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Salt Stress Highlights the Relevance of Genotype × Genotype Interaction in the Nitrogen-Fixing Symbiosis between *Sinorhizobium meliloti* and Alfalfa

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Abstract: Sustainable-forage production is globally increasing, especially in marginal areas where the edaphic conditions for plant growth are not optimal. Soil salinization influences the symbiotic interaction between alfalfa and rhizobia. The efficiency of different symbiotic pairs (*Sinorhizobium meliloti—Medicago sativa*) was evaluated in relation to NaCl application (100 mM) on two different alfalfa cultivars (Marina and Etrusca) and 21 *S. meliloti* strains isolated in Algeria. At 100 mM NaCl, it was observed that there was a higher variability of plant dry weight compared to the control. The strains able to improve plant growth at 100 mM NaCl were different and specific for each alfalfa cultivar, highlighting that (symbiont) G × (host) G interaction is magnified under stressed (saline) conditions (E). Three strains were then identified as candidate inoculants for *M. sativa* cv Marina and used for an in-field experiment with induced stress (no irrigation), together with *S. meliloti* GR4 (a highly competitive strain). In-field experiments, showed a high variability, and a significant difference of plant biomass was observed only for those inoculated with *S. meliloti* GR4. Obtained results suggest that multiple traits should be considered for inoculant-strain selection, and for an efficient translation from lab to field, it requires extensive comprehension of the mechanisms driving G × G × E interaction.

Keywords: rhizobia–legume symbiosis; G × G interactions; sustainable economy

1. Introduction

The demand for fodder is increasing worldwide due to the growing population and the rising demand for animal products [1]. However, in arid, semi-arid, and saline areas, the cultivation of forage crops is a challenge due to water scarcity and high soil salinity [2]. Climate change exacerbates these problems; by decreasing precipitation and increasing

temperatures, the likelihood of extreme weather events increases, which in turn leads to a rapid decline in crop productivity [3]. Ongoing anthropic activities are also contributing to the decrease of soil's water availability, and soil's nutritional value. In particular, the combination of climate change and intensive land use exerts a strong impact on soil fertility and leads to soil erosion and degradation [4,5]. It is estimated that almost 30% of the world's croplands are at risk of land degradation with soil salinity erasing arable lands at a rate of 0.3–1.5 million ha every year [6]. In these areas, farmers are often forced to rely on low-quality forage, resulting in lower livestock productivity and health. Thus, there is a need for improved forage-crop-production practices to mitigate the effects of climate change and ensure the sustainability of livestock production [7].

The use of drought-tolerant plant varieties and their combination with effective bioinoculants are promising strategies to meet the challenges of climate change with sustainable solutions. They represent an emerging technology that involves the combined use of drought-tolerant plant genotypes and beneficial microorganisms to promote plant growth and improve soil health [8]. Such microorganisms (plant-growth-promoting bacteria, PGPB), can colonize plant roots, promote plant growth by enhancing nutrient uptake, improving soil structure, and induce systemic resistance to pathogens. Among PGPBs, symbiotic rhizobia are known for their ability to form nitrogen-fixing symbioses with legumes [9], with them being able to overcome many abiotic stresses that leguminous crops may face in drought areas [10]. In addition, rhizobia can improve soil structure and water-holding capacity, reduce water loss through evaporation, and improve water-use efficiency [8], being thus capable of alleviating salt-stress conditions.

Alfalfa (*Medicago sativa* L.) is a perennial leguminous forage crop with a high biomass yield, resistance to harvest, and high protein content [11]. Its symbiotic association with the nitrogen-fixing *Sinorhizobium melitoti* helps nitrogen incorporation to agricultural systems, reducing the application of synthetic nitrogen fertilizers [12]. Alfalfa is extensively cultivated worldwide, especially in the arid and semi-arid areas, for pasture, hay, and silage making [13]. It is regarded as moderately tolerant to salinity. However, its growth and productivity are greatly reduced by salt stress [14]. *S. meliloti* and its symbiotic interaction with alfalfa (*M. sativa*) are of great agricultural interest and represent a well-established model for the study of symbiosis and nitrogen fixation [15]. This relationship is not exclusive, and different *S. meliloti* strains may symbiotically colonize the same plant cultivar, but produce different, often suboptimal, crop performances. The selection of elite inoculant strains for the optimization of symbiotic-efficiency optimization is, therefore, crucial for sustainable-production development [7]. The effectiveness of the symbiosis between rhizobia and the plant is influenced by a variety of factors, including the genotype of the plant, the specific strain of bacterial symbiont, and the surrounding environmental conditions [16]. Indeed, there is a growing body of research focused on the effect that differential partnerships of symbiotic rhizobia and host plants may have on the overall plant phenotypes. Such symbiont genotype x host genotype (G × G) interactions have been observed in *Medicago* species (as alfalfa), highlighting the great influence they have on the success of the crop [17,18]. Indeed, gene expression in alfalfa may change in response to the infection with different rhizobial strains, possibly reflecting differences in symbiotic performance [19]. An additional level of complexity is provided by the environmental factors (E), which can influence both organisms involved in the symbiotic process. Therefore, all these factors (G × G and G × E) may contribute to the overall efficiency of the interaction [17].

In this study, our primary objective was the identification of elite symbiotic rhizobia with the aim of enhancing alfalfa productivity, particularly in conditions of environmental stress. Initial investigations involved the assessment of genotype-by-genotype (G × G) interactions within the *S. meliloti-M. sativa* symbiotic system. Our experimental design specifically addressed the hypothesis that the influence of G × G interactions varies in response to salinity levels: *S. meliloti* (G) × *M. sativa* (G), and the surrounding environment (E). To achieve this, a unique panel of strains, sourced from Algerian soils, was employed with the intent of pinpointing the most efficacious strains under saline conditions for subse-

quent bioinoculant formulation. Following the identification of optimal strain and cultivar combinations, our study transitioned from laboratory assessments to field applications. We conducted experiments under conditions of water stress, following the selection of the best carrier for the inoculants, thus bridging the gap between controlled environments and practical field applications.

2. Materials and Methods

2.1. Isolation of Alfalfa-Root-Nodule Rhizobia

Rhizobial strains were isolated from root nodules of the alfalfa plants *Medicago sativa* grown in four sites differing for their geographic location and salt content (Table 1). Salt content of each site was determined using a conductivity meter. Plants were grown for 21 days and then nodules were excised from each plant. Surfaces were sterilized with sodium hypochlorite solution for 1 min, and then were thoroughly rinsed with sterile distilled water, and then crushed in NaCl 0.8%. Crushed nodule suspensions were serial diluted, plated on Yeast Mannitol Agar YMA [20] and incubated at 28 °C for 3 days. Colonies obtained were selected and streaked 5 times consecutively to verify the absence of contaminants. Axenic strain cultures were stored in 25% (*v*/*v*) glycerol at −80 °C.

Table 1. Geographic information and electrical conductivity of four sites where *M. sativa* plants were collected for rhizobia isolation.

Region Name	Location	Electrical Conductivity (dS/m)	Geographic Coordinates
Fleuris	Municipality of Ben Freha (Wilaya of Oran)	2	35°41′39.968″ N, 0°24′47.605″ E
Es-Senia	Wilaya of Oran	1.53	35°38′11.675″ N, 0°37′56.653″ E
El Malah	Wilaya of Ain Temouchent	2.5	35°23′23.513″ N, 1°5′51.691″ E
Oued Sebbah	Wilaya of Ain Temouchent	3	35°22′15.229″ N, 0°48′24.655″ E

2.2. 16S rRNA Gene Sequence Amplification and RAPD Analysis

Bacterial cell lysis was performed using a single colony for each isolate and resuspending it in 50 µL of deionized sterile water. The cellular suspension was then kept at 100 °C (10 min), iced (2 min), and then centrifuged (10000× *g*; 5 min). For the amplification reaction, we used the following primers: 27F 5′-(AGAGTTTGATCMTGGCTCAG)-3 and 1387R 5′-(GGGCGGWGTGTACAAGGC)-3′ [21]. The reaction mixture (final volume 20 µL) was composed of 1 × reaction buffer (Thermo Scientific, Waltham, MA, USA), 200 µM of each dNTP, 0.1 mg/mL of BSA (Thermo Scientific, Waltham, MA, USA), 0.1 µM of each primer, and 0.05 U of Taq DNA polymerase (Thermo Scientific, Waltham, MA, USA); 2 µL of lysate was used as the template. Thermal-cycling conditions were 94 °C for 5 min, followed by 30 cycles at 95 °C (30 s), 55 °C (30 s), and 72 °C (90 s), and 72 °C (5 min). The Genejet PCR purification kit (Thermo Scientific, Waltham, MA, USA) was used for amplicons purification. Purified amplicons were then sequenced (Applied Biosystems 3730XL DNA Analyzer). Sequence data of the 16S rRNA gene amplicons are available at GenBank database with the following accession numbers: OQ621319–OQ621339 (Table S1). Random amplification of polymorphic DNA (RAPD) was performed using two different primers: P7 5′-(CCAAGCTGCC)-3′ [22] or AP5 5′-(TCCCGCTGCG)-3′ [23]. The reaction mixture was the same as that reported for the 16S rRNA gene amplification, except for the primer (1 µM). Thermal cycling conditions were 95 °C (5 min), followed by 40 cycles at 95 °C (1 min), 35 °C (1 min), and 72 °C (1 min); the final extension was carried out at 72 °C for 7 min. A 2% agarose gel was used to separate the amplicons; staining was performed with Midori Green Advance (Nippongenetics); and fragments size was estimated through comparison with the marker (GeneRuler 1kb DNA ladder, Thermo Scientific, Waltham, MA, USA). RAPD fingerprints obtained with both primer AP5 and P7 were recorded in the binary form. Cluster analysis was performed joining the RAPD matrices of both primers

(P7 and AP5) and using the Jaccard similarity coefficient (J) [24] and the algorithm UPGMA (unweighted pair-group method, with arithmetic mean) with the PAST v3.25 software [25].

2.3. Growth Kinetics of Rhizobial Strains under Saline Conditions

Sinorhizobium meliloti strains were grown on a tryptone yeast (TY) agar medium and incubated at 28 °C for 48 h; next, colonies were picked up with a sterile cotton swab and suspended in 15 mL of TY liquid medium. Cell density was adjusted to OD_{600} = 0.1. To carry out the experiment, 125 µL of 2 × NaCl solutions, for the different final concentrations (0 and 100 mM), were added to the wells of 96-well plates. Then, each well of a 96-well microplate was added with 5 µL of 50 × Dye Mix A (Biolog, Hayward, CA, USA) and 125 µL of 2X bacterial suspension in TY liquid medium (OD_{600} = 0.1) (5 g l^{-1} tryptone, 3 g l^{-1} yeast extract, and 0.4 g l^{-1} CaCl2). Plates were incubated at 30 °C in an OmniLog Reader (Biolog) for 96 h. Readings were recorded for 96 h every 15 min, and data were analyzed using OmniLog-PM software (Biolog, Hayward, CA, USA). The metabolic activities of strains were recorded as Arbitrary OmniLog Units (AOU), which represent the area under the kinetic curves. Each condition was performed in triplicate. For each replicate, the AOU normalized value was calculated based on the ratio of the AOU detected for each NaCl concentration and the AOU value at the concentration of 0 mM. Data were expressed as the average of normalized values for each condition.

2.4. Germination of M. sativa Cultivars on NaCl Gradient

Twenty-five seeds of each cultivar were placed in 100-mm sterile plastic Petri dishes to test the germination under five concentrations of NaCl: 0%, 0.5%, 1.0%, 1.5%, and 2.0% (wt/v) in distillate water. These concentrations are equivalent to NaCl solutions of 0 mM, 85.6 mM, 171.1 mM, 256.7 mM, and 342.2 mM, respectively [26]. Two pieces of Whatman #1 filter paper were placed at the bottom of each plate, and 4.5 mL of the appropriate NaCl solution was added at the initiation of the test [27]. Then, the seeds were placed on top of the filter paper and each plate was sprayed with a solution of Tiosol 80W fungicide (2 g/L) and sealed with parafilm to prevent moisture loss. The plates were placed in a dark growth chamber at 25 °C, and, after 7 days, the number of germinated and non-germinated seeds were counted. Five replicates for each NaCl concentration per cultivar were performed [27].

2.5. Growth of M. sativa Cultivars on NaCl Gradient

Plants were tested at the concentrations of 0, 100, 200 and 300 mM of NaCl. Seeds were surface sterilized (2.5% NaOCl; 5 min) and then washed twenty times with sterile distilled water. Seeds were then placed in Petri dishes for 4 days at 25 °C in the dark to allow germination. The obtained seedlings were then moved to plastic pots (500 mL volume) and allowed to grow in a sterilized mix of sand and vermiculite (ratio 2:3) and supplied with 120 mL of Fahraeus medium prepared with the addition of 5 mM KNO_3 and 1 mM NH_4NO_3 (Fahraeus G., 1957). NaCl stock solution was added to the Fahraeus medium for a final concentration of 100, 200, and 300 mM, respectively. The concentration of 100 mM NaCl is equivalent to about 9.8 dS/m [28], which could be considered an extreme saline soil according to Durand [29]. Whereas, 200 mM and 300 mM NaCl concentration are equivalent to about 20.4 dS/m and 30,6 dS/m, respectively. Eight plants for each condition were grown in a growth chamber maintained at 25 °C with a 16 h photoperiod. After 5 weeks, the epicotile length (expressed in centimeters), number of nodules, and dry weight (expressed in grams) were measured. Mortality was calculated as the ratio between the number of dead plants and total plants expressed as percentage.

2.6. S. meliloti—M. sativa Nodulation assay in Saline Conditions

Sterilized seeds were let germinate for 3 days in the dark and then 3 days in a growth chamber (25 °C with a 16 h photoperiod). Seedlings were transferred into plastic pots (30 mm × 117 mm, diameter × length) containing a sterilized mixture of sand and vermiculite (ratio 2:3) and supplied with 20 mL of sterilized Fahraeus N-free solution [30] or with

Fahraeus N-free solution with NaCl (100 mM). *S. meliloti* strains were grown to the late exponential phase (OD_{600} = 0.6 to 0.8) and washed twice in Fahraeus N-free solution and then adjusted to an OD_{600} = 0.1 in Fahraeus N-free solution. Five-day-old seedlings were inoculated with 1 mL of rhizobial cell suspension (10^8 cfu/mL) and grown in a growth chamber (25 °C, 16 h photoperiod).

In non-inoculated seedlings (negative control), 1 mL of Fahraeus N-free solution was added at 0 and 100 mM. From six to nine replicates for each strain and each condition were tested for both *Medicago* cultivars, and after 5 weeks the same parameters described in Section 2.6 were recorded.

2.7. Determination of Strains Survival on Carriers

Rhizobial cells' survival was tested on one strain, as representative of the collection (strain SM15(1)). Three different carriers were evaluated: perlite; compost obtained with vegetable data blend and made from untreated lumber; and Biochar obtained from pyrolysis of pruning of urban trees in a syngas plant at temperatures between 700 and 800 °C. All the carrier materials were finely ground, then passed through a 104 µm sieve. Triplicate samples of perlite and Biochar, and triplicate samples of compost were sterilized within a glass bottle at 120 °C for 20 min (compost for 40 min). To ensure good growth and survival of the strain, the final moisture content of the carriers was 33% for Biochar and 50% for perlite and compost, as previously reported [31]. *S. meliloti* SM15(1) was grown at 30 °C in liquid TY to the early stationary phase (OD_{600} = 1). Appropriate volume of the cellular suspension was pelleted and re-suspended in an appropriate volume of 0.8% NaCl solution according to the moisture content. The cellular suspension was aseptically injected and mixed with the carriers. After the preparation, the inoculants with Biochar and Perlite were immediately transferred to 4–6 °C, whereas the inoculants with compost were incubated for 15 days at 28 °C, and then stored at 4–6 °C until the sampling time. The survival of the strain SM15(1) was assessed for each formulation at different time intervals (0, 30, 60, and 90 days after inoculum preparation). Viable bacteria were estimated by plating 10-fold serial dilutions on TY plates. The mean values of the viable number of rhizobia per g of inoculant were calculated at each different time point and plotted on a logarithmic scale. Due to the preparation process, the initial loads (cfu g technique) of the three types of inoculants were different. In particular, the initial load of the Biochar inoculant was significantly lower in respect to the other two carriers.

2.8. Small-Scale in-Field Experiment

M. sativa cv Marina (Continental Semences) was used in the field trial. The *S. meliloti* strains GR4, MA10(1), MO15(1), MO35(1) and MO56(1) were used as single-strain inoculants of *M. sativa* seeds. The inoculants were obtained using compost as the carrier; 2 g of inoculants were mixed with 30 g of *M. sativa* seeds previously covered with 0.7 mL Arabic gum 40% (*w*/*v*) through mixing. Seeds mixed with non-inoculated compost served as the negative control for the in-field experiment. The average number of rhizobial cells *per* seed was evaluated as follows: 50 inoculated seeds were mixed with 500 µL NaCl 0.8% and vortexed for 3 min. The obtained suspension was serially diluted 1:10 and each dilution was plated on the TY agar medium. The field experiment was located in San Casciano (coordinates 43°40′15.2″ N, 11°08′06.6″ E). The physical-chemical features of the soil type at the growing site are reported in Table S2 and were determined according to Migliorini et al. [32]. The field trial started on 20 April 2022, and ended on 8 September 2022. The meteorological conditions of the period at the experimental site were recorded at Montespertoli, Florence meteo station, and partially obtained at www.sir.toscana.it (accessed on 29 September 2023). The experimental design was a randomized complete block with four replications (7 × 3). The total experimental area was 598 m^2 (19.5 × 26 m), and the plots were 2 m by 5 m (10 m^2), with 1.5 m between the plots and 2 m between the blocks. The treatments consisted of (i) Seeds mixed with non-inoculated compost, (ii) non-inoculated seeds sowed in soil amended with chicken

manure (400 kg ha^{-1}) as the positive control, and (iii) seeds inoculated with the following *S. meliloti* strains: MA10(1), MO35(1), MO15(1), GR4, MO56(1). The sowing was performed by hand to a depth of 1.5 cm, with a planting rate of 30 kg ha^{-1}. No irrigation treatment was applied in the experiment. Weeds were removed by hand when necessary. Data collection started on 8 September 2022. Each plot was divided into two areas of 5 mq^2. Then, the total biomass was harvested from each area and directly weighted using a dynamometer to determine the wet weight (ww). From the harvested biomass of each area, two random samples of about one kilogram were selected, weighted to determine the wet weight (ww), then transferred into paper bags and dried at 60 °C for 4 days to subsequently measure the dry weight (dw). The ratio between dry weight and wet weight of each subsample (four per plot) was calculated and expressed as a percentage. Then, the average percentage of dry weight was calculated, and, for each area, this value was multiplied to the wet weight (g) to estimate the total dry weight. The wet weight and the estimated dry weight were reported in kilograms *per* hectare. For the two area of each experimental plot, the wet weight and (estimated) dry weight were considered as parameters to evaluate the symbiotic performances. The number of indigenous rhizobia present in the soil of the experimental site, and able to nodulate *M. sativa* cultivar Marina, was determined using the most-probable number (MPN) technique [33]. *M. sativa* cv Marina seeds were surface sterilized as previously described and allowed to germinate on the cover of sterile plastic Petri dishes upside down for 4 days in the dark at room temperature. Seedlings were transferred in 500 mL capacity plastic pots containing a sterilized mixture of sand and vermiculite (ratio 2:3) and supplied with 120 mL of sterilized N-free Fahraeus solution [30]. Then, 1 g of three independent soil samples obtained from three different positions in the experimental field were mixed. The obtained 3 g of mixed soil was diluted in 30 mL of sterilized NaCl 0.8% (*w*/*v*) and shaken vigorously for 3 min. The obtained soil suspension was diluted up to 10^{-8}. A total of 10 mL of each dilution (10^{-1}–10^{-8}) was used to inoculate *M. sativa* seedlings (n = 3) grown on sterilized substrates. Sterile ddH_2O alternated with N-free Fahraeus solution was used for plant watering. The number of nodulated plants was counted 45 days after inoculation and the MPN rhizobia g^{-1} was estimated according to the MPN table [33].

2.9. Statistical Analysis

All statistical analyses were performed with the R software 4.2.3 [32], and the Shapiro test was applied to all datasets to evaluate the normality of data distribution. In the germination test, an analysis of variance (ANOVA) and post hoc Tukey's test were performed among (i) all conditions tested, (ii) among salt concentrations for each cultivar, and (iii) among two cultivars for each salt concentration. For each parameter estimated in the growth experiments of *M. sativa* cultivars on NaCl gradient, the non-parametric Kruskal–Wallis was performed among all conditions tested (all cultivars—salt concentration associations) followed by the post hoc Dunn test.

Similarly, in the nodulation assay in saline conditions, a statistical analysis among the strains tested was performed using non-parametric Kruskal–Wallis for each parameter and condition tested (cultivar Marina at 0 mM NaCl, cultivar Etrusca 0 mM NaCl, cultivar Marina 100 mM NaCl, cultivar Etrusca 100 mM NaCl). For each growth parameter, pairwise comparisons were performed for each tested strain with a non-parametric Wilcoxon test between (i) 0 mM NaCl and 100 mM NaCl conditions and (ii) the cultivars used.

The effects of the rhizobial strain, cultivar, and strain × cultivar interaction on the tolerance of the plant to salt stress were assessed using PERMANOVA and PERMDISP analysis based on the decomposition of the Bray–Curtis dissimilarity. First, for each replicate, normalized values of dry weights and epicotyl length were calculated at each condition as the ratio of values obtained for each strain and the mean values of the respective parameters obtained in the non-inoculated control. Then, Bray–Curtis distances were calculated based on normalized values of growth parameters for each strain and condition tested with the function of vegdist of the vegan R package [34]. A permutational multivariate analysis

of variance (PERMANOVA) of Bray–Curtis dissimilarity metrics was executed with the adonis2 function in the vegan package (v. 2.5–4) [34]. Since PERMANOVA outputs could be influenced by non-homogeneous within-group dispersion of data [35], the analysis of multivariate homogeneity (PERMDISP) was also fulfilled to ensure that groups of samples did not differ in dispersion and that PERMANOVA results were entirely due to differences in location [36]. PERMDISP analysis was conducted using the functions betadisper and permutest implemented in the vegan package (v. 2.5–4) [34]. The number of permutations was set to 9999 in all analyses, and a p-value threshold of 0.05 was considered significant.

In the determination of *S. meliloti* strains' survival on different carriers, statistical analysis was performed at 30, 60, and 90 days with non–parametric Kruskal-Wallis and post hoc Dunn tests. Also, survival only in compost was tested for the strains MA10(1), MO15(1), MO35(1), MO56(1), and GR4. A statistical analysis was performed for three independent replicates at 0, 14, 40, 84, and 130 days with the ANOVA and Tukey's post hoc test. In the small-scale in-field experiment, the statistical analysis was performed with ANOVA for each parameter, and pairwise comparisons among strains tested were performed using the *t*-test.

3. Results

3.1. Isolation and Identification of Rhizobial Strains

A total of 21 rhizobial strains were isolated from root nodules of alfalfa plants grown in four different sites in Algeria (Fleuris, Es-Senia, El Malah, and Oued Sebbah) differing for their salt content (electrical conductivity ranging from 1.53 to 3 mmhos/cm; Table 1). The 16S rRNA gene sequence confirmed that all the isolates belonged to the *S. meliloti* species (Table S2). Bacterial-strain typing was performed using RAPD analyses. According to the RAPD results, all 21 isolates showed different profiles indicating that they were all different *S. meliloti* strains (Figure S1).

All strains were assayed for growth kinetics at 0 mM and 100 mM NaCl, with NaCl previously added to the tryptone yeast (TY) agar medium. The metabolic activities of strains expressed as normalized values of Arbitrary OmniLog Units (AOU) (i.e., AOU at 100 mM NaCl/the average value of AOU at 0 mM of the related strain) suggested that no difference in growth between the two concentrations was observed for all strains (Table S3). Indeed, all the strains tested thrived well at a concentration of 100 mM NaCl, with an insignificant reduction of the metabolic activity ranging from 10% (0.900 metabolic activity of MO35(1)) to 2% (0.980 metabolic activity of MA13(2)) (Table S3).

3.2. Salt Tolerance of Medicago sativa Cultivars Etrusca and Marina under Increasing Levels of NaCl Concentration

For the selection of the appropriate saline concentration for the following nodulation test, the effect of saline stress on the germination and growth of two *M. sativa* cultivars (Etrusca and Marina) under controlled conditions was evaluated (Figures S2 and S3). In general, both the cultivars Etrusca and Marina tolerate well salt stress in the germination stage. However, the mean percentage of germination was stable until the concentration of 171.1 mM NaCl for both cultivars, which rapidly decreased from a concentration higher than 256.7 mM NaCl. However, no significant differences in the ability to germinate were observed between *M. sativa* cultivars at each NaCl tested concentration (Figure S2, Table S4). Concerning the ability to tolerate NaCl, for each parameter (dry weight, root and stem lengths, plant mortality) statistical differences were evaluated among all the conditions tested of the two cultivars (all cultivars—salt concentration associations) with nonparametric Kruskal–Wallis and post hoc Dunn tests (Figure S3). Both cultivars showed no differences with controls at 100 mM NaCl for the four parameters considered (Figure S3). However, Marina displayed a higher tolerance to increasing NaCl concentrations up to 200 mM and 300 mM NaCl compared to Etrusca in all three parameters. Moreover, the cultivar Marina showed reduced mortality with respect to Etrusca at 100 mM NaCl, equal to

50% and 62.5%, respectively (Figure S3C, Table S5). Moreover, the cultivar Marina reached a mortality equal to 62.5% at 200 mM NaCl (Figure S3C, Table S5).

3.3. Effect of Sodium Chloride and Symbiosis on M. sativa (Cultivars Etrusca and Marina) Growth

The 21 *S. meliloti* Algerian strains were used for the nodulation test with *M. sativa* in the presence of NaCl (Figure S4). We selected the concentration of 100 mM NaCl, which was well tolerated by both Etrusca and Marina cultivars (Figure S3) and did not induce growth kinetics change in the rhizobial strains (Table S3). Differences in dry weight, epicotyl length and the number of nodules were observed among *S. meliloti* strains tested at 0 and 100 mM NaCl and for both cultivars tested (Kruskal–Wallis test, p-value < 0.05; Table 2).

Table 2. Statistical analysis among *S. meliloti* strains for values of dry weight, epicotyl length and number of nodules of the *M. sativa* cultivars Marina and Etrusca at 0 mM and 100 mM NaCl. p-values of dataset of 0 mM—Marina, 100 mM—Marina, 0 mM—Etrusca, 100 mM—Etrusca are reported.

	Marina		**Etrusca**	
	0 mM NaCl	**100 mM NaCl**	**0 mM NaCl**	**100 mM NaCl**
Dry weight	$<2 \times 10^{-16}$	$<2 \times 10^{-16}$	$<2 \times 10^{-16}$	$<2 \times 10^{-16}$
Epicotyl length	0.00025	7.1×10^{-5}	9.7×10^{-9}	1.5×10^{-7}
Number of nodules	4.9×10^{-8}	0.00059	5.3×10^{-10}	9.0×10^{-8}

Specifically, the plant parameters were affected differently by *S. meliloti* inoculation under 100 mM NaCl compared to the control (Figures 1 and 2, Table 2, File S1). The number of nodules were mostly unvaried between 0 and 100 mM NaCl, indicating that the presence of NaCl at this concentration did to not have a major impact on nodule formation in the condition tested (Figure S1C,D, File S1); however, we cannot exclude variations in the nodulation kinetics, but this was not observable with the current experimental setup. For the cultivar Marina, the only exceptions were the significantly lower number of nodules in plants inoculated with the MO28(1) strain at 100 mM NaCl compared to the control, and the significantly higher number of nodules in plants inoculated with the MO55(2) strain at 100 mM NaCl, with respect to the control (Figure 1C, File S1). For cultivar Etrusca, a higher number of nodules were detected at 100 mM compared to the control following the inoculation of strains MO20(2) (Figure 1D, File S1). A decrease in alfalfa-nodule numbers in relation to increased salt concentrations is often observed [37]. However, the few differences observed in our trial for most of the symbiotic couples tested could be due to the use of two alfalfa cultivars tolerant to the salt concentration tested and to *S. meliloti* strains isolated from alfalfa plants grown mostly in slightly saline soils (Table 1). The epicotyl lengths of both cultivars were unchanged between control and 100 mM NaCl following the inoculation of almost all strains (Figure 1A,B, File S1). However, significantly higher values of epicotyl length were detected in plants of the cultivar Marina inoculated with the strain MO4(2) in the control compared to 100 mM NaCl (Figure 1A, File S1), and in plants of the cultivar Etrusca inoculated with strains MO1(1) and SS19(1) (Figure 1B, File S1).

Differently, and more relevantly, plant dry weights showed more marked differences between the control and 100 mM NaCl through the inoculation with 12 and 3 strains in the Marina and Etrusca cultivar, respectively (Figure 2A,B, File S1). This evidence indicates that rhizobial symbiosis may have a synergistic effect with NaCl on plant growth; moreover, the symbiotic efficiency of the *S. meliloti–M. sativa* partnership (viz. plant growth promotion in our experimental conditions) under the presence of salt could depend on the genotype of the inoculated strain and of the host plant (Figure 2A,B, File S1). In detail, in association with the cultivar Marina, eight strains promoted increased plant growth in the presence of salt. Plant dry weights at 100 mM NaCl were significantly higher compared to the controls with the strains MA10(1), MO15(1), MO19, MO35(1), MO53(2), MO55(2), SO14(2), SS19(1) (Figure 2A, File S1). Specifically, the strains MA10(1), MO15(1), and MO35(1) showed an increment of 60%, 83.5%, and 73.5%, respectively, at 100 mM NaCl compared to the control (Figure 2A, File S1). On the contrary, the dry weights of plants were significantly decreased

under salt conditions when in symbiosis with the strains MO1(1), MO34(1), MO4(2), and MO47(2) (Figure 2A, File S1). For the cultivar Etrusca, fewer differences were observed, and the only strain that increased plants' dry-weight values in the presence of NaCl was MO19, with an increment of 19.2% (Figure 2B, File S1) compared to control. The same strain was also able to promote the Marina dry weight but to a further extent (29.7% vs. 19.2%) (Figure 2A,B, File S1).

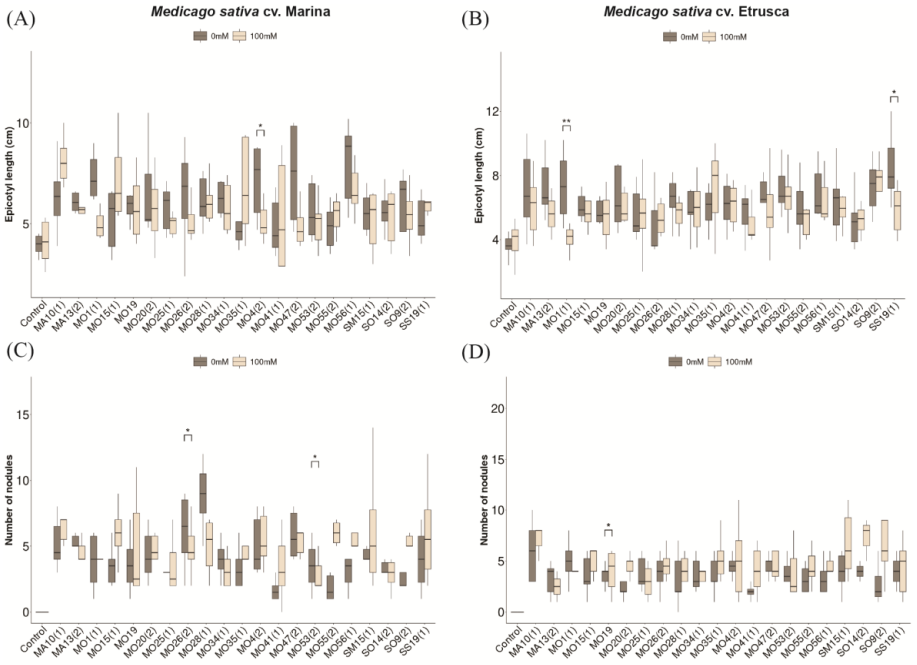

Figure 1. Epicotyl length and nodule numbers of *M. sativa* cultivar Marina and Etrusca in association with 21 Algerian *S. meliloti* strains. Boxplot of epicotyl length (cm) and number of nodules in cultivar Marina (**A**,**C**) and Etrusca and (**B**,**D**) at 0 mM NaCl (dark grey) and 100 mM NaCl (light grey). Horizontal lines of boxes represent the median, whereas the whiskers represent the maximal and minimal values. Asterisks indicates statistically significant differences between 0 and 100 mM NaCl for each tested strains (Kruskal–Wallis, post hoc test: Wilcoxon test, * for $p < 0.05$; ** for $p < 0.01$.

To evaluate the quantitative relevance of the symbiotic strain and strain × cultivar combinations to plant phenotypes under the presence of salt, PERMANOVA and PERMDISP analyses were performed. Significant differences in symbiotic phenotype depending on strain, cultivar, and strain–cultivar combinations were found in both 0 mM and 100 mM NaCl (Table 3).

Table 3. Strain-by-cultivar variation in symbiotic phenotype in presence of NaCl. Statistical differences of growth plant parameters at 0 mM and 100 mM NaCl assessed through permutational multivariate analysis of variance (PERMANOVA) and analysis of multivariate homogeneity (PERMDISP) between strains tested, cultivars used and in-strain × cultivar interaction. F-ratio (F) for PERMDISP and (R^2) for PERMANOVA are reported together with the level of significance (ns, not significant; *** $p < 0.001$).

	0 mM NaCl Dataset					**100 mM NaCl Dataset**				
	PERMANOVA			**PERMDISP**		**PERMANOVA**			**PERMDISP**	
	R^2	**F**	***p***	**F**	***p***	**R^2**	**F**	***p***	**F**	***p***
Strains	0.17920	4.0737	1×10^{-4} ***	1.4752	0.084	0.25123	5.3664	1×10^{-4} ***	1.1292	0.301
Cultivar	0.04389	19.9531	1×10^{-4} ***	0.1532	0.671	0.04802	20.5154	1×10^{-4} ***	0.0321	0.874
Strains:Cultivar	0.21824	4.9611	1×10^{-4} ***	2.0221	0.001 ***	0.22792	4.8685	1×10^{-4} ***	1.323	0.13
Residual	0.55867					0.47283				
Total	1.00000					1.00000				

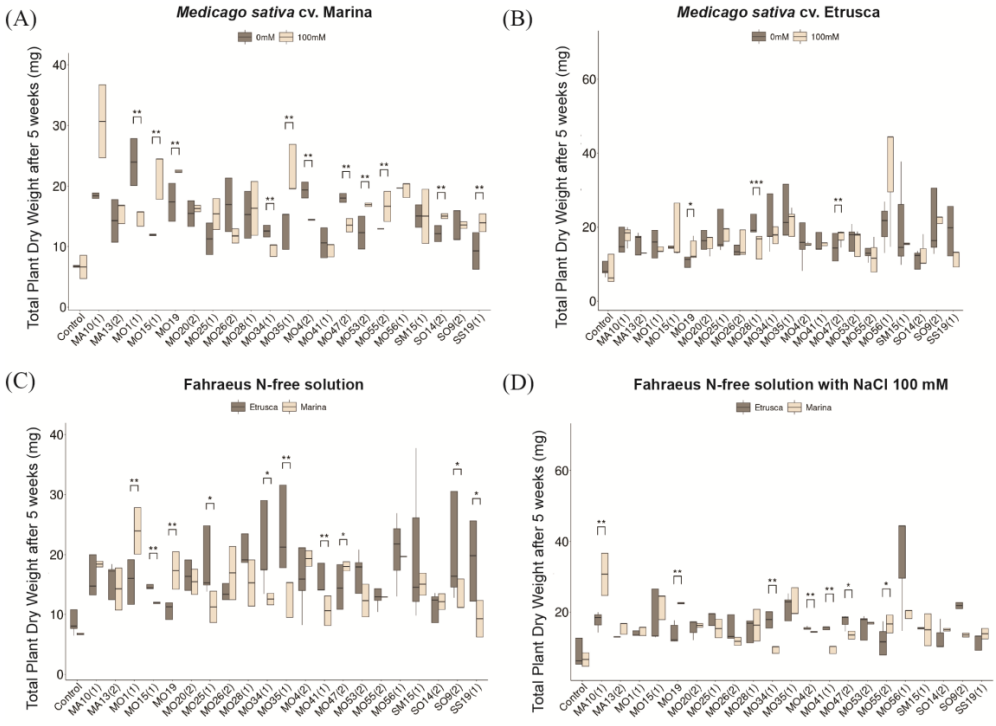

Figure 2. Effects of rhizobial symbiosis and salinity on the growth of two *Medicago sativa* cultivars. The coupled effect of rhizobial nodulation (using 21 different Algerian *S. meliloti* strains) and salinity (0 and 100 mM) in *M. sativa* cultivars (Marina and Etrusca). Dry weight (g) in cultivars (**A**) Marina and (**B**) Etrusca; dry weight (**C**) at 0 mM NaCl and (**D**) at 100 mM NaCl. Horizontal lines of boxes represent the median, whereas the whiskers represent the maximal and minimal values. Asterisks indicates (i) statistically significant differences between 0 and 100 mM NaCl for each tested strain (**A**,**B**) and (ii) statistically significant differences between cultivars for each tested strain (**C**,**D**) (Kruskal–Wallis, post hoc test: Wilcoxon test, * for $p < 0.05$; ** for $p < 0.01$; *** for $p < 0.001$).

Indeed, though plant cultivars and rhizobial strains significantly impacted plant phenotypes, confirming the importance of strain and plant genotypes over symbiotic phenotypes, the highest R^2 values were found for strain x cultivar combinations (0.218 in 0 mM NaCl and 0.251 in 100 mM NaCl) indicating that there is a high level of importance to the interaction between a given strain genotype and a specific host-plant genotype on symbiotic phenotypes, irrespective of salt treatment. However, the higher R^2 values at 100 mM NaCl compared to 0 mM NaCl, coupled with the high and significant average within-group dispersion of samples at 0 mM NaCl (F-value = 2.0221, *p*-value = 0.001; Table 3), indicate that this G × G interaction is more relevant in unmasking differences in plant phenotypes when the plants are grown under salt conditions than in unsalted controls. These findings further suggest that elite combinations of strains and plant cultivars could be developed to increase alfalfa productivity under field conditions. Indeed, examples of strains which increased plant performances under salty conditions were found (e.g., MA10(1), MO35(1) for cv. Marina), which can be further exploited as possible elite bioinoculants.

3.4. Evaluation of Carriers for the Formulation of S. meliloti Inoculants and Preliminary in-Field Trial

Rhizobial inoculants for seed coating are produced by adding rhizobial strains to specific substrates (carriers). A pre-selection of carriers for the further development of rhizobial inoculants was performed to reject those which did not have adequate compatibility with *S. meliloti*. Three different carriers (perlite, biochar, and compost) were tested for their capacities to maintain adequate survival of rhizobial strains over time (after 30, 60, and 90 days). At 30 days after, biochar showed significantly lower load than the other carriers, with a total loss of viability of about 50% (Figure 3). On the contrary, perlite and

compost were effective in maintaining a high number of viable cells with few variations compared to initial viable counts (Figure 3). At the end of the period of storage (90 days), the formulation with the compost showed the most stable values of viable cells (Figure 3).

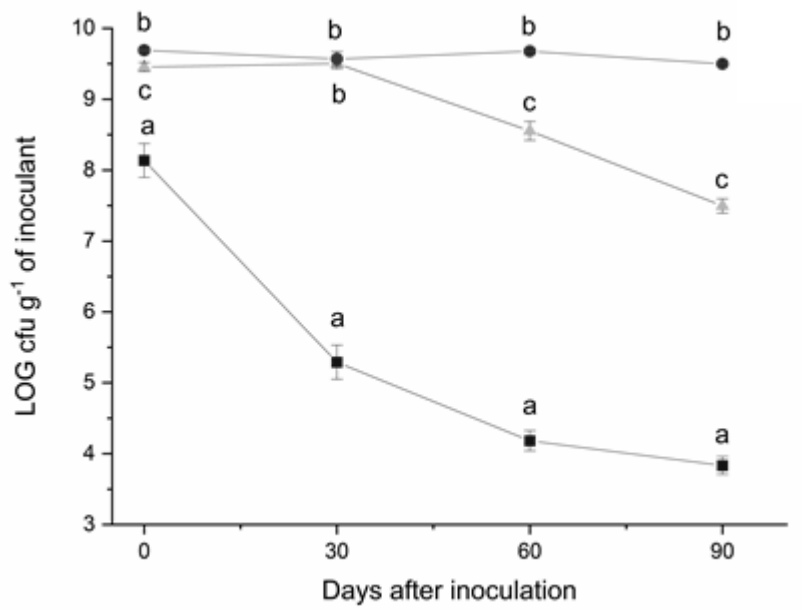

Figure 3. Survival of *S. meliloti* Algerian strain SM15(1) in different carrier materials. Compost: (black circle), perlite (grey triangle), biochar (black square). Each point represents average value of decimal logarithmic of viable cells for g^{-1} inoculant calculated on three replicates. The error bars indicate the standard errors. Different letters indicate statistically significant differences (Kruskal–Wallis, post hoc test: Dunn's multi-comparative test, $p < 0.05$).

To confirm the suitability of compost as carrier to be used in the formulation of rhizobial inoculants, the survival of the additional selected strains further used in in-field experiment was also estimated. The starting load of about 10^9 cfu g^{-1} did not significantly change up to 120 days for all the strains tested (Figure S5, Table S6). To test the effectiveness of *S. meliloti* strains to promote host-plant growth under low input of water, an in-field trial was performed. *Medicago sativa* cv. Marina was used as the host plant, due to its higher tolerance to salt in respect to cv. Etrusca (Figure S3), and the subset *S. meliloti* strains previously analyzed were selected. Specifically, the strains MA10(1), MO15(1), MO35(1) were chosen for their ability to promote plant growth at 100 mM NaCl (Figure 2A, File S1), while MO56(1) was chosen as the negative control (Figure 2A, File S1). Moreover, *S. meliloti* GR4 was included in the panel; this strain has previously shown to be highly competitive for alfalfa-root-nodule occupancy [38,39]. Rhizobial inoculants, the titres of seed coat and the main physico-chemical and microbiological features of the soil, are reported in Tables S4 and S5. During the period of the trial (from April to September), the soil was generally in a condition lacking water due to the absence of irrigation, high temperatures, and low rainfall (Figure S6). At the harvest time, wet weight (kg/ha), and dry weight (kg/ha) of alfalfa in plots receiving different inoculation treatments showed a similar pattern (Figure S7). As expected, due to the local heterogeneity of plots, a large variability among parcels was observed, with uninoculated and fertilized controls being not significantly different from the other tested inoculants. This result was possibly in relation to the presence of native rhizobial populations, which were able to effectively nodulate alfalfa. However, among the inoculant formulations tested, GR4 and MO35(1) showed values higher than the others, with GR4 producing a significant increase of alfalfa biomass (Figure S7).

4. Discussion

The cultivation of forage crops is essential to sustain livestock production, but it is often challenging in arid, semi-arid, and salt-rich areas, especially under the growing threats of climate change and anthropic activities. The use of bio-inoculants, specifically rhizobia, represents a promising strategy to improve forage-crop productivity in these challenging environments [40]. However, the development of effective bio-inoculants should take into account the many variables present in nature, including abiotic factors (e.g., soil physico-chemical parameters and climatic conditions) and the biotic components (e.g., the soil and plant microbiome, the presence of pests, etc.) [41,42]. The first factors to consider are those related to the genetics of the bio-inoculant and the host plant, which give rise to the nitrogen-fixing symbiosis. Plant genetics, i.e., the selection of plant varieties that are highly productive and/or tolerant to stressing abiotic and biotic conditions is the pivotal factor in modern agricultural production [43]. Rhizobial inoculants are usually screened with respect to their nitrogen-fixation effectiveness. However, edaphic factors (e.g., soil salinity) also play an important role in the symbiotic process. For instance, soil salinity affects multiple steps of this interaction [37], inducing signal exchange alteration, deformation of root hairs, and nitrogenase-activity reduction [44,45]. The effect of edaphic factors could be more or less pronounced depending on the genotype of the symbiotic pair tested, reflecting differences in the genomic loci that may enhance the relationship between the two symbiotic partners and/or their adaptation to the environment [46]. Salt stress affects alfalfa biomass, and N-fixation efficiency [37]. However, it has been shown that inoculation with *S. meliloti* at moderate salt concentration partially counteracts the effects of salt stress on *Medicago truncatula* gene expression [47].

The results of our study provide clear evidence for G × G interaction. Both permutational multivariate analysis of variance and analysis of multivariate homogeneity outputs showed the rhizobial strains and the strain × cultivar combinations as the main contributors to the overall plant phenotypic differences, with and without increased salinity in the medium. Interestingly, the cv. Marina, which displayed a higher NaCl tolerance than cv. Etrusca, also had higher phenotypic (plant dry weight) diversity, following the symbiosis with different strains in the presence of NaCl. This result may indicate that symbiotic rhizobia may have better impact over plant yield under salinity conditions when associated with salt-tolerant plant genotypes. Consequently, we can hypothesize the presence of an important modulation of plant metabolism using symbiotic rhizobia, which can go beyond the sole fixed-nitrogen supply [48]. The higher impact of symbiotic rhizobia in modulating plant metabolism under stress conditions with respect to unstressed ones was already shown at the proteomic level, also [49]. This results strongly suggest that the development of novel cultivars resistant to harsh environmental conditions should consider the use of symbiotic rhizobia during the various stages of screening [50]. Microbiome-guided plant breeding has recently been highlighted as the key for the development of crop cultivars more adapted to sustainable agriculture practices as for bioinoculant application [51,52]. Rhizobia–alfalfa symbiosis, given the knowledge acquired on the symbiotic process and over the whole plant microbiome, could constitute a model system to such microbiome-oriented breeding [42].

In the perspective of a lab-to-the-field translation, we also evaluated the viability of the analyzed *S. meliloti* strains with different carriers used for the production of bio-inoculants. Bio-inoculants are produced by mixing rhizobia with powdered or granular carrier materials that provide a habitat for long-term survival and growth of the bacterial strains. Peat is one of the most commonly used carriers, but being a dwindling non-renewable resource, alternative carrier materials for rhizobial inoculants have been proposed [53–56]. Although the three tested materials (perlite, biochar, compost) have been previously proposed as suitable carriers for rhizobia inoculant production [54,57–59], we observed a varying degree of compatibility with the tested *S. meliloti* strain. Only compost had no effect on rhizobial survival at 3 months of storage, while perlite and biochar caused a slow and rapid decrease of bacterial survivability, respectively. However, the actual compatibility of

the tested carriers with *S. meliloti* needs to be further investigated [59] and linked to the diverse physico-chemical properties. These can affect bacterial survival differently and may vary depending on the production processes and initial substrates. Indeed, rhizobia survival in biochar can be influenced by several factors: pore diameter of the material, moisture-absorption capacity, C/N ratio, and pH [59]. Considering that the acceptable shelf life of rhizobia in inoculant formulations should range from 5×10^7 to 1×10^9 rhizobia cells g^{-1} [60], both perlite and compost can be considered as suitable carriers for *S. melitoti*, as it has already been established for *S. fredii* or *Bradyrhizobium japonicum* [54]. However, compost showed to be the carrier with the longer shelf-life, and it was selected for the following in-field test.

The in-field trial was carried out under harsh climatic conditions, with high temperatures and lack of irrigation to mimic drought condition. No significant differences were observed between the parcels sowed with inoculated seeds (with the Algerian strains) with respect to the parcels sowed with un-inoculated seeds, as both positive (fertilized) and negative controls. The presence of nodules on plants harvested from fertilized parcels (positive control), and the lack of significant differences between the fertilized and inoculated parcels, indicate that fertilization had no influence on the nodulation processes. It has been previously investigated that rhizobia N fixation abilities were not affected by N enrichment [61–64], highlighting that legumes still gain growth benefits from symbiosis with rhizobia. Alfalfa harvested from the negative control parcels (un-inoculated seeds and unfertilized soil) yielded a similar biomass to that from the inoculated/fertilized parcels. In addition, the plants harvested from the negative control parcels were also nodulated at the end of experiment. This result suggests that soil-indigenous rhizobia may have symbiotic performances similar to the selected strains, or, more likely, they may outcompete the strains of the inoculated seeds for nodule occupancy [65]. Consistent with the latter hypothesis, the *S. meliloti* GR4, previously characterized as a good competitor for nodulation [39], was the only strain for which the corresponding inoculated seed yielded a significantly higher biomass compared to the negative control parcels. This highlighted how competitiveness should be a key feature of newly formulated inoculants to ensure the success and efficacy of rhizobial inoculants in non-native soils [39,66].

5. Conclusions

Soil salinity exerts a profound impact on agricultural productivity by inducing water stress and diminishing crop yields, presenting a formidable challenge for farmers. The symbiotic relationship between legumes and rhizobia, crucial for nitrogen fixation, is also susceptible to high salt concentrations. Within this symbiotic association, diverse rhizobial strains may nodulate the same legume species with markedly different outcomes. This variation is influenced not only by the nitrogen-fixing efficiency of the rhizobial strain but also by the intricate interplay between the partners, *S. meliloti* (G) × *M. sativa* (G), and the surrounding environment (E). Our investigation revealed that, under salt stress and with varying plant hosts (*M. sativa* Etrusca or Marina), distinct rhizobial strains exhibited disparate behaviors, resulting in divergent plant yields. Laboratory assessments facilitated the identification of robust symbiotic pairs for growth under stressed conditions. However, in-field experiments yielded limited increases in plant yield compared to control samples nodulated with indigenous rhizobia. Notably, only the GR4 strain, previously recognized as a strong competitor, demonstrated a significant enhancement in plant biomass. Given the complexity of the soil environment and its myriad of influencing factors, our findings underscore the importance of considering multiple traits in strain selection for a successful translation from laboratory to field conditions. The competition with local indigenous rhizobia emerges as a potential challenge for large-scale production of inoculated seeds, necessitating the selection of locally adapted elite strains. Furthermore, additional research is needed to unravel the mechanisms underlying genotype-by-genotype-by-environment (G × G × E) interactions in this symbiotic system.

Supplementary Materials: The following supporting information can be downloaded at https://www.mdpi.com/article/10.3390/soilsystems7040112/s1. Figure S1: The random amplified polymorphic DNA (RAPD) profile of *S. meliloti* Algerian isolates; Figure S2: Germination of *M. sativa* cultivars Etrusca and Marina under different concentrations of NaCl; Figure S3: Plant parameters of *M. sativa* cultivars Etrusca and Marina influenced by NaCl gradient; Figure S4: Experimental set-up for nodulation assays; Figure S5: Survival of *S. meliloti* Algerian strains in compost at 4–6 °C; Figure S6: Temperature and rainfall recorded during the field experiment; Figure S7: Alfalfa yield with different inoculant treatments in the field trial; Table S1. List of *S. meliloti* strains used in this work; Table S2. Main soil physico-chemical characteristics and count of indigenous nodulating rhizobia (Most Probable Number) of field experiments. Table S3. Growth profiles of the rhizobia strains at 100 mM NaCl detected with Biolog Technology; Table S4. Tuckey test post hoc outputs on percentage of germination of *M. sativa* cultivars Marina and Etrusca at different NaCl concentrations; Table S5. Mortality of *M. sativa* cultivars Marina and Etrusca in association with 21 *S. meliloti* in presence of salt; Table S6. Count of viable cells of *S. meliloti* strains on the *M. sativa* cultivar Marina seed coated with compost; File S1: Pairwise comparisons among *S. meliloti* Algerian strains tested in *M. sativa* cultivars Marina and Etrusca.

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