K.P., D.F., G.T. (Georgios Tsaniklidis), V.A.T., F.B., E.S., K.K., F.J.D., I.I., G.T. (Georgios Tsoktouridis) and T.M.; software, K.P., D.F., G.T. (Georgios Tsaniklidis), V.A.T., I.I. and T.M.; validation, D.F., V.A.T., F.B., E.S., K.K., F.J.D., I.I., T.M. and N.K.; formal analysis, K.P., D.F., G.T. (Georgios Tsaniklidis), V.A.T., F.B., E.S., I.I. and T.M.; investigation, K.P., D.F., G.T. (Georgios Tsoktouridis), V.A.T., F.B., E.S., K.K., F.J.D., I.I., G.T. (Georgios Tsaniklidis), T.M.; resources, K.P., T.M., I.I. and G.T. (Georgios Tsoktouridis); data curation, K.P., D.F., G.T. (Georgios Tsaniklidis), V.A.T., F.B., E.S., K.K., F.J.D., I.I., G.T. (Georgios Tsoktouridis) and T.M.; writing—original draft preparation, K.P., D.F., G.T. (Georgios Tsoktouridis), V.A.T. and N.K.; writing—review and editing, K.P., D.F., G.T. (Georgios Tsoktouridis), V.A.T., F.B., E.S., K.K., F.J.D., I.I., G.T. (Georgios Tsaniklidis), T.M. and N.K.; visualization, V.A.T., D.F. and N.K.; supervision, T.M., D.F., G.T. (Georgios Tsoktouridis); project administration, G.T. (Georgios Tsoktouridis); funding acquisition, K.P., N.K., T.M. and G.T. (Georgios Tsoktouridis). All authors have read and agreed to the published version of the manuscript.

Funding: This research has been co-financed by the European Union and Greek national funds through the Operational Program Competitiveness, Entrepreneurship and Innovation, under the call RESEARCH—CREATE—INNOVATE (project code: T1EDK-05380), entitled "Conservation and sustainable utilization of rare threatened endemic plants of Crete for the development of new products with innovative precision fertilization".

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

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

Acknowledgments: We are grateful to the laboratory staff and the undergraduate students Eleutheria Liapaki, Sofia Vouraki, Menel Ben Saleh Jouini, Mihail Kokotakis, Konstantinos Dermitzakis, Haralampos Liapakis, Emmanouella Vasilaki, Ioannis Papadakis, Emmanouil Haralampakis and Georgios Stylianakis of the Hellenic Mediterranean University for their contributions, continued diligence, and dedication to their craft. The valuable comments of the editor and two anonymous reviewers are greatly appreciated.

Conflicts of Interest: The authors declare no conflict of interest.

Abbreviations

a*: Green to red range intensity; b*, blue to yellow range intensity; CEC, cation exchange capacity; EC_{se}, electrical conductivity of the saturation extract; F_v/F_m, ratio of variable to maximum chlorophyll fluorescence; GAE, gallic acid equivalent; IAD, index of absorbance difference; L*, lightness; RUE, rutin equivalent; SAR, sodium absorption ratio; SLA, specific leaf area.

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