SCIENTIFIC REPORTS

OPEN

SUBJECT AREAS:
APPLIED MICROBIOLOGY
SYMBIOSIS

Received
5 June 2014

Accepted
18 August 2014

Published
17 September 2014

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Micromonospora from nitrogen fixing nodules of alfalfa (*Medicago sativa* L.). A new promising Plant Probiotic Bacteria.

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Biotic interactions can improve agricultural productivity without costly and environmentally challenging inputs. *Micromonospora* strains have recently been reported as natural endophytes of legume nodules but their significance for plant development and productivity has not yet been established. The aim of this study was to determine the diversity and function of *Micromonospora* isolated from *Medicago sativa* root nodules. *Micromonospora*-like strains from field alfalfa nodules were characterized by BOX-PCR fingerprinting and 16S rRNA gene sequencing. The ecological role of the interaction of the 15 selected representative *Micromonospora* strains was tested in *M. sativa*. Nodulation, plant growth and nutrition parameters were analyzed. Alfalfa nodules naturally contain abundant and highly diverse populations of *Micromonospora*, both at the intra- and at interspecific level. Selected *Micromonospora* isolates significantly increase the nodulation of alfalfa by *Ensifer meliloti* 1021 and also the efficiency of the plant for nitrogen nutrition. Moreover, they promote aerial growth, the shoot-to-root ratio, and raise the level of essential nutrients. Our results indicate that *Micromonospora* acts as a Rhizobia Helper Bacteria (RHB) agent and has probiotic effects, promoting plant growth and increasing nutrition efficiency. Its ecological role, biotechnological potential and advantages as a plant probiotic bacterium (PPB) are also discussed.

Nodules are new organs generated mainly in roots of leguminous plants, in cooperation with alpha and beta proteobacteria developed for biological nitrogen fixation. It was initially thought that only symbiotic nitrogen-fixing bacteria could exist inside healthy N_2 fixing nodules. Recent studies have shown that they are frequently populated by a broad and heterogeneous range of both gram-positive and gram-negative bacteria[1–4]. Recently, the first intranodular actinobacteria have been described[5,6], but from the first description in this environment, the number of actinomycetes found has increased and in fact even new species have been described. Examples of these new findings inside nodules are *Curtobacterium* in *Trifolium* and *Ornithopus*[7,8]; *Microbacterium* in *Acacia*, *Glycyrrhiza*, *Medicago* and *Ornithopus*[7–11]; *Micromonospora* in several legumes[12–16]; *Streptomyces* in *Sphaerophisa*[17] and others. Notably *Micromonospora*, which has been isolated from more than 20 different widely distributed plant species seem to have good potential as a plant-probiotic bacteria (PPB), although this remains to be studied in depth.

At our laboratory, strains of *Micromonospora* have been isolated from healthy plant nodules in a variety of genera of leguminous plants including *M. sativa* (alfalfa)[12,13,15,16]. Alfalfa is one of the most widely adapted agronomic crops and a cheap source of protein-rich forage with high digestibility, which is a valuable trait in economical animal husbandry. Alfalfa should be considered a key component of sustainable agricultural systems for the future because of its high yield, nutritional quality, pest resistance, and its value in soil conservation and improvement[18].

One of the major challenges for the twenty-first century will be sustainable crop production. Agricultural practices derived from the green revolution, defined by the use of pesticides, fertilizers and herbicides of chemical origin, together with the genetic improvement of plant germoplasm, produced an increase in agricultural productivity. Decades ago, the cost and risks derived of this kind of agriculture were elucidated and as a consequence[19,20] a new agricultural revolution is now starting to develop in which probiotic microorganisms have become an alternative to chemicals[21]. The possibilities for influencing plant growth-promoting potential applying microorganisms as Plant Probiotic Bacteria (PPB) agents have been largely explored[22–25]. The interest of these

microorganisms is clear, and today inoculants can be found on the market in several countries. Based on recent surveys, interest in the use of inoculants is also rising, suggesting that the market potential of bioinoculants will increase further in coming years[26].

However, it is necessary to study their ecological role and make an adequate analysis, evaluation and selection of the microbial strains used in order to obtain the desired effect and, unfortunately, the beneficial plant-microbe interaction has often been ignored in breeding strategies, even after their importance in soil ecosystems was confirmed (reviewed by Smith and Goodman[27]).

In light of the foregoing, the main goal of this study was to determine the diversity and ecological function of *Micromonospora* and analyze its plant probiotic capabilities since there is little information about it even though its biotechnological potential and also its impact in this new agricultural revolution are relevant.

Results

Bacterial isolation and morphological characterization. *Micromonospora*-like colonies were isolated from surface sterilized root nodules of naturally occurring alfalfa plants on yeast mannitol agar, along with rhizobia-like bacteria after 3-week incubation at 28°C. *Micromonospora* strains were recovered in almost all of the nodules sampled. In all, 66 strains were isolated from the sampling sites: Aldearrubia (AL) 21 strains, Babilafuente (ALFb) 11 strains, Palaciosrubios (ALFpr) 19 strians, San José (ALFr) 4 strains and Tormes riverbank (ALF) 11 strains. All 66 actinobacterial strains had the morphology described for the genus *Micromonospora*; they were Gram+, filamentous, lacked aerial mycelium, and presented orange or brown colonies that darkened after around 3 weeks due to sporulation.

Genetic diversity of the *Micromonospora*-like isolates. High-resolution BOX-PCR fingerprints were obtained for the 66 actinomycetes isolated from the nitrogen-fixing root nodules of *M. sativa* (Figure 1). The amplified fragments ranged from 0.1 to 2.2 kb. Clusters based on the similarity matrix generated with Pearson's coefficient and the UPGMA algorithm were defined at the 60% similarity level, affording 10 groups and revealing the high genetic diversity of the isolates. Figure 1 shows the diversity of the genetic profiles of the strains studied. Fifty-five strains were distributed in 10 clusters containing 2–13 strains; the remaining 11 isolates had a unique profile. No clones were found even in the strains from the same nodule. With respect to the isolation site, the strains isolated from Aldearubia (21 strains) and the 19 strains recovered from Palaciosrubios were distributed along the entire dendrogram, they have representatives in almost every cluster; the 11 strains from Babilafuente were detected in 6 groups; the 11 strains from Tormes River bank in 8 groups and the 4 strains from San José in 4 groups. Two groups contained strains from the five different sampling sites (cluster 1 and 3). Clusters 9 and 10 (2 strains each) only contain strains from Aldearrubia, the rest of the clusters contained strains from 2 or more of the locations sampled.

According to the genetic diversity (BOX-PCR fingerprinting) and geographical origin of the isolates, we selected fifteen strains for *in planta* interaction studies.

Phylogenetic analysis and functional characterization of selected *Micromonospora* strains. Nearly complete 16S rRNA gene sequences (≥1434 nt) were obtained for the fifteen selected strains. NCBI and Eztaxon nucleotide blast searches revealed that 100% of the sequenced microorganisms were identified as belonging to the genus *Micromonospora* as suggested by their morphological characteristics.

Sequence similarities between the new isolates and currently described *Micromonospora* species ranged from 97.78 to 100%. A significant number of the isolates sequenced (approx. 87%) showed > 99% sequence similarity with already described *Micromonospora* species (Table 1). The inferred phylogenetic tree based on 16S rRNA gene sequences using maximum likelihood (Figure 2) and neighbour-joining methods (Figure S1) showed that six of the isolates clustered with already described *Micromonospora* species. The tree topology generated by both maximum likelihood and neighbour joining methods was strongly supported by bootstrap values which were similar for both methods. However, nine of the strains did not group closely with any of the currently recognized species (AL2, ALFb4, ALFpr18c, ALFpr19a, ALFb1, AL16, ALF4, ALFr4 and ALFr5; Figure 2). Further taxonomic work will be required to elucidate the status of these last strains.

With the exception of strain AL2 lacking pectinase activity, all of the other *Micromonospora* strains showed the ability to degrade plant cell wall components, namely cellulose, pectin and xylan (Table 2). Even though, the cellulose activity was weak in all the strains. Other components of organic matter such as proteins (caseinase and gelatinase activities) and starch were also degraded by all the strains, being the only exception the strain ALF1, which could not degrade gelatine. Moreover, all of the tested strains showed lipase activity. They were able to degrade Tween 80. Tween 20 was strongly degraded by two strains (ALFr5 and ALFb7), weakly by nine and no hydrolytic activity was detected in four of them. Neutral and alkaline phosphatase activities were detected in all the strains but none showed acid phosphatase activity (Table 2).

Thirteen *Micromonospora* strains were able to produce IAA. AL16 and AL20 were the strains with the highest production levels (≥74.8 μg/mL) whereas the strain ALF7 showed the lowest (2.9 μg/mL), the remaining had IAA production ranging from 11.3 to 47.0 μg/L (Table 2).

The ability to grow at different pH (from 4.5 to 9) was tested. All the fifteen *Micromonospora* strains grew well at a pH range of 7 to 8. None of the strains grew at pH below 5.5 nor at pH 9. We found high variability when grown at pH 6.5 (Table 2).

Effect of *Micromonospora* on plant growth and nutrient content of alfalfa. Investigating putative plant growth-promoting effects on alfalfa of the fifteen *Micromonospora* strains alone and in co-inoculation with the model strain *E. meliloti* 1021 was addressed in this part of the study. A mesocosm experiment was conducted in a greenhouse in pots containing a sandy-clay soil (Table S1) under controlled conditions of temperature, photoperiod and humidity. At harvest, shoot and root biomass, number of nodules and shoot nutrient contents were determined (Figure S2, Tables S2 and S3). The measured plant growth and nutrient content parameters were grouped together to form a data matrix of 2,560 data points (8 parameters × 32 inoculation treatments × 10 replicates).

We first used an indirect analysis (PCA) of the data [excepting number of nodules (Nod) data] to summarize the variation across all the 320 alfalfa plants tested. Figure 3a shows the distance biplot resulting from the PCA analysis. The first PCA axis explained 70.6% of the variance in the data, while the second axis accounted for 20.3% only. We calculated the relative amount of the total variability that each of the two first axes should explain under the null model of random variation by using the broken-stick approach[28]. The values predicted for the first and second axes are 37.0% and 22.8%. Therefore, only the fraction of variability explained by the first axis surpasses the values predicted by the null model, indicating that the first axis describes non-random, interpretable variation in the data while the second does not. With the exception of Rdw (root dry weight) and S:R (shoot to root ratio), the remaining five response variables [Sdw (shoot dry weight), C, N, P and K] had high positive correlation (> 0.9) with the PC1 scores. Further examination of the PCA biplot, focusing on the disposition the centroids of the 32 dummy independent variables (inoculation treatments) projected *post hoc* into the ordination space, reveals that the first principal

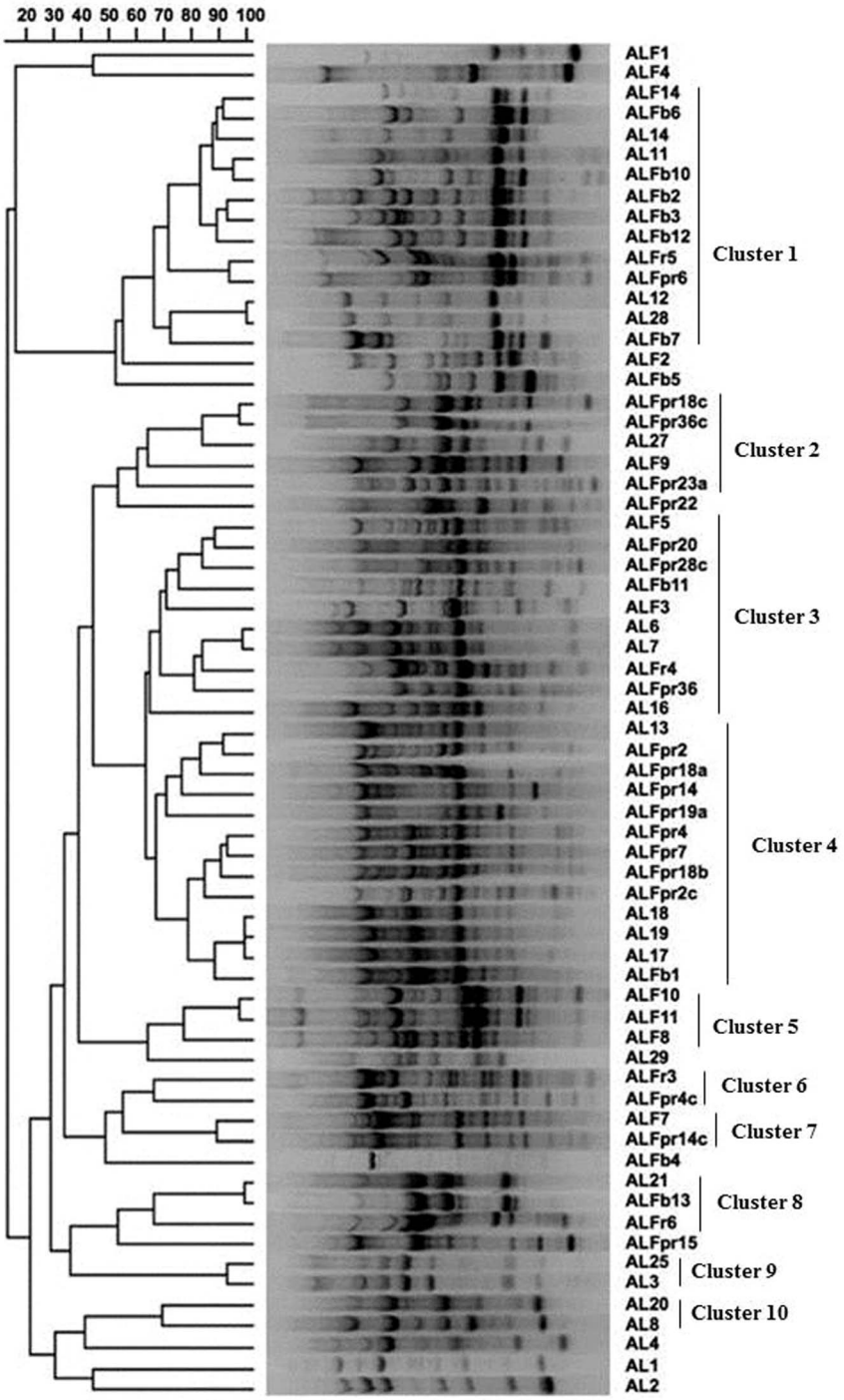

Figure 1 | Dendrogram showing genetic relatedness of 66 *Micromonospora* strains isolated from *M. sativa* determined by analysis of BOX–PCR fingerprints using the Pearson's coefficient and UPGMA cluster methods.

component is related to *E. meliloti* 1021 inoculations (with and without *E. meliloti* 1021). Plants inoculated with *E. meliloti* 1021 tended to have Sdw and shoot contents of C, N, P and K higher than the *E. meliloti* 1021-free plants. Similarly, within the cohorts of plants inoculated and non-inoculated with *E. meliloti* 1021, the inoculation with specific *Micromonospora* strains tended to produce higher or lower values of these parameters comparing to other *Micromonospora* inoculation treatments and to the controls (Figure 3a). Redundancy analyses (RDA) testing for the significance of effects of the inoculation with *E. meliloti* 1021, the inoculation with *Micromonospora* and their interaction revealed statistical significance of all the three factors (Table S4).

Given the significance of the interaction effect of *E. meliloti* 1021 and *Micromonospora* inoculations (F-ratio= 2.090, P-value = 0.002; Table S4), we performed separate RDA analyses for the cohorts of plants inoculated and non-inoculated with *E. meliloti* 1021. In the cohort of *E. meliloti* 1021-free plants, redundancy analysis (RDA) revealed that the explanatory effect of the *Micromonospora* inoculations was highly significant according to the Monte Carlo test for significance of all canonical axis (F-ratio= 9.920, P= 0.0010). Figure 3b shows the distance biplot resulting from this RDA analysis. The proportion of variability explained by all the constrained canonical axes was 50.8%, and 30.9% and 13.7% by, respectively, the first and second canonical axes, both being significant (Figure 3b). We

Table 1 | Geographical origin and 16S rRNA gene sequence analysis of strains selected for *in planta* trials

Strain	Origin	# Accession	Most similar *Micromonospora* type strain. (Accession number)	Similarity (%)	Source
AL2	Aldearrubia	KF876220	*M. chaiyaphumensis* MC5-1 (AB196710)	99.72	This work
AL4	Aldearrubia	KF876221	*M. viridifaciens* DSM 43909T (X92623)	99.52	This work
AL16	Aldearrubia	KF876222	*M. saelicesensis* Lupac 09 (AJ783993)	99.65	This work
AL20	Aldearrubia	KF876223	*M. chokoriensis* 2-19/6 (AB241454)	99.79	This work
ALF1	Tormes riverbank	KF876224	*M. humi* P0402 (GU459068)	99.51	This work
ALF4	Tormes riverbank	KF876225	*M. coxensis* 2-30-b/28 (AB241455)	99.31	This work
ALF7	Tormes riverbank	KF876233	*M. saelicesensis* Lupac 09 (AJ783993)	99.86	This work
ALFb5	Babilafuente	KF876226	*M. aurantiaca* ATCC 27029 (CP002162)	99.72	[37]
ALFb7	Babilafuente	KF876227	*M. tulbaghiae* TVU1 (EU196562)	99.93	This work
ALFb1	Babilafuente	KF876228	*M. saelicesensis* Lupac 09 (AJ783993)	99.58	This work
ALFb4	Babilafuente	KF876229	*M. echinospora* ATCC 15837 (U58532)	97.78	This work
ALFpr18c	Palaciosrubios	KF876230	*M. lupini* Lupac 14N (AJ783996)	99.31	[37]
ALFpr19a	Palaciosrubios	KF876231	*M. saelicesensis* Lupac 09 (AJ783993)	99.51	This work
ALFr5	San José	KF876232	*M. cremea* CR30 (FN658654)	98.62	This work
ALFr4	San José	KF876234	*M. saelicesensis* Lupac 09 (AJ783993)	99.51	This work

undertook pair-wise RDA comparisons in order to determine which of the *Micromonospora*-inoculated treatments produced statistically significant differences when compared with the uninoculated control treatment. Table S5 summarizes the results of this set of multivariate tests. Results indicated that only three out of the 15 *Micromonospora* strains (namely AL16, ALFb1 and ALFb7) produced non-significant differences with respect to the uninoculated control treatment (Table S5). Univariate (ANOVA) analyses on the response variables were

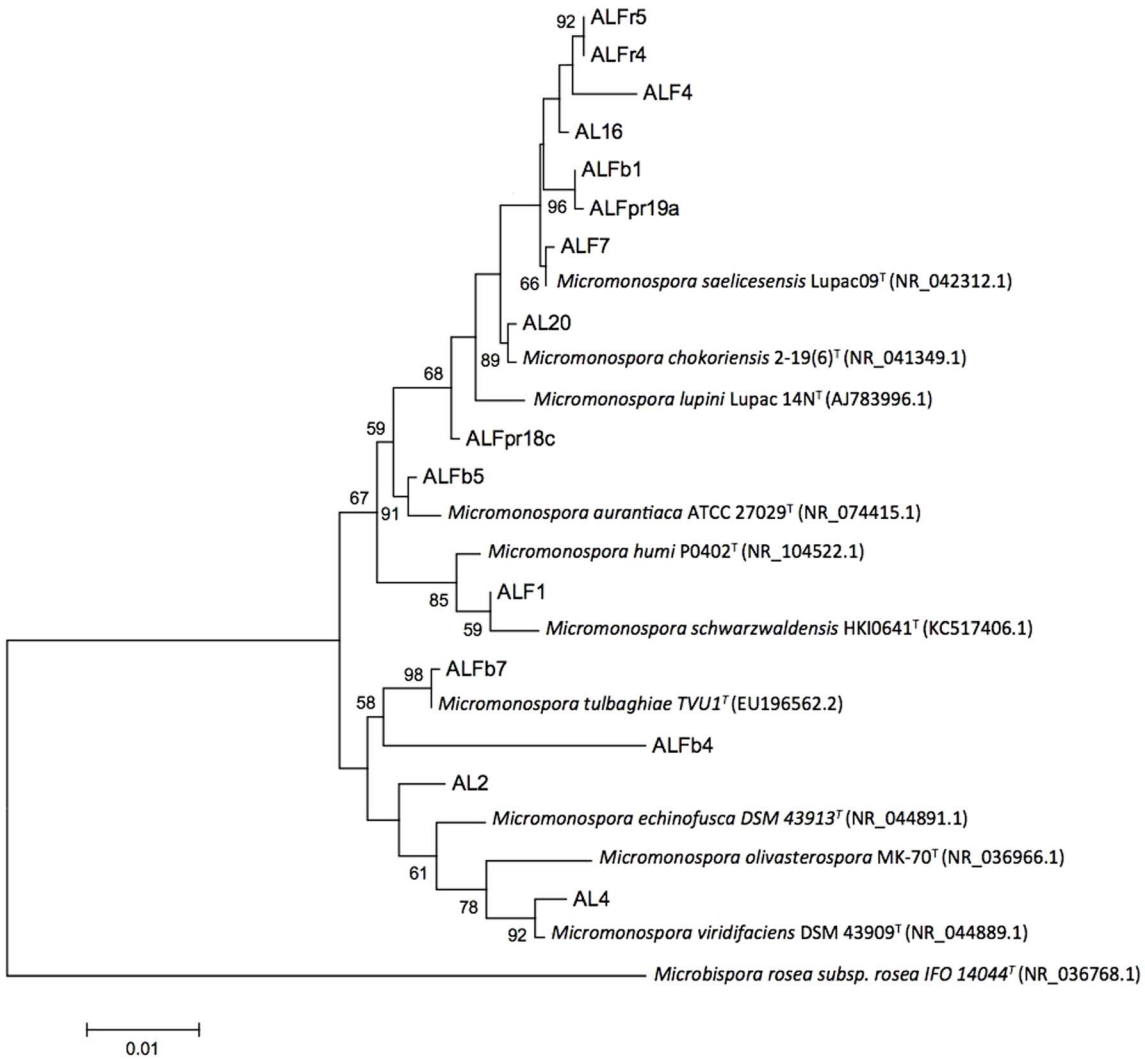

Figure 2 | Maximum likelihood phylogenetic tree based on 16S rRNA gene sequences showing the relationship between the *Micromonospora* isolates and the closest recognized *Micromonospora* species. Bar, 0.01 substitutions per nucleotide position. Bootstrap percentages (1000 replicates) above 50% are shown at nodes.

Table 2 | Ecological, PPB related enzimatic activities and indolacetic acid production in selected *Micromonospora* strains

Strains/Activity	AL2	AL4	AL16	AL20	ALFb1	ALFb4	ALFb5	AL b7	ALF1	ALF4	ALF7	ALFpr18c	ALFpr19a	ALFr4	ALFr5
Cellulase	w	w	w	w	w	w	w	w	w	w	w	w	w	w	w
Xilanase	w	w	+	+	+	+	+	+	+	+	+	+	+	+	+
Pectinase	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Caseinase	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Gelatinase	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+
Amilase	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Phosphatase (Acid)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Phosphatase (Neutral)	+	+	+	+	+	+	+	+	+	+	+	+	+	w	+
Phosphatase (Alkaline)	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Tween20	w	w	w	w	-	w	w	+	w	w	-	-	w	-	+
Tween80	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Indolacetic Acid	8.0	nd	86.4	74.8	34.4	28.4	22.9	16.7	15.0	nd	2.9	27.2	11.3	47.0	27.0
pH 6.5	w	+	+	w	-	w	+	+	+	-	-	+	-	-	-

(+) positive, (-) negative and (w) weak. Indolacetic acid production is expressed in µg/ml. In the case of phosphatases, positive activity was considered when the absorbance reading was 0.2 above that of the controls.
nd, not detected.

performed to compare differences within the control treatment and those *Micromonospora*-inoculated treatments showing significant differences in the pairwise RDA comparisons (Table 3). It was found that shoot biomass production (Sdw) of alfalfa plants inoculated with *Micromonospora* ALFb5, ALFr5 or AL4 was significantly different ($P \leq 0.01$) to that of the uninoculated control plants. The mean shoot dry weight (Sdw) of plants that were inoculated with *Micromonospora* ALFb5 or ALFr5 was, respectively, 19% and 35% greater than that of control plants, while in plants inoculated with the strain AL4 was 20% lower. However, root dry weights (Rdw) in four of the inoculation treatments were significantly lower ($P \leq 0.01$) than in the control treatment, and only the inoculation with *Micromonospora* ALFb5 produced an increase marginally significant ($P \leq 0.1$). Therefore, S:R ratios in treatments inoculated with *Micromonospora* were similar to, or higher ($P \leq 0.01$) than that in the control treatment (Table 3). Regarding to the shoot nutrient contents, significant decreases respect to the control were only observed for carbon in plants inoculated with *Micromonospora* AL4 and ALFr4. Plants inoculated with *Micromonospora* AL20, ALFb5, ALFpr19a or ALFr5 had higher ($P \leq 0.1$) shoot contents of N, P and K than the control plants. Besides, K shoot contents were also higher in the treatments inoculated with *Micromonospora* AL2, ALF4 or ALFb4 than in the control treatment ($P \leq 0.05$). It is noteworthy that inoculation with any of these twelve *Micromonospora* strains yielded higher ($P \leq 0.1$) shoot N contents than the control treatment (Table 3), with increases ranging from 22% (ALF7) to 101% (ALFr5).

In the cohort of *E. meliloti* 1021-inoculated plants, redundancy analysis (RDA) revealed that the explanatory effect of the *Micromonospora* inoculations was significant according to the Monte Carlo test for significance of the first axis (*F*-ratio= 18.046, *P*= 0.017) and all canonical axes (*F*-ratio= 2.207, *P*= 0.001). However, the second canonical axis was non-significant (*F*-ratio= 8.209, *P*= 0.458), indicating that it explained no more variation than random and, thus, does not need to be further considered in the interpretation of the results[29]. The proportion of variability explained by all the constrained canonical axes was 18.7%. Figure 3c shows the distance biplot resulting from this RDA analysis. Pair-wise RDA comparisons indicated that only *Micromonospora* ALFb5 and ALFpr18c produced significant differences with respect to the *Micromonospora*-free control treatment (Table S5). Univariate (ANOVA) analyses indicated that inoculation with *Micromonospora* ALFb5 or ALFpr18c produced significantly ($P \leq 0.05$) more nodules and higher shoot biomass (Sdw) than the control treatment (Table 3). The mean Sdw of plants co-inoculated with *E. meliloti* 1021 and either *Micromonospora* ALFb5 or ALFpr18c were, respectively, 26% and 24% greater than that of plants only inoculated with *E. meliloti* 1021 (control treatment). These two co-inoculated treatments also showed higher shoot-to-root (S:R) ratios than the control treatment ($P \leq 0.1$). In comparison with control plants, co-inoculated plants afforded significantly higher ($P \leq 0.05$) shoot contents of C (24% and 20%), N (10% and 21%), P (21%) and K (35% and 28%).

Discussion

The number and diversity of *Micromonospora* strains recovered from alfalfa nodules strongly suggest that this actinobacteria is commonly associated with the symbiotic organ of legumes. Besides other microorganisms, almost all nodules selected had a population of one or more *Micromonospora* strains. Moreover, for each isolation experiment two sterile, non-crushed nodule was rolled over YMA agar and incubated under the same conditions as the homogenized samples in order to assess the effectivity of the sterilization procedure. No colonies appeared on any YMA plate indicating that sterilization was effective.

BOX–PCR fingerprinting has been shown to be a useful tool to discriminate highly related strains and has been applied to study the

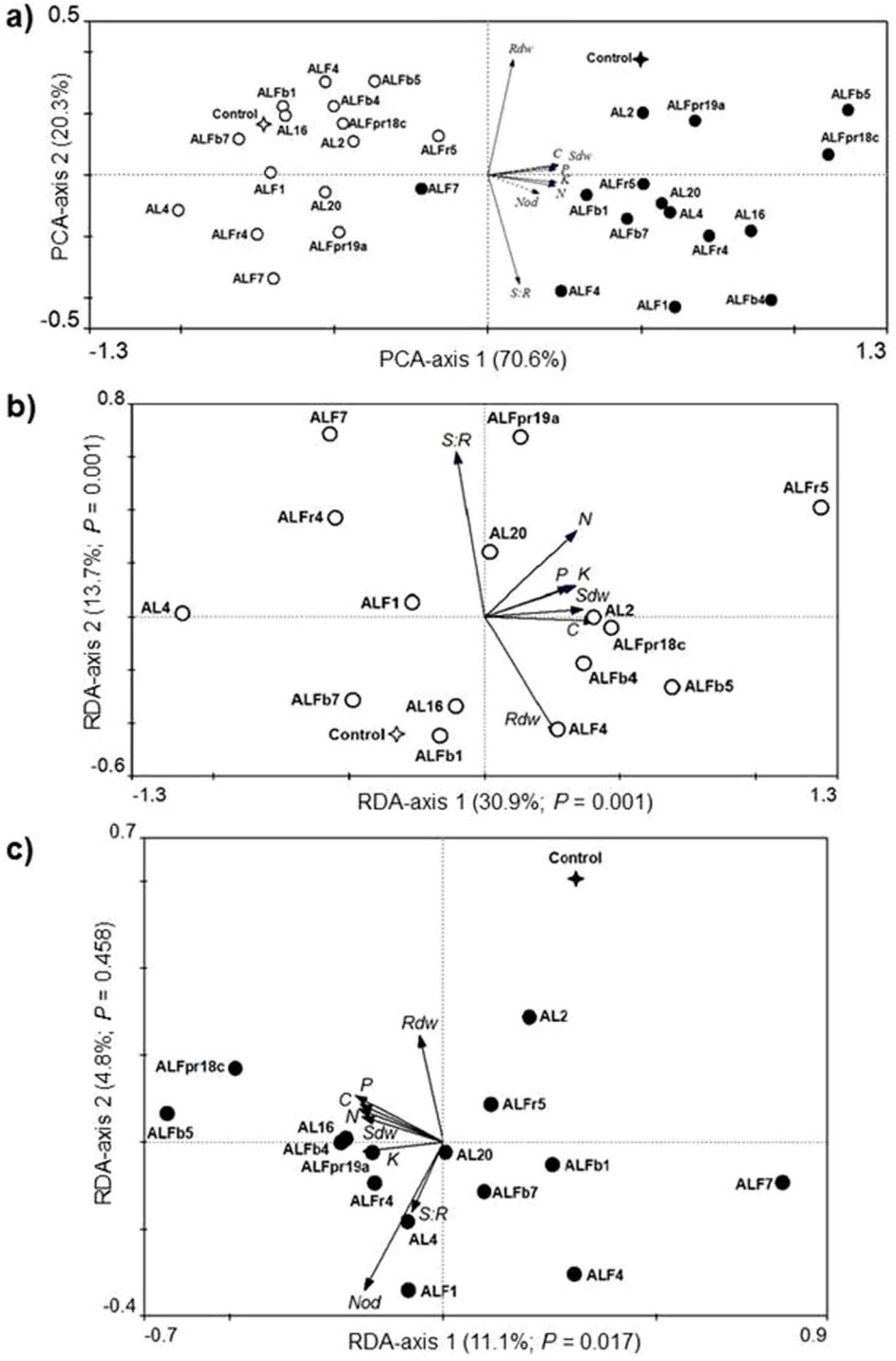

Figure 3 | Biplot representations of the results of PCA and RDA analyses. (a) PCA performed on the matrix with plant growth parameters and shoot nutrients content. RDA performed on the matrix of plant growth parameters and shoot nutrients content constrained by the matrix of bacterial inoculation treatments, either in (b) the cohort of plants singly inoculated with different strains of *Micromonospora* spp. and in (c) the cohort of plants co-inoculated with *Micromonospora* and *E. meliloti* 1021. Open circles represent the centroids of the treatments inoculated singly with each of the fifteen *Micromonospora* strains while full circles represent those of the treatments also inoculated with *E. meliloti* 1021. Star symbols represent the centroids of the control treatments without any microbial inoculation (open star) and inoculated with *E. meliloti* 1021 only (full star). Arrows represent variables measured on individual alfalfa plants: shoot dry weight (*Sdw*), root dry weight (*Rdw*), shoot to root ratio (*S:R*), number of nodules (*Nod*); and shoot contents of carbon (*C*), nitrogen (*N*), phosphorus (*P*), and potassium (*K*). The variable *Nod* is passively projected into the PCA diagram (a) but it was not included in the calculation (dotted arrow). Values on the axes indicate percentages of total variation explained by each axis and *P*-values of significance for the RDA canonical axes (b and c) obtained by Monte Carlo permutation tests (999 permutations).

genetic diversity of different bacterial taxa, including *Micromonospora*[30,31]. A high degree of genetic variation was observed among the 66 isolates, when analysed by BOX–PCR fingerprinting, indicating that they represented different bacterial genotypes. It should also be noted that none of them were clones of one another, supporting the idea of the existence of high genetic diversity among *Micro-*

Table 3 | Growth parameters and shoot contents of C, N, P and K of alfalfa plants inoculated with those *Micromonospora* strains that alone or in co-inoculation with *Ensifer meliloti* 1021 showed significant multivariate (RDA) differences respect to the *Micromonospora*-free controls

		(mg plant^{-1})						(no. plant^{-1})
Strain	S:R[a]	Sdw	Rdw	Shoot C	Shoot N	Shoot P	Shoot K	Nodules
Single inoculation with *Micromonospora* spp.								
Control[b]	*1.03*	*956*	*948*	*408*	*9.9*	*2.68*	*18.6*	N/A
AL2	1.18	1067	943	556	**13.6*****	3.22	**25.4*****	N/A
AL4	1.22	763***	635***	278***	**12.2***	2.20	15.3	N/A
AL20	**1.35*****	1015	763	399	**12.8*****	**3.42****	**22.9***	N/A
ALF1	1.20	939	802	373	**14.3*****	2.75	19.2	N/A
ALF4	0.92	1032	1129	405	**13.1*****	3.17	**23.6****	N/A
ALF7	**1.81*****	992	559***	359	**12.1***	2.68	19.6	N/A
ALFb4	1.01	1016	1049	423	**15.5*****	3.15	**23.6****	N/A
ALFb5	1.00	**1140*****	**1161***	457	**14.6*****	**3.47****	**24.9*****	N/A
ALFpr18c	1.07	1030	978	**475****	**16.4*****	3.12	22.4	N/A
ALFpr19a	**1.51*****	972	662***	384	**16.6*****	**3.37***	**25.5*****	N/A
ALFr4	**1.45*****	880	619***	347***	**13.1*****	2.63	20.7	N/A
ALFr5	**1.31****	**1288*****	1027	**515*****	**19.9*****	**3.69*****	**26.4*****	N/A
Co-inoculation with *Ensifer meliloti* 1021								
Control[c]	*1.20*	*1572*	*1381*	*668*	*41.7*	*4.62*	*32.0*	*42*
ALFb5	**1.60***	**1980*****	1312	**826****	**52.1*****	**5.57****	**43.2*****	**87*****
ALFpr18c	**1.85*****	**1948****	1207	**805****	**50.5****	**5.59****	**40.9*****	**71*****

[a]S:R, shoot to root ratio; Sdw, shoot dry weight; Rdw, root dry weight.
[b]Control treatment corresponds to uninoculated alfalfa plants.
[c]Control treatment corresponds to alfalfa plants inoculated only with *E. meliloti* 1021.
Means (N= 10) are shown. Within columns, treatment means in bold type or underlined were, respectively, higher or lower than their respective control treatment (in italics) according to Dunnett's one-tailed tests at $P \leq 0.1$ (*), $P \leq 0.05$ (**) and $P \leq 0.01$ (***).

monospora strains in legume root nodules. Our results are coherent with data from *Lupinus angustifolius* and *Pisum sativum* root nodules[13,15].

The BOX grouping provided a useful background for determining the taxonomic relationship of the strains isolated since these groups served to select strains for 16S rRNA gene sequencing. Even though several strains have more than 99% similarity with described species, others had 16S rRNA gene sequence similarities below 99%, indicating that they were not related to any of the already known species of *Micromonospora* and probably represent new ones. This case has been observed previously by Trujillo and co-workers, who described two new *Micromonospora* species: *M. lupini* and *M. saelicesensis*, whose 16S rRNA genes were highly similar to already described species[12]. Our results (BOX–PCR fingerprinting and 16S rRNA gene sequences) also suggest that the diversity of *Micromonospora* was independent of the location where they were isolated since in several BOX-PCR groups there are strains from more than one of the five locations sampled (Figure 1).

The high diversity and ubiquity of this actinobacteria inside legume root nodules suggest that its presence is not fortuitous, but that *Micromonospora* might have an important ecological role in nature. To discern ecological roles of *Micromonospora* in interaction with alfalfa, we evaluated in a mesocosm experiment their effects on plant growth and nutrition, both in nodulated and non-nodulated alfalfa plants. Multivariate statistics showed that *E. meliloti* 1021-nodulated plants tended, of course, to have higher values of aerial dry matter and nutrient content than the non-nodulated ones (Figure 3a). But we also found a significant effect of the inoculation with *Micromonospora* as well as a significant interaction between both *E. meliloti* 1021 and *Micromonospora* inoculations, indicating different behaviour of the *Micromonospora* strains according to the nodulation status of the plant (Table S4). In non-nodulated plants, twelve out of the fifteen *Micromonospora* strains produced significant multivariate differences with respect to the uninoculated control (Table S5; Figure 3b), while in nodulated plants only the treatments co-inoculated with the strains ALFb5 and ALFpr18c differed significantly from the *Micromonospora*-free control treatment (Table S5; Figure 3c). Several actinobacteria, including strains of *Micromonospora* sp., had been shown to promote both shoot and root growth and nodulation in alfalfa as well as in the actinorhizal plant species *Ochetophila (Discaria) trinervis* when co-inoculated with the corresponding nitrogen-fixing micro-symbiont, *Ensifer* or *Frankia*[32–34]. Contrary to our results, these authors found that actinobacteria alone exerted no effect on plant growth. It should be emphasized that, unlike us, Solans and co-workers grew the plants in soilless, gnotobiotic conditions[32–34].

Three main empirical facts from our greenhouse experiment must be highlighted. The first one is that *Micromonospora* does not induce larger root systems. The most common effect of PPBs on plant is the formation of larger root systems, which allow exploring a greater volume of soil for water and nutrients[35]. Root biomasses (Rdw) in *Micromonospora*-inoculated treatments were similar to or lower than in control plants (Table 3; Figure 3a,b). The second fact is that most *Micromonospora* strains increased the N shoot content in non-nodulated plants (Table 3; Figure 3b). It has been reported in recent years the existence of putative N_2-fixing *Micromonospora*[13,36]. Therefore, in a previous work we conducted an exhaustive experimental study centred on two of the representative strains here assayed, namely ALFb5 and ALFpr18c, in order to discern if they could fix N_2 either as free-living diazotrophs or in symbiosis with alfalfa plants[37]. Neither of the strains grew in nitrogen-free media or reduced acetylene under micro-aerobic conditions. Incorporation of ^{15}N into the microbial biomass or alfalfa tissues was not detected. Also, attempts to amplify putative *nif*H genes in these strains were unsuccessful. Besides, we tracked the presence of structural genes for N_2-fixation in two other *Micromonospora* strains that have their genome sequenced[38,39], finding no evidence for them[37]. These results seem to rule out N_2 fixation by *Micromonospora* as source of nitrogen for plants, focusing the explanation for higher N shoot contents on enhanced nutrient uptake efficiency and/or more plant-available nitrogen in soil.

Although there are few published studies on the impact of PPB on nutrient uptake systems, concomitant improvement of mineral nutrition (including N, P and K) and increase of root surface area

has been described in several plant species[35]. With regards to N nutrition, it has been hypothesized that PPB could directly stimulate nitrate transport systems in plants[40], but recent genetic studies on *Arabidopsis thaliana* indicate that while there are two NO_3^- transporter genes (*NRT2.5* and *NRT2.6*) that are strongly upregulated in response to inoculation with the PPB *Phyllobacterium brassicacearum* strain STM196, plant growth promotion is not linked to changes in NO_3^- uptake rate or NO_3^- distribution between roots and shoots[41]. However, most actinobacteria are saprophytes able to produce a wide range of extracellular hydrolytic enzymes[2,42–44]. All the strains we studied synthesize hydrolytic enzymes able to cleave complex nitrogen-containing polymeric substrates, such as caseinase and gelatinase (Table 2), strongly suggesting that *Micromonospora* can favour plant nutrition by enhancing nitrogen mineralization in soils. Nonetheless, further research is needed to fully explain the rationale for improved nitrogen nutrition in plants inoculated with *Micromonospora*. Moreover, all the fifteen *Micromonospora* showed neutral and alkaline phosphatase activities (Table 2), which can enhance the mineralization of organic phosphate in neutral or alkaline soils[45] like the one used in our greenhouse experiment (pH 7.47; Table S1), thus making soil P more available to plants as suggested by higher shoot P content in some *Micromonospora*-inoculated treatments than in the controls (Table 3; Figure 3b, c).

In the cohort of plants nodulated by *E. meliloti* 1021 only two strains of *Micromonospora* (ALFb5 and ALFpr18c) produced statistically significant multivariate differences with respect to the *Micromonospora*-free control group (Table S5; Figure 3c). The success of the interaction between a PPB strain and the plant relies on a set of adaptation mechanisms by both partners, among which the phytochemical profile of the root exudates plays a fundamental role in the bacterial colonization of the root as well as in the regulation of PPB plant beneficial properties[46]. The composition of root exudates has been shown to differ in legumes depending on their nodulation status[47–49], so that the biochemical environment in the rhizosphere of *E. meliloti* 1021-nodulated alfalfa plants might be less advantageous for *Micromonospora* compared with that of non-nodulated plants. Considering the soil pH, legumes are known to acidify the rhizosphere because of the release of protons following excess uptake of cations over anions during N_2 fixation[50–52]. Only six out of the 15 *Micromonospora* strains tested in this study grew vigorously *in vitro* at pH 6.5 (Table 2) and none at lower pH values (4.5 or 5.5). Indeed, strains ALFb5 and ALFpr18c are among those able to grow at acidic pH (6.5) while the strain ALFr5, a strain that only excelled in the cohort of non-nodulated plants, does not (Table 2; Figure 3b,c). Therefore, a more acidic rhizosphere in the N_2-fixing plants may partially explain differential effects of certain *Micromonospora* strains on growth of nodulated and non-nodulated plants. Nonetheless, given the great influence that the nodulation has on N acquisition capacity and growth of legumes, it is plausible that the effect of most of the *Micromonospora* is not marked enough to be statistically significant in symbiotically N_2-fixing alfalfa plants.

And third, although in plants nodulated by *E. meliloti* 1021 only *Micromonospora* ALFb5 and ALFpr18c were found to have significant, globally positive effects on plant growth and nutrition (Table 3), it was observed a trend towards the improvement in nodulation intensity (number of nodules) with the presence of *Micromonospora* (Figure 3c; Figure S2c). Furthermore, all the fifteen *Micromonospora* strains could be re-isolated from nodules of random plants of each co-inoculated treatment, suggesting that none of the *Micromonospora* strains here assayed had incompatibility with *E. meliloti* 1021.

Plant growth-promoting bacteria can increase nodulation in legumes through different mechanisms, including the production or degradation of phytohormones involved in nodule initiation and organogenesis[53], or by affecting the interaction between plant and rhizobia[54–56]. Auxins are involved in the initiation and normal development of both determinate[57] and indeterminate nodules, like *Medicago* root nodules[58]. IAA production has been associated with the induction of increased nodule numbers in *Medicago truncatula* plants inoculated with an *E. meliloti* strain that overproduces IAA[59] and also with nodule-like structures even in non-leguminous plants[60–62]. Moreover, rhizobial cellulases have been shown to be crucial for legume nodulation[63]. The ability of *Micromonospora* strains to produce cellulases could thus explain the increase in the number of nodules observed in co-inoculated plants compared to the control plants only inoculated with *E. meliloti* 1021. However, IAA production by *Micromonospora* may not be directly related in our study to an increase in nodulation despite of the literature.

Conclusion. In this study 66 *Micromonospora* strains were isolated, characterized using BOX-PCR and sequencing of 16S rRNA genes and selected some of them for studying their interaction with alfalfa. Our results, together with those from other authors, indicate that *Micromonospora* are ubiquitous in legume root nodules, presenting a very high genetic diversity. Most of them exhibit *in vitro* a great ability to degrade organic polymers as well as presenting a direct mechanism for plant growth promotion (IAA production). We have shown that *Micromonospora* could play an important ecological role in interaction with the host plant by enhancing aerial growth and nutrient contents, being an increase of N uptake by the plant a general phenomenon in the *Micromonospora*-alfalfa interaction. It remains to be elucidated whether these positive effects also occur in other plant species. *Micromonospora* engaged in tripartite interactions with *E. meliloti* 1021 and alfalfa increase nodulation, and some of their strains can also significantly promote the growth and nutrition of N_2-fixing plants. Contrary to most of plant growth-promoting bacteria, beneficial effects of *Micromonospora* do not rely on induction of plant root growth. All the above data suggests that, in general, *Micromonospora* can be considered as excellent PPB, although a correct selection of strain is of capital importance because of the detrimental effect that some *Micromonospora* may have for plant growth (i.e. strain AL4 in non-nodulated plants; Table 3). Additionally, *Micromonospora* is a sporulating bacterium so that it can endure in soil and harsh environments. Thus, some of their strains seem to be excellent candidates for the production of bioinoculants, which would make the use of environmentally unfriendly chemical fertilizers less intensive in a broad range of agroecosystems.

Methods

Isolation and ecological characterization of *Micromonospora* strains. Isolations of *Micromonospora* were done from surface sterilized root nodules of naturally occurring alfalfa plants from five different regions of Castilla y León (Spain). Functional characterization of the isolated strains included: hydrolytic activities toward casein, starch, gelatin, xylan, Tween 80 and 20, cellulose and pectin; presence of acid, neutral and alkaline phosphatase activities; production of indole acetic acid (IAA); and growth under different environmental conditions. For further details on isolation and functional characterization of the isolates, see Materials and Methods in Supplementary Information.

Genetic and phylogenetic characterization of *Micromonospora* strains. BOX–PCR fingerprinting profiles from bacterial genomic DNA were obtained according to Trujillo *et al.*[13]. Similarity matrices of electrophoretic band profiles were calculated using the Pearson Correlation Coefficient followed by dendrogram construction using the UPGMA algorithm. Strain clusters were defined at the 60% level of similarity. Three different strains were used as probes, processing them in every PCR and electrophoresis run in our experiments. When gel patterns were analyzed with the software BioNumerics (Applied Maths, Sint-Martens-Latem, Belgium), probe strains band patterns always were observed to be identical.

One representative strain of each BOX cluster was selected for 16S rDNA sequencing and phylogenetic analysis. The sequences were aligned and compared with those deposited in public databases, and then neighbour-joining and maximum-likelihood phylogenetic trees were constructed. Sequence data has been submitted to the GenBank database under accession numbers from KF876220 to KF876234. See Materials and Methods in Supplementary Information for full details.

***Micromonospora-Ensifer meliloti* 1021-alfafa interaction assay.** The fifteen representative *Micromonospora* strains (Table 1) were tested in interaction with

alfalfa plants, either alone or in co-inoculation with *E. meliloti* 1021. Alfalfa plants were individually grown in pots (1L volume) containing tyndallized soil in a greenhouse under controlled environmental conditions. The experimental design included 32 treatments with 10 replicates per treatment. Treatments were defined by a factorial combination of two *E. meliloti* 1021 inoculation treatments (non-inoculated or inoculated) and sixteen *Micromonospora* inoculation treatments (non-inoculated or inoculated with each of the fifteen representative strains). Plants were harvested 14 weeks after inoculations and the following parameters were determined: Shoot (Sdw) and root (Rdw) dry weight; shoot content in carbon (C), nitrogen (N), phosphorus (P) and potassium (K); and number of root nodules (Nod). See Materials and Methods in Supplementary Information for full details.

Statistical analysis. Plant growth and nutrient content data were analysed using multivariate (PCA and RDA) and univariate (ANOVA) analyses with the CANOCO 4.5 (Microcomputer Power, Ithaca, NY) and SPSS for Windows v21.0 (IBM Corp., Armonk, NY) programs. The inoculation treatments were coded as dummy variables and used as independent variable in the multivariate analyses. Significance in RDA analyses was tested by Monte Carlo permutation tests (999 unrestricted permutations) for the first canonical axis as well as for the sum of all canonical axes. In univariate comparisons, post-hoc Dunnett's one-tailed t-tests were used to identify inoculation treatments with means significantly different from the control at $P \leq 0.1$, $P \leq 0.05$ an d $P \leq 0.01$. For further details on the statistical analyses, see Materials and Methods in Supplementary Information.

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Aknowledgements

This work was supported by Junta de Castilla y León Grant SA306A11-2 and MICINN Grant AGL2010-17380. P.M.-H. was supported by a fellowship from CSIC JAE-PRE. We thank N. Skinner for revising the English version of the manuscript.

Author contributions

Conceived and designed the experiments: P.M.-H., E.M.-M., J.M.I. Performed the experiments: P.M.-H. Analyzed the data: P.G.-V. Wrote the paper: P.M.-H., E.M.-M., J.M.I.

Additional information

Supplementary information accompanies this paper at http://www.nature.com/scientificreports

Competing financial interests: The authors declare no competing financial interests.

How to cite this article: Martínez-Hidalgo, P., Galindo-Villardón, P., Igual, J.M. & Martínez-Molina, E. *Micromonospora* from nitrogen fixing nodules of alfalfa (*Medicago sativa* L.). A new promising Plant Probiotic Bacteria. *Sci. Rep.* **4**, 6389; DOI:10.1038/srep06389 (2014).