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Inoculation and N Fertilization Affect the Dry Matter, N Fixation, and Bioactive Compounds in Sulla Leaves

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Abstract: Sulla (*Sulla coronaria* [L.] Medik), a Mediterranean short-lived legume with tolerance to drought-prone environments, requires inoculation outside its natural habitat. Its leaves are appreciated for the bromatological composition and content of bioactive compounds. However, no information is available regarding the distinct effects of inoculation and nitrogen (N) applications on leaf dry matter (DM), fixed N, and bioactive compounds. Sulla leaves were sampled from the vegetative stage to seed set in Sardinia (Italy) during 2013–2014 and leaf DM, N content, and fixed N were determined. Compared to the best performing inoculated treatments, DM yield and fixed N values of the control only represented 8% to 20% and 2% to 9%, respectively. A significant relationship between fixed N and leaf DM yield was established, reaching 30 kg fixed N t⁻¹ at seed set. Significant variations in leaf atom% ¹⁵N excess and %Ndffa quantified decreases in leaf N fixation coupled with N application. Moreover, the petiole content of phenolic compounds markedly increased in the uninoculated control, suggesting deeper investigations on the relationship between bioactive compounds and inoculation treatments. Results highlighted substantial variation in DM, N yields, N-fixation ability, and content of bioactive compounds of sulla leaves caused by inoculation and N fertilization.

Keywords: *Sulla coronaria*; inoculation; N supply; leaf biomass; N fixation; phenolic compounds

1. Introduction

The development of sustainable and alternative cropping systems may benefit from wider exploitation of legume crops, which supply high-quality food and feed and also deliver multiple services [1,2]. In fact, the symbiotic associations of legumes with compatible rhizobia fix the atmospheric nitrogen (N) saving of fossil energy inputs, release high-quality organic matter in the soil, contribute to reduce the emission of greenhouse gases, and allow carbon sequestration in soils [3–5].

Sulla coronaria (L.) Medik syn. *Hedysarum coronarium* L. [6] is a perennial legume originating in the Mediterranean Basin and usually grown as a rainfed biennial forage crop due to its notable adaptation to marginal and drought-prone environments [7–9]. Sulla has a remarkable potential in fixing atmospheric N and improving soil fertility and structure, as well as a valuable role in supporting multifunctional agriculture [9–12].

Sulla produces a bloat-safe and high-quality forage with a proper content of condensed tannins that is beneficial for improving animal health and welfare [13,14]. Variations in total phenolic, non-tannic

phenolic, and condensed tannin contents in sulla forage have been observed, depending on plant phenological phases and organs investigated [15]. Indeed, leaves are the biomass fraction of sulla with the highest content of phenolic compounds that range from 40.9 to 48.8 g kg⁻¹, whereas in stems the range is from 13.5 to 17.6 g kg⁻¹ [16].

It should be considered that the localization of bioactive compounds in plant tissues and changes in their concentration associated with plant maturity affect the palatability and intake of forage consumed by animals. Selmi et al. [17], investigated the leaves of *Hedysarum coronarium*, *Medicago truncatula*, *Pisum sativum*, and *Vicia sativa*; they found greater crude protein content and much lower fiber fraction (NDF and ADF) concentration, with related good performance in vitro, when compared to the values obtained by Gasmi-Boubaker et al. [18] using whole shoots of the same species. According to Gasmi-Boubaker et al. [18], this may be explained by the low potential feeding value of the stem, which represents from 50% to 70% of the biomass. Di Trana et al. [19] evidenced that polyphenols content of ingested sulla can affect the antioxidant activities and polyphenol contents of goat milk and also found a positive correlation between goat plasma antioxidant capacity and condensed tannins intake. These findings highlight the antioxidant activity of diets based on sulla forage, which appears as a promising strategy for improving product quality. Additionally, phenolic compounds are known to play multifunctional roles in rhizospheric plant-microbe interactions and, in particular, in plants establishing intimate symbiosis with N-fixing bacteria [20]. On the other hand, plant phenolics and tannins are synthesized to meet ordinary plant physiological demands but also as a response to biotic and abiotic stresses [14,21].

Sulla leaves represent an important shoot fraction not only for their valuable bromatological composition but also as a main source of bioactive compounds. Therefore, variation in its contribution to the total shoot dry matter (DM) are important. Previous studies have shown that, in traditional environments of cultivation for sulla, leaf contribution might be affected by genotype, cutting frequency, and year of life cycle [7,11,22,23].

It is known that sulla cropping outside its natural habitat and where it has not been grown before requires mandatory inoculation with the host-specific *Rhizobium sullae* [24,25]. Several papers have documented the importance of proper inoculation methods with selected strains for increasing total plant dry matter [26–31]. Recently, a commercial inoculant specific for sulla has been made available, favoring the inoculation of sulla at a larger scale than in the past [32], making the exploitation of this valuable legume in new environments easier.

Another key issue in legume establishment, when the N fixation process might not be active yet, deals with the use of a starter N dose at sowing, especially in low fertility soils. However, this issue is still controversial and contrasting conclusions can also be found for the same legume species (e.g., faba bean (*Vicia faba* L.) [33,34]. To date, no information is available regarding the distinct effects of inoculation or the application of starter N on DM and fixed N of sulla leaves, as well as the precise effect of inoculation on the content of bioactive compounds of sulla leaves. We hypothesized that leaf proportion and traits might be affected by inoculation and fertilization treatments.

Within a framework aimed at promoting the valorization of legumes' multiple benefits in Mediterranean farming systems and by extending sulla to a new area of cultivation, the specific aims of this research were to (i) investigate the contribution to aerial dry matter, nitrogen yield, and fixed nitrogen from leaves harvested in sulla plants subjected to different inoculation treatments and N fertilization rates, and (ii) determine the effect of inoculation on leaf bioactive compounds and antioxidant capacity compared to an uninoculated treatment.

2. Materials and Methods

2.1. Locations, Experimental Design, and Crop Management

The research was performed in Sardinia (Italy) during 2013–2014 in an extensive traditional silvopastoral area within a private dairy sheep enterprise (40°49'74" N, 8°28'14" E, elevation 357 m

a.s.l.) mainly based on the exploitation of semi-natural pastures, where sulla crop was introduced for the first time as a novel option for improving seasonal feeding sources. The soil, classified as Lithic Xerorthents [35], has neutral pH (7.2) and loamy texture with adequate contents of N (0.2%), phosphorous (29 ppm), and organic matter (3.9%). Moreover, the soil has been left uncultivated for the last 40 years, and sulla was not present in the area as a spontaneous plant. In autumn 2013, after soil ploughing and seedbed preparation, the sulla crop was established at a sowing density of 300 seeds m⁻². Before sowing, the soil was fertilized with 100 kg ha⁻¹ of P₂O₅ and sulla seeds were surface sterilized by immersion in ethanol (90%). The experimental design was a split-plot with three replications, inoculation treatment in the main plot, and N applications in the subplot. Each main plot consisted of three sulla rows, each 15 m in length and 1 m apart. Different inoculation methods for sulla were applied as described in detail and with related issues by Sulas et al. [31]. Briefly, the following treatments were evaluated: (i) the ancient inoculation practice by mixing seed with soil from an existing sulla field (soil inoculant, SIN), (ii) the liquid inoculant (LIN), supplied by Sassari University; (iii) the current Australian commercial peat inoculant (P1IN) strain, applied at the recommended rate (iv) and double rate peat inoculant (P2IN); (v) an uninoculated control (UNI). Additionally, a subplot was left unfertilized (N0), whereas a subplot was fertilized in November with an ammonium nitrate fertilizer at a rate of 100 kg ha⁻¹ of N (N100).

Moreover, to quantify the N-fixation ability in sulla leaves by using *Avena sativa* (oats) as a non-fixing reference plant, enriched ¹⁵N fertilizer (10 atom% ¹⁵N enriched ammonium sulfate) was applied to 3 m² (1 m × 3.0 m) areas at a rate of 4 kg N ha⁻¹ at seedling emergence. The ¹⁵N-enriched fertilizer was diluted in water and uniformly hand-sprayed at a rate of 1 L m⁻².

2.2. Measurements and Sampling

Representative shoot samples (30 cm length) within the 3 m² ¹⁵N labeled area along the row containing plants were cut at vegetative, flowering, and seed set stages, respectively, according to the phenological scale by Borreani et al. [8]. Shoot samples were partitioned into components and fresh leaf subsamples were oven-dried at 60 °C until constant weight and dry matter (DM) content was calculated. Dry sub-samples of leaves were ground finely enough to pass through a 1 mm mesh and submitted by dry combustion to elemental analyzer isotope ratio mass spectrometry to determine N content (%N) and the atom% ¹⁵N. Leaf nitrogen yield (kg N ha⁻¹) was calculated by multiplying leaf DM yield (kg ha⁻¹) per its N content (%). The proportion of N derived from the atmosphere (%Nd_{fa}) in sulla leaves was calculated according to the ¹⁵N isotopic dilution method [36] using the following equation:

$$\%Nd_{fa} = (1 - \text{atom}\% \text{ } ^{15}\text{N}_{\text{sulla}} / \text{atom}\% \text{ } ^{15}\text{N}_{\text{oats}}) \times 100 \quad (1)$$

where = (atom% ¹⁵N sample – atom% ¹⁵N N₂ air) and atom% ¹⁵N of air N₂ = 0.3663.

The amount of N fixed (N_{fix}) by sulla leaves was then obtained:

$$N_{\text{fix}} (\text{kg ha}^{-1}) = \text{sulla N} (\text{kg ha}^{-1}) \times \%Nd_{fa} / 100 \quad (2)$$

2.3. Content of Bioactive Compounds and Antioxidant Capacity in Sulla Leaves

For unfertilized peat inoculation (P1IN) and uninoculated treatments (UNI), additional leaf samplings were performed at the vegetative and flowering stage, respectively. Sulla leaves were subdivided into leaflets and petioles and their subsamples were kept on ice, freeze dried, and ground to a fine powder. Ground leaflet and petioles subsamples (50 mg) were treated with 2.5 mL acetone/water (7:3) mixture and shaken for 60 min. The samples were then centrifuged for 10 min at 3900 rpm and the supernatant was stored at 4 °C until use for the following determinations. Total phenolic (TotP) and non-tannic phenolic (NTP) content of the extracts were determined using the Folin–Ciocalteu reagent according to Singleton and Rossi [37], with some modifications by Piluzza and Bullitta [15].

Results were expressed as g gallic acid equivalent per kg dry weight of leaf material (g GAE/kg DW). The butanol assay of Porter et al. [38] was adapted [15] for quantification of extractable condensed tannin contents. The condensed tannins content was expressed as g delphinidin equivalent per kg dry weight (g DE/kg DW). Total flavonoids (TotF) were quantified by colorimetric assay with the AlCl_3 method in accordance with procedures reported previously [39]. Catechin was used as a standard and the flavonoid content was expressed as g catechin equivalent per kg dry weight of leaf material (g CE/kg DW).

Antioxidant capacity was determined by means of the ABTS ((2,2'-azinobis (3-ethylbenzothiazoline-6-sulphonic acid) diammonium salt)) and by DPPH (1,1-diphenyl-2-picrylhydrazyl) assays [40] with some modifications [41]. Trolox, a water-soluble analogue of vitamin E was used as the reference standard. The results were expressed in terms of Trolox equivalent antioxidant capacity (TEAC), as mmol Trolox equivalents per 100 g dry weight of leaf material (mmol TEAC/100 g DW).

2.4. Statistical Analysis

Statistical analysis was performed using R [42]. To homogenize the variance, after the Bartlett test, percentages were arcsin transformed [43]. The data reported in the tables were back transformed. For each sampling, data (<http://dx.doi.org/10.17632/t9yvrwbc89.1>) were subjected to a two-way analysis of variance (ANOVA) to test the effect of inoculation and N rates and their interaction on leaf contribution to total shoot DM yield, leaf %N, total N, atom% ^{15}N excess, %Ndfa, and fixed N. Means were compared based on Tukey's HSD (honestly significant difference) test at the 0.05 probability level.

Regarding the content of bioactive compounds and antioxidant capacity, Fisher's least significant difference (LSD) was applied for means separation to find significant differences between UNI and P1IN treatments within leaflets and petioles.

3. Results

3.1. Leaf Contribution, DM Yield, Nitrogen Content, and Yield

Annual rainfall during 2013–2014 reached 600 mm (10% higher than long-term value), whereas temperatures were similar to mean values. From vegetative to seed set stage, relative leaf contribution on average progressively halved from 96% to 23% of total shoot DM (Table 1). Leaf contribution was irrespective of inoculation, whereas it was affected by N rates at the later stage, when N100 induced lower relative leaf contributions particularly in SIN and P2IN treatments.

Table 1. Average ($\pm$ standard error) leaf contribution to sulla shoot.

Leaf Contribution (%)	Vegetative		Flowering		Seed Set		<i>p</i> > F
	N0	N100	N0	N100	N0	N100	
SIN	0.94 $\pm$ 0.02	0.94 $\pm$ 0.02	0.49 $\pm$ 0.0	0.41 $\pm$ 0.02	0.25 $\pm$ 0.01	0.19 $\pm$ 0.01	**
LIN	0.97 $\pm$ 0.01	0.97 $\pm$ 0.01	0.80 $\pm$ 0.1	0.56 $\pm$ 0.003	0.23 $\pm$ 0.003	0.21 $\pm$ 0.02	ns
P1IN	0.96 $\pm$ 0.01	0.96 $\pm$ 0.01	0.50 $\pm$ 0.1	0.57 $\pm$ 0.20	0.24 $\pm$ 0.01	0.21 $\pm$ 0.01	ns
P2IN	0.97 $\pm$ 0.003	0.97 $\pm$ 0.003	0.45 $\pm$ 0.03	0.53 $\pm$ 0.02	0.31 $\pm$ 0.05	0.22 $\pm$ 0.01	**
UNI	0.97 $\pm$ 0.01	0.97 $\pm$ 0.01	0.67 $\pm$ 0.2	0.55 $\pm$ 0.10	0.23 $\pm$ 0.03	0.22 $\pm$ 0.003	ns
Average	0.96		0.55		0.23		
	<i>p</i> > F		<i>p</i> > F		<i>p</i> > F		
Inoculation (I)	0.242		0.055		0.244		
N rates (N)	1.000		0.246		0.007		
I $\times$ N	1.000		0.268		0.273		

In the rows, Tukey honestly significant difference (HSD) test for N fertilizer effect on each type of inoculation.

** *p* $\leq$ 0.01; ns, not significant. SIN, soil inoculant; LIN, liquid inoculant; P1IN peat inoculant; P2IN, double rate peat inoculant; UNI, uninoculated control.

Absolute leaf DM yield was significantly affected by both inoculation and fertilization, except at the seed set stage for the latter when the interaction I $\times$ N was significant (Table 2). On average, leaf DM yield (Y) varied from about 1400 kg ha⁻¹ at the vegetative stage to substantially stable values

of 2300–2400 kg ha⁻¹ in the remaining two stages, even with substantial different leaf contribution to the total shoot DM (Table 1). Within N0 and at the first sampling, P1IN and SIN produced 3- to 4-fold leaf biomass than UNI and LIN treatments. Leaf dry matter yield (DMY) differed significantly between N0 and N100 for LIN and UNI. This trend, indicating lowest leaf DMY and a significant increase due to N fertilization, was maintained in UNI until the seed set stage. Additionally, it is worth noting that in absence of fertilization, leaf DMY of UNI only represented 21%, 12%, and 8% of the best inoculated treatment at the vegetative, flowering, and seed set stages, respectively. On the other hand, fertilization induced remarkable increases in leaf DMY of UNI, which was from 3- to 4-fold as high in N100 as it was in N0.

Table 2. Average ($\pm$ standard error) leaf dry matter yield (kg ha⁻¹).

Dry Matter (kg ha ⁻¹)	Vegetative			Flowering			Seed Set	
	N0	N100	<i>p</i> > F	N0	N100	<i>p</i> > F	N0	N100
SIN	2164 $\pm$ 340a	1844 $\pm$ 74a	ns	2003 $\pm$ 178b	3432 $\pm$ 433b	*	2867 $\pm$ 461b	3434 $\pm$ 171a
LIN	465 $\pm$ 21b	1626 $\pm$ 271a	**	1460 $\pm$ 223c	1407 $\pm$ 234c	ns	1365 $\pm$ 227c	2199 $\pm$ 367b
P1IN	1230 $\pm$ 225ab	1243 $\pm$ 89b	ns	2282 $\pm$ 380a	5015 $\pm$ 713a	**	3096 $\pm$ 530b	1916 $\pm$ 109c
P2IN	1678 $\pm$ 280a	1843 $\pm$ 162a	ns	2471 $\pm$ 323a	3256 $\pm$ 463b	*	4744 $\pm$ 791a	2458 $\pm$ 268b
UNI	459 $\pm$ 67b	1529 $\pm$ 254ab	**	309 $\pm$ 52d	1427 $\pm$ 86c	***	373 $\pm$ 62d	1554 $\pm$ 49c
Average		1408			2306			2401
		<i>p</i> > F			<i>p</i> > F			<i>p</i> > F
Inoculation (I)		0.014			<0.001			<0.001
N rates (N)		0.049			<0.001			0.622
I $\times$ N		0.107			0.098			0.024

In the columns N fertilizer applications within each stage (a–d), means followed by different letters are significantly different at *p* < 0.05 level. In the rows, Tukey HSD test for N fertilizer effect on each type of inoculation. * *p* $\leq$ 0.05; ** *p* $\leq$ 0.01; *** *p* $\leq$ 0.001; ns, not significant. SIN, soil inoculant; LIN, liquid inoculant; P1IN peat inoculant; P2IN, double rate peat inoculant; UNI, uninoculated control.

Both inoculation and fertilization significantly affected leaf N percentage at the vegetative stage (Table 3). Under both fertilization treatments, the leaf N content of SIN, P1IN, and P2IN was significantly higher than UNI. Within N0, N content of SIN was twice as high as the value of UNI. Compared to N0, N100 treatment significantly increased N content of LIN and UNI by 40% and 67%. Irrespective of inoculation and fertilization, the leaf N content reached, on average, 3.16% at flowering, whereas it dropped at 2.64% at seed set stage, when the highest value for UNI in N0 was presumably linked to the delayed and reduced plant development caused by the absence of inoculation.

Table 3. Average ($\pm$ standard error) nitrogen content of sulla leaves (% dry matter (DM)).

N Content (%)	Vegetative			Flowering		Seed Set	
	N0	N100	<i>p</i> > F	N0	N100	N0	N100
SIN	2.00 $\pm$ 0.1a	2.27 $\pm$ 0.0a	ns	3.09 $\pm$ 0.1	3.55 $\pm$ 0.1	2.50 $\pm$ 0.2c	2.24 $\pm$ 0.3c
LIN	1.27 $\pm$ 0.1c	1.77 $\pm$ 0.1c	**	2.72 $\pm$ 0.1	3.21 $\pm$ 0.2	2.03 $\pm$ 0.1d	2.42 $\pm$ 0.2b
P1IN	1.93 $\pm$ 0.1a	2.23 $\pm$ 0.1a	*	3.22 $\pm$ 0.2	2.76 $\pm$ 0.1	2.90 $\pm$ 0.0b	2.73 $\pm$ 0.1a
P2IN	1.73 $\pm$ 0.1b	2.03 $\pm$ 0.1b	*	2.93 $\pm$ 0.3	3.73 $\pm$ 0.3	2.85 $\pm$ 0.2b	2.79 $\pm$ 0.2a
UNI	1.03 $\pm$ 0.2d	1.73 $\pm$ 0.0c	**	3.41 $\pm$ 0.2	2.99 $\pm$ 0.3	3.29 $\pm$ 0.1a	2.68 $\pm$ 0.1a
Average		1.80			3.16		2.64
		<i>p</i> > F			<i>p</i> > F		<i>p</i> > F
Inoculation (I)		<0.001			0.257		<0.001
N rates (N)		<0.001			0.191		0.184
I $\times$ N		0.250			0.016		0.063

In the columns N fertilizer applications within stage (a–d), means followed by different letters are significantly different at *p* < 0.05 level. In the rows, Tukey HSD test for N fertilizer effect on each type of inoculation. * *p* $\leq$ 0.05; ** *p* $\leq$ 0.01; ns, not significant. SIN, soil inoculant; LIN, liquid inoculant; P1IN peat inoculant; P2IN, double rate peat inoculant; UNI, uninoculated control.

Across the investigated stages, leaf N yield was significantly affected by inoculation and fertilization, except at seed set for the latter, when the interaction was significant (Table 4). As a general

average, leaf N yield varied from 26.8 to 72.6 kg ha⁻¹ at the vegetative and flowering stage, respectively. Under N0, leaf N yield markedly decreased without inoculation where it was always the lowest and only represented 11%, 15%, and 9% of the best inoculated treatment at the three consecutive stages. However, N100 fertilization determined a 4- to 5-fold increase in leaf N yield of UNI.

Table 4. Average ($\pm$ standard error) leaf nitrogen (N) yield (kg ha⁻¹).

N Content (kg ha ⁻¹)	Vegetative			Flowering			Seed set	
	N0	N100	<i>p</i> > F	N0	N100	<i>p</i> > F	N0	N100
SIN	42.7 $\pm$ 5.6a	42.1 $\pm$ 2.0a	ns	62.3 $\pm$ 7.5ab	122.0 $\pm$ 16.5a	***	72.5 $\pm$ 12.1b	77.7 $\pm$ 13.8a
LIN	5.8 $\pm$ 0.4c	28.4 $\pm$ 4.7b	***	40.1 $\pm$ 6.7b	45.6 $\pm$ 7.6b	ns	27.7 $\pm$ 4.7c	51.4 $\pm$ 8.6b
P1IN	23.3 $\pm$ 2.9b	27.8 $\pm$ 2.7b	ns	70.8 $\pm$ 11.7a	137.9 $\pm$ 17.8a	***	89.8 $\pm$ 15.0b	52.6 $\pm$ 5.6b
P2IN	29.3 $\pm$ 7.8b	37.2 $\pm$ 4.4a	*	70.6 $\pm$ 11.3a	123.3 $\pm$ 23.3a	***	136.4 $\pm$ 22.7a	67.8 $\pm$ 5.4a
UNI	4.7 $\pm$ 0.4c	26.7 $\pm$ 6.2b	***	10.8 $\pm$ 1.8c	42.3 $\pm$ 3.1b	*	12.1 $\pm$ 2.0d	41.8 $\pm$ 2.9c
Average		26.8			72.6			63.9
		<i>p</i> > F			<i>p</i> > F			<i>p</i> > F
Inoculation (I)		<0.001			<0.001			0.001
N rates (N)		0.004			<0.001			0.363
I $\times$ N		0.161			0.310			0.027

In the columns N fertilizer applications within stage (a–d), means followed by different letters are significantly different at *p* < 0.05 level. In the rows, Tukey HSD test for N fertilizer effect on each type of inoculation. * *p* $\leq$ 0.05; *** *p* $\leq$ 0.001; ns, not significant. SIN, soil inoculant; LIN, liquid inoculant; P1IN peat inoculant; P2IN, double rate peat inoculant; UNI, uninoculated control.

3.2. Leaf Atom% ¹⁵N Excess, %Nd_{fa}, Fixed N, and Relationships between Leaf Biomass and Fixed Nitrogen

Inoculation and fertilization significantly affected leaf atom% ¹⁵N excess which, on average, reduced markedly from 0.061% to 0.007%, indicating a progressive reduction across the season (Table 5). The leaf atom% ¹⁵N excess in UNI (N0) showed the highest values, except at seed set. Irrespective of inoculation treatments, the N100 fertilization marked increased leaf atom% ¹⁵N excess values at the vegetative stage, whereas in SIN, LIN, P1IN it was at the flowering stage. At seed set, leaf atom% ¹⁵N excess values of only P1IN and P2IN were affected by fertilization.

Table 5. Average ($\pm$ standard error) atom% ¹⁵N excess of leaf sulla (%¹⁵N).

¹⁵ N Excess (%)	Vegetative			Flowering			Seed Set	
	N0	N100	<i>p</i> > F	N0	N100	<i>p</i> > F	N0	N100
SIN	0.014 $\pm$ 0.01c	0.080 $\pm$ 0.01b	***	0.003 $\pm$ 0.001b	0.013 $\pm$ 0.002b	*	0.005 $\pm$ 0.001	0.006 $\pm$ 0.002
LIN	0.038 $\pm$ 0.01b	0.135 $\pm$ 0.03a	***	0.009 $\pm$ 0.001b	0.029 $\pm$ 0.001a	**	0.007 $\pm$ 0.003	0.007 $\pm$ 0.002
P1IN	0.026 $\pm$ 0.003b	0.048 $\pm$ 0.02c	*	0.003 $\pm$ 0.001b	0.033 $\pm$ 0.01a	**	0.004 $\pm$ 0.001	0.007 $\pm$ 0.0003
P2IN	0.017 $\pm$ 0.004c	0.043 $\pm$ 0.01c	*	0.003 $\pm$ 0.001b	0.009 $\pm$ 0.002b	ns	0.004 $\pm$ 0.001	0.011 $\pm$ 0.002
UNI	0.086 $\pm$ 0.01a	0.123 $\pm$ 0.002a	**	0.024 $\pm$ 0.01a	0.031 $\pm$ 0.02a	ns	0.009 $\pm$ 0.0004	0.010 $\pm$ 0.001
Average		0.061			0.016			0.007
		<i>p</i> > F			<i>p</i> > F			<i>p</i> > F
Inoculation (I)		<0.001			0.027			0.050
N rates (N)		<0.001			0.003			0.018
I $\times$ N		0.040			0.370			0.153

In the columns N fertilizer applications within stage (a–d), means followed by different letters are significantly different at *p* < 0.05 level. In the rows, Tukey HSD test for N fertilizer effect on each type of inoculation. * *p* $\leq$ 0.05; ** *p* $\leq$ 0.01; *** *p* $\leq$ 0.001; ns, not significant. SIN, soil inoculant; LIN, liquid inoculant; P1IN peat inoculant; P2IN, double rate peat inoculant; UNI, uninoculated control.

On average, leaf %Nd_{fa} varied from an initial value of about 58% to 91% at seed set (Table 6). Inoculation significantly affected %Nd_{fa} at each investigated stage, whereas effects of fertilization were noticeable at vegetative and flowering stages only. Within N0, leaf %Nd_{fa} of uninoculated treatment always had the lowest values. N100 significantly reduced leaf %Nd_{fa} of SIN, P2IN, and LIN at vegetative stage and of P1IN and LIN at flowering stage.

Table 6. Average ($\pm$ standard error) proportion of leaf N derived from the atmosphere (%Nd_{fa}).

Nd _{fa} (%)	Vegetative			Flowering			Seed set	
	N0	N100	<i>p</i> > F	N0	N100	<i>p</i> > F	N0	N100
SIN	89.8 $\pm$ 3.1a	51.6 $\pm$ 3.9b	*	97.4 $\pm$ 0.8a	91.6 $\pm$ 1.2a	ns	93.1 $\pm$ 1.3a	92.4 $\pm$ 2.2a
LIN	64 $\pm$ 2.3ab	25.4 $\pm$ 1.0c	*	92.1 $\pm$ 1.6a	79.8 $\pm$ 3.5b	*	91.8 $\pm$ 2.0a	91.9 $\pm$ 2.4a
P1IN	77 $\pm$ 5.1a	62.1 $\pm$ 6.0a	ns	97.7 $\pm$ 0.4a	75.0 $\pm$ 10.6b	*	94.7 $\pm$ 1.0a	92.0 $\pm$ 0.5a
P2IN	87.3 $\pm$ 0.5a	62.1 $\pm$ 1.3a	*	97.5 $\pm$ 0.9a	94.4 $\pm$ 1.0a	ns	93.2 $\pm$ 3.2a	86.2 $\pm$ 1.9b
UNI	38.5 $\pm$ 1.2b	25.7 $\pm$ 0.8c	ns	76.9 $\pm$ 8.5b	75.2 $\pm$ 12.5b	ns	86.8 $\pm$ 2.3b	87.8 $\pm$ 0.9b
Average		58.3			87.8			91.0
	<i>p</i> > F			<i>p</i> > F			<i>p</i> > F	
Inoculation (I)	<0.001			0.043			0.033	
N rates (N)	<0.001			0.034			0.141	
I $\times$ N	0.497			0.495			0.284	

In the columns N fertilizer applications within stage (a–c), means followed by different letters are significantly different at $p < 0.05$ level. In the rows, Tukey HSD test for N fertilizer effect on each type of inoculation. * $p \leq 0.05$; ns, not significant. SIN, soil inoculant; LIN, liquid inoculant; P1IN peat inoculant; P2IN, double rate peat inoculant; UNI, uninoculated control.

Fixed N in leaves was significantly affected by inoculation across all stages and was always lower for UNI (Table 7). Within N0, fixed N in leaves of UNI only represented about 4%, 13%, and 9% of the best inoculated treatment in the three consecutive stages. N100 significantly affected fixed N in leaves of all inoculated treatments, except LIN at flowering when fixed N in leaves exceeded 100 kg ha^{−1}. At seed set interaction was also significant, ($p < 0.05$) as UNI and LIN showed a similar trend in being significantly higher within the N100 treatment.

Table 7. Average ($\pm$ standard error) fixed N in sulla leaves (kg ha^{−1}).

Fixed N (kg ha ^{−1})	Vegetative		Flowering			Seed Set	
	N0	N100	N0	N100	<i>p</i> > F	N0	N100
SIN	38.5 $\pm$ 5.5a	21.6 $\pm$ 1.5a	55.6 $\pm$ 9.3b	111.7 $\pm$ 16.0a	***	68.5 $\pm$ 11.4c	71.9 $\pm$ 12.1a
LIN	3.8 $\pm$ 1.0c	3.1 $\pm$ 1.9b	36.9 $\pm$ 6.2c	38.2 $\pm$ 6.4b	ns	27.0 $\pm$ 4.5d	47.8 $\pm$ 8.0b
P1IN	18.2 $\pm$ 3.5b	17.6 $\pm$ 4.5a	66.9 $\pm$ 10.7a	102.1 $\pm$ 11.6a	**	86.0 $\pm$ 14.6b	45.8 $\pm$ 6.6b
P2IN	25.6 $\pm$ 4.3a	24.1 $\pm$ 6.5a	64.0 $\pm$ 6.8a	113.9 $\pm$ 20.6a	**	128.3 $\pm$ 21.4a	62.7 $\pm$ 3.6a
UNI	1.5 $\pm$ 0.2c	3.3 $\pm$ 0.8b	8.9 $\pm$ 1.5d	34.5 $\pm$ 5.2b	**	11.0 $\pm$ 1.8e	36.3 $\pm$ 2.0c
Average		15.7		63.3			58.5
	<i>p</i> > F		<i>p</i> > F			<i>p</i> > F	
Inoculation (I)	<0.001		<0.001			0.002	
N rates (N)	0.173		<0.001			0.271	
I $\times$ N	0.178		0.346			0.034	

In the columns N fertilizer applications within stage (a–e), means followed by different letters are significantly different at $p < 0.05$ level. In the rows, Tukey HSD test for N fertilizer effect on each type of inoculation. ** $p \leq 0.01$; *** $p \leq 0.001$; ns, not significant. SIN, soil inoculant; LIN, liquid inoculant; P1IN peat inoculant; P2IN, double rate peat inoculant; UNI, uninoculated control.

By taking together data from the best inoculation treatments (SIN, P1IN, and P2IN), the regressions of the fixed N for each leaf biomass component on the corresponding DM yield varied significantly among the stages considered, indicating about 18, 26, and 30 kg fixed N t^{−1} leaf under N0 (Figure 1). Moreover, when comparing these values with the corresponding ones obtained under N100, different trends and reduced rates of fixed N were observed, attesting the absence or low relationship between fixed N and the corresponding leaf DM yield.

(a)

(b)

Figure 1. The relationship between fixed N and leaf dry matter yield in inoculated and unfertilized (a) or N100 fertilized (b) sulla plants. Black circles, black squares, and empty circles indicate leaf sampling carried out at vegetative, flowering, and seed set stage, respectively.

3.3. Leaf Antioxidant Capacity and Bioactive Compounds

At the vegetative stage, ABTS reached 30 mmol TEAC/100 g DW in UNI leaflets and it was 21% higher than in P1IN treatment (Table 8). The total antioxidant capacity determined through the DPPH assay followed the same trend. Again, total phenolics, non-tannic phenolics, tannic phenolics, and condensed tannins of UNI showed slightly higher values by 35%, 27%, 36%, and 32%, respectively. On the contrary, all parameters referred to petioles evidenced 2- to 4-fold higher values in the uninoculated petioles.

Table 8. Trolox equivalent antioxidant capacity (TEAC) by ABTS ((2,2'-azinobis (3-ethylbenzothiazoline-6-sulphonic acid) diammonium salt)) and DPPD (1,1-diphenyl-2-picrylhydrazyl) assays, total phenolics (TotP), non-tannic phenolics (NTP), tannic phenolics (TP), total flavonoids (TotF), and extractable condensed tannins (CT) of sulla leaf fractions (mean $\pm$ standard deviation).

		TEAC (mmol/100 g DW)		TotP (gGAE/kg DW)	NTP (gGAE/kg DW)	TP (gGAE/kg DW)	TotF (gCE/kg DW)	CT (gDE/kg DW)
		ABTS	DPPH					
Vegetative								
Leaflets	UNI	30.3 $\pm$ 1.0a	32.7 $\pm$ 1.5a	50.5 $\pm$ 0.4a	12.8 $\pm$ 0.1a	37.6 $\pm$ 0.5a	17.2 $\pm$ 1.2a	25.1 $\pm$ 0.6a
	P1IN	25.0 $\pm$ 0.6b	26.2 $\pm$ 0.7b	37.4 $\pm$ 0.8b	10.1 $\pm$ 0.4b	27.7 $\pm$ 0.5b	14.8 $\pm$ 1.2a	18.9 $\pm$ 1.3b
<i>p</i> > F		<0.05	<0.001	<0.001	<0.001	<0.001	0.07	0.01
Petioles	UNI	22.3 $\pm$ 0.2a	22.8 $\pm$ 0.5a	29.8 $\pm$ 0.6a	13.0 $\pm$ 0.3a	16.9 $\pm$ 0.6a	9.8 $\pm$ 0.2a	10.2 $\pm$ 0.4a
	P1IN	5.5 $\pm$ 0.7b	5.3 $\pm$ 0.1b	10.6 $\pm$ 0.7b	6.8 $\pm$ 0.3b	3.8 $\pm$ 0.7b	3.6 $\pm$ 0.2b	3.5 $\pm$ 0.2b
<i>p</i> > F		<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
Flowering								
Leaflets	UNI	34.9 $\pm$ 1.5a	31.4 $\pm$ 1.1a	50 $\pm$ 3.2a	18.1 $\pm$ 0.6a	31.9 $\pm$ 2.7a	18.5 $\pm$ 1.0a	23.8 $\pm$ 1.8a
	P1IN	28.8 $\pm$ 1.3b	28.9 $\pm$ 2.73b	43.2 $\pm$ 1.0b	14.8 $\pm$ 0.8b	28.5 $\pm$ 0.5a	18.2 $\pm$ 0.8a	25.3 $\pm$ 0.8a
		<0.01	0.21	<0.05	<0.01	0.09	0.06	0.28
Petioles	UNI	25.3 $\pm$ 2.03a	25.7 $\pm$ 2.4a	39.4 $\pm$ 0.6a	12.9 $\pm$ 0.4a	26.5 $\pm$ 0.9a	14.3 $\pm$ 0.3a	17.7 $\pm$ 1.2a
	P1IN	8.7 $\pm$ 0.1b	5.8 $\pm$ 0.4b	12.9 $\pm$ 0.3b	6.8 $\pm$ 0.3b	6.1 $\pm$ 0.5b	5.0 $\pm$ 0.4b	3.5 $\pm$ 0.2b
<i>p</i> > F		<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001

In the columns within each leaf fraction, means followed by different letters are significantly different. P1IN, peat inoculant; UNI, uninoculated control.

At flowering, the similarly high differences between petioles were recorded, indicating high values for total phenolic, non-tannic phenolic, tannic phenolic, total flavonoid, and condensed tannin concentrations. In leaflets, the values of tannic phenolics, total flavonoids, and condensed tannins did not differ significantly between treatments.

4. Discussion

It has already been acknowledged that sulla inoculation with its specific rhizobial strain is a necessary practice for establishment outside the natural distribution area of its genus [24–26]. Previous reports documented the positive influence acted by a proper inoculation in terms of total shoot DM yield, without indications regarding the effects on distinct biomass components of the sulla plant [26–29]. Our research specifically addressed variations in leaf quantitative and qualitative traits caused by inoculation and fertilization factors. Previous studies evidenced that genotype, cutting regime, and year of life cycle were able to affect leaf traits and its contribution to total shoot DMY [7,11,22]. Additionally, our paper focused on the distinct effects of inoculation and fertilization on leaf contribution to sulla shoots in plants grown under undisturbed conditions. Summarizing the results achieved within inoculation treatments, the different parameters analyzed showed very consistent outcomes; only slight variations were observed but likely due to the different phases accounted for (namely vegetative, flowering, seed set). In fact, the commercial peat-based inoculant, used at the recommended rate, at a double rate or in both cases, showed significantly more promising results in terms of leaf N yield, %Ndfa, and fixed N but also in N content in leaves and DM yield. These results were somewhat overlapping for the two considered reproductive phases (flowering and seed set).

Similar to our study, a decline in leaf proportion associated with increased maturity was described by Minneé et al. [23] in New Zealand, whereas higher leaf proportions were reported at the same stage of growth investigated in Sicily [11].

The more relevant effect deals with wide differences in leaf DM yield when comparing inoculated and uninoculated sulla plants. Among inoculated treatments, overall LIN showed reduced performances compared to SIN, P1IN, and P2IN. However, inoculated sulla produced 3- to 8-fold more leaf DM than it did the uninoculated control, confirming the pivotal role of inoculation in a new area of cultivation. Moreover, the non-significant effect of N100 fertilization on leaf DM of SIN, P1IN, and P2IN indicated that N was not a limiting factor in the same treatments for leaf growth at

the vegetative stage. Our results cannot be directly compared with previous findings from different countries, where productive gains ascribed to inoculation were expressed in term of total shoot DMY, without differentiation into single components (leaves vs. stems and inflorescences) [26–30]. Even if absolute values of leaf DMY differed when compared with values reported by other authors [11,23], in accordance with our findings the same authors also reported a similar trend, indicating substantially constant values of leaf DMY with maturity. At the vegetative stage only, the N content of the inoculated and unfertilized control (UNI, N0) was half that of the P1IN and SIN treatments. Moreover, N fertilization applied with 100 units increased the N content of inoculated treatments, except SIN, indicating a different effect of SIN treatment on N content. However, absolute differences for N content within SIN, P1IN, and P2IN were substantially limited and not maintained at the subsequent stages of flowering and seed set. It is important to point out that unlike peat- and liquid-based inoculants, which are obtained following sterilization procedures, SIN is based on soil particles. Soil might include other microorganisms and, therefore, this might represent a possible explanation for the different result obtained for SIN. A recent study by Muresu et al. [44] reported that the microbial composition in *sulla* nodules (which was out of the aims of our research) is mainly affected by the different status (wild or cropped) in which *sulla* plants can be found. Moreover, the same authors ascertained in nodules of wild plants a higher endophytic occurrence that is potentially useful to promote plant growth in harsh conditions.

At flowering, the significant interaction of inoculation and fertilization presumably indicated a nitrogen concentration mainly due to a lower absolute value of leaf biomass in UNI. Of course, leaf N yield values reflected coupled variations of both yield and N content.

It is worth nothing that both fertilization and inoculation affected significantly the atom% ^{15}N excess of leaves, which is a trait independent from biomass yield level. The recorded leaf atom% ^{15}N excess values were lower or similar than available atom% ^{15}N excess values of entire *sulla* shoots as reported from previous studies [10,31]. Additionally, across the three investigated stages, N100 fertilization induced higher leaf atom% ^{15}N excess values, which means a lower dilution of atmospheric N, and concurrently lower N fixation rates compared to N0. This effect might be explained by the increased level of mineral soil N resulting from fertilization, which is preparatory to possible inhibitory action on nodule formation and N-fixation delay, as reported for some legume species [45–48]. Therefore, it represents a “collateral effect” of the use of high N applications, which sometimes is not considered, and not only by farmers. This effect and the marked differences due to inoculation were clearer at the vegetative stage, whereas they were less evident at flowering and seed set, when %Nd_{fa} was not affected by fertilization and only UNI still differed but also reached about 87%. This last result was presumably caused by contamination among inoculated and uninoculated plots, due to soil disturbances caused by wild boars. Moreover, the insignificant effect of N fertilization on %Nd_{fa} at the last stage is explained by the progressive reduction of soil nitrate availability at the end of the *sulla* growth cycle. A similar result was reported by Naudin et al. [49] for peas. Interestingly, the relationship of the fixed N for the corresponding leaf DM yield outlined an increasing trend with plant maturity, ranging from 18 (vegetative) to 30 (seed set) kg of fixed N t⁻¹ leaf. With respect to the latter values, a previous study carried out at two Italian locations reported a significant and not site-specific equivalence of about 1.8 kg ha⁻¹ of fixed N per 100 kg ha⁻¹ of total aerial DM [10], in line with the performances of white clover but higher than that achieved by pure stands of red clover [50]. Therefore, we investigated the relationship between fixed N and DM in *sulla* leaves and our results reported for the first time specific relationships for *sulla* leaves when harvested at different plant stages.

Additionally, when comparing N0 values with the corresponding ones obtained under N100, different trends and reduced rates of fixed N were observed, indicating the absence or a low relationship between fixed N in leaves and absolute lower rates ranging from 10 (flowering) to 18 (seed set) kg of fixed N t⁻¹ leaf. These contrasting findings represent additional clues confirming the depressive effect on N fixation efficiency acted by N application, particularly at vegetative and flowering stages, which were closer to the time of N supply. Only few papers still deal with N-fixation studies in *sulla* [10,12,22]

and no other information is available regarding the effects of inoculation on DM yields and traits of sulla leaves, to the best of our knowledge.

The important issue regarding the depressive effect on N fixation arising from the use of N rates in legume crops is still not duly considered. In fact, other reports based on the comparison of different inoculants or strains coupled with N applications in different legume species such as lentil, snap bean, and faba bean are available [34,51,52]. However, the same authors did not measure or consider that higher yields supported by N fertilization might reduce the N fixation efficiency of the same symbiosis. Therefore, future research must be addressed to clarify this important topic, which could be very relevant every time mineral N is still supplied to N fixing legume crops. On the contrary, the full efficiency of legumes should be pursued without N fertilizer applications while optimizing biological N sources with proper inoculants containing high effective and efficient rhizobial strains and also optimal combination with host genotypes. Symbiotic nitrogen fixation is a more sustainable and environmentally friendly process of acquiring N and its efficiency relies on considerable efforts by researchers and producers in matching suitable legume hosts with their compatible microsymbionts [53,54]. In this direction, recent investigations have addressed the diversity of rhizobia nodulating sulla, selection of inoculant strains, molecular phylogenetic analysis, strain fingerprinting and species identification, extracellular polysaccharides produced by *R. sullae* strains, etc. [55–59].

In addition to the limited information already available regarding bioactive compounds in sulla biomass under standard conditions of growth in traditional cultivation areas, our results reported new insights regarding peculiar variations in the concentration of phenolic compounds due to inoculation treatments. The concentration of total phenolics, non-tannic phenolics, tannic phenolics, and condensed tannins differed greatly between leaflets and petioles, confirming previous findings on the same legume species, indicating that leaflets are plant organs with the highest content of phenolic compounds [15]. Surprisingly, the concentration of the above-mentioned phenolic compounds differed greatly in leaf components of uninoculated plants compared to inoculated plants. In particular, wider differences due to inoculation were found in petioles (up to 3–5-fold concentrations) than in leaflets. This result represents novel information, to our knowledge. Even if we are not able to fully explain this finding, the higher values in petioles can be, presumably, associated to a plant reaction against stress conditions arising by the absence of the specific sulla microsymbiont and subsequent failure of the nodulation process. In fact, phenolic compounds usually act as signaling molecules in the initiation of legume-rhizobia symbiosis, which requires signaling and recognition by both partners; they can also act as agents in plant defense conferring disease resistance [60].

However, the absolute lower leaf DM yields in the uninoculated treatment might counterbalance important benefits potentially arising from higher content of the bioactive compounds that are usually contained in sulla leaves. On the other hand, it is important to point out that the peculiar content of bioactive compounds in sulla forage has been recognized to be linked to important benefits for ruminants and the environment such as bloating save, faster growth in young sheep, higher protein use efficiency in rumen, and decreased gastrointestinal parasite infection, nitrogen restitution, and methane production in ruminants, etc. [61,62].

Overall, our results suggest to better investigate the possible relationship between bioactive compounds and inoculation treatments, as, to date, inoculation benefits are usually discussed in terms of increases in DM and N yields, not considering the possible variation on qualitative parameters of forage biomass induced by inoculation. For example, proper tannin contents could be potentially exploited in the mitigation of methane emissions from ruminants in forage-based feeding systems and as a natural and ecologically friendly resource for improvement of nutrient utilization and environmental sustainability in meat and dairy farming [14]. Additionally, considering the potential multifunctional role from bioactive compounds, further research should be addressed for the identification of individual phenolic compounds.

5. Conclusions

Different inoculation treatments and N fertilization rates significantly affected leaf DM, N yields, and N-fixation ability of sulla plants from vegetative to seed set stages.

Without N fertilization, remarkable differences for leaf DM yield and fixed N values were found between inoculated and uninoculated treatments.

The variations recorded in leaf atom% ^{15}N excess and %Ndfa values quantified significant decreases in leaf N fixation capacity caused by N fertilization, leading to a reduced efficiency of the symbiotic association of sulla with related rhizobia that must be considered.

When considering the best unfertilized inoculation treatments, significant relationships between fixed N and leaf DM yield were established with peak values recorded at seed set.

Inoculation significantly affected the concentration of phenolic compounds, 3–5-fold higher in petioles of uninoculated than inoculated plants, whereas variations were smaller in the corresponding leaflets.

Results support novel and detailed information regarding quantitative and qualitative traits of sulla leaves.

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References

1. Rochon, J.J.; Doyle, C.J.; Greef, J.M.; Hopkins, A.; Molle, G.; Sitzia, M.; Scholefield, D.; Smith, C.J. Grazing legumes in Europe: A review of their status, management, benefits, research needs and future prospects. *Grass Forage Sci.* **2004**, *59*, 197–214. [\[CrossRef\]](#)
2. Deligios, P.A.; Tiloca, M.T.; Sulas, L.; Buffa, M.; Caraffini, S.; Doro, L.; Sanna, G.; Spanu, E.; Spissu, E.; Urracci, G.R. Stable nutrient flows in sustainable and alternative cropping systems of globe artichoke. *Agron. Sustain. Dev.* **2017**, *37*, 54. [\[CrossRef\]](#)
3. Peoples, M.; Brockwell, J.; Herridge, D.; Rochester, I.; Alves, B.; Boddey, R.; Dakora, F.; Bhattari, S.; Maskey, S.; Sampet, C.; et al. The contributions of nitrogen fixing crop legumes to the productivity of agricultural systems. *Symbiosis* **2009**, *48*, 1–17. [\[CrossRef\]](#)
4. Sinclair, T.R.; Vadez, V. The future of grain legumes in cropping systems. *Crop Pasture Sci.* **2012**, *63*, 501–512. [\[CrossRef\]](#)
5. Stagnari, F.; Maggio, A.; Galieni, A.; Pisante, M. Multiple benefits of legumes for agriculture sustainability: An overview. *Chem. Biol. Technol. Agric.* **2017**, *4*, 2. [\[CrossRef\]](#)
6. Choi, B.H.; Ohashi, H. Generic criteria and infrageneric system for Hedysarum and related genera (Papilionoideae-Leguminosae). *Taxon* **2003**, *52*, 567–576. [\[CrossRef\]](#)
7. Sulas, L.; Re, G.A.; Ledda, L.; Careda, S. The effect of utilization frequency on the forage production of sulla (*Hedysarum coronarium* L.). *Ital. J. Agron.* **1997**, *2*, 89–94.
8. Borreani, G.; Roggero, P.P.; Sulas, L.; Valente, M.E. Quantifying morphological stage to predict the nutritive value in sulla (*Hedysarum coronarium* L.). *Agron. J.* **2003**, *95*, 1608–1617. [\[CrossRef\]](#)
9. Sulas, L. The future role of forage legumes in the Mediterranean climatic areas. In *Grasslands: Developments Opportunities Perspectives*; Reynolds, S.G., Frame, J., Eds.; FAO: Rome, Italy; Science Publishers: Enfield, NH, USA, 2005; pp. 29–54, ISBN 1-57808-359-1.
10. Sulas, L.; Seddaiu, G.; Muresu, R.; Roggero, P.P. Nitrogen fixation of sulla under Mediterranean conditions. *Agron. J.* **2009**, *101*, 1470–1478. [\[CrossRef\]](#)

11. Amato, G.; Giambalvo, D.; Frenda, A.S.; Mazza, F.; Ruisi, P.; Saia, S.; Di Miceli, G. Sulla (*Hedysarum coronarium* L.) as potential feedstock for biofuel and protein. *Bioenerg. Res.* **2016**, *9*, 711–719. [\[CrossRef\]](#)
12. Saia, S.; Urso, V.; Amato, G.; Frenda, A.S.; Giambalvo, D.; Ruisi, P.; Di Miceli, G. Mediterranean forage legumes grown alone or in mixture with annual ryegrass: Biomass production, N₂ fixation, and indices of intercrop efficiency. *Plant Soil* **2016**, *402*, 395–407. [\[CrossRef\]](#)
13. Bonanno, A.; Di Miceli, G.; Di Grigoli, A.; Frenda, A.S.; Tornambè, G.; Giambalvo, D.; Amato, G. Effects of feeding green forage of sulla (*Hedysarum coronarium* L.) on lamb growth and carcass and meat quality. *Animal* **2011**, *5*, 148–154. [\[CrossRef\]](#)
14. Piluzza, G.; Sulas, L.; Bullitta, S. Tannins in forage plants and their role in animal husbandry and environmental sustainability: A review. *Grass Forage Sci.* **2013**, *69*, 32–48. [\[CrossRef\]](#)
15. Piluzza, G.; Bullitta, S. The dynamics of phenolic concentration in some pasture species and implications for animal husbandry. *J. Sci. Food Agric.* **2010**, *90*, 1452–1459. [\[CrossRef\]](#)
16. Re, G.A.; Piluzza, G.; Sulas, L.; Franca, A.; Porqueddu, C.; Sanna, F.; Bullitta, S. Condensed tannin accumulation and nitrogen fixation potential of *Onobrychis viciifolia* Scop. grown in a Mediterranean environment. *J. Sci. Food Agric.* **2014**, *94*, 639–645. [\[CrossRef\]](#)
17. Selmi, H.; Gasmi-Boubaker, A.; Mehdi, W.; Rekik, B.; Salah, Y.B.; Rouissi, H. Chemical composition and in vitro digestibility of leaves of *Hedysarum coronarium* L., *Medicago truncatula* L., *Pisum sativum* L. and *Vicia sativa* L. *Livestock Res. Rural Dev.* **2010**, *6*, 22.
18. Gasmi-Boubaker, A.; Selmi, H.; Losada, R.M.; Losada, R.M.; Youssef, S.B.; Zoghalmi, A.; Mehdi, W.; Rigueiro-Rodriguez, A. Nutritive value of whole plant (stem and leaves) of *Hedysarum coronarium* L., *Medicago truncatula* L., *Vicia sativa* L. and *Pisum sativum* L. grown under Mediterranean conditions. *Livestock Res. Rural Dev.* **2012**, *24*, 1–6.
19. Di Trana, A.; Bonanno, A.; Cecchini, S.; Giorgio, D.; Di Grigoli, A.; Claps, S. Effects of Sulla forage (*Sulla coronarium* L.) on the oxidative status and milk polyphenol content in goats. *J. Dairy Sci.* **2015**, *98*, 37–46. [\[CrossRef\]](#)
20. Chagas, F.O.; de Cassia Pessotti, R.; Caraballo-Rodríguez, A.M.; Pupo, M.T. Chemical signaling involved in plant–microbe interactions. *Chem. Soc. Rev.* **2018**, *47*, 1652–1704. [\[CrossRef\]](#)
21. Alonso-Amelot, M.E.; Oliveros-Bastidas, A.; Calcagno-Pisarelli, M.P. Phenolics and condensed tannins of high altitude *Pteridium arachnoideum* in relation to sunlight exposure, elevation, and rain regime. *Biochem. Syst. Ecol.* **2007**, *35*, 1–10. [\[CrossRef\]](#)
22. Sulas, L.; Re, G.A.; Stangoni, A.P.; Ledda, L. Growing cycle of *Hedysarum coronarium* L. (sulla): Relationship between plant density, stem length, forage yield and phytomass partitioning. *Cahiers Options Méditerranéennes* **2000**, *45*, 147–152.
23. Minneé, E.M.K.; Bluett, S.J.; Woodward, S.L. Harvesting sulla for yield and quality. *Agron. Soc. NZ* **2004**, *34*, 83–88.
24. Casella, S.; Gault, R.R.; Reynolds, K.C.; Dyson, J.R.; Brockwell, J. Nodulation studies on legumes exotic to Australia: *Hedysarum coronarium*. *FEMS Microbiol. Lett.* **1984**, *22*, 37–45. [\[CrossRef\]](#)
25. Thami Alami, I.; El Mzouri, E.H. Study of the efficacy and persistence of Sulla rhizobium strains. *Cahiers Options Méditerranéennes* **2000**, *45*, 321–325.
26. Gurfel, D.; Löbel, R.; Schiffmann, J. Symbiotic nitrogen-fixing activity and yield potential of inoculated *Hedysarum coronarium* in Israel. *ISR J. Bot.* **1982**, *31*, 296–304.
27. Rodriguez-Navarro, D.N.; Temprano, F.; Orive, R. Survival of *Rhizobium* sp. (*Hedysarum coronarium* L.) on peat-based inoculants and inoculated seeds. *Soil Biol. Biochem.* **1991**, *23*, 375–379. [\[CrossRef\]](#)
28. Yates, R.J.; Howieson, J.G.; Carr, S.J. The role of root-nodule bacteria in the adaptation of two long lived forage legumes from the Mediterranean basin to Western Australia. In Proceedings of the 11th N-fixation Conference, Perth, Australia, 22–27 September 1996; pp. 144–145.
29. Sulas, L.; Re, G.A.; Loi, A.; Howieson, J.G. The selection of optimal root-nodule bacteria inoculants increases the forage yield of *Hedysarum coronarium* (sulla). In Proceedings of the 17th General Meeting of the European Grassland Federation, Debrecen, Hungary, 18–21 May 1998; pp. 899–904.
30. Ewing, M.; Poole, C.; Skinner, P.; Bennett, A. *Sulla and Other Forage Species for Southern Australia*; RIRDC Publication: Perth, Australia, 2001.
31. Sulas, L.; Piluzza, G.; Salis, M.; Deligios, P.A.; Ledda, L.; Canu, S. Cropping systems sustainability: Inoculation and fertilisation effect on sulla performances in a new cultivation area. *Ital. J. Agron.* **2017**. [\[CrossRef\]](#)

32. Yates, R.J.; Howieson, J.H.; De Meyer, S.E.; Tian, R.; Seshadri, R.; Pati, A.; Woyke, T.; Markowitz, V.; Ivanova, N.; Kyrpides, N.; et al. High-quality permanent draft genome sequence of *Rhizobium sulae* strain WSM1592; a *Hedysarum coronarium* microsymbiont from Sassari, Italy. *Stand. Genom. Sci.* **2015**, *10*, 1. [[CrossRef](#)]
33. Di Paolo, E.; Garofalo, P.; Rinaldi, M. Irrigation and nitrogen fertilization treatments on productive and qualitative traits of broad bean (*Vicia faba* var. minor L.) in a Mediterranean environment. *Legume Res.* **2015**, *38*, 209–218. [[CrossRef](#)]
34. Youseif, S.; Abd El-Megeed, F.; Saleh, S. Improvement of faba bean yield using *Rhizobium/Agrobacterium* inoculant in low-fertility sandy soil. *Agronomy* **2017**, *7*, 2. [[CrossRef](#)]
35. *Soil Survey Staff*; Usda-Nrcs: Lincoln, NE, USA, 2000.
36. Unkovich, M.J.; Herridge, D.; Peoples, M.; Cadisch, G.; Boddey, B.; Giller, K.; Alves, B.; Chalk, P. Measuring plant-associated nitrogen fixation in agricultural systems. *ACIAR Monogr.* **2008**, *136*, 1–258.
37. Singleton, V.L.; Rossi, J.A. Colorimetry of total phenolics with phosphomolybdic-phosphotungstic acid reagents. *Am. J. Enol. Vitic.* **1965**, *16*, 144–158.
38. Porter, L.J.; Hristich, L.N.; Chan, B.G. The conversion of procyanidins and prodelphinidins to cyanidin and delphinidin. *Phytochemistry* **1986**, *25*, 223–230. [[CrossRef](#)]
39. Sulas, L.; Re, G.A.; Bullitta, S.; Piluzza, G. Chemical and productive properties of two Sardinian milk thistle (*Silybum marianum* L. Gaertn.) populations as sources of nutrients and antioxidants. *Genet. Resour. Crop Evol.* **2016**, *63*, 315–326. [[CrossRef](#)]
40. Surveswaran, S.; Cai, Y.Z.; Corke, H.; Sun, M. Systematic evaluation of natural phenolic antioxidants from 133 Indian medicinal plants. *Food Chem.* **2007**, *102*, 938–953. [[CrossRef](#)]
41. Piluzza, G.; Bullitta, S. Correlation between phenolic content and antioxidant properties in twenty-four plant species of traditional ethnoveterinary use in the Mediterranean area. *Pharm Biol.* **2011**, *49*, 240–247. [[CrossRef](#)]
42. R Development Core Team. *R: A Language and Environment for Statistical Computing*; R Found. Stat. Comput.: Vienna, Austria, 2014.
43. Gomez, K.A.; Gomez, A.A. *Statistical Procedures for Agricultural Research*, 2nd ed.; John Wiley & Sons: Hoboken, NJ, USA, 1984; ISBN 0-4714-87092-7.
44. Muresu, R.; Porceddu, A.; Sulas, L.; Squartini, A. Nodule-associated microbiome diversity in wild populations of *Sulla coronaria* reveals clues on the relative importance of culturable rhizobial symbionts and co-infecting endophytes. *Microbiol. Res.* **2019**, *221*, 10–14. [[CrossRef](#)]
45. MacDuff, J.H.; David, S.C.; Davidson, I.A. Inhibition of N₂ fixation by white clover (*Trifolium repens* L.) at low concentrations in NO₃ in flowing solution culture. *Plant Soil* **1996**, *180*, 287–295. [[CrossRef](#)]
46. Waterer, J.G.; Vessey, J.K. Effect of low static nitrate concentrations on mineral nitrogen uptake nodulation, and nitrogen fixation in field pea. *J. Plant Nutr.* **1993**, *16*, 1775–1789. [[CrossRef](#)]
47. Voisin, A.S.; Salon, C.; Munier-Jolain, N.G.; Ney, B. Quantitative effects of soil nitrate, growth potential and phenology on symbiotic nitrogen fixation of pea (*Pisum sativum* L.). *Plant Soil* **2002**, *243*, 31–42. [[CrossRef](#)]
48. Omrane, S.; Chiurazzi, M. A variety of regulatory mechanisms are involved in the nitrogen-dependent modulation of the nodule organogenesis program in legume roots. *Plant Signal. Behav.* **2009**, *4*, 1066–1068. [[CrossRef](#)]
49. Naudin, C.; Corre-Hellou, G.; Pineau, S.; Crozat, Y.; Jeuffroy, M.H. The effect of various dynamics of N availability on winter pea-wheat intercrops: Crop growth, N partitioning and symbiotic N₂ fixation. *Field Crop Res.* **2010**, *119*, 2–11. [[CrossRef](#)]
50. Carlsson, G.; Huss-Danell, K. Nitrogen fixation in perennial forage legumes in the field. *Plant Soil* **2003**, *253*, 353–372. [[CrossRef](#)]
51. Beshir, H.; Walley, F.; Bueckert, R.; Tar'an, B. Response of snap bean cultivars to *Rhizobium* inoculation under dryland agriculture in Ethiopia. *Agronomy* **2015**, *5*, 291–308. [[CrossRef](#)]
52. Tena, W.; Wolde-Meskel, E.; Walley, F. Symbiotic efficiency of native and exotic *Rhizobium* strains nodulating lentil (*Lens culinaris* Medik.) in soils of Southern Ethiopia. *Agronomy* **2016**, *6*, 11. [[CrossRef](#)]
53. Howieson, J.G.; Yates, R.J.; Foster, K.; Real, D.; Besier, B. Prospects for the future use of legumes. In *Leguminous Nitrogen-Fixing Symbioses*; Dilworth, M.J., James, E.K., Sprent, J.I., Newton, W.E., Eds.; Elsevier: London, UK, 2008; pp. 363–394; ISBN 978-1-4020-3548-7.
54. Sprent, J.I. *Legume Nodulation: A Global Perspective*; Wiley-Blackwell: Oxford, UK, 2009; ISBN 978-1-4051-8175-4.

55. Fitouri, S.D.; Trabelsi, D.; Saïdi, S.; Zribi, K.; Jeddi, F.B.; Mhamdi, R. Diversity of rhizobia nodulating sulla (*Hedysarum coronarium* L.) and selection of inoculant strains for semi-arid Tunisia. *Ann. Microbiol.* **2012**, *62*, 77–84. [[CrossRef](#)]
56. Liu, W.Y.Y.; Ridgway, H.J.; James, T.K.; Premaratne, M.; Andrews, M. Characterisation of rhizobia nodulating *Galega officinalis* (goat's rue) and *Hedysarum coronarium* (sulla). *N. Z. Plant Prot.* **2012**, *65*, 192–196.
57. Razika, G.; Amira, B.; Yacine, B.; Ammar, B. Influence of carbon source on the production of exopolysaccharides by *Rhizobium sullae* and on the nodulation of *Hedysarum coronarium* L. legume. *Afr. J. Microbiol. Res.* **2012**, *6*, 5940–5946. [[CrossRef](#)]
58. Aliliche, K.; Beghalem, H.; Landoulsi, A.; Chriki, A. Molecular phylogenetic analysis of *Rhizobium sullae* isolated from Algerian *Hedysarum flexuosum*. *A Van Leeuw. J. Microb.* **2016**, *109*, 897–906. [[CrossRef](#)]
59. Mandal, S.M.; Chakraborty, D.; Dey, S. Phenolic acids act as signaling molecules in plant-microbe symbioses. *Plant Signal. Behav.* **2010**, *5*, 359–368. [[CrossRef](#)]
60. Woodward, S.L.; Waghorn, G.C.; Lassey, K.R.; Laboyrie, P.G. Does feeding sulla (*Hedysarum coronarium*) reduce methane emissions from dairy cows? *Proc. N. Z. Soc. Anim. Prod.* **2002**, *66*, 227–230.
61. Ramirez-Restrepo, C.A.; Barry, T.N. Alternative temperate forages containing secondary compounds for improving sustainable productivity in grazing ruminants. *Anim. Feed Sci. Technol.* **2005**, *120*, 179–201. [[CrossRef](#)]
62. Molle, G.; Decandia, M.; Giovanetti, V.; Cabiddu, A.; Fois, N.; Sitzia, M. Responses to condensed tannins of flowering sulla (*Hedysarum coronarium* L.) grazed by dairy sheep: Part 1: Effects on feeding behaviour, intake, diet digestibility and performance. *Livest. Sci.* **2009**, *123*, 138–146. [[CrossRef](#)]

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