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A microcosm study on the influence of pH and the host-plant on the soil persistence of two alfalfa-nodulating rhizobia with different saprophytic and symbiotic characteristics

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Abstract The acid tolerance of *Sinorhizobium meliloti* in culture media and in soils is considered a useful criteria to select for strains with improved survival in agricultural acidic soils. Using a glass tube system with gamma-irradiated soil at different pH values, we analysed the survival of two different alfalfa-nodulating rhizobia: *S. meliloti* (pH_{limit} for growth 5.6–6.0) and the acid-tolerant *Rhizobium* sp. LPU83, closely related to the strain *Rhizobium* sp. Or191 (pH_{limit} for growth below 5.0). Although the acid-tolerant rhizobia showed a slightly better survival during the first months in acid soil (pH=5.6), none of the strains could be detected 2 months after inoculation (bacterial counts were below 10^3 colony-forming units (cfu)/30 g of soil). The inclusion of two alfalfa plants/glass tube with soil, however, supported the persistence of both types of rhizobia at pH 5.6 for over 2 months with counts higher than 9×10^6 cfu/30 g of soil. Remarkably, in the presence of alfalfa the cell densities reached by *S. meliloti* were higher than those reached by strain LPU83, which started to decline 1 week after inoculation. Although more acid-sensitive in the culture medium than the Or191-like rhizobia, in the presence of the host plant the *S. meliloti* strains showed to be better adapted to the free-living condition, irrespective of the pH of the soil.

Keywords Glass tube system · Soil acidity · Alfalfa

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Introduction

The inoculation of alfalfa seeds with efficient and competitive *Sinorhizobium meliloti* has long been practised to increase plant production and to preserve the nitrogen fertility of soils. In acid soils, however, inoculation of alfalfa with rhizobia frequently shows poor results, mainly due to the limited survival of *S. meliloti* under acidity and a restricted symbiosis with alfalfa at low pH. Experiments in the field and in the greenhouse have shown that the nodulation and production of alfalfa decreases sharply as the soil pH decreases below pH 6.0 (Rice et al. 1977), *S. meliloti* being more affected by acidity than the host plant (Clarke et al. 1993). While the number of rhizobia in neutral soils may frequently reach 10^5 colony-forming units (cfu)/g, the number of rhizobia in moderately acidic soils does not usually exceed 10^2 cfu/g (Brockwell et al. 1991). In addition, it has been reported that in acid soils there is an increase in the proportion of ineffective nodules (Barber 1980). It has been suggested that either the acidic environment may cause a loss of diversity in the population of efficient rhizobia, or formerly efficient rhizobia may have lost their symbiotic capacity (Barber 1980).

In general, fast-growing rhizobia (e.g., *S. meliloti*, *S. medicae*, *Rhizobium etli*, *R. leguminosarum*) are less tolerant to acidity than slow-growing rhizobia, such as some species of *Bradyrizhobium*. However, this does not seem to be a general observation. While strains of *R. tropici* can grow well in culture even at pH 4.0, *S. meliloti* and *S. medicae* are poorly tolerant to acidity, even when compared with most other fast-growing rhizobia (they usually do not grow below pH 5.8; Graham 1992).

Most studies on rhizobial tolerance to acidity focus on either the analysis of bacterial growth at low pH in culture media (Howieson et al. 1988; Tiwari et al. 1992, 1996a, 1996b; Graham et al. 1994; Del Papa et al. 1999) or the evaluation of bacterial tolerance to acidity in soils (Brockwell et al. 1991; Lowendorf and Alexander 1983). The living conditions in the natural environment include a fairly complex set of biotic (i.e., predators) and abiotic factors (i.e., concentration of heavy metals), some of which are dependent on the type of soil and the soil pH (Graham 1992; Bordeleau and Prévost 1994). The physicochemical complexity of soils and the saprophytic competence of the indigenous biota make evaluation of the specific effects of acidity a difficult task. Thus, the fact that a rhizobium is "acid-tolerant" in culture media does not necessarily warrant an outstanding survival of the same rhizobium in soil under comparable acid conditions. Even more uncertain is the correlation between the rhizobial ability to persist in acid soils and the capacity of the rhizobia in acidity to express their symbiotic phenotype (Bromfield and Jones 1980; Graham et al. 1982; Rice 1982; Hartel and Alexander 1983; Howieson et al. 1988). Nonetheless, acid tolerance in artificial media is considered a positive characteristic when selecting rhizobia for the improvement of inoculant products for acid soils (Howieson and Ewing 1986; Glenn and Dilworth 1994). The selection of moderately acid-tolerant *S. meliloti* strains from the Mediterranean basin (Greece, Italy) helped the establishment of medic pastures in the acidic soils of Western Australia (Howieson and Ewing 1986; Howieson et al. 1988). The rationale of such a practical approach relies on the assumption that the more metabolically active a rhizobium is at low pH, the higher is its ability to adapt to the unfavourable environmental condition.

We previously reported the presence of at least two different populations of alfalfa-nodulating rhizobia in moderately acid soils of Argentina (Del Papa et al. 1999; Wegener et al. 2001): *S. meliloti*, which represents the major group, and a second group of ineffective and less predominant rhizobia related to the acid-tolerant strain *Rhizobium* sp. Or191 (Eardly et al. 1985, 1992). Unfortunately, the Or191-like ineffective rhizobia proved to be more competitive for nodulation at low pH than *S. meliloti* (Segundo et al. 1999). Such a phenotype under low pH conditions might favour the establishment of the inefficient symbionts of alfalfa. Since there are currently no studies on the saprophytic behaviour of the Or191-like rhizobia, in this work we investigated the free-living persistence of these rhizobia under moderately acid conditions. To that aim, we used a microcosm system (glass tube format) containing gamma-irradiated soil samples at different pH values. The results showed that, although more sensitive to acidity, the N_2-fixing symbiont *S. meliloti* survived better in the presence of alfalfa than the acid-tolerant Or191-like rhizobia, a result that suggests an early preferential effect of the host plant on *S. meliloti*.

Materials and methods

Bacterial strains and culture media

The strains used in this work were *S. meliloti* 2011 (from J. Dénarié, France), *S. meliloti* LPU63 (moderately tolerant to acidity; Del Papa et al. 1999) and the acid-tolerant *Rhizobium* sp. LPU83 (Del Papa et al. 1999), closely related to the previously reported strain *Rhizobium* sp. Or191 (Eardly et al. 1985, 1992). The strains were grown in liquid TY medium (Beringer 1974) at 28 °C. The pH of the culture media was adjusted to 7.00 (±0.05 units) with a glass-electrode pH meter prior to sterilisation.

Glass tube assays

Microcosm tubes were set up as described by Hagen et al. (1997). Briefly, 30 g of sterile soil were introduced into an open glass column with a length of 20 cm and a diameter of 3 cm. A fritted glass filter served as a base, to fix the soil within the glass tube. In order to achieve a constant water supply to the soil, a cotton thread was passed through a hole in the middle of the glass filter and introduced into a water reservoir (Dresing et al. 1998).

The soil samples used to prepare the glass tube were collected from an experimental field at the Instituto Nacional de Tecnología Agropecuaria, Castelar, Argentina, and presented the following physicochemical characteristics: pH 5.6, humidity factor 1.1, organic matter 2.76%, organic carbon 1.60%, total nitrogen 0.81%, C/N ratio 8.9, available phosphate 28.1 ppm, paste condition 0.51 ms/Cm, water saturation 51.3%. The collected soil was air-dried at room temperature and sieved to a size of 5 mm. Sterilisation was made by exposing the soil to 5 Mrad of γ radiation (Ionics, Argentina). The tests were carried out at both the original soil pH (5.6) and at pH 6.8, which was achieved by adding 1 M KOH to the soil. Soil samples within the glass tubes were rewetted to about 30% moisture content and kept at room temperature for 4–6 h. The glass tubes were inoculated with one of one of the following alfalfa-nodulating rhizobia in the late log phase of growth (approx. 10^5–10^6 cfu/g): *S. meliloti* 2011, *S. meliloti* LPU63, or the acid tolerant *Rhizobium* sp. LPU83. Inoculated glass tubes were incubated in a plant-growth chamber (16 h light, day/night temperature 20/16 °C) until sampled. Enumeration of rhizobia was performed weekly by plating appropriate soil dilutions from two glass tube replicates. In order to suspend the bacteria from the soil samples, the glass tube content was shaken for 1 h at 100 rpm in 100 ml of sterile PBS (8 g NaCl l, 1 g Na_2HPO_4l, pH 8.2). The soil pH of each sampled glass tube was measured at the time of plating.

Microcosm assays with alfalfa plants (glass tube format)

Surface-sterilized alfalfa seeds (*Medicago sativa*) cv. CUF101 were germinated on 1.5% water/agar plates and two seedlings (2 days old) were planted in each glass tube before inoculation. Before rhizobial enumeration, both plants were carefully removed from the soil, avoiding the release of root nodules. The nodules were excised when present and the remaining root system was returned to the soil.

Results

Soil persistence of alfalfa-nodulating rhizobia under different pH conditions in a glass tube system

In order to investigate the effect of acidity on the soil persistence of alfalfa-nodulating rhizobia, we used a glass tube system with gamma-irradiated soil. The number of rhizobia/glass tube was monitored over time. Table 1

Table 1 Effect of soil pH on the survival and persistence of different alfalfa-nodulating rhizobia. Rhizobial counts were assessed by plating and data represent the average ±SD of three independent samples. Experiments were performed using gamma-irradiated soil at pH 6.8 and pH 5.6 (see Materials and methods). *R. Rhizobium*, *S. Sinorhizobium*

Day	Number of rhizobia (colony-forming units in 30 g of soil)					
	S. meliloti 2011		*S. meliloti* LPU63		*R.* sp. LPU83	
	pH 6.8	pH 5.6	pH 6.8	pH 5.6	pH 6.8	pH 5.6
0	$1.1\times10^5\pm2\times10^4$	$6.9\times10^4\pm1\times10^4$	$9.9\times10^4\pm2\times10^4$	$1.2\times10^5\pm3\times10^4$	$2.0\times10^4\pm8\times10^3$	$1.0\times10^4\pm3\times10^2$
39	$5.0\times10^9\pm1\times10^9$	$2.5\times10^4\pm1\times10^4$	$4.9\times10^9\pm3\times10^8$	$7.5\times10^3\pm2\times10^3$	$1.3\times10^6\pm7\times10^4$	$3.5\times10^4\pm2\times10^4$
56	$8.1\times10^8\pm3\times10^8$	$<1.0\times10^3$	$2.3\times10^9\pm4\times10^7$	$<1.0\times10^3$	$1.8\times10^9\pm4\times10^8$	$<1.0\times10^3$

shows the number of rhizobia in the soil at pH 6.8 at different time intervals post-inoculation. Under this condition, the *S. meliloti* 2011 and LPU63 strains behaved similarly, with a pronounced growth that resulted in approx. 10^9 cfu/glass tube in the second month post-inoculation. Although the acid-tolerant strain *Rhizobium* sp. LPU83 reached similar counts by the end of the assay, at approx. 5 weeks (39 days) post-inoculation the strains of *S. meliloti* showed higher cell densities (10^9 cfu/glass tube) than the Or191-like strain LPU83 (approx. 10^6 cfu/glass tube; Table 1, see day 39).

Under the acidic condition (pH 5.6), all rhizobia showed a different behaviour (Table 1). At 2 months post-inoculation, none of the strains exceeded 10^3 cfu/glass tube. However, while the *S. meliloti* 2011 and LPU63 strains declined in counts from the beginning of the assay, strain *Rhizobium* sp. LPU83 initially increased, to reach 3×10^4 cfu/glass tube and only then declined to less than 10^3 cfu/glass tube, like the other rhizobia.

Soil persistence of *S. meliloti* and the acid-tolerant *Rhizobium* sp. LPU83 at different pH in the presence of alfalfa plants

In order to evaluate whether the presence of the host plant modifies the rhizobial persistence under acid stress, we carried out glass tube assays in the presence of two alfalfa plants (see Materials and methods). Figure 1A, B (black symbols) shows that, at pH 6.8, *S. meliloti* 2011 and *S. meliloti* LPU63 both reached their maximum cell densities at 10 days post-inoculation. Rhizobial numbers were steadily maintained at the maximum value throughout the next 2 months. A slightly different progress of cell densities was observed for the Or191-like strain *Rhizobium* sp. LPU83 (Fig. 1C, black squares) which reached nearly the same counts than *S. meliloti* but began to decline from day 10 post-inoculation.

In the acid glass tubes with alfalfa (Fig. 1A–C, white symbols), all strains presented a short phase of initial death (5 days). Thereafter, strains *S. meliloti* 2011 and *S. meliloti* LPU63 grew steadily, keeping titres higher than 10^8 cfu/glass tube (such cell densities contrast with those observed for the same strains in the absence of plants, at less than 10^3 cfu/glass tube; Table 1). It is noteworthy that , at pH 5.6, the acid-tolerant strain *Rhizobium* sp. LPU83 did not reach the same cell densities as *S. meliloti* (acid-sensitive; Del Papa et al. 1999) and declined gradually

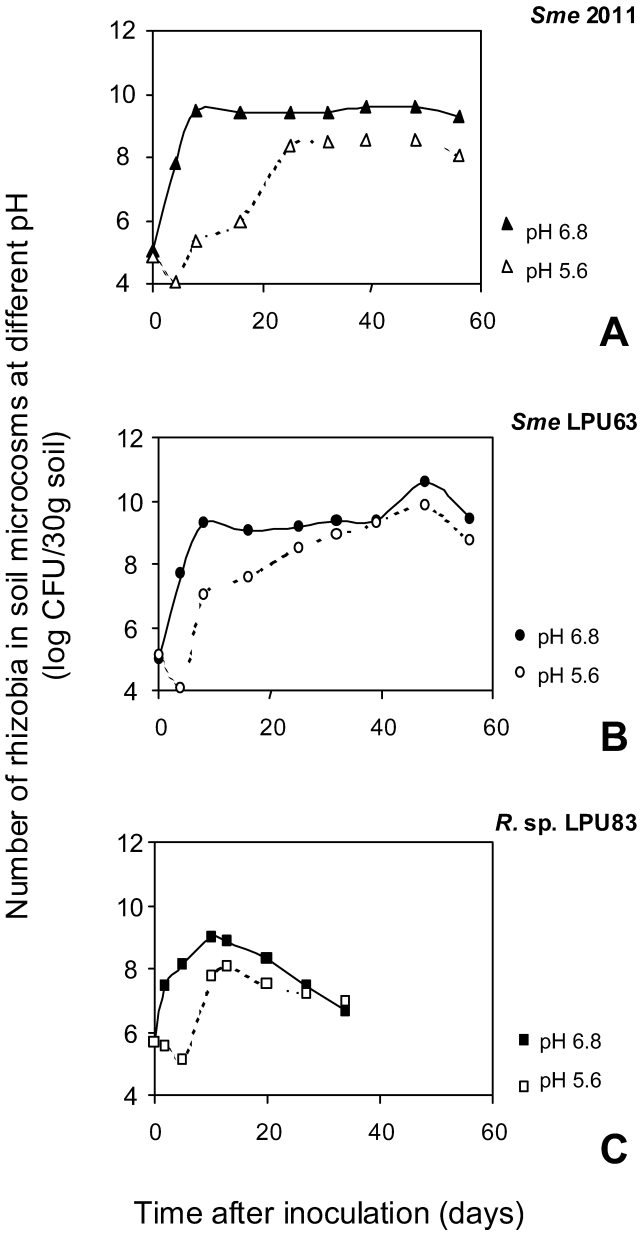

Fig. 1A–C Soil persistence of alfalfa-nodulating rhizobia at different pH in the presence of alfalfa plants. The strains assayed were as indicated: **A** *Sinorhizobium meliloti* 2011, **B** *S. meliloti* LPU63, **C** *Rhizobium* sp. LPU83. Rhizobial counts were assessed by plating and correspond to the average of three independent samples ±SD (CV% <10). Experiments were performed using gamma-irradiated soil at pH 6.8 and pH 5.6 (see Materials and methods). *CFU* Colony-forming units

after day 10 post-inoculation. A comparable phase of death was also observed at the nearly neutral pH 6.8 for strain *Rhizobium* sp. LPU83 (Fig. 1C, black squares).

Discussion

Using a glass tube system, we evaluated the soil survival of two distinct alfalfa-nodulating rhizobia: *S. meliloti* and *Rhizobium* sp. LPU83 at different pH conditions. As we mentioned in the Introduction, strain LPU83 belongs to an acid-tolerant, promiscuous and inefficient group of alfalfa-nodulating rhizobia closely related to the previously described strain, *Rhizobium* sp. Or191 (Eardly et al. 1985; Del Papa et al. 1999; Wegener et al. 2001).

In agreement with results from Lowendorf and Alexander (1983), the evidence from this work indicates that the critical pH for growth in culture media is not the only factor influencing rhizobial survival under acid stress in soil. The striking acid-tolerant phenotype of strain LPU83 in culture media (Del Papa et al. 1999) was not much reflected in the survival rate of this rhizobium in acidic soil. Without plants at pH 5.6, the acid-tolerant LPU83 showed only a slightly better survival than *S. meliloti* during the initial weeks post-inoculation. Other traits appear to be relevant to overcome the effects of acidity within the soil environment. An important characteristic of the rhizobia seems to be associated with the ability of the strains to make use of the protective environment of alfalfa roots. In the presence of alfalfa, the multiplication of rhizobia probably occurred in the plant rhizosphere quite independently of the influence of soil pH. Interestingly, both *S. meliloti* strains showed a better response to the presence of alfalfa than the strain *Rhizobium* sp. LPU83, displaying higher and sustained counts over time (Fig. 1). The differential behaviour between *S. meliloti* and the acid-tolerant and inefficient strain LPU83 is consistent with a report by Barber (1980) on the relevance of the host plant to keep a field population of *S. meliloti* against another group of inefficient and acid-tolerant isolates, which grew up in the laboratory at pH 5.0. The report by Barber (1980) described a positive correlation between the age of alfalfa stands in Oregon and the number of inefficient and acid-tolerant rhizobia. Since there are no known *S. meliloti* strains with the ability to grow at a pH below 5.6 (O'Hara et al. 1989; Graham et al. 1994; Del Papa et al. 1999), it is very likely that the acid-tolerant isolates reported by Barber corresponded to the group of Or191-like rhizobia. Thus, it is possible that the promotion of acid-sensitive and efficient *S. meliloti* by alfalfa in the field might have been partially due to a differential ability of these rhizobia to benefit from the presence of the host root, as we demonstrated in this study. Using a simple glass tube assay, we demonstrated a major effect of alfalfa roots in promoting the free-living population of *S. meliloti*, compared with the much reduced growth promotion of the acid-tolerant Or191-like competitors. It is thus an open question as to how *S. meliloti* can take better advantage of the presence of the host plant, a characteristic that appeared to be expressed under the moderate acid conditions used in this work.

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