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Short Communication

Influence of Culture Medium and Growth Stage on the Survival of *Bradyrhizobium japonicum* during Desiccation and Storage at Two Relative Humidities

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Abstract

The influence of culture medium and growth stage of *Bradyrhizobium japonicum* strain G49 on survival during desiccation was studied with liquid cultures grown for 5 and 14 days on glucose- or glycerol-based media, with or without Na-glutamate. They were exposed to short-term desiccation and storage at 0% and 75% relative humidity (RH). Average death rate during the experiment was similar at 0% and 75% RH, but after moisture equilibration, death rate was higher at 75% than at 0% RH. The composition of the culture medium influenced survival and death rate of *B. japonicum*. In general, glucose alone in the medium appeared to allow a higher survival than the glycerol-based medium. Survival of the bradyrhizobial cells decreased when glutamate was added to glucose, and increased when glutamate was added to glycerol. Five-day old cultures survived as well or better than 14-day old cultures.

Keywords: *Bradyrhizobium japonicum*, culture medium desiccation, growth stage, survival

1. Introduction

Liquid inoculants of *Bradyrhizobium japonicum* are an alternative to the use of peat-based bradyrhizobia for soybean inoculation. These liquid inoculants

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can be produced as pure bacterial cultures without the need to find, and process any adapted carrier (Wadoux, 1991). These liquid inoculants can be used for seeds and for seed-bed inoculation (Brockwell et al., 1980; Wadoux, 1991).

As with the other types of inoculants, bradyrhizobia applied as liquid inoculants may experience desiccation on the seeds or in the soil during the inoculation process. Short-term decreases of several orders of magnitude of *Bradyrhizobium japonicum* were reported to occur during desiccation in soils (Al-Rachidi et al., 1982; Mahler and Wollum, 1980).

Several factors have been reported which influence the survival of bradyrhizobia during desiccation. When the rate of drying was increased, it was found to decrease the survival of cowpea bradyrhizobia (Jansen van Rensburg and Strijdom, 1980) and *B. japonicum* (Pena-Cabriales and Alexander, 1979). Water availability can also influence survival. *B. japonicum* has been reported to have a lower survival rate in a loamy soil when the water potential decreased (Mahler and Wollum, 1980). Additionally, survival of dried *B. japonicum* cells entrapped in polysaccharide gels was found to be optimal at very low water activity (Mugnier and Jung, 1985). Survival of *B. japonicum* during desiccation can be improved by adding ingredients to the inoculant such as adhesives (Davidson and Reuszer, 1978; Elegba and Rennie, 1984), sugars (Bushby and Marshall, 1977; Mugnier and Jung, 1985) or other chemicals, PVP (Bushby and Marshall, 1977) or oil (Hoben et al., 1991). Survival has also been reported to vary between strains of *B. japonicum* (Al-Rashidi et al., 1982, Jansen van Rensburg and Strijdom, 1980; Mahler and Wollum, 1980).

However, the effect of growth stage, culture medium and exopolysaccharides (EPS) on survival are not well-documented for liquid inoculants of *B. japonicum*.

The purpose of this work was to assess the possible effect of culture medium and growth stage of *B. japonicum* on its survival as liquid inoculants during desiccation and storage at 0% and 75% relative humidity (RH). Culture media able to support intense *B. japonicum* growth required for inoculant manufacture and allowing the production of different amounts of EPS, were selected.

2. Materials and Methods

Strain

The *B. japonicum* strain used for this study was G49 (IARI SB16, New Delhi), currently used in France for soybean inoculation.

Cultures

The four culture media were adapted from Cliquet et al. (1992). Per litre, they contain: Yeast extract (Difco Laboratories), 0.5 g; NH_4Cl, 0.6 g; KH_2PO_4,

0.8 g; $MgSO_4$, $7H_2O$, 0.49 g; NaCl, 0.1 g; $CaCl_2$, $2H_2O$; 0.14 g, $FeCl_3$, 29 mg. MES (2-(N-morpholino)ethanesulfonic acid) at 52 mM was used for buffering, and the pH adjusted to 6 before autoclaving.

Two carbon sources were selected, glucose and glycerol, as they were reported to allow, respectively, low and high EPS production (Tully, 1985) and to produce high numbers of bacteria (Balatti et al., 1991; Cliquet et al., 1992). Glutamate, which improves the growth of *B. japonicum*, was used as a supplementary organic C and N source (Cliquet et al., 1992).

(Glu)-medium contained glucose (10 g l^{-1}) and (Glu-G)-medium, glucose (10 g l^{-1}) and Na glutamate (1.2 g l^{-1}). (Gly)-medium contained glycerol (10.2 g l^{-1}) and (Gly-G)-medium, glycerol (10.2 g l^{-1}) and Na glutamate (1.2 g l^{-1}).

Sterilization was done by autoclaving (120° C) for 20 min, the media containing carbon, nitrogen sources and yeast extract, except for glucose which was sterilized by filtration.

Cultures were grown in 100 ml conical flasks containing 10 ml medium, inoculated with 0.5 ml of a suspension in sterile water of *B. japonicum* cells from a fresh agar slant. They were held at 28° C and shaken at 200 rpm. Two growth stages were considered: the end of exponential growth, 5 days (5 d) and the stationary phase, 14 days (14 d).

Cultures used for drying experiments contained the following log number of viable *B. japonicum* per ml after 5 days growth, (Glu)-medium, 9.78±00.7; (Glu-G)-medium, 9.9±00.9; (Gly)-medium, 9.63±0.20; (Gly-G)-medium, 9.80±00.5, and after 14 days growth, (Glu)-medium, 9.97±0.09; (Gly)-medium, 9.76±0.05.

Exopolysaccharide (EPS) production was studied in another experiment with the same media and growth conditions as for culture production.

Drying experiments

After 5 and 14 days growth, 0.5 ml of each culture was mixed with 5 g of sterilized glass beads (0.25–0.30 mm diameter) in a 30 ml cotton-plugged vial. Vials were stored at 20° C in 6.5 l desiccators (42 vials per desiccator), each with 79 g of anhydrous $CaCl_2$ or 1000 ml of a saturated solution of NaCl, in order to be in equilibrium with 0% and 75% RH, respectively. The decrease in moisture content of the vials is reported in Table 1.

Analysis

Plate counts of viable *B. japonicum* were done in triplicate by serial dilution in sterile water and plating on Bergersen culture medium (Bergersen, 1961),

Table 1. Moisture content of vials during desiccation

Relative humidity (%)			
0		75	
Time (d)	Moisture content (%)	Time (d)	Moisture content (%)
0	10.5	0	10.5
4	6.4	7	9.8
6	2.8	19	4.1
8	1.0	25	4.2
10	0.4	30	3.8
16	0.4		

using the Spiral™ system. To monitor the moisture equilibration of the cultures during desiccation and storage, water contents of vials were determined by weighing.

Samples were analyzed after 0, 4, 6, 8, 10 and 16 days drying at 0% RH, and after 0, 7, 19, 25, 30 days drying at 75% RH. Exopolysaccharides were determined by the method described by Tully (1985).

In order to compare cultures with different zero-time numbers of viable *B. japonicum*, survival was calculated as N/N_0 and expressed as log N/N_0, where N_0 is the zero-time colony count and N the number of survivors after exposure to drying. Log N/N_0 expresses the decrease in log number of viable cells.

Statistical analysis and regressions were performed using STAT-ITCF software (ITCF, 1991), following Dagnélie (1970). Results are expressed at the $P = 0.05$ level.

The first linear regression considered (formula 1) was $\log N/N_0 = k_1 t$ were t is the exposure time to drying in days, and k_1 a coefficient expressing the average bacterial death rate during the whole experiment, in log viable cells d^{-1}. The second regression (formula 2) was $\log N/N_0 = -k_2 t + e$, where t is the exposure time to drying, in days, k_2, a coefficient expressing the bacterial death rate after moisture equilibration, in log viable cells d^{-1}, and e, the estimated value of log N/N_0 for $t = 0$.

3. Results and Discussion

The log numbers of viable *B. japonicum* and EPS produced after 5 and 14 days growth in the different culture media are reported in Table 2. All the media allowed the production of 10^{10} viable *B. japonicum* per ml. Glycerol was found to allow the production of more EPS than glucose, as reported by Tully

Table 2. Growth of *B. japonicum* and EPS production of (5 d) and (14 d) cultures. (Glu) = glucose; (Glu-G) = glucose + glutamate; (Gly) = glycerol; (Gly-G) = glycerol + glutamate. Data from the same column followed by the same letters are not statistically different.

Medium	Log viable cells (ml^{-1})		EPS ($\mu g\ ml^{-1}$)	
	(5 d)	(14 d)	(5 d)	(14 d)
(Glu)	10.07_b	10.24_a	219_b	259_b
(Glu-G)	10.22_a	ND	211_b	232_b
(Gly)	10.10_b	10.06_b	101_c	1078_a
(Gly-G)	10.17_a	ND	482_a	ND

(1985), especially for (14 d) and (5 d) cells grown with glutamate. These cells had a higher survival rate than those grown without glutamate and produced 4 times as much EPS. However, it is difficult to draw conclusions about the respective effects of glutamate and EPS produced. Thus, our results, as those of Hartel and Alexander (1986) for *Bradyrhizobium* sp. do not confirm the hypothesis that EPS protects microorganisms from desiccation.

In these experiments, cultures of *B. japonicum* were exposed to conditions that varied with time: a phase 1 of drying to constant moisture content, and a phase 2 of storage at constant RH. In order to take these conditions into account, we used two models to evaluate the average death rate, k_1 (phases 1 and 2) and the death rate at constant RH, k_2 (phase 2).

Losses in log numbers of viable *B. japonicum* after dessication at 0 and 75% RH are reported in Tables 3 and 4, respectively, only when moisture equilibration was achieved (8 and 16 days at 0% RH and 19 and 30 days at 75% RH). On the average, losses at 0% RH, were, in log number of viable cells, of 1.85 after 8 days, and 3.48 after 16 days, for all the media and culture ages. At 75% RH, average losses were of 1.11 log number of viable cells after 19 days and 7.35 log after 30 days for all the media and culture ages.

Linear regressions calculated with formula 1 at 0% RH (0.8, 10 and 16 days) and 75% RH (0, 19, 25 and 30 days) were all found to be significant, and statistically different only at 0% RH. The average bacterial death rate k_1 was of 0.205 and 0.225 log cells d^{-1} for 0 and 75% RH, respectively. When pooled, cultures were found to have average death rates k_1, which were not statistically different at 0% and 75% RH. Observed k_1 values are in accordance with values estimated from previously reported results for *B. japonicum*: 0.06–0.23 in sand (Jansen van Rensburg and Strijdom, 1980), or 0.15–0.22 in soils (Pena-Cabriales and Alexander, 1979).

Regressions calculated with formula 2 at 0% RH (8, 10 and 16 days) and

Table 3. Survival and bacterial death rate at 0% RH. Data from the same column followed by the same letters are not statistically different. Bacterial death rates k_1 and k_2 are followed by (R-squared) of the linear regression. NS = not statistically different at P = 0.05.

Culture	Survival (log N/N_0)		Bacterial death rate ($\log_{10} d^{-1}$)	
	8 d	16 d	k_1	k_2
(Glu)-(5 d)	-1.42_b	-2.26_a	-0.143_b (0.98)	-0.116_c (0.74)
(Glu)-(14 d)	-1.18_b	-3.09_a	-0.177_b (0.95)	-0.276_b (0.89)
(Glu-G)-(5 d)	-2.79_d	-2.51_a	-0.205_b (0.89)	NS
(Gly)-(5 d)	-0.10_a	-4.88_b	-0.222_b (0.77)	-0.606_a (0.98)
(Gly)-(14 d)	-3.75_e	-4.98_b	-0.374_a (0.95)	NS
(Gly-G)-(5 d)	-1.84_c	-3.13_a	-1.90_b (0.96)	-0.178_{bc} (0.64)
Mean	−1.85	−3.48	−0.225 (0.81)	−0.229 (0.28)

Table 4. Survival and bacterial death rate at 75% RH. Data from the same column followed y the same letters are not statistically different. Bacterial death rates k_1 and k_2 are followed by (R-squared) of the linear regression.

Culture	Survival (log N/N_0)		Bacterial death rate ($\log_{10} d^{-1}$)	
	19 d	30 d	k_1	k_2
(Glu)-(5 d)	-1.17_b	-5.02_b	−0.161 (0.88)	-0.360_a (0.69)
(Glu)-(14 d)	-1.72_b	-7.76_b	−0.231 (0.90)	-0.576_{ab} (0.92)
(Glu-G)-(5 d)	-1.23_d	-7.89_b	−0.216 (0.89)	-0.611_b (0.97)
(Gly)-(5 d)	-0.41_a	-7.64_b	−0.192 (0.77)	-0.741_{bc} (0.94)
(Gly)-(14 d)	-1.99_c	-7.97_b	−0.228 (0.92)	-0.531_{ab} (0.93)
(Gly-G)-(5 d)	-0.96_b	-7.81_b	−0.199 (0.84)	-0.624_b (0.91)
Mean	−1.11	−7.35	−0.205 (0.86)	−0.574 (0.91)

75% RH (19, 25 and 30 days) were all found to be significant and statistically different, except at 0% RH for (5 d) culture on (Glu-G)-medium and (14 d) culture on (Gly)-medium. The average death rate after moisture equilibration, k_2, was significantly lower at 0% than 75% RH, 0.229 and 0.574 log d^{-1}, respectively. This result is in accordance with the results of Mugnier and Jung (1985) and Mary et al. (1993), who found a higher survival rate of dried *B. japonicum* at low % RH than at 75% RH.

The composition of the growth medium influenced both survival and death rates of *B. japonicum*. Glucose alone allowed a significantly higher survival at 0% RH, of both (5 d) and (14 d) cultures after 16 days desiccation and a significantly lower death rate k_2 than glycerol alone. The same trends were observed at 75% RH, with a significantly higher survival after 30 days drying, and a lower death rate k_2 for (5 d) cultures, with glucose-based, compared to other media. These results are in accordance with the better survival of glucose than glycerol-grown *B. japonicum* reported during storage of dried cells (Mugnier and Jung, 1985).

When added to glucose, glutamate decreased the survival after 8 days, but not after 16 days. It has no effect on death rates k_1 or k_2. When glutamate was added to glycerol, the survival of (5 d) cultures decreased after 8 days, but increased after 16 days, and the death rate k_2 decreased.

These results must be taken into account in the improvement of liquid inoculants. As desiccation can cause very important losses during inoculation, a compromise must be found between culture media allowing the best cell production and the best survival on desiccation. As an illustration, we have previously found (Cliquet et al., 1992), that glutamate improves the growth of *B. japonicum* on glucose, but not on glycerol-based media. From a survival point of view, it may be interesting to add, for inoculant production, glutamate to the glycerol-medium, and to suppress it in the glucose-medium.

The culture age did not influence the survival nor the average death rate k_1, for the (Glu)-medium at 0% RH. However, (5 d) cultures had a lower death rate k_2 than the (14 d) ones after moisture equilibration. For (Gly)-medium, the (5 d) cultures survived better than the (14 d) ones after 8 and 10 days, but not after 16 days at 0% RH. The (14 d) cultures on (Gly)-medium had a significant higher average death rate k_1 than by the other treatments. After moisture equilibration at 0% RH, the (5 d) cultures on (Gly)-medium had the highest observed death rate k_2. At 75% RH, (5 d) cultures survived better than the (14 d) ones, after 19 and 30 days of (Glu)-medium and after 19 days, only of (Gly)-medium. At 75% RH, death rates k_1 and k_2 were not influenced by the age of cultures of (Glu) and (Gly)-media. Thus, (5 d) cultures (end of exponential growth), survived as well or better than the (14 d) ones, whatever

the culture medium. These results differ from the data reported for *Rhizobium meliloti* stationary cells which survived desiccation better than young cells (Mary et al., 1986). This point must also be considered if cultures are to be dried for inoculant manufacture.

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