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Potential of *Rhizobium sullae–Sulla coronaria* Symbiotic Biological Nitrogen Fixation to Supplement Synthetic Mineral Nitrogen in Olive Tree Fertilization

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Abstract: The aim of the present work is to compare olive tree nitrogen fertilization over two years of trials, using synthetic chemical fertilizers along with organic fertilizers composed of the green manure of sulla (*Sulla coronaria*) inoculated with the symbiont *Rhizobium sullae* or left uninoculated. The tests indicated that symbiotic nitrogen fixation promoted by the sulla–*R. sullae* symbiosis represents an important source of nitrogen that can replace or supplement synthetic nitrogen fertilizers for olive tree cultivation when sulla is inoculated with *R. sullae* in a soil already populated by the symbiont. Integration of the indigenous population of *R. sullae* via sulla inoculation with a selected strain yielded nodule formation in 100% of plants and produced a sufficient amount of biomass rich in nitrogen with a low C/N ratio. On the contrary, olive tree fertilization using the green manure of sulla that was not inoculated with the symbiont supplied significantly less organic nitrogen in 2017 and 2018, respectively, compared to the control. Optimal management of the multi-factorial approaches involved in green manure olive fertilization are also reported.

Keywords: green manure; fertilization; olive; rhizobia; sulla

1. Introduction

Legumes associate with specific rhizobia to fix atmospheric nitrogen to ammonia, thereby providing fixed nitrogen to plants. Adding green manure from legumes to soil also results in organic nitrogen fertilization, which reduces the emission of greenhouse gases, improves the sequestration of carbon in soils, and decreases fossil energy inputs in the system due to N fertilizer reduction [1]. Sulla (*Sulla coronaria* L. Medik syn. *Hedysarum coronarium* L.) [2] is a perennial legume originating in the Mediterranean basin that is known for its broad tolerance to various environmental stresses. In addition to its native occurrence in calcareous pliocenic clay soils, sulla is used as a forage crop because of its pronounced drought resistance, strong tolerance to alkaline soils, and high agronomical yield [3–5]. Sulla is specifically colonized by the microsymbiont *Rhizobium sullae* [6], which induces root nodules within which biological nitrogen fixation occurs. Biological nitrogen fixation is the primary source of nitrogen for sulla, which is cultivated for forage production. However, it also represents an important source of nitrogen when the sulla is prematurely cut and used to supply nitrogen to olive plants as green manure. Sulla is particularly suitable for green manure fertilization because it not only supplies organic nitrogen but also mobilizes phosphorus and iron in alkaline soils, also making these nutrients available to olive trees [7]. However, traditional fertilization with green manure often does not yield the same results as chemical nitrogen fertilization and, thus, green manure fertilization may require supplemental chemical nitrogen fertilizer application to reduce the risk of yield reduction in olive. One

of the main causes that often limit the successful use of green manure in tree fertilization is the low amount of organic nitrogen incorporated into the soil due to the low concentration of proteins and the amount of biomass used for fertilizer. Both protein concentration and sulla biomass are related to nodulation and nitrogen fixation efficiency of the sulla–*R. sullae* symbiosis. These processes are strongly influenced by the indigenous *R. sullae* population in the soil and by the genetic characteristics of the symbiont strains [8,9]. Compared with chemical fertilizers, green manure fertilization with sullas offer auxiliary benefits, such as increased soil humus concentration, improved soil structure, and increased water retention during the summer months [10,11]. The integration or the replacement of nitrogen fertilizers with sulla–rhizobia green manure could lead to a sustainable agricultural system that protects the environment in olive orchards. However, despite the fact that sulla has been studied extensively as a forage legume, studies on its use as green manure for olive fertilization are rare. The main objective of this research is to study the most appropriate green manure technologies that would allow the exploitation of biological nitrogen fixation by the sulla–*R. sullae* symbiosis to replace or supplement the use of synthetic nitrogen fertilizers.

2. Materials and Methods

2.1. Soil and Site Characteristics

The research was performed in a farmed orchard with 400 olive tree ha^{-1} planted in a clay-loam soil at 450 m elevation above sea level. The area is characterized by an annual rainfall of 400–600 mm and is located in middle-eastern Italy (Molise region, 41°46′ N, 14°32′ E). The soil has a neutral pH (7.3) with a medium content of nitrogen (1600 mg Kg^{-1}), phosphorus (32 mg Kg^{-1}), potassium (350 mg Kg^{-1}), and organic matter (1.6%). The olive trees of cv. Leccino are 35 years old and were planted at a distance of 5 × 5 m in the orchard. The experiment was performed during 2016–2018, and the preliminary tests performed in 2016 were used to establish the subsequent two-year trial. In the three-year period before 2016, nitrogen fertilization was implemented using 400 g of urea per plant, which is equal to 184 g N per plant [12].

2.2. Seed Density Tests

During 2016, preliminary tests were conducted to obtain useful information on the most suitable seeding density for the production of sufficient sulla biomass to be used in the biennial test on nitrogen fertilization of olive using green manure. In these preliminary tests, three seeding density levels of 20, 40, and 60 Kg ha^{-1} were used. The lowest seeding densities represent the doses of seed that are normally used in different areas of Italy when sulla is grown for forage production [4]. Seeds were sown under the olive tree canopies occupying an area of 12 m^2 per plant, randomly chosen within the orchard. The olive plants chosen for the tests were not fertilized with inorganic nitrogen fertilizers. Each seeding density was repeated 4 times using a total of 36 olive plants. Although the soil that hosted the trial contained the symbiont *R. sullae*, the first sulla seeds to be sown were inoculated with a 0.2% (*v/w*) dose of a suspension of *R. sullae* containing 10^9 colony forming units (CFU) mL^{-1} that was prepared according to the procedure described below. The seeds of sulla sown in September 2016 were buried by hand with a rake at a depth of approximately 2–3 cm. In the following spring, at the end of February, March, and April, the sulla plants consisting of stems with leaves were cut at ground level, transported to the laboratory and analyzed. Each month, the sulla samples harvested separately from each seeding density were obtained from 4 olive plants chosen at random within the test zone. The laboratory analyses focused on evaluating the fresh and dry weight of the sulla biomass collected from the field trial as well as the evaluation of the C/N ratio according to the procedure described below.

2.3. Field Trials

Field trials were accomplished in the olive orchard, as described above. The trials were performed during 2017 to 2018 using the same olive plants. The olive trees used for the biennial experimentation

were randomly chosen within the orchard, and the annual pruning remains were removed from the field. The groups for comparison included green manure fertilization with uninoculated sulla, green manure fertilization with inoculated sulla, and synthetic nitrogen fertilization with 400 g plant^{-1} of urea (control with 184 g N olive plant^{-1}). Each group had 4 replicates. In 2017 and 2018, seeds of the sulla cv. Grimaldi were sown at the beginning of September under the canopy of olive plants occupying a circular area of approximately 12 m^2. The seeds were buried approximately 2–3 cm deep using common metal rakes. Immediately before sowing, seeds used for the uninoculated treatment were washed twice with an equal volume of sterile distilled water, whereas inoculated seeds were treated with 0.2% (*v*/*w*) of a suspension of *R. sullae* containing 10^9 CFU mL^{-1}. The sowing density of the sulla localized under the canopy of each olive tree was equal to 72 g (60 Kg ha^{-1}). This value was chosen considering the biomass yield of sulla produced from different densities of sowing in 2016, as shown in Table 1.

Table 1. Aerial sulla biomass yield and C/N ratio obtained with different seed densities in different months.

Seed Density	Sulla Plants	February		March		April	
(Kg ha^{-1})	(No m^{-2})	Dry Biomass [1]	C/N	Dry Biomass	C/N	Dry Biomass	C/N
20	540 ± 25 [c]	197 ± 9 [b]	12	320 ± 34 [c]	12	410 ± 45 [c]	20
40	725 ± 28 [b]	224 ± 6 [b]	11	430 ± 11 [b]	13	534 ± 25 [b]	20
60	935 ± 60 [a]	432 ± 27 [a]	11	595 ± 32 [a]	12	686 ± 42 [a]	19
Significance	**	*		**		*	

[1] g m^{-2} *, ** significant at $p \leq 0.05$, 0.01, respectively. Different letters within each column indicate significant differences according to Duncan's test ($p \leq 0.05$).

2.4. Inoculum Preparation with R. sullae

The inoculum of *R. sullae* was produced in the laboratory using strain number 2202 of the Agriculture Department (Molise University) collection previously isolated from the nodules of the sulla grown in the same area as the field used for experimentation, which were selected for good nodulation activity and nitrogen-fixing efficiency. The bacterial culture, preserved at −40 °C in 20% (*v*/*v*) glycerol, was transferred to yeast mannitol agar (YMA) composed of 0.25 g KH_2PO_4, 0.50 g K_2HPO_4, 0.1 g NaCl, 0.6 g yeast extract (Biolife, Milan, Italy), 10 g mannitol (Sigma-Aldrich, St. Louis, MO, USA), 0.01 g Congo red, and 1000 mL distilled water, pH 6.8 [13]. After 48 h of incubation at 30 °C, an aliquot of the bacterial culture was transferred to the same substrate reported above but without agar (YM broth) and incubated with agitation (50 rpm) for an additional 48 h. At the end of the incubation period, bacterial cells were collected by centrifugation at 6000× *g* for 5 min and then resuspended in a physiological solution of 0.9% (*w*/*v*) NaCl. The concentration of the bacteria in the inoculum was adjusted to approximately 10^{10} cells mL^{-1} using a Thoma counting camera (Brand, Germany). The inoculum was stored at 4 °C, and then the concentration of viable cells was assessed immediately before use by evaluating the number of CFU mL^{-1} by microbiological analysis of the inocula plated on YMA.

2.5. Nodulation and Nitrogen Fixation Assay

Sulla nodulation was evaluated at the end of March before digging in the green manure biomass. From each experimental area where the sulla had been cultivated, the roots were collected along with approximately 5 kg of soil obtained at a depth of 5 to 30 cm. The samples were placed individually in plastic containers with water in which the roots were gently separated from the soil. After brief exposure to the air, the roots of the nodulated sulla plants were used for the analysis of the nitrogenase activity measured by the acetylene reduction assay according to Haider et al. [14]. Nitrogenase activity was expressed in µmol ethylene g^{-1} nodules h^{-1}, the root nodules were counted detached from the host plant, and weighed. In total, 20 plants from each treatment were analyzed.

2.6. Aerial Biomass Analysis and Green Manure Execution

The biomass from all treatments was composed of the aerial part of the herbaceous plants grown under the canopy of the olive trees used in the experiments. For olive plants that hosted the sulla, 98% of the biomass was composed of this legume, while the biomass from the control was composed of grasses, which was mainly represented (80%) by Italian ryegrass (*Lolium multiflorum* L.). The fresh biomass samples of each treatment were handpicked from a 1 m^2 surface. After weighing, they were oven-dried at 60 °C until a constant weight was reached, and the dry matter content was calculated. Dry sub-samples of biomass material were finely ground and used for the subsequent chemical analyses. Total nitrogen concentration was evaluated with the Kjeldahl method [15], while total carbon was determined by oxidizing organic carbon with $K_2Cr_2O_7$ (1.5 N) in an acid environment with H_2SO_4 and a subsequent reading in a spectrophotometer [16]. The amount of organic nitrogen incorporated into the soil with the green manure was calculated by multiplying the total nitrogen concentration of each type of dry biomass per the amount of aerial dry biomass produced under the canopy of each olive tree. In both years of trials (2017 and 2018), the green manure was applied at the end of March. This period was chosen based on the sufficient biomass production of sulla and the favorable low C/N ratio obtained from the preliminary tests performed during 2016, shown in Table 1. Urea fertilizer was added to the control immediately before the green manure. The sulla plants and the Italian ryegrass (control) grown under the canopy of the olive trees used for the tests were finely cut with a mulching green, and care was taken not to disperse the fresh biomass outside the experimental area of 12 m^2 of each olive plant. Immediately afterwards, the fresh biomass was buried at a depth of 5 to 15 cm using a small milling machine (Honda Motor Co. Ltd., Japan).

2.7. Soil and Olive Leaf Analysis

At the end of spring (May–June), when olive plants were in full bloom from each experimental area, soil samples were taken at depths of 5 to 30 cm using a manual probe. Upon arrival in the laboratory, the samples were divided into two sub-samples to be subjected separately to chemical and microbiological analysis. The samples used for chemical analysis were freed from plant roots, air-dried, ground with a pestle and sieved (2 mm mesh) for subsequent analysis. The total nitrogen concentration of the soil was determined with the Kjeldahl method, as previously noted [15]. The soil sub-samples used for microbiological analysis were used for the quantification of the symbiont *R. sullae*, as described below. In the May–June period, foliar diagnosis of the olive plants was performed during the full bloom to ascertain the nitrogen concentration. From the canopy of the olives corresponding to each treatment, approximately 80–100 adult leaves were randomly obtained and transferred to the laboratory. After air-drying in a dark room, the samples were finely ground and used for the determination of total nitrogen using the Kjeldahl method, as reported above [17]. The results of the chemical analyses of all the treatments were recorded and compared. The fruits were harvested manually from each olive plant at the beginning of their maturation, which represents the optimal time for oil production.

2.8. Numerical Counting of R. sullae in the Soil

The soil samples were carefully freed from the roots of the plants using a sterile metal forceps, and then the mass was stirred with a sterile spatula and finally used for enumeration of *R. sulla* via the most probable number (MPN) method [13], using plastic growth pouches [18]. Clean sulla seeds (cv. Grimaldi) were surface-sterilized in 70% (*v*/*v*) alcohol for 3 min and rinsed thoroughly in several changes of sterile distilled water. The seeds were pre-germinated on 1% water agar until the radicles were approximately 2 cm long. Three seedlings were planted per pouch. A ten-fold dilution of each soil sample with three replicates per dilution was used to inoculate the pouches containing an N-free nutrient solution [19]. Then, 1 mL of soil inoculants was used to inoculate each pouch. The pouches

were randomly arranged in wooden racks and kept in the greenhouse. The plants were assessed for nodules after 12 weeks, and the MPN of *R. sullae* was calculated [13].

2.9. Statistical Analysis

All data obtained during 2016, 2017, and 2018 experimental seasons were subjected to analysis of variance (ANOVA) according to Snedecor and Cochran [20] using the MSTAT program. Least significant range (LSR) was used to compare between means of treatment according to Duncan [21].

3. Results

The preliminary tests performed in 2016 provided useful information for setting up the next tests performed in 2017 and 2018. These consisted of preliminary data on the best seeding density of the sulla seed and the timing of green manure fertilization application to the olive plants. Seed sowing performed with 60 kg ha^{-1} of seeds resulted in the highest yield of sulla dry biomass, between 432 and 686 g m^{-2} in February–April (Table 1). However, in contrast to yield, the C/N biomass ratio was quite low before April. In fact, the values recorded in April were significantly higher than those recorded in February and March (Table 1). Sulla biomass characterized by a higher C/N ratio is less suitable for the fertilization of olive plants with green manure [22,23]. Based on the best experimental results obtained in 2016, the subsequent biennial trial of olive tree fertilization with green manure used a seeding density equal to 60 kg of seed per hectare, and the month of March was chosen as the best time to bury the biomass.

The biennial test performed for olive tree fertilization with green manure provided fairly homogeneous results in 2017 and 2018. The results of the microbiological analysis outcome from the greenhouse experiments performed with the samples taken at the end of March before the application of green manure revealed an increased presence of *R. sullae* in the soils that hosted the sulla inoculated with its specific symbiont. In detail, in the soil cultivated with the uninoculated sulla, the MPN of *R. sullae* cells ranged from $2{\cdot}10^3$ to $8{\cdot}10^3$ g^{-1} of soil in 2017 and 2018, respectively. In contrast, in the soil containing sulla inoculated with the symbiont, the MPN of *R. sullae* varied from $6{\cdot}10^4$ to $9{\cdot}10^4$ in the two years of trials (Table 2).

Table 2. Sulla nodulation with *Rhizobium sullae* and nitrogenase activity evaluated through two consecutive years of experimentation.

Treatment	*Rhizobium sullae* Content (MPN g^{-1} soil) [1]		Nodulated Plants (%)		Nodules Per Plant (*n*)		Nodule Biomass Per Plant (mg)		ARA [2] (μmol C_2H_4 $h^{-1}g^{-1}$ nodules)	
	2017	2018	2017	2018	2017	2018	2017	2018	2017	2018
Uninoculated	$2{\cdot}10^3 \pm 77^{b}$	$8{\cdot}10^3 \pm 110^{b}$	60 ± 10^{b}	72 ± 9^{b}	6.54 ± 0.53^{b}	8.51 ± 0.24^{b}	40 ± 6^{b}	52 ± 3^{b}	3.64 ± 0.73^{b}	4.71 ± 0.50^{b}
Inoculated	$6{\cdot}10^4 \pm 240^{a}$	$9{\cdot}10^4 \pm 186^{a}$	100^{a}	100^{a}	10.80 ± 0.44^{a}	15.33 ± 0.82^{a}	65 ± 9^{a}	105 ± 12^{a}	5.90 ± 0.92^{a}	7.21 ± 0.34^{a}
Significance	**	*	**	**	*	**	**	**	**	**

[1] MPN, Most Probable Number. [2] ARA, Nitrogenase activity measured by the acetylene reduction assay. *, **, significant at $p \leq 0.05$, 0.01, respectively. Different letters within each column indicate significant differences according to Duncan's test ($p \leq 0.05$).

The increased presence of *R. sullae* in the soil that was cultivated with the symbiont-inoculated sulla positively influenced legume nodulation and nitrogen fixation in both years of experimentation. During the trial, the number of nodulated plants shifted from 60%–72% in the soil when uninoculated sulla was used to 100% in soil with sulla inoculated with *R. sullae*. The number of nodules per plant and their biomass were increased in the second year of tests but, nevertheless, significantly increased values were recorded in the plants of sulla inoculated with *R. sullae* in both years. Moreover, the greater biomass of nodules per plant recorded in the sulla inoculated with *R. sullae* significantly increased the nitrogenase activity of each plant in both experimental seasons (Table 2). The total nitrogen concentration present in the sulla biomass was greater compared with the control (Italian ryegrass) and, even in this case, the best results were recorded when the legumes were inoculated with *R. sullae***.** Similarly, the C/N ratio was decidedly reduced both in the uninoculated sulla and the

control (Table 3). The dry biomass yield was higher in sulla compared to the control regardless of the use of the inoculation practice. The annual production of biomass under the canopy of the olives and its concentration of total nitrogen were determined (Table 3), and the average quantity of nitrogen supplied to each olive plant via green manure fertilization was estimated (Table 4).

Table 3. Production of aerial biomass under the olive plant canopy.

Treatment	Dry Biomass Yield ($g\ m^{-2}$)		Dry Biomass Used per Olive Tree (g)		Nitrogen Content of Dry Biomass (%)		C/N	
	2017	2018	2017	2018	2017	2018	2017	2018
Uninoculated	517 ± 49 ^{a}	521 ± 18 ^{a}	6,200 ± 225 ^{a}	6256 ± 432 ^{a}	3.20 ± 0.20 ^{b}	3.10 ± 0.41 ^{b}	13 ^{b}	15 ^{b}
Inoculated	592 ± 90 ^{a}	588 ± 87 ^{a}	7,102 ± 114 ^{a}	7050 ± 97 ^{a}	3.87 ± 0.17 ^{a}	3.60 ± 0.22 ^{a}	11 ^{c}	12 ^{c}
Control[1]	354 ± 84 ^{b}	321 ± 12 ^{b}	4, 248 ± 96 ^{b}	3848 ± 84 ^{b}	1.38 ± 0.10 ^{c}	1.27 ± 0.15 ^{c}	22 ^{a}	25 ^{a}
Significance	*	*	**	**	**	**	**	**

[1] The dry biomass of the control refers to Italian ryegrass. *, **, significant at $p \leq 0.05$, 0.01, respectively. Different letters within each column indicate significant differences according to Duncan's test ($p \leq 0.05$).

Table 4. Nitrogen supplied to each olive plant with different green manure fertilization.

Year	Nitrogen Forms	Uninoculated Sulla (g)	△ [1]	Inoculated Sulla (g)	△	Control [2] (g)
2017						
	organic	198.40 ^{b}		274.85 ^{a}		58.62 ^{c}
	mineral	0.00		0.00		184.00
	N total	198.40 ^{b}	−44.22	274.85 ^{a}	32.23	242.62 ^{a}
2018						
	organic	193.94 ^{b}		253.80 ^{a}		48.87 ^{c}
	mineral	0.00		0.00		184.00
	N total	193.94 ^{b}	−38.93	253.80 ^{a}	20.93	232.87 ^{a}

[1] Difference between the total N supplied with the sulla green manure compared to the total N of the control.[2] The control's organic nitrogen category refers to the Italian ryegrass while the N mineral, to mineral nitrogen fertilization. Values in the same line having different letters indicate significant differences according to Duncan's test ($p \leq 0.05$).

In both harvests, the organic nitrogen treatment supplied with the green sulla manure was superior to the Italian ryegrass manure of the control, particularly when this legume was inoculated with *R. sullae*. The results concerning the total nitrogen (organic + mineral) supplied to each olive plant revealed significantly low values in the treatment group where sulla was left uninoculated (Table 4). The average of the productive yield of the orchard did not exhibit noteworthy changes, reaching approximately 18–21 kg of fruits per plant. On the contrary, the total nitrogen concentration evaluated in the soil in the year 2017 and 2018, was significantly higher in the treatment done with sulla inoculated with *R. sullae* compared to the control and the uninoculated sulla, respectively. Similarly, the total nitrogen concentration evaluated in the olive leaves showed the best values in 2017 compared to the uninoculated sulla, while in 2018, it was significantly higher than that of the other treatments (Table 5).

Table 5. Productive yield of the orchard and concentration of total nitrogen in the soil and the leaves of the olive plants fertilized with sulla green manure.

Treatment	Fruit Yield (Kg plant $^{-1}$)			Soil Total N (%)			Leaves Total N (%)		
	2017	2018	mean	2017	2018	mean	2017	2018	mean
Uninoculated	16 ± 4 [1]	20 ± 5	18	0.16 ± 0.01^{ab}	0.17 ± 0.02^{b}	0.17	1.67 ± 0.42^{b}	1.72 ± 0.51^{b}	1.70
Inoculated	17 ± 3	25 ± 4	21	0.18 ± 0.04^{a}	0.19 ± 0.05^{a}	0.19	1.85 ± 0.31^{a}	1.90 ± 0.50^{a}	1.88
Control	16 ± 2	21 ± 4	19	0.15 ± 0.02^{b}	0.18 ± 0.04^{ab}	0.17	1.78 ± 0.44^{ab}	1.73 ± 0.30^{b}	1.76
Significance	NS	NS		*	*		*	*	

[1] Mean ± standard deviation. NS, not significant. *, significant at $p \leq 0.05$. Different letters within each column indicate significant differences according to Duncan's test ($p \leq 0.05$).

4. Discussion

Nitrogen is the most important and irreplaceable element for vegetative growth and fruit production of olives. Nitrogen is normally distributed to agricultural soils in mineral forms by adding synthetic fertilizers. However, the long-term application of inorganic nitrogen fertilizers alters the residual fertility of the soil and the abundance and diversity of the functional soil microbial population [24]. Fertilizing olive trees with organic nitrogen is quite common in the Mediterranean area because it is a traditional technique that has been developed over many years. In this method, legumes are finely cut with a shredder and then left on the ground as biomass to be incorporated into the soil as green manure. However, as a source of nitrogen for tree crops, the expected results from using green manure are often not achieved, resulting in a lack of nitrogen in the soil, which requires supplementation with inorganic fertilizers [25]. In addition to the low amount of organic nitrogen embedded in the soil described above, other causes limit the successful outcome of using green manure in tree fertilization. The most important limitations concern the dynamics of the organic nitrogen mineralization in the soil exploited by the olive roots, which is mainly linked to the C/N ratio of biomass and the synchrony between the period of maximum availability of mineral nitrogen produced in the soil by the microorganisms and the phenological phase of the olive plants in which the maximum nutrient concentrations are required. Rodrigues et al. [26] recommended some caution in the use of pure legumes as biomass providers in olive orchards due to the reduced transfer of nitrogen from the legumes to the olive trees. However, the results of the present study indicate that olive tree fertilization with organic nitrogen from sulla green manure can compete with synthetic fertilization, but only if additional factors such as legume sowing density, use of an inoculum with a specific symbiont, and time of adding the green manure are correctly managed. The data reported in Table 1 show that the sowing density normally used for the production of forages, i.e., 20–40 Kg ha^{-1} [4], is not suitable for green manure fertilization because it does not produce sufficient sulla biomass. Increased seeding density and aerial biomass produce an increased number of root nodules active in the soil during the first months of sulla development. Sulla, like other legumes, achieves complete nodulation of the root system during the months of May and June when it is in full bloom. To achieve an increased number of active nodules per unit area of soil before the end of March (green manure application), the number of nodulated plants per m^2 needs to be increased. Taking into consideration the highest number of plants of sulla per m^2 reported in Table 1 and extent of nodulation of plants inoculated with *R. sullae* (Table 2), an average number of nodules per m^2 of land equal to approximately 9000 and 13000 for 2017 and 2018, respectively, was estimated. Thus, rhizobial inoculation is a fundamental technique even when the soil is populated by indigenous strains of *R. sullae* [27,28]. The integration of the indigenous population of *R. sullae* together with seed inoculation resulted in 100% nodulated plants, which were characterized by a higher nitrogenase activity and a sufficient amount of biomass rich in nitrogen with a low C/N ratio suitable for green manure (Tables 2–4). On the contrary, olive tree fertilization using green manure of uninoculated sulla resulted in nitrogen deficiency equal to 44.22 and 38.93 g per plant in 2017 and 2018, respectively, compared with the control (Table 4). The higher nitrogen concentrations of inoculated sulla biomass, in addition to better nitrogen fixation, could be a response also of other factors as well such as plant growth-promoting factors triggered by the inoculation with *R. sullae*. The low C/N ratio of the biomass used in the green manure at the end of March (Table 1) favored the rapid mineralization of organic nitrogen after the soil was applied, making it available to olive trees during the critical phases of flowering and fruiting. In fact, the different nitrogen concentrations found both in the soil and leaves of the olive trees two months after the addition of the green manure (Table 5) can be explained by concluding that the olive trees fertilized with the biomass of sulla inoculated with the symbiont received more nitrogen (Table 5). Ultimately, since olive fruit production did not experience significant changes, we can deduce that olive tree fertilization with sulla green manure did not damage production. In fact, foliar diagnosis showed sufficient total nitrogen following all treatments [17]. However, it should be noted that based on the results of soil analyses, green manure treatment with uninoculated sulla may require both organic nitrogen and a mineral nitrogen fertilizer supplement.

5. Conclusions

Green manure fertilization using sulla biomass that has been inoculated with a symbiont can completely replace synthetic fertilizers without further integration. This goal, which may also be extended to other tree crops, can only be achieved by optimally managing the following parameters: legume sowing density, use of the inoculum with its specific symbiont, time of addition of the green manure, and synchrony between the rate of mineralization of organic nitrogen incorporated in the soil with the highest requirement of nitrogen by the olive plants. In conclusion, the reduction of nitrogenous inorganic fertilization in favor of biological nitrogen fixation using multi-factorial approaches, as noted in the present research, remains an important goal to be pursued in the future.

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