Survival of *Rhizobium leguminosarum* biovar *viceae* subjected to heat, drought and salinity in soil

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Abstract

Two strains (RCR 1001 and 1044) and a commercial inoculant (Okadin) of *Rhizobium leguminosarum* biovar *viceae* were tested for their ability to survive in autoclaved clay soil for up to four months under heat, salinity and drought stress. Resistance to heat was tested by incubating rhizobia in soil at 27, 37 and 42 °C. Tolerance of rhizobia to salinity was investigated by growing rhizobia in soil salinized with 1 and 2 % NaCl (m/m). Drought resistance was tested by subjecting bacteria to soil moisture contents of 20, 10 and 5 %. Strain RCR 1001 was more resistant to heat and nodulated faba bean better than other tested strains. A commercial inoculant Okadin survived more (plate count method) and nodulated faba bean (plant infectivity, most probable number, MPN) at moisture content of 5 % and 2 % NaCl. Although, strains RCR 1001 and 1044 resisted these stress conditions (plate count) they lost their abilities to nodulate faba bean (MPN-test). There is a possibility for selection of effective rhizobia which are more tolerant to harsh conditions.

Key words: NaCl, nodulation, tolerance, *Vicia faba*

Introduction

Environmental stresses especially during the period between inoculation of seeds and germination impose severe problems to establishment of a successful symbiosis (Weaver and Holt 1990, Weaver *et al.* 1985). Survival of *Rhizobium leguminosarum*, the microsymbiont of faba bean, may be affected mainly by salinity, low water potential and extremes in temperature.

Differences in adaptation to high temperatures have been demonstrated from different climatic zones. Eaglesham and Ayanaba (1984) reported that more than 90 % of cowpea rhizobia isolated from hot dry Sahelian Savanna in Niger were able to grow at 40 °C while the rhizobia isolated form cooler humid regions of West Africa did not grow at this temperature (Bowen and Kennedy 1956, Munevar and Wollum 1981).

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The numbers of rhizobia in soil decline drastically as soil dries; the slow growing rhizobia are more tolerant to desiccation than the fast growing ones (Bushby 1982). Other workers, however, could not establish such relation (Foulds 1971, Mahler and Wollum 1981). Rhizobia survived less on drying soils which contain only small amounts of clay or organic matter (Chao and Alexander 1982, Salema *et al.* 1982, Shoushasi and Pepper 1985).

There are marked differences between strains of rhizobia in adaptation to saline conditions and in fact the host legumes are much more sensitive to salt stress than their *Rhizobium* partners (Abd-Alla 1992, Sprent and Sprent 1990). Some strains of rhizobia especially those isolated from arid lands can actually grow in solutions with salinity as high as the salinity of sea water (Singleton *et al.* 1982).

Most studies on the survival of rhizobia species under environmental stresses were carried in broth cultures or peat (Abdel Wahab and Zahran 1979, Busse and Bottomley 1989, Elsheikh and Wood 1990). Few studies reported the persistence and distribution of *Rhizobium/Bradyrhizobium* under stress in soil (Fuhrmann *et al.* 1986, Pena-Cabriales and Alexander 1979).

Studies of root nodule bacteria under stress conditions in soil might mimic their survival in their natural habitats. This is because bacterial strains under carbon and energy limited sources in soil may be more sensitive to environmental stress than they would be in rich laboratory media. In addition, it is generally agreed that the plate count method to assess the viability of root bacteria under stress conditions is not a sufficient criterion for their symbiotic ability, since rhizobia may withstand and successfully multiply under these conditions but their infectibility and nodulating ability may be changed (Somasegaran and Hoben 1985).

The objective of this study was to compare the total count and most probable number (as assessed by the infectibility test) aiming at the selection of *Rhizobium leguminosarum* strains which are better adapted to varying temperatures, salinity or moisture regimes. The capability of rhizobia to produce successful nodulation was also tested.

Materials and methods

Rhizobial strains: Two strains of *Rhizobium leguminosarum* bv. *viceae* RCR 1001 and 1044 and a commercial strain "Okadin" were used in this study. Bacterial cultures were grown in yeast extract mannitol medium for 3 d.

Soils: The agricultural clay soil used had the following characteristics: pH 7.7, total soluble salts 0.25 %, organic matter 2.3 % and total nitrogen 0.18 %. The soil was air-dried for 1 week, thoroughly mixed and passed through a 2 mm sieve. 5 g samples were placed into serum bottles (25 cm^3), capped with cotton plugs and autoclaved twice for 2 h (121 °C) at two consecutive days. Each bottle was inoculated with 2 cm^3 bacterial suspension containing 10^6 cells cm^{-3} for each of the strains used.

For drought stress experiments, the water content of soil in bottle was adjusted to 0.5, 0.25 and 0.125 % of maximal capillary capacity (soil moisture 20, 10 and 5 %). The level of water content was maintained constant by weighing bottles twice a week and water lost was replaced by the addition of an appropriate quantity of sterile water. Bottles were incubated at 27 °C for 4 months. For the heat tolerance test, soil moisture was adjusted to 20 % and samples were held at 27, 37 and 42 °C for 4 months. To examine the effect of salt stress, strains of rhizobia were grown in soils containing 0, 1 and 2 % NaCl and incubated at 27 °C for 4 months.

Survival of rhizobia was monitored after 1, 2, 3 and 4 months by plating serially diluted samples of the treatments on congo red yeast extract mannitol agar using the pour plate method (Somasegaran and Hoben 1985). These authors suggested that the plate count method may be inadequate to test the survival of rhizobia under stress. Accordingly, the most probable number technique (Toomsan *et al.* 1985) was employed to assess the ability of each strain to nodulate faba bean after 4 months of treatments. Most probable number values were estimated by inoculating seeds with aliquots from a decimally diluted sample and determining nodulation at 28 d after seedling emergence. The faba bean (*Vicia faba* cv. Giza 3) was used in the present study.

Statistical analysis: All data were subjected to one-way analysis of variance using computer programs (PC-stat). Means were compared by Duncan's multiple range test.

Results

Temperature: Populations of the three strains either increased or remained constant as estimated by plate count at 27 and 37 °C. Significant reduction were detected at 42 °C in the numbers of both strain RCR 1001 and the commercial inoculant. However, survival of RCR 1044 remained constant for 3 months after which its population decreased significantly (Table 1). Also MPN noticeably decreased after exposure to high temperature (42 °C) and more than 39 % of the surviving population lost their ability to nodulate faba bean.

RCR 1044 adapted to elevated temperature and survived better than RCR 1001 and commercial inoculant.

Table 1. Survival of *Rhizobium leguminosarum* biovar *viceae* strains RCR 1001 (R1), RCR 1044 (R2) and Okadin (R3) incubated in sterile soil under different temperatures. Plate count was monitored after 1, 2, 3 and 4 months, MPN after 4 months of incubation. Each value represents the mean of four replicates. Means without the same letter in each column were significantly different at the 5 % level by the Duncan's multiple range test.

Temperature [°C]	Plate count Log$_{10}$ No. of cells g^{-1}(soil)												MPN		
	1			2			3			4			4		
	R1	R2	R3	R1	R2	R3	R1	R2	R3	R1	R2	R3	R1	R2	R3
27	6.3a	7.8a	7.4a	7.3a	7.9a	7.4a	7.4a	8.1a	7.6a	7.1a	7.8a	7.1a	6.0a	6.5a	6.0a
37	6.0a	6.5a	6.6a	6.3a	7.3a	6.8a	6.8a	7.4a	6.9a	6.4a	7.4a	6.4a	5.0a	6.3a	5.8a
42	4.8b	6.2b	5.7b	3.8b	6.1b	4.1b	3.5b	5.7b	3.7b	2.1b	5.4b	2.0b	1.8b	4.0b	1.2b

Drought stress: Plate counts and MPN determinations show a drastic decline of the population of all strains with increasing desiccation (a water content of 5 %). Population survival of the commercial inoculant was significantly higher than RCR 1001 and 1044 strains (Table 2).

Table 2. Survival of *Rhizobium leguminosarum* biovar *viceae* strains RCR 1001 (R1), RCR 1044 (R2) and Okadin (R3) incubated in sterile soil subjected to drought stress. (-) No available estimate since none of the seedlings used in the dilution count nodulated.

Water content [%]	Plate count Log$_{10}$ No. of cells g^{-1} (soil) 1			2			3			4			MPN 4		
	R1	R2	R3	R1	R2	R3	R1	R2	R3	R1	R2	R3	R1	R2	R3
20	6.2a	6.1à	7.3a	6.9a	6.0a	7.2a	5.4a	5.2a	6.3a	5.1a	4.9a	6.1a	4.8a	4.5a	5.2a
10	3.1b	3.0b	5.2b	2.1b	1.3b	4.2b	1.4b	1.1b	3.1b	1.5b	1.3b	2.8b	1.5b	1.2b	1.7b
5	1.1c	1.2c	2.1c	1.0c	0.9c	2.1c	0.8c	0.3c	2.0c	0.3c	0.1c	1.2c	-	-	1.0c

Salt stress: Examination after 4 months for bacterial persistence indicated that salinity at 1 and 2 % reduced the survival of all strains. Salt tolerant populations of RCR 1001 and 1044 were significantly less than the commercial inoculant (Table 3).

Table 3. Survival of *Rhizobium leguminosarum* biovar *viceae* strains RCR 1001 (R1), RCR 1044 (R2) and Okadin (R3) incubated in sterile saline soil.

NaCl [%(m/m)]	Plate count Log$_{10}$ No. of cells g^{-1} (soil) 1			2			3			4			MPN 4		
	R1	R2	R3	R1	R2	R3	R1	R2	R3	R1	R2	R3	R1	R2	R3
0	6.4a	6.3a	7.3a	6.5a	6.2a	7.4a	6.5a	6.4a	7.5a	6.5a	6.2a	7.1a	6.0a	5.8a	6.8a
1	4.7b	5.3b	6.1b	3.0b	3.1b	5.1b	2.1b	2.2b	4.9b	2.2b	1.2b	3.1b	2.0b	0.8b	3.0b
2	2.3c	2.1c	3.2c	2.2c	2.1c	2.3c	0.5c	0.4c	3.1c	0.3c	0.2c	2.0c	-	-	1.2c

Discussion

Strains of rhizobia vary greatly in their ability to persist in soil and nodulate legumes under stress conditions (Busse and Bottomley 1989). Graham (1991) recently reported that environmental stress limits *Rhizobium/Bradyrhizobium* distribution and persistence in soil and can reduce nodulation and nitrogen fixation in the field. Strains of *Rhizobium leguminosarum* used in the present study responded differently to desiccation, salt and temperature. Thus the commercial inoculant Okadin was more tolerant to drought and salt stress than strains RCR 1001 and 1044 (Tables 2, 3). Upon exposure to elevated temperature (42 °C), however strain RCR 1044 could survive and adapt better to high temperature (Table 1) than the other two strains.

Gewaily *et al.* (1991) similarly reported that survival of *Rhizobium leguminosarum* was significantly reduced at 40 °C when inoculated in sterile and non sterile soils. Baldani and Weaver (1992) attributed heat tolerance of *Rhizobium leguminosarum* biovar *trifolii* strains to cryptic plasmids. These plasmids induce the synthesis of heat shock proteins upon exposure of bacteria above normal growth temperatures (Sen *et al.* 1990).

In the present study, faba bean plants inoculated with the strains RCR 1001 and 1044 subjected to severe drought (soil moisture content of 5 %) and high salinity did not form nodules (MPN test, Table 3). This confirms that the success of the MPN method is independent on the viability of the cell but on its infectibility, which may include attachment and subsequent development of infection and nodule formation. Many, if not all, of these steps may be inhibited by stress conditions.

Al-Rashidi *et al.* (1982) suggested that strains which survive under greater water stress are those which retain less water within the cells. Recently Pillai and Pepper (1990) reported that certain genes play a major role in the ability of *Rhizobium leguminosarum* bv. *phaseoli* to survive in symbiosis with host under water stress.

Salt tolerant rhizobia which might include the commercial strain in the present study (Table 3) survive in saline environments by osmotic adjustments. Elsheikh and Wood (1990) reported that a 2 % mixture of salts, more representative of conditions in saline soil, had no effect on survival of chickpea and soybean rhizobial strains which initially multiplied in 2 % NaCl but then died.

Zahran and Lindstrom (1990) reported an alteration of synthesis patterns of lipopolysaccharides and proteins in rhizobia subjected to salinity stress and suggested that this might be a mechanism for adaptation to salt stress. Zahran (1991) demonstrated some modification in the morphological and physiological properties of the long-time persisting rhizobia under saline conditions. This also might affect their infectibility. Since less than 40 % of surviving population lost their ability to nodulate faba bean (Table 3) when subjected to high salt stress (2 %), it could thus be concluded that while rhizobia might be able to withstand and multiply under salt stress, the infectibility and nodulating ability may be changed.

Because differences do exist between strains of the same species in tolerance to different environmental stress conditions, our study suggests that strains should be screened for their survival during heat, water and salt stress before added to inoculants intended for use in arid and semi arid regions or for use in saline soils.

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