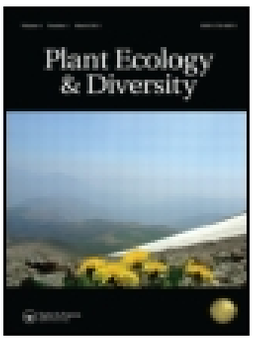

Plant Ecology & Diversity

ISSN: 1755-0874 (Print) 1755-1668 (Online) Journal homepage: http://www.tandfonline.com/loi/tped20

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To cite this article: Cláudia S. L. Vicente & María A. Pérez-Fernández (2016): Broad environmental tolerance of native root-nodule bacteria of Biserrula pelecinus indicate potential for soil fertility restoration, Plant Ecology & Diversity, DOI: 10.1080/17550874.2016.1182226

To link to this article: http://dx.doi.org/10.1080/17550874.2016.1182226

Published online: 22 Jun 2016.

Plant Ecology & Diversity, 2016
http://dx.doi.org/10.1080/17550874.2016.1182226

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Broad environmental tolerance of native root-nodule bacteria of *Biserrula pelecinus* indicate potential for soil fertility restoration

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(*Received 1 January 2016; accepted 20 April 2016*)

Background: *Biserrula pelecinus* is an annual legume native to the Mediterranean basin, found in pastureland, alone or in association with other legumes (Leguminosae) and grasses (Poaceae). It has been used in revegetation programmes of mining wastes showing phytoremediation potentials and thus becoming potentially highly attractive for plant ecology and restoration management of natural ecosystems.
Aims: To characterise native root-nodule bacteria isolated from *B. pelecinus* from the Iberian Peninsula, and to select suitable N fixers for field-application and soil rehabilitation.
Methods: Strains were isolated and molecularly identified by 16S rRNA amplification and sequencing. Strains were phenotypically characterised in different abiotic conditions (acidity, salinity and heavy metals) and tested for their ability to fix atmospheric N_2. The most suitable N fixers were applied in greenhouse experiments with *B. pelecinus* under different fertilization levels to assess their tolerance to fertilized and polluted soils, commonly encountered in restoration projects.
Results: *B. pelecinus* root-nodule isolates tolerated pH from 4.5 to 9.5 grew in saline conditions (2.5% of NaCl), and tolerated 50 μM of Al^{3+} and Mn^{2+}. Three isolates efficient in N_2 fixation, relative to the reference *Mesorhizobium* strain, were considered excellent candidates for the amelioration of nutrient poor sites.
***Conclusions*:** These results provide valuable information for the potential use in soil restoration of *B. pelecinus* in a wide-range of conditions, exploiting the natural variability of its root-nodule bacteria.

Keywords: acidity; *Biserrula pelecinus*; biological nitrogen fixation; fertilization; heavy metals; salinity

Introduction

Native to the Mediterranean Basin, *Biserrula pelecinus* L. is a pasture legume that grows in acidic sandy soils (Howieson et al. 1995). *B. pelecinus* is a prolific hard-seed producer, which form persistent soil seed banks (Malo and Suárez 1995). About 40% of seed survive cattle ingestion, and hence, the summer grazing is unlikely to harm the stand density or long-term persistence (Malo and Suárez 1995; Loi et al. 1999). Cattle tend to avoid grazing *B. pelecinus* in spring, preferentially targeting other plants, including weeds (Nichols et al. 2007), thus making *B. pelecinus* a potential species for herbicide-free weed management (Loi et al. 1999). Moreover, this legume has been exploited for its phytoremediation potential in revegetation programmes of mining wastes (Perrineau et al. 2011).

The symbiotic bacteria associated with *B. pelecinus* L. were identified as belonging to *Mesorhizobium* genus in the family Rhizobiaceae (Nandasena et al. 2001). The first reports related to the specificity of *B. pelecinus*-rhizobia defined the symbiosis as extremely specific (Nandasena et al. 2001). Nandasena et al. (2004) found that strains originated from *B. pelecinus* had a specific host range and did not nodulate legumes, such as *Amorpha fruticosa* L., *Astragalus sinicus* L., *Cicer arietinum* L., *Hedysarum spinosissimum* L., *Lotus parviflorus* Desf., *Macroptilium atropurpureum* (DC) Urb and *Trifolium lupinaster* L. Nevertheless, more recent studies have identified a new diversity of isolates associated to *B. pelecinus* with a high degree of promiscuity (Nandasena et al. 2006, 2009; Vicente et al. 2009). The novel biovar assigned for *B. pelecinus, Mesorhizobium ciceri* biovar *biserrulae*, differs from *Mesorhizobium ciceri* by significant differences in growing conditions, antibiotic resistance and carbon source utilisation, as well as in similarity between symbiotic genes (Nandasena et al. 2009). Moreover, Nandasena et al. (2009) described two novel species belonging to the genus *Mesorhizobium*, *Mesorhizobium australicum* sp. nov. and *Mesorhizobium opportunistum* sp. nov.

Biological nitrogen fixation (BNF), the process by which rhizobia convert atmospheric nitrogen (N_2) into a plant-usable form, is influenced by biotic and abiotic constraints (e.g. soil acidity and salinity, or use of N fertilizers) that affect either rhizobia, the plant host or their symbiotic interaction (Bohlool et al. 1992). Low pH and high salinity were found to negatively influence the colonisation and infection of rhizobia by affecting the expression of nodulation genes (Ferguson and Gresshoff 2015; Plá and Libertad 2015). Additionally, acidity and salinity were also found to increase heavy metal availability (McLaughlin et al. 1994; Giller et al. 1998), which is highly toxic, even at low concentrations (Giller and Cadisch 1995, to both free-living N_2-fixing organisms and rhizobia; for a complete review,

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see Giller et al. 1998). In legume–rhizobial systems, BNF can be also suppressed by mineral fertilization (Waterer et al. 1994), though a wide variation in rhizobia tolerance to ammonium or nitrate in soil (Sprent 1999) is observed. Curiously, some actinorhizal symbioses (i.e. *Frankia*) have a weak or no feedback regulation from inorganic N in the environment, even when N availability is high (Vitousek et al. 2013). BNF inhibition in legume–rhizobia systems varies depending on the form of N applied (Dart and Wildon 1970), season (Pate and Dart 1961), light intensity, temperature and concentration (Gibson 1971) and other environmental factors (Pankhurst 1981). In temperate pastureland, the application of low rates of N fertilizer promotes nodulation and, consequently, an early and rapid crop growth (Ledgard et al. 2001; Namvar and Sharifi 2011). Still, accumulation of excessive N in soils contribute to diminish rhizobial diversity, unless resistant strains are isolated and used in the recovery the microbiota of heavily fertilized soils (Herridge and Danso 1995). The main aims of this study were (1) to characterise native root-nodule bacteria from *B. pelecinus* in terms of *in vitro* tolerance to three main stressful conditions of ecosystems (acidity; salinity and heavy metals); and (2) to evaluate the BNF of selected rhizobia isolates under fertilization as well as their resistance to elevated concentrations of N. From an ecological point of view, we hypothesised that the growing phenotype of root-nodule bacteria (i.e. fast-, moderate- and slow-growers) associated with *B. pelecinus* may affect adaptation to the abiotic environment. Our results show that slow-growers fixed efficiently N_2 in the presence of metal contaminants and N fertilization, which translated into a greater production of plant biomass.

Materials and methods

Bacterial isolation and characterisation

Forty-two native root-nodule bacteria with different rep-PCR (repetitive element palindromic PCR) fingerprints (PCR amplification of repetitive sequences within bacteria genome) (De Bruijn 1992), which have surveyed in geographically distinct locations across northern Spain and southern Portugal (Vicente et al. 2009), were used in this study. Pure cultures of each root-nodule bacteria were visually characterised according to time of growth (days), visual assessment of production of extracellular polysaccharide (EPS) in yeast extract-mannitol (YEM) and colony morphology (colour/opacity, size, shape, elevation and margins). All isolates were maintained in YEM slants at 4 °C for routine use, and −80 °C for long-term conservation with 20% glycerol. The ability of root-nodule bacteria to change pH medium was determined by using YEM supplemented with 25 µg of bromothymol blue (Frioni 1999). The mean doubling time (t_D) of each *B. pelecinus* root-nodule isolate was estimated through growth curves in YEM broth at 28 °C and 120 rpm, and time-point OD_{600} measures. The t_D was determined by the inverse of the slope of $\log_2$ OD plotted in function of time.

Tolerance to different abiotic stresses

Tolerance to pH 4.5, 5.5 and 9.5 was evaluated according to Oliveira and Magallhães (1999). A single colony of a fresh culture (5 days old) was streaked onto YEM with pH adjusted from 4.5 to 9.5 (1 M HCl for acid pH and 1 M NaOH for alkali). The YEM pH 7 was used as control. Tolerance to salinity was evaluated by streaking rhizobial isolates in YEM supplemented with 0.5%, 2.5% and 5% NaCl (w/v). The ability of root-nodule bacteria to grow in the presence of metals was assessed by the agar dilution method (Hayat et al. 2002). Aluminium ($AlCl_3$) and manganese ($MnCl_2$), known metals in agricultural lands (Kochian et al. 2004), were tested at concentrations ranging between 50 and 1000 µM. Metal solutions were sterilised separately from YEM, incorporated at 45°C and poured onto the plates. Each plate was divided into four equal sectors. Growth of root-nodule bacteria was carried out in YEM at 28 °C and 120 rpm for 48 h. A single drop (10 µl aliquot) was inoculated in each sector of the plate. A control plate was prepared without metals added. After 7 days of incubation at 28 °C, the diameter of the drop was measured. The ratio between the growth area in metal treatment (*At*) and the growth area in control (*Ac*) was calculated as an indicator of the tolerance level of root-nodule isolates. The minimum inhibitory concentration (MIC) was determined as the smallest concentration necessary to inhibit total bacteria growth. A three-system category was also used to classify bacterial growth: (1) sensitive (acidity and salinity: 0–2.00; heavy metals: $At/Ac < 1$); (2) moderate tolerance (acidity and salinity: 2.06–3.00; heavy metals: $1.00 \geq At/Ac > 1.50$ and (3) tolerant (acidity and salinity: 3.06–4.00; heavy metals: $1.50 \geq At/Ac > 2.00$). For all tests, three biological replicates were carried out.

Estimation of symbiotic N_2-fixation

Symbiotic effectiveness of *B. pelecinus* root-nodule isolates was tested under controlled greenhouse conditions (average temperature of 20 ± 2 °C; 80% humidity and 14 h daylight and 10 h dark photoperiod). Three treatments were established: (1) N-symbiotic, *B. pelecinus* seedlings independently inoculated with each root-nodule isolate (n = 42); (2) *Mesorhizobium, B. pelecinus* inoculated with commercial reference strain *M. ciceri* biovar *biserrulae* and (3) reference plant *B. pelecinus* without inoculation. Three complete randomised blocks with seven seedlings each were established for each treatment.

Seeds of *B. pelecinus* cv. Casbah were routinely scarified and surface sterilised for 2 min in 70% (v/v) EtOH, 20 min in 10% (v/v) NaClO, followed by six rinses with sterile distilled water. Seeds were germinated in Petri dishes with 1.5% (w/v) agar and aseptically transplanted into pots. Plastic pots (0.2 dm^3), surface sterilised with 10% (v/v) NaClO, were filled up with steam-sterilised mixture of 2:1 soil–river sand, overlaying an autoclaved absorbent cloth to prevent rapid drainage and possible contaminations from running water. Inoculation of *B. pelecinus* seedlings took place after the emergence of the first pair of real leaves

(5 days after transplanting). Starter cultures of each root-nodule isolate were grown until stationary phase in YEM broth, incubated at 28 °C and 120 rpm. Each seedling received 4 ml of the appropriate bacterial inoculant. Pots were completely covered with sterile polyethylene beads (Aulabor Industries, S.A. Barcelona, Spain) to prevent airborne rhizobia contamination. Twice a week, seedlings were watered through a sterile watering pipe (Vincent 1970). Harvest was conducted 7 weeks after inoculation. Each single plant was thoroughly washed with tap water to remove substrate residues from the roots. Nodulation was scored as Nod^+ and Nod^-, respectively, depending on the presence or absence of nodules. Shoots were oven dried at 70 °C for 48 h, weighted in a precision balance and powdered with a mill ball IKA Labortechnik A10 (Janke & Kunkel GmbH & CO). Total nitrogen from shoot dry biomass was quantified by Kjeldahl technique (Guebel et al. 1991), carried out in the *Laboratório Químico Agrícola Rebelo da Silva* (Lisbon, Portugal). A sample of 2 mg shoot dry biomass was weighted into tin capsules and sent for isotope ratio mass spectrometry analysis (IRMS) (Brenna et al. 1997) at the University of the Balearic Islands, Balearic Islands, Spain.

The efficiency of each root-nodule bacteria was assessed by ^{15}N natural abundance methodology. For ^{15}N natural abundance, the fraction of N derived entirely from N_2 fixation (N_{dfa}) in the N_2-fixing plants (Högberg 1997) was calculated by $\%N_{dfa} = [(\delta^{15}N_{ref} - \delta^{15}N_{fix})/(\delta^{15}N_{ref} - B)] \times 100$, where $\delta^{15}N_{ref}$ is the $\delta^{15}N$ from a non-fixing N_2 reference plant, and B is the $\delta^{15}N$ from N_2-fixing plant when totally dependent on N_2 as the only N source. The B-value used was −3.53‰ calculated elsewhere (Vicente 2010). The total amount of N in the plant derived from N_2 fixation (N_{fix}) was determined by $N_{fix} = N_{dfa} \times$ N content.

Tolerance to mineral fertilization

Under controlled greenhouse conditions, five inoculation treatments (inoculation with AjuPt16, SafPt6, SafPt12, *M. ciceri* biovar *biserrulae* and N-free) were applied. Plants inoculated with the five inoculants were additionally treated with three fertilization levels (FL) (F0, no nutrients available; F25, moderate nutrient supply; and F75, high nutrient supply). All plants grew randomly located in a light, temperature and humidity growth chamber, with five plants per $T \times$ FL. FL ranged between 5 g per 10 dm^3 (F25) and 10 g per 10 dm^3 (F75) of 16N:7P:15K + 2MgO (Floranid® Permanent, Germany) slow-release fertilizer (doses selection based on manufacture recommendation). Seed surface-sterilisation and sowing of *B. pelecinus* cv. Casbah was prepared as above. Mesorhizobia inoculants were prepared as described in Vincent (1970). After 8 weeks of growth plants were harvested. Shoot and root biomass were oven-dried at 70 °C (48 h), and weighted. Shoot dry mass was milled with a ball mill (IKA Labortechnik A10, Janke & Kunkel GmbH & CO) for the analyses of total N from shoot dry-weight (SDW, mg $plant^{-1}$); N analyses were conducted at the Laboratório Químico Agrícola Rebelo da Silva, Lisbon, Portugal. Shoot N-content was determined by Shoot N = %N × SDW. Dry biomass samples were analysed for isotopic composition ($\delta^{15}N$) through IRMS at the Universidad de Las Islas Baleares, Islas Baleares, Spain.

Statistical analyses

Statistical analyses were made using the SPSS software version 15.0 for Windows. Homogeneity of variances was checked by Levene's test. Data (SDW, N content and N_{fix}) were subjected to analysis of variance (ANOVA) to test for differences in biomass production, ^{15}N and total N production among treatments and strains. A *post-hoc* Tukey's test at 95% confidence level was used after significant ANOVA.

Results

Tolerance to abiotic conditions

Phenotypic characterisation of all isolates is summarised in Table 1. All isolates were considered tolerant to severe acidity ($4.5 < pH < 5.5$), with the exception of isolate AjuPt9 (Table 1). Eight isolates were sensitive to pH 9.5 (MonPt2, MonPt8, TerPt9, SafPt1, ElvPt18, AjuPt11, AjuPt12, AjuPt21 and ArrPt20) (Table 1). *M. ciceri* biovar *biserrulae* grew in the whole pH range. The increase of NaCl concentrations resulted in a decrease of bacterial growth. At 0.5% NaCl, all root-nodule isolates grew abundantly with copious production of EPS, whereas no growth was recorded at 5% NaCl. Around 40% of *B. pelecinus* isolates were able to tolerate NaCl concentrations up to 2.5% (w/v) (Table 1). The ability of *B. pelecinus* root-nodule isolates to tolerate Al^{3+} and Mn^{2+} was screened at concentrations ranging from 50 to 1000 µM (Table 1). The cation that exerted the most severe effect on bacterial growth was Mn^{2+}, with a MIC of 50 µM. Nearly 43% of *B. pelecinus* isolates only resisted the concentration of 50 µM, five isolates could grow until 250 µM (fast growers MontPt16, and moderate growers TerPt6, AjuPt10, SafPt5 and ElvPt10), and only the slow-grower AjuPt8 could tolerate 500 µM. It was not possible to determine the tolerance of 17 isolates (i.e. ElvPt15) due to swarm-like phenomena (irregular bacterial growth in solid medium supplemented with Mn^{2+}). Regarding Al^{3+}, seven isolates (slow grower MonPt2; moderate growers TerPt9, MonPt10, AjuPt16, ArrPt11 and fast growers MonPt13, AjuPt19) were not able to grow in the presence of this cation. The most tolerant root-nodule bacteria were the fast growers SafPt1, ElvPt15 and MonPt8, growing in YEM supplemented with 500 µM Al^{3+}. *M. ciceri* biovar *biserrulae* presented a MIC_{Al} and MIC_{Mn} of 50 µM.

Table 1. Phenotypic characterisation of root-nodule bacteria from *Biserrula pelecinus* and the *Mesorhizobium* reference strain.

Root-nodule isolate		t_D	pH	NaCl (%w/v)	MIC Metals (μM)	
					Al^{3+}	Mn^{2+}
AjuPt1	Fast growth	0.92	4.5–9.5	2.5	50	ND
AjuPt12		1.26	4.5–7.0	0.5	50	50
MonPt16		1.40	4.5–9.5	0.5	250	250
ElvPt15		1.49	5.5–9.5	2.5	500	ND
ArrPt17		1.62	4.5–9.5	2.5	50	ND
SafPt1		1.72	5.5–7.0	0.5	500	ND
MonPt8		1.73	5.5–7.0	2.5	500	ND
AviSp1		1.75	4.5–9.5	2.5	50	50
ArrPt12		1.81	4.5–9.5	2.5	50	50
ArrPt16		1.89	4.5–9.5	2.5	50	50
AjuPt25		1.92	4.5–9.5	0.5	50	50
AjuPt27		1.97	4.5–9.5	2.5	50	50
ArrPt14		2.00	4.5–9.5	2.5	50	ND
AjuPt11		2.00	4.5–7.0	0.5	50	50
ArrPt20		2.01	5.5–7.0	2.5	50	ND
SafPt7		2.21	5.5–9.5	0.5	250	ND
ArrPt10		2.41	4.5–7.0	2.5	500	ND
AjuPt30		2.44	4.5–9.5	2.5	50	50
AjuPt13		2.68	4.5–9.5	2.5	50	ND
AjuPt15		2.73	4.5–9.5	2.5	50	50
SafPt12		2.83	4.5–9.5	0.5	50	50
AjuPt19		3.04	7.0–9.5	0.5	0	50
ArrPt15		3.23	4.5–9.5	2.5	50–250	ND
MonPt13		3.57	4.5–9.5	2.5	ND	50
ElvPt17	Moderate growth	4.29	5.5–9.5	2.5	50	ND
AjuPt16		4.31	4.5–9.5	2.5	ND	50
TerPt6		4.31	4.5–9.5	2.5	250	250
ArrPt11		4.50	4.5–7.0	2.5	ND	50
MonPt10		4.56	4.5–9.5	0.5	ND	ND
Mesorhizobium		5.38	4.5–9.5	2.5	50	50
AjuPt10		5.41	4.5–9.5	2.5	50	250
TerPt9		5.65	5.5–7.0	2.5	ND	ND
SafPt6		5.67	4.5–9.5	0.5	50	ND
AjuPt21		6.18	5.5–7.0	2.5	50	50
ElvPt18		6.46	5.5–7.0	2.5	50	50
SafPt5		6.74	4.5–9.5	0.5	500	250
TerPt7		6.88	4.5–9.5	2.5	50	50
TerPt19		7.39	4.5–9.5	2.5	250	ND
TerPt17		7.56	5.5–7.0	0.5	50	50
ElvPt10		8.05	4.5–9.5	2.5	250	250
SafPt8		8.31	5.5–9.5	2.5	50	50
MonPt2	Slow growth	12.47	5.5–7.0	0.5	ND	ND
AjuPt8		12.50	4.5–9.5	0.5	50	500

The characteristics analysed are mean doubling time (tD) to evaluate growth ability (fast growth, t_D = 0.92–3.57; moderate growth, t_D = 4.29–8.31; and slow growth, t_D = 12.47–12.50), and tolerance levels under abiotic conditions [salinity (NaCl), pH and heavy metals (Al^{3+} and Mn^{2+})]. Tolerance levels not determined are indicated by ND. MIC indicates minimal inhibitory concentration determined as smallest concentration necessary to inhibit total bacteria growth.

Estimation of symbiotic N_2 fixation

All isolates were NOD^+, forming indeterminate shape nodules in *B. pelecinus* seedlings (data not shown). No nodules were present in the control treatment (reference plant *B. pelecinus* without inoculation). The $\delta^{15}N_{ref}$ for the reference plant was 7.160‰ (Table 2). According to ^{15}N natural abundance, 32% of the isolates showed poor N_2 fixation (N_{dfa} lower than 50%). The best N_2 fixers (SafPt8, SafPt5, AjuPt21, AjuPt16, SafPt6 and SafPt12) reached $\delta^{15}N$ value between 0.03‰ and −0.60‰, and $\%N_{dfa}$ between 67% and 73%, respectively. Highly significant differences ($P < 0.01$) between these isolates were observed in terms of SDW, N content and N_{fix} (Table 3). *B. pelecinus* plants inoculated with *Mesorhizobium* strain had an SDW average of 70 mg $plant^{-1}$, with a %N of 3.36, resulting in a value of N_{fix} of 2.35 mg^{-}(Table 3). SafPt12 and SafPt6 rhizobial isolates produced high values of SDW and N_{fix}, respectively, an SDW average of

Table 2. Estimation of biological nitrogen fixation (BNF), using ^{15}N natural abundance.

Root-nodule isolate	SDW (mg plant^{-1}) (±SD)	($\delta^{15}N/\delta^{14}N$)‰ (±SD)	N_{dfa} (%)
TerPt9	31.40 (10.00)	7.05 (0.19)	1
MonPt10	45.10 (10.00)	6.18 (0.18)	9
AjuPt11	45.80 (30.00)	6.23 (0.18)	9
MonPt16	42.60 (10.00)	6.08 (0.18)	10
ElvPt10	55.00 (30.00)	5.79 (0.29)	13
MonPt13	41.20 (10.00)	5.41 (0.18)	16
AviSp1	64.40 (30.00)	4.60 (0.23)	24
AjuPt8	53.60 (40.00)	4.56 (0.25)	24
ElvPt15	64.30 (30.00)	4.12 (0.20)	28
AjuPt30	33.20 (40.00)	3.35 (0.18)	36
AjuPt1	41.60 (30.00)	2.62 (0.30)	42
AjuPt15	52.70 (40.00)	2.72 (0.18)	42
ArrPt10	52.30 (70.00)	2.32 (0.18)	45
AjuPt27	28.80 (20.00)	1.92 (0.18)	49
ArrPt20	44.30 (20.00)	1.84 (0.19)	50
ElvPt18	55.30 (20.00)	1.63 (0.18)	52
ArrPt11	25.10 (20.00)	1.64 (0.18)	52
ArrPt14	26.20 (10.00)	1.57 (0.18)	52
AjuPt19	23.40 (10.00)	1.49 (0.18)	53
TerPt17	37.60 (20.00)	1.51 (0.19)	53
ElvPt17	50.30 (30.00)	1.37 (0.19)	54
ArrPt15	26.80 (10.00)	1.26 (0.19)	55
TerPt19	27.00 (10.00)	1.24 (0.19)	55
MonPt2	47.80 (0.00)	1.16 (0.23)	56
TerPt7	39.40 (20.00)	0.90 (0.25)	59
ArrPt12	41.00 (10.00)	0.73 (0.18)	60
ArrPt16	20.60 (10.00)	0.79 (0.19)	60
SafPt7	28.90 (20.00)	0.65 (0.19)	61
AjuPt10	26.40 (20.00)	0.49 (0.18)	62
AjuPt25	31.50 (20.00)	0.52 (0.18)	62
ArrPt17	21.60 (20.00)	0.44 (0.19)	63
TerPt6	28.70 (10.00)	0.36 (0.25)	64
AjuPt12	23.60 (10.00)	0.20 (0.18)	65
SafPt1	29.70 (20.00)	0.17 (0.25)	65
MonPt8	57.20 (20.00)	0.10 (0.25)	66
AjuPt13	35.90 (20.00)	0.14 (0.18)	66
AjuPt16	58.50 (20.00)	−0.04 (0.25)	67
AjuPt21	50.80 (20.00)	0.03 (0.18)	67
SafPt8	30.30 (10.00)	−0.22 (0.18)	69
SafPt5	29.90 (10.00)	−0.48 (0.18)	71
SafPt6	53.80 (10.00)	−0.41 (0.19)	71
SafPt12	64.50 (30.00)	−0.60 (0.18)	73
Mesorhizobium	70.00 (40.00)	−3.53 (0.19)	100
Reference plant	17.40 (0.00)	7.16 (0.19)	0

BNF: Biological nitrogen fixation; N_{dfa}: nitrogen derived entirely from BNF; SDW: shoot dry-weight; SD: standard deviation. Bacterial isolates in bold indicate best BNF fixers according to ^{15}N natural abundance.

64.50 mg plant^{-1} and a N_{fix} of 1.41 mg; and an SDW average of 53.80 mg plant^{-1}and a N_{fix} of 1.24 mg.

Tolerance to mineral fertilization

The selected root-nodule bacteria (SafPt6, AjuPt16, SafPt12) and *Mesorhizobium* commercial strain were tested for tolerance to mineral fertilization (Table 4). Increasing N fertilization resulted in better developed plants in all treatments, in comparison with F0. In F25 and F75, no statistical differences were observed in parameter LSA (leaf surface area) between the plants from different inoculation treatments and N-free control plants. The increase in the level of nutrients resulted in an increase of SDW, shoot N content and number of nodules (NN). Differences among treatments (without inoculation, N-free; with inoculation, SafPt6, AjuPt16, SafPt12 and *Mesorhizobium*) were observed in regimes F25 and F75, for those parameters. NN increased in treatments with SafPt12, SafPt6 and *Mesorhizobium* under F25 and F75 treatments, while nodules dry-weight (NDW) was maintained, suggesting thus the increase of small-size nodules. *B. pelecinus* seedlings inoculated with AjuPt16 were poorly nodulated under any of the nutrient regimes. No nodules were presented in the control plants (N-free plants) indicating absence of airborne contamination during the experimental period.

Table 3. Shoot dry-weight (SDW) N concentration (%) and amount of N_2 fixed (N_{fix}) in seedlings of *Biserrula pelecinus* inoculated with root-nodule isolates AjuPt16, AjuPt21, SafPt5, SafPt6, SafPt8, SafPt12 and *Mesorhizobium* reference strain. SDW: Shoot dry-weight; N_{fix}: N_2 fixed; SD: standard deviation. Different letters indicate significant differences between isolates after Tukey's test ($P < 0.05$).

Treatments	SDW (mg plant^{-1} ± SD)	N (%)	Nfix (mg N SDW^{-1})
SafPt8	30.10 (10.00)^{b}	2.64 (0.00)^{g}	0.55 (0.14)^{b}
SafPt5	29.90 (10.00)^{b}	2.89 (0.00)^{f}	0.62 (0.25)^{b}
AjuPt21	50.80 (20.00)^{ab}	2,32 (0,00)^{e}	0.79 (0.34)^{ab}
AjuPt16	58.50 (20.00)^{a}	2.66 (0.00)^{d}	1.05 (0.32)^{ab}
SafPt6	53.80 (10.00)^{ab}	3.26 (0.00)^{c}	1.24 (0.14)^{ab}
SafPt12	64.50 (30.00)^{a}	3.01 (0.00)^{b}	1.41 (0.60)^{b}
Mesorhizobium	70.00 (30.00)^{a}	3.36 (0.00)^{a}	2.35 (1.30)^{a}

The effects of the different levels of fertilization (F0, F25 and F75) on BNF of the isolates tested were evaluated using ^{15}N natural abundance (Figure 1). Inoculated seedlings reduced their N_2 fixation as the level of nutrients increased from F25 to F75, thus indicating an effect on the N_2 fixing apparatus. Both $\%N_{dfa}$ and N_{fix} were highly influenced by treatments and nutrient regimes ($P \leq 0.01$) (Figure 1). *M. ciceri* biovar *biserrulae* was the best fixing strain at F0 and F25 (55% and 36%, respectively) (Figure 1). SafPt12 isolate was the second best fixing strain (%Ndfa between 47% in F0 and 26% in F25), followed by SafPt6 (F0, 44%; F25, 18%). The increased fertilization drastically reduced or even suppressed the $\%N_{dfa}$ in all seedlings treated with any of the isolates. However, in the intermediate FL (F25), all isolates and *M. ciceri* biovar *biserrulae* increased in fourfold the percentage of N fixed compared with that obtained in F0 treatment.

Discussion

Legume-rhizobia systems contribute to the sustainable development of healthy terrestrial ecosystems (Vitousek et al. 2013). The long evolutionary history of these

Table 4. Effect of fertilization levels in seedlings of *Biserrula pelecinus* inoculated with root-nodule isolates AjuPt16, SafPt6, SafPt12 and *Mesorhizobium* reference strain. LSA: Leaf surface area; SDW: shoot dry-weight; N content: leaf nitrogen content; NN: number of nodules; NDW: nodules dry-weight. Different letters indicate significant differences between isolates after Tukey's test ($P < 0.05$).

Regime	Treatments	LSA (mm^2)	SDW (mg $plant^{-1}$ ± SD)	N content (mg $plant^{-1}$ ± SD)	NN	NDW (mg $nodule^{-1}$ ± SD)
	SafPt12	18.53 (1.87)^{a}	48.233 (23.792)^{a}	1.56 (0.03)^{a}	4 (2.3)^{a}	4 (2.8)^{a}
	SafPt6	16.24 (7.29)^{a}	55.907 (34.122)^{a}	1.77 (1.11)^{a}	5 (2.9)^{a}	5 (4.4)^{a}
F0	AjuPt16	1.44 (0.63)^{b}	5.407 (1.767)^{b}	0.11 (0.04)^{b}	3 (1.7)^{b}	1 (1.7)^{b}
	Mesorhizobium	17.04 (6.31)^{a}	52.840 (20.209)^{a}	1.77 (0.68)^{a}	5 (2.2)^{a}	6 (3.6)^{a}
	N-free	1.51 (0.63)^{b}	7.239 (1.629)^{b}	0.15 (0.03)^{b}		
	SafPt12	48.27 (18.91)^{a}	158.927 (51.060)^{b}	6.20 (2.14)^{a}	15 (4.5)^{b}	4 (1.8)^{a}
	SafPt6	54.00 (15.44)^{a}	176.187 (53.225)^{a}	6.27 (1.77)^{a}	17 (4.8)^{b}	5 (2.4)^{a}
F25	AjuPt16	51.46 (21.26)^{a}	160.327 (56.401)^{ab}	5.66 (2.25)^{ab}	6 (4.0)^{c}	6 (2.6)^{a}
	Mesorhizobium	46.68 (17.00)^{a}	173.853 (49.143)^{ab}	5.82 (1.72)^{ab}	23 (10.4)^{a}	8 (2.8)^{a}
	N-free	47.09 (13.89)^{a}	157.080 (31.183)^{b}	4.24 (1.33)^{b}		
	SafPt12	72.01 (30.95)^{a}	203.120 (36.593)^{b}	8.24 (1.30)^{ab}	14 (8.0)^{c}	3 (2.5)^{a}
	SafPt6	71.91 (23.95)^{a}	236.587 (54.544)^{ab}	8.82 (1.62)^{ab}	21 (11.2)^{b}	4 (2.8)^{a}
F75	AjuPt16	70.59 (28.64)^{a}	209.300 (74.763)^{b}	7.53 (1.95)^{b}	5 (3.0)^{d}	5 (2.5)^{a}
	Mesorhizobium	71.64 (30.36)^{a}	278.400 (50.436)^{a}	9.79 (2.07)^{a}	30 (9.7)^{a}	5 (2.8)^{a}
	N-free	81.42 (41.36)^{a}	258.853 (88.711)^{ab}	8.93 (2.15)^{ab}		
F_{regime}		0.679	346.866**	445.527**	83.689**	3.542*
$F_{treatments}$		166.594**	5.648**	7.683**	124.869**	2.021
$F_{regime \times treatments}$		1.421	2.887**	2.652**	14.186**	3.561**

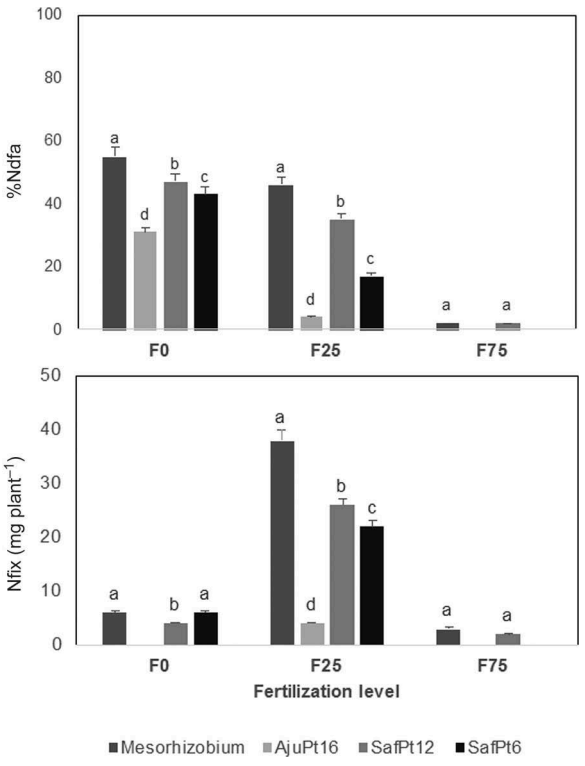

Figure 1. Estimation of nitrogen derived from biological N_2 fixation ($\%N_{dfa}$) and N_2 fixed (N_{fix}) in three N-fertilization levels (F0, no fertilization; F25, medium fertilization and F75, high-fertilization level) in seedlings of *Biserrula pelecinus* inoculated with root-nodule isolates AjuPt16, SafPt6, SafPt12 and *Mesorhizobium* reference strain. Different letters indicate significant differences between isolates after Tukey's test ($P < 0.05$).

symbiotic relationships reflects a highly specialised adaptation of bacteria to the constrictions imposed by the environment (Young 2006). In the present study, our main aim was to characterise native root-nodule bacteria from *B. pelecinus* from the Iberian Peninsula, and the selection of suitable N-fixers for field-application and soil restauration. All *B. pelecinus* root-nodule bacteria have presented similar phenotypic characteristics of a rhizobia culture, with different generation times ranging within the interval referred to this genus (Maâtallah et al. 2002; Chen et al. 2005; Nandasena et al. 2007). They were able to grow at 0.5–2.5% (w/v) NaCl, decreasing severely at 5% (w/v) NaCl. Previous results showed that *B. pelecinus* symbionts could withstand 1.5% (w/v) NaCl but not at 2.0% (w/v) NaCl (Nandasena et al. 2007), displaying sensitivity to salinity similar to that of its plant host (Nichols et al. 2007). Also, almost all bacteria were able to grow at severe acidity (pH 4.5 and 5.5), as shown also by Howieson et al. (1995). This result was expected since the majority of these isolates came from soils with low pH therefore showing a relatively high proportion of tolerance to acidity in their native environments (Vicente et al. 2009). *M. ciceri* was also found to be moderate acidophilic (Brígido et al. 2007). The results from extreme alkalinity conditions (pH 9.5) showed that, at least, 75% *B. pelecinus* isolates were also capable to grow copiously as previously seen in other strains from the same plant (Howieson et al. 1995; Nandasena et al. 2007). The majority of the isolates were sensitive to Al^{3+} and Mn^{2+}, with a MIC of 50 μM for both metals. These results are

consistent with others that have reported Al^{3+} as the most stressful metal for rhizobial growth (Murphy et al. 1984; Vargas and Graham 1988; Roy and Chakrabartty 2000). The study of the effects of Mn^{2+} on rhizobia growth dates back 1938, when Steinberg first reported that the absence of Mn^{2+} and other microelements reduced growth of *Rhizobium trifolii*, and similarly, when it was applied at a concentration of 10 µM Mn^{2+} the growth of *Rhizobium meliloti*, *Rhizobium japonicum* and *Rhizobium phaseoli* was considerably increased (Wilson and Reisenauer 1970). The tolerance to Mn^{2+} for 17 *B. pelecinus* bacteria isolates was not determined due to swarm-like phenomena as reported by Kearns (2010). Swarming phenomena in N_2-fixing bacteria has been poorly characterised (Tambalo et al. 2010), and hypothesised to play a role in bacteria colonisation of natural environments (Salvetti et al. 2009), regulated by quorum sensing mechanisms (Daniels et al. 2004). Adverse conditions may stimulate swarming motility in search of a new condition, less harmful for bacterial establishment (Daniels et al. 2004). The swarming effect of only these strains could indicate an avoidance behaviour in the presence of Mn^{2+}. More studies are needed to investigate this observation.

A correct evaluation of symbiotic effectiveness of root-nodule bacteria is critical in the selection of strains for the development of elite inoculants to be used in sustainable agriculture (Slattery and Pearce 2002; Crews and Peoples 2004; Álvarez et al. 2014). In this study, ^{15}N natural abundance approach was used to determinate N_2 fixation. Relatively few isolates were considered to efficiently fix N, emphasising the extremely specific relationship between *B. pelecinus* and its micro-symbiont (Howieson et al. 1995; Nandasena et al. 2004). The *B*-value, $\delta^{15}N$ from N_2 fixing-plant when totally dependent on N_2 as the only N source, was determined as −3.53‰ (Vicente 2010) and the N_{dfa} (the fraction of N derived entirely from N_2 fixation) estimated from the more efficient *B. pelecinus* root-nodule bacteria ranged between 67% and 73%. The difference between $\delta^{15}N$ in the reference crop ($\delta^{15}N_{ref}$ = 7.16‰) and *B-value* ($\delta^{15}N$ = −3.53‰) was more than five $\delta^{15}N$ units, ensuring the other precondition for NA application (Högberg 1997). The *Mesorhizobium* reference strain and isolates SafPt12, SafPt6 and AjuPt16 presented an effectiveness index ranging between 60 and 87% (Ferreira and Marques 1992). In terms of $\%N_{dfa}$, SafPt12 reached 73% with 1.41 mg N SDW^{-1} of N fixed, followed by SafPt6 with a $\%N_{dfa}$ of 71% (N_{fix} = 1.24 mg N SDW^{-1}) and AjuPt16 with a $\%N_{dfa}$ of 67% (N_{fix} = 1.05 mg N SDW^{-1}). There is no information regarding the application $\delta^{15}N$ natural abundance to the pasture legume *B. pelecinus*. For other legumes such as *Medicago sativa, Trifolium pratense* and *Trifolium repens*, Carlsson (Carlsson and Huss-Danell 2003; Carlsson 2005; Carlsson et al. 2006) reported a large proportion of N gained from N_2 fixation (high N_{dfa}) under glasshouse conditions and field studies, usually more than 80%.

Differences in the fixing ability between fast- and slow-growing types of rhizobia have been reported (Trinick 1968; Pérez-Fernández et al. 2015), mostly related with competitive abilities to occupy nodules. In general terms, we observed that slow-to-moderate growers had greater tolerance to stressful conditions, and were among the ones fixing greater amounts of N_2. This is in accordance with observations reported on the sensitivity to low pH. Fast growers were uniformly sensitive to the low pH and tolerant of the high pH, whereas the reverse occurs for slow growers (Sadowsky et al. 1983). On the other hand, it has been found that fast-growing *Sinorhizobium* strains were more resistant to Al, Mo and Fe than slow growers of *Bradyrhizobium* (Flis et al. 1992; Arora et al. 2009). Our study has shown that fast-growing rhizobia vary in their tolerance to metals and salinity, as well as in their tolerance to high levels of fertilization. From an ecological point of view, we believe that slow-growing strains from *B. pelecinus* are both more tolerant to metal contaminants and excessive fertilization and they are better able to efficiently fix N_2, which, in turn, translates into a greater plant biomass production.

The effect of combined N fertilization in BNF of rhizobia associated with *B. pelecinus* was studied under greenhouse conditions. The increase in the level of fertilization resulted in an increase of SDW, shoot N content and NN and statistical differences were seen between treatments (without inoculation, N-free; with inoculation, SafPt6, AjuPt16, SafPt12 and *Mesorhizobium*). NN increased for SafPt12, SafPt6 and *Mesorhizobium* under F25 and F75 treatments, while NDW was maintained, thus proving that low N can trigger *B. pelecinus* nodulation and biomass production (Ledgard et al. 2001; Mrkovacki et al. 2008). The increase on the level of fertilization led to a drastic decrease of $\%N_{dfa}$ and N_{fix}. However, in the medium-FL (F25), all isolates and *M. ciceri* biovar *biserrulae* presented a higher N fixed percentage, almost fourfold of the N fixed in F0, again indicating the triggering effect of the F25 mineral concentration. These small amounts of starter N are required to satisfy the N demands of plants during the period before the nodules are developed to supply plant needs. Simultaneously, fertilization improved the physical proprieties of the soil, promoting higher uptake by the crop increasing dry matter and seed yield (Basu et al. 2008) (Table 4).

B. pelecinus root-nodule bacteria SafPt12, SafPt6 and AjuPt6 were considered suitable N_2-fixers when compared with the *Mesorhizobium* reference strain, encouraging further investigation in their ability to tolerate and to promote legume growth under stressful conditions, characteristic from the pasturelands of the Mediterranean systems.

Conclusions

Our results are instrumental in defining management practices in which *B. pelecinus* may be used for restoration of soil fertility. The tolerance of native *B. pelecinus* root-nodule bacteria to abiotic stresses indicates the ecological competence of these bacteria, their ability to persist under

adverse edaphic conditions, to migrate within the soil and to colonise the soil from the rhizosphere of the plant host. Such ability could be related to growth phenotype of the bacteria, although further studies are needed.

Acknowledgements

This work was supported by the doctoral research grant (SFRH/BD/17252/2004) from the Portuguese Foundation for Science and Technology (FCT).

Disclosure statement

No potential conflict of interest was reported by the authors.

Funding

This work was supported by The Portuguese Foundation for Science and Technology (FCT) [SFRH/BD/17252/2004].

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Cláudia Vicente's main research interests are related to environmental microbiology, mostly the characterisation of microbial communities associated with agricultural and forest soils, and research on their biotechnological potential.

María Pérez-Fernández's main research interests include plant–soil–microorganisms interactions in forests along altitudinal and latitudinal gradients, evaluating the effect of climate change in the dynamics of the soil microorganisms, their activities and how they modulate tree establishment and putative changes in the tree-line.

References

Álvarez R, Steinbach HS, De Paepe JL. 2014. A regional audit of nitrogen fluxes in pampean agroecosystems. Agriculture, Ecosystems & Environment. 184:1–8.

Arora NK, Khare E, Singh S, Maheshwari DK. 2009. Effect of Al and heavy metals on enzymes of nitrogen metabolism of fast and slow growing rhizobia under explanta conditions. World Journal of Microbiology & Biotechnology. doi:10.1007/s11274-009-0237-6

Basu M, Bhadoria PB, Mahapatra SC. 2008. Growth, nitrogen fixation, yield and kernel quality of peanut in response to lime, organic and inorganic fertilizer levels. Bioresource Technology. 99:4675–4683.

Bohlool BB, Ladha JK, Garrity DP, George T. 1992. Biological nitrogen fixation for sustainable agriculture: A perspective. Plant and Soil. 141:1–11.

Brenna JT, Corso TN, Tobias HJ, Caimi RJ. 1997. High-precision continuous-flow isotope ratio mass spectrometry. Mass Spectrometry Reviews. 16:227–258.

Brígido C, Alexandre A, Laranjo M, Oliveira S. 2007. Moderately acidophilic Mesorhizobia isolated from chickpea. Letters in Applied Microbiology (*Cicer arietinum* L.) 44:168–174.

Carlsson G. 2005. Input of nitrogen from N_2 fixation to Northern grasslands [PhD thesis]. [Umeå Sweden]: Swedish University of Agricultural Sciences.

Carlsson G, Huss-Danell K. 2003. Nitrogen fixation in perennial forage legumes in the field. Plant and Soil. 253:353–372.

Carlsson G, Palmborg C, Huss-Danell K. 2006. Discrimination against ^{15}N in the three N_2-fixing *Trifolium* species as influenced by *Rhizobium* strain and plant age. Acta Agriculturae Scandinavica, Section B – Soil & Plant Science. 56:31–38.

Chen WX, Wang ET, Keuykendall D. 2005. Genus VI, *mesorhizobium*. In: Don J, Krieg NR, Stalyey JT, editors. Bergey's manual of systematic bacteriology. 2nd ed., Vol. II. New York (NY): Springer.

Crews TE, Peoples MB. 2004. Legume versus fertilizer sources of nitrogen: ecological tradeoffs and human needs. Agriculture, Ecosystems & Environment. 102:279–297.

Daniels R, Vanderleyden J, Michiels J. 2004. Quorum sensing and swarming migration in bacteria. FEMS Microbiology Reviews. 28:261–289.

Dart PJ, Wildon DC. 1970. Nodulation and nitrogen fixation by Vigna sinensis and Vicia atropurpurea: The influence of concentration, form, and site of application of combined nitrogen. Australian Journal of Agricultural Research. 21:45–65.

De Bruijn FJ. 1992. Use of repetitive (repetitive extragenic palindromic and enterobacterial repetitive intergeneric consensus) sequences and the polymerase chain reaction to fingerprint the genomes of *Rhizobium meliloti* isolates and other soil bacteria. Applied and Environmental Microbiology. 58:2180Y2187.

Ferguson BJ, Gresshoff PM. 2015. Physiological implications of legume nodules associated with soil acidity. In: Sulieman S, Than PLS, editors Legume Nitrogen Fixation in a changing environment – achievements and challenges. Switzerland: Springer International Publishing. p. 113–125.

Ferreira EM, Marques JF. 1992. Selection of Portuguese *Rhizobium leguminosarum* bv. *trifolii* strains for production of legume inoculants. Plant and Soil. 147:151–158.

Flis SE, Glen AR, Dilworth MJ. 1992. The interaction between aluminium and root nodule bacteria. Soil Biology and Biochemistry. 13:403–417.

Frioni L. 1999. Procesos microbianos, Tomo I y II. Argentina: Fundación de la Universidad Nacional de Río Cuarto. p. 282–286.

Gibson AH. 1971. Factors in the physical and biological environment affecting nodulation and nitrogen fixation by legumes. Plant and Soil. 35:139–152.

Giller KE, Cadisch G. 1995. Future benefits from biological nitrogen fixation: an ecological approach to agriculture. Plant and Soil. 174:255–277.

Giller KE, Witter E, McGrath SP. 1998. Toxicity of heavy metals to microorganisms and microbial processes in agricultural soils: a review. Soil Biology and Biochemistry. 30:1389–1414.

Guebel DV, Nudel BC, Giulietti AM. 1991. A simple and rapid micro-Kjeldahl method for total nitrogen analysis. Biotechnology Techniques. 5:427–430.

Hayat S, Ahmad I, Azam ZM, Ahmad A, Inam A, Samiullah S. 2002. Effect of long-term application of oil refinery wastewater on soil health with special reference to microbiological characteristics. Bioresource Technology. 84:159–163.

Herridge DF, Danso SKA. 1995. Enhancing crop legume N_2 fixation through selection and breeding. Plant and Soil. 174:51–82.

Högberg P. 1997. Tansley Review No. 95. ^{15}N natural abundance in soil-plant systems. New Phytologist. 137:179–203.

Howieson JG, Loi A, Carr SJ. 1995. *Biserrula pelecinus* L.-a legume pasture species with potential for acid, duplex soils which is nodulated by unique root-nodule bacteria. Australian Journal of Agricultural Research. 46:997–1009.

Kearns D. 2010. A field guide to bacterial swarming motility. Nature Reviews – Microbiology. 8:634–644.

Kochian LV, Hoekenga OA, Piñeros MA. 2004. How do crop plants tolerate acid soils? Mechanisms of aluminum tolerance and phosphorous efficiency. Annual Review of Plant Biology. 55:459–493.

Ledgard SF, Sprosen MS, Penno JW, Rajendram GS. 2001. Nitrogen fixation by white clover in pastures grazed by dairy cows: Temporal variation and effects of nitrogen fertilization. Plant and Soil. 229:177–187.

Loi A, Howieson JG, Cocks PS, Carr SJ. 1999. Genetic variation in populations of two Mediterranean annual pasture legumes (Biserrula pelecinus L. and Ornithopus compressus L.) and associated rhizobia. Australian Journal of Agricultural Research. 50:303–313.

Maâtallah J, Berraho EB, Muñoz S, Sanjuán J, Lluch C. 2002. Phenotypic and molecular characterization of chickpea rhizobia isolated from different areas of Morocco. Journal of Applied Microbiology. 93:531–540.

Malo JE, Suárez F. 1995. Cattle dung and the fate of *Biserrula pelecinus* L. (Leguminosae) in a Mediterranean pasture: seed dispersal, germination and recruitment. Botanical Journal of the Linnean Society. 118:139–148.

McLaughlin MJ, Palmer LT, Tiller KG, Beech TA, Smart MK. 1994. Increased soil salinity causes elevated cadmium concentrations in field grown potato tubers. Journal of Environmental Quality. 23:1013–1018.

Mrkovacki N, Marnkovic J, Acimorvic R. 2008. Effect of N fertilizer application on growth and yield of inoculated soybean. Notulae Botanicae Horti Agrobotanici Cluj-Napoca. 36:48–51.

Murphy HE, Edwards DG, Asher CJ. 1984. Effects of aluminium on nodulation and early growth of four tropical pasture legumes. Australian Journal of Agricultural Research. 35:663–673.

Namvar A, Sharifi RS. 2011. Phenological and morphological response of chickpea (*Cicer arietinum* L.) to symbiotic and mineral nitrogen fertilization. Zemdirbyste.98:121–130.

Nandasena K, O'Hara GW, Tiwari RP, Yates RJ, Kishinevsky BD, Howieson JG. 2004. Symbiotic relationships and root nodule ultrastructure of the pasture legume *Biserrula pelecinus* L. – A new legume in agriculture. Soil Biology and Biochemistry. 36:1309–1317.

Nandasena KG, O'Hara GW, Tiwari RP, Howieson J. 2006. Symbiotically diverse root-nodule bacteria able to nodulate *Biserrula pelecinus* L. emerge six years after introduction of this legume to Australia. Applied Environmental Microbiology. 72:7365–7367.

Nandasena KG, O'Hara GW, Tiwari RP, Willems A, Howieson JG. 2009. *Mesorhizobium australicum* sp. nov. and *Mesorhizobium opportunistum* sp. nov., isolated from *Biserrula pelecinus* L. in Australia. International Journal of Systematics and Evolutionary Microbiology. 59:2140–2147.

Nandasena KG, O'Hara GW, Tiwari RP, Yates RJ, Howieson JG. 2001. Phylogenetic relationships of three bacterial strains isolated from the pasture legume *Biserrula pelecinus* L. International Journal of Systematics and Evolutionary Microbiology. 51:1983–1986.

Nandasena KG, O'Hara GW, Tiwari RP, Willlems A, Howieson JG. 2007. *Mesorhizobium ciceri* biovar *biserrulae*, a novel biovar nodulating the pasture legume *Biserrula pelecinus* L. International Journal of Systematics and Evolutionary Microbiology. 57:1041–1045.

Nichols PGH, Loi A, Nutt BJ, Evans PM, Craig AD, Craig AD, Pengelly BC, Dear BS, Lloyd DL, Revell DK, et al. 2007. New annual and short-lived perennial pasture legumes for Australian agriculture-15 years of revolution. Field Crops Research. 104:10–23.

Oliveira LA, Magallhães HP. 1999. Quantitative evaluation of acidity tolerance of root nodule bacteria. Revista De Microbiologia. 30:203–208.

Pankhurst CE. 1981. Effect of plant nutrient supply on nodule effectiveness and *Rhizobium* strain competition for nodulation of *Lotus pedunculatus*. Plant and Soil. 60:325–339.

Pate JS, Atkins CA. 1983. Nitrogen uptake, transport and utilization in Nitrogen fixation. In: Broughton WJ, editor. Oxford: Legumes. Claredon Press. p. 245–298.

Pate JS, Dart PJ. 1961. Nodulation studies in legumes. IV, The influence of inoculum strain and time of application of ammonium nitrate on symbiotic response. Plant and Soil. 15:329–346.

Pérez-Fernández MA, Hill YJ, Calvo-Magro E, Valentine A. 2015. Competing Bradyrhizobia strains determine niche occupancy by two native legumes in the Iberian Peninsula. Plant Ecology. 216:1537–1549.

Perrineau MM, Le Roux C, De Faria SM, de Carvalho Balieiro F, Galiana A, Prin Y, Béna G. 2011. Genetic diversity of symbiotic Bradyrhizobium elkanii populations recovered from inoculated and non-inoculated Acacia mangium field trials in Brazil. Systematic and Applied Microbiology. 34:376–384.

Plá CL, Libertad CP. 2015. Salinity physiological impacts on legume nitrogen fixation. In: Sulieman S, Than PLS, editors Legume Nitrogen Fixation in a changing environment – achievements and challenges. Switzerland: Springer International. p. 35–655.

Roy N, Chakrabartty PK. 2000. Effect of aluminum on the production of siderophore by Rhizobium sp. (Cicer arietinum). Current Microbiology. 41:5–10.

Sadowsky MJ, Harold HK, Bohlool BB. 1983. Biochemical characterization of fast-and slow-growing rhizobial that nodulate soybeans. International Journal of Systematic Bacteriology. 33:716–722.

Salvetti S, Celandroni F, Ceragioli M, Senesi S, Ghelardi E. 2009. Identification of non-flagellar genes involved in swarm cell differentiation using a *Bacillus thuringiensis* mini T-10 mutant library. Microbiology. 155:912–921.

Slattery JF, Pearce DJ. 2002. Development of elite inoculant strains of *Rhizobium* in southeastern. In: Herridge D, editor. Inoculants and Nitrogen Fixation of legumes in Vietnam. proceedings of a workshop proceedings. ACIAR proceedings,no. 109e. Brisbane: PK Editorial Services. p. 86–94.

Sprent J. 1999. Nitrogen fixation and growth of non-crop legume species in diverse environments. Perspectives in Plant Ecology, Evolution and Systematics. 2:149–162.

Tambalo D, Yost C, Hynes M. 2010. Characterization of swarming motility in *Rhizobium leguminosarum* bv *viciae*. FEMS Microbiology Letters. 307:165–174.

Trinick MJ. 1968. Nodulation of tropical legumes I. specificity in the rhizobium symbiosis of *Leucaena leucocephala*. Experimental Agriculture. 4:243–253.

Vargas AAT, Graham PH. 1988. *Phaseolus vulgaris* cultivar and *Rhizobium* strain variation in acid-pH tolerance and nodulation under acid conditions. Field Crops Research. 19:91–101.

Vicente C. 2010. Evaluation of biological nitrogen fixation of *Biserrula pelecinus* L. under stressful conditions [PhD Thesis]. [Seville, Spain]: University of Pablo of Olavide.

Vicente CSL, Pérez-Fernández MA, Pereira G, Tavares-de-Sousa MM. 2009. Biodiversity of root-nodule bacteria associated with the leguminous plant *Biserrula pelecinus*. Soil Science. 174:424–429.

Vincent JM. 1970. A manual for the practical study of root nodule bacteria. IBP Handbook 15. Oxford: Blackwell.

Vitousek PM, Menge PM, Reed SC, Cleveland CC. 2013. Biological nitrogen fixation: rates, patterns and ecological controls in terrestrial ecosystems. Philosophical Transactions of the Royal Society B: Biological Sciences. 368:1–9.

Waterer JG, Vessey JK, Stobbe EH, Soper RJ. 1994. Yield and symbiotic nitrogen fixation in a pea-mustard intercrop as influenced by N fertilizer addition. Soil Biology and Biochemistry. 26:447–453.

Wilson DO, Reisenauer HM. 1970. Effect of manganese and zinc ions on the growth of *Rhizobium*. Journal of Bacteriology. 102:729–732.

Young KD. 2006. The selective value of bacterial shape. Microbiology and Molecular Biology Reviews. 70:660–703.