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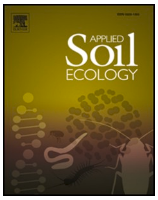

Soil bacteria and symbiotic rhizobia synergistically promote nitrogen fixation and biomass production of alfalfa (*Medicago sativa* L.) plants even under water shortage conditions without altering the native rhizospheric microbiota

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ABSTRACT

In modern and sustainable agriculture, the exploitation of biological nitrogen fixation (BNF) is an alternative to synthetic nitrogen fertilizers which can lead to groundwater contamination, reduction of soil quality, and greenhouse gas emissions. This study aimed to exploit the synergistic effects of nitrogen-fixing symbiotic rhizobia and nonsymbiotic soil bacteria on alfalfa (*Medicago sativa* L) yield in low-input farming. Two microbial consortia (Mix1 and Mix2) containing a nitrogen-fixing alfalfa symbiont *Sinorhizobium meliloti* were tested along with a different number of soil bacteria whose PGP (Plant Growth Promoting) characteristics (IAA production, nitrogen fixation, and phosphate solubilization) led to synergistic effect. The two consortia and the *S. meliloti* strain were tested under increasingly complex conditions, through in vitro strain culture testing, growth chamber, pot trials, and field trials. In the laboratory growth chamber, the alfalfa plants inoculated with the consortium Mix2, containing more strains with different activities, showed increased nitrogenase activity compared to those inoculated with *S. meliloti* alone. Field experiments showed that biomass production and assimilation of nitrogen due to nitrogen fixation (^{15}N test) of Mix2-inoculated plants was significantly increased compared to Mix1-inoculated and uninoculated ones (native microbiota), even under water shortage. Moreover, no significant impact of consortia on the native rhizospheric microbiota was detected. Our findings indicated that consortia containing both rhizobia and non-rhizobia PGP strains, which don't alter soil ecology, could be used for the enhancement of growth and nitrogen fixation of legume plants and that diversity and synergistic interaction of the consortium could be a good index for predicting success in field trials.

1. Introduction

Microbial inoculants contain beneficial microorganisms, such as bacteria, fungi, or algae, enhancing plant growth and resilience (Gómez-Godínez et al., 2021). They contribute to plant nutrient uptake, tolerance to stress, and soil health improvement by enhancing root architecture, stimulating antioxidant defence, and fostering symbiotic relationships with plant roots. Bioinoculants can also suppress plant pathogens by competing for resources, producing antimicrobial compounds, and inducing systemic resistance in plants (Haq et al., 2024).

In recent years, many microbial strains have been identified as good candidates for plant inoculation under laboratory conditions, and the global market for bioinoculants has developed significantly. However, the efficacy of these bioinoculants has not often been confirmed under

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field conditions, raising the question of lab-to-the-field issues (Li et al., 2022). The lack of effectiveness under field conditions relies on many factors, including variable pedoclimatic conditions, differences in the response by plant genotypes, and competition of inoculated strains over the native soil microbiota. To partially overcome these problems, microbial consortia and synthetic communities have been proposed (Arnault et al., 2024). Such bioinoculants are formulated to mimic the function of native microbiota (synthetic communities) or include various functions (microbial consortia) ranging from nutrient uptake to phytohormone modulation. However, while synthetic communities are made up of many different strains and species, which hinder the cost-effective production of bioinoculants based on these strains, consortia are formed by a few strains, allowing more efficient bioinoculants production (Khan, 2022; Negi et al., 2024). Commonly developed consortia may include strains that can fix atmospheric nitrogen, solubilize phosphorus, and produce growth-promoting substances like hormones (e.g., auxins and cytokinins), thus reducing the need for chemical fertilizers (Santoyo et al., 2021; Khan, 2022; Negi et al., 2024). Consortia can be assembled using microbes usually colonizing the soil and plant rhizosphere but may also include endophytic strains colonizing the internal plant tissue (Maheshwari et al., 2023; Moretti et al., 2024). Since endophytic strains colonize the internal plant tissues, which constitutes a low-diversity and highly specialized environment where only specific strains or species can be retrieved, they offer the potential advantage of reducing the need for high competition over the native soil microbiota (Riva et al., 2022).

Alfalfa (*Medicago sativa* L.) is one of the most widely cultivated forage legumes worldwide (Kulkarni et al., 2018). It is a perennial and self-fertile legume with a short life cycle, high natural diversity, high nutritional quality, and high nitrogen (N) content. The best growth and productivity of alfalfa plants occur when there are high temperatures (such as in the spring-summer period in the Mediterranean environment) and good water availability. The latter is particularly critical in arid and semi-arid pastoral regions, where this crop can only be grown if irrigated. As a leguminous plant, alfalfa can assimilate atmospheric N_2 through biological nitrogen fixation (BNF) thanks to the activity of endophytic symbiotic bacteria called rhizobia (Lindström and Mousavi, 2020). However, considering the variety preference in the host-*Rhizobium* symbiosis and the high heterogeneity of soil rhizobia populations (Cangioli et al., 2022a, 2022b), the inoculation of alfalfa with highly performing selected (elite) *Rhizobium* strains, effective even in conditions of limited water availability, is a relevant issue.

Indeed, alfalfa establishes symbiosis with the rhizobial species *Sinorhizobium meliloti*, but strain and host plant variation due to genotypic preferences often induce the formation of suboptimal symbiosis, particularly under harsh conditions (Bellabarba et al., 2021; Fagorzi et al., 2021; Bellabarba et al., 2023). Reports on consortia combining rhizobia and non-rhizobial strains have shown important potentialities for the promotion of plant growth (Santoyo et al., 2021). Therefore, the selection of the most efficient rhizobial strain (or consortium) and identification of non-rhizobial partners that could be used as co-inoculants to enhance rhizobia activity, is becoming relevant. Moreover, guidelines to reduce the time for consortia development and the lab-to-the-field gap are needed.

In this study, we hypothesize that the combination and redundancy of plant growth-promoting (PGP) abilities in a microbial consortium may lead to higher consortium success under field conditions.

To evaluate this assumption on the forage legume crop, we assembled two microbial consortia containing a symbiotic *Rhizobium* strain and a different number (and redundancy) of soil bacteria with PGP properties.

To achieve our goal, we planned to: i) build two consortia containing nitrogen-fixing symbiotic rhizobial strains and non-rhizobial soil bacteria with PGP features; ii) provide a lab-scale indication of overall plant nitrogen fixation; and iii) analyse biomass production and nitrogen fixation under field conditions.

2. Experimental procedures

2.1. Bacterial strains and growth conditions

The bacterial strains used in this study are reported in Table 1. The strain *Sinorhizobium meliloti* BL225C was originally isolated from alfalfa in Lodi, Italy (Carelli et al., 2000). Significant effects on alfalfa plants nodulation, tolerance to abiotic stress, ACC deaminase activity, and competitiveness for nodule colonization were observed for BL225C compared to other *S. meliloti* strains (Biondi et al., 2009; Checcucci et al., 2016; Checcucci et al., 2017; Bellabarba et al., 2021). The genome sequence and genome phylogeny of *S. meliloti* BL225C have been reported previously (Galardini et al., 2011; Galardini et al., 2013). The strains *Enterobacter asburiae* RCA24 and *Kosakonia sacchari* RCA25 were isolated from Volano rice plants grown in Italy (south Milan) (Defez et al., 2017; Andreozzi et al., 2019), the strains *Phytobacter diazotrophicus* BDA59–3*,* *Klebsiella quasipneumoniae* BDA62–2*,* *Kosakonia pseudosacchari* DBA62–3*,* *Klebsiella pasteurii* BDA134–6*,* and *Microbacterium laevaniformans* BDA137–13 were isolated from the African rice *Oryza glaberrima*, grown with low external input in the tropical region of Mali (West Africa), characterized by strong variation in the water regime and temperatures (Bianco et al., 2021). Of these seven strains, four were N-fixers (RCA25, BDA59–3, BDA62–3, and BDA134–6), two were IAA producers (RCA24 and BDA62–2), two were at same time N-fixers and IAA producers (BDA62–3 and BDA134–6), and one was positive in the genetic screening for 1-aminocyclopropane-1-carboxylate (ACC) deaminase (*acdS)* gene.

All PGP strains were aerobically grown at 30 + 2 °C in Luria Bertani medium, while *S. meliloti* BL225C was grown in TYR (tryptone 5 g l^{-}, yeast extract 3 g l^{-1}, $CaCl_2$ 6 mM) medium (Defez et al., 2019). The following antibiotics, for which strains are naturally resistant, were included in the medium: vancomycin (20 μg ml^{-1}) for RCA24 and RCA25, ampicillin (60 μg ml^{-1}) for BDA59–3 and BDA62–2, carbenicillin (25 μg ml^{-1}) for DBA62–3 and BDA134–6, and neomycin (15 μg ml^{-1}) for BDA137–13.

Table 1
Plant growth promoting (PGP) traits of strains used to assemble the Mix1 and Mix2 consortia.

Strain	Native host plant	PGP trait	Mix	Reference for original isolation
Sinorhizobium meliloti BL225C	*Medicago sativa* L.	IAA production*, Nitrogen-fixation and *acdS* positivity**	1, 2	Carelli et al., 2000
Klebsiella quasipneumoniae BDA62–2	*Oryza glaberrima* L.	IAA production*	1, 2	Bianco et al., 2021
Microbacterium laevaniformans BDA137–13	*Oryza glaberrima* L.	IAA production* and *acdS* positivity**	1, 2	Bianco et al., 2021
Phytobacter diazotrophicus BDA59–3	*Oryza glaberrima* L.	Nitrogen-fixation	2	Bianco et al., 2021
Kosakonia pseudosacchari BDA62–3	*Oryza glaberrima* L.	Nitrogen-fixation and IAA production*	2	Bianco et al., 2021
Klebsiella pasteurii BDA134–6	*Oryza glaberrima* L.	Nitrogen-fixation and IAA production*	2	Bianco et al., 2021
Enterobacter asburiae RCA24	*Oryza sativa* L. cv. *Volano*	IAA production*	2	Defez et al., 2017
Kasakonia sacchari RCA25	*Oryza sativa* L. cv.	Nitrogen-fixation	2	Defez et al., 2017

[*] IAA: indole-3-acetic acid.

[**] *acdS*: gene coding the 1-aminocyclopropane-1-carboxylate (ACC) deaminase enzyme.

2.2. *Analysis of bacterial salt tolerance*

Isolates were evaluated for their ability to grow under saline conditions. For each strain, cells were cultured on LB medium at 30 °C with shaking at 130 rpm. The optical density (OD_{600}) of bacterial cultures was measured using a spectrophotometer and adjusted to $OD_{600} = 0.5$ with LB medium. For the growth assay, 135 μl of LB medium supplemented with 1 M NaCl was dispensed into each well of a 96-well microtiter plate. An aliquot (15 μl) of the prepared inoculum was then added, resulting in a final well volume of 150 μl and an OD_{600} of 0.05 for each strain. Bacterial growth was automatically monitored for 72 h using a microplate reader (Tecan Infinite 200 PRO, Tecan, Switzerland). For each strain, 3 biological replicates were kept.

2.3. *Compatibility test by spot-on-lawn assay*

Before assembling the microbial consortia, a spot-on-lawn assay was performed to exclude cross-inhibition between strains. An aliquot (25 μl) of the target strain ($OD_{600} = 1$) was inoculated into molten agar cooled at 45 °C and poured into 140 mm diameter Petri dishes to create a uniform lawn. After solidification of the agar, 15 μl of each test strain was spotted on the surface. The plates were incubated at the appropriate temperature for 24 h. After incubation, interactions between the strains were assessed: zones of inhibition around the spots indicated antagonism, while co-growth without inhibition suggested compatibility.

2.4. *Genome sequencing and bioinformatic analysis*

DNA was extracted from cultures grown overnight in LB medium using PowerSoil DNA Isolation Kit (Qiagen, Hilden, Germany). After gel electrophoresis and fluorometric quantification (Qubit, ThermoFisher Scientific, Waltham, MA, USA), genomic DNA was fragmented with g-TUBE (Covaris Inc., Woburn, MA, USA) to average 15 Kbp size and used for library preparation using Pacific Biosciences SMRTbell Express Template Prep Kit 2.0 (Pacific Biosciences, Menlo Park, CA, USA). Samples were sequenced with the Sequel Sequencing Kit 3.0 in a Sequel apparatus (Pacific Biosciences, Menlo Park, CA, USA) by SMRT technology (Eid et al., 2009). Obtained reads were analysed using HGAP v. Microbial Assembly 8 within SMRT Link software ver. 8.0.0.80529 (Pacific Biosciences, Menlo Park, CA, USA) producing *oriC*-oriented assembly by running the "microbial multiplexing" pipeline with default options. Annotation was performed by the NCBI Prokaryotic Genome Annotation Pipeline (PGAP) (Tatusova et al., 2016). The taxonomic identification of strains from genome sequence was performed using the Type (Strain) Genome Server (Meier-Kolthoff et al., 2013; Meier-Kolthoff and Göker, 2019). The mapping of KEGG pathways was performed using KAAS on the KEGG web server (Moriya et al., 2007). Unique genes for the design of primer pairs which specially target the sequenced strains were identified by pangenome analysis using Roary software (Page et al., 2015) run on Galaxy web server (https://usegalaxy.eu/). Sequences were deposited in NCBI database under Bioproject PRJNA670042 (BDA59–3, BDA134–6, BDA62–3, BDA137–13) and PRJNA1120238 (RCA24, RCA25, BDA62–2).

2.5. *Amplicon sequencing analysis of the rhizosphere microbiota*

For field experiment, 9 samples were collected (3 inocula × 3 irrigation levels) for each block, for a total of 54 samples. After digging up the entire plant (10 cm radius around the plant and down to 20 cm soil depth) and shaking off the plant roots, the soil remaining attached to the root was detached with a sterile scalpel and considered rhizospheric soil. Soil samples were collected in triplicate at a depth of 15 cm, and then mixed. Environmental DNA (eDNA) was extracted from 250 mg of soil using the DNeasy PowerSoil Pro kit (Qiagen). The bacterial V4 region of 16S rRNA gene was amplified from the extracted DNA using the primers 515F (5′-GTGCCAGCMGCCGCGGTAA-3′) and 806R (5′-GACTACHVGGGTATCTAATCC-3′) following the protocol previously described (Cangioli et al., 2022a). PCR products were sequenced by Biomarker Technologies (BMK) GmbH (Germany) in a single run on Illumina Novaseq 6000 apparatus with flow cell SP (Illumina, USA). The bioinformatic and statistical analysis of obtained reads was performed through the pipeline previously described (Cangioli et al., 2022a, 2022b). Shortly, Illumina reads were trimmed and paired-end sequences were clustered into amplicon sequence variants (ASVs), following the DADA2 pipeline (version 1.16) (Callahan et al., 2016). Sequences were filtered and dereplicated to collapse identical reads into unique sequences, and chimeras were removed. The taxonomy assignment was carried out against the SILVA NR99rel138 database of bacteria (Quast et al., 2013). Annotated ASV count tables were processed using the Phyloseq package in R environment version 4.0.5 (McMurdie and Holmes, 2013) Sequence reads were deposited on SRA database and can be accessed through the Bioproject PRJNA1163085.

2.6. *Plant inoculation*

The procedure described below was applied to inoculate alfalfa seeds (cv Gea, provided by Continental Semences S.p.A., Italy). Under laboratory conditions, alfalfa seeds were surface sterilized and incubated at 21 °C for germination as described by Defez et al. (2017). After three days, seedlings were incubated with the bacterial suspensions Mix 1 and Mix 2 under constant gentle stirring for 2 h (RT) in Petri dishes. Bacterial suspensions contained the strain BL225C at 10^4 bacteria ml^{-1} and each endophyte at 10^6 bacteria ml^{-1} in 1× PBS solution. In preliminary inoculation experiments we verified that these concentrations were the ones leading to the best results. Seedlings incubated only with the strain BL225C in 1× PBS were used as a reference. Inoculated seedlings were then transferred into pots containing sand-perlite soil (2:1 ratio). Each planting unit was kept in the growth chamber under log daylight (16 h), 21 °C temperature, and 75 % relative humidity. Once a week medium containing both macronutrients and micronutrients was added to the plants (Jensen, 1942).

For the field experiment, unsterilized seeds were incubated under constant gentle stirring for 2 h (RT) with the bacterial suspensions Mix 1 and Mix 2 as above described. Seeds for control plots were incubated in 1× PBS solution. After incubation, the bacterial suspensions were removed and the seeds dried under a laminar flow hood for two hours. Dried seeds were then sown in the plots prepared for the field trials.

2.7. *Alfalfa plants grown in the laboratory*

2.7.1. *Re-isolation of bacterial strains from inoculated plants*

The colonization of alfalfa plants was assessed at 15 days after inoculation for both unsterilized and sterilized plants. The surface of the whole plant was sterilized using the following procedure: 1 min in 70 % EtOH, 1 min in 5 % sodium hypochlorite solution, and several washing with sterilized distilled water. Unsterilized and sterilized plants were then homogenized using a sterile mortar and pestle in 5 ml of 1× PBS. Dilutions of the homogenates were spread onto rich medium agar plates containing the antibiotic specific for the strain BDA62–3 presents in the consortium. The plates were incubated for 2 days at 30 °C for bacterial growth. Randomly colony were selected from the plates and grown in LB medium at 30 °C, on a shaker at 200 r/m, for 24 h. Aliquots of cultures were processed and used to amplify the 16S rRNA fragment (~1.4 Kb) as described in Amenta et al. (2024). The homogenate was also filtered with sterile gauze and used as DNA source to amplify specific sequences of the strains RCA24 and RCA25 thought a PCR-based method. PCR was carried out using the following primers: for RCA24, 5′-ACACTGACTTGAATGCTGCA-3′ and 5′-TGCTCTTTTGACACTTTCTTGGA-3′; for RCA25, 5′- CATTCTCAGCAATACGGGCC -3′ and 5′-CTCTGTTCAGGTGGCCAAAT –3′, which target unique genes in the respective bacterial genomes (NCBI database under Bioproject PRJNA1120238). For PCR reaction, 0.03 U of DreamTaq Hot Start DNA

Polymerase (Thermo Scientific, Walthman, MA, USA) were used following the procedure reported in Bianco et al. (2021). The PCR products were purified, and their nucleotide sequences determined through DNA fragments sequencing by fluorescent chain termination and capillary electrophoresis method (Eurofins Genomics, Ghent, Belgium).

2.7.2. Measurement of nitrogenase activity and biomass production

To analyse the effect of bacterial inoculation on the activity of the nitrogenase enzyme, alfalfa plants at 42 Days After Inoculation (DAI) were carefully removed from the pots, and the roots were rinsed with tap water. Nodulated roots were cut and transferred into glass tubes (10 ml) that were airtight, with a serum cap. The tubes containing an atmosphere of 10 % acetylene were then incubated for 30 min at room temperature (Camerini et al., 2008). Three samples (1 ml) were taken at different times and the amount of ethylene produced was evaluated using a gas chromatograph (Perkin-Elmer, Clarus 580, Walthan, MA, USA) equipped with a TG-BOND Alumina (Na_2SO_4 deactivation) column (30 m × 0.53 mm, Thermo Scientific, Walthan, MA, USA) and a hydrogen flame detector. The carrier gas (helium) flow rate was 48 cm/s, and the oven program was isocratic for 3 min at 130 °C. Activity data are the mean ± SD of at least twenty biological replicates.

For the evaluation of aboveground biomass production, shoots of three-week-old plants were cut down and weighted for fresh biomass measurement. The same plants were then dried at 60 °C until constant weight and weighed for dry matter determination. Data are the mean ± standard deviation of at least twenty biological replicates.

2.7.3. Analysis of root architecture

To analyse the effect of bacterial inoculation on root architecture, alfalfa seeds were inoculated as above described and transferred to Seed Growth Pouches (Mega Internationals, Minneapolis) containing Jensen' s nitrogen-free medium (Jensen, 1942). Two weeks after inoculation roots were photographed and the images processed with the software ImageJ v1.54p to measure root length, number of secondary roots, and number of nodules.

2.8. Field experiment

2.8.1. Experimental site

The experiment started in autumn 2021 at the Pietranera experimental farm about 30 km north of Agrigento, Sicily, Italy (37°30′N, 13°31′E; 178 m a.s.l.) and was carried out for three growing seasons (2021–22; 2022–23; 2023–24). The soil was an Entisol (Typic Xerorthent) and its physico-chemical properties was provided by the University of Palermo (clay 209 g kg^{-1}, silt 461 g kg^{-1}, and sand 330 g kg^{-1}; pH 7.84; total nitrogen 1.20 g kg^{-1}; total organic carbon 9.25 g kg^{-1}; electrical conductivity 1.88 dS m^{-1}; cation exchange capacity 18.4 cM kg^{-1}). The climate of the experimental site is semiarid Mediterranean with a mean annual rainfall of 582 mm, mostly in the autumn-winter period (September–February; 72 %) and in the spring (March–May; 23 %). There is a dry period from May to September. The mean air temperatures are 18.6 °C in autumn, 9.8 °C in winter, 14.2 °C in spring, and 24.4 °C in summer.

2.8.2. Experimental design and crop management

The experiment was set up in a strip-plot design with six replications. The treatments included: 1) control without inoculation (i.e. plants growing with natural soil microbiota) 2) alfalfa seeds inoculated with Mix1 consortium (Table 1); 3) alfalfa seeds inoculated with Mix2 consortium (Table 1). The sub-treatment consisted of three water supply levels, i.e. water reintegration of 50 % (I50), 75 % (I75), and 100 % (I100) of maximum evapotranspiration.

The previous crop grown without fertilization in the selected field was durum wheat. The soil was plowed in August and harrowed after the first autumn rainfalls; 69 kg P_2O_5 ha^{-1} was applied in all treatments before harrowing. Crops were sown in late October 2021 with a density of 600 viable seeds per square meter. Plots were 6.0 × 1.44 m (8 rows, 0.18 cm apart and 6 m long). At the beginning of spring 2022, all plots were hand-weeded. All plots were harvested by hand-cutting plants at 5 cm from soil level on the same day; in 2022, four cuts were made (from the beginning of May to the end of August); in 2023 four cuts (a cut in December and three cuts in April–June) and in 2024, due to the dry conditions, only one cut was made (in May). The soil water content of the 0–40 cm layer was measured in each plot using the gravimetric method from April 2022 to August 2022. Irrigation was implemented using a reduced-intensity sprinkle method. In the spring-summer of 2022, 2500, 3750, and 5000 m^3 ha^{-1} were applied for I50, I75, and I100 treatments, respectively. Irrigation was halted in September 2022, resulting in all plots receiving only natural rainfall during the 2022–23 and 2023–24 growing seasons. Weather data were collected from a weather station within 100 m of the experimental site.

To investigate the effects of microbial consortia inoculation on root system development, a pot experiment was carried out. The pot trial was conducted in a wire house with a transparent plastic roof and open sides, shielding the pots from natural rain while allowing for natural temperature variations. Each pot (diameter 8 cm, height 60 cm) was filled with soil collected from the site of the field experiment, and one plant was grown per pot. Six replicates were included for each treatment (Ctrl, Mix1, and Mix2). Sowing occurred on the third decade of October 2021. All pots were harvested manually by cutting the plants 5 cm above the soil surface at two time points: 150 and 195 days after sowing. At the second harvest, root biomass was carefully extracted by sieving and washing. Shoot and root biomass were subsequently oven-dried at 40 °C to constant weight to determine dry matter content. Throughout the growing period, soil moisture was monitored twice a week using the gravimetric method and was maintained between 70 % and 95 % of the soil's water-holding capacity.

2.8.3. Estimation of fixed nitrogen using ^{15}N dilution methodology

The ^{15}N isotope dilution technique was used to estimate N fixation by the alfalfa. ^{15}N fertilizer ($[NH_4]^2SO_4$ with an isotopic composition of 10 atom%^{15}N) (Sigma–Aldrich Inc. - St. Louis, MO, USA) was uniformly applied, at a rate of 11.5 kg N ha^{-1}, in liquid form to a 0.81 m^2 micro plot in the middle of each plot. To enhance the stability of the ^{15}N enrichment in the soil, the total amount of ^{15}N fertilizer was divided into four applications: one at the end of winter and the other three immediately following the first three cuts. After each application of labelled fertilizer, all plots were irrigated (5 l m^{-2}) to prevent plant leaves from retaining the ^{15}N fertilizer and to facilitate a more uniform distribution of the added ^{15}N in the soil profile. For each irrigation treatment, in each replication, a plot of mixture of three model perennial grass species (*Dactylis glomerata, Lolium perenne* and *Festuca arundinacea*) was also included, as reference species to calculate the biological nitrogen fixed by the legume crop. The three reference crops selected for our study are commonly used to estimate BNF in *Medicago sativa* (Hardarson et al., 1988; Carlsson and Huss-Danell, 2003; Goh, 2007) and are widespread in the Mediterranean region. Since no single species can exactly match the phenology, rooting depth, and N-uptake patterns of alfalfa, we used a mix of species as a reference crop, rather than a single species. This approach helped minimize potential estimation errors. The grass mixture plots were managed in the same way as alfalfa plots (same irrigation, same cutting, and same microplots with the addition of ^{15}N fertilizer).

A sample of aboveground biomass was taken at each cut of the first growing season within each test area where the labelled fertilizer was applied, dried at 40 °C for 36 h, weighed, ground to a fine powder (sieved using a 0.1-mm mesh size) in a fast-running mill, and analysed for enrichment in total N and ^{15}N. Total N and ^{15}N concentrations were determined using elemental analyser–isotope ratio mass spectrometry (Carlo Erba NA1500).

Data on ^{15}N enrichment of biomass at the first growing season were used to calculate the percentage of legume N derived from symbiotic N_2 fixation (%Ndfa), as follows:

$$\%Ndfa = \left(1 - \frac{atom\%^{15}Nleg}{atom\%^{15}Ngrass}\right)$$

where atom$\%^{15}$Nleg represents the atom$\%^{15}$N excess of legume tissue and atom$\%^{15}$Ngrass represents the atom$\%^{15}$N excess of unfertilized grass mixture. The ^{15}N-natural abundance of the atmosphere (0.3663 % ^{15}N) was used to calculate the atom$\%^{15}$N excess of both crops at each cut.

The amount of N fixed (Nfix) by alfalfa was estimated as follows:

$$Nfix = \frac{Ntot \times \%Ndfa}{100}$$

where Ntot represents the total N in the legume aboveground biomass.

2.8.4. *Plant growth parameters*

At each cut, the following measurements were done on each sub-plot: plant height, specific cover, and fresh aboveground biomass production. A representative sample (1 Kg) of fresh aboveground biomass was taken, dried at 60 °C until constant weight, and weighed, for dry matter determination.

2.8.5. *Statistical analysis*

Data on aboveground biomass production (t DM ha^{-1}), N uptake (kg N ha^{-1}) under different irrigation levels, total nitrogen in the aboveground biomass derived from the atmosphere (%Ndfa), and aboveground biomass production in three growing seasons were analysed with R software (R Core Team, 2020) using a generalized least-squares model in the "nlme" package (Pinheiro et al., 2021), with the implemented "varIdDent()" function to account for the variance heterogeneity. Model residuals were checked for heteroscedasticity and normal distribution. Data from each growing season were analysed separately. Within each irrigation level, we compared all response variables of both Mix1 and Mix2 with control using the dabestr package (Ho et al., 2019) to calculate effect sizes as unpaired mean differences and generate bias-corrected and accelerated bootstrapped 95 % confidence intervals. We used this combined approach given the increasing recognition of the limitations of using only the *P*-value and to avoid dichotomous cutoffs (Ho et al., 2019; Wasserstein and Lazar, 2020). Statistical analysis of nitrogenase activity, biomass production, primary root length, secondary roots, and number of nodules of laboratory grown alfalfa was performed by applying the one-way analysis of variance (ANOVA) and Tukey's HSD post-hoc test. Data of twenty biological replicates are approximately normally distributed. The VassarStats ANOVA program available at http://vassarstats.net/index.html (accessed on 8 June 2024) was used. The results were considered statistically significant when $p \leq 0.05$.

3. Results

3.1. *Characteristics of the bacterial strains used for the assembly of consortia*

The bacterial consortia contained the *Rhizobium* strain *S. meliloti* BL225C and 7 non-rhizobial endophytes expressing different traits (Table 1). Results reported in the Supplementary Fig. S1 showed the absence of cross-inhibition of growth among the strains, allowing us to hypothesize their compatibility when used for plant co-inoculation. We also showed that all strains grew well in 1 M NaCl, indicating tolerance to this salt concentration (Supplementary Fig. S2).

Two consortia were assembled from the tested strains: Mix 1 and Mix 2 (Table 1). Both consortia contained the symbiotic nitrogen-fixing *Rhizobium* strain *S. meliloti* BL225C, which allowed the establishment of nitrogen-fixing nodules on *Medicago sativa* plants. The consortium Mix1 (Table 1) included the endophytes BDA62–2 and BDA137–13, both IAA producers, and one *acdS* positive strain (BDA137–13). The consortium Mix2 (Table 1) was assembled with all eight selected strains and thus contained five N-fixers (BL225C, RCA25, BDA59–3, BDA62–3, BDA134–6), five IAA-producers (RCA24, BDA62–2, BDA62–3, BDA134–6, BDA137–13) and the *acdS* positive BDA137–13.

3.2. *Genome sequences of the consortia*

The genome sequences of four strains (BL225C, BDA59–3, BDA62–3, BDA134–6) were already available and published (Galardini et al., 2011; Bianco et al., 2021), while the genome sequencing of four isolates (RCA24, RCA25, BDA62–2, BDA137–13) was performed during this work to provide a complete information dataset on the genetics of the consortia. The accession numbers of all isolates are reported in Table 2. The strain *S. meliloti* BL225C was also de novo sequenced using SMRT sequencing technology and genome information has been updated in GenBank database. Table 2 reports the main genome features of all isolates. Results of genome-based taxonomic assignment of *K. sacchari* RCA25, *E. asburiae* RCA24, *M. laevaniformans* BDA137–13, *K. quasipneumoniae* BDA62–2 based on dDDH are reported in Supplementary Table S1. For BDA137–13, evidence for TYGS results suggested it belonged to a potential novel species of *Microbacterium*. However, ANI based on GenBank analysis (https://ftp.ncbi.nlm.nih.gov/genomes/all/GCF/042/668/555/GCF_042668555.1_ASM4266855v1/) supports an assignment to *M. laevaniformans* (ANI = 93.509 on ASM3953998v1). In Supplementary Table S2, composition of the *nif* gene cluster and presence of the ACC deaminase gene cassette in *K. sacchari* RCA25 and *M. laevaniformans* BDA137–13 strains were reported. The mapping on KEGG pathways of the annotation for the presence of reactions for IAA was reported in Supplementary Fig. S3.

3.3. *Effects of bacterial consortia on nitrogenase activity*

The effect of inoculation on the symbiotic phenotype of nodulated alfalfa plants was studied by measuring the activity of the nitrogenase enzyme through an acetylene reduction assay (ARA), as many of the selected strains were N-fixers. Alfalfa plants inoculated with Mix1 showed a slight increase in total nitrogenase activity, while a more than twofold increase was observed for plants inoculated with Mix2 (Table 3).

Table 2
Main genome characteristics of strains.

Strain	Genome size (Mbp)	Number of genes	N. of replicons/contigs	Assembly accession no.
Sinorhizobium meliloti BL225C	7.0	6′692	3	ASM2889512v1
Klebsiella quasipneumoniae BDA62–2	5.4	4′313	13	ASM4319543v1
Microbacterium laevaniformans BDA137–13	3.2	3′025	8	ASM4266855v1
Phytobacter diazotrophicus BDA59–3	5.3	5′074	1	ASM1516765v1
Kosakonia pseudosacchari BDA62–3	5.0	4′783	2	ASM1516741v1
Klebsiella pasteurii BDA134–6	6.1	5′664	2	ASM1560134v1
Enterobacter asburiae RCA24	4.7	4′554	2	ASM4012233v1
Kosakonia sacchari RCA25	5.1	4′844	4	ASM4012231v1

Table 3

Nitrogenase activity of *M. sativa* plants co-inoculated with the *Rhizobium S. meliloti* BL225C and different PGP bacterial endophytes.

Sample	Nitrogenase activity (nmol ethylene/plant/min)	Ratio[b]
Ms + BL225C	0.76 ± 0.25	1
Ms + BL225C + RCA24	1.28 ± 0.17	1.7
Ms + BL225C + RCA25	1.41 ± 0.37	1.8
Ms + BL225C + BDA59–3	0.60 ± 0.14	0.8
Ms + BL225C + BDA62–2	1.44 ± 0.17	1.9
Ms + BL225C + BDA62–3	1.45 ± 0.47	1.9
Ms + BL225C + BDA134–6	0.89 ± 0.12	1.2
Ms + BL225C + BDA137–13	1.50 ± 0.40	2.0
Ms + Mix1[a]	1.10 ± 0.32	1.4
Ms + Mix2[a]	1.8 ± 0.60	2.4

[a] The composition of Mix1 and Mix2 was as reported in Table 1.

[b] Ratio calculation was based on alfalfa plants inoculated only with *S. meliloti* BL225C.

3.4. Evaluation of consortia effect on alfalfa growth and re-isolation of endophytic bacteria from inoculated plants

Roots development and biomass production of alfalfa plants were evaluated to see whether the in vitro assessment of nitrogenase activity could be reflected in plant growth increase. Plants inoculated with Mix2 showed a statistically significant increase in primary root length and number of secondary roots (Fig. 1 and Table 4). For these plants a significant increase (up to 27 %) in biomass production was also measured compared to plants inoculated only with the rhizobium *S. meliloti* BL225C. When unsterilized and surface-sterilized inoculated plants were used for the re-isolation of strains BDA62–3, RCA24, and RCA25 contained in Mix2, the sequence of the 16S rRNA gene fragment obtained from PCR-colony assay confirmed the presence of the strain BDA62–3 in both plants (Fig. S4). Furthermore, results of PCR analysis carried out using specific primer pairs for the strains RCA24 and RCA25 showed that the two strains were abundant in the extract of unsterilized plants and weakly present in the sterilized ones (Fig. S5).

3.5. Response of inoculation in the field

The effects of the two consortia on biomass production and fixed nitrogen transferred to the plant were assessed in optimal soil water availability and water shortage under field conditions.

Total precipitation during the first growing season (2021−22) was 897 mm, 54 % higher than the long-term average for the area (Fig. 2). Most precipitation fell between October and December (609 mm, 68 % of the total). Rainfall during the second growing season (2022−23) was 672 mm, 15 % more than the long-term average, with a relatively even distribution from autumn to spring. The total rainfall for 2023–24 was 398 mm (−31 % compared to the long-term average); the annual mean temperature was 17.9 °C compared to 16.8 °C of the long-term average.

In the first growing season, inoculation with the Mix2 microbial consortium led to a significant increase in aboveground biomass compared to the uninoculated control (Fig. 3). This increase was more evident for plants subjected to water shortage (+17 %, +18 %, and + 10 % respectively in I50, I75, and I100). The Mix1 microbial consortium did not significantly affect the aboveground biomass in any irrigation treatments applied compared to the control.

Pot trial experiment showed that the inoculation with Mix2 led to 12.5 % and 12.3 % increase of shoot and root biomass compared to the control (Supplementary Fig. S6). For inoculation with Mix1, an 8 % and 3 % increase in biomass production was measured for shoots and roots, respectively (Supplementary Fig. S6).

Mix2-inoculated plants also showed increased total nitrogen accumulated in the above ground phytomass in all irrigation treatments (increments between 11.5 and 16.9 %; Fig. 4) compared to the control ones. As with the aboveground biomass, Mix1 did not lead to a significant alteration in N uptake.

For the non-inoculated control plants, the percentage of nitrogen derived from the atmosphere (%Ndfa) was, on average, 71.5 %, with no appreciable differences in the different irrigation treatments (Fig. 5). The inoculation with the Mix1 consortium did not significantly affect this parameter, whereas the Mix2 consortium led to a significant increase in the %Ndfa (+7.5 %, on average) compared to the control. No appreciable differences for the different irrigation treatments were

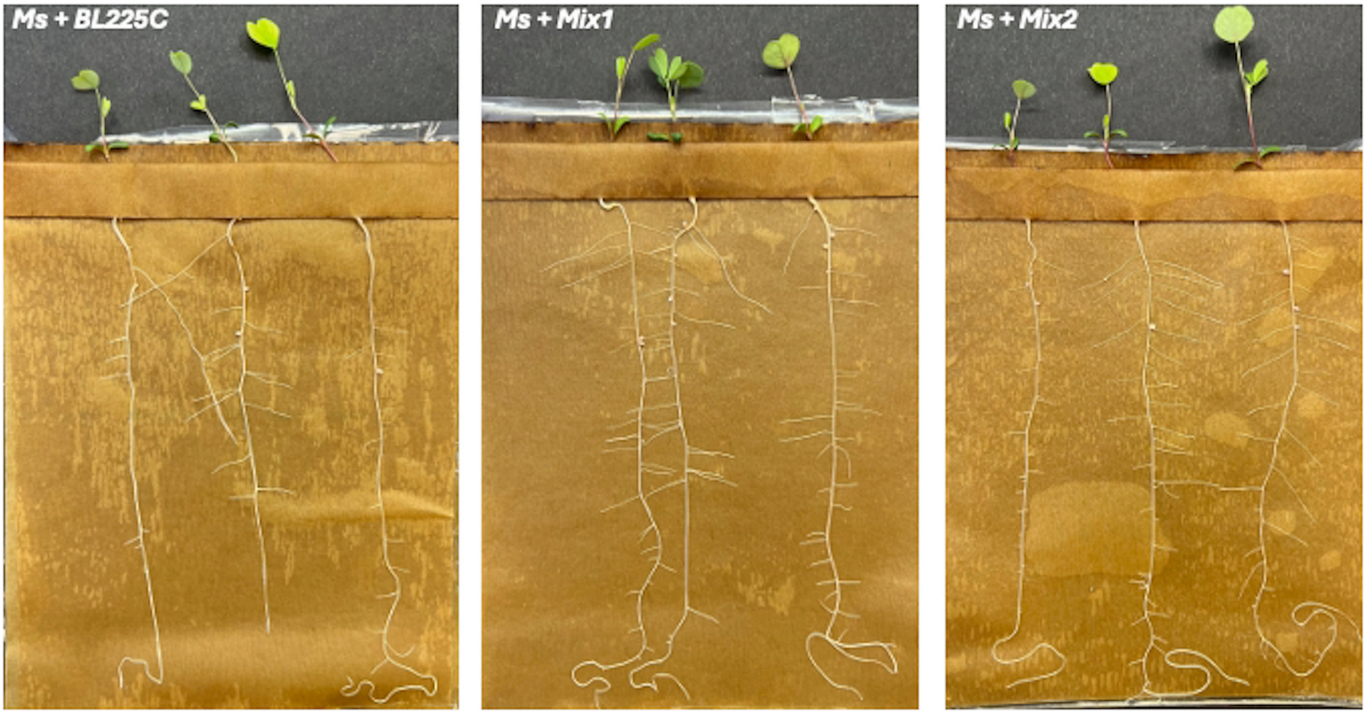

Fig. 1. Root phenotype of two-week old alfalfa plants inoculated with the *rhizobium* alone and co-inoculated with Mix1 and Mix2.

Table 4
Effect of microbial inoculation on roots phenotype and biomass production for *M. sativa* plants.

Sample	Primary root length (cm)	Number of secondary roots	Number of root nodules	FW (g)	DW (g)
Ms + BL225C	15.0 ± 3.3	12.8 ± 1.9	2.3 ± 0.5	0.066 ± 0.018	0.011 ± 0.003
Ms + Mix1	16.9 ± 1.9*	16.5 ± 3.8**	2.7 ± 0.8	0.065 ± 0.011	0.011 ± 0.002
Ms + Mix2	17.0 ± 2.4**	17.3 ± 3.9***	2.6 ± 0.8	0.077 ± 0.017*	0.014 ± 0.003**

Asterisks indicate significant differences according to a one-way ANOVA followed by post hoc Tukey's test.

* $p < 0.05$.

** $p < 0.01$.

*** $p < 0.001$.

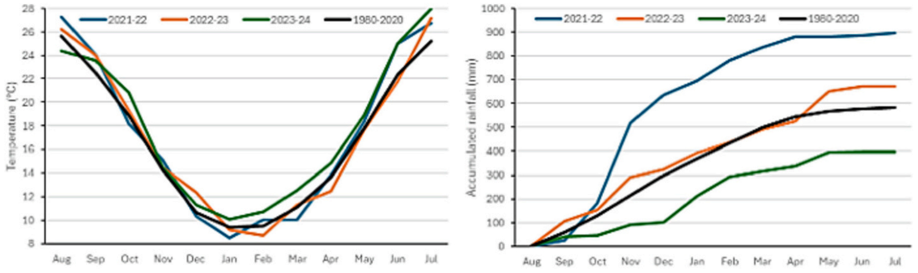

Fig. 2. Monthly mean air temperature (left) and accumulated rainfall (right) at the experimental site during the three growing seasons (2021–2022, 2022–23, and 2023–2024); 40-year (1980–2020) average monthly temperatures and accumulated rainfall are also included.

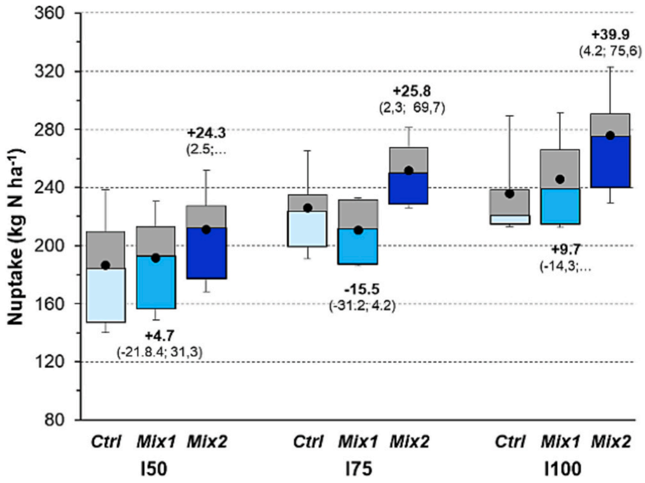

Fig. 3. Effects of microbial inoculation on above ground biomass production (t DM ha^{-1}) under different irrigation levels at the first growing season. The lower and upper segments of each rectangle show the 25th and 75th percentiles; the central line and the circle show the median and mean of the data, respectively. The vertical segments extending from the rectangles represent the range of data variation. The plots show the differences between the means of Mix1-inoculated and uninoculated plants (Ctrl) and between the means of Mix2-inoculated and Ctrl plants within the same irrigation treatment and the estimated 95 % confidence intervals of mean differences (in brackets).

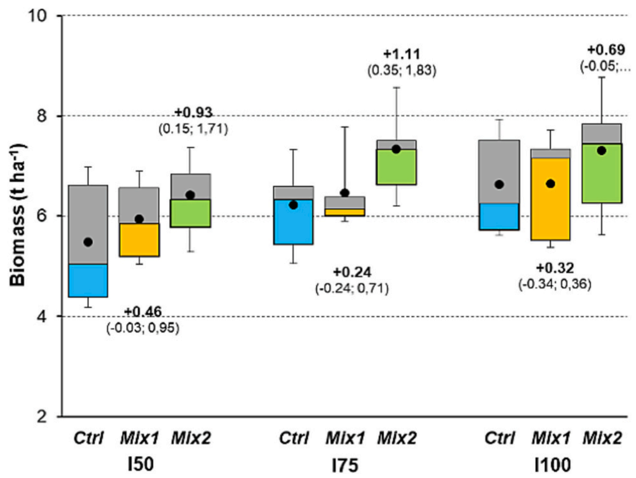

Fig. 4. Effect of microbial inoculation on alfalfa N uptake (kg N ha^{-1}) under different irrigation levels at the first growing season. The lower and upper segments of each rectangle show the 25th and 75th percentiles; the central line and the circle show the median and mean of the data, respectively. The vertical segments extending from the rectangles represent the range of variation of the data. The plots show the differences between the means of Mix1-inoculated and uninoculated plants (Ctrl) and between the means of Mix2-inoculated and Ctrl plants within the same irrigation treatment and the estimated 95 % confidence intervals of mean differences (in brackets).

observed (between +6.5 and + 8.5 %). Data on ^{15}N enrichment of biomass at the first growing season were reported in Supplementary Fig. S7.

The positive effect of Mix2-inoculation observed for the above ground biomass of alfalfa in the first growing season (up to 12.1 % compared to the control) was also observed in the two following ones but with a progressive attenuation (Fig. 6). In the second growing season, the average increase was up to 9.1 % compared to the control, while in the third one the increase was not statistically significant. The Mix1 inoculation did not determine any effect on above ground biomass production in the growing season analysed.

3.6. *Impact of consortia on the native soil microbiota*

After sequencing of rhizospheric sample soils defined in Supplementary Table S3, a satisfactory number of reads was obtained from the 16S rRNA gene library results for all rhizospheric samples (Supplementary Dataset S1). The analysis of amplicon sequence variants showed that all samples had Goods' coverage values above 0.99, nearly reaching saturation in a rarefaction analysis (Table 5). The obtained results indicated, within a small variation of the taxonomic composition, that consortia (Mix1 and Mix2) and irrigation levels did not significantly affect the microbiota ASV diversity (Table 6, Fig. 7).

Concerning the taxonomic characteristics of the microbiota, the

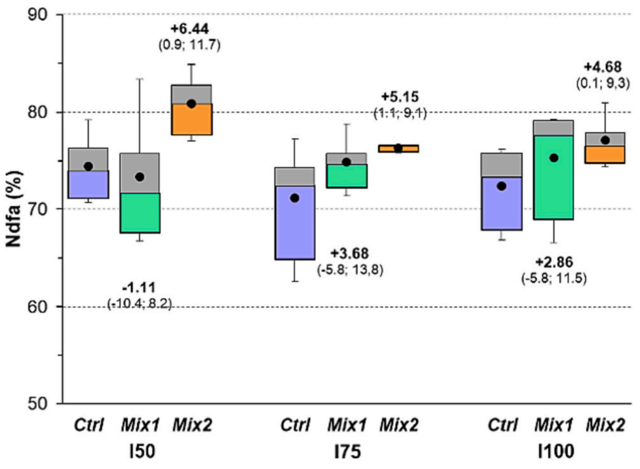

Fig. 5. Effect of microbial inoculation on the percentage of total nitrogen in the above ground biomass derived from the atmosphere (%Ndfa) under different irrigation levels at the first growing season. The lower and upper segments of each rectangle show the 25th and 75th percentiles; the central line and the circle show the median and mean of the data, respectively. The vertical segments extending from the rectangles represent the range of variation of the data. The plots show the differences between the means of Mix1-inoculated and uninoculated plants (Ctrl) and between the means of Mix2-inoculated and Ctrl plants within the same irrigation treatment and the estimated 95 % confidence intervals of mean differences (in brackets).

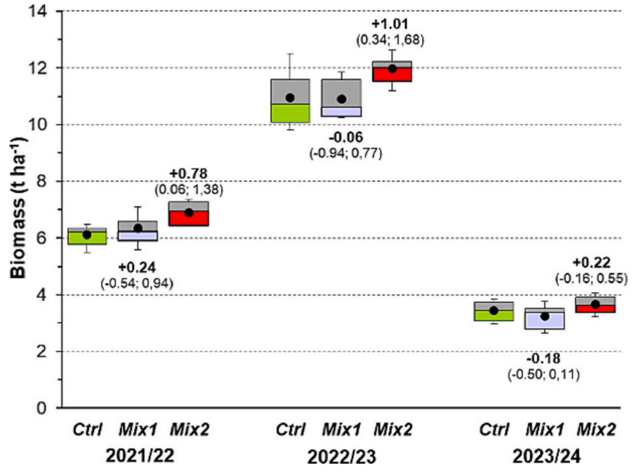

Fig. 6. Effect of microbial inoculation on above ground biomass production in three growing seasons. The t DM ha^{-1} values are the average of the irrigation levels. The lower and upper segments of each rectangle show the 25th and 75th percentiles; the central line and the circle show the median and mean of the data, respectively. The vertical segments extending from the rectangles represent the range of variation of the data. The plots show the differences between the means of Mix1-inoculated and uninoculated plants (Ctrl) and between the means of Mix2-inoculated and Ctrl plants within the same irrigation treatment and the estimated 95 % confidence intervals of mean differences (in brackets).

amplicon sequence variations (ASVs) associated with Actinobacteriota, Proteobacteriota, Firmicutes, Verrucomicrobiota, Chloroflexi, and Acidobacteriota were found to be the most abundant (alpha <0.05) (Fig. 7).

To evaluate beta diversity, the clustering of samples due to their taxonomic composition was performed through a nonMetric Multidimensional Scaling (NMDS) (Fig. 8). Although a possible pattern related to Mix1 inoculation could be hypothesized, PerMANOVA analysis showed no significant differences between control, Mix1 inoculation, and Mix2 inoculation (p value >0.05) (Table 7).

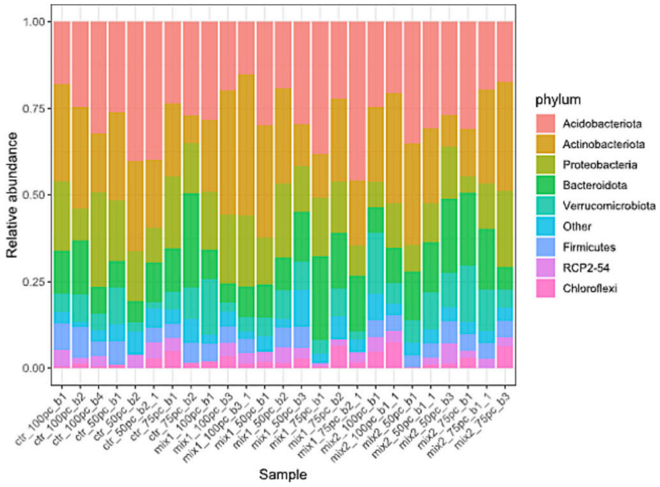

Fig. 7. Taxonomic composition of sampled microbiota. Concerning the taxonomic characteristics of the microbiota, the amplicon sequence variations (ASVs), those associated with Actinobacteriota, Proteobacteriota, Firmicutes, Verrucomicrobiota, Chloroflexi, and Acidobacteriota were found to be the most abundant (alpha <0.05) (Fig. S5). Codes are as in Table S3.

Table 5

Good's coverage of 16S rRNA gene amplicon sequencing rhizosphere samples.

Sample[a]	no.sing	no.seqs	goods
ctr_100pc_b1	0	16,920	100
ctr_100pc_b2	0	26,304	100
ctr_100pc_b4	0	15,252	100
ctr_50pc_b1	0	19,543	100
ctr_50pc_b2_1	0	10,092	100
ctr_50pc_b2	0	15,160	100
ctr_75pc_b1	0	34,197	100
ctr_75pc_b2	0	15,544	100
mix1_100pc_b1	0	21,106	100
mix1_100pc_b3_1	0	18,470	100
mix1_100pc_b3	0	18,565	100
mix1_50pc_b1	0	16,238	100
mix1_50pc_b2	0	23,138	100
mix1_50pc_b3	0	19,032	100
mix1_75pc_b1	0	24,173	100
mix1_75pc_b2_1	0	7349	100
mix1_75pc_b2	0	23,127	100
mix2_100pc_b1_1	0	16,075	100
mix2_100pc_b1	0	23,294	100
mix2_50pc_b1_1	0	14,001	100
mix2_50pc_b1	0	17,020	100
mix2_50pc_b3	0	6969	100
mix2_75pc_b1_1	0	7777	100
mix2_75pc_b1	0	12,221	100
mix2_75pc_b3	0	16,663	100

[a] Ctr, uninoculated control; 50pc, 75pc, and 100pc, water reintegration of 50 %, 75 %, and 100 % of maximum evapotranspiration.

4. Discussion

Microbial inoculants, containing beneficial microorganisms enhance plant growth, nutrient uptake, and stress resilience. They exert these effects by improving root structure, promoting symbiotic relationships, and boosting plant defences. Despite laboratory successes, field trials with bioinoculants often deal with challenges due to variable soil conditions and competition with native microbiota (Sessitsch et al., 2019). To address this issue, combination of microbial communities or microbial consortia, which mirror part of the functions carried out by plant growth-promoting native microbiota, have been developed (Mousa et al., 2024). It has been demonstrated that consortia combining rhizobia with non-rhizobial strains enhance the growth of alfalfa, a

Table 6
Wilcoxon test performed on alpha diversity analysis. Values refer to the mean and the standard deviation (SD).

Sample[a]	Richness		Shannon		Simpson	
	Mean	SD	Mean	SD	Mean	SD
ctr_100pc	43.33	9.29	3.49	0.19	0.96	0.01
ctr_50pc	37.33	8.96	3.33	0.26	0.96	0.01
ctr_75pc	54.50	24.75	3.64	0.50	0.96	0.02
mix1_100pc	41.33	3.06	3.36	0.10	0.96	0.01
mix1_50pc	40.67	7.64	3.41	0.22	0.96	0.01
mix1_75pc	48.67	19.73	3.46	0.61	0.95	0.04
mix2_100pc	45.50	7.78	3.53	0.22	0.96	0.01
mix2_50pc	46.33	30.66	3.19	0.40	0.94	0.02
mix2_75pc	31.67	15.53	3.10	0.42	0.94	0.02

[a] Ctr, uninoculated control; 50pc, 75pc, and 100pc, water reintegration of 50 %, 75 %, and 100 % of maximum evapotranspiration.

leguminous crop capable of nitrogen fixation through rhizobial symbiosis (Lu et al., 2017). In this study, two consortia containing different nitrogen-fixing and auxin (indole-3-acetic acid)-producing strains, were tested.

The genome sequencing of the isolates RCA24, RCA25, BDA62–2, and BDA137–13 provided a complete information dataset on the genetics of the consortia. For BDA137–13, evidence for TYGS results suggested it belonged to a potential novel species of *Microbacterium*. However, ANI based on GenBank analysis (https://ftp.ncbi.nlm.nih.gov/genomes/all/GCF/042/668/555/GCF_042668555.1_ASM4266855v1/) supports an assignment to *M. laevaniformans* (ANI = 93.509 on ASM3953998v1). Considering these results, we decided to indicate BDA137–13 as *M. laevaniformans* BDA137–13. The annotation of genome sequences confirmed the presence of the *nif* gene cluster in *K. sacchari* RCA25 and the *acdS* gene with its transcriptional regulator (Checcucci et al., 2017) in *M. laevaniformans* BDA137–13. The mapping on KEGG pathways of the annotation confirmed the presence of reactions for IAA production in *M. laevaniformans* BDA137–13 and *E. asburiae* RCA24. On the contrary, the annotation failed to identify the presence of responses for *K. quasipneumoniae* BDA62–2, suggesting the need to manually curate genome annotation for this strain. However, since multiple pathways leading to IAA can be present in bacteria (Duca and Glick, 2020), we cannot rule out that *K. quasipneumoniae* BDA62–2 can produce IAA via different reactions. The IAA-producing reaction from indole-3-acetaldehyde identified in *M. laevaniformans* BDA137–13 and *E. asburiae* RCA24 genome annotation was predicted to be carried out by other enzymes, an indole-3-pyruvate monooxygenase for BDA137–13 and an aldehyde dehydrogenase for RCA24.

The effects of the two consortia (Mix1 and Mix2) on alfalfa growth were analysed and compared with an inoculum of specific alfalfa symbiont *S. meliloti* in laboratory growth chamber and with native microbiome in field trials.

When the strains were used individually for the inoculation of alfalfa plants and the nitrogen fixation was measured in vitro (ARA assay), a positive effect on nitrogenase activity was observed in almost all cases, even if the selected strain was not a nitrogen-fixer, as in the case of BDA62–2 and BDA137–13. These results suggested that the two strains were able to stimulate the activity of the N-fixing rhizobium one. The enhancement in nitrogen fixation measured for Mix2-inoculated plants reflected the increase in the length of primary root, the number of secondary roots, and biomass production observed for these plants. In addition, in line with these results, the level of N acquired from the

Table 7
PerMANOVA test on beta-diversity of microbiota performed among treatments and irrigation levels.

Treatment with consortia[a]	Df	Sums of Squares	F.Model	R^2	p. value	p. adjusted
Ctr vs Mix 1	1	0.480894	0.963845	0.060377	0.91	1
Ctr vs Mix 2	1	0.500359	1.0046398	0.066955	0.437	1
Mix 1 vs Mix 2	1	0.486299	0.976035	0.061094	0.78	1
Irrigation[b]						
100pc vs 50pc	1	0.502419	1.012117	0.063209	0.359	1
100pc vs 75pc	1	0.504576	1.014765	0.067584	0.323	0.969
50pc vs 75pc	1	0.515239	1.037550	0.064695	0.092	0.276

[a] Ctr, uninoculated control.
[b] 50pc, 75pc, and 100pc, water reintegration of 50 %, 75 %, and 100 % of maximum evapotranspiration.

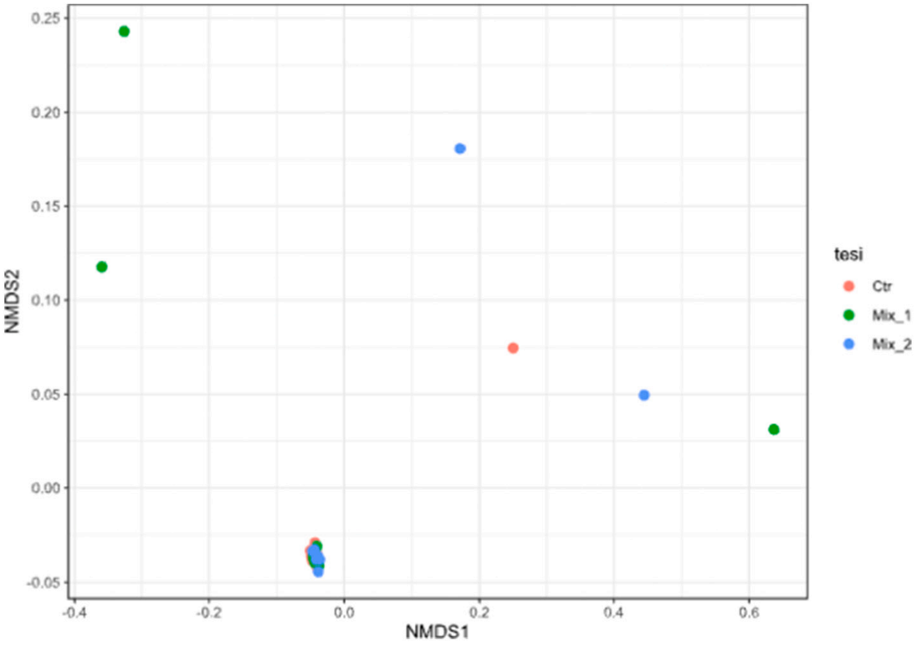

Fig. 8. nonMetric Multidimensional Scaling of alfalfa samples, divided by treatment. Ctr refers to the uninoculated control.

atmosphere measured for Mix2-inoculated plants in field trials (^{15}N assay) significantly increased. These data suggested that the nitrogen fixed by the N-fixer strains present in the consortium was transferred to alfalfa plants from the initial inoculation phase until the determination of ^{15}N enrichment in plant biomass at the first growing season. In field conditions, only Mix2 consortia led to significant benefits in phytomass production and nitrogen fixation compared to the uninoculated control. These benefits are in line with results previously obtained for other legume species and cereals (Defez et al., 2019; Andreozzi et al., 2019) and could derive from the presence in the Mix2 of 3 additional IAA-producers.

Numerous studies have demonstrated that drought stress significantly reduces nitrogen fixation in leguminous plants (Dollete et al., 2024; Hungria and Vargas, 2000; Lumactud et al., 2023; Valentine et al., 2010), often more markedly than it does on biomass accumulation (Aranjuelo et al., 2007). In this study, the benefits observed for Mix2-inoculated plants, especially on nitrogen fixation, were more evident under water-limited conditions. This finding is particularly relevant, as identifying effective strategies to sustain high nitrogen fixation levels in water-limited conditions is crucial to mitigating yield losses in leguminous plants (de Freitas et al., 2022). We hypothesized that the Mix2 consortium, by promoting an effective benefit for host plants in open fields, even under water-limited conditions, was able to overcome the highly complex conditions of the rhizosphere, one of the main obstacles related to the transfer of results from the laboratory to the field (Trivedi et al., 2021).

Our findings could result from i) the stimulatory activity of IAA-producing strains on root system development, allowing the inoculated plants to better explore the surrounding soil and more efficiently acquire the essential nutrients for BNF, even under water shortage conditions; ii) a synergistic action of the non-rhizobial soil bacteria on the strain *S. meliloti* BL225C, leading to an increase of nitrogenase activity by bacteroids inside root nodules; iii) an additive effect of non-rhizobial N-fixing strains present in Mix2: under the tested conditions, these strains may have contributed with their nitrogenase activity. The hypothesis of a synergistic effect could be plausible for Mix1 containing only the *S. meliloti* BL225C as an N-fixing strain and two other strains, both IAA-producers. However, the first hypothesis is strengthened by the results obtained in this study: Mix2-inoculated plants showed longer primary roots, a greater number of secondary roots, and increased biomass production. This hypothesis is also supported by previous studies in which it has been demonstrated that the overexpression of IAA in both rhizobia and bacterial endophytes significantly increased nitrogenase activity of the host plants (legumes and cereals) (Defez et al., 2019; Andreozzi et al., 2019). Such an inducing effect was also observed when cereal plants were co-inoculated with N-fixing and IAA-producer endophytes. In this case, the increase in nitrogen fixation was connected to higher nitrogen content and biomass production measured for the inoculated plants compared to the control ones (Andreozzi et al., 2019).

The effects observed for Mix2-inoculated plants demonstrated that although the single strains showed variable effects, their combination was effective in improving the overall microbial activity promoting the growth of the host plants even under water stress conditions. Similar results were also obtained for other crops (Saleem et al., 2021; Liu et al., 2023; Méndez-Bravo et al., 2023). Therefore, our findings confirm the potential of combining bacteria with multiple beneficial traits for enhancing plant growth. The superior activity of Mix2, which contained strains belonging to the same phylum but with different activity, allowed the consortium to perform more complex tasks than single organisms. These results highlighted that the assembly of a microbial consortium containing bacteria that act synergistically does not necessarily require that the strains belong to different species (Timofeeva et al., 2023).

The addition of nonindigenous microbes to soil resulting from the use of bioinoculants could affect the indigenous rhizosphere population in several ways: i) it can increase the population of a given taxa; ii) it can promote the colonization of already present taxa if the inoculant can increase the availability of certain resources; iii) it can reduce native microbial diversity if the inoculant becomes dominant over native taxa. However, if changes in species richness are small it is difficult to observe these effects due to the complexity of soil ecosystems. (Cornell et al., 2021; Huang et al., 2024).

Furthermore, current soil metagenomic sequencing is not deep enough to detect small differences. To assess these differences, a collection of cultured isolates should be produced considering the type of microbe to be isolated based on taxonomy, functional group, etc., and therefore the type of soil to be used for isolation.

The data obtained from the amplicon sequencing of rhizospheric sample soils showed that our endophytic inoculants did not significantly alter the native rhizosphere biodiversity and taxonomic composition. Indeed, the *Actinobacteriota*, *Proteobacteriota*, *Firmicutes*, *Verrucomicrobiota*, *Chloroflexi*, and *Acidobacteriota* were the dominant phyla in all the tested rhizospheric samples, including the uninoculated control.

In our study, we analysed the impact of inoculation on the soil microbiota by separating it from the effect of the plant alone. From an ecological perspective, this result indicates that the observed beneficial effects were likely due to the stimulatory activity of IAA-producing strains on root system development, rather than to a broad restructuring of the native microbiome. This indicates that the introduced consortia may have integrated into existing microbial networks without causing ecological disturbances. However, analyses following repeated inoculation with consortia over the years and a collection of cultured isolates should be carried out to assess the long-term impacts of our consortia.

5. Conclusions

Commercial rhizobia-based inoculants are typically propagated in a high-nutrient environment without environmental stresses. Thus, under conditions where the agroecosystem's nutrients are limiting and different stress conditions hinder plant growth, they may fail to compete with resident communities and effectively promote plant growth.

The presented study demonstrates that the selected microbial consortia (Mix2), containing rhizobial and non-rhizobial N-fixing and IAA-producing strains, showed superior activity, allowing the consortium to be more efficient than single strains in N-uptake enhancement and drought stress protection. These effects were more evident in the first growing season, in which increased aboveground biomass of inoculated alfalfa plants was observed. We demonstrated that our consortia did not significantly alter the composition of the rhizosphere microbial community structure under field conditions.

We hypothesized that the beneficial effects observed were due to the stimulatory activity exerted by the IAA-producing strains on the development of root systems. Inoculated plants with more developed root systems were able to better explore the surrounding soil, acquiring more efficiently both the essential nutrients for growth and the water resources present in the soil. Moreover, the selected consortium integrated into the native microbial species without altering the soil ecology.

The results presented in this study are promising and suggest that microbial consortia, containing strains with different activities, offer greater benefits than single strains. They can reduce problems related to synthetic fertilizers and abiotic stresses, such as drought in agricultural plants, in an environmentally responsible manner.

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CRediT authorship contribution statement

Lisa Cangioli: Data curation, Methodology, Writing – review & editing. **Camilla Fagorzi:** Data curation, Methodology. **Francesca Vaccaro:** Methodology, Data curation. **Stefano Varriale:** Methodology,

Data curation. **Maria Laura Amenta:** Data curation, Methodology. **Alessio Mengoni:** Writing – review & editing, Conceptualization, Supervision, Funding acquisition, Project administration. **Rosolino Ingraffia:** Methodology, Data curation. **Antonella Lo Porto:** Methodology, Data curation. **Gaetano Amato:** Writing – review & editing. **Dario Giambalvo:** Writing – review & editing. **Jean Rodrigue Sangaré:** Methodology. **Roberto Defez:** Writing – review & editing. **Carmen Bianco:** Supervision, Funding acquisition, Writing – review & editing, Project administration, Conceptualization, Writing – original draft.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Data availability

For the analysis of rhizosphere microbiota, sequence reads are deposited on SRA database and can be accessed through the Bioproject PRJNA1163085.

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