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*Article*

# Conditions Affecting Shelf-Life of Inoculated Legume Seed

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**Abstract:** Microbial inoculants are becoming more available as sustainable alternatives to fertilizers and other agrichemicals in broad-acre cropping. However, with the exception of legume inoculants little is understood about effective delivery and survival of the inoculum. Legume inoculants are applied to both seed and soil but seed inoculation is the most economical technique. Large quantities of pasture seed in Australia are inoculated by commercial seed coating companies, but the long-term survival of seed-applied inoculum is variable and monitoring of viability requires specialist microbiology skills and facilities. The aim of our research was to define optimum storage conditions for survival of rhizobia on legume seed and evaluate water activity as a means of monitoring shelf-life. The relationship between survival and water activity varied according to seed species, inoculum preparation, coating ingredients, initial water activity and time suggesting that storage conditions would need to be defined for each different combination. Although drying seeds after coating significantly reduced viable numbers of rhizobia, survival of rhizobia on dried commercially coated lucerne seed after 11 weeks was less variable than seeds that had not been dried. The highest numbers were maintained when seeds remained dry with water activities of between 0.47 and 0.38. The quality of inoculated seed could be improved by reducing the death rate of inoculum during preparation and providing optimum storage conditions for long-term survival.

**Keywords:** inoculants; legume-seed inoculation; rhizobia; survival; water activity

## 1. Introduction

Interest in application of microorganisms to agricultural crops and pastures as a means of supplying a sustainable source of nutrients, increasing nutrient-use efficiency or controlling disease has increased in recent years. Culture collections hold many thousands of potentially beneficial microorganisms but very little attention has been given to their commercial formulation or large-scale application to broad-acre crops [1]. Conversely, legumes have been inoculated for more than a century and the limitations of current legume inoculant formulation and application technology have been well studied providing a useful model system for other microbial inoculants.

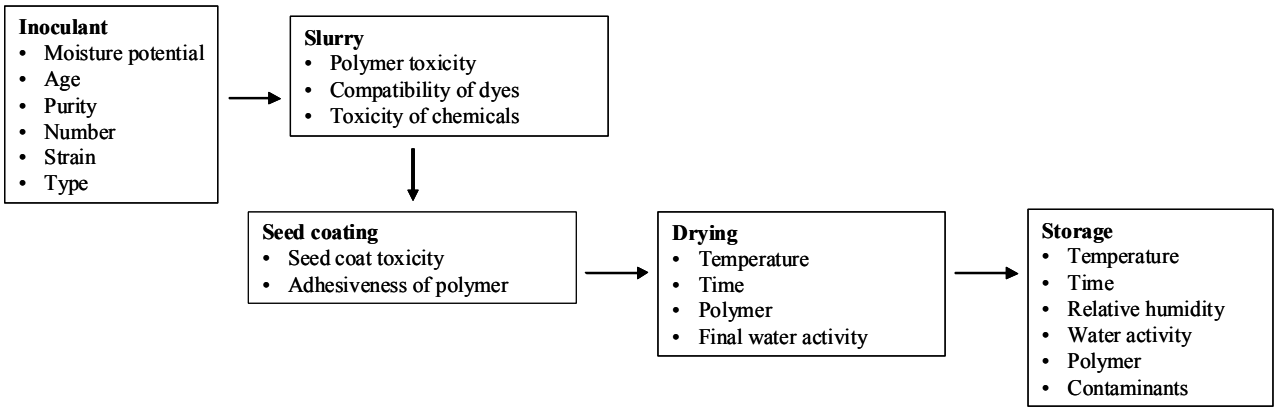
The aim of legume inoculation is to provide high numbers of viable effective rhizobia to the rhizosphere to allow rapid colonization, nodulation and nitrogen fixation by the selected inoculant strain in order to maximize legume yield potential [2,3]. In 2005, nitrogen fixed annually by inoculated legumes in Australia was valued at $3 billion N fertilizer equivalents [4]. The need to inoculate is dependent on the number of effective rhizobia present in soil where legumes are to be sown. Rhizobial numbers in soil may be adequate if the host legume has been sown previously but can decline under both permanent and transient abiotic edaphic stress [5]. Where stress-related suppression of rhizobial numbers has not occurred, intraspecific competition can be a problem; particularly where less effective populations are present in soil or have emerged through horizontal transfer of nodulation genes [6,7]. Therefore, inoculation may be a considered a cost-effective form of insurance against ineffective or suboptimal nodulation.

Inoculants may be introduced to either the seed or directly to soil. However, soil application requires large volumes of inoculant for adequate distribution of rhizobia so inoculation of legume seed is a more economical way of introducing rhizobia to the rhizosphere. Seed inoculation by commercial seed coaters, either on demand after sale of the seed (custom inoculation) or prior to sale (pre-inoculation), provides farmers with a convenient ready-to-use product. Application of inoculant by commercial seed coaters also has the potential to improve the reliability of inoculated seed quality by allowing for better control and standardisation of coating methods. However, problems facing the seed coating industry are poor survival of the inoculant rhizobia, particularly on clover seed where surveys have indicated that numbers rarely satisfy numerical standards [8,9], and the lack of accessible tools for rapid monitoring of rhizobial viability.

Viable cell numbers of all rhizobia decline on seed but the rate of decline is dependent on environmental conditions and varies between rhizobial species and strains [2,10,11]. There are many conditions throughout the seed coating process where rhizobial viability may be compromised (Figure 1). Desiccation is one of the major factors affecting survival of rhizobia on seed [12,13]. The extent and rate of desiccation is dependent on the ambient relative humidity during inoculation and storage of inoculated seed. There are many reports in the literature about the effect of relative humidity [12,14–16] and water activity on survival of rhizobia [17]. There is evidence to suggest that survival is better when the difference between the intracellular and extracellular water status is

minimised. Freeze-dried cells survive better when stored at low relative humidities [11,18] whereas survival of cells that are fully hydrated declines when relative humidity is rapidly reduced [14]. Slow rehydration of cells is also important for survival [16]. Polymer adhesives used to apply inoculants to seeds may reduce the effects of fluctuations in relative humidity by mediating moisture sorption and desorption improving survival over time [11].

**Figure 1.** Factors affecting survival of rhizobia during commercial inoculation of legume pasture seed.




Survival is affected by the initial condition of the cells in the inoculant, particularly the moisture status, age, purity, the initial number, the strain and the type of inoculant [13]. Changes in the physiological and morphological characteristics of cells during the maturation of the peat inoculant have been shown to affect survival [19–21]. Inoculants are usually suspended in polymeric adhesive, often containing other additives such as dyes or pigments, plant nutrients and seed protection agents, before being applied to seed. However, polymers and other seed-applied additives may be chemically incompatible with rhizobia [22] and toxic water-soluble legume-seed exudates may also reduce rhizobial viability [23].

Optimum long-term storage conditions for rhizobial survival on inoculated seed are not well defined. As with inoculants, it is assumed that temperature and moisture status is important for rhizobial viability over time [24]. Polymers also play a role in reducing exposure of cells to environmental stress but protective properties vary with polymer [11]. In addition, as contaminants in inoculant carriers are known to suppress growth of rhizobia during inoculant production [24], it is assumed that contaminants on inoculated seed may also reduce cell numbers or effectiveness of rhizobial colonization of the rhizosphere.

Rhizobial viability on seed is usually determined by dilution plate counting or plant infection methods. While counting viable cells is the most direct method of determining the microbiological quality of inoculated seed, the methods are time consuming and require specialist expertise and facilities. There is a need for the development of techniques for rapid measurement of the microbiological quality of inoculated seed that may be easily adopted by the seed coating industry.

Water activity is defined as the energy status or availability of water and is affected by colligative properties of dissolved solutes and capillary and surface interactions with solid matrices. It is measured as the vapour pressure above a sample relative to the vapour pressure above pure water and this varies with changes in temperature. The water activity of processed food is regularly monitored to detect

conditions that may support the growth of food spoilage organisms and thus to determine shelf-life [25]. As it is strongly associated with bacterial growth and survival, water activity may be a useful measure of the conditions that support survival of rhizobia on inoculated seed. Shelf-life of inoculated seeds could then be determined for a specified range of water activities. The aim of our research was to determine optimum conditions for the storage of pre-inoculated seed products and evaluate water activity measurement as a rapid and accessible means of monitoring the microbiological quality and shelf-life of inoculated seeds.

## 2. Results and Discussion

### *2.1. Survival of Rhizobia on Laboratory Coated Seed after Storage at Different Relative Humidity*

The relationship between water activity and survival at constant temperature varied according to seed species, polymer and time (Figure 2). When polyvinyl alcohol GL05 was used to apply peat cultures of *Rhizobium leguminosarum* bv. *trifolii* TA1 to seeds of subterranean clover (*Trifolium subteraneum*), survival of TA1 was not affected by water activity of the seeds after 24 h and 1 week storage at different relative humidities (Figure 2A). However, after two weeks storage there was a significant decrease in viable numbers when water activity had fallen below 0.3 ($P < 0.05$). Blending canola oil with GL05 improved survival at low water activities indicating that oil may protect dry cells (Figure 2B). Improved survival of desiccated rhizobia in vegetable oil has been demonstrated previously [26].

**Figure 2.** Viable number of *Rhizobium leguminosarum* bv. *trifolii* TA1 on seeds of *Trifolium subterraneum* (**A**, **B** and **C**) and *Trifolium repens* (**D** and **E**) relative to water activity after storage at different relative humidities. Peat cultures of rhizobia were applied to seed using different polyvinyl alcohol polymers, GL05 (**A** and **D**), GL05 + oil (**B**) and KL05 (**E**). In one experiment colonies were suspended directly from the surface of YMA in polyvinyl acetate latex (**C**).

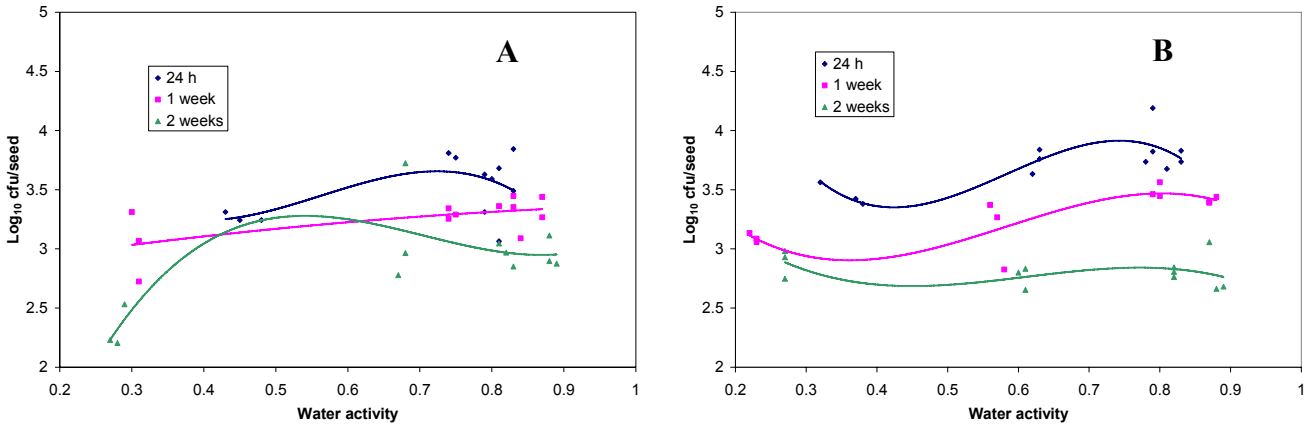
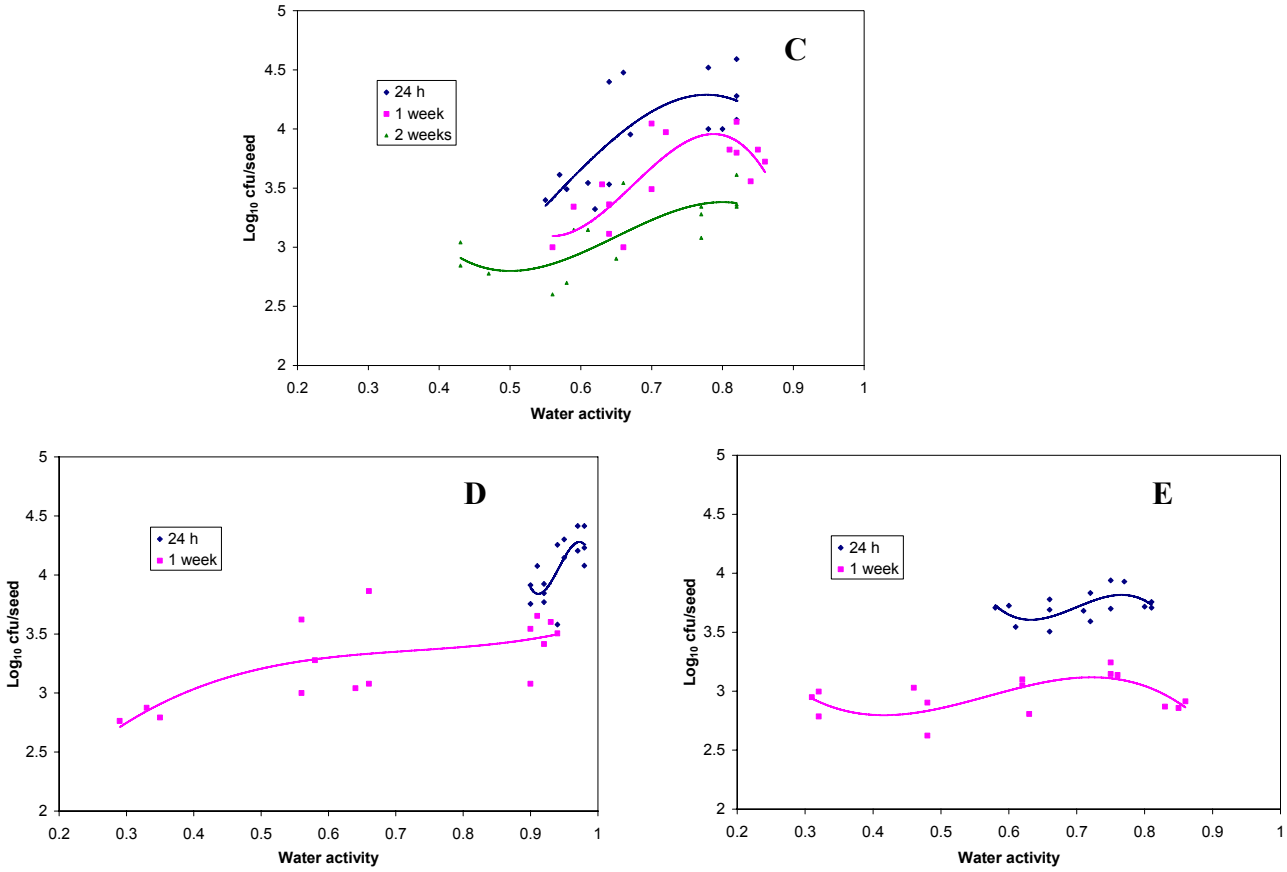


**Figure 2.** *Cont.*




Seeds coated with the GL05-oil blend equilibrated to relative humidity conditions faster than the seeds treated with GL05. Seeds stored at the lowest relative humidity reached water activities of less than 0.4 after 24 h, whereas the water activities of seeds coated with GL05 were all greater than 0.4 after 24 h. Oil may be acting as a plasticizer in this case, reducing the glass transition temperature of GL05, which would increase the rate of water loss. This increased rate of dehydration and change in glass transition properties of GL05 may have contributed to the reduced overall survival of TA1 after application with the GL05-oil blend (average percent survival from 24 h to 1 week = 36.5% and from 24 h to 2 weeks = 11.6%) compared with GL05 over the two week period (average percent survival from 24 h to 1 week = 51.8% and from 24 h to 2 weeks = 29.6%). Survival of rhizobia is improved when the rate of dehydration and rehydration is slow [14,16].

The relationship between water activity and survival of TA1 on seeds of white clover (*T. repens*) after application with GL05 was comparable with that on subterranean clover seed with generally better survival at high water activities than low water activities (Figure 2D). Seeds coated with GL05 were slower to equilibrate with relative humidity conditions than seeds coated with KL05 indicating that KL05 dried more rapidly on seeds (Figure 2E). Moisture sorption properties of polyvinyl alcohol varies according to the degree of hydrolysis and KL05 has a higher rate of moisture sorption/desorption compared with GL05 at water activities above 0.7 [11]. This faster rate of dehydration may account for the generally lower numbers on white clover seed after application with KL05 ($5.4 \times 10^3$ cfu/seed at 24 h and $9.7 \times 10^2$ at 1 week) than with GL05 ($1.3 \times 10^4$ cfu/seed at 24 h and $2.5 \times 10^3$ cfu/seed at 1 week). Initial numbers for both polymer treatments were similar. The death

rate of TA1 on white clover between 24 h and 1 week was, however, similar for both polymer treatments (overall percent survival between 24 h and 1 week = 18.8% for GL05 and 17.9% for KL05 treatments) but higher than the death rate on subterranean clover; thus confirming data from pre-inoculated seed surveys indicating poorer quality of inoculated white clover compared with subterranean clover species [9].

Survival of suspended YMA-grown colonies of TA1 on subterranean clover after application with polyvinyl acetate latex was more affected by low water activity than the other treatments (Figure 2C). This was probably due to the use of suspended YMA colonies rather than peat cultures. The superiority of peat cultures over agar or broth cultures for survival on seed was demonstrated previously [27]. However, survival at high water activities is comparable with survival of peat cultures on subterranean clover seeds. Interestingly, the water activity did not drop as low with polyvinyl acetate as with the other polymers, showing this property varies with different polymers.

The relationship between water activity and survival may be useful in determining favourable storage conditions for inoculated seeds. While TA1 survived well on clover seed with high water activities, storage of seeds with high water activity is not practical. High water activities (above 0.7) may encourage the growth of contaminants and there is less flexibility of temperature for storage. The atmosphere inside a sealed packet of seeds with a water activity of 0.9 at 15 °C would reach dew point with a decrease in temperature of approximately 2–3 °C [28], which may encourage germination. Lower water activities for storage are more desirable and may be maintained through the use of modified atmosphere packaging. Maintenance of low water activity may also be useful for the storage of seeds coated with freeze-dried inoculants better able to withstand high temperatures [18]. While survival of TA1 may be adequate at water activities between 0.5 and 0.7, longer term studies are required to further establish if rhizobial viability on commercially inoculated clover seed could be maintained at low water activities.

### *2.2. Survival of Rhizobia on Commercially Coated Legume Seed*

Commercially inoculated lucerne (*Medicago sativa)* and subterranean clover seed were provided by two different seed companies. Lucerne and subterranean clover seeds were inoculated with peat cultures of current commercial strains *Sinorhizobium meliloti* RRI128 and *Rhizobium leguminosarum* bv. *trifolii* WSM1325 respectively. Inoculants were applied using proprietary polymeric adhesive blend including dyes, plant nutrients and seed protection agents. Seed batches were collected and transported to the University of Sydney for analysis to determine optimum storage conditions. However, the initial number of rhizobia on the commercially coated seed was very low (Table 1) and in all cases the rhizobia did not survive long enough to be able to determine the relationship with water activity. The experiment was continued with only one batch of lucerne seed (sample F) on which the number of viable rhizobia had been maintained above the current numerical standard of 1000 cfu/seed one week after coating.

**Table 1.** Number of viable rhizobia/seed on seed inoculated using commercial processes. Samples were collected from small experimental batches except sample E which was a commercial batch. TCTC = too contaminated to count.

| Sample | Seed | Seed company | Time since coating | $Log_{10}$ no./seed | Contaminants |
|---|---|---|---|---|---|
| A | Lucerne | 1 | 2 weeks | 1.10 | No |
| B | Lucerne | 1 | 2 weeks | 1.93 | No |
| C | Lucerne | 1 | 2 weeks | 2.10 | No |
| D | Lucerne | 1 | 2 weeks | 2.17 | No |
| E | Lucerne | 2 | Unknown | TCTC | Yes |
| F | Lucerne | 2 | 1 week | 4.12 | Yes |
| G | Subteranean clover | 2 | 1 week | TCTC | Yes |

No contaminants were observed on samples from seed company 1. Poor survival was possibly due to low initial numbers of rhizobia, incompatibility of seed coating materials or lack of protection by seed coating materials from detrimental effects of drying on rhizobial cells. Large numbers of contaminants were present on samples from seed company 2 and in two cases (samples E and G) the level of contamination was so high that initial plate counts were not possible.

The number of colonies of *Sinorhizobium meliloti* RRI128 on YMA plates recovered from lucerne seed (sample F) after seven weeks of storage were reduced when contaminants were present (data not shown). The number of contaminants on seed also increased the variation in the number of viable rhizobia, masking any effect of water activity. It may be assumed that contaminants would be competitive and suppress rhizobial growth in the rhizosphere if conditions were ideal for their growth. They may inhibit legume root nodulation, decrease the potential shelf life of inoculated seed and increase the difficulty in performing quality control procedures. Vincent suggested that low dilution 'skips' in plant infection MPN tests may be due to inhibition of nodulation by contaminants [29].

Although it was not possible to determine the effects of storage conditions on rhizobial survival with these seed samples, the results are presented here to illustrate the problems associated with rhizobial viability on commercially pre-inoculated seed. However, it should be noted that only one out of the seven samples was from a batch of seed coated using standard commercial practice (sample E). The other samples were from small experimental batches where coating methodology may have been modified and therefore not representative of commercial coating conditions. Nevertheless, it was not possible to determine the number of viable rhizobia on lucerne seed from sample E because of heavy contamination and the time since coating was unknown. In Australia, there is no requirement for pre-inoculated seed to be labeled with a date of production or expiry.

### *2.3. Survival of RRI128 on Commercially Coated Lucerne after Storage at Different Relative Humidity*

Subsequent to the previous attempt to measure the effect of storage conditions on commercially coated seed, lucerne seed was collected again from commercial seed coating company number 2 at three points in the coating process. Seed was collected immediately after coating from the coating drum, before drying and after drying to determine if drying seed after coating had an effect on rhizobial viability during storage at different relative humidities. Seeds were packaged in sealed plastic bags and transported from the seed company to the Australian Inoculants Research Group (AIRG)

where the viable number of rhizobia per seed was counted three days after inoculation and then to the University of Sydney laboratories where viable numbers after sixteen days were determined. The seed was then placed in relative humidity chambers for long-term studies of viability.

After three days the number of viable rhizobia on seeds that had not been dried was significantly higher than the number on seeds that had been dried ($P < 0.05$, Table 2) indicating that drying inoculated seeds has a detrimental effect on rhizobial viability. After storage in sealed bags for 16 days the difference in viable rhizobial numbers on seed had disappeared. However, the water activity of each batch reflected the extent of drying. Seeds that were collected from the coating drum had the highest water activity, followed by seeds collected just before drying. Dried seeds had the lowest water activity. The lower water activity of the seeds collected before drying indicated some drying must have occurred in the six minutes it took for the seeds to move from the drum to the drying table. Water activity also indicates that the moisture status of the coated seeds was maintained in sealed plastic bags. Greater cell death at high water activities between three and 16 days suggests the product is unstable when there is excess moisture. However, drying seed during production reduced the number of viable cells by about 3000 per seed, highlighting the need to carefully refine the seed-drying process to improve rhizobial survival.

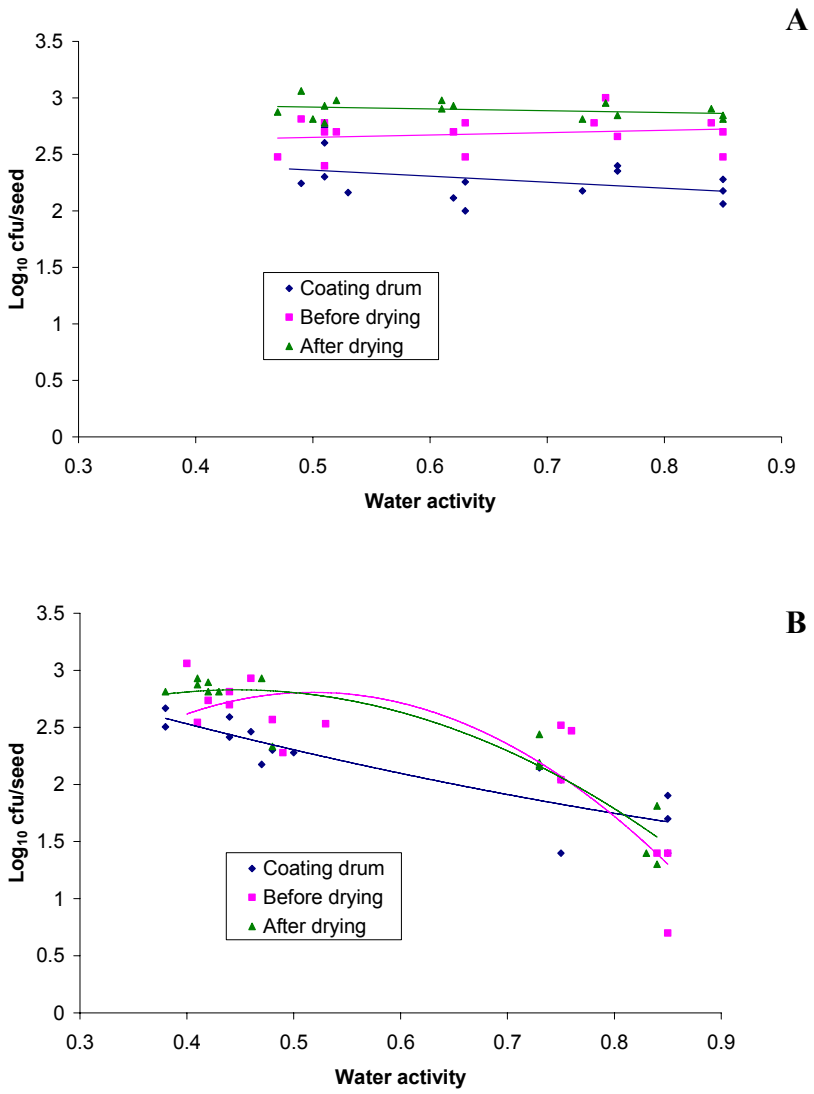
**Table 2.** Survival of rhizobia and water activity of coated seed before storage at different relative humidities. Data are averages of three replicates. Means followed by different letters are significantly different ($P < 0.05$).

| Seed sample | $Log_{10}$ cfu/seed 3 days after coating | $Log_{10}$ cfu/seed 16 days after coating | Percent survival (%) | Water activity 16 days after coating |
|---|---|---|---|---|
| Coating drum | 4.08a | 3.11a | 12.3 | 0.84a |
| Before drying | 4.03a | 2.91a | 9.6 | 0.79b |
| After drying | 3.87b | 3.24a | 24.3 | 0.59c |

Similar to the previous results with clover seed, survival of rhizobia on lucerne seed after storage at different relative humidities for three weeks suggested there was little relationship between equilibrium water activity and survival (Figure 3A). However, the initial water activity of seeds had a significant effect on survival ($P < 0.05$). The number of viable cells per seed was significantly lower on seeds with an initially high water activity (0.84) than with dried seeds after storage at all relative humidities. Survival on seeds collected just before drying, with a slightly lower water activity (0.79), was also generally but not significantly lower than on dried seeds ($P > 0.05$).

Survival of rhizobia on lucerne seed after storage for nine weeks indicated a clear relationship between water activity and survival (Figure 3B). Generally, survival was better on seeds stored at low relative humidity, with an equilibrium water activity below 0.5. Initial water activity was still a factor in survival after nine weeks, with rhizobia surviving more poorly on seed that initially had the highest water activity. Survival of rhizobia on seeds collected just before drying was similar to survival on dry seeds, indicating that even a small reduction in water activity from 0.84 to 0.79 is beneficial for long term survival. However, viable numbers of rhizobia on these semi-dried seeds were more variable than on the dried seeds.

**Figure 3.** Survival of rhizobia on lucerne seed as a function of water activity after three (**A**) and nine (**B**) weeks storage at different relative humidities. (**A**) $R^2$ values: 0.1344 (coating drum), 0.0326 (before drying), 0.0845 (after drying). 2-way ANOVA: Seed batch $P$ = <0.001, RH $P$ = 0.418, Seed batch x RH $P$ = 0.340; (**B**) $R^2$ values 0.7623 (coating drum), 0.7664 (before drying), 0.8756 (after drying). 2-way ANOVA: Seed batch $P$ = 0.02, RH $P$ = <0.001, Seed batch x RH $P$ = 0.068.




The lowest equilibrated water activity of commercially coated lucerne seeds after nine weeks storage at low relative humidity was 0.38 which was higher than equilibrated water activity of clover seed inoculated with polyvinyl alcohol polymers after two weeks. However, the lowest water activity of the lucerne seed after three weeks of storage at low relative humidity (0.47) was similar to the lowest water activity of clover seeds coated with the polyvinyl acetate latex polymer after two weeks storage at low relative humidity (0.43). This indicates that equilibrated water activity of seeds is dependent on the moisture sorption properties of the polymer adhesive which varies between solution and latex polymers.

The data indicates that when water activity of seeds is high for extended periods of time, survival of rhizobia is poor. When water activity of seeds is reduced, survival is dependent on the extent to which the water activity changed (Table 3). In previous research, desiccated cells of TA1 survived better when stored at low relative humidity, indicating that survival is best when changes in moisture status of cells are minimized [11]. In that case, the relationship between survival of dried polymer-embedded TA1 and water activity changed with different polymers, highlighting the need to determine this relationship for different coating materials used by seed companies.

Coatings on seeds that had not been dried had less integrity than the coating on seeds that had been dried. Material from coatings that had not been dried was loose and easily removed whereas dried coatings were intact. This may also account for lower and more variable numbers of viable rhizobia on seeds that had not been dried. Drying coated seeds is an important step in the seed coating process so that seeds can be packaged and the coating remains intact. High integrity of seed coatings is important to prevent sloughing-off during transport and seed drilling operations [10]. However, the extent and process of drying should be monitored to minimise loss of cells during this process. Despite stable numbers of rhizobia per seed over the nine week storage period when water activity was below 0.5, the number was below the standard of 1000 per seed. Reducing loss of viability during drying would increase the likelihood of seed batches passing standards.

**Table 3.** Recovery of RRI128 from *M. sativa* seed after 9 weeks storage at high and low relative humidity (RH) and corresponding change in water activity. Values were calculated using cell numbers and water activities at 16 days and 9 weeks after storage.

| Seed sample | Recovery at highest RH (%) | Change in Aw at highest RH | Recovery at lowest RH (%) | Change in Aw at lowest RH |
|---|---|---|---|---|
| Coating drum | 0.49 | +0.01 | 3.07 | −0.42 |
| Before drying | 0.18 | +0.06 | 8.29 | −0.36 |
| After drying | 0.51 | +0.25 | 10.95 | −0.18 |

## 3. Experimental Section

### *3.1. Survival of Rhizobia on Coated Seed Stored at Different Relative Humidities*

Survival of rhizobia was determined after storage of inoculated seed at different relative humidities and a constant temperature of 15 °C. Experiments were carried out using inoculated seed samples prepared in the laboratory and seed collected from seed companies after inoculation using commercial seed coating processes. Seed species included subterranean clover (*Trifolium subterraneum*), white clover (*Trifolium repens*) and lucerne (*Medicago sativa*).

### *3.2. Inoculation of Seed in the Laboratory*

In the laboratory, subterranean and white clover species were coated with either peat cultures of *Rhizobium leguminosarum* bv. *trifolii* TA1 or TA1 colonies suspended from the surface of YMA and pelleted with fine lime. Suspensions of peat cultures and YMA colonies were prepared using solutions of different polymers and were applied to seeds at recommended commercial rates (1 L/50 kg seed for

subterranean clover and 1 L/25 kg seed for white clover). Polymers used included 20% (w/v) solutions of polyvinyl alcohol GL05 and KL05 (Gohsenol, Nippon Goshei, Osaka, Japan), a 20% solution of GL05 blended with canola oil (5% w/v) and a polyvinyl acetate latex prepared by the Key Centre for Polymers and Colloids (School of Chemistry, University of Sydney). Peat cultures were mixed with polymers at a rate of 25 g peat/100 mL polymer or colonies were suspended from four YMA plates in 30 mL polymer. Seeds were inoculated with polymer-cell suspensions at a rate of 1 mL/50 g seed in a sterile plastic container and mixed well until all seeds were wet (approximately 30 seconds). Wet seed was then pelleted using finely milled limestone (SeedCote™, Becker Underwood, Somersby, Australia) applied at a rate of 12 g/50 g seed and mixed until seeds were coated evenly. Viable numbers of rhizobia were counted immediately after coating and seeds were distributed to relative humidity chambers. Viable numbers of rhizobia were determined on stored seed after 24 h, 1 week and 2 weeks.

### *3.3. Seed Collected from Commercial Seed Companies*

Commercially coated seeds were collected from two seed coating companies. One company provided four samples from experimental batches of lucerne coated with *Sinorhizobium meliloti* RRI128 (commercial peat inoculant). A second company provided two samples of lucerne seeds coated with commercial peat inoculant containing RRI128 (one sample from a commercial batch and one from a small custom batch) and a sample of subterranean clover seed coated with commercial peat inoculant containing *Rhizobium leguminosarum* bv. *trifolii* WSM1325 from a small custom batch.

Commercially coated lucerne seed was collected at a later date from the second seed company at three points during the seed coating process. Seed was collected directly from the seed coating drum, after transfer on a conveyer belt to the drying table and after drying. Seeds were packaged in sealed plastic bags and transported to laboratories at AIRG and the University of Sydney for analysis. The number of viable rhizobia on coated seed was counted at three days (at AIRG) and 16 days (at the University of Sydney) after coating. At 16 days after coating the seed was distributed to relative humidity chambers.

### *3.4. Storage of Inoculated Seed at Different Relative Humidities*

Triplicate samples of coated seeds were stored in a refrigerated incubator at 15 °C. A constant temperature was required to maintain stable relative humidity conditions so that experiments could be replicated. Laboratory coated seeds were stored at relative humidities of 23%, 33%, 54%, 75%, 85% and 100% and commercially coated seeds were stored at 10%, 33%, 54%, 75% and 85%. In the later experiments with commercially coated seed, 10% relative humidity was substituted for 23% to provide a lower range as ambient relative humidity can often be low in rural regions of Australia. The highest relative humidity of 100% was removed as a treatment as coated seeds were likely to be contaminated and rhizobia would be difficult to count if growth of non-rhizobial species was encouraged. Relative humidity chambers were prepared using desiccators containing super-saturated salt solutions listed in Table 4 [30]. Relative humidity and temperature were monitored over the course of the experiments using TinyTag™ relative humidity and temperature data loggers (Hastings Data Loggers, Wauchope, Australia).

**Table 4.** Saturated salt solutions and equilibrium relative humidities.

| Saturated salt solution (g/L) | Relative humidity (%) |
|---|---|
| Potassium hydroxide (2000) | 10 |
| Potassium acetate (2500) | 23 |
| Magnesium chloride (2800) | 33 |
| Magnesium nitrate (2200) | 54 |
| Sodium chloride (500) | 75 |
| Potassium chloride (500) | 85 |

### *3.5. Determination of Viable Numbers of Rhizobia and Measurement of Water Activity*

Rhizobial cells were resuspended from 100 seeds in 100 mL of sterile phosphate peptone buffer (PPB, 1.21 g $K_2HPO_4$, 0.34 g $KH_2PO_4$, 1.0 g peptone and 1 L water) by shaking for 10 min on a wrist-action shaker. The number of viable rhizobia was determined using spread plate counting on YMA after 10-fold dilution in PPB and water activity was measured at various time intervals using a PawKit™ water activity meter (Decagon Devices Inc, Pullman, WA, USA).

### *3.6. Statistical Analysis*

Significant differences in viable numbers of rhizobia on laboratory and commercially coated seed was determined by one-way analyses of variance at each time using GenStat (14th Edition, VSN International Ltd, Hemel Hempstead, UK). The effect of seed drying and storage at different relative humidity on survival was analysed by two-way analysis of variance. Polynomial functions were fitted and $R^2$ values calculated using Microsoft Office Excel 2003 (Microsoft Corporation, Redmond, WA, USA).

## 4. Conclusions

Relationships between survival of rhizobia on legume seed and water activity of coated seeds were established after storage of inoculated seeds at different relative humidities and constant temperature. However, this changed over time and was dependent on seed species, inoculum preparation, coating ingredients and whether inoculated seeds had been dried during production. While strains were not compared directly, it is assumed that results would also vary with different rhizobial strains because of inherent variability in survival [11]. This indicates that the relationship between water activity and survival would need to be calibrated over time for each combination of seed-rhizobial species, inoculant type, temperature range, coating ingredients and coating techniques before water activity could be used to monitor shelf-life.

If storage conditions are defined, shelf life may be more accurately determined and indicated on packaging. Longer-term studies will further establish relationships between storage conditions and survival on commercially coated seed. Inoculated seeds may then be easily monitored by seed coating companies using measurements such as water activity as an indicator that storage conditions are optimized for rhizobial survival. More research is needed to refine drying techniques and coating materials that reduce the death rate of rhizobia on seed. Survival of inoculant microorganisms on seed

must be better understood and improved if inoculated seed is to be an effective means of delivering microbial inoculants to broad-acre crops.

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