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Characterisation of symbiotically efficient alfalfa-nodulating rhizobia isolated from acid soils of Argentina and Uruguay

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Abstract

The diversity, growth and symbiotic behaviour of symbiotically efficient alfalfa-nodulating rhizobia isolated from acid soils of Argentina and Uruguay were analysed. Partial sequencing of the 16S rDNA indicated that these isolates belong to *Sinorhizobium meliloti* species. IS-fingerprinting analysis revealed a high diversity among the isolates but some of them appear related to inoculant strains currently used in the region. The *S. meliloti* isolates showed a decreased growth rate with increasing acidity. They were, however, able to nodulate alfalfa at pH 5.6, but showed a delayed nodulation and decreased nodule number typical of *S. meliloti* strains. The impaired nodulation of *S. meliloti* at pH 5.6 did not result in a reduction of alfalfa dry matter production or nitrogen content. However, significant differences were observed for the relative symbiotic effectiveness of the strains analysed. LPU63 (Argentina) was the most effective among the isolates and exhibited a high nodulation competitiveness at both neutral and acidic pH. These results suggest that the isolate LPU63 may be a potential efficient inoculant for alfalfa in acid soils. © 1999 Federation of European Microbiological Societies. Published by Elsevier Science B.V. All rights reserved.

Keywords: Competitiveness; Insertion sequence; Fingerprinting; pH; Symbiosis

1. Introduction

The growth and persistence of alfalfa (*Medicago sativa*) a perennial legume capable of producing high yields of high quality forage, are impaired in moderately acid soils. The low performance of alfalfa and other leguminous plants in soils with low pH is due to several factors that affect the host plant, their rhizobia and the symbiotic interaction [1–3].

Sinorhizobium meliloti is among the more acid sensitive rhizobia, hence the selection of acid tolerant *S. meliloti* strains has been considered as a possible approach to solve alfalfa growth and persistence at low pH. Howieson et al. [4] found a poor correlation between growth rate in acidic media and acid toler-

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ance in the field. Nevertheless, the selection of acid tolerant rhizobial strains isolated from *Medicago* spp. in acid soils of Sardinia has greatly improved the establishment of medic pastures in mildly acidic soils in Western Australia [4–6]. In Latin America, there are more than 800 millions ha of oxisols and ultisols which are inherently acidic [7,8]. In addition to the naturally occurring acid soils, intensive agricultural practices in Argentina and Uruguay are leading to a progressive acidification of vast regions where the alfalfa crop production is constrained [9,10].

Naturalised alfalfa-nodulating rhizobia have recently been isolated from acid soils of central Argentina and Uruguay and a collection of 465 isolates has been established (A. Lagares and G. Martínez-Drets, unpublished data). Initial characterisation of the isolates has revealed two main groups: (1) symbiotically efficient isolates unable to grow at pH 5.0 and below; and (2) acid-tolerant (able to grow at pH 5.0 and lower) but symbiotically inefficient isolates, closely related to *Rhizobium* spp. strain Or191 originally isolated from moderately acid soils in Oregon [11,12].

The aim of this work was to characterise the group of symbiotically efficient isolates and to select among them potential efficient inoculants that could be used to improve alfalfa production in the local acid soils of Argentina and Uruguay.

2. Materials and methods

2.1. Rhizobial strains

Alfalfa nodulating rhizobia isolated from acid soils of Argentina and Uruguay used in this work, are listed in Table 1. Additionally, the following bacteria were used for comparison: commercial inoculants for alfalfa currently used in Uruguay (U45, U137, and U143) and Argentina (B36, B58, B399, B401), wild type *S. meliloti* strains, GR4 [13], 2011 [14], and rhizobial strains isolated from *Medicago* spp., in acid soils of Southern Europe, WSM419 [5], WSM826, WSM879 and WSM922 (J. Howieson Centre for *Rhizobium* Studies Murdoch University South St, Murdoch, Australia). All strains were routinely maintained in tryptone yeast (TY) medium [15].

2.2. Sequencing 16S rDNA

A DNA region corresponding to nucleotides 20–338 of *Escherichia coli* 16S rDNA was amplified with

Table 1
Alfalfa nodulating rhizobia isolated from acid soils of Argentina and Uruguay used in this work

Strains	Characteristics	Soil location	Soil pH	Source
S. meliloti				
LPU7, 18	isolated in the field	INTA Castelar, Prov. BsAs, Argentina	5.6	A. Lagares
LPU11	isolated in the field	INTA Castelar, Prov. BsAs, Argentina	5.96	A. Lagares
LPU30	isolated using pots	Rafaela km 170. Prov Santa Fe	6.04	A. Lagares
LPU63	isolated using pots	INTA Castelar, Prov. BsAs, Argentina	5.89	A. Lagares
LPU283	isolated using pots	Santa Fe, Argentina	5.68	A. Lagares
LPU119	isolated using pots	Arrecifes, Prov. BsAs, Argentina	6.64	A. Lagares
CE17	isolated in the field	Colonia, Uruguay	6.14	G. Martinez
CE21	isolated in the field	Colonia Cosmopolita, Uruguay	6.02	G. Martinez
CE31	isolated in the field	Paysandú, Uruguay	6.23	G. Martinez
CE32	isolated in the field	Río Negro, Uruguay	6.21	G. Martinez
CE47	isolated using pots	Colonia, Puerto del Rosario, Uruguay	5.72	G. Martinez
CE56	isolated using pots	San Ramón, Uruguay	6.01	G. Martinez
CE65C4	isolated using pots	Colonia 33, Paraje San Gabriel, Uruguay	5.78	G. Martinez
Rhizobium spp.				
LPU83	isolated using pots; acid tolerant able to grow at pH 5.0	INTA Castelar, Prov. BsAs, Argentina	6.08	A. Lagares

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the universal primers Y1 (5′-TGG CTC AGA ACG AAC GCT GGC GGC-3′) and Y2 (5′-CCC ACT GCT GCC TCC CGT AGG AGT-3′) as previously described [16]. The PCR products were directly sequenced using an Automatic Laser Fluorescent DNA sequencer (Applied Biosystems).

2.3. DNA hybridisation and IS-fingerprinting

Total DNA was isolated as described previously [17]. After *Eco*RI digestion, 2 μg of DNA were electrophoretically separated in an 0.8% Tris-borate agarose gel and vacuum blotted onto nylon membranes, positively charged (Boehringer Mannheim). DNA probe for IS*Rm2011-2* [18] was obtained by PCR amplification of an internal fragment using plasmid pRmNT40 [19] as template. Oligonucleotides used in the amplification reaction were: 2011B1 (5′-TGGACGAAGACGAACATGG-3′)/2011B2 (5′-TTGAAGTAGGCTGCGCATT-3′). PCR fragments were isolated from the agarose gel and labelled with digoxigenin-11-dUTP (Boehringer Mannheim). Hybridisation was carried out at high stringency conditions (68°C, 5×SSC) according to the supplier's instructions. Washing was carried out twice, 5 min each, in 2×SSC, 0.1% SDS at room temperature and twice, 15 min each, in 0.1×SSC, 0.1% SDS at 68°C. Chemiluminiscent detection of the hybridisation signals was performed as specified (Boehringer Mannheim).

Southern hybridisation blots were scanned and fingerprint patterns were imported into a database. Comparison of these patterns was carried out with the AQ-Image software (Bio Image) using the DICE correlation method. For comparison, bands between 500 and 23 000 bp were considered with a band size tolerance of 4%. For derivation of the dendrogram, the UPGMA (unweighted pair group with mathematical averaging) method was applied.

2.4. pH-tolerance of alfalfa nodulating rhizobia

Starter cultures were grown in minimal medium (MM) [20] containing 1.0 mM $CaCl_2 \cdot 2H_2O$ at pH 7.0. Tubes containing 3 ml of MM at pH 7.0, buffered with 20 mM MOPS (2-[*N*-morpholino]propanosulfonic acid), and at pH 6.0 and 5.6, buffered with 20 mM MES (3-[*N*-morpholino]ethanesulphonic acid), were inoculated to a final density of 5×10^6 cell ml^{-1}. Tubes were incubated in a gyratory shaker (225 rpm) at 28°C and periodically sampled for a determination of viable cells by plate count on MM (pH 7.0).

2.5. Nodulation and competition assays

Alfalfa (*Medicago sativa* cv. Aragon) plants were grown, in a controlled environmental chamber, on nitrogen-free medium as described [21] with 20 mM MOPS (3-[*N*-Morpholino]propanesulfonic acid; Sigma), for pH 7.0 and 20 mM MES (2-[*N*-morpholino]ethanesulfonic acid; Sigma), for pH 5.6. With these buffers, pH changed only 0–0.2 units over 30 days. The pH values of the media were checked, at room temperature, in a Crison (MicropH 2000) pH-meter. The medium was adjusted to the required pH with KOH prior to autoclaving (115°C, 20 min). Nodulation kinetic assays were performed in hydroponic buffered culture with a 0.7 mM $CaCl_2 \cdot 2H_2O$ concentration. For each bacterial strain, 24 individual plants were inoculated with 10^6 cells ml^{-1}. The experiments were followed during 31 days. During the assay and periodically the number of nodules and the percentage of nodulated plants were recorded.

For competition assays, 10–12 tubes with single alfalfa plants each were inoculated with mixtures (1:1 ratio) of the corresponding bacterial strains (10^6 cells ml^{-1}). To facilitate strain identification, one of the two coinoculated strains harboured plasmid pGUS-3 (F.M. García-Rodriguez, unpublished), a pBI101 (Clontech) derivative carrying a translational fusion of *nfeD* promoter [22] with *gusA* (β-glucuronidase). To determine nodule occupancy, the plants were collected 14–15 days after inoculation and briefly washed with water. Roots were incubated in the dark overnight at 37°C in 1 mM X-Gluc (5-bromo-chloro-3-indolyl-β-D-glucuronide, Apollo Scientific, UK) in 50 mM sodium-phoshate buffer (pH 7.5) with 1% SDS. The nodule occupancy was determined by counting blue and white nodules.

2.6. Symbiotic effectiveness

Plants were harvested 30 days after inoculation and the shoot dry matter weight was measured, the

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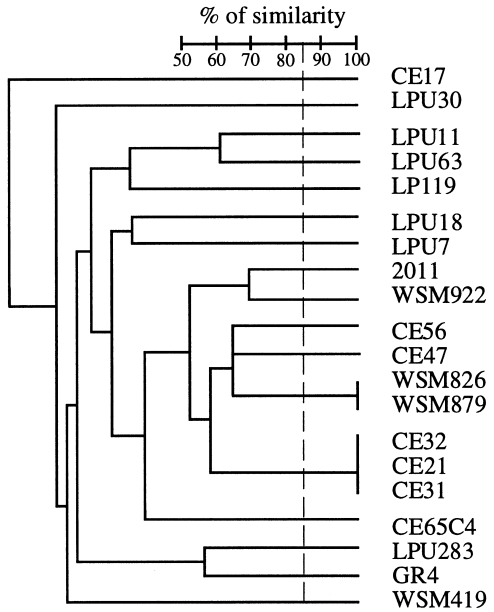

Fig. 1. IS-fingerprinting grouping of the *S. meliloti* isolates from acid soils of Argentina and Uruguay. *S. meliloti* strains GR4, 2011, and the acid soil tolerant rhizobia strains WSM419, WSM922, WSM826 and WSM879 were also included. IS*Rm2011-2* DNA was used as hybridisation probe. The DNA fingerprints of the commercial inoculant strains currently used in Uruguay, U45, U137 and U143 were identical to that of the isolates CE21, CE31 and CE32. The threshold of 85% was chosen since analysis of independent fingerprint hybridisations of the same strain yielded similar values in the range of 85–100%. The comparison was done using Dice test correlation and the UPGMA method was used for calculating the corresponding dendrogram.

same plants were used to determine the total nitrogen (N) according to previously described procedures [23]. For these assays 24 plants were used.

3. Results and discussion

3.1. The symbiotically efficient alfalfa nodulating rhizobia in acid soils of Argentina and Uruguay belong to S. meliloti species and show a high genetic diversity

Fourteen symbiotically efficient alfalfa nodulating rhizobia isolated from acid soils of Argentina (LPU strains) and Uruguay (CE strains) with pH values between 5.6 and 6.64 (Table 1) were analysed for their 16S rDNA nucleotide sequence. For all of them, the determined sequence was identical to the 16S rDNA of species of *S. meliloti* (Genbank accession numbers D14509 and D12783) indicating that these isolates belong to the former *Rhizobium* species.

The diversity of the *S. meliloti* isolates was assayed by IS-fingerprinting techniques using as DNA probe, the insertion sequence IS*Rm2011-2* [18]. The IS*Rm2011-2* element is widely distributed and abundant within *S. meliloti* indigenous populations, being found in all tested strains. Based on the strain-specific fingerprint pattern, a dendrogram was obtained (Fig. 1), showing a high diversity among the isolates.

S. meliloti-based inoculants are applied for growing alfalfa in Argentina and Uruguay, hence, we tested whether some of the isolates were related to these inoculant strains. Isolates CE21, 31 and 32 (Table 1) share the same IS-fingerprint pattern with inoculant strains U45, U137 and U143 currently used in Uruguay (data not shown). Strain U45 was isolated in Uruguay 30 years ago whereas strains U137 and U143 were recently isolated in Colonia

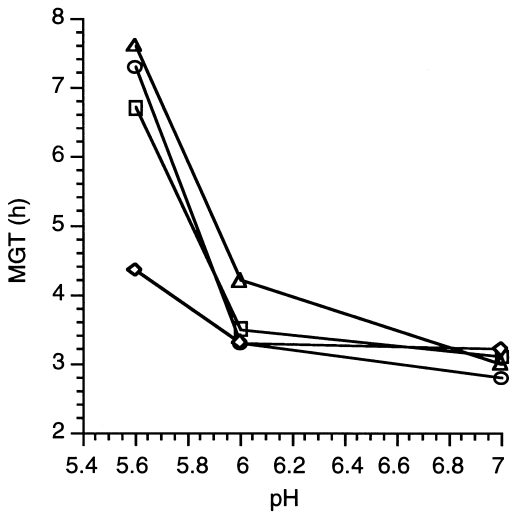

Fig. 2. The effect of pH on the mean generation times (MGT) of *S. meliloti* strains GR4 (□), 2011 (○) and the isolates LPU63 (△) and *Rhizobium* spp. LPU83 (◇). Given values are the numerical average of the results obtained from two independent assays.

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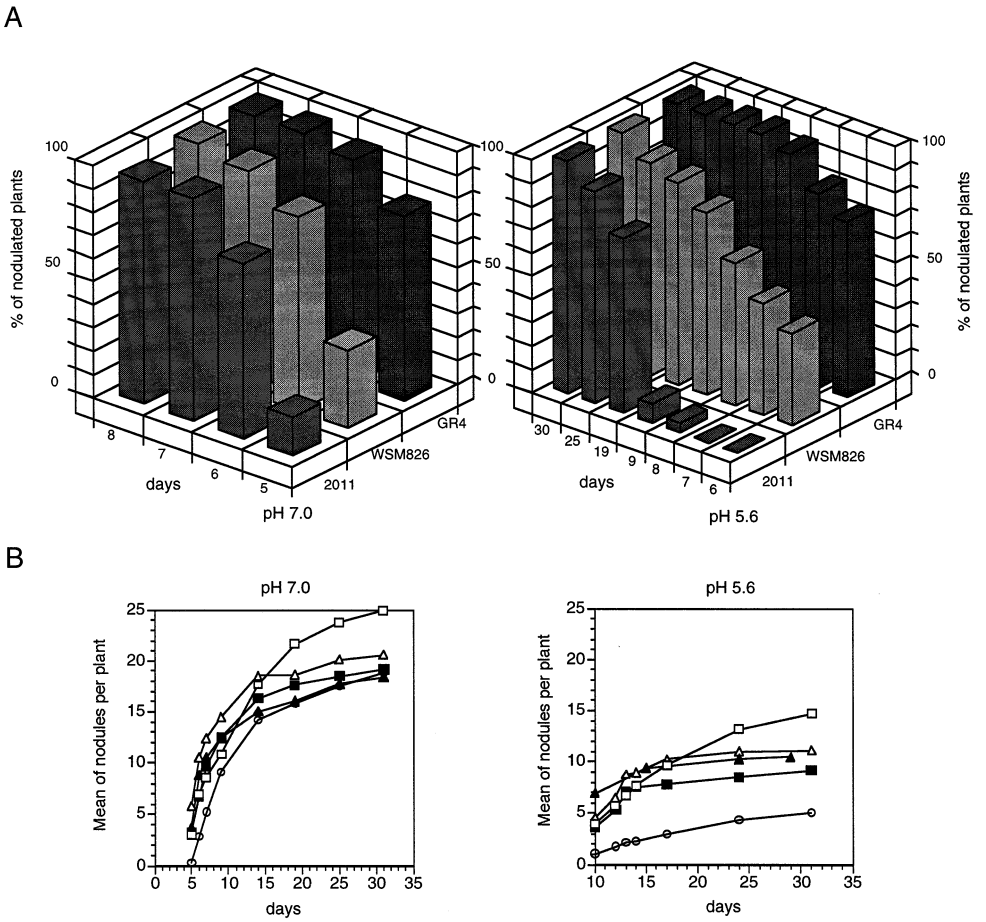

Fig. 3. The effect of pH on nodulation by alfalfa nodulating rhizobia. (A) Percentage of nodulated plants. The different behaviour of the isolates and strains tested are represented by that of *S. meliloti* strains GR4, 2011 and WSM826. (B) Nodulation kinetics of *S. meliloti* strains GR4 (■), 2011 (○), the isolates CE31 (△), LPU63 (▲), and *Rhizobium* spp. LPU83 (□). Given values are the average number of nodules per plant. Results are taken from a representative experiment among three independent assays.

(Uruguay) from old alfalfa cultures (G. Martinez-Drets, personal communication). Our results suggest that CE31-type isolates are currently abundant in the acid fields of Uruguay, perhaps as a result of the inoculation practice. The isolates from Argentina (LPU strains) all showed distinct IS*Rm2011-2* profiles and were different to those exhibited by alfalfa inoculants currently used in the region (data not shown). The *S. meliloti* strains WSM826 and 879 used in this work for comparison, appear to be close derivatives as deduced from their identical IS-fingerprint pattern (Fig. 1).

3.2. *The S. meliloti isolates show a decreased growth rate with increasing acidity*

In defined buffered media, the *S. meliloti* isolates from Argentina and Uruguay exhibited growth curves at pH 7.0, 6.0 and 5.6, showing a decreasing growth rate with increasing acidity as occurs with other *S. meliloti* strains, such as GR4 and 2011. The mean generation time (MGT) for all these bacteria (Fig. 2) was close to 3 h at pH 7.0 increasing to around 7 h at pH 5.6. The different behaviour of the isolates and strains tested are represented in Fig. 2

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Table 2
The effect of pH on alfalfa nodules number, shoot dry matter and nitrogen content, 30 days after inoculation with different alfalfa nodulating rhizobia

Strains	pH	Nodules/plant[a]	Shoot-dry matter[a,b]	% of N in shoot[c]
2011	7.0	18.79 ± 1.31	11.42 ± 0.92	2.15 ± 0.32
	5.6	5.08 ± 0.60	10.09 ± 0.87	3.12 ± 0.01
GR4	7.0	19.17 ± 1.54	15.08 ± 1.10	2.69 ± 0.55
	5.6	9.13 ± 0.63	17.09 ± 0.87	3.83 ± 0.01
CE31	7.0	20.58 ± 1.43	18.04 ± 0.69	3.65 ± 0.15
	5.6	11.13 ± 0.85	16.82 ± 0.79	3.71 ± 0.55
LPU63	7.0	18.38 ± 1.02	20.25 ± 0.74	3.84 ± 0.22
	5.6	10.21 ± 0.61	21.41 ± 0.81	4.28 ± 0.04
LPU83	7.0	24.92 ± 1.5	8.67 ± 0.68	1.82
	5.6	14.67 ± 2.10	5.23 ± 0.35	1.37

[a]Values are the mean ± S.E. of 24 plants.
[b]Shoot dry matter is expressed as mg per plant.
[c]The % is the mean of two sets of 12 plants each. In the case of strain LPU83, due to the low N content, the 24 plants were pooled for estimation.

by that of *S. meliloti* strains GR4, 2011 and LPU63. The isolate LPU7 as well as the acid soil tolerant strains WSM419 and WSM826 grew very poorly at pH below 6.0 in the MM media used in this work (data not shown). All the *S. meliloti* isolates showed a slower generation time at pH 5.6 than the acid tolerant strain *Rhizobium* spp. LPU83 (Fig. 2).

3.3. The S. meliloti isolates are able to nodulate alfalfa at pH 5.6, but exhibit a delayed nodulation and decreased nodule number

Earlier reports from Munns [24,25] using two *S. meliloti* strains (U45 and SU47) showed that acidity led to a delay in alfalfa nodulation and a reduction in nodule numbers. The nodulation kinetics of the *S. meliloti* isolates from Argentina and Uruguay were compared at two different pH values (7.0 and 5.6) with those of *Rhizobium* spp. LPU83, and *S. meliloti* strains WSM826, WSM879, WSM922, 2011 and GR4. At pH 7.0, strains GR4, WSM879, LPU83 and the *S. meliloti* isolates showed similar behaviour (represented in Fig. 3A by strain GR4), nodulating 80% of the plants 5 days after inoculation, whereas this percentage was lower for strains WSM826 (30%) and 2011 (15%). At neutral pH, 100% of the plants appeared nodulated 7 days after inoculation (Fig. 3A). At pH 5.6, a general delayed nodulation was observed for all strains (Fig. 3A). At this acidic pH, strains GR4, WSM879, LPU83 and the *S. meliloti* isolates required 9 days to nodulate 100% of the plants (represented in Fig. 3A by strain GR4), whereas strains WSM826 and 2011 required between 25 and 30 days (Fig. 3A).

The acidic pH also resulted in a reduction of the number of nodules elicited on alfalfa roots. The different behaviour of the isolates and strains tested are

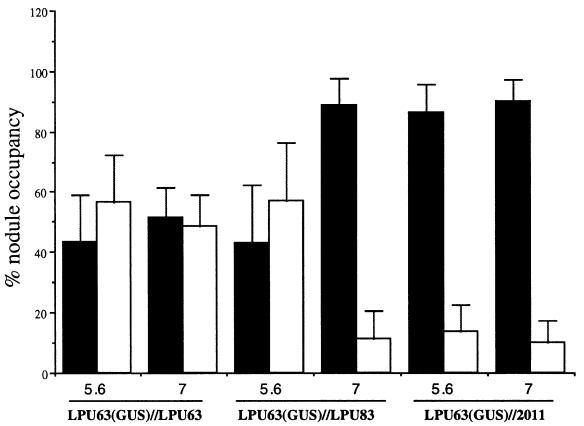

Fig. 4. The effect of pH on the nodulation competitiveness of *S. meliloti* LPU63. The competitive ability of the isolate LPU63 carrying plasmid pGUS-3 was compared to that of *S. meliloti* 2011 and *Rhizobium* spp. LPU83. Results are given as mean of the percentage of nodules occupied per plant by LPU63 carrying GUS (blue nodules) and the corresponding coinoculated strain in the mixture (white nodules), shown by black and white bars, respectively. Errors bars at 95% confidence interval are also shown. Results are taken from a representative experiment among three independent assays.

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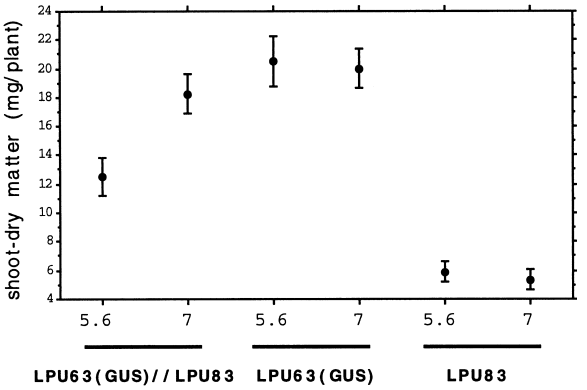

Fig. 5. The effect of coinoculation of *S. meliloti* and *Rhizobium* spp. LPU83 on alfalfa yield. The shoot-dry matter was measured 30 days after inoculation as indicated in Section 2. The given values are the mean of 24 plants. Errors bars at 95% confidence interval are shown.

represented by that of *S. meliloti* strains GR4, 2011, the isolates CE31, LPU63, and *Rhizobium* spp. LPU83 (Fig. 3B). The *S. meliloti* isolates elicited roughly 50% less nodules at pH 5.6 than at pH 7.0, but the same behaviour was observed for strain GR4 (isolated from a neutral soil) and for the acid tolerant strain *Rhizobium* spp. LPU83.

Our results indicate that the impaired nodulation phenotype at acidic pH is a general characteristic for alfalfa nodulating rhizobia which is not dependent on the acid tolerance of the microsymbiont.

3.4. Symbiotic effectiveness and nodulation competitiveness of the S. meliloti isolates

We measured the shoot dry matter and nitrogen content of alfalfa plants 30 days after inoculation at both neutral and acidic pH. As shown in Table 2, *Rhizobium* spp. LPU83 induces poor nitrogen-fixing root nodules on alfalfa roots at both, neutral and acidic pH. Surprisingly, as indicated in Table 2, neither the dry matter nor the relative concentration of nitrogen were significatively reduced at pH 5.6 when alfalfa was inoculated with *S. meliloti*. However, significant differences were observed for the relative symbiotic effectiveness of the strains and isolates analysed (Table 2). At neutral pH, the dry matter of alfalfa inoculated with the isolates CE31 and LPU63 was significatively ($P < 0.05$) higher than that of strains 2011 and GR4. At pH 5.6, the inoculation with the isolate LPU63 resulted in higher alfalfa dry matter production.

An efficient inoculant should also be able to compete for nodulation. Therefore, we tested the nodulation competitiveness of the isolate LPU63. As indicated in Fig. 4, *S. meliloti* LPU63 exhibits a high competitive ability when coinoculated with strain 2011 at both pH values, neutral and acidic, but only at pH 7.0 when coinoculated with *Rhizobium* spp. LPU83. As expected, coinoculation of LPU63 and LPU83 (ratio 1:1) at pH 5.6 resulted in a reduction of alfalfa dry matter which was not observed at neutral pH (Fig. 5). Although, neither the growth rate nor the nodulation phenotype at pH 5.6 differentiate clearly *S. meliloti* strains, the symbiotic effectiveness and nodulation competitiveness data indicate that isolate LPU63 may be a potential efficient inoculant for alfalfa in acid soils.

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