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Desiccation Responses and Survival of *Sinorhizobium meliloti* USDA 1021 in Relation to Growth Phase, Temperature, Chloride and Sulfate Availability

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ORIGINAL ARTICLE

Desiccation responses and survival of *Sinorhizobium meliloti* USDA 1021 in relation to growth phase, temperature, chloride and sulfate availability

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chloride, desiccation, growth phase, matrix, *Sinorhizobium meliloti*, sulfate, temperature.

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Abstract

Aims: To identify physical and physiological conditions that affect the survival of *Sinorhizobium meliloti* USDA 1021 during desiccation.
Methods and Results: An assay was developed to study desiccation response of *S. meliloti* USDA 1021 over a range of environmental conditions. We determined the survival during desiccation in relation to (i) matrices and media, (ii) growth phase, (iii) temperature, and (iv) chloride and sulfate availability.
Conclusions: This study indicates that survival of *S. meliloti* USDA 1021 during desiccation is enhanced: (i) when cells were dried in the stationary phase, (ii) with increasing drying temperature at an optimum of 37°C, and (iii) during an increase of chloride and sulfate, but not sodium or potassium availability. In addition, we resolved that the best matrix to test survival was nitrocellulose filters.
Significance and Impact of the Study: The identification of physical and physiological factors that determine the survival during desiccation of *S. meliloti* USDA 1021 may aid in (i) the strategic development of improved seed inocula, (ii) the isolation, and (iii) the development of rhizobial strains with improved ability to survive desiccation. Furthermore, this work may provide insights into the survival of rhizobia under drought conditions.

Introduction

The response of bacteria to desiccation is of interest to the general public as survival and spread of most bacteria depends in part on their ability to survive desiccation. Rhizobiaceae, a bacterial family of enormous agricultural importance, occur naturally in most agricultural soils and their survival is affected directly by both drought and salinity (Zahran 1999). To make optimal use of the process of nitrogen fixation, seed inoculation companies apply *Rhizobium* strains to the seed surface that are selected for their ability to efficiently fix dinitrogen. However, survival of viable cells after dry storage of seed material is challenged by adverse factors such as salinity, temperature, seed coat toxicity, and desiccation (Deaker *et al.* 2004).

Many conditions have been identified that affect the survival of rhizobia during desiccation. These include, differences in drying methods such as forced drying using vacuum *vs* air drying (VanRensburg and Strijdom 1979) and the media used (Dye 1982; Estrella *et al.* 2004). Survival also depends on the speed and severity of drying (Mary *et al.* 1985), the extent and speed of rehydration (Bushby and Marshall 1977; Kosanke *et al.* 1991), the physiological state (Mary *et al.* 1986), the drying temperature (Kremer and Peterson 1983), and NaCl, and osmoprotectant availability (Mary *et al.* 1986; Kosanke *et al.* 1999). Furthermore, the carrier material used can affect survival, and reports range from granulated peat, bentonite, corn oil, and charcoal (Kremer and Peterson 1983) to biopolymer gels (Chenu 1993; Kaci *et al.* 2005).

Different studies have focused on different rhizobial strains. Due to the high degree of intrageneric differences to cope with desiccation stress (Sadowski and Graham 1998) full insight into factors that determine the survival and response to desiccation is hindered. For example (i) depending on the relative humidity and drying conditions, bradyrhizobia survive desiccation better than sinorhizobia when dried fast; however, when dried slow, the opposite is seen (VanRensburg and Strijdom 1979; Mary *et al.* 1994). (ii) Some *S. meliloti* strains respond positively to the addition of NaCl during drying while others do not (Mary *et al.* 1986), (iii) According to Trotman and Weaver (1995), the ability to withstand high temperature is not related to the ability to withstand desiccation in all rhizobia, and (iv) an increase in survival during the stationary phase is barely or not significant in *Sinorhizobium*, and not significant in *Bradyrhizobium* (Boumahdi *et al.* 1999). The goals of this study were to identify factors that affect the ability of *S. meliloti* USDA 1021 to survive desiccation in relation to matrix, growth phase, temperature, as well as chloride and sulfate availability. We have chosen this strain because its genome has been sequenced (Galibert *et al.* 2001).

Materials and methods

Materials, growth and drying media

The media used were tryptone yeast extract (TY) (Beringer 1974), yeast extract mannitol broth (YMB) (Atlas and Parks 1993), phosphate mannitol medium (PMM) (based on GTS medium, Milcamps *et al.* 1998), in which succinate and glucose were replaced with 0·4% mannitol, and the Tris buffer was replaced with 0·454% (w/v) K_2HPO_4, or $MgSO_4/KH_2PO_4$ buffer (Mary *et al.* 1985). The matrices used were: nitrocellulose filters (0·45 μm, Millipore, HA 02500, Bedford, MA, USA), Ottawa Sand Standard (20–30 mesh; Fisher Scientific, Fairlawn, NJ, USA) and alfalfa seeds (Outsidepride, BS-ALFALFA-5; Lot No: A2N-1769-3; Outsidepride, Salem, OR, USA).

Assay to measure survival during desiccation

Tryptone yeast medium (Meade *et al.* 1982) was inoculated with a single *S. meliloti* USDA 1021 colony from a TY plate and incubated for 3 days at 28°C while agitating at 220 rev min^{-1} until full cell density was reached ($OD_{595\ nm} \sim 2{\cdot}5$). A quantity of 50 μl of this culture were transferred to tubes containing each 5 ml YMB. These cultures were incubated at 28°C and agitated at 220 rev min^{-1} until $OD_{595\ nm}$ values of 0·1–0·2 were reached. Pellets were washed and resuspended in 1 ml of sterile drying/resuspending medium, and colony-forming units (CFUs) were determined with the plate drop method (Hoben and Somasegaran 1982) using sterile water for dilution series. Unless noted otherwise, six parallel samples of each 100 μl cell suspension were transferred onto nitrocellulose filters, or alternative matrices, which were each placed in a microcentrifuge tube. The open microcentrifuge tubes were transferred to a 450-ml gas-tight jar containing 100 ml super-saturated potassium acetate (KAc) which yields a relative humidity (RH) of 22% in the air phase (Potts 1994). The sealed jars were stored for 3 days to 1 week at 20°C in the dark. To test survival in sand, microcentrifuge tubes were first filled with 1 g of Ottawa sand or 0·5 g alfalfa seeds and autoclaved. A 100 μl of cell suspension in water was added to the matrix. Exposure to desiccation conditions was maintained for 5 days. Relative humidity was determined using a hygrometer probe (Traceable, VWR, Bristol, CT, USA). In order to determine the number of surviving cells, the filters in the microcentrifuge tubes were removed from the jars and exposed to 100% RH for 1 h inside an enclosed container at room temperature. One millilitre YMB medium was added to a filter inside a microcentrifuge tube and CFUs in these samples were estimated as described above.

Physiological studies

To compare desiccation survival during different growth stages, YMB tubes were inoculated as described above and incubated until exponential phase ($OD_{595\ nm} \sim 0{\cdot}28$) or stationary phase ($OD_{595\ nm} \sim 1{\cdot}1$). Equal amounts of cells were harvested based on the standardized optical density. Cells were resuspended in water and dried on a nitrocellulose filter. To study the influence of NaCl on survival during desiccation, water, YMB and PMM were supplemented with 200 or 400 mmol NaCl (w/v) to resuspend the cells. When PMM was chosen to determine a response, cells were first grown in PMM and consequently washed and resuspended in PMM, and in PMM amended with NaCl, KCl, Na_2SO_4 or K_2SO_4. To study the effect of temperature on survival during desiccation, the desiccation jars were incubated at different temperatures (5, 28, 37, 42 and 60°C).

Mathematical methods

The percentage of surviving cells are displayed as the ^{10}Log of this percentage, where 2 = 100% survival and 0 = 1% survival. Calculation of the 'fold increase' in survival was achieved by dividing the percentage of surviving cells in a sample by the percentage of surviving cells in a parallel control experiment based on nonamended conditions [exponential growth phase, 0 or 400 mmol (w/v)

NaCl, or 5°C respectively]. When six samples were used, the sample with the most deviating result was deleted. To determine the significance of the observed differences in survival during desiccation, a one-sided Student's *t*-test assuming equal distribution was applied to both data sets (Dytham 2003).

Results

Responses of *S. meliloti* USDA 1021 to desiccation

Initially, desiccation speed was evaluated by comparing the use of a matrix such as nitrocellulose filters against drying without a matrix. Sample volumes of 100 μl were visibly dry after 24 h when dried on a nitrocellulose filter, while 72 h were required for a sample to dry without a filter based on weight reduction. The drying rates were 4·6 ($R^2 = 0{\cdot}94$) and 1·4 ($R^2 = 0{\cdot}99$) μl h^{-1} respectively (data not shown). At 100% RH cells stayed viable for the course of the experiment (Fig. 1a). A decline in viable counts was observed as soon the samples were visibly dry, resulting in a decline in viable counts after 1 day when dried on a filter and 3 days when dried without a filter. The decline in viable counts cannot be explained by irreversible binding to the filter as, using direct counts, 100% of the cells were released from the filter upon rewetting (data not shown). We found a strong negative correlation of survival with the initial cell number at the start of the experiment when dried without a filter (Table 1, Exp. A). Fewer cells may dry faster during the critical phase of drying and may expose cells for a shorter time to conditions of detrimental water content (VanRensburg and Strijdom 1979). Thus, for studying responses to desiccation, the use of nitrocellulose filters is preferred.

Survival curves of filter-dried bacteria were produced to compare the effect of drying in water *vs* YMB. More CFUs appeared when cells were resuspended in YMB compared with water. When stored in YMB at 100% RH (Fig. 1a) the number of survivors did not change after an initial 350% increase in CFUs during the first day. This initial growth of 1·9 cell divisions may explain the 2·4-fold difference in survival when YMB was compared with

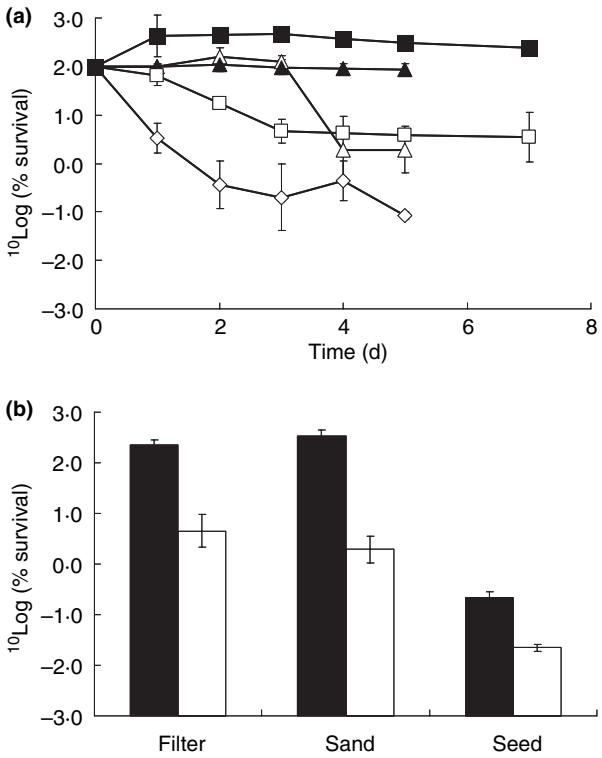

Figure 1 Survival (CFUs) of *Sinorhizobium meliloti* USDA 1021 cells from different matrices and different relative humidities (RH) after 1 week of storage. Error bars represent standard deviation. (a) Survival of *S. meliloti* USDA 1021 at 100% *vs* 22% RH, in yeast extract mannitol broth (YMB) or water, and dried on a filter *vs* without a filter ($n = 6$, except drying on a filter in YMB, $n = 4$). (■) YMB with a filter stored at 100% RH; (□) YMB with a filter stored at 22% RH; (◇) water with a filter stored at 22% RH; (▲) water without a filter stored at 100% RH; (△) water without a filter stored at 22% RH. (b) Viability (in CFUs) of cells from the matrices; nitrocellulose filters, Ottawa sand and alfalfa seeds after storage at 22% RH ($n = 2$). (■) Stored at 100% RH; (□) stored at 22% RH.

Table 1 The effect of drying medium and matrix on survival during desiccation of *Sinorhizobium meliloti* USDA 1021

Exp.*	Drying matrix	Drying medium‡	Initial CFUs§	Average % survival	95% range¶
A	No matrix†	Water	$4{\cdot}7 \times 10^6$	7·07	4·9–10·2
			$1{\cdot}1 \times 10^7$	1·85	1·60–2·14
			$1{\cdot}4 \times 10^7$	0·31	0·22–0·44
B	Filter	Water		0·37	0·23–0·60
		YMB		0·88	0·55–1·41
C	Filter	Water		1·2	0·95–1·51
		NaCl (400)		0·11	0·10–0·11

*Experiment number.

†In the bottom of a microcentrifuge tube.

‡Numbers in parentheses indicate the concentration of NaCl (mmol, w/v).

§Procedural number prior to drying.

¶Statistical differences were based on $n = 6$ repeats, except for experiment B ($n = 12$).

water as the drying medium ($P = 0{\cdot}029$, $n = 12$, Table 1, Exp. B). No significant differences in survival could be observed when cells were resuspended in TY, YMB, or $MgSO_4/KH_2PO_4$ buffer (data not shown). However, an 11-fold decline in CFUs was observed when dried in the presence of NaCl compared with water alone (Table 1, Exp. C), indicating a toxic effect for NaCl in water.

To determine a suitable matrix for the measurement of the desiccation response of *S. meliloti* USDA 1021, we investigated three different matrices: (i) nitrocellulose filters, (ii) Ottawa sand and, (iii) alfalfa seeds. As the data in Fig. 1b indicate, survival results from filter and sand were comparable, but survival on alfalfa seeds was low even without drying. Thus alfalfa seeds do not provide a suitable matrix to study the response to desiccation of *S. meliloti* USDA 1021. We continued to work with nitrocellulose filters to further study desiccation responses.

Physiological experiments

Stationary phase

We tested the survival of strain *S. meliloti* USDA 1021 during desiccation in relation to growth phase. At 100% RH survival was not affected. However, when dried at 22% RH cells in the stationary phase survived 2·9-fold ($n = 5$, $P = 1{\cdot}5 \times 10^{-3}$) better than those cells dried in the exponential growing phase (Fig. 2a).

Temperature

Survival of *S. meliloti* USDA 1021 was tested for a range of temperatures. The finding of a temperature optimum (Fig. 2b) and the decrease in desiccation survival at temperatures higher than 37°C ($P = 3{\cdot}9 \times 10^{-4}$ compared with 5°C) may present the limit of a physiological response.

The effect of salt

The effect of NaCl on the survival of filter-dried cells during desiccation was determined by resuspending cells in water, or in water containing NaCl, followed by drying at 22% RH (Table 1, Exp. C). When cells were dried in YMB amended with 200 or 400 mmol (w/v) NaCl, a 3·1-($P = 1{\cdot}8 \times 10^{-3}$) and 4·1-fold ($P = 3{\cdot}9 \times 10^{-4}$) increase in survival was observed compared with YMB without the addition of NaCl respectively (Fig. 2c). Similarly, when survival in PMM was compared with PMM amended with 400 mmol l^{-1} NaCl, a fivefold increase was observed for *S. meliloti* USDA 1021. The observed increase in survival after NaCl-mediated desiccation may be induced by an increase in sodium or chloride. Therefore, we tested the response to desiccation by exposing cells to sodium, potassium, chloride and sulfate ions. Data in Table 2 (Exp. A–C) indicate that (i) chloride increased survival

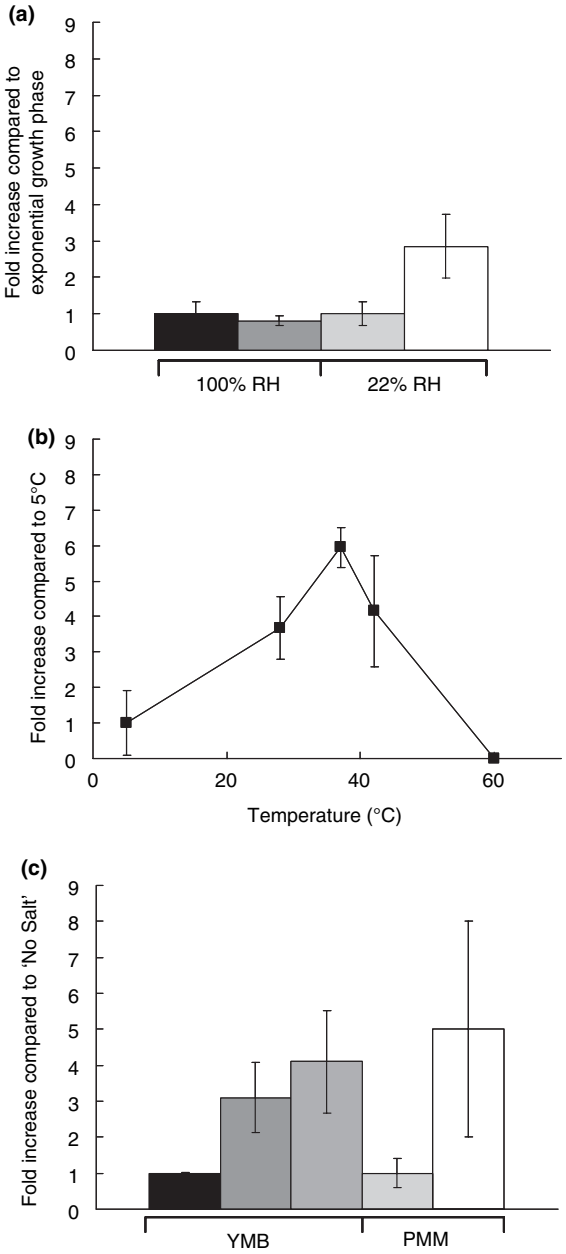

Figure 2 Survival of *Sinorhizobium meliloti* USDA 1021 after desiccation during different growth phases, over a range of temperatures, and under salt stress. Error bars represent the standard deviation. $P < 0{\cdot}01$ in all situations compared with the normalized condition. (a) Survival during exponential and stationary phase ($n = 5$) at 100% and 22% RH; (■) exponential phase, 100% RH; (■) stationary phase, 100% RH; (▒) exponential phase, 22%; (□) stationary phase, 22% RH. (b) Survival after desiccation at increasing temperatures. (c) Survival during desiccation in the presence of different NaCl concentrations. YMB (■) compared with YMB amended with 200 (■) or 400 (▒) mmol l^{-1} NaCl ($n = 6$), and PMM (▒) compared with PMM with 400 (□) mmol l^{-1} NaCl ($n = 9$).

Table 2 The effects of sodium, potassium, chloride and sulfate ions in phosphate mannitol medium (PMM) on the response to desiccation of *Sinorhizobium meliloti* strain USDA 1021

Exp.*	Dried in†	Average % survival	95% range‡	Fold difference§
A	PMM	0·15	0·08–0·26	1
	NaCl (400)	0·60	0·45–0·81	4·0
	Na_2SO_4 (200)	1·21	0·71–2·08	8·1
B	PMM	0·45	0·15–1·35	1
	K_2SO_4 (200)	2·51	2·25–2·79	5·6
C	PMM	0·41	0·32–0·51	1
	KCl (400)	1·55	0·84–2·88	3·8
	Na_2SO_4 (200)	4·01	2·59–6·22	9·8

*Experiment number.
†Numbers in parenthesis indicate the concentrations of NaCl, KCl, Na_2SO_4, or K_2SO_4.
‡Statistical differences were based on five repeats.
§Based on average.

approx. fourfold compared with the control, and (ii) sulfate, despite a concentration of only 200 mmol (w/v), increased the ability of *S. meliloti* USDA 1021 to survive desiccation compared with the control six- to 10-fold. An effect of sodium and potassium on survival cannot be ruled out.

Discussion

Responses to desiccation of *S. meliloti* USDA 1021 to drying media and drying speed

Saturated potassium acetate provides a relative humidity of 22% in the airphase. Previous studies have shown that the survival of *S. meliloti* RCR2011 is highest between a relative humidity of 67% and 22% (Mary *et al.* 1985). Thus 22% RH should lead to severe desiccation conditions without the negative effects of leaving the range of tolerable relative humidity and drying too severely. We found that drying for 1 day resulted in a similar decline in CFUs as drying for 3 days. We assume this decline corresponds to the last phase of the drying process. This is in accordance with other drying studies (Bushby and Marshall 1977; Salema *et al.* 1982; Mary *et al.* 1985, 1986). We have chosen to focus only on short-term survival during desiccation, as even long-term storage leads to a mere 10-fold reduction in CFUs over 78 days (data not shown). Thus, the effect of physiological and environmental conditions during drying on surviving cells can best be measured immediately after drying.

Effect of the matrix

As the data in Fig. 1c indicate, survival on alfalfa seeds is low even prior to drying, which can be explained either by low recovery or seed coat toxicity caused by polyphenolics (Salema *et al.* 1982; Smith 1992). Toxicity is not likely to be caused by heat labile compounds, as we autoclaved the seeds prior to use. Drying further lowers survival, thus, at least two factors determine survival on alfalfa seeds as described before (Salema *et al.* 1982). Interestingly, storage and drying on a nitrocellulose filter and in Ottawa sand results in similar survival rates for both matrices. We chose nitrocellulose filters over sand to further study desiccation responses: (i) to prevent differential drying conditions for cells as sand provides a three-dimensional matrix while nitrocellulose is easier to control as a two-dimensional matrix and, (ii) to prevent possible shearing forces during rewetting which are caused by sand.

Physiological experiments

Stationary phase

Generally, bacterial cells are more stress resistant in the stationary phase. Maturation of seed inocula also increases survival on seeds (Materon and Weaver 1985). However, studies have reported variable results on survival of *S. meliloti* in relation to growth phase. Using freeze drying, Dye (1982) did not find an increase in survival in the stationary phase. Boumahdi *et al.* (1999) found a twofold, yet not significant, increase in survival during the stationary phase for *S. meliloti* RCR2011, but not for the bradyrhizobia tested. Mary *et al.* (1986), however, found an increase in survival after drying in the stationary phase of *S. meliloti* RCR2011. We tested the survival of strain *S. meliloti* USDA 1021 during desiccation in relation to growth phase. Cells in the stationary phase survived 2·9-fold ($n = 5$, $P = 1{\cdot}5 \times 10^{-3}$) better than cells dried in the exponential growing phase (Fig. 2a). An increase in survival in the stationary phase may be caused by the accumulation of trehalose, as trehalose accumulates in rhizobia during the stationary phase and has a protective role in survival during desiccation (Potts 1994). Similarly, polyhydroxybutyrate (PHB) accumulates during the stationary phase in rhizobia (Manna *et al.* 2000). In *Azospirillum*,

strains that accumulate PHBs are more resistant to desiccation (Okon and Itzigsohn 1992).

Temperature

The finding of a temperature optimum (Fig. 2b) and the decrease in desiccation survival at temperatures higher than 37°C ($P = 3{\cdot}9 \times 10^{-4}$ compared with 5°C) may present the limit of a physiological response. This is supported by the fact that *S. meliloti* USDA 1021 does not grow at 42°C. Drying of cells is accompanied by a change in midpoint temperature of the cell membranes during the drying process, and can, in combination with the ambient temperature, cause the membranes to leak (Potts 1994). As *S. meliloti* USDA 1021 cells are commonly grown at 28°C, we expect that drying at lower temperatures decreases membrane integrity and lowers survival. Similarly, drying at higher, but nonlethal temperatures would prevent membrane transition and increase survival (Potts 1994). As this effect is supported by our findings, we conclude that the response to desiccation under a temperature range includes not only a physiological response, but also depends on a physical factor. Trotman and Weaver (1995) showed that temperature resistance does not correlate with desiccation resistance, thus this correlation is strain specific.

The effect of NaCl

Cells dried in YMB or PMM amended with NaCl, survive desiccation better. Thus YMB and PMM not only counter the toxic effect of NaCl, these media also increase survival (Fig. 2c). This observation is supported by the work of Mary *et al.* (1986) who showed that *S. meliloti* RCR 2011 could better survive desiccation when it was first grown in media containing NaCl. Mary *et al.* observed a 5·5-fold increase in survival when using 530 mmol l^{-1} NaCl. However, in the same study, survival of *S. meliloti* 1·5-fold did not increase, indicating that protection by external solutes can be excluded for the increase in survival of RCR2011. The positive effect of NaCl can be explained by (i) *de novo* synthesis of trehalose and betain in osmotically stressed *Rhizobium* cells. Trehalose protects against desiccation and betain counters NaCl toxicity in dry seed inocula, even after 4 months of storage (Kosanke *et al.* 1999) and, (ii) the accumulation of PBH in osmotically stressed cells. PBH accumulation has been related to desiccation stress resistance in *Azospirillum* (Okon and Itzigsohn 1992).

The observed increase in survival after NaCl-mediated desiccation can be induced by an increase in sodium or chloride. As the data in Table 2 (Exp. A–C) indicate (i) chloride causes a response, (ii) sulfate, despite a concentration of only 200 mmol (w/v), increases the ability of *S. meliloti* to survive desiccation compared with chloride, and (iii) the differences in the increase in survival are not caused by sodium or potassium ions. It is unlikely that osmotic stress is responsible for the observed phenomenon as survival does not correlate with the calculated osmotic strength of the medium. Polysaccharides can protect against desiccation (Chenu 1993; Kaci *et al.* 2005), and differential responses in polysaccharide production under NaCl and Na_2SO_4 stress were reported (Lloret *et al.* 1995). However, negative as well as no effects of the addition of polysaccharides on survival during desiccation have been observed (Bushby and Marshall 1977; Pena-Cabriales and Alexander 1979; Osa-Afiana and Alexander 1982; Mary *et al.* 1986). If production of different polysaccharides under osmotic stress by NaCl and Na_2SO_4 leads to differences in survival of *S. meliloti* USDA 1021 remains to be investigated.

Conclusions

We conclude that the survival of *S. meliloti* USDA 1021 was increased under the following conditions: (i) 37°C compared with 5°C, during the stationary phase compared with the exponential phase, and under NaCl availability. Chloride, but mainly sulfate availability did increase survival of *S. meliloti* USDA 1021.

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