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Crossing the Limits of *Rhizobium* Existence in Extreme Conditions

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Abstract. An ecological survey was conducted to characterize 5000 *Rhizobium* sp. sesbania strains of diverse geographical origin, isolated from the root nodules of *Sesbania aculeata* growing in neutral (pH 7) and alkaline (pH 8.5 and above) soils. The rhizobia from the alkaline soil showed significantly higher salt tolerance than those isolated from neutral soil. Upper limits of stress survival of rhizobial isolates, *Rhizobium* sp. NBRI0102 sesbania selected from neutral soil, and *Rhizobium* sp. NBRI2505 sesbania selected from alkaline soil, were studied under free living conditions. *Rhizobium* sp. NBRI0102 sesbania and *Rhizobium* sp. NBRI2505 sesbania tolerated yeast extract mannitol broth (YEB) containing 10% and 28% salt (NaCl, wt/vol) for up to 18 h of incubation at 30°C. Growth of *Rhizobium* sp. NBRI0102 sesbania and *Rhizobium* sp. NBRI2505 sesbania at pH 7, 11, and 12 was identical, except for a lag period of about 10 h in the growth of *Rhizobium* sp. NBRI0102 sesbania at pH 11 and 12, as compared with pH 7. *Rhizobium* sp. NBRI0102 sesbania and *Rhizobium* sp. NBRI2505 sesbania survived at 50°C and 65°C, in YEB at pH 7 for up to 4 and 2 h, respectively. To our knowledge, this is the first report of rhizobia demonstrating survival of *Rhizobium* sp. NBRI2505 sesbania, estimated by counting viable cells, to such extreme conditions of salt and temperature, individually. In contrast to *Rhizobium* sp. NBRI0102 sesbania, high temperature was tolerated efficiently by *Rhizobium* sp. NBRI2505 sesbania, in the presence of salt at higher pH. Our results suggest that the possession of the trait of high salt tolerance might be of some evolutionary significance for the survival of rhizobia in alkaline soils, at high pH and temperature.

Leguminous plants are frequently used for degraded soil sites in arid and semiarid regions because they can grow in barren soils that are unsuitable for most crops. These sites are hyper-saline and suffer from high salt, pH, and temperature stresses. In developing countries, fast-growing legumes are required for both fodder and sustained fuelwood production. In agriculture, leguminous biological nitrogen fixation is used to improve infertile soils, especially those affected by salinity [22, 26] and high soil temperatures [16]. Rhizobial strains are very sensitive to soil environmental factors like high salt, pH, and temperature stresses, which affect their dinitrogen fixation capacity and hence the productivity of legumes [2, 6, 8, 9, 11, 12, 20]. *Rhizobia* growing in alkaline soils in India during summer season are subjected to high temperature, pH, and salt stress [22]. An understanding of the growth of *Rhizobium* isolated from the root nodules of legumes is likely only when the physiology of these organisms has been carefully studied under these suboptimal conditions. *Rhizobium* with the genetic potential for increased tolerance to high salt, pH, and temperature stresses could enhance production of food and forage legumes in semiarid and arid regions of the world [7, 20].

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Salinity stress is one of the most serious factors limiting the productivity of agricultural crops. The detrimental effects of salt on plants are a consequence of both a water deficit, resulting in osmotic stress, and effects of excess sodium ions on critical biochemical processes [4]. Salt may affect symbiosis by its effects on the growth and survival of rhizobia in soil, restriction on root colonization, inhibition of processes of infection and nodule development, or impairment of active nodule functioning. The presence of high sodium chloride (NaCl) concentration has been reported to cause a reduction in the number of *Rhizobium* in legume inoculants. Thus, tolerance to salt stress is an important part of saprophytic competence and competitiveness in *Rhizo-*

bium [24]. These effects may be mediated through an effect of salt on the host or through a specific effect on the micro-symbiont itself [1].

Temperature dependence of nodulation and symbiotic nitrogen fixation has been reported to depend on the plant cultivar and the nodulating strain [5, 16, 17]. They are characterized by the presence of high levels of neutral salts in the surface layers resulting from capillary rise of water when evaporation exceeds precipitation. In the flood plains of rivers, low-lying lake margins and coastal plains, saline groundwater within a few meters of the soil surface can be a major contributory factor [2]. Reclamation and revegatation of saline lands, occupying approximately 7% of the earth's land surface, are a high priority in many nations within tropical latitudes. Many legumes appear to offer potential for planting on saline sites where secondary salinity is causing loss of agricultural land. In the developing countries, fast-growing legumes are required for both fodder and sustained fuelwood production [2, 22]. Leguminous plants growing in highly saline environments require both the free-living rhizobia and the host to be tolerant to salt. Generally, the rhizobia are more tolerant to salt stress than their leguminous hosts, although some tree species, like *Acadia* and *Prosopis*, and, among agricultural crops, *Medicago* and *Sesbania* are known to be salt tolerant [5, 9, 10, 17].

In view of the sensitivity of rhizobia to the abiotic stresses frequently encountered in the tropics and subtropics, an investigation was conducted to better protect or enhance stress tolerance of rhizobia. Since alkalinity is often associated with high salt and high pH in semiarid and arid regions, we sought to isolate and identify strains of *Rhizobium* sp. sesbania tolerant to high salt from root nodules of *Sesbania aculeata* growing in neutral and alkaline soils and to determine whether the same strains were also tolerant to high pH and high temperature. We report here studies done to elucidate the nature of salt tolerance in strains of *Rhizobium* sp. sesbania isolated from neutral and alkaline soils and assess the role of the pH and temperature stresses in contributing to salt tolerance.

Materials and Methods

In the present survey, 5000 *Rhizobium* sp. sesbania strains were screened from individual root nodules of *S. aculeata* growing in neutral and alkaline soils from Agra, Dholpur, Jhansi, Kanpur, and Lucknow (500 *Rhizobium* sp. sesbania strains each from neutral and alkaline soils). *Rhizobium* strains were isolated from the root nodules (20 nodules per plant) of *S. aculeata*, growing in neutral and alkaline soils.

Nodules were disinfected and individually crushed in a small amount of sterile saline (0.85% wt/vol NaCl), and the suspension was streaked on yeast extract mannitol agar solid medium (YEM) as described earlier [22]. *Rhizobium* strains, unless otherwise stated, were grown and maintained on YEM that contained (per liter): 10 g mannitol, 1 g yeast extract, 0.5 g K_2HPO_4, 0.2 g $MgSO_4 \cdot 7H_2O$, and 0.1 g NaCl with 1.5% (wt/vol) agar and 0.025% (wt/vol) Congo red. The tolerance of rhizobia to salt was measured by their growth on YEM plates at 0, 2, 4, and 6% NaCl (wt/vol) in triplicate.

Growth was visually assessed after the plates were incubated at 30°C for 10 days. All the 500 *Rhizobium* sp. sesbania strains grew on the Congo red-incorporated YEM plates (0% salt) as white, translucent, glistening, and elevated colonies with entire margins [23]. The strains growing on 2, 4, and 6% NaCl-supplemented YEM plates were scored accordingly, as tolerant to salt. The ability of the strains to grow in broth was tested with YEM medium without agar (YEB) in 150-ml Erlenmeyer flasks containing 50 ml YEB, with the initial inoculation of about 1×10^7 CFU/ml. The pH of the medium was adjusted to 7, and the control cultures were grown at 30°C. The flasks were incubated on a New Brunswick Scientific (Edison, NJ, USA) Innova Model 4230 refrigerated incubator shaker at 180 rpm.

Plants were grown in a sterile sand containing N-free supplemental nutrients, as described earlier [18]. Seeds were surface sterilized by gently shaking a B. Braun Certomat. WR reciprocal shaker (Melsungen, Germany) Model D-3508 at 100 strokes/min at 28°C with 70% ethanol (5 min), 20% Clorox bleach (10 min), followed by three rinses in sterile Milli-Q-water (MQW). After surface sterilization, seeds were soaked in the bacterial suspension for 4 h at 28°C on a B. Braun Certomat. WR reciprocal shaker at 100 strokes/min. Control seeds (uninoculated) were soaked in 0.85% saline MQW washed from the YEM plate. Thermocol trays (35×35 cm) with 16 (4×4) holes per tray (each space was 7 cm wide, 10 cm deep, and 1 cm apart from the others) were used to grow plants. Each hole was filled 6 cm with vermiculite. N-free supplemental nutrient solution (25 ml) was added to each hole before planting seeds to adjust the soil to 20% moisture. Plants were grown in a greenhouse as described earlier [19]. After 2 months of plant growth, the plants were harvested carefully from trays. Contrary to inoculated plants, nodules were not observed in uninoculated control plants (data not provided). The nodulation test further confirms the identity of the *Rhizobium* strains used in the present study [23].

The stress tolerance of *Rhizobium* strains towards salt (NaCl), pH, and temperature was tested by growing them on YEB under various stress conditions, as indicated. Viable cells were counted by removing samples at various times in the presence or absence of stress, as indicated. Serial dilutions of each sample were spotted (25 μl) onto YEM plates and incubated at 30°C in triplicate as described earlier [22]. Viable cells were counted after 2–3 days. Populations at each time point in the figures represent the means of three independent experiments. A standard deviation of ± 0.25 log CFU/ml was found for the viable cell counts. For the temperature stress treatment where no survival of *Rhizobium* sp. NBRI0102 sesbania and *Rhizobium* sp. NBRI2505 sesbania in YEB was observed, the cultures were transferred to an incubator shaker maintained at 30°C for 3 days to determine the capability of the culture to grow after the removal of temperature stress. There was no recovery of cell growth in such survival tests.

Results and Discussion

Symbiotic nitrogen fixation is commonly limited by soil infertility conditions, including salinity. Optimization of the benefits of legume inoculation with *Rhizobium* depends on the survival of rhizobia in soil. The introduction and persistence ability of a strain are affected by a number of abiotic factors like high salt, high pH, and high temperature [3, 13, 22]. Therefore, tolerance to high

Table 1. Salt tolerance of *Rhizobium* sp. sesbania strains from root nodules of *Sesbania aculeata* growing in neutral and alkaline soils[a]

Salt (NaCl, % wt/vol)	*Rhizobium* sp. sesbania strains			
	Neutral soil	% salt tolerance over control	Alkaline soil	% salt tolerance over control
0	2500 ± 0	100	2500 ± 0	100
2	1053 ± 45	42	2284 ± 72	91
4	180 ± 9	7	1926 ± 36	77
6	0 ± 0	0	1458 ± 59	58

[a] *Rhizobium* sp. sesbania strains were isolated from individual root nodules of *Sesbania aculeata* growing in neutral and alkaline soils. Growth was visually assessed after yeast extract mannitol agar solid medium (YEM) plates were incubated at 30°C for 10 days. The tolerance of rhizobia to salt was determined by growth on YEM plates at 0, 2, 4, and 6% NaCl (wt/vol). Values represent the means of three replicates ± S.D.

salt, pH, and temperature stresses may be important in the survival, multiplication, and spread of *Rhizobium* in soils. An understanding of the growth of *Rhizobium* isolated from the root nodules of legumes growing under such stressed conditions is likely when the physiology of these organisms has been carefully studied under these suboptimal conditions [22]. Such rhizobial strains could be used on stressed sites as an inoculum to promote leguminous plant growth in the tropics and subtropics.

Salt-tolerant rhizobia are likely to be found in environments affected by osmotic and temperature stress [25]. The strains screened in this study seemed generally well adapted to the environments from which they had been isolated, i.e., hot, dry, high pH, and salt-affected ecosystems. Significant positive correlations were found between the possession of high salt tolerance and the adaptation of rhizobia in alkaline soils. In neutral soils, the upper salt tolerance limit of *Rhizobium* sp. sesbania strains was only 4% (Table 1). The salt tolerance limit of rhizobia from alkaline soil was significantly higher than that of the rhizobia isolated from neutral soil. In the presence of 2, 4, and 6% salt, the tolerance of rhizobial strains isolated from neutral soils was 42, 7, and 0% respectively as compared with 91, 77, and 58% respectively, of the rhizobial strains from the alkaline soils (Table 1). It seemed, therefore, that the possession of high salt tolerance might be of some evolutionary significance for the survival of rhizobia in alkaline soils.

Upper limits of stress survival of one rhizobial isolate, *Rhizobium* sp. NBRI0102 sesbania, selected from neutral soil, and one *Rhizobium* sp. NBRI2505 sesbania, selected from alkaline soil, were studied further under free living conditions. Survival of the *Rhizobium* sp. NBRI0102 sesbania was monitored for up to 24 h at 30°C, in the presence of 0, 2, 4, 6, 8, and 10% salt, at pH 7 (Fig. 1A). The strain survived in YEB containing 10% salt for up to 18 h (Fig. 1A). Survival of the *Rhizobium* sp. NBRI2505 sesbania was monitored for up to 24 h at 30°C, in the presence of 0, 4, 8, 16, 20, and 28% salt, at pH 7 (Fig. 1B). The strain survived in YEB containing 28% salt for up to 18 h (Fig. 1B). Survival of *Rhizobium* sp. NBRI0102 sesbania (Fig. 1C) and *Rhizobium* sp. NBRI2505 sesbania (Fig. 1D) was monitored at 30°C up to 24 h at pH 7, 11, and 12. There was a lag period of about 10 h in the growth of *Rhizobium* sp. NBRI0102 sesbania at pH 11 and 12, as compared with that at pH 7 (Fig. 1C), while growth of *Rhizobium* sp. NBRI2505 sesbania at pH 7, 11, and 12 was identical (Fig. 1D). The effect of high temperature on the viability of *Rhizobium* sp. NBRI0102 sesbania at 30, 40, 42.5, 45, and 50°C was determined up to 24 h. The strain was able to survive for up to 4 h incubation in YEB at 50°C, at pH 7 (Fig. 1E). The effect of high temperature on the viability of *Rhizobium* sp. NBRI2505 sesbania at 30, 40, 50, 55, 60, and 65°C was determined up to 24 h. The strain survived a 2 h incubation in YEB at 65°C, at pH 7 (Fig. 1F).

To our knowledge, this is the first report of *Rhizobium* sp. NBRI2505 sesbania demonstrating survival estimated by counting cells viable to such extreme conditions of salt and temperature, individually. Zahran and colleagues [25] reported visual increase in the growth of *Rhizobium* sp. (*Lupinus*) LK5 at 10% (1.7 M NaCl) salt. Maximum survival temperature reported for *R. japonicum* by Munvar and Wollum [17] ranged from 33.7° to 48.7°C. Shenbagarathai [21] reported *Sesbania procumbens Rhizobium* SBS-R100 capable of growing at pH 11 by demonstrating increase in optical density. We reported the variability among *Rhizobium* strains isolated from different nitrogen fixing trees (NFTs) in growth response to high temperature, pH, and salt concentrations. *Rhizobium* strains isolated from *Albizia lebbek* survived at 50°C, while *Rhizobium* strains isolated from *S. formosa*, *Acacia farnesiana*, and *Dalbergia sissoo*

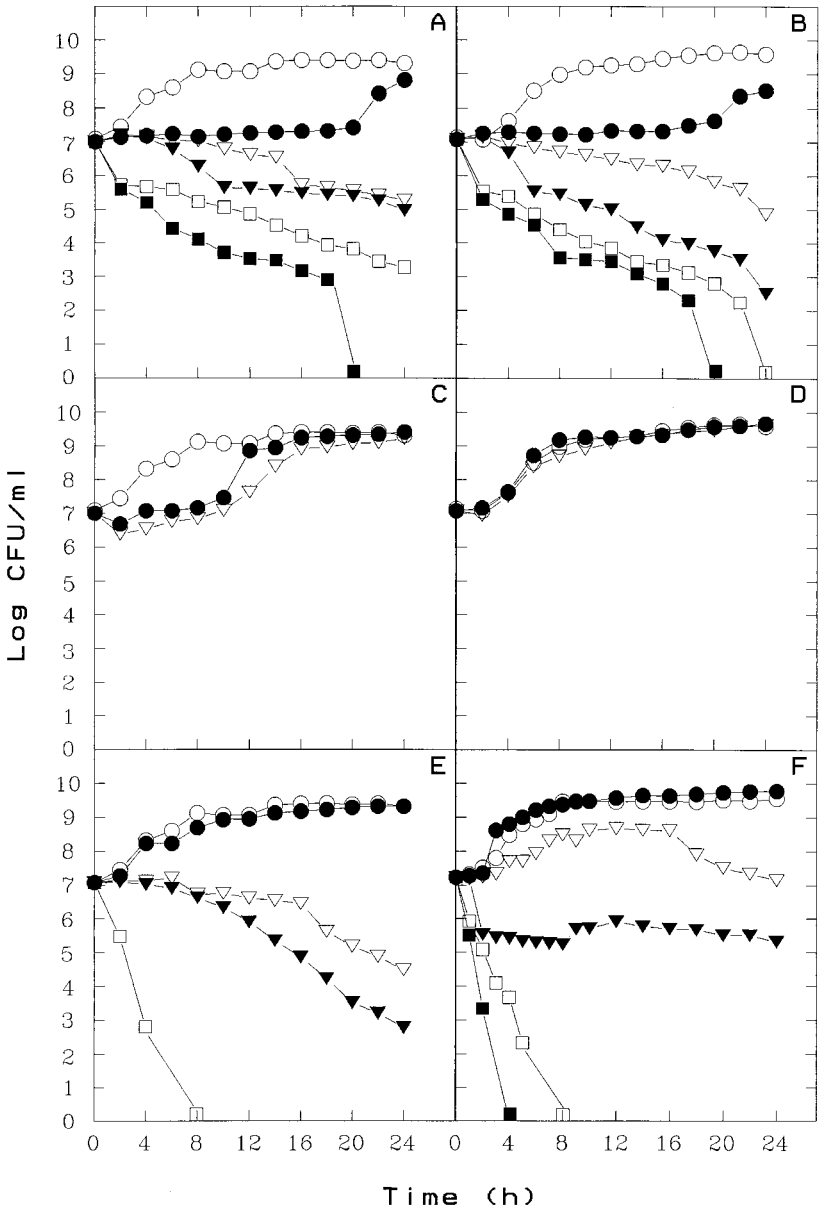

Fig. 1. Effect of salt (NaCl), pH, and temperature on the survival of *Rhizobium* sp. NBRI0102 sesbania. (A) Survival at 30°C, in the presence of 0 (○), 2 (●), 4 (▽), 6 (▼), 8 (□), and 10% (■) salt, at pH 7. (C) Survival at 30°C in the presence of 0% salt at pH 7 (○), 11 (●), and 12 (▽). (E) Survival at 30 (○), 40 (●), 42.5 (▽), 45 (▼), and 50°C (□), in the presence of 0% salt, at pH 7, of *Rhizobium* sp. NBRI2505 sesbania. (B) Survival at 30°C, in the presence of 0 (○), 4 (●), 8 (▽), 16 (▼), 20 (□), and 28% (■) salt, at pH 7. (D) Survival at 30°C in the presence of 0% salt at pH 7 (□), 11 (■), and 12 (▽). (F) Survival at 30 (○), 40 (●), 50 (▽), 55 (▼), 60 (□), and 65°C (■), in the presence of 0% salt, at pH 7. Bacterial survival was determined at the indicated times in triplicate, and the results are the means of three independent experiments. A standard deviation of ±0.25 log CFU/ml was found for the viable cell counts.

were well adapted to grow at pH 12. All the *Rhizobium* strains tolerated salt concentrations up to 5% [22].

We have recently developed a method for fast screening and selection of high temperature-tolerant rhizobial strains from root nodules of *Prosopis juliflora* growing in alkaline soils [14]. The high temperature-tolerant rhizobia were selected from 2500 *Rhizobium* isolates with similar growth pattern on YEM plates after 72 h incubation at 30°C, 45°C, and followed by second screening at 47.5°C. Seventeen high-temperature-tolerant rhizobial strains having distinguishable protein band patterns were finally selected for further screening by subjecting them to temperature stress up to 60°C in YEB for 6 h. The high-temperature-tolerant strains were NBRI12, NBRI329, NBRI330, NBRI332, and NBRI133. With this procedure, a large number of rhizobia from root nodules of *P. juliflora* were screened for high temperature tolerance. Assimilation of several carbon sources, tolerance to high pH and salt stress and ability to nodulate *P. juliflora* growing in glasshouse and nursery of the strains, was studied. All five isolates had higher plant dry weight in the range of 29.9–88.6% as compared with uninoculated nursery-grown plants. It was demonstrated that it is possible to screen in nature for superior rhizobia, exemplified by the isolation of temperature-tolerant strains, which established effective

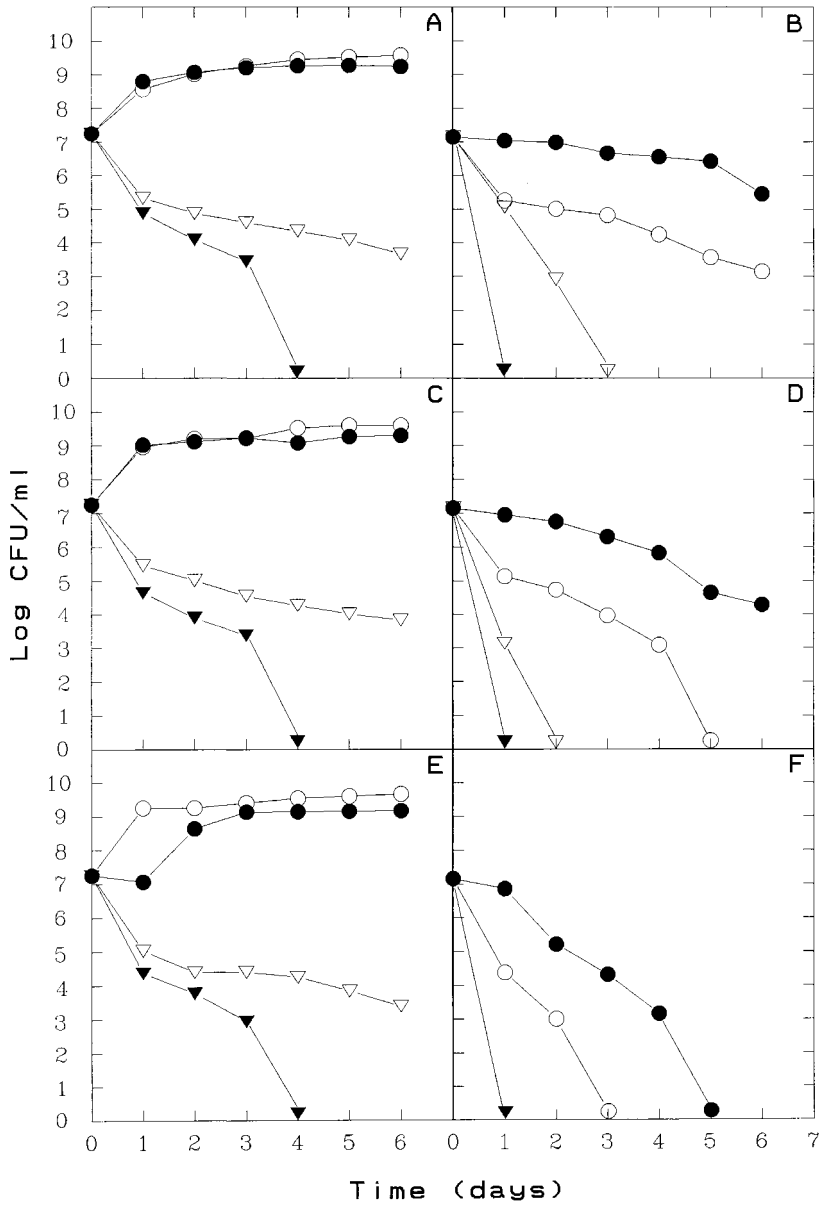

Fig. 2. Effect of salt (NaCl), pH, and temperature on the growth of *Rhizobium* sp. NBRI0102 sesbania. Survival was monitored at 30°C at pH 7 (A), 8 (C), and 9 (E), at 42.5°C at pH 7 (B), 8 (D), and 9 (F) in the presence of 0 (○), 2 (●), 4 (▽), and 6% (▼) salt (NaCl). Bacterial survival was determined at the indicated times in triplicate, and the results are the means of three independent experiments. A standard deviation of ±0.25 log CFU/ml was found for the viable cell counts.

symbiosis with nursery-grown *P. juliflora*. These findings indicate that there is a correlation between strain performance under *in vitro* stress in pure culture and strain behavior under symbiotic condition. Pure culture evaluation may be a useful tool in search for *Rhizobium* strains better suited for soil environments where high temperature, pH, and salt stress constitutes a limitation for symbiotic biological nitrogen fixation [14]. The results of the present investigation confirm our earlier observation that, based on pure culture studies, it is possible to identify *Rhizobium* strains that are tolerant to high salt, pH, and temperature stresses [14]. Using this method, we isolated a high-temperature-tolerant rhizobial strain *Rhizobium* sp. NBRI330 from root nodules of *P. juliflora* growing in alkaline soil [15]. The strain had the ability to nodulate *P. juliflora*. Nursery-grown plants inoculated with *Rhizobium* sp. NBRI330 had 60.6% higher plant dry weight as compared with uninoculated plants. The individual stress survival limit of a rhizobial strain *Rhizobium* sp. NBRI330 isolated from alkaline soil in a medium containing 32% (wt/vol) salt was 8 h, and at 55°C up to 3 h. High temperature (45°C) was tolerated efficiently by *Rhizobium* sp. NBRI330 in the presence of salt at pH 12, as compared with pH 7 [15].

Alkaline soils, besides the effect of high salt, are also known to be affected by high temperature and pH

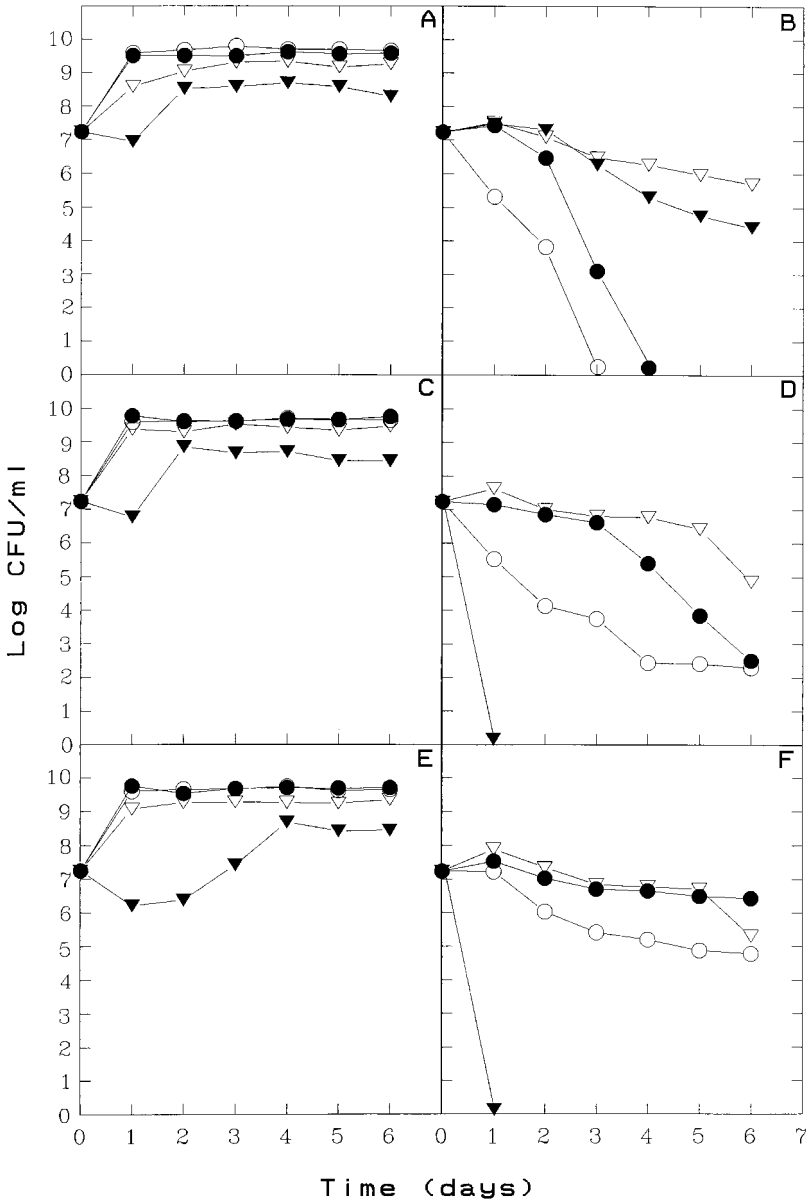

Fig. 3. Effect of salt (NaCl), pH, and temperature on the growth of *Rhizobium* sp. NBRI2505 sesbania. Survival was monitored at 30°C at pH 7 (A), 8 (C), and 9 (E), at 55°C at pH 7 (B), 8 (D), and 9 (F) in the presence of 0 (○), 2 (●), 4 (▽), and 6% (▼) salt (NaCl). Bacterial survival was determined at the indicated times in triplicate, and the results are the means of three independent experiments. A standard deviation of ±0.25 log CFU/ml was found for the viable cell counts.

stress [15, 22]. Since in alkaline soils more salt-tolerant rhizobia were detected, it was of interest to compare the survival of *Rhizobium* sp. NBRI0102 sesbania and *Rhizobium* sp. NBRI2505 sesbania in the presence and absence of salt, when challenged with temperature and pH stresses. *Rhizobium* sp. NBRI0102 sesbania grew at 30°C in the presence of 0 and 2% salt at pH 7 (Fig. 2A), at pH 8 (Fig. 2C), and at pH 9 for 6 days (Fig. 2E). However, at 42.5°C, *Rhizobium* sp. NBRI0102 sesbania survived in the presence of 0, 2, 4, and 6% salt at pH 7 for 6, 6, 2, and 0 days respectively (Fig. 2B); at pH 8 for 4, 6, 1, and 0 days respectively (Fig. 2D); and at pH 9 for 2, 4, 0, and 0 days respectively (Fig. 2F).

Rhizobium sp. NBRI2505 sesbania grew at 30°C in the presence of 0, 2, 4, and 6% salt at pH 7 (Fig. 3A), at pH 8 (Fig. 3C), and at pH 9 (Fig. 3E), for 6 days. However, at 55°C *Rhizobium* sp. NBRI2505 sesbania survived in the presence of 0, 2, 4, and 6% salt at pH 7 up to 2, 3, 6, and 6 days, respectively (Fig. 3B). On the contrary, at pH 8 (Fig. 3D), and pH 9 (Fig. 3F), the strain survived for up to 6, 6, 6, and 0 days, respectively. In contrast to *Rhizobium* sp. NBRI0102 sesbania, high temperature was tolerated efficiently by *Rhizobium* sp. NBRI2505 sesbania at pH 8 (Fig. 3D) and pH 9 (Fig. 3F), as compared to pH 7 (Fig. 3B). In general, 55°C was tolerated efficiently by *Rhizobium* sp. NBRI2505 sesba-

nia in the presence of 2, 4, and 6% salt at pH 7 (Fig. 3B) and in the presence of 2% and 4% salt at pH 8 (Fig. 3D) and pH 9 (Fig. 3F), compared with the absence of salt. The observed enhanced survival at higher temperature of *Rhizobium* sp. NBRI2505 sesbania in the presence of 2% and 4% salt and at pH 8 and 9, as compared with *Rhizobium* sp. NBRI0102 sesbania, indicates that the trait of high salt tolerance at higher pH may have an ecological significance in alkaline soils. These results confirm our earlier observation that the possession of the trait of high salt tolerance might be of some evolutionary significance for the survival of rhizobia in alkaline soils, at high pH, and high temperature. The overall results of the study that *Rhizobium* efficiently tolerated high temperature in the presence of salt and high pH were in agreement with our recent reports [14, 15]. Additional studies are needed to verify whether the stress-tolerant *Rhizobium* sp. NBRI2505 sesbania has a competitive advantage over *Rhizobium* sp. NBRI0102 sesbania in the soil and as a symbiont for *S. aculeata* plants in high salt, pH, and temperature-stressed ecosystems.

The results suggest that *Rhizobium* in alkaline soils has been able to evolve with the ability to grow in high salt, pH, and temperature-stressed ecosystems. The stress-tolerant *Rhizobium* sp. NBRI2505 sesbania should serve as an excellent model to study the physiological, biochemical, and molecular mechanism(s) of salt, pH, and temperature tolerance. *Rhizobium* sp. NBRI2505 sesbania, having previously undescribed stress tolerance levels to high salt and temperature, may be useful in the genetic improvement of plant-associated bacteria. The strain may also be used as a bacterial host to propagate new or improved characters for ecosystems having problems of high salt, pH, and temperature stresses. More extensive ecological survey of *Rhizobium* collected from stressed sites should broaden the genetic background available for exploitation of this agriculturally important microorganism. Our group has initiated molecular studies of stress sensing and response by using promoterless reporter gene insertions to have a good understanding of the basis for host-strain interaction under stress. The study may be helpful in minimizing the impact of high salt, pH, and temperature stresses, currently limiting crop production under low-input conditions, and giving rise to a more sustainable agriculture. Further study of the environmental factors affecting growth and survival of the rhizobia in soil should suggest the basis for obtaining inoculants that are able to give greater nitrogen fixation on crops of economic or agricultural importance, in tropical and subtropical areas.

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