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Konstantinos A Paschalidis *, Dimitrios Fanourakis, Georgios Tsaniklidis, Ioannis Tsichlas, Vasileios A. Tzanakakis, Fotis Bilias, Eftichia Samara, Ioannis Ipsilantis, Katerina Grigoriadou, Ioulietta Samartza, Theodora Matsi, Georges Tsoktouridis *, Nikos Krigas

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Molecular DNA Barcoding and Fertilization Effects on Growth and Herbal Quality Features of Cultivated *Sideritis syriaca* subsp. *syriaca*, a Local Endemic Plant of Crete with High Medicinal Value

Konstantinos Paschalidis [1,*], Dimitrios Fanourakis [1†], Georgios Tsaniklidis [2†], Ioannis Tsichlas [1], Vasileios A. Tzanakakis [1], Fotis Bilias [3], Eftihia Samara [3], Ioannis Ipsilantis [3], Katerina Grigoriadou [4], Ioulietta Samartza [4], Theodora Matsi [3], Georgios Tsoktouridis [4,5,*] and Nikos Krigas [4]

[1] Department of Agriculture, School of Agricultural Sciences, Hellenic Mediterranean University, 71410, Heraklion, Crete, Greece; kpaschal@hmu.gr (K.P.); dfanourakis@hmu.gr; (D.F.); vtzanakakis@hmu.gr.t (V.A.T.); giannistsixlas@gmail.com (I.T.)

[2] Hellenic Agricultural Organization (ELGO-DIMITRA), Institute of Olive Tree, Subtropical Crops and Viticulture, 73134, Chania, Crete, Greece; G.T (Georgios Tsaniklidis)

[3] Soil Science Laboratory, School of Agriculture, Aristotle University of Thessaloniki, 54124 Thessaloniki, Greece; thmatsi@agro.auth.gr (T.M.); iipsi@agro.auth.gr (I.I.); fbilias@agro.auth.gr (F.B.); edsamara7@gmail.com (E.S.)

[4] Institute of Plant Breeding and Genetic Resources, Hellenic Agricultural Organization Demeter, P.O. Box 60458, Thermi, GR-57001 Thessaloniki, Greece; katgrigoriadou@elgo.gr (K.G.); nkrigas@elgo.gr (N.K.); gtsok@elgo.gr (Georgios Tsoktouridis); isamartza@gmail.com (I.S.)

[5] Theofrastos Fertilizers, Industrial Area of Korinthos, Irinis & Filias, Ikismos Arion, Examilia, 20100 Korinthos, Greece

† Equal contribution to this study.

* Correspondence: correspondence: Georgios Tsoktouridis (gtsok@elgo.gr) and Konstantinos Paschalidis (kpaschal@hmu.gr)

Abstract: Herein, we applied DNA Barcoding for the genetic characterization of *Sideritis syriaca* subsp. *syriaca* (Lamiaceae; threatened local Cretan endemic plant), using seven molecular markers of cpDNA. Five fertilization schemes were evaluated comparatively in a pilot cultivation in Crete. Chemical fertilizers (ChF), integrated nutrient management fertilizers (INM) and two biostimulants were utilized (foliar and soil application). Plant growth, leaf chlorophyll fluorescence and colour were assessed, and leaf content of chlorophyll, key antioxidants (carotenoids, flavonoids, phenols), and minerals were evaluated. Fertilization regime did not affect leaf colour but induced distinct differences in leaf shape altering quality characteristics. INM-foliar and ChF-soil application promoted yield, without affecting tissue water content or biomass partitioning to inflorescences. ChF-foliar application was the most stimulatory treatment for enhanced antioxidant compound content, while INM-biostimulant was the least effective one. In conclusion, adverse fertilization options appear as optimal when considering yield or antioxidant compound content. New DNA sequence datasets for the plastid regions of *pet*B/*pet*D, *rpo*C1, *psb*K-*psb*I and *atp*F/*atp*H have been deposited in the GenBank for *S. syriaca* subsp. *syriaca* while the molecular markers *rbc*L, *trn*L/*trn*F, *psb*A/*trn*H were compared to those of another 15 *Sideritis* species retrieved from the GenBank, constructing a phylogenetic tree to show their genetic relatedness.

Keywords: molecular markers; genetic characterization; antioxidant; carotenoids; flavonoids; greece; phenols; biostimulant; malotira; mountain tea

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Abbreviations: a*, green to red range intensity; b*, blue to yellow range intensity; CEC, cation exchange capacity; ECse, saturation extract electrical conductivity; F_v/F_m, ratio of variable to maximum chlorophyll fluorescence; GAE, gallic acid equivalent; I_{AD}, index of absorbance difference;

INM, integrated nutrient management; L*, lightness; MAPs, medicinal-aromatic plants; RUE, rutin equivalent; SAR, sodium absorption ratio

1. Introduction

Genomic approaches supported by molecular biology and bioinformatics are of critical importance to document and conserve biodiversity [1]. DNA sequences used as "barcodes" to define genetically biological entities (species) represent a fast, low-cost, reliable, and uncomplicated solution for plant species identification, further allowing insight into their phylogenetic relations [2,3]. In various terrestrial medicinal-aromatic plants (MAPs), molecular barcoding has been widely applied, using short regions of nuclear or chloroplast DNA to genetically characterize and identify taxa at species or even at subspecies level [4]. Previous studies in members of Lamiaceae (major family of MAPs worldwide) have used various molecular markers and DNA tools for successful identification of many members belonging to different genera of Lamiaceae with some genera receiving more attention than others due to complex taxonomical issues, such as members of the genus *Sideritis* [5–17].

The first molecular DNA study for *Sideritis* taxa was initiated in 1999 using RAPDs for fingerprinting 15 plant populations of *S. pusilla* (Lange) Pau and their putative parental species *S. hirsuta* L. and *S. leucantha* Cav. [5], and the first DNA sequences submitted in GenBank were back in 2002 for the *trn*L gene of cpDNA for five different *Sideritis* species [6]. Another significant phylogenetic study has deposited in GenBank datasets focused on 23 Macaronesian *Sideritis* taxa (species and subspecies) using the ITS, *trn*L and *trn*T-*trn*L molecular markers [9], and more recently another 93 specimens of Greek *Sideritis* taxa have been deposited for ITS, *psb*A-*trn*H (PopSet: 972386283), *rbc*L (PopSet: 972385939) and *mat*K (PopSet: 972386103) molecular markers [18]. The nuclear molecular marker ITS has been broadly used in such studies [6,14,15,17], whereas multiple regions from the cpDNA have also been used for species identification [6,10–13,16]. Many fingerprinting studies to date have employed genetic analyses using Amplified Fragment Length Polymorphisms (AFLPs) for several subpopulations of the local Greek endemic *S. euboea* Heldr. [8], Random Amplified Polymorphic DNA (RAPD) markers [19,20] and 12 URP to investigate inter-population genetic diversity of *S. raeseri* Boiss. & Heldr. [7].

Species-wise, this investigation was focused on a perennial local endemic plant of Crete (Greece), namely *S. syriaca* L. subsp. *syriaca* (Lamiaceae), commonly called in Crete 'malotira' (or Cretan Mountain tea). This taxon is wild-growing on rocky substrates at high altitudes (1000-2200 m) of the major mountain massifs of Crete, and flowers in June and July [21]. Malotira represents a contemporary yet traditional herbal medicinal product which is widely used for tea preparation and as food ingredient [22] with well-documented therapeutic indications [23] approved by the European Medicines Agency (https://www.ema.europa.eu/en/medicines/herbal/sideritis-herba, accessed 30 November 2023). Although it has been recently cultivated at small scale in Omalos plateau (Chania, Crete) as a valuable locally native MAP [24], it is however threatened with extinction [25] due to over-harvesting of sizeable volumes of plant material directly sourced from wild-growing populations of Lefka Ori in Chania, Crete (https://flashnews.gr/post/694054/ena-ntokimanter-gia-tin-kritiki-malotira-eftase-mechri-ti-germania-vinteo/, accessed 30 November 2023); such harvested (or cultivated at lesser extent) material is traded in local markets of Crete and is also exported as a local MAP product [26].

In many countries, traditional and complementary medicine is still a major health resource with herbal materials used for therapeutic purposes [27,28]. In China alone, ca. 40% of the administered medicines are of herbal origin [29]. The latter, is also coupled with an emerging global trend of using MAPs and natural products in Western societies [30]. Notably, several industrially produced (pharmaceutical) drugs are still based on MAPs [28] and the market and public demand on a wide range of plant-derived chemicals (e.g., fragrances, cosmetics, color/ flavor additives, etc.) continues to rise [29,31–33]. In this context, the growing request for MAPs, however, inevitably imperil their wild-growing populations [29,34] like those of the threatened focal taxon in this study. It is known that unmonitored or poorly-controlled trade, coupled with unsuitable harvesting methods, and over-

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exploitation pressure on wild-growing populations may imperil several range-restricted species such as the local endemic *S. syriaca* subsp. *syriaca* [17,26] as well as may jeopardize the ecological equilibrium at habitat/ecosystem level [34]. Nonetheless, introducing focal species of conservation concern and economic interest in agricultural settings such as the focal taxon herein can satisfy the growing demand for specific MAPs without further depleting wild-growing phytogenetic resources. To this end, applied research is needed such as documentation of origin and consolidated taxonomic identity of plant material used, species-specific propagation protocols, cultivation guidelines, and fertilization regimes, thus facilitating conservation actions and enabling sustainable exploitation in different economic sectors [24,35].

Cultivation-wise, any plant demand for nutrients is often conventionally met by chemical (inorganic) fertilizers. Accumulative evidence, however, suggests that over-application or inefficient utilization of chemical fertilizers is a major environmental pollution driver with cumulative alarms [36,37]. Typical examples include degradation of soil quality, and nutrient loading of water bodies with deleterious effects on aquatic ecosystems [36,38]. A more eco-friendly and more sustainable mode of satisfying plant nutrient needs is using organic fertilizers which produce a rather limited environmental impact compared to chemical ones [39]. Fertilization management can be further improved by applying biostimulants [40] which may increase root nutrient acquisition, enhancing in this way fertilization efficiency [40]. In this perspective, the joint addition of chemical fertilizers, organic fertilizers and biostimulants (collectively termed as integrated nutrient management; INM) can potentially ensure plant growth and productivity at a lower environmental cost [41,42]. Such applied research lines have also been suggested as indispensable stepping stones for the sustainable exploitation and pilot cultivations of neglected and underutilized local Cretan endemic plants with potential economic interest [24].

Market-wise, the herbal material quality is initially shaped by the fertilization scheme applied [39] and includes a wide range of features, the relative importance of which is determined by the intended use [32,33]. The level of antioxidant compounds (often referred as antioxidants) is commonly an important index of plant material quality [38,43]. Antioxidants are widely recognized as potential health promoters, and they have well-documented preventative and therapeutic effects against several chronic diseases [44,45]. These include carotenoids, flavonoids, and phenolics, which are characterized by powerful antioxidant ability [38], and the herein focal taxon has also rich content in such beneficial compounds [23]. It has been suggested that organic fertilizers and integrated nutrient management (INM) strategies may stimulate the herbal material antioxidant profile more than chemical fertilizers [44,46–50]. Furthermore, as these strategies help in avoiding societal charges of nitrogen losses, the paybacks of their use might considerably shade their expenses, rendering them extremely suggested for incorporation in fertilization schemes [51]. Such fertilization regimes have also been associated with enhanced chlorophyll content [52]. Chlorophyll content determines leaf greenness, which is another measure of herbal material quality, being traditionally employed by processors, distributors, and consumers [32].

Context-wise, the present investigation aimed to genetically characterize the local endemic *S. syriaca* subsp. *syriaca* (malotira or Cretan Mountain tea) using seven plastid molecular markers, four of which are employed for the first time in members of genus *Sideritis*. The molecular markers selected as suitable plant DNA barcodes are widely used, are suggested by several studies and have been routinely tested in terms of amplification and sequencing success (universality), intra- / inter-specific variation, and resolving power [3,53,54]. These new DNA sequences can be used for reliable taxonomic identification and traceability of commercial products of *Sideritis herba* [23], also supporting the genetic authentication and identity consolidation of related commercial products. Another aim of this study in response to absence of species-specific data was to comparatively investigate the effects of different fertilization treatments and application techniques in a pilot field cultivation of *S. syriaca* subsp. *syriaca* in Heraklion, Crete; the latter was used to define a species-specific fertilization protocol able to promote plant growth and/or key herbal quality features (i.e., colour intensity, antioxidant metabolite content) for sustainable and commercial exploitation.

2. Results

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2.1. Molecular characterization of the studied Sideritis syriaca subsp. syriaca

The DNA sequences of *S. syriaca* subsp. *syriaca* GR-1-BBGK-15,5939 generated in the present study for the molecular markers *pet*B/*pet*D (999 bp), *rbc*L (718 bp), *trn*L/*trn*F (695 bp), *rpo*C1 (476 bp), *psb*A/*trn*H (534 bp), *psb*K/*psb*I (329 bp) and *atp*F/*atp*H (498 bp) were deposited in the Genbank obtaining the accession numbers OR909054-OR909060.

No data was available in the GenBank for *Sideritis* taxa for the molecular markers *pet*B/*pet*D, *rpo*C1, *psb*K/*psb*I and *atp*F/*atp*H, thus first-time furnished herein for *S. syriaca* subsp. *syriaca*. However, there were 161 entries available on the Genbank for the *trn*L/*trn*F molecular marker corresponding to 49 *Sideritis* taxa, 96 entries belonging to 8 *Sideritis* taxa for *psb*A/*trn*H, and 45 entries of 22 Sideritis taxa for *rbc*L [18]. Therefore, a unified phylogenetic tree was constructed for the molecular markers *trn*L/*trn*F and *rbc*L for a total of 15 *Sideritis* taxa (Figure 1). The concatenated sequence used for this phylogenetic tree had a length of 1233 bp; within this sequence, a total of 43 single nucleotide polymorphisms (SNPs), a polyT (9 Ts) region and one indel (insertion of TGAA for *S. romana* L.) have been identified (Supplementary Material Table S2). In the phylogenetic tree, *S. syriaca* subsp. *syriaca* was clearly separated from closely related species such as *S. euboea* and *S. scardica* (Figure 1).

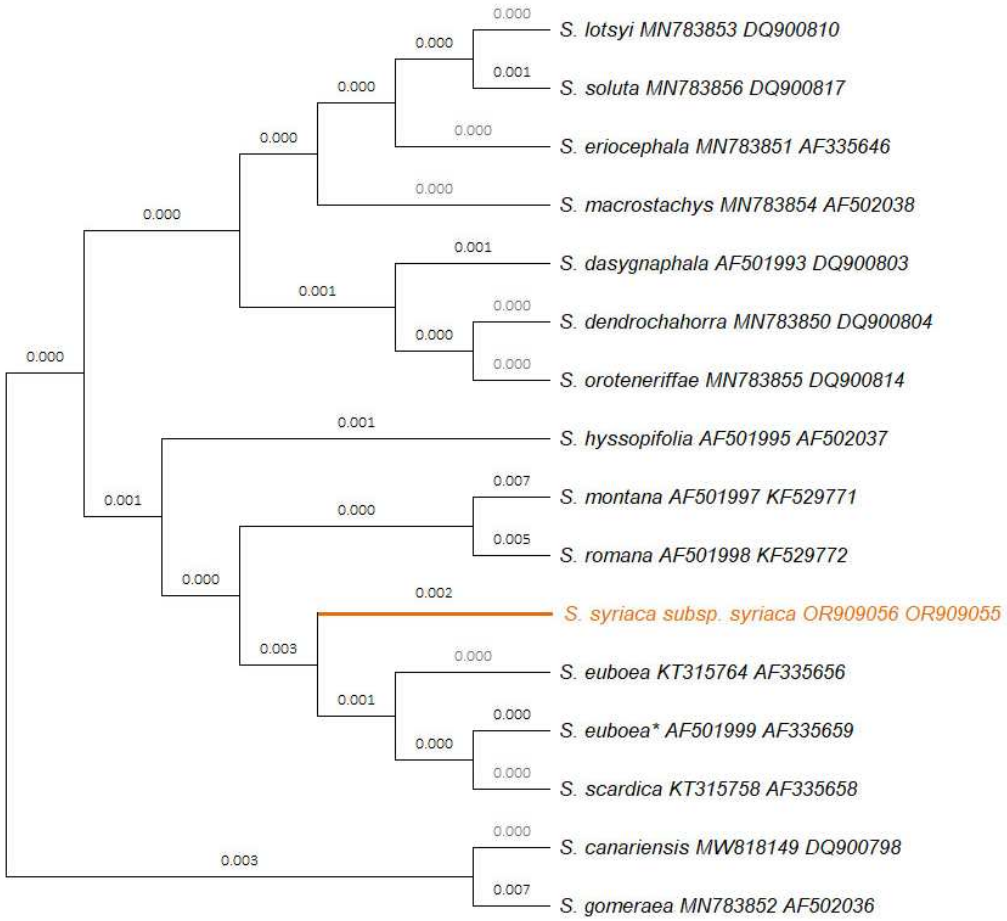

Figure 1. Phylogenetic tree illustrating the relationships among 15 *Sideritis* species based on the molecular plastid markers *rbc*L and *trn*L/*trn*F. The tree was constructed using the Neighbor-Joining method and Maximum Composite Likelihood substitution model. Accession numbers of DNA sequences obtained in this study are indicated next to taxon names. The clade marked in orange represents *Sideritis syriaca* subsp. *syriaca* GR-1-BBGK-15,5939 studied herein. The taxon marked with asterisk (*) is characterized as *S. syriaca* in the database, however, based on its original collection area (Mt. Dirphys in Evia, Greece), it should be identified as the unique local endemic mountain tea plant of this mountain namely *S. euboea* and not as *S. syriaca* subsp. *syriaca* which is a single-island endemic of Crete Island, Greece [55].

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The DNA sequence of the present study (OP909056) for *rbc*L was identical (100%) to *S. syriaca* subsp. *syriaca* (KT315757) and identical (100%) to another 19 specimens corresponding to *S. raeseri* subsp. *raeseri*, *S. euboea* and *S. scardica* Griseb. originated from Greece; it was also similar (99.86%) to *S. euboea* AF501999 from Mt Dirphys which was wrongly labeled as *S. syriaca* as well as to *S. hyssopifolia* L. (AF501995) (Supplementary Material Table S3.A). The DNA sequence OP909054 of the present study for *psb*A-*trn*H was identical (100% similarity) to the respective sequences of *S. syriaca* subsp. *syriaca* deposited in the GenBank (KT633327, KT633328, KT633350) [18]. Surprisingly, there were nucleotide differences among 90 more *Sideritis* taxa originated from Greece with results shown to be highly problematic (Supplementary Material 3.B).

Basic Local Alignment (BLAST) results showed that the *pet*B/*pet*D molecular marker exhibited a 98.60% similarity with *Stenogyne haliakalae* Wawra (NC_029817). The sequence generated for the *trn*L/*trn*F region in this study matched with *S. scardica* (AF335658) by 99.81%, whereas the *rpo*C1 sequence matched to *Stachys byzantina* K.Koch (NC_029825) by 99.37%. The sequence for the *psb*K/*psb*I intergenic spacer matched with *Anisomeles indica* (L.) Kuntze (NC_046781) and *Leucosceptrum canum* Sm. (NC_051966) by 94.84%. Finally, the sequence for *atp*F/*atp*H matched *Stachys byzantina* (NC_029825) by 98.20% [18].

2.2. Fertilization scheme exerted minor effects on leaf colour

At all three growth stages, leaf SPAD value was not affected by the fertilization treatment (Figure 2). At vegetative stage, plants receiving INM-fa had higher IAD value (i.e., being less green) from all treatments besides INM-sa (Suppl. Figure S1A). At early flowering stage, plants receiving ChF-sa showed higher IAD value from plants treated with ChF-fa and MPE-sa (Suppl. Figure S1B). At full flowering stage, plants receiving ChF-sa had higher IAD value as compared to plants treated with ChF-fa, INM-sa or MPE-sa (Suppl. Figure S1C).

At all three growth stages, leaf L value was not affected by the fertilization regime (Suppl. Figure S2). At vegetative stage, plants receiving ChF-sa had lower a* value as compared to controls plants or plants receiving ChF-fa or INM-sa (Suppl. Figure S3A). At early flowering stage, plants receiving INM-sa or ChF-sa showed lower a* value than control plants or plants treated with ChF-fa (Suppl. Figure S3B). At full flowering stage, plants receiving INM-sa had lower a* value than plants receiving INM-fa or ChF-fa (Suppl. Figure S3C).

At early flowering stage, plants receiving INM-sa had higher b* value as compared to control plants (Suppl. Figure S4B). At vegetative and full flowering stages, the fertilization treatment did not affect leaf b* value (Suppl. Figure S4A, C).

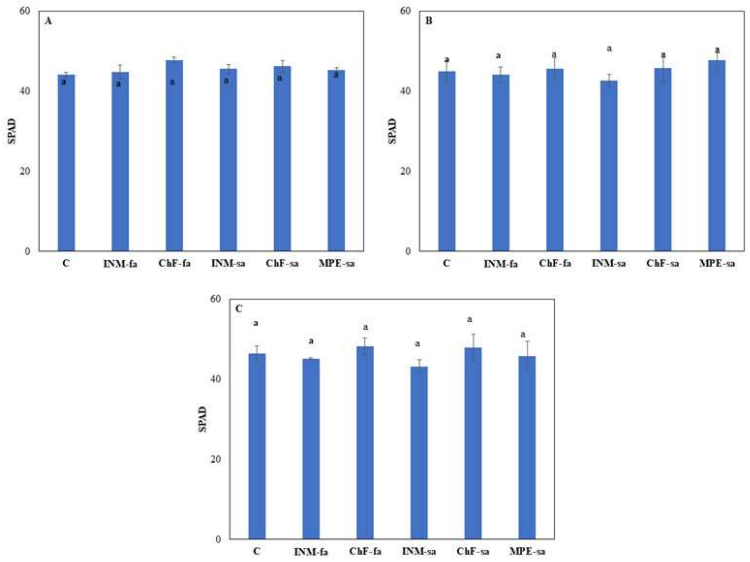

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Figure 2. Effect of fertilization regime through different (root/foliar) application methods on leaf SPAD value of *Sideritis syriaca* subsp. *syriaca* at vegetative (A), early flowering (B), and full flowering (C) stage. C: Control (water); INM-fa: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

2.3. Fertilization scheme induced limited effects on leaf photosynthetic performance

At three growth stages, the effect of fertilization treatment on overall photosynthetic efficiency was assessed by *in situ* F_v/F_m measurements (Figure 3). At vegetative stage, plants receiving INM-fa had the highest F_v/F_m value (Figure 3A). At early flowering stage, plants receiving INM-sa had higher F_v/F_m value, as compared to control plants (Figure 3B). At full flowering stage, leaf F_v/F_m value was not affected by the fertilization regime (Figure 3C).

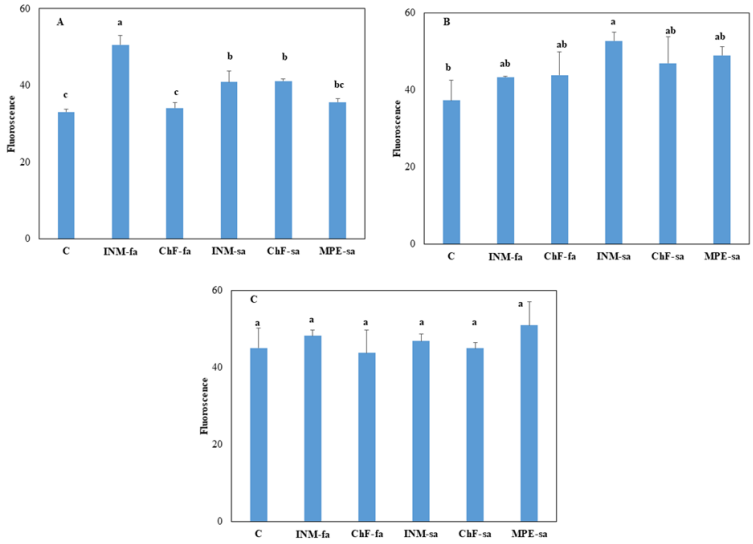

Figure 3. Effect of fertilization regime through different (root/foliar) application methods on chlorophyll fluorescence value of *Sideritis syriaca* subsp. *syriaca* at vegetative (A), early flowering (B), and full flowering (C) stage. C: Control (water); INM-fa: Integrated nutrient management (INM)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

2.4. Fertilization induced distinct leaf shape profiles

To determine the effect of fertilization regime on leaf form, four shape indicators (aspect ratio, circularity, roundness, solidity) were employed. Control plants showed a higher aspect ratio as compared to plants receiving ChF-sa or MPE-sa (Figure 4A). The lowest circularity was noted in control plants (Figure 4B). Plants receiving MPE-sa had the highest circularity from all treatments, besides ChF-sa (Figure 4B). Plants under MPE-sa had higher roundness than control plants (Figure 4C). The lowest solidity was noted in control plants (Figure 4D). Plants treated with MPE-sa had the highest solidity of all treatments, besides plants receiving INM-sa (Figure 4D).

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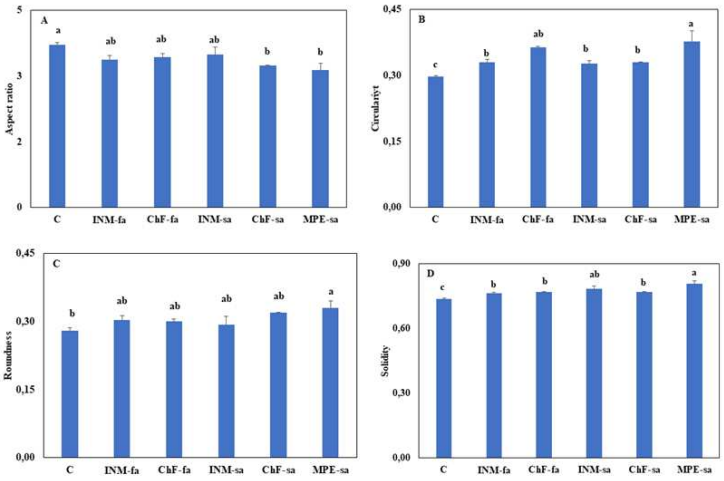

Figure 4. Effect of fertilization regime through different (root/foliar) application methods on four leaf shape factors of *Sideritis syriaca* subsp. *syriaca*. C: Control (water); INM-fa: Integrated nutrient management (INM) by foliar application; ChF-fa: Chemical fertilization by foliar application;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

2.5. INM and ChF-sa stimulated plant growth, without affecting biomass allocation to generative organs

INM with either application method (i.e., INM-fa or INM-sa) and ChF-sa improved biomass accumulation as compared to the remaining treatments including the controls (Figure 5A). These differences in above-ground dry mass were not associated with variation in tissue relative water content (Figure 5B). Plants receiving INM-sa had higher partitioning to generative organs, as compared to plants receiving ChF-fa (Figure 5C).

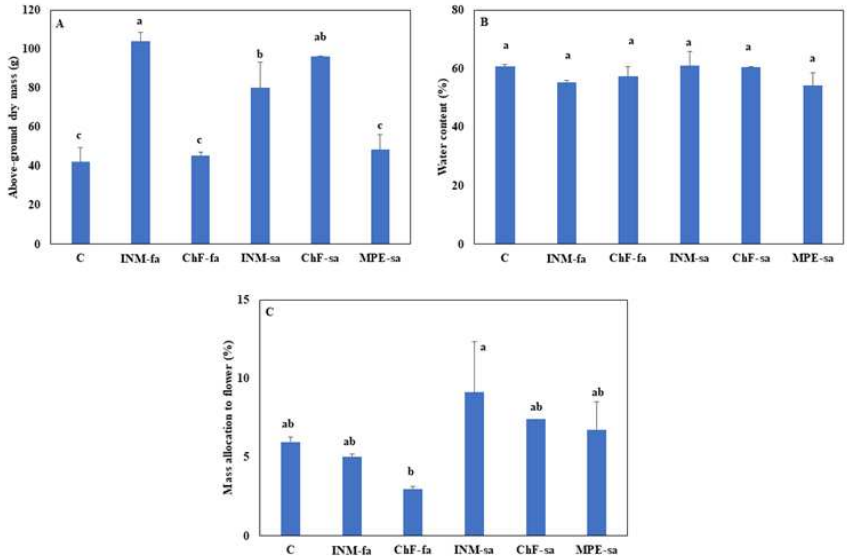

Figure 5. Effect of fertilization regime through different (root/foliar) application methods on above-ground dry weight (A), water content (B) and dry weight partitioning to inflorescences (C) of *Sideritis syriaca* subsp. *syriaca*. C: Control (water); INM-fa: Integrated nutrient management (INM) by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

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2.6. Fertilization regime affected leaf chlorophyll content

Plant receiving foliar fertilization (i.e., INM-fa, ChF-fa) or INM-sa had higher leaf chlorophyll content as compared to controls (Figure 6A). Plants receiving ChF-sa or MPE-sa had lower leaf chlorophyll content as compared to controls, while the latter had the lowest content (Figure 6A).

2.7. Fertilization regime affected leaf antioxidant compound content

Carotenoids, flavonoids, and phenols are critical non enzymatic antioxidants. Plant receiving foliar fertilization (i.e., INM-fa, ChF-fa) or INM-sa had higher leaf carotenoid content as compared to controls (Figure 6B). Plants receiving MPE-sa had lower leaf carotenoid content as compared to controls (Figure 6B).

The highest leaf total phenolic content was noted in plants receiving ChF-fa, followed by plants treated with INM-sa or ChF-sa (Figure 6C). Plants treated with INM-sa or ChF-sa had higher total phenolic content as compared to controls, whereas the ones receiving MPE-sa had the lowest total phenolic content (Figure 6C).

Plants receiving INM-fa, INM-sa or ChF-sa had lower leaf flavonoid content, as compared to controls (Figure 6D). The lowest leaf flavonoid content was noted in plants receiving MPE-sa (Figure 6D).

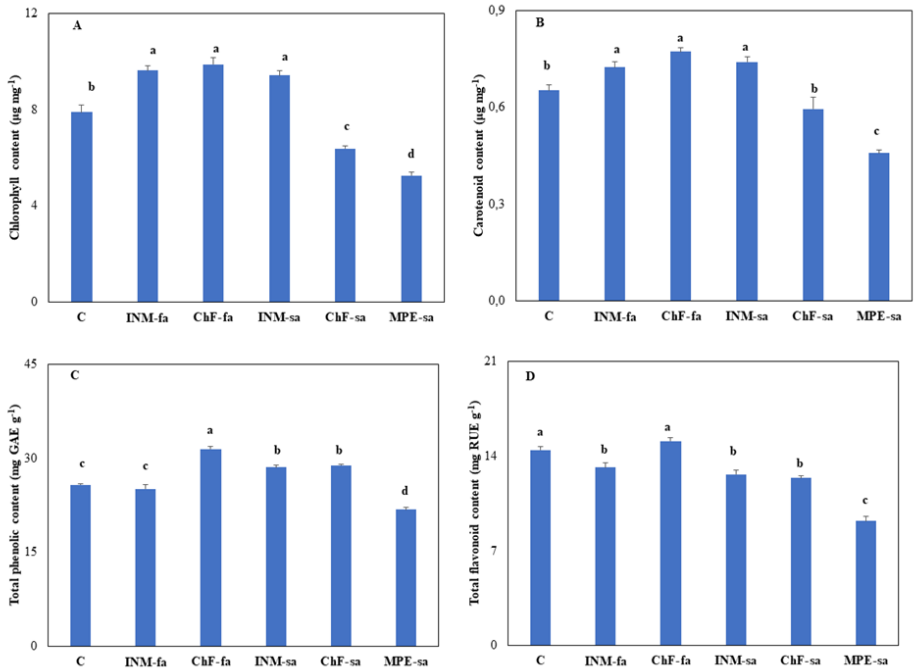

Figure 6. Effect of fertilization regime through different (root/foliar) application methods on leaf chlorophyll (A), carotenoid (B), total phenol (C) and total flavonoid (D) content of *Sideritis syriaca* subsp. *syriaca*. C: Control (water); INM-fa: Integrated nutrient management (INM) by foliar application;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

2.8. Fertilization regime affected leaf soluble sugar content

Plants receiving ChF-sa had higher leaf soluble sugar content than controls, whereas the ones receiving INM-sa or MPE-sa had lower leaf soluble sugar content (Figure 7).

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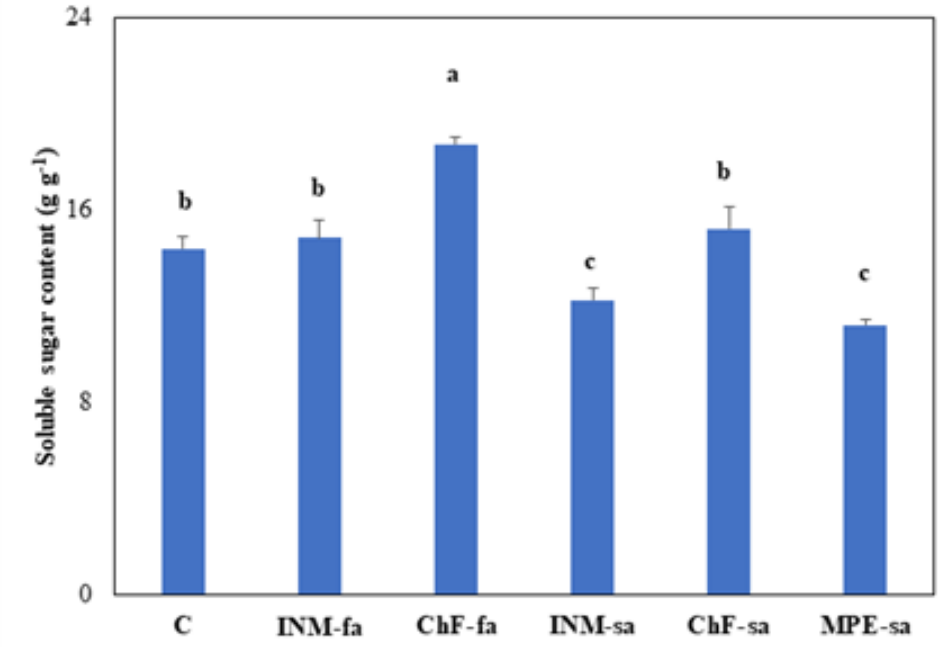

Figure 7. Effect of fertilization regime through different (root/foliar) application methods on leaf soluble sugar content of *Sideritis syriaca* subsp. *syriaca*. C: Control (water); INM-fa: Integrated nutrient management (INM) by foliar application; ChF-fa: Chemical fertilization by foliar application;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

2.9. Leaf and inflorescence mineral analysis

Among all treatments (including control), the highest N concentration in leaves was observed when plants cultivated under integrated nutrient management fertilization applied to soil (INM-sa). Conversely, the highest K concentration was noted by foliar application of conventional fertilizers (ChF-fa). This trend was also observed for P, although it is noteworthy that similar results were also seen with the foliar application of INM fertilization (INM-fa). In contrast, no significant differences were observed in Ca or Mg concentrations with any of the applied treatments (Table 1).

On the other hand, the results regarding the leaf concentration of micronutrients in *S. syriaca* subsp. *syriaca* were less conclusive. Specifically, no treatment effects were evident for Zn, and the same held true for Cu, where although ANOVA results indicated significant differences between treatments, none of them significantly differed from the control. On the contrary, the integrated nutrient management (INM) fertilization along with the biostimulant treatment (MPE) applied through soil application exhibited the highest concentrations for Fe, while the conventional fertilization by foliar application (ChF-fa) proved to be more effective than all other treatments in supplying the highest Mn leaf concentration (Table 2).

ANOVA of floral mineral analysis data showed differences among treatments (Tables 3 and 4). The highest N content was observed in INM-sa, whereas regarding P the highest contents were found in INM-sa and ChF-sa. The latter also had the highest K content (Table 3).

Regarding inflorescence micronutrient content of *Sideritis syriaca* subsp. *syriaca* (Table 4), the highest B content was observed in INM-sa and the lowest value in ChF-sa treatment, which were similar to control (Table 4).

Table 1. Effect of fertilization regime through different (root/foliar) application methods on leaf essential macronutrient content of *Sideritis syriaca* subsp. *syriaca*. C: Control (water); INM-fa: Integrated nutrient management (INM) by foliar application; ChF-fa: Chemical fertilization by foliar application; INM-sa: INM by soil application; ChF-sa: Chemical fertilization by soil application; MPE-

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sa: Mixture of plant extracts as biostimulator by soil application (THEOMASS). Values represent the mean of three replicates ± SE.

Treatment	N	P	K	Ca	Mg
			(g kg^{-1})		
C	13.4 ± 0.4c*	2.3 ± 0.1b	18.9 ± 0.3b	23.0 ± 1.4a	3.2 ± 0.1a
INM-fa	21.8 ± 1.7ab	3.4 ± 0.0a	18.7 ± 0.4b	18.7 ± 0.5a	2.7 ± 0.0a
ChF-fa	18.3 ± 0.3abc	3.5 ± 0.0a	22.4 ± 0.5a	22.9 ± 1.8a	3.0 ± 0.1a
INM-sa	22.8 ± 0.8a	2.5 ± 0.1b	18.7 ± 0.5b	22.1 ± 1.7a	2.9 ± 0.1a
ChF-sa	12.8 ± 1.6c	2.7 ± 0.0b	20.4 ± 0.4ab	23.3 ± 1.5a	4.2 ± 0.5a
MPE-sa	16.0 ± 1.5bc	2.3 ± 0.2b	19.1 ± 0.5b	25.6 ±1 .0a	3.2 ± 0.1a
p F-test	0.021	< 0.001	0.029	NS#	NS#

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

Table 2. Effect of fertilization regime through different (root/foliar) application methods on leaf essential micronutrient content of *Sideritis syriaca* subsp. *syriaca*. C: Control (water); INM-fa: Integrated nutrient management (INM) by foliar application; ChF-fa: Chemical fertilization by foliar application; INM-sa: INM by soil application; ChF-sa: Chemical fertilization by soil application; MPE-sa: Mixture of plant extracts as biostimulant by soil application (THEOMASS). Values represent the mean of three replicates ± SE.

Treatment	Cu	Zn	Fe	Mn	B
			(mg kg^{-1})		
C	14.9 ± 0.1abc*	26.5 ± 1.4a	1058 ± 89cd	52.0 ± 1.6c	27.5 ± 0.8b
INM-fa	13.2 ± 0.4c	23.8 ± 0.5a	1057 ± 49cd	62.8 ± 1.3bc	19.7 ± 0.3d
ChF-fa	14.5 ± 0.5bc	27.5 ± 0.4a	1277 ± 79bc	83.0 ± 5.0a	26.9 ± 0.3bc
INM-sa	15.8 ± 0.3ab	24.7 ± 0.6a	1703 ± 111a	68.4 ± 1.1b	25.8 ± 0.3bc
ChF-sa	15.5 ± 0.6ab	23.3 ± 0.1a	871 ± 19d	37.0 ± 0.4d	24.1 ± 0.8c
MPE-sa	16.9 ± 0.2a	26.8 ± 1.0a	1578 ± 24ab	54.0 ± 1.5c	35.0 ± 0.6a
p F-test	0.033	NS#	0.003	< 0.001	< 0.001

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

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

Treatment	**N**	**P**	**K**	**Ca**	**Mg**	**Na**
			(g kg^{-1})			
C	8.65±0.00c	1.31±0.45b	12.67±2.22ab	1.84±0.19bc	1.04±0.20ab	0.52±0.11b
INM-fa	10.14±0.36bc	2.40±0.38a	13.95±0.44ab	2.75±0.31ab	1.28±0.01a	0.85±0.02a
ChF-fa	10.28±2.07bc	1.72±0.12ab	9.19±1.24b	1.22±0.01c	0.61±0.12b	0.51±0.08b
INM-sa	16.63±1.68a	1.26±0.31b	12.21±1.16ab	3.20±0.69ab	1.27±0.29a	0.55±0.16b
ChF-sa	10.46±0.00bc	2.64±0.00a	16.84±0.00a	3.49±0.00a	1.40±0.00a	0.68±0.00ab
MPE-sa	13.38±1.92ab	1.61±0.53ab	12.52±2.52ab	2.56±1.04ab	1.36±0.35a	0.44±0.10b
***p* F-test**	0.014	0.075	0.081	0.083	NS#	NS

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

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

Treatment	B	Zn	Fe
		mg kg^{-1}	
C	6.93±0.12a	14.81±3.20ab	149.86±43.79ab
INM-fa	5.60±0.20b	15.32±1.41ab	201.35±42.26a
ChF-fa	6.60±0.34ab	10.78±2.93b	49.94±23.79b
INM-sa	6.82±0.78a	14.30±1.20ab	135.70±38.84ab
ChF-sa	5.49±0.00b	20.20±1.00a	157.32±10.00ab
MPE-sa	6.29±0.18ab	17.51±4.47ab	148.68±56.23ab
***p* F-test**	0.068	NS	NS

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

3. Discussion

3.1. Molecular characterization of the studied Sideritis syriaca subsp. syriaca

The genetic identification of plant species combining taxonomic identification and modern molecular techniques based on DNA sequences has the power to effectively characterize both morphologically and genetically given specimens [56]. To date, DNA barcoding has been widely used as a powerful tool in combination with bioinformatic analysis in ecological, taxonomic, comparative phylogenetic and biodiversity conservation studies across various taxonomic levels [3] including distinction of members of the Lamiaceae family [15,57], and more specifically of the genus *Sideritis* [11,13,14].

DNA barcoding was a valid technique for the discrimination of the herein studied *S. syriaca* subsp. *syriaca* genotype, further enhancing the classical taxonomic identification based on morphological features and offering insight into the phylogenetic relationships of closely related taxa. In the frame of this study, seven new sequences (i.e., *pet*B/*pet*D, *rbc*L, *trn*L/*trn*F, *rpo*C1, *psb*A/*trn*H, *psb*K-*psb*I and *atp*F/*atp*H) were generated and deposited in GenBank for *S. syriaca* subsp. *syriaca* GR-1-BBGK-15,5939, obtaining unique accession numbers and creating a genetic documentation for the focal taxon while four DNA sequences of the plastid molecular markers *pet*B/*pet*D, *rpo*C1, *psb*K-*psb*I and *atp*F/*atp*H have been reported and deposited to the GenBank for the first time. The phylogenetic analysis with the molecular markers *rbc*L and *trn*L/*trn*F confirmed the identity of the specimen and indicated its phylogenetic position compared to other *Sideritis* taxa (Figure 1). *S. syriaca* subsp. *syriaca* GR-1-BBGK-15,5939 was classified in a group together with *S. montana* L., *S. romana, S. euboea* and *S. scardica*, which are also found in Greece ([55]; Figure 1). The *Sideritis* specimen annotated with an asterisk in Figure 2 is denoted as *S. syriaca* in the GenBank database; however, a reevaluation is warranted for this accession. Considering its collection locality (Greece: Mt. Dirfys; [6]), it is suggested that the appropriate identification should be *S. euboea* which is the local endemic mountain tea species of Mt. Dirphys. This recommendation is substantiated by the restricted geographical distribution of *S. syriaca* subsp. *syriaca* on the island of Crete as a local single-island endemic plant [55]. Similar issues of misidentification or inaccurate taxonomy of specimens related with the focal taxon is extensively addressed in [17] for the case of the ITS2 molecular marker.

The Basic Local Alignment (BLAST) results indicated the highest similarity of the new DNA sequences generated for *S. syriaca* subsp. *syriaca* GR-1-BBGK-15,5939 to the sequences retrieved from the GenBank. Since there was no previous information on sequence data for any *Sideritis* taxon for the cpDNA regions *pet*B/*pet*D, *rpo*C1, *psb*K-*psb*I and *atp*F/*atp*H, the BLAST showed only other closely related taxa. The *trn*L/*trn*F showed the highest similarity to *S. scardica* (99.81%). The *rbc*L molecular

marker of the present study was identical (100%) to *S. syriaca* subsp. *syriaca* and to another 19 specimens corresponding to the Balkan endemics *S. raeseri* subsp. *raeseri* and *S. scardica* and the local Greek endemic *S. euboea*, all originated from Greece; in addition, high similarity was shown to the *S. euboea* and *S. hyssopifolia* which is wild-growing in France, Italy, Sicilia, Spain and Switzerland [55]. It is worth mentioning that the molecular marker *rbc*L exhibited identical sequences across a notable array of *Sideritis* taxa such as *S. perfoliata* L. subsp. *perfoliata, S. scardica, S. syriaca* subsp. *syriaca, S. clandestina* (Bory & Chaub.) Hayek subsp. *clandestina* and *S. euboea*, provided that all the available sequences for this molecular marker pertain to merely eight taxa. Although *psb*A/*trn*H was excluded from the phylogenetic analysis herein, its utilization is rather anticipated to yield inconclusive outcomes in terms of species differentiation. This assumption comes in contrast with previous studies [13] suggesting that the *mat*K and *psb*A/*trn*H could serve as potential single-region barcodes for Lamiaceae species. Additional phylogenetic analysis for another 94 specimens of genus *Sideritis* from Greece showed identical sequences of three specimens of *S. syriaca* subsp. *syriaca*, however, the rest of these specimens presented controversial matching and nucleotide differences to the assigned *Sideritis* species and subspecies (Supplementary material Table S3.B). Recently, a new *Sideritis* species has been described from Bulgaria namely *S. elica* Aneva, Zhelev & Bonchev that was clearly distinct from *S. scardica* based on morphological and molecular data [16]; in that case, the *trnH-psbA* molecular marker showed a 6.8% polymorphism [16]. Previous studies have also investigated the phylogeny of selected *Sideritis* taxa using only one nuclear molecular marker [14,17,58], thus compromising the genetic information for comparison with the current investigation.

The computation herein suggests that the combination of two or more molecular markers provides the potential for more informative species' differentiation. In this study, we furnished for the first time genetic characterization based on seven molecular markers, thus consolidating the identity and authenticating the studied Cretan local endemic germplasm of *S. syriaca* subsp. *syriaca* which is threatened with extinction [25].

3.2. Fertilization effects supporting the sustainable exploitation of S. syriaca subsp. syriaca

Plant species become extinct at a rate which is two to three orders higher than the expected natural one [59]. Overexploitation and habitat destruction or alteration currently threaten over 15,000 plant taxa with extinction [60], with a large part of their wild-growing populations currently depleted [61]. For species with increasing demand, adaptation to agricultural environments stands out as a sustainable conservation option. Successful cultivation, however, depends on the development of cultivation protocols, a major part of which is the fertilization scheme. The present field study reports the primary stages of establishing *S. syriaca* subsp. *syriaca* (endemic to Crete, Greece) into systematic cultivation, and evaluates the fertilization regime which is optimal for improving plant growth as well as in terms of critical features of herbal material quality. In this perspective, emphasis was placed into the use of fertilizers having low environmental footprint, which is of vital importance in the respective MAP market area [62].

As compared to controls, INM and ChF-sa improved plant growth (Figure 5A). In INM, this effect was more prominent under foliar application (Figure 5A). This increase in biomass accumulation was not associated with variation in tissue relative water content (Figure 5B). In this way, the efficiency of processing (moisture content reduction through drying [32,33] was not affected by the fertilization regime. The partitioning to the inflorescences was not significantly different among the three growth-promoting fertilization treatments (INM-fa, INM-sa, ChF-sa; Figure 5C). Considering yield, the current findings suggest that INM (especially through foliar application) and ChF-sa ought to be employed in *S. syriaca* subsp. *syriaca* cultivation.

Recent research on the pilot cultivation of Cretan endemic species in northern Greece (including *S. syriaca* subsp. *syriaca*) has also revealed noteworthy yield improvements with the implementation of comparable fertilization methods [63]. However, such enhanced yield was accompanied by distinct concentration patterns of macro- and micro-nutrients during the harvest stage. Specifically, a previous own study have highlighted that chemical fertilization through foliar application (ChF-fa) may lead to elevated levels of metallic micronutrients at the harvest stage, specifically Cu and Zn

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[63]. Conversely, the same treatment when applied to the soil has been shown to result in a significant increase in Fe and Mn [63]. Interestingly, N concentration is reported to show minimal variation across different treatments compared to the control, whereas P concentration may rise with foliar application using conventional fertilizers, and K can exhibit positive responses with the integrated nutrient management (INM) fertilization approach through foliar application [63].

The dissimilarities between the present study's findings in Crete and the aforementioned pilot case of cultivation in northern Greece could be attributed to the respective differences on properties of the initial soil used as a medium, especially in terms of soil fertility. The soil in the present study (Crete) had elevated concentrations of soil-available macro- and micronutrients, exceeding their sufficiency levels [64,65]. In contrast, the soil utilized in the previously published own study [63] in northern Greece lacked sufficient soil-available potassium, falling below critical sufficiency levels, while the soil phosphorus content was only marginally sufficient [63]. The above indicates that any fertilization scheme suggestion in the future should be carefully adjusted with the initial amounts of soil available nutrients taking also into consideration the complexity of soil-soil solution interactions that may interfere in the uptake process by plant's root system.

Regarding the mineral analysis of the inflorescences consisting the major part of *Sideritis herba* [23], it was found that all treatments tended to increase the macronutrient content with the exception of P. The higher N content was observed in INM-sa, whereas INM-fa and ChF-sa showed the highest P values. The latter tended also to had higher K and Ca content, suggesting overall that ChF-sa and INM-fa may favor the accumulation of macronutrients in the inflorescences of *S. syriaca* subsp. *syriaca*. Regarding inflorescence micronutrient content, it seems that no significant enrichment of inflorescences can be referred in terms of Fe, Zn and B contents. No relative information was found in literature to compare and evaluate the macro- and micro-nutrient contents in inflorescences of *S. syriaca* subsp. *syriaca*. Thus, further work is needed for a greater period of time and under different experimental conditions to evaluate the partitioning of nutrients to inflorescences and the response of *S. syriaca* subsp. *syriaca* to fertilization.

Leaf green colour intensity is an important visually perceived quality index throughout the supply chain [32]. In this study, leaf coloration was evaluated for the first time by three devices (SPAD meter, DA meter, Chroma Meter), indicating a different potential in discriminating the minor fertilization treatment effects (Figure 2, and Suppl. Figs. S1–S4). At full flowering stage, for instance, no difference among treatments was apparent when examining leaf SPAD value (Figure 2), L value (a measure of lightness; Suppl. Figure S2C) or b value (a measure of blue to yellow range intensity; Suppl. Figure S4C). Although little differences were noted in I_{AD} value (Suppl. Figure S1C) and a value (a measure of green to red range intensity; Suppl. Figure S3C) among treatments, plant receiving fertilization did not differ with control plants. Therefore, it is concluded that the fertilization scheme did not affect leaf colour aspects in *S. syriaca* subsp. *syriaca*. Besides colour, visual inspection in herbal material grading includes leaf form. As compared to control plants, fertilization increased both circularity and solidity (Figure 4B, D). Differences in circularity are translated to variation in lobing and serration, while differences in solidity to deep lobes or marked petiole morphology [66]. In this regard, trained personnel, or automated identification protocols [33,67] are expected to be capable of visually discriminating *S. syriaca* subsp. *syriaca* herbal material that has received fertilization during cultivation based on shape indicators, since colour differences were either absent or minor.

Since the beneficial effects of antioxidant intake are increasingly recognized by consumers, the antioxidant content of natural products (such as *Sideritis herba*) is becoming more prominent as an index of herbal material quality [38,68]. In this investigation, three critical antioxidants (carotenoids, flavonoids, phenols) were quantified and comparatively evaluated for the first time. MPE-sa was associated with reduced content in all three metabolites (Figure 6B, C, D), thus consistently downgrading quality. Fertilization mostly promoted carotenoid and total phenolic contents, whereas it decreased flavonoid content (Figure 6B, C, D). The fertilization regime stimulating antioxidant content was metabolite-specific (Figure 8B, C, D). Considering all three antioxidants together, ChF-

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fa was the most stimulatory scheme. Contrary to our results, INM has been previously associated with enhanced antioxidant compound content in other taxa [49,50].

Although the present study focused only on one growth cycle in Crete, it offered for the first time reference data for growth/yield aspects and several herbal quality aspects in *S. syriaca* subsp. *syriaca*. When the primary target is yield, INM (especially through foliar application) and ChF-sa ought to be employed. When antioxidant compound content is the primary goal, ChF-fa is required while MPE-sa ought to be avoided in *S. syriaca* subsp. *syriaca* cultivation.

4. Materials and Methods

4.1. Plant material

Several botanical expeditions were conducted in the mountain ranges of Crete in the frame of the 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) with the aim to locate wild-growing populations of *S. syriaca* subsp. *syriaca* (Figure 8). Seed collections for ex situ conservation were authorized through a special permit of 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) obtained yearly by the Greek Ministry of Environment and Energy. The collected seeds and voucher samples were taxonomically identified, and consequently unique IPEN (International Plant Exchange Network) accession numbers were assigned to each of them (GR-1-BBGK-08,4544; GR-1-BBGK-13,5743; GR-1-BBGK-13,5738; GR-1-BBGK-13,5907; GR-1-BBGK-14,5798; GR-1-BBGK-15,5939; GR-1-BBGK-17,6029; GR-1-BBGK-19,1100) according to the protocols of the Balkan Botanic Garden of Krousia, Institute of Plant Breeding and Genetic Resources, Hellenic Agricultural Organization, Demeter. Adequate number of plants were raised ex situ for the field experiment from seedlings and/or cuttings from mother plants of GR-1-BBGK-15,5939 with the aid of a specific propagation protocol [14]. All plant material employed in the experimental procedure were transplanted in 2 L plastic pots by the company AFI GLAVAKI KE SIA OE Tree & Plant Nurseries, Aridea, PELLAS, GR-58400, Greece.

Figure 8. (A) *Sideritis syriaca* subsp. *syriaca* wild-growing individual at full flowering on rocky crevices of Sfakianes Madares in Lefka Ori, Chania, Crete (Greece); (B) Densely hairy leaf indumentum and (C) Pilot field with *S. syriaca* subsp. *syriaca* experimental individuals cultivated at the premises of the Hellenic Mediterranean University, Heraklion, Crete (Greece).

4.2. DNA barcoding and molecular analysis

Molecular analyses were conducted using fresh juvenile leaves of *S. syriaca* subsp. *syriaca* GR-1-BBGK-15,5939. The methodology employed for DNA extraction, amplification, and subsequent DNA sequence analysis adhered to the procedures outlined by [69]. The oligonucleotide primers (5′ to 3′) (InVitrogen Inc., Paisley, Scotland, UK) used for PCR amplification of *S. syriaca* subsp. *syriaca* were the following: ACTCGCACACACTCCCTTTCC and GCTTTTATGGAAGCTTTAACAAT for *atp*F/*atp*H [70], TTGACYCGTTTTTATAGTTTAC and AATTTAGCYCTTAATACAGG for *pet*B/*pet*D [71], CGCGCATGGTGGATTCACAATCC and GTTATGCATGAACGTAATGCTC for *trn*H/*psb*A

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[72], TTAGCCTTTGTTTGGCAAG and AGAGTTTGAGAGTAAGCAT [73] for *psb*K-*psb*I, ATGTCACCACAAACAGAGACTAAAGC and CTTCTGCTACAAATAAGAATCGATCTC for *rbc*L [74], GGCAAAGAGGGAAGATTTCG and CCATAAGCATATCTTGAGTTGG for *rpo*C1 [75], ATTTGAACTGGTGACACGAG and CGAAATCGGTAGACGCTACG for *trn*L/*trn*F [76]. Annealing temperature for each pair of primers was 54 to 60°C, depending on sequence temperature estimation.

Alignment of each generated sequence was completed by employing the Basic Local Alignment Tool (BLAST), comparing them with existing sequences available in the GenBank. DNA sequences corresponding to the above-mentioned molecular markers for other *Sideritis* taxa were retrieved from GenBank and were subsequently aligned for each marker using Mega11 software [77]. A unified phylogenetic tree was constructed using the Neighbor-Joining statistical method and Maximum Composite Likelihood substitution model. To ensure accessibility and transparency, all newly generated sequences were submitted to the GenBank obtaining the accession numbers OR909054-OR909060. The evaluation of Evolutionary Divergence between Sequences for each molecular marker involved the calculation of pair-wise distances, conducted within the Mega11 software [77].

4.3. Field experiment and experimental design

The field experiment was established at the beginning of March 2021 within a 20 × 25 m fenced area of the campus of the Hellenic Mediterranean University, Heraklion, Crete (35° 19' N, 25° 6' E) at low altitude (60 m above sea level). The planting distance between individuals was 40 cm and 80 cm between separate rows 20 m long arranged at east-west direction and including as "guard plants" other local Cretan endemics, i.e., plant individuals of *Origanum dictamnus* L., *Origanum microphyllum* (Benth.) Vogel, *Carlina diae* (Rech.f.) Meusel & A.Kástner, and *Verbascum arcturus* L.

The experimental design incorporated completely randomized blocks of 10 plants of *S. syriaca* subsp. *syriaca* per block, and three blocks per treatment randomly arranged in different rows. To provide the same starting point for all experimental plants, all individuals were trimmed at 5 cm above ground level at the end of April. At the end of May (i.e., 30 d after trimming), six fertilization treatments were introduced weekly till final harvest. An automatic irrigation system was employed with 2 L h^{-1} adjustable drippers to supply water to the plants three times per week. Pest and disease control was not deemed necessary during experimentation, but removal of weeds was regularly required and manually performed. Final harvest was carried out at the end of June 2021. The soil properties of the pilot field are described in previous own studies [65,78].

4.4. Fertilization treatments

The pilot cultivation of *S. syriaca* subsp. *syriaca* followed weekly fertilization treatments using water (control), chemical (ChF), biostimulant and INM in liquid or soluble granule fertilizers administered with foliar and soil applications (Supplementary materials Methods S1). The foliar applications were performed using a 5 L plastic handheld sprayer (low pressure) until apparent wetness, and the soil applications were manually performed (100 mL nutrient solution per plant). The INM and biostimulants were supplied with four special fertilizers from THEOFRASTOS company, Industrial area of Korinthos, GR-20100 Korinthos, Greece (Supplementary materials Methods S1). These polysaccharide-based semi-organic fertilizers are made from high quality organic edible raw materials and plant extracts, resulting in a full supplement of plants with amino acids, vitamins, sugars, and macro-microelements. The INM polysaccharides are aimed to contribute to the formation of stable soil aggregates formed in connection with active soil microorganisms; INM polysaccharides when foliar-sprayed, they are expected to be rapidly absorbed by the plants. The chemical fertilizers were all in soluble powder or granule form, except the liquid fertilizer for micronutrients (Plex Mix, AGRI.FE.M. LTD Fertilizers, Greece). Two foliar fertilization treatments (INM and chemical), three soil application treatments (INM, chemical and a biostimulator) and a control for the pilot cultivation of *S. syriaca* subsp. *syriaca* in the field were employed in the experimentation (Supplementary materials Methods S1).

4.5. Plant measurements

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All plant and leaf level measurements were regularly conducted until 25 May 25 2021, ending with completion of flowering. Regarding leaf measurements, fully-expanded leaves grown under direct light were sampled within 15 min. When this was not feasible, samples were placed in vials, were flash-frozen in liquid nitrogen and were transferred to a freezer (-80°C) for storage. All replicate leaves were sampled from separate plant individuals.

4.5.1. Non-invasive evaluation of leaf coloration at three growth stages

Since leaf coloration depends on the fertilization regime affecting both photosynthetic capacity and visually perceived quality [79], this variable was assessed by employing three methods:

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

(ii) Index of absorbance difference (I_{AD}) accurately evaluating fruit ripeness, since it is closely associated with outer mesocarp chlorophyll content [80]; I_{AD} was computed as the difference between the absorbance values at 670 and 720 nm, near the chlorophyll absorbance peak [80]. The potential of I_{AD} in reflecting respective differences in leaves has not been previously evaluated. In this investigation, I_{AD} was determined in leaves by using the DA meter (tr DA Meter, T.R. Turoni, Italy).

(iii) Leaf colour quantified 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; L* [a measure of lightness, ranging from 0 (black) to 100 (white)], a* (a measure of intensity in the green to red range, where negative values refer to green and positive to red), and b* (a measure of representing intensity in the blue to yellow range, where negative values refer to blue and positive to yellow) [33].

These measurements were *in situ* conducted in attached leaves of intact plant individuals, at three different growth stages (vegetative, early flowering, and full flowering). Three points were recorded per replicate leaf and were further averaged. Three replicate leaves were assessed per treatment.

4.5.2. Non-invasive evaluation of photosynthetic performance in growth stages

As a valid indicator of leaf photosynthetic performance [81,82], the ratio of variable to maximum chlorophyll fluorescence (F_v/F_m) was assessed. Measurements were performed by using a chlorophyll fluorometer (OS-30P, Op-tiSciences, Hudson, NH, USA). Prior to evaluation, leaves were dark-adapted (≥ 20 min) by employing leaf clips. F_v/F_m was assessed by applying a saturated photosynthetic photon flux density of 3000 μmol m^{-2} s^{-1}.

These measurements were *in situ* conducted in attached leaves of intact plant individuals, and at vegetative, early flowering, and full flowering stages. Three points were recorded per replicate leaf and were further averaged. Three replicate leaves were assessed per treatment.

4.5.3. Leaf shape indicators

A morphometric analysis was performed by analyzing leaf form. 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 estimate the following four (dimensionless) metrics of leaf form: (a) aspect ratio [(major axis) / (minor axis); axes of the best-fitted ellipse], (b) circularity [(4π × area) / $(\text{perimeter})^2$], (c) roundness [(4 × area) / [4π × $(\text{major axis})^2$]], and (d) solidity [(area) / (convex area)] [64,83]. Each metric was used to quantify a distinct feature of leaf shape. Aspect ratio and roundness are affected by the length to width ratio, while circularity and solidity are depend on serration and lobing [66]. Aspect ratio ranges from 1 (circle) to 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 employed to detect leaves lacking such structures [66]. Solidity, unlike

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circularity, is not greatly influenced by serrations and minor lobing [66] . Thirty leaves (5 per plant × 6 plant individuals) were analyzed per treatment.

4.5.4. Plant growth and biomass partitioning to generative organs

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

4.5.5. Leaf chlorophyll and carotenoid contents

Chlorophyll content is important for leaf coloration and photosynthetic performance and carotenoids represent key non enzymatic antioxidants [68,84,85]. The effect of growth conditions on chlorophyll and carotenoid contents was therefore assessed. Following fine chopping, leaf portions (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, extraction took place in a dark room [84,85]. 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 [86]. Three leaves were assessed per treatment. For each replicate, four samples (collected from different plant individuals) were pooled, and the assay was performed twice.

4.5.6. Leaf total phenolic and total flavonoid contents

Phenols and flavonoids are critical non enzymatic antioxidants [68,84,85] . Leaf samples (0.1 g) were extracted with 1 mL of 80% aqueous methanol in an ultrasonic bath (10 min) and were then centrifuged (15000 g for 10 min). The contents of total phenolics and total flavonoids were determined by using the Folin-Ciocalteu assay and the aluminum chloride colorimetric assay, respectively [68]. The absorbance against prepared reagent blank was determined using a microplate reader (Infinite 200 PRO, TECAN, 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 fresh mass. Three leaves were assessed per treatment. For each replicate, four samples (collected from different plant individuals) were pooled, and the assay was performed twice.

4.5.7. Leaf soluble sugar content

The carbohydrate status is related to photosynthetic activity. The effect of fertilization scheme on carbohydrate status was therefore assessed. Leaf samples (0.1 g) were incubated with 1 mL deionized water in a water bath (100°C for 30 min). The homogenate was centrifuged (15000 g for 15 min) at room temperature (25°C). Then, 0.1 mL of the solution was mixed with anthranone ethyl acetate and sulphuric acid. Soluble sugar content was assayed in the supernatant according to [87] by measuring the absorbance at 630 nm using a spectrophotometer (Mapada UV-1800; Mapada Instruments Co., Ltd., Shanghai, China). These measurements were conducted on three replicates per treatment. For each replicate, four samples (collected from different plant individuals) were pooled, and the assay was performed twice.

4.5.8. Leaf and inflorescence mineral analysis

To assess the role of fertilization regime on mineral uptake by plants, leaf and inflorescence mineral analysis was conducted. The samples were washed with distilled water, dried at 70°C, weighed, ground, and then analyzed for total N by the Kjeldahl method [88]. In addition, sub-samples were ash-burned at 500°C for at least 4 h (Mills and Benton-Jones, 1996); 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, flame photometry, atomic absorption spectromentry and UV-Vis spectrometry, depending on the element.

Mineral content was expressed on a dry weight basis. Three replicates were evaluated per treatment. For each replicate, four samples (collected from different plant individuals) were pooled, and the assay was performed twice.

4.6. Statistical analysis

Data analyses of the fertilization experiment were carried out using the SAS statistical software (SAS Institute, Cary, NC, USA). Data were tested for homogeneity of variances (Duncan's test). Subsequently, estimated least significant differences (LSD) of treatment effects were determined (P = 0.05).

5. Conclusions

In this study, a consolidated genetic fingerprinting based on seven molecular markers (four markers used for the first time in members of genus *Sideritis*) and DNA barcoding using two molecular markers was generated for the first time for *S. syriaca* subsp. *syriaca,* a threatened local endemic plant of Crete (Greece) with depleting wild-growing populations due to overharvesting from the natural environment, thus consolidating its molecular identity, and enabling the traceability of related commercial products. Due to limited cultivation/fertilization data, the optimal fertilization regime for plant growth/yield and herbal material quality was investigated in *S. syriaca* subsp. *syriaca* in a field study to facilitate its local cultivation in Crete as a valuable MAP with strong medicinal value approved by the European Medicines Agency. Five fertilization regimes were examined including chemical fertilizers (foliar/ root application), INM (foliar/ root application), and INM with biostimulant (root application). Leaf colour was not altered by the fertilization scheme, but leaf shape was indeed affected. In this way, the visually perceived quality was influenced by fertilization. INM (especially through foliar application) and chemical fertilizer (by soil application) improved yield and did not alter tissue water content or biomass partitioning to generative organs. Chemical fertilizers (foliar application) were the best treatment for enhanced antioxidant compound content, whereas INM with biostimulant was the worst one. In conclusion, different fertilization choices may be employed when targeting either yield or antioxidant compound accumulation in *S. syriaca* subsp. *syriaca*. For the former, INM (foliar application) and chemical fertilization (soil application) were found suitable, whereas for the latter the best scheme found was chemical fertilization (foliar application). The data furnished herein are aimed to further facilitate the sustainable exploitation of this valuable medicinal-aromatic plant still suffering from overharvesting directly from wild-growing populations threatened with extinction.

Supplementary Materials: The following supporting information can be downloaded at the website of this paper posted on Preprints.org. **Methods S1.** Chemical composition of fertilizers used in the experimental procedure (Theofrastos company, Korinthos, Greece) and fertilization treatments applied in the pilot cultivation of *Sideritis syriaca* subsp. *syriaca* with other Cretan endemic plants [64,65] at the premises of the Hellenic Mediterranean University; **Table S2.** Alignment presenting the nucleotide differences among 15 *Sideritis* species based on the molecular plastid markers *rbc*L and *trn*L/*trn*F. Accession numbers of DNA sequences obtained in this study from the GenBank are indicated next to taxon names. The clade marked in orange represents *S. syriaca* subsp. *syriaca* GR-1-BBGK-15,5939 studied herein. The taxon marked with asterisk (*) is characterized as *S. syriaca* in the database, but based on the origin of the specimen, it should be identified as *S. euboea* which is local endemic of Mt Dirphys and not as *S. syriaca* subsp. *syriaca* which is a local single-island endemic of Crete (Greece); **Table S3.A.** Phylogenetic analysis of *Sideritis* taxa using the plastid molecular marker *rbc*L. Alignment of 20 *Sideritis* DNA sequences retrieved from GenBank compared to *Sideritis syriaca* subsp. *syriaca* GR-1-BBGK-15,5939 specimen of the current study (OP909056); the conserved areas (100%) are toggled; **Table S3.B.** Phylogenetic analysis of *Sideritis* taxa using the plastid molecular marker *psb*A-*trn*H. Alignment of 94 *Sideritis* DNA sequences retrieved from GenBank compared to *Sideritis syriaca* subsp. *syriaca* GR-1-BBGK-15,5939 specimen of the current study (OP909054); the conserved areas (100%) are toggled; **Figure S1.** Effect of fertilization regime through different (root/foliar) application methods on leaf index of absorbance difference of *Sideritis syriaca* subsp. *syriaca* at vegetative (A), early flowering (B), and full flowering (C) stage; **Figure S2.** Effect of fertilization regime through different (root/foliar) application methods on leaf L value of *Sideritis syriaca* subsp. *syriaca* at vegetative

(A), early flowering (B), and full flowering (C) stage; **Figure S3.** Effect of fertilization regime through different (root/foliar) application methods on leaf a value of *Sideritis syriaca* at vegetative (A), early flowering (B), and full flowering (C) stage; **Figure S4.** Effect of fertilization regime through different (root/foliar) application methods on leaf b value of *Sideritis syriaca* subsp. *syriaca* at vegetative (A), early flowering (B), and full flowering (C) stage.

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