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Desiccation tolerance of rhizobia when protected by synthetic polymers

Rosalind Deaker*, Rodney J. Roughley, Ivan R. Kennedy

Faculty of Agriculture, Food and Natural Resources, Ross St. Building A03, University of Sydney, NSW 2006, Australia

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Abstract

Survival of rhizobia applied to the surface of legume seeds is poor due to factors such as desiccation. Poor survival of rhizobia results in poor nodulation and yield of legumes. Selecting polymeric adhesives for inoculation of legume seed with rhizobia that provide protection during desiccation may improve survival and increase the potential for maximum legume yields. Vacuum-drying cells after suspension in selected polymers proved an effective method for screening the potential of polymers to improve the desiccation tolerance of rhizobia. The effect of different polymers on survival of desiccated rhizobia could be attributed to their different chemical and physical properties. The specific protective properties of polymers have been difficult to determine due to the variation in the chemical nature of polymers often compared. In this research polyvinyl alcohol (PVA) with varying degrees of hydrolysis provided a useful range of measurable physical properties against which bacterial survival could be measured. PVA with a percent hydrolysis in the range 86.5–89% was better able to protect desiccated cells of a range of rhizobial strains than polymers with higher (98.5%) or lower (78.5–82%) degrees of hydrolysis. The percent hydrolysis affected the moisture properties of PVA and survival of rhizobia was not maximised with high moisture sorption or low water activity by the polymer but rather when moisture properties were at an intermediate level. In comparison, survival was poorest in highly hygroscopic polymers methyl cellulose (MC) and polyvinyl pyrrolidone (PVP). The survival profile of desiccated rhizobia stored at different relative humidities was altered when cells were embedded in different polymers and is probably related to moisture sorption by those polymers. The percent hydrolysis also affects the extent to which PVA is able to stabilise colloids against the precipitating action of KCl. The colloid-stabilising property and survival was highest at 86.5–89% indicating that this property may be manipulated to achieve better survival. There is an indication that highly stabilising PVA may lead to more evenly dispersed cells providing more colony forming units rather than better survival. However, survival was not strongly correlated to the colloid-stabilising properties of the other polymers and was very poor after suspension in highly stabilising MC indicating a strong interaction between factors. Synthetic polymers designed to improve survival of rhizobia exposed to desiccation stress should include properties that combine high stabilisation and optimum moisture sorption properties.

Keywords: Rhizobia; Polymers; Desiccation; Moisture sorption; Colloidal stabilisation; Polyvinyl alcohol

1. Introduction

Desiccation is one of the major factors causing poor rhizobial survival on legume seeds (Vincent et al., 1962; Thompson, unpub. Ph.D. thesis, University of Sydney, 1964). Rhizobia are susceptible to drying on surfaces particularly where individual cells are exposed to low ambient relative humidity. This has been demonstrated on various surfaces such as glass (Vincent et al., 1962), sand (Bushby and Marshall, 1977a, b) and membrane filters (Mary et al., 1985).

Several investigations have focused on the effects of the suspending medium on the recovery of cells after dehydration. Sugars, sugar alcohols, amino acids, synthetic and natural polymers and clay minerals have improved recovery of dried bacterial cells. Sucrose was superior to sorbitol, lysine, amino acids, milk and yeast mannitol broth for preserving rhizobia during freeze-drying (Vincent, 1958). Annear (1962) found peptone mixed with glucose or sorbitol was effective for the preservation of vacuum-dried bacteria on cellulose fibres at room temperature. He concluded that a drying medium must be assessed with

*Corresponding author. Tel.: +61 2 9036 9385; fax: +61 2 9351 5108.
E-mail address: r.deaker@usyd.edu.au (R. Deaker).

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regard to the protection it affords during drying, the protection it affords during storage, the simplicity and definability of its composition and the range of organisms with which it is effective.

Colloid-stabilising properties of suspending media were found to be important for survival. When streptococci were dried in the presence of a range of colloidal substances, peptone offered more protection than starch, gum tragacanth and gastric mucin (Heller, 1941). The improved protection by the colloids was positively correlated with their hydrophilicity and their ability of the colloids to stabilise colloidal gold against the precipitating action of salt (calculated as the gold number, Zsigmondy, 1917).

Water activity and solvent properties of water in biopolymers has also been implicated in survival of bacteria during desiccation (Mugnier and Jung, 1985). Survival of biopolymer-entrapped *Bradyrhizobium japonicum* decreased when the water activity was increased from 0.06 through to 0.75. Moisture sorption by polymers affects the water available to microorganisms at different relative humidities.

Polymers are commonly used to improve the adhesion of peat-based rhizobial legume inoculants and other seed-coating materials to the surface of seeds (for a review of legume-seed inoculation technology see Deaker et al., 2004). Gum arabic and methyl cellulose (MC) are common ingredients of the commercial inoculant adhesives often sold with inoculants. Polyvinyl pyrrolidone (PVP) was patented as a seed coating adhesive for microorganisms following reports that the insoluble form, polyvinyl polypyrrolidone (PVPP), bound water-soluble legume-seed-coat exudates some of which were toxic to rhizobia (Hale and Mathers, 1977; Lloyd, 1983). Since then these properties have been exploited by the incorporation of vinyl pyrrolidone co-polymers into seed–inoculant formulations (Williams, 1992; Williams and Day, 1994)

Despite efforts to identify properties of polymers that improve survival of rhizobia under desiccation, little progress has been made because of the complex and disparate nature of the materials often compared. The aims of the experiments reported in this paper were to screen chemically similar polymers and identify key properties that enhance survival of rhizobia under desiccation.

2. Materials and methods

2.1. Polymer selection

Selected polymers are listed in Table 1. Polymers MC (Bio-Care Technology Pty. Ltd.) and PVP (BDH) were chosen as reference polymers. The cellulosic polymer MC is commonly used as a commercial adhesive for coating legume seeds with peat inoculants and the soluble form of PVP was shown to be an effective adhesive for the commercial inoculation of white clover (Lloyd, 1983).

Three different grades of polyvinyl alcohol (PVA, Nippon Gohsei) were selected to provide a set of polymers that were essentially the same chemically but varied in the extent to which acetate groups on the original polyvinyl acetate had been replaced with alcohol groups. The percent hydrolysis of polyvinyl alcohol alters the moisture characteristics, a property shown to effect survival (Mugnier and Jung, 1985). Percent hydrolysis values were provided by the manufacturer and are listed in Table 1. With this basis, survival patterns could then be directly related to this single property. Percent hydrolysis is a property not typically specified for MC and PVP. All polymers selected were also water soluble, an important factor for the eventual release of encapsulated rhizobia into the seedling rhizosphere.

Table 1
Polymers selected for survival analysis

Polymer	Abbreviation	Description and supplier
Methylcellulose	MC	Commercially available seed pelleting adhesive (Bio-Care Technology Pty Ltd., 2001)
Polyvinyl pyrollidone	PVP	MW = approx. 44,000, soluble (BDH)
Polyvinyl alcohol[a]	PVA—KL05	78.5–82% hydrolysis, MW = 29,000
Polyvinyl alcohol[a]	PVA—GL05	86.5–89% hydrolysis, MW = 29,400
Polyvinyl alcohol[a]	PVA—NL05	98.5% hydrolysis, MW = 22,000

[a]All polyvinyl alcohols were Gohsenol grade manufactured by Nippon Gohsei.

2.2. Screening of polymers for protection of desiccated rhizobia

Polymers were screened for their ability to protect rhizobia during the process of vacuum-drying and storage of the dried cells in controlled conditions of temperature and relative humidity (RH). Bacterial cells were suspended in aqueous polymer solutions and dried in a mini freeze-dryer (Dynavac, Australia) allowing consistency in the drying process. The suspensions were contained within cotton wool-plugged glass ampoules to reduce the variation due to differences in adhesion that may be experienced with beads or seeds. Except for MC, where only 1.5% solutions could be used because of high viscosity, 5% solutions of the polymers were chosen for screening. Bacterial cells were washed before drying to remove excess exopolysaccharides that may mask the effect of the polymers being tested. Following drying, the cotton wool plugs were removed and the polymer-embedded cells were exposed to air at 20 °C and 54% RH to simulate average atmospheric conditions experienced by rhizobia on the seed surface during storage before sowing.

Table 2
Rhizobium strains and places of isolation

Strain No.	Strain name	Isolated from
TA1[a]	*Rhizobium leguminosarum bv. trifolii*	Bridport, Tasmania, Australia
SU277/1[b]	*Sinorhizobium meliloti*	*Leeton, NSW, Australia*
WU425[a]	*Bradyrhizobium* sp. *(Lupinus)*	Western Australia
CB1015[a]	*Bradyrhizobium* sp.	Hort Haringhata, India.
CB1809[b]	*Bradyrhizobium japonicum*	Beltsville, USA.
SU343[b]	*Mesorhizobium loti*	Nodogen® peat, USA.

[a]Strains were selected from the culture collections of SUNFix Centre for Nitrogen Fixation, University of Sydney.
[b]Bio-Care Technology Pty. Ltd, Sommersby, NSW.

2.3. Growth and preparation of cells

All rhizobial strains used in this work and their origin of isolation are listed in Table 2. The strains were obtained from the culture collections of SUNFix Centre for Nitrogen Fixation (University of Sydney) and BioCare Technologies Pty. Ltd. Except where different rhizobial strains were compared, *Rhizobium leguminosarum* bv. *trifolii* TA1 was used in all the experiments described in this paper to test the effect of polymers on desiccation tolerance. Unless otherwise stated, cultures of TA1 were prepared in sucrose/glucose broth (SGB) containing 0.75 g of K_2HPO_4, 0.4 g of $MgSO_4 \cdot 7H_2O$, 0.4 g of $CaCO_3$, 2.5 g of sucrose, 2.5 g of glucose, and 3 g of yeast extract per litre and other rhizobial strains in yeast mannitol broth (YMB, Vincent, 1970). All cultures were shaken on a rotary shaker and incubated at 28 °C. Broth cultures were centrifuged for 10 min at 5000 rpm, washed twice with phosphate buffer (1.21 g of K_2HPO_4 and 0.34 g of KH_2PO_4/L, pH 7.2) and re-suspended in solutions of different polymers. Aliquots (100 μL) of the suspension were transferred to sterile ampoules fitted with a non-absorbent cotton wool plug and dried under vacuum in the freeze-drier for 4 h to a final pressure of 0.1 Torr to apply a standard desiccation stress. The final pressure was reached within the first hour.

2.4. Enumeration of viable cells

Numbers of viable cells were counted on initial suspensions, immediately after drying and after 24 h of storage. To count viable cells from the dry suspensions, the ampoules were surface sterilised with 70% ethanol and broken in two using a glass file. The entire ampoule was placed into 10 mL of phosphate buffer containing 1 g peptone per litre (PPB) and shaken on a wrist-action shaker for 10 min. Serial dilutions to 10^{-3} were plated using a spread plate technique for TA1 and a drop-plate technique for other strains. Plates were incubated at 28 °C until colonies were sufficiently large to be counted. An analysis of variance was performed on $\log_{10}$ transformed data using GenStat (v 8.1) to determine any significant differences between polymer treatments at each time.

2.5. Moisture sorption and water activity of polymers

Approximately 1 g of each polymer was weighed into pre-weighed sample cups, vacuum-dried (0.1 Torr) in the mini freeze-drier and re-weighed. Duplicate samples of each polymer were then placed in a desiccator and stored over a saturated potassium dichromate solution resulting in 98% RH. Samples were weighed periodically until the mass stabilised (approx. 2 weeks). The gravimetric moisture content of the equilibrated polymer was calculated as the percentage mass of water per dry weight of polymer lost after oven drying at 80 °C for 48 h (% adsorbed moisture/g dry weight polymer). The water activity of equilibrated polymers was measured using a water activity meter (Aqualab model CX3, Decagon Devices Inc., Pullman, Washington, USA) as an indication of the energy status of water in the polymer matrix. It is defined as the vapour pressure above a sample at a particular temperature divided by the vapour pressure above pure water at the same temperature.

2.6. Stabilisation properties of polymers

The critical coagulation coefficients (CCC) of the polymers were determined as a measure of their ability to stabilise another colloid against the precipitating action of KCl (Matijevic et al., 1961). A solution of each polymer (0.5 mL of 0.01%) was added to colloidal polystyrene latex (0.5 mL of a 4.9×10^{-4} w/v solution 450 nm diameter with sulphate groups on the surface) in a cuvette and mixed. Solutions of different KCl concentrations (1 mL of 0.4–3.0 M in 0.1 M increments) were added and absorbance was measured at 620 nm every 3 s for 3 min on a diode array spectrophotometer (Hewlett-Packard model 8452A, Avondale, Pennsylvania, USA).

The rate of initial transmission (based on the absorbance data) was calculated and a stability plot was produced by plotting $\log_{10} W$ against KCl concentration (where W is the maximum initial transmission rate/initial transmission rate at each concentration of KCl). The CCC was calculated as the highest concentration of KCl where coagulation of the latex no longer occurs (i.e. where $\log_{10} W = 0$).

Particle size of the latex was measured to ensure that coagulation was not occurring instantly upon addition of

polymer. Polymer solutions (1 mL of 0.01%) were added to the polystyrene latex solution in a cuvette and placed in a Photon Correlation Spectrometer (Malvern, Precision Devices and Systems Ltd, Malvern, Worcestershire, UK). Particle size was measured by the scattering of a 50 mW laser beam.

2.7. Survival of rhizobia at different relative humidities

Ampoules containing dried suspensions of TA1 in polymers were placed at 20 °C and at a range of relative humidities. Relative humidity chambers were produced using saturated salt solutions potassium hydroxide (10%), magnesium chloride (33%), magnesium nitrate (54%), sodium chloride (75%), potassium chloride (85%) and potassium dichromate (98%). The numbers of viable cells were counted in the initial suspensions and in four replicate samples immediately after drying, after 24 h and after 25 days of storage. An analysis of variance was performed on $\log_{10}$ transformed data using GenStat (v 8.1) to determine any significant differences between relative humidity treatments for each polymer and time.

2.8. Distribution of rhizobial cells in polymer matrices

The effect of stabilising polymers on recovery of rhizobia was determined by comparison of the distribution of cells within the polymer matrices. YMB cultures of TA1 were diluted to achieve approximately 10^4 viable cells/mL washed and suspended in polymer solutions (5%) as described above. Aliquots (100 μL) were transferred to sterile ampoules and vacuum-dried for 4 h. The cells were immediately re-suspended in phosphate–peptone buffer (10 mL). Multiple aliquots (20×100 μL) were plated onto YMA and allowed to grow at 28 °C until colonies were visible. Colonies were counted, the distribution of counts within each sample tube was calculated and the variance analysed.

3. Results

3.1. The effect of stabilisation and moisture sorption properties on survival

The loss of viability of TA1 is presented as net loss of mean $\log_{10}$ viable cells after vacuum-drying for 4 h and after storage for 24 h at 20 °C and 54% RH (Table 3). The results are listed in decreasing order of survival after 24 h. Also listed is the CCC of the polymers and the moisture content and water activity after equilibration at 98% relative humidity. There was a significant trend in the number of viable cells recovered from PVA after 24 h ($P < 0.05$). The highest recovery of viable cells was from GL05 followed by NL05 then KL05. This trend was not apparent immediately after drying although survival was significantly lower in KL05. Although MC offered some protection to cells after 4 h of drying, after 24 h of storage the number of viable cells embedded in MC and PVP were below the limit of detection. Without polymer, cell numbers also rapidly declined.

There was no clear relationship between moisture sorption and survival. The most highly hygroscopic polymers MC and PVP were the least protective. Of the PVA, the most protective (GL05) had a moisture content intermediate to the other two. To find an optimum a quadratic function was fitted to a scatter plot in Microsoft Excel®. The moisture content of PVA at 98% RH for optimum survival was found to be 33.2% by calculating the point at which the slope of the tangent to the curve was equal to zero. The trend in water activity of PVA was inversely related to moisture content with the most hygroscopic having the lowest water activity.

The stabilising properties of polymers were measured by calculating the CCC where polymers with the largest CCC were the most stabilising. MC and GL05 conferred the greatest stability to the latex against precipitation by KCl. Of the three PVAs, GL05 had the highest stabilising effect followed by KL05 and NL05. Survival was highest when

Table 3
Survival of *Rhizobium leguminosarum* bv. *trifolii* TA1 after vacuum-drying in polymers and polymer properties critical coagulation coefficients and moisture properties

Polymer	Net loss $\log_{10}$ viable cells		Critical Coagulation Coefficient (CCC)	Moisture properties at 98% relative humidity	
	(0–4 h)	(0–24 h)		Moisture content (% dry weight)	Water activity
GL05	1.6a	1.7a	1.41	35.6	0.87
NL05	1.7a	3.6b	0.61	29.3	0.90
KL05	3.3b	5.7c	0.91	38.1	0.85
No polymer	3.7b	6.7d	0.21	ND	ND
MC	2.9a	>6.9d	1.41	68.0	ND
PVP	3.1b	>6.9d	0.91	75.4	ND

Net losses of viable bacteria ($\log_{10}$) were calculated for 0–4 and 0–24 h from the means of 4 replicates. An analysis of variance was performed using $\log_{10}$ values of viable cells at 4 h and 24 h. There was no difference between $\log_{10}$ viable cells at each time where net loss values are followed by the same letter ($P > 0.05$). ND where no determination was made.

the CCC was maximum in the case of PVA. However, there was no direct correlation between stabilisation by all three PVAs and survival. Similiarly, the high stabilising ability of MC did not result in a high survival rate of desiccated TA1.

To validate the CCC measurement, particle diameters of polystyrene latex mixed with polymers were also measured by photon correlation spectroscopy. This ensured coagulation of the latex did not occur immediately upon addition of the polymer which would have falsely indicated high stabilising ability. The average diameter of the latex without polymer was 425.4 nm (se 4.2 nm) and addition of the polymers increased the diameter differentially. While mixing the latex with PVA had relatively little affect on the latex diameter, there was a trend in the size of the diameter, the largest with GL05 (478.9 nm; se 4.9) followed by KL05 (468.0 nm; se 5.2) then NL05 (441.8 nm; se 3.6). Although mixing with MC resulted in a large latex diameter (659.7 nm; se 6.2), the diameters were all less than double the polymer-free latex diameter indicating that coagulation of the latex had not occurred within the first few minutes of polymer addition. Thus stable transmission indicating low coagulation rates at high KCl concentration are true measurements and are not an artefact of rapid coagulation of the latex by the polymer.

3.2. Survival of different strains of rhizobia suspended in PVA

The survival of five different strains of rhizobia was measured after desiccation in suspensions of PVA (Table 4). Net losses of viable cells were calculated from the difference in the means of $\log_{10}$ cfu/mL at 0 and 24 h of four replicate samples. With the exception of WU425 which survived well in all polymers, survival of the strains was significantly improved after suspension in GL05 ($P<0.05$). In most cases the poorest survival occurred after suspension in KL05 as was the case with TA1. However, the difference in survival between KL05 and NL05 was not significant ($P>0.05$).

3.3. Survival of dried, polymer-embedded rhizobia at different relative humidities

The loss of viable cells after storing vacuum-dried, polymer-embedded TA1 at 20 °C and at different relative humidities is listed in Table 5. Net losses of viable cells in MC and the water control are between 1000 and 10,000 times higher than those of the PVA after 24 h. There is no difference in survival of TA1 embedded in any of the three polymers over the range of relative humidities after 24 h, but there is a significant trend ($P<0.05$) when there is no polymer present. Net losses of viable cells in the water control after 24 h increased from low relative humidities to high. The numbers of viable cells at 10% relative humidity were significantly higher than that at 33% and 54% and at higher relative humidities, cells were not recoverable. No cells were recovered after 600 h from the MC and water treatments where the minimum level of detection was 100 cfu/mL.

Net losses of viable cells after 600 h in NL05 indicate a significant decrease in survival from the low relative

Table 4
Survival of different vacuum-dried rhizobial strains in PVA

Suspension medium	Net loss of viable bacteria/mL ($\log_{10}$ 0–24 h)				
	SU277/1	SU343	WU425	CB1015	CB1809
GL05	0.72b	2.88b	2.16a	3.12b	2.16b
NL05	1.92a	4.08a	2.40a	3.84a	2.88a
KL05	1.68a	4.32a	2.88a	4.32a	3.12a

Variances were analysed for each strain. Results were calculated from the means of four replicates. All polymer concentrations were 5%. Values within strains followed by the same letter are not significantly different ($P>0.05$).

Table 5
Survival of vacuum-dried polymer-embedded TA1 after storage at different relative humidities and 20 °C

RH	Net loss of viable bacteria ($\log_{10}$)							
	GL05		NL05		MC		Water	
	0–24 h	0–600 h	0–24 h	0–600 h	0–24 h	0–600 h	0–24 h	0–600 h
10%	3.36a	6.00d	3.36a	6.00a	7.92a	>8.40	6.24a	>8.40
33%	3.36a	5.40c	3.36a	6.00a	7.92a	>8.40	7.68b	>8.40
54%	3.12a	4.80bc	3.60a	5.40a	7.68a	>8.40	>7.92b	>8.40
75%	3.36a	4.20ab	3.12a	>8.40b	7.20a	>8.40	>8.16c	>8.40
85%	3.12a	4.20ab	3.84a	>8.40b	7.44a	>8.40	>8.16c	>8.40
98%	3.84a	3.60a	3.84a	>8.40b	7.68a	>8.40	>8.16c	>8.40

Net losses of viable bacteria ($\log_{10}$) were calculated for 0–24 h and 0–600 h from the means of 4 replicates. An analysis of variance was performed separately using $\log_{10}$ values for each treatment and time. Values followed by the same letter are not significantly different ($P>0.05$).

humidities (10–54%) to the high relative humidities (75–98%) where cells were not able to be recovered. Net losses of viable cells of TA1 in GL05 after 600 h follow the opposite trend; survival tends to be better at the higher relative humidities with a gradual but significant trend downwards to the lower relative humidities. Although the net losses of viable cells at the low relative humidities were similar to NL05, cells from GL05 were recovered at all relative humidities whereas cells from NL05 were not recovered at high relative humidities.

3.4. Distribution of rhizobial cells in polymer suspensions

The ability of polymers to disperse suspended cells evenly was determined by counting the number of viable cells in 20 sub-samples of suspensions from polymer-embedded cells. Cell distribution was calculated as the mean standard error, calculated by averaging the standard errors of four means of replicated cell counts. The mean standard error of numbers of cells dried in GL05 was 0.014 and NL05 was 0.033. Although the mean standard error from GL05 was half that from NL05 indicating more evenly distributed cells, the results were not statistically significant ($P > 0.05$).

4. Discussion

Using PVA varying in percent hydrolysis allowed the systematic definition of important polymer properties that affect rhizobial survival. The percentage of hydroxyl groups on the PVA molecule affects moisture sorption and stabilisation properties of the polymers. Variations in moisture sorption affect survival when cells are exposed to desiccation and different relative humidities, supposedly by altering the rate of dehydration and rehydration so that the water status of cells is more balanced with that of the surrounding environment. Our results indicate that when optimal moisture sorption is combined with the polymer's ability to stabilise the cells against the precipitating action of increasing concentrations of salt, typical of dry environments, the polymer provides conditions more favourable for the survival of rhizobia.

The improved survival of TA1 when suspended in the G- and N-series PVA suggests that higher degrees of hydrolysis of the polymer provides conditions that protects cells against damage from desiccation. The N- and G- series PVA and to a lesser extent MC provide the best protection to the cells during drying. However, only the N- and G-series PVA continued to protect the cells during storage. Clearly, as suggested by Annear (1962), protection during both drying and storage should be considered when assessing the suitability of the suspension media. Variations in response to polymer treatment by other rhizobial species may be due to differences in their inherent desiccation tolerance. Differences between polymers may be masked when cells are more tolerant of desiccation.

In our research rhizobial cells were subjected to extreme conditions of desiccation. Cells were washed and suspended in polymer solutions followed by rapid drying under vacuum and dried cells were exposed to air. This resulted in relatively low survival. The percentage of TA1 cells that survived 4 h of primary drying in GL05, NL05 and water was only 1%, 1.6% and 0.02%, respectively. Whereas previously, Vincent (1958) reported that the percentage survival of the same species of rhizobia after primary drying was improved from 0.1% in water to 24–44% in the presence of sucrose. The relative percentages indicate that the polymers are not as protective as sucrose after the primary stage of drying. However, cell washing, exposure to air and the concentration of sucrose (not reported by Vincent) may be critical factors affecting survival. As a result comparisons between the two systems are difficult. Poor survival in the vacuum-drying system described here, while not ideal for commercial application, was sufficient to highlight differences in the properties of the polymers. Drying on seeds is much less rapid than in the freeze-drying system. Vincent et al. (1962) reported that water loss from coated beads was linear to 18 h and had equilibrated by 24 h whereas in this research cells were dried under vacuum over a 4 h period. It is expected that survival on seed after air drying would be higher than that after vacuum drying and would be dependent on the atmospheric relative humidity as well as the moisture sorption characteristics of both the polymeric adhesive and the seed.

Moisture sorption by polymers is clearly important for survival. It is affected by the availability of sites within the polymer molecule for the specific binding of water and the spaces within the polymer matrix allowing multilayer formation (Bell and Labuza, 2000). Moisture sorption by the different grades of PVA may be explained by the high degree of hydrolysis of NL05 which allows for intra- and intermolecular hydrogen bonding in the polymer matrix resulting in tighter packing of the molecule reducing specific and multilayer binding of water. KL05 and GL05 contain higher percentages of larger acetate groups which restrict packing and expose more specific binding sites which in turn allow multilayer binding of water. Similarly, the high amounts of water adsorption of PVP and MC may be explained by their large pyrrolidone and methyl groups restricting packing and allowing entry of water. Interestingly, despite suggestions that survival may be related to low water activity (Mugnier and Jung, 1985) the best survival did not occur in polymers with the lowest or highest water activity but rather in polymers with an intermediate water activity indicating that there is an optimum level for this property. For PVA used in this study, optimum moisture content was estimated to be 33.2% after equilibration of the polymer at 98% relative humidity. This may have significance for the development of new polymers designed specifically for improved desiccation tolerance of rhizobia.

In our research survival of rhizobia varied according to species and relative humidity, as found by many other studies (Mugnier and Jung, 1985; Mary et al., 1985, 1994). Variations due to species are probably due to inherent differences in physiological tolerance to desiccation such as the ability to accumulate compatible solutes and exopolysaccharides. Dried TA1 cells without polymer survived best if their dehydrated state was maintained but the numbers of viable cells declined as the relative humidity increased. This would have resulted in an increasing differential in the hydration status of the cell's internal and external environments. Interestingly, the reverse pattern occurred when hydrated cells were exposed to different relative humidities (data not shown). Bushby and Marshall (1977a) observed similar effects and speculated that slow-growing rhizobia survive better than fast-growing strains under desiccation stress because they retained less internal water thus reducing the differential between the internal and external water content. From our results it appears that the low moisture sorption of NL05 is not adequate to control the hydration status of cells at high relative humidities, perhaps exposing the cells to water too rapidly. However, GL05 may act to slow the rate of dehydration and rehydration found to be important for survival (Kosanke et al., 1992) by moderating changes to the hydration status of cells. It follows that survival of dry rhizobia at high RH is enhanced by lower water availability in the suspending polymer.

These results are further complicated by the toxic nature of oxygen below 70% RH (reviewed in H. V. A. Bushby, unpub. PhD Thesis, University of Tasmania, 1974). Lien found that oxygen transmission through PVA films decreased with increasing percent hydrolysis and increased with increasing RH (L. Lien, unpub. Hons. Thesis, University of Sydney, 2000). It is more likely that differences in survival are related to the combined effect of these properties. The combination of high water availability and low oxygen transmission at high RH in NL05 may account for poor survival relative to GL05. Intermediate levels of water activity and oxygen transmission are conducive to better survival suggesting that these properties may be further optimised. Poor survival in the highly hygroscopic MC and PVP may be due to large and rapid relative water loss on drying. Poor film formation or open structures of these polymers may result in the formation of macro-cracks or channels in the dried film exposing cells to detrimental levels of oxygen and rapid changes in hydration with fluctuations in RH.

Stabilisation by the suspending medium contributes to improved survival (Heller, 1941). The stabilisation of bacterial cells and macromolecules during drying is necessary to reduce damage due to the precipitating action of increasing salt concentrations. Stabilisation improves the cell's structural integrity during hydration and rehydration and thus improves survival. Comparison of different grades of PVA suggests that improved survival in GL05 is positively correlated with a high CCC. However, this relationship is not general and the CCC's of NL05 and KL05 do not follow the trend. The cellulosic polymers also have a high CCC but survival is poor in these polymers due to other confounding factors. This highlights the complexity of the system in that high stabilisation of the polymer must be combined with other favourable attributes to be of benefit.

5. Conclusion

Clearly, improved survival of rhizobia cannot be attributed to one single polymer property. However, moisture sorption and colloidal stabilisation properties are important and should be considered in combination with other factors. It is difficult to compare polymers with vastly different chemical structures and a systematic approach using graded sets of similar polymers such as PVA will assist in further defining polymer properties that improve desiccation tolerance of rhizobia. It is predicted that the construction of survival response curves to various polymer properties will allow for more directed synthesis of beneficial polymers for improved survival of rhizobia on legume seed.

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