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EDITED BY

Desouza Blaise,
Central Institute for Cotton Research (ICAR),
India

REVIEWED BY

Esther Menendez,
University of Salamanca, Spain
Endalkachew Woldemeskel,
World Agroforestry, Ethiopia

*CORRESPONDENCE

Danilo Scordia

✉ danilo.scordia@unime.it

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Enhancing Mediterranean forage legume production through inoculation with elite rhizobia

Aurelio Scavo¹, Aurora Maio¹, Francesca Calderone¹,
Tommaso La Malfa¹, Calvin Trostle², Ronald J. Yates³,
Stefania Toscano¹, Carmelo Cavallo¹, Marianna Oteri¹,
Maria Elena Furfaro⁴, Antonino Nazareno Virga⁴,
Danilo Scordia^{1*} and Fabio Gresta¹

¹Department of Veterinary Sciences, University of Messina, Messina, Italy, ²Soil and Crop Science Department, Texas A&M AgriLife Extension, Lubbock, TX, United States, ³Legume and Rhizobium Sciences, Murdoch University, Murdoch, WA, Australia, ⁴Consorzio di Ricerca Filiera Carni, University of Messina, Messina, Italy

Forage legumes play a fundamental role in the sustainability of cropping systems, as rotating species with grain crops, intercrops, or winter cover crops. However, their compatibility with rhizobial inoculants needs context-specific studies. The objectives were to evaluate the effectiveness of three species-specific inoculants [Australian granular (AUG), Australian peat (AUP), and American peat (USP)], compared with a non-inoculated control (CNT). These were applied at the recommended and double dose on five Mediterranean forage legumes (*Vicia sativa*, *Medicago polymorpha*, *Trifolium michelianum*, *T. subterraneum*, and *T. pratense*). Plant growth, nodulation, and relative N₂ fixation were measured. Species-specific variations were observed for each inoculant. Across the average of legume species, AUG demonstrated the highest growth- and nodulation-promoting effects at both standard and double inoculum doses. The USP was the worst inoculant at the standard dose but induced positive effects at double dose. The relative N₂ fixation was only improved at double dose, especially by USP and AUG, whereas only AUP provided significant N₂ fixation enhancements at standard dose. Overall, the double dose was the best strategy for all tested forage legumes. These findings suggest that inoculating Mediterranean forage legumes with selected inoculants, especially at double dose, may be an effective solution to increase their N₂ fixation ability, reduce the use of mineral N fertilizers, and identify the optimal forage legume × inoculant combinations for intercropping systems with cereals.

KEYWORDS

agroecology, *Rhizobium*, biological nitrogen fixation, plant growth, nodulation, sustainability

1 Introduction

Agroecosystems are facing a significant decline in soil fertility due to the intensification of agricultural practices and climate change. This is especially true in semi-arid regions where erratic rainfall, frequent soil erosion events, increased salinity, and severe weed pressure are characteristic features (Abdelhak, 2022). Under these conditions of low soil organic matter, nitrogen (N) is generally the most limiting soil macronutrient. It is estimated applied N for plant growth is effectively incorporated into agricultural products at a rate of 40%–50%. While the remaining is subjected to losses by nitrate leaching (NO₃), ammonia volatilization (NH₃), and nitrous oxide (N₂O) emissions, causing environmental burdens (Mahmud et al., 2021). The environmental impact, coupled with increasing costs of mineral N fertilizers, demands the scouting for alternative sources of N (Allito et al., 2020).

The symbiotic biological N fixation (BNF) by N-fixing bacteria, especially those of the family *Rhizobiaceae*, is a sustainable and alternative source of available N. The bacteria allow reduced N mineral fertilizer application while maintaining high crop yields, in agreement with the European *Green Deal* and the United Nations (UN) Sustainable Development Goals (United Nation (UN), 2015; European Commission (EC), 2019). Effective root nodule symbiosis not only allows legumes to grow in N-poor soils but also increases the soil N levels for the subsequent rotational cash crops or for companion crops in polycultural systems (Drevon et al., 2015; Schwember et al., 2019; Scordia et al., 2024). However, BNF is species specific, and its efficiency depends on legume genotype and bacterial strain (Allito et al., 2021; Kohlmeier et al., 2023). Soil bacteria associated with legumes for BNF are commonly termed rhizobia. The family *Rhizobiaceae* includes at least 168 species in 17 genera, of which those with the highest number of described species are *Rhizobium*, *Bradyrhizobium*, *Mesorhizobium*, and *Sinorhizobium* (Kuzmanović et al., 2022). Rhizobial strains are supplied through legume inoculants, crop species-specific products containing isolates of live rhizobia protected by organic carrier material (Lupwayi et al., 2000). Although large-scale production of legume inoculants is complex, the legume inoculant industry is now well established (O'Callaghan et al., 2022), with several different commercial inoculant formulations such as peat, granular, liquid, and freeze-dried powders (Howieson and Dilworth, 2016). Inoculant quality depends on the cell numbers of a selected rhizobial strain, an easy-to-apply and effective formulation, an adequate shelf life, and the dose of application (Lupwayi et al., 2000). Nonetheless, it has been demonstrated that applying inoculants at higher than recommended rates could be beneficial, especially where forage legumes are grown in adverse soil conditions such as low pH (Farquharson et al., 2022; Frame and Laidlaw, 2005).

Forage legumes are a key component for the sustainability of pastures and are key to livestock production, BNF, soil organic matter levels, and soil erosion mitigation (Sheaffer and Seguin, 2003). They are commonly cultivated in rotation with grain crops, but in recent years, they also emerged as intercrops or winter cover crops (Holman et al., 2018; Vujić et al., 2021; Scordia et al., 2024).

Carlsson and Huss-Danell (2003), who investigated the N₂ fixation in three perennial forage legumes primarily relating to ungrazed northern temperate/boreal areas, reported BNF rates up to 545 kg N ha⁻¹ year⁻¹ in white clover (*Trifolium repens*), 350 kg N ha⁻¹ year⁻¹ in alfalfa (*Medicago sativa* L.), and 373 kg N ha⁻¹ year⁻¹ in red clover (*T. pratense*). In Australia, it is estimated forage legumes fix 3.5 million tonnes of N annually on about 45 million hectares, equivalent to a national value of up to Aus\$ 3.5 billion annually (Farquharson et al., 2022). Forage legumes are a traditional component of Mediterranean grassland communities such as Syria, Greece, Sardinia, Sicily, Morocco, and Tunisia, coevolving over the last 1,000 years with native rhizobial populations (Howieson et al., 2000). Unfortunately, the high soil temperatures of arid and semi-arid Mediterranean regions, coupled with severe salinity levels and desertification processes that are increasing under the climate change effects, may affect negatively forage legume-rhizobia associations (Rejili et al., 2012). Furthermore, most farmers of legumes in the Mediterranean region and elsewhere assume that their fields nodulate and fix nitrogen as they have not been adequately trained; thus, they have never examined the roots for active nodulation. This, too, presents a risk to productive farming if legumes are undernodulated. Hence, there is a need for legume inoculation with elite rhizobia to provide and to ensure optimal BNF, which in turn is required to offset increasing agronomic challenges. These include farming practices such as monoculture plantings, low-frequency (over 5 years) legume rotational break crops, soil acidification, detrimental residual soil herbicides, lack of certainty in field nodulation, and uncertain climatic conditions (Yates et al., 2024). However, the compatibility of new forage legume species or cultivars to agriculture with commercial rhizobial inoculants requires ongoing research support to optimise the symbiosis (Rigg et al., 2021; Shi et al., 2023).

In this preliminary work, considering the increasing diffusion of cereal-legume double cropping, five forage legumes common to Mediterranean regions (*Vicia sativa* L., *M. polymorpha*, *Trifolium michelianum* Savi, *T. subterraneum*, and *T. pratense*) were screened in pot bioassays under edaphic and climatic uncontrolled conditions to select the optimal inoculant formulation and dose of application for each legume-rhizobial strain combination for testing in future intercropping systems. These forage legumes exhibit different rhizobial associations and are highly specific to the micro-symbiont they nodulate to achieve an effective symbiosis (Kohlmeier et al., 2025). For instance, *V. sativa* forms a symbiosis with the micro-symbiont *Rhizobium leguminosarum* bv. *viciae*, although differences in host plant preference for specific rhizobial genotypes within natural populations have been observed (Laguerre et al., 2003). Clovers and medics are nodulated and can form an effective symbiosis with *R. leguminosarum* biovar *trifolii* and *Sinorhizobium* spp., respectively (Charman and Ballard, 2004; Farquharson et al., 2022). Our goals were (1) to evaluate the effect of inoculant formulations and rhizobial strains on plant growth, nodulation, and N₂ fixation of selected forage legumes, (2) to assess whether increasing the inoculant rate could further improve the dependent variables, and (3) whether the phenological growth stage could affect the inoculation efficiency.

2 Materials and methods

2.1 Experimental design

Two different pot trials under natural conditions, hereafter referred to as Experiments A and B, were set up in 2022/2023 in a private farm located in Milazzo (Messina, 38°11'25" N, 15°14'28" E) according to a complete randomized block design with three replications. In Experiment A, four forage legumes were inoculated with three species-specific different inoculants (an Australian granular, AUG; an Australian peat, AUP; an American peat from Visjon Exceed®, USP), versus a non-inoculated control (CNT). Inoculants were seed applied at the recommended application method and dose at sowing, and crops were harvested at the vegetative growth stage (just prior to reproductive growth). In Experiment B, five forage legumes were inoculated with the same Experiment A inoculants but at double dose, and crops were harvested in the reproductive growth stage to respectively assess the second and third research objectives.

2.2 Plant material

The forage legumes employed in Experiment A were *Vicia sativa* L. var. Buza, *M. polymorpha* var. Scimitar, *Trifolium michelianum* Savi (local ecotype), and *T. subterraneum* var. Urana. In Experiment B, *T. pratense* var. Rozeta was added. *M. polymorpha*, *T. subterraneum*, and *T. michelianum*, annual self-seeding species with autumn-winter-spring cycle (Charman and Ballard, 2004; Scavo et al., 2021), were purchased from Padana sementi (Tombolo, Padua, Italy). *V. sativa* and *T. pratense* were two local ecotypes. Although the legume species investigated in the present study differ in growth/biomass production potentials, growth habits, and life cycle length due to inherent genetic differences, they are adapted to semiarid environments and were selected based on predominant winter growth in Mediterranean climates (Blackwell et al., 2018; Enriquez-Hidalgo et al., 2020; Scavo et al., 2021).

2.3 Pot trials

Experiments were conducted in dark plastic pots (diameter 20 cm; height 20 cm; volume 5 L) filled with a substrate composed

of 3 cm of stones at the base to prevent soil saturation, soil from the transition zone between natural pastures and forest in Messina mountains, and peat (Pindstrup Mosebrug A/S, Denmark). The substrate components are described in Table 1. Ten seeds pot⁻¹ for each species were sown at 1 cm depth and then thinned to ensure a homogeneous population density of three plants pot⁻¹. Detailed information on sowing, thinning, and harvest dates is shown in Table 2. Starting from sowing, pots were drip irrigated every 2 days for a total of 12,600 ml pot⁻¹ in Experiment A and 9,400 ml pot⁻¹ in Experiment B. No fertilization or pest control treatments were applied.

Inoculation was carried out at sowing on 21 August 2023 for Experiment A and 7 September 2023 for Experiment B following the recommendations reported in the product labels. In detail, AUG and AUP inoculants were kindly provided by Murdoch University (Australia). AUG and AUP specific strains were *Rhizobium leguminosarum* bv. viciae (Australian Group E – WSM4643) for common vetch, *Sinorhizobium* spp. (Australian Group AM – WSM1115) for burr medic and *Rhizobium leguminosarum* bv. trifolii (Australian Group C – WSM1325) for clovers. USP

TABLE 1 Main physico-chemical characteristics of the soil at the beginning of Experiments A and B.

Soil characteristic	Unit of measurement	Value
Sand	%	40
Clay	%	40
Silt	%	20
Organic matter	g kg ⁻¹	4.9
Total CaCO ₃	g kg ⁻¹	9
Total N	g kg ⁻¹	1.5
Available P	mg kg ⁻¹	86
pH		7.6
Electrical conductivity	mS cm ⁻¹	0.18
Cation exchange capacity	meq 100 g ⁻¹	4.7
CaO	mg kg ⁻¹	772
MgO	mg kg ⁻¹	27
Extractable K	mg kg ⁻¹	21

TABLE 2 Sowing, thinning and harvest dates, and length of the biological cycle expressed as days after sowing (DAS) for each legume species in Experiments A and B.

Forage legume species	Experiment A				Experiment B			
	Sowing	Thinning	Harvest	DAS	Sowing	Thinning	Harvest	DAS
<i>Vicia sativa</i>	21/08/2023	18/09/2023	20/12/2023	121	07/09/2023	26/09/2023	26/03/2024	201
<i>Medicago polymorpha</i>	21/08/2023	14/09/2023	20/12/2023	121	07/09/2023	06/10/2023	09/04/2024	215
<i>Trifolium michelianum</i>	21/08/2023	14/09/2023	27/12/2023	128	07/09/2023	06/10/2023	23/03/2024	201
<i>Trifolium subterraneum</i>	21/08/2023	14/09/2023	27/12/2023	128	07/09/2023	06/10/2023	09/04/2024	215
<i>Trifolium pratense</i>					07/09/2023	06/10/2023	15/04/2024	221

consisted of three species-specific inoculants containing either *Rhizobium leguminosarum* bv. viciae or bv. trifolii depending on the legume species. Australian inoculants at standard doses (Experiment A) were inoculated as slurry to coat the seed by mixing 1.5 g (for AUP) or 2.5 g (for AUG) of each inoculant strain with 30 ml of water and injecting it into a moist seedbed (25 g of peat substrate with 500 ml of water) at sowing. For double doses (Experiment B), double inoculant rates were applied in the same amount of water as the standard rate. The USP was seed applied by mixing 0.75 g of inoculum on 100 g of seeds for each forage legume

previously soaked with 1.1 ml of water. Detailed information about inoculants is summarized in Table 3.

Weather conditions were obtained from a weather station of the SIAS (Servizio Informativo Agrometeorologico Siciliano, www.sias.regione.sicilia.it), close to the experimental site. Total rainfall and reference evapotranspiration (ET_0) during the experimental period (August–April) were 469 and 780.2 mm, respectively (Table 4). Except for the November–February period, the remaining months experienced water deficits, especially August (−149 mm) and October (−97 mm). In general, weather conditions

TABLE 3 Description of the inoculant treatments adopted in Experiments A and B pot trials.

Inoculant formulation and source	Treatment code	<i>Rhizobium</i> inoculum	Forage legume	Inoculum potential	Application rate (g pot ^{−1})
Australian granular (Murdoch Univ.)	AUG.1	<i>Rhizobium leguminosarum</i> bv. viciae	<i>Vicia sativa</i> var. Buza	(1 × 10 ⁹ CFU g ^{−1})	2.5 (Exp. A) and 5.0 (Exp. B)
	AUG.2	<i>R. leguminosarum</i> bv. trifolii	<i>Trifolium michelianum</i> , <i>T. subterraneum</i> var. Urana, † <i>T. pratense</i> var. Rozeta	(1 × 10 ⁹ CFU g ^{−1})	2.5 (Exp. A) and 5.0 (Exp. B)
	AUG.3	<i>Sinorhizobium</i> spp.	<i>Medicago polymorpha</i> var. Scimitar	(1 × 10 ⁹ CFU g ^{−1})	2.5 (Exp. A) and 5.0 (Exp. B)
Australian peat (Murdoch Univ.)	AUP.1	<i>R. leguminosarum</i> bv. viciae	<i>V. sativa</i>	(1 × 10 ⁹ CFU g ^{−1})	1.5 (Exp. A) and 3.0 (Exp. B)
	AUP.2	<i>R. leguminosarum</i> bv. trifolii	<i>T. michelianum</i> , <i>T. subterraneum</i> , <i>T. Pratense</i>	(1 × 10 ⁹ CFU g ^{−1})	1.5 (Exp. A) and 3.0 (Exp. B)
	AUP.3	<i>Sinorhizobium</i> spp.	<i>M. polymorpha</i>	(1 × 10 ⁹ CFU g ^{−1})	1.5 (Exp. A) and 3.0 (Exp. B)
American peat (Visjon Biologics)	USP.1	Exceed® Pea/Vetch/Lentil: <i>R. leguminosarum</i> bv. viciae	<i>V. sativa</i>	(2 × 10 ⁸ CFU g ^{−1})	1.5 (Exp. A) and 3.0 (Exp. B)
	USP.2	Exceed® Subterranean Clover: <i>R. leguminosarum</i> bv. trifolii (subterranean)	<i>T. subterraneum</i>	(2 × 10 ⁸ CFU g ^{−1})	1.5 (Exp. A) and 3.0 (Exp. B)
	USP.3	Exceed® True Clover: <i>S. meliloti</i> and <i>R. leguminosarum</i> bv. trifolii	‡ <i>M. polymorpha</i> , <i>T. michelianum</i> , <i>T. Pratense</i>	(2 × 10 ⁸ CFU g ^{−1})	1.5 (Exp. A) and 3.0 (Exp. B)
Non-inoculated control	CNT	None	All	Unknown	None

†*T. pratense* in Exp. B only. ‡Visjon Biologics does not market a crop-specific inoculant for *M. polymorpha*, *T. michelianum* and *T. pratense*, but suggested trying Exceed “Alfalfa/True Clover”, a blend of *S. meliloti* and *R. leguminosarum* bv. trifolii (clover).

TABLE 4 Weather conditions [maximum and minimum temperatures, mean relative humidity (RH), rainfall, and reference evapotranspiration (ET_0)] at the experimental site (Milazzo, 38°11'25" N, 15°14'28" E) during the 2023/2024 growing seasons of Experiments A and B.

	Aug	Sep	Oct	Nov	Dec	Jan	Feb	Mar	Apr
T _{max} (°C)	31.0	29.9	28.4	22.8	18.5	17.2	17.7	19.8	22.3
T _{min} (°C)	20.4	19.1	17.5	12.9	9.8	8.0	8.6	9.9	10.4
RH _{mean} (%)	53.1	53.4	57.1	60.6	63.3	64.2	63.4	60.6	53.5
Rainfall (mm)	14.0	32.8	1.2	77.6	63.8	77.0	121.2	55.6	25.8
ET ₀ (mm)	162.9	119.3	98.4	59.9	41.2	44.1	54.8	88.8	110.9

were optimal for the growth of forage legumes and supplemented with drip irrigation when required.

2.4 Measurements

After harvest (Table 1), soil substrates were removed from pots, the roots were gently washed with tap water, and the aboveground and belowground plant parts were carefully separated. Aboveground and belowground fresh weight, root nodulation (nodule counts), and total nodule fresh weight were measured on the three plants pot⁻¹. Dry matter was determined by oven-drying biomass samples at 65°C up to constant weight.

Aboveground and belowground samples were ground to pass a 1-mm sieve (CyclotecTM 1093 Sample Mill, Foss, Denmark), and the total nitrogen was determined according to the Kjeldahl method (UDK 169, Velp Scientifica, Italy).

2.5 Estimation of relative N₂ fixation

N₂ fixation was estimated by the N-difference method, in which the N yields of fixing plants are compared with an uninoculated legume of the same species (control). This is a simple and low-cost method based on the arbitrary assumption that plants absorb the same amount of N from the soil and translocate equal amounts of soil-derived N (Hardarson and Danso, 1993). Here, we adapted the method by considering the uninoculated legumes of the same species as control plants, aware that uninoculated control plants can nodulate due to the presence of native rhizobial communities in the experimental soils. We did not use sterile soil in control pots because it would have changed the soil microbiome and thus the inoculation efficiency, given that they act in synergism with plant-growth-promoting rhizobacteria (Tilak et al., 2006). N yields of aboveground (ANY) and belowground (BNY) plant parts were calculated as:

$$\text{ANY} = (\text{aboveground DW}) \times (\% \text{N}_{\text{shoots}})$$

$$\text{BNY} = (\text{belowground DW}) \times (\% \text{N}_{\text{roots}})$$

where %N_{shoots} and %N_{roots} are the total nitrogen in above and belowground of legume species, respectively. The amount of relative

N₂ fixation was quantified in accordance with the equation proposed by Howieson and Dilworth (2016):

$$\text{Nfixed} = (\text{total N yield inoculated}) \\ - (\text{total N yield non-inoculated control})$$

where total N yield is the sum of aboveground and belowground N yields. Given that inoculated and non-inoculated plants grew with the same pedo-climatic conditions and management, we assume that the difference in N fixed could be attributable to the inoculation effect.

2.6 Statistical analysis

Data for each experiment were analyzed separately by two-way analysis of variance (ANOVA), according to the complete randomized design. The legume species and the inoculant type (rhizobial strain × inoculant formulation combination) were the fixed effects. Before the ANOVA, the homogeneity of variance was assessed with the Bartlett's test and normality by a graphical inspection of the residuals. Percentage data were arcsine √% transformed before the analysis. The significance of differences among groups was tested using the Tukey's HSD test at $p \leq 0.05$. All analyses were conducted using CoStat[®] software version 6.003 (Cohort Software, Monterey, CA, USA).

3 Results

3.1 Experiment A

3.1.1 Plant growth and nodulation

The ANOVA showed that legume species contributed the most to the overall variance for all the investigated parameters (Table 5). The effect of inoculant formulation was significant for root nodulation and total nodule weight, whereas aboveground and belowground biomass were significantly affected only by legume species. Moreover, a significant 'species × inoculant' effect was found for belowground biomass and root nodulation.

The effects on aboveground and belowground biomass, which were the highest in *T. subterraneum* (17.2 and 16.6 g pot⁻¹, respectively) and *T. michelianum* (13.0 and 15.4 g pot⁻¹), were

TABLE 5 Analysis of variance (ANOVA) according to the complete randomized design for main factors and their interactions in Experiment A for aboveground dry weight (ADW), belowground dry weight (BDW), root nodulation (RN), total nodule weight (NW), aboveground nitrogen yield (ANY), belowground nitrogen yield (BNY) and relative N₂ fixation.

Source	df	ADW	BDW	RN	NW	ANY	BNY	Relative N ₂ fixation
Species (S)	3	346.01 ***	602.66 ***	238884.74 ***	1.21 ***	1938.3 ***	1029.5 ***	717.2 ***
Inoculant (I)	3	21.88 ns	13.95 ns	53613.73 ***	0.74 **	167.9 ns	11.6 ns	330.7 ***
(S) × (I)	9	8.46 ns	21.97 **	52824.78 ***	0.14 ns	67.6 ns	26.7 **	121.4 ***
Error	30	8.94	6.53	1292.36	0.10	65.1	8.5	18.8

Values are given as mean square (MS); df, degrees of freedom; *** and ** indicate significance at $p \leq 0.001$ and $p \leq 0.01$, respectively (Tukey's HSD test); ns, not significant.

species specific (Figure 1). In fact, all inoculants investigated decreased belowground biomass of *T. subterraneum* and *T. michelianum* compared with the control (CNT), while an opposite trend was observed for *V. sativa*. In *M. polymorpha*, except for USP that reduced belowground biomass, AUG and AUP showed not significant differences compared to CNT.

Root nodulation was significantly improved by AUG (+60% than control), followed by AUP (+23.6%) and USP (+11.5%) (Figure 1). The greatest root nodulation was found in *V. sativa* and *T. subterraneum* inoculated with AUG (555 and 547 number pot^{-1} , respectively). AUP induced the highest root nodulation in *T. michelianum* (445 number pot^{-1}), while no positive effect was observed in *M. polymorpha*.

Total nodule fresh weight showed a similar trend to root nodulation (Figure 1). AUG determined the highest total nodule weight (1.1 g pot^{-1}), while both AUP and USP caused lower values than CNT. Across the average of inoculants, no significant differences were observed between *V. sativa*, *T. michelianum*, and

T. subterraneum, while *M. polymorpha* had the lowest total nodule weight (0.3 g pot^{-1}).

3.1.2 Relative N_2 fixation

From the ANOVA emerged that the “species $\times$ inoculant” interaction was significant on BNY and N_2 fixation (Table 5). ANY, BNY, and relative N_2 fixation were mostly affected by legume species, and the inoculant effect was not significant for ANY and BNY.

Across the average of inoculants, *T. subterraneum* and *T. michelianum* showed the highest ANY (43.5 and 33.0 mg N pot^{-1} , respectively) and BNY (24.9 and 18.2 mg N pot^{-1}), while no significant effects were observed between the inoculants (Figure 2). Concerning BNY, the two-way interaction showed that it was increased by all inoculants in *V. sativa* and decreased in *T. michelianum*, although without significant differences. No significant differences among the inoculants were also detected in *T. subterraneum*. USP was the worst inoculant for all forage legumes.

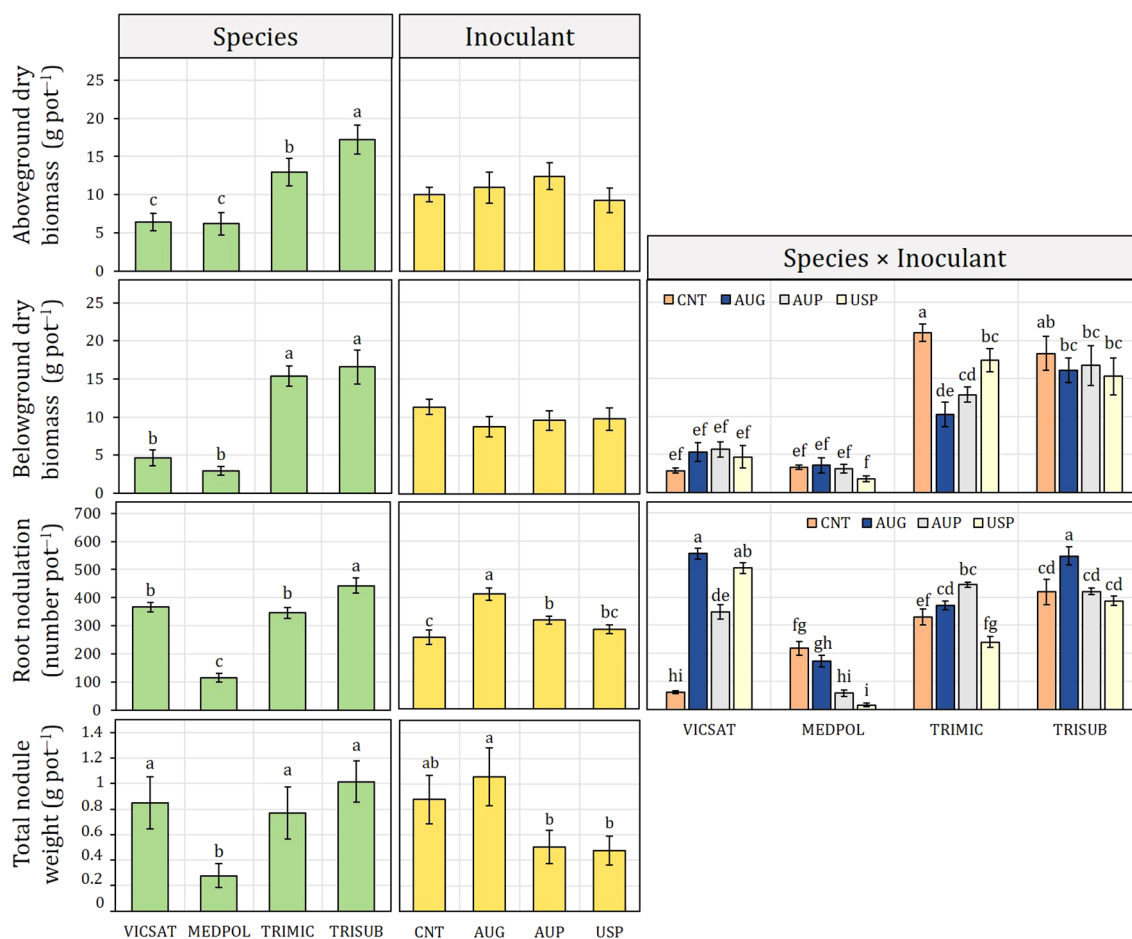


FIGURE 1

Inoculant formulation effect (non-inoculated control, CNT; Australian granular, AUG; Australian peat, AUP; American peat from Visjon Exceed[®], USP) on aboveground and belowground dry biomass, root nodulation, and total nodule fresh weight of *Vicia sativa* (VICSAT), *Medicago polymorpha* (MEDPOL), *Trifolium michelianum* (TRIMIC), and *Trifolium subterraneum* (TRISUB) from Experiment A. Bars are standard error. Different letters indicate statistical significance at $p \leq 0.05$ (Tukey's HSD test).

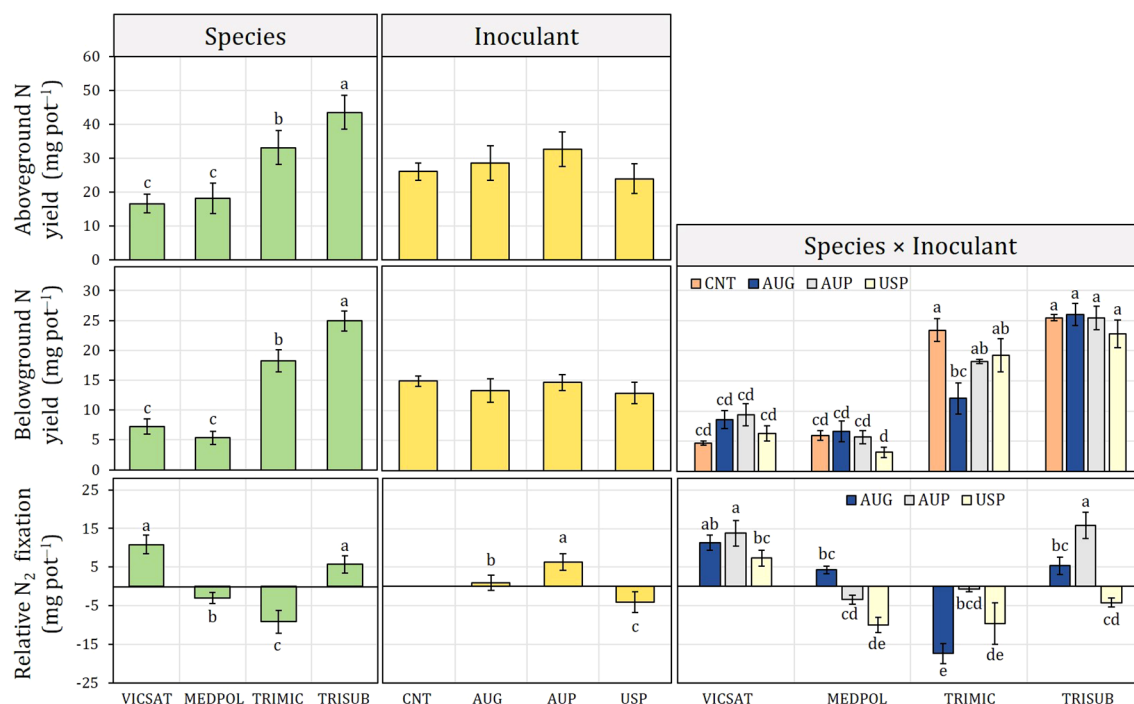


FIGURE 2

Inoculant formulation effect (non-inoculated control: CNT; Australian granular: AUG; Australian peat, AUP; American peat from Visjon Exceed®, USP) on aboveground N yield, belowground N yield and relative N_2 fixation of *Vicia sativa* (VICSAT), *Medicago polymorpha* (MEDPOL), *Trifolium michelianum* (TRIMIC), and *Trifolium subterraneum* (TRISUB) from Experiment A. Bars are standard error. Different letters indicate statistical significance at $p \leq 0.05$ (Tukey's HSD test).

Across the average of species, AUP induced the greatest relative N_2 fixation ($6.4 \text{ mg N pot}^{-1}$), followed by AUG ($0.8 \text{ mg N pot}^{-1}$), whereas USP fixed less than CNT (Figure 2). *V. sativa* was the best fixing species among the forage legumes investigated ($10.8 \text{ mg N pot}^{-1}$), followed by *T. subterraneum* ($5.7 \text{ mg N pot}^{-1}$), while *M. polymorpha* and *T. michelianum* fixed less than CNT with the investigated inoculants at the standard application rate. In detail, the inoculants improved the relative N_2 fixation in *V. sativa* and *T. subterraneum*, excluding USP for the latter species, with AUP that determined the highest values for both species (13.7 and $15.7 \text{ mg N pot}^{-1}$, respectively). Only AUG induced relative N_2 fixation in *M. polymorpha* ($4.3 \text{ mg N pot}^{-1}$), while none of the inoculants was effective in *T. michelianum*.

3.2 Experiment B

3.2.1 Plant growth and nodulation

Except for the aboveground biomass, the ANOVA highlighted a significant “species $\times$ inoculant” effect for all the investigated parameters (Table 6). Legume species were confirmed as the major factor affecting variance.

Across the average of species, USP had the highest stimulating effect on aboveground biomass (23.3 g pot^{-1} , $+63.8\%$ than CNT), followed by AUG (19.5 g pot^{-1}) (Figure 3). Concerning belowground biomass, no significant differences were detected between inoculants and the control for *V. sativa*. The highest belowground biomass for *M. polymorpha* was induced by USP

TABLE 6 Analysis of variance (ANOVA) according to the complete randomized design for main factors and their interactions in Experiment B for aboveground dry weight (ADW), belowground dry weight (BDW), root nodulation (RN), total nodule weight (NW), aboveground nitrogen yield (ANY), belowground nitrogen yield (BNY), and relative N_2 fixation.

Source	df	ADW	BDW	RN	NW	ANY	BNY	Relative N_2 fixation
Species (S)	4	1252.32 ***	381.45 ***	258531.50 ***	5.00 ***	5740.8 ***	2118.9 ***	393.7 **
Inoculant (I)	3	227.13 ***	65.54 ***	48700.21 ***	1.19 ***	906.7 ***	340.2 ***	404.7 *
(S) $\times$ (I)	12	56.28 ns	58.20 ***	29260.87 ***	0.64 ***	353.9 **	235.1 ***	734.9 ***
Error	38	32.71	5.70	2778.34	0.12	124.3	28.1	80.7

Values are given as mean square (MS); df, degrees of freedom; ***, **, and * indicate significance at $p \leq 0.001$, $p \leq 0.01$, and $p \leq 0.05$, respectively (Tukey's HSD test); ns, not significant.

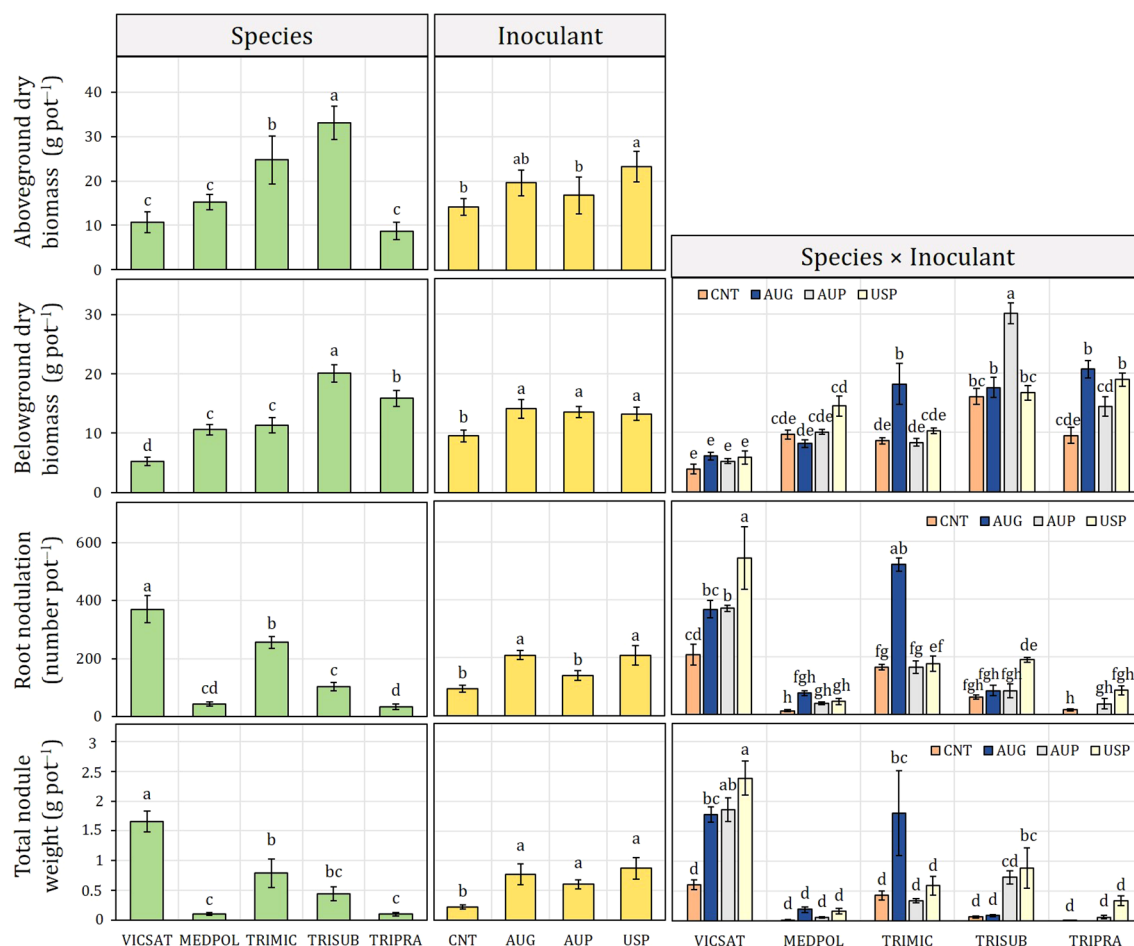


FIGURE 3

Inoculant formulation effect (non-inoculated control, CNT; Australian granular, AUG; Australian peat, AUP; American peat from Visjon Exceed®, USP) on aboveground and belowground dry biomass, root nodulation, and total nodule fresh weight of *Vicia sativa* (VICSAT), *Medicago polymorpha* (MEDPOL), *Trifolium michelianum* (TRIMIC), *Trifolium subterraneum* (TRISUB) and *Trifolium pratense* (TRIPRA) from Experiment B. Bars are standard error. Different letters indicate statistical significance at $p \leq 0.05$ (Tukey's HSD test).

(14.5 g pot⁻¹), for *T. michelianum* and *T. pratense* by AUG (18.2 and 20.6 g pot⁻¹, respectively), and for *T. subterraneum* by AUP (30.1 g pot⁻¹). All inoculants increased the belowground biomass versus the control, with no significant differences among inoculant types. Across the average of inoculants, *T. subterraneum* had the highest belowground biomass (20 g pot⁻¹), followed by *T. pratense* (15.9 g pot⁻¹), and *V. sativa* the lowest (5.1 g pot⁻¹).

Both root nodulation and total nodule weight were highly affected by the “species × inoculant” interaction (Table 6). USP induced the highest values in *V. sativa* (543 number pot⁻¹ and 2.4 g pot⁻¹), *T. subterraneum* (190 number pot⁻¹ and 0.3 g pot⁻¹) and *T. pratense* (84 number pot⁻¹ and 0.1 g pot⁻¹), while AUG in *M. polymorpha* (75 number pot⁻¹ and 0.2 g pot⁻¹) and *T. michelianum* (520 number pot⁻¹ and 1.8 g pot⁻¹) (Figure 3). Interestingly, AUG inhibited root nodulation on *T. pratense*. *V. sativa* had the highest root nodulation and total nodule weight, followed by *T.*

michelianum. Regardless of legume species, all inoculant formulations increased both parameters versus the control.

3.2.2 Relative N₂ fixation

The “species × inoculant” interaction was significant for ANY ($p \leq 0.01$), BNY ($p \leq 0.001$), and relative N₂ fixation ($p \leq 0.001$) (Table 6). Legume species had the greatest influence on variance for ANY and BNY, while N₂ fixation was more affected by inoculant formulation.

ANY and BNY showed the same trend, respectively, observed for aboveground and belowground biomass (Figure 4). Concerning ANY, the highest value was found in *T. subterraneum* (73.9 mg N pot⁻¹) and *T. michelianum* (47.9 mg N pot⁻¹) for legume species, and in USP (48.3 mg N pot⁻¹, +58.7% than CT) and AUG (43.3 mg N pot⁻¹, +42.4% than CT) for inoculant formulations. In particular, USP induced the highest ANY for *T. subterraneum* (96.8 mg N

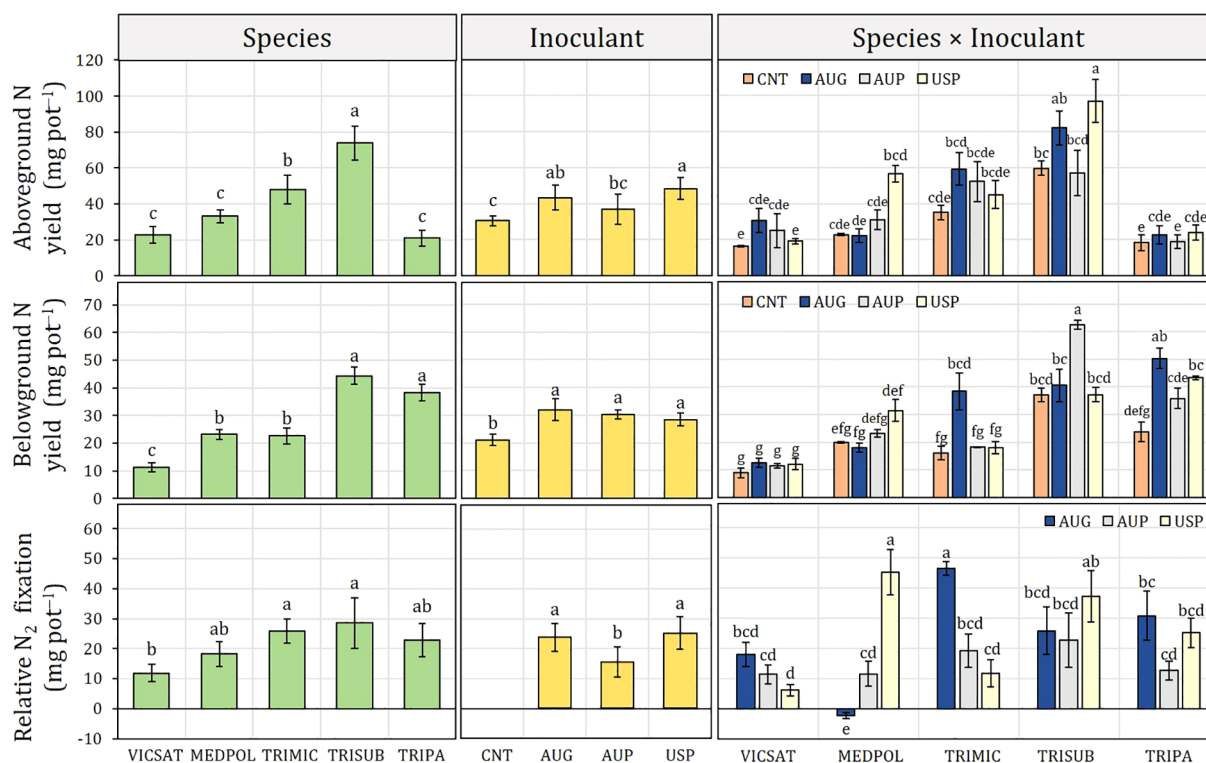


FIGURE 4

Inoculant formulation effect (non-inoculated control, CNT; Australian granular, AUG; Australian peat, AUP; American peat from Vision Exceed®, USP) on aboveground N yield, belowground N yield and N₂ fixation of *Vicia sativa* (VICSAT), *Medicago polymorpha* (MEDPOL), *Trifolium michelianum* (TRIMIC), *Trifolium subterraneum* (TRISUB), and *Trifolium pratense* (TRIPA) from Experiment B. Bars are standard error. Different letters indicate statistical significance at $p \leq 0.05$ (Tukey's HSD test).

pot⁻¹), *M. polymorpha* (56.6 mg N pot⁻¹), and *T. pratense* (23.9 mg N pot⁻¹), whereas AUG was the best inoculant for *V. sativa* (30.7 mg N pot⁻¹) and *T. michelianum* (59.2 mg N pot⁻¹). Concerning BNY, *T. subterraneum* and *T. pratense* showed the greatest values (44.2 and 38.2 mg N pot⁻¹, respectively), while *V. sativa* confirmed as the poorest legume species (11.1 mg N pot⁻¹). Across the average of species, no significant differences were found between inoculant formulations, as observed for belowground biomass, all of which have increased BNY compared to CNT. In accordance with the belowground biomass trend, AUG determined the highest BNY in *T. michelianum* (38.4 mg N pot⁻¹) and *T. pratense* (50.3 mg N pot⁻¹), USP in *M. polymorpha* (31.4 mg N pot⁻¹), and AUP in *T. subterraneum* (62.4 mg N pot⁻¹), whereas no significant differences were observed in *V. sativa*.

Across the average of inoculant formulations, *T. subterraneum* and *T. michelianum* showed the highest relative N₂ fixation rates (28.6 and 25.7 mg N pot⁻¹, respectively) and *V. sativa* the lowest one (11.8 mg N pot⁻¹) (Figure 4). On the average of legume species, the relative N₂ fixation of AUG and USP inoculants was similar and higher than AUP. From the “species × inoculant” interaction emerged that AUG in *V. sativa*, *T. michelianum*, and *T. pratense* fixed N₂ at levels greater than the other inoculants (18.0, 46.5, and 30.7 mg N pot⁻¹), but performed worse than CNT in *M. polymorpha* (−2.3 mg N pot⁻¹). At the same time, *M. polymorpha* and *T. subterraneum* inoculated with USP had significantly higher

relative fixed N₂ than the other inoculants, with fixation rates of 45.4 and 37.2 mg N pot⁻¹, respectively.

4 Discussion

Positive effects of rhizobia inoculants on forage legume growth and nodulation have been previously reported (Carlsson and Huss-Danell, 2003; Shockley et al., 2004), including common vetch (Albayrak et al., 2006) and annual medics (Materon, 1988). The enhancement of plant growth induced by rhizobial inoculation could be attributed to the direct fixed N provided by rhizobia strains, the increase in plant nutrient uptake, and the production of plant growth-promoting hormones such as indole-3-acetic acid (Yadav and Verma, 2014; Allito et al., 2021). Although background rhizobial populations are common in Mediterranean regions due to the long history of forage legume cultivation, their N-fixing effectiveness varies widely (Rejili et al., 2012). Two main strategies can be pursued to enhance forage legumes N-fixation: inoculating with commercial elite strains or inoculating with selected native rhizobia. This research focused on the former strategy, given that most of these strains, such as the Australians, were selected for high effectiveness, desiccation tolerance, and persistence in harsh conditions, which are in part exacerbated by climate change and can adversely impact legume-rhizobia associations.

In the present study, inoculation with elite species-specific rhizobia strains and different inoculant formulations significantly improved plant growth and nodulation of the forage legumes investigated only in Experiment B.

The native rhizobial populations likely present in the substrate may have been sufficient in Experiment A, as evidenced by nodulation in the control plants. However, assuming that native rhizobia are always effective is misguided. Some fields lack a history of specific legumes, and certain rhizobial strains can form nodules without efficiently fixing N, as indicated by nodules that lack pink or red coloration, signifying low leghemoglobin content. From a practical perspective, farmers often assume adequate nodulation without examining root nodules, risking undernodulation and reduced N-fixation. Insufficient inoculation, combined with challenges such as monoculture, limited crop rotation, soil acidification, residual herbicides, and unpredictable climates, can significantly reduce legume productivity and N-fixation (Yates et al., 2024).

Only root nodulation and total nodule weight were affected in Experiment A, in which the AUG and the American peat inoculant (USP) showed, respectively, the best and the worst promoting effect among the inoculants. The optimal performance of AUG may have a dual explanation. First, higher efficacy of granular inoculants compared to liquid or peat-based powder inoculation treatments has been demonstrated by Kyei-Boahen et al. (2002) for chickpea, Rice and Olsen (1992) for alfalfa, and Ocumpaugh (1991) for arrowleaf clover, especially under unfavourable conditions for rhizobia survival. Furthermore, the long transport distance from Australia and the United States could have compromised the quality of peat inoculants, whereas granules could have maintained stable cell numbers (1×10^9 CFU g⁻¹). Second, native rhizobia strains may exert better performances than non-native commercial inoculants. Batista et al. (2015), for example, indicated that red clovers inoculated with the native isolate of *R. leguminosarum* bv. *trifolii* (strain 317) in Uruguay grasslands produced more biomass than those inoculated with the commercial strain U204 thanks to the increased nodulation competitiveness of indigenous isolate 317 than U204. A similar finding was reported by Roughley et al. (1976) for subterranean clover across five sites in New South Wales (Australia). However, the symbiotic performance of naturalised soil rhizobia is not always constant and can be compromised if diverse rhizobia populations have naturalised in soils, as demonstrated by Drew et al. (2011) for several annual clover species of Mediterranean origin.

Rhizobial inoculation was ineffective in its nodulating and growth promotion effects of *M. polymorpha*, in agreement with Charman and Ballard (2004), who concluded that none of the 222 screened lines of burr medic formed effective symbioses with a wide range of soil rhizobia. The reasons for poor nodulation are numerous and can be attributed to the high specificity of burr medic rhizobial strains, the inoculation of inappropriate rhizobia strains, the presence of competing indigenous rhizobia in the commercial soil substrate, or the decline of viable rhizobia contained in the inoculant. About the former aspect, it is known that rhizobia strains differ in nodulation effectiveness. Hence,

rhizobiologists are continuing to search rhizobia strains for improved N fixation and subsequent forage quality (Yates et al., 2021). More specifically, for an efficient translation to the field of the inoculant-strain selection, it should be considered the overall symbiont genotype $\times$ host genotype $\times$ environment interaction (Bellabarba et al., 2023). In this regard, selecting legumes with inherent diversity in growth potential, biomass production, growth habits, and life cycle length offers a range of options to fit specific management goals, such as forage production, soil cover, or improved BNF.

In Experiment B, a greater influence of the “species $\times$ inoculant” interaction than Experiment A was observed, denoting a higher species- and inoculant-dependent effect. Overall, the trend was similar to Experiment A, but the effects were more pronounced, probably by virtue of the double dosage of inoculant application on the one hand, and the different phenological growth stage on the other hand. Doubling the rate of application of inoculants is often reported as a good strategy to improve the nodulation effectiveness (Jakhar et al., 2018; Jesus et al., 2018). This improvement is generally suggested under poor soils with no legume history and soil pH ≥ 5.5 or where host plants are stressed (Farquharson et al., 2022). Increasing the application rates of peat inoculants on seeds enhances, in turn, the likelihood of sufficient rhizobia survival until plant germination. Moreover, it is known that the effects of rhizobial inoculation are exacerbated at flowering. Our results are consistent with Lamptey et al. (2014), who found that inoculated soybean plants harvested at flowering recorded higher fresh and dry shoot matter versus plants harvested at the vegetative stage. Hossain and Solaiman (2004) reported a similar finding for mungbean varieties.

Overall, AUG and USP showed the best growth-promoting effects and N fixation rates in Experiment B, with species-specific results. According to Zdor and Pueppke (1990), peat formulation may help protect rhizobial strains from adverse environmental effects versus liquid carriers. Moreover, granular inoculants, which contain less moisture, could offer even greater protection. Therefore, although all inoculants under investigation were applied directly to the seeds, granular and peat inoculants could have increased the strain survival. The worse results provided by AUP compared to USP may be related to the use of native rhizobia strains.

Interestingly, AUG inhibited *T. pratense* root nodulation. We suppose this negative effect may be attributed to the not-appropriate choice of rhizobial strain. In this regard, Valverde et al. (2005) isolated and described from *T. pratense* a novel *Trifolium*-nodulating species (*Phyllobacterium trifolii* sp. nov.) that produces nodules on *Trifolium* spp. and *Lupinus* spp. Rodríguez-Navarro et al. (2022) selected and characterized *Trifolium*-nodulating rhizobia for pasture inoculation in Spain, indicating that several *Rhizobium* species can nodulate *Trifolium* spp. better than *R. leguminosarum* bv. *trifolii* and that some *Trifolium* species growth could be improved through appropriate rhizobial selection.

In both experiments, aboveground and belowground N yields were respectively consistent with aboveground and belowground dry weights, with significant differences between forage legume species. *T. subterraneum* showed the highest aboveground and belowground N yields among the investigated forage legumes.

Similar N shoot concentrations were reported by Ovalle et al. (2006). To the best of our knowledge, root N content of forage legumes has been poorly investigated. It was estimated to be 15–111 kg N ha⁻¹ for temperate species. Nnadi and Haque (1988) reported root N contents of 2.43% for *V. benghalensis*, 2.25% for *V. dasycarpa*, 2.08% for *M. truncatula*, 2.01% for *T. steudneri* cv. Shola, 1.23% for *M. scutellata*, 1.10% for *Lablab purpureus* cv. Rongai, and 0.87% for *L. purpureus* cv. Highworth, with positive but not significant interactions between N concentrations in the roots and aerial parts. Most studies estimate N₂ fixation only from aboveground plant parts, but according to Danso et al. (1988) up to 60% of the total fixed N of forage legumes may derive from roots. Indeed, the variation in N₂ fixation may vary based not only on the method adopted but also on the plant parts analyzed, dry matter yields, and C/N ratio (Carlsson and Huss-Danell, 2003). In general, N₂ fixation is positively correlated to dry weights and high C/N ratios (Carlsson and Huss-Danell, 2003; Zhang et al., 2022), which explains why aboveground and belowground N yields did not reflect the N₂ fixation in the present work. In addition, considering the significant contribution of belowground N, as demonstrated by the high root:shoot ratios here obtained (data not shown), we consider our estimation of the total N₂ fixation more realistic than the sole shoots N₂ fixation. Although we did not use sterile soil in the present experiments for BNF estimation, a natural soil with its native microbiota allows for evaluating the effectiveness of the additional inoculum in a more realistic context. Indeed, a sterile soil not only eliminates natural rhizobia but also other beneficial microorganisms, altering microbial interactions and making it difficult interpreting data in relation to real agricultural soil conditions.

5 Conclusions

From this preliminary study, we concluded that inoculation of selected forage legumes (*V. sativa*, *M. polymorpha*, *T. michelianum*, *T. subterraneum*, and *T. pratense*) with specific inoculants at double the recommended dose may be an efficient approach to enhance plant growth, nodulation, and N₂ fixation. Unfortunately, there is still a low availability of elite commercial strains across the Mediterranean area due to the lack of rhizobia inoculant companies in Europe. The implementation in the Mediterranean agriculture of elite rhizobia strains supported by high-quality research, such as from Australia and the United States, could be a partial solution, avoiding long transport distances that could compromise the quality of inoculants. The cost of increased inoculant use is minimal compared to the potential risk in yield and profit due to unknown nodulation (lack of field knowledge by the farmer), poor nodulation, and N deficiency. Given the ongoing decline in the fertility of Mediterranean soils, this strategy could enhance legume production under restrictive conditions while reducing the need for mineral N fertilizers for subsequent crops. Future research steps will focus on field trials to validate the optimal forage legume × inoculant combinations identified here, particularly in intercropping with cereals, as it represents a promising agroecological practice for Mediterranean cropping systems.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Author contributions

AS: Data curation, Formal analysis, Investigation, Software, Validation, Visualization, Writing – original draft, Writing – review & editing. AM: Data curation, Investigation, Methodology, Writing – review & editing. FC: Data curation, Investigation, Methodology, Writing – review & editing. TM: Data curation, Investigation, Methodology, Writing – review & editing. CT: Conceptualization, Investigation, Methodology, Writing – review & editing. RY: Conceptualization, Investigation, Methodology, Writing – review & editing. ST: Formal analysis, Investigation, Writing – review & editing. CC: Data curation, Investigation, Writing – review & editing. MO: Conceptualization, Funding acquisition, Investigation, Project administration, Resources, Writing – review & editing. MF: Writing – review & editing, Data curation, Formal analysis. AV: Data curation, Writing – review & editing. DS: Methodology, Project administration, Resources, Validation, Writing – review & editing, Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation. FG: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Supervision, Validation, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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