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Legume seed inoculation technology—a review

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Abstract

Inoculation of legume seed is an efficient and convenient way of introducing effective rhizobia to soil and subsequently the rhizosphere of legumes. However, its full potential is yet to be realised. Following widespread crop failures, the manufacture of high quality inoculants revolutionised legume technology in Australia in the 1960s. Many improvements to inoculants and the advent of an inoculant control service ensured that quality was optimised and maintained. Minimum standards for the number of rhizobia per seed were set after consideration of several factors including seed size and loss of viability during inoculation. Despite manufacturers' recommendations for storage and application of inoculants, there is a distinct lack of control over the inoculation process; hence the full potential of high quality products may not always be achieved. The efficacy of inoculation varies depending on several factors, all of which affect the number of viable rhizobia available for infection of legume roots. Increased numbers of viable rhizobia per seed by application of inoculant above the commercially recommended rate, results in a continued linear increase in nodulation and yield. Several studies have reported yield increases of up to 25%. However, applying higher quantities of inoculant is uneconomical and technically difficult. Alternatively, higher numbers of viable rhizobia per seed may be achieved by improving survival during seed inoculation. Despite recognition of the factors affecting survival of rhizobia on seed and a substantial demand for commercially pre-inoculated legume seed, poor survival is still a major concern. Desiccation, temperature and seed coat toxicity all influence survival of rhizobia on seed. Their adverse effects may be ameliorated by selecting tolerant rhizobial strains and legume seed cultivars with low toxicity or artificially, by the use of additives in the seed coating. The accumulation of the desiccant protectant trehalose in strains of rhizobia, may result in better survival under desiccation stress. Similarly, the accumulation of exopolysaccharide (EPS) may act as a barrier reducing excessive water loss. Polymeric adhesives such as gum arabic, methyl cellulose and polyvinyl pyrollidone (PVP) have improved survival. However, studies of additives used in inoculation have been ad hoc and little of their mode of action is understood. A better understanding of the mechanisms involved in the protection of rhizobia from adverse conditions will assist in defining the optimum conditions for seed inoculation and storage to ensure a higher quality product for farmers at the time of sowing.

Keywords: Legume inoculation; Rhizobia; Desiccation; Survival; Polymers; Seed coating

1. Introduction

We review the literature relating to legume inoculation technology and propose research areas to improve the results of legume seed inoculation. In 1887, Hellriegel and Wilfarth showed in their classical experiments that N_2 fixation was associated with nodulated legume roots and was essentially the role of the infective agents of nodules. These agents varied in their ability to nodulate different groups of plants. One year later, Beijerinck isolated and described the root nodule bacteria and by 1896 the definition of the 'cross inoculation groups' was in practical use (Fred et al., 1932). Such groups of legumes (usually closely related taxonomically) are nodulated by the same species of *Rhizobium*. It became common knowledge that there could be a need to inoculate legumes that had not been grown in a particular soil or for some years. This was particularly the case in Australia, where the legumes cultivated for agriculture were all introduced (Davidson and Davidson, 1993). Scientists later recognised there was a much more specific relationship between bacterial strains and legume-hosts in terms of infectiveness (the ability to nodulate) and effectiveness (the ability to fix N_2). To improve pasture establishment in Australia and to support an ever-growing grazing industry, research was initiated from the 1930s at several institutions to isolate effective strains and determine their relationships with specific hosts. The aim of inoculation is to provide sufficient numbers of viable effective rhizobia to induce rapid colonisation of the rhizosphere allowing nodulation to take place as soon as possible after germination and produce optimum yields (Thompson, 1988; Catroux, 1991).

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2. Legume inoculation

2.1. Production and quality control of legume inoculants

Legume seed is commonly inoculated with peat cultures of rhizobia. Their commercial production in Australia began in 1953 using finely milled peat as the bacterial carrier (Davidson and Davidson, 1993). The quality of inoculants was improved, following widespread nodulation failures, by the amelioration of five main factors affecting survival in peat (Roughley and Vincent, 1967). Firstly, the origin of the peat was shown to be important. Survival of clover, lucerne and cowpea rhizobia varied according to the location and depth of the peat source. The peats tested varied according to their colour and texture but no explanation was given by the authors as to the cause of variation in survival. Secondly, pH was shown to be critical and acid peats could be amended with calcium or magnesium carbonate. Thirdly, peat sterilisation, preferably by gamma irradiation, was considered essential particularly for the growth and survival of slow-growing rhizobia presumably allowing them to out-compete faster-growing contaminants. Fourthly, when rhizobia were added to peat previously dried at 100 °C, they survived poorly due to both the heat of wetting generated upon inoculation and the production of inhibitory substances originating from the heat treatment. Finally, moisture contents of 40–50% proved optimal for growth and survival of a range of rhizobial strains prepared as peat cultures. Later, accumulation of salt in the peat deposit, caused by several dry seasons, was found to adversely affect rhizobial survival (Steinborn and Roughley, 1975). The implementation of these findings to inoculant production was a direct result of an initiative in inoculant quality control undertaken jointly by the University of Sydney and NSW Department of Agriculture Laboratory Service (UDALS). In 1971 this function passed to the Australia-wide Australian Inoculants Research and Control Service (AIRCS), since renamed in 2000 the Australian Legume Inoculants Research Unit (ALIRU) (Date, 1983; Roughley et al., 1984; Herridge et al., 2002). Inoculants must pass ALIRU standards based on the number of effective rhizobia in the peat that will result in a minimum number of cells per seed after application at the manufacturers recommended rate. These minimum standards are 500 for white clover, 10^3 for small-seeded legumes (e.g. lucerne, subterranean clover), 10^4 for medium seeds (e.g. mungbean, pigeon pea) and 10^5 for large seeds (e.g. lupins and soybean). These are widely accepted minimum standards although they may vary slightly in some countries (Lupwayi et al., 2000). There are also standards for the number of contaminants in commercial peat cultures. In Australia contaminants must number less than 10^7 cfu per gram of peat whilst in France there must to be no contamination throughout storage (Lupwayi et al., 2000; Catroux, 1991). However, over-crowding of plates at dilutions lower than 10^{-6} would make contaminants difficult to detect in such concentrated suspensions. Inoculant regulatory bodies may be supported by legislation such as in Canada, Brazil, France and Uruguay or have voluntary participation by manufacturers such as in Australia, South Africa and New Zealand. In the United Kingdom and United States product quality is left to the discretion of the manufacturers (Gault, 1978; Lupwayi et al., 2000; Catroux et al., 2001).

2.2. The need for inoculation

The decision to inoculate is usually based on a demonstrated need from experimental plots or as an insurance against crop or pasture failure. Results of an investigation into inoculation of *Trifolium subterraneum*, grown in soils in New South Wales where nodulation failures had occurred, emphasised the difficulty in predicting the need to inoculate (Roughley and Walker, 1973). Of the 32 sites tested, 14 showed no response to inoculation and strains of effective rhizobia were found to occur naturally. No obvious relationship was observed between the soil types and the presence of effective strains. However, at the other 18 sites at least one of the inoculation methods tested improved nodulation. Generally, lime-pelleting (addition of superfine limestone) produced more reliable results and was superior to slurry inoculation in the soils where the pH was below 5.5.

Ireland and Vincent (1968) showed that high numbers of naturalised *R. leguminosarum* bv. *trifolii*, effective on white clover but not on subterranean clover, severely restricted nodulation of subterranean clover by an introduced effective strain. In a soil containing 10^5 ineffective rhizobia per g^{-1}, yield was doubled with a 10-fold increase in the inoculation rate of the introduced strain. Application of 10^6 rhizobia per seed was necessary to ensure 90% effective nodulation. Interestingly, this is 1000 times higher than the rate recommended by the ALIRU. Furthermore, Thies et al. (1991a) reported a positive effect of inoculation on eight legume crops where the soil population of rhizobia was between 10 and 100 g^{-1} of soil. Elsewhere, they proposed a model to predict the response to inoculation and found that 59% of the variation-in-yield could be explained by the number of rhizobia present in soils at sowing (Thies et al., 1991b). Therefore, there is a need for inoculation with high numbers of effective rhizobia to out-compete populations of ineffective rhizobia in the soil or to build up populations where soil conditions have limited their persistence.

2.3. Inoculation techniques

Rhizobia may be introduced to legumes by inoculation of the seed or soil. Seed may be inoculated by farmers immediately prior to sowing or custom inoculated by local seed merchants with coating facilities to be sown within a week. Alternatively, legume seed may be commercially inoculated and stored prior to its sale. This product is commonly referred to as pre-inoculated seed. However,

despite a growing demand for pre-inoculated seed since its introduction in Australia in 1971, testing in both 1972–1974 and 1999–2002 revealed poor survival of the rhizobial inoculum raising questions as to the value of this technology (Brockwell et al., 1975; Gemmell et al., 2002). Alternative methods to seed coating include direct inoculation of the soil using peat inoculants suspended in water or inoculants formulated as liquids or granules.

Although inoculation techniques are highly variable, the fundamental practices are listed in Table 1. After inoculating seeds by dusting with peat culture, most of the inoculant is removed when seed passes through machinery. When the moist inoculum dries out, it is easily dislodged from the seed and settles to the bottom of the seed hopper (Gault, 1978). However, when peat inoculant is applied with adhesive, more peat is retained on the seed coat. The adhesive should be able to prevent sloughing-off of coating material preventing blockages in seed drilling equipment and not cause damage to cotyledons (Brockwell, 1977). Nodulation of soybeans after sprinkling inoculant on the seed in the seed box of the planter shortly before sowing was consistently inferior when compared to slurry inoculation (Brockwell et al., 1988).

Lime-pelleting of inoculated legume seed with superfine limestone ($CaCO_3$) was introduced to counteract the acidic effects of soil or superphosphate on the survival of the rhizobia. Several rhizobial species including *R. leguminosarum* bv. *trifolii* (Jensen, 1943) and *Sinorhizobium meliloti* (Amarger, 1980) are particularly sensitive to acid conditions. Cass Smith and Pittman (1939) neutralised superphosphate by drilling with limestone. Loneragan et al. (1955) found that pelleting seeds with limestone followed by dusting with peat culture could be as effective as drilling inoculated seed with limestone and was much more economical. Brockwell (1962) improved this process by incorporating the inoculum in an adhesive under the limestone coat. Roughley et al. (1966) reported that locating the inoculum within a pellet provided better conditions for the survival of rhizobia than slurry-inoculation. Not only did it protect rhizobia against soil and fertiliser acidity but also there were several other advantages of lime-pelleting. These included improved ballistics of seed sown aerially, better survival of rhizobia when there were delays between inoculating and sowing (there was a 10-fold improvement in survival when compared with slurry-inoculated seed stored at room temperature for 1 week), and prompt nodulation when germination was delayed. Date (1968) investigated the effect of limestone on survival of *R. leguminosarum* bv. *trifolii* TA1 and found gum arabic and methyl cellulose alone were better than when seeds were pelleted with limestone up to 42 d after inoculation. He concluded that the low pH of the adhesive was less detrimental to survival than the high pH of the limestone. Norris (1972) recommended coating tropical legume seeds with rock phosphate because the rhizobia which nodulate these legumes were likely to be sensitive to high pH. Clearly, the limestone used in these studies were high in pH, a recent survey of powdered pelleting materials revealed that different limestones can vary in pH from 6.5 to 9.7 (Gary Bullard, pers. comm.). The use of a limestone with a more neutral pH may ameliorate the problem of pH sensitivity.

Direct inoculation of the seedbed at time of sowing using liquid and granular inoculants avoids damage to fragile seed coats and overcomes the adverse effect of pesticides and fungicides applied to seed on rhizobia. It also reduces the risk of losing viable bacteria through seed drilling equipment or when the seed coat is lifted out of the ground during germination. Small-seeded legumes have benefited from liquid inoculation by allowing higher application rates of inoculum than possible with seed inoculation (Brockwell, 1977). Liquid inoculation of soybeans at sowing was equally as successful as slurry inoculation of seed; however, slurry inoculation produced earlier nodulation (Brockwell et al., 1988). They attributed this to the higher concentration of rhizobia in the vicinity of the seed leading to more rapid colonisation of the rhizosphere than when rhizobia were distributed throughout the seedbed by liquid inoculation. A granular inoculant for lucerne was patented in New Zealand in 1971 using a marble-chip core, coated in peat culture. Peat and clay granules were used in the United States (Gault, 1978). Peat granules were specifically developed for use in the peanut industry. Seed drilling equipment could be adjusted to locate the granules beside, with or below the seed in the seedbed. Gault (1978) states that granular inoculant was not an accepted technology in Australia. Unlike the United States where seed-drilling equipment was modified for the application of other granular technologies such as pesticides and herbicides, there was no need for

Table 1
Legume inoculation techniques

Technique	Description
Seed inoculation	
Dusting	Peat inoculant is mixed with the seed without re-wetting
Slurry	Seed is mixed with a water solution of peat often with the addition of an adhesive
Lime or phosphate pelleting	Seed is treated with a slurry peat inoculant followed by a coating of calcium carbonate (superfine limestone) or rock phosphate
Vacuum impregnation	Rhizobia is introduced into or beneath the seed coat under vacuum
Soil inoculation	
Liquid inoculation	Peat culture mixed with water or liquid inoculant applied to the seedbed at the time of sowing (liquid inoculants may also be applied to seed)
Granular inoculation	Granules containing inoculum sown with seed in seedbed

Summarised from Brockwell, J., 1977; Bio-Care Technology Pty. Ltd. Inoculant Brochure 1998; Thompson, J., 1988).

granular attachments on seed drilling equipment in Australia. Inoculant production in Australia was a relatively minor industry and production of granular inoculants would require large economic input particularly for distribution over vast distances. There is nevertheless a renewed enthusiasm for granular inoculant technology in Australia. Recent field trials where clay granules applied at a rate of 10 kg ha^{-1} have given successful field nodulation and N_2 fixation in peanuts, lupins, peas and faba beans (Gary Bullard, pers. comm.).

It is clear that both seed and soil inoculation techniques have advantages and disadvantages. Selection of a method depends on the availability of equipment, seed size and fragility of cotyledons, the presence of seed applied fungicides and convenience. Current research is focussed on optimising these fundamental delivery techniques in order to improve survival of rhizobia and subsequent nodulation.

2.4. Seed coating process

The process to produce consistently evenly coated seeds needs to be controlled and reproducible. Seeds are coated using a variety of machinery and some examples of these are outlined below. The processes used by individual farmers and commercial seed coaters may vary but the principles are essentially the same (for an extensive review of seed coating processes and machinery see Gault (1978)).

On farms prior to sowing, the seed is usually dusted with peat inoculum, or mixed with either a water or adhesive slurry, often followed by superfine limestone for pasture seeds and allowed to dry. Here the method of application of inoculant varies according to individual practices and legume species. Although mixing is mostly done manually in the seed box or cement mixer, inoculation of large-seeded pulses may also be automated using seeders fitted with an inoculant tank, pump and seed/inoculant-mixing chamber. While these are well suited to applying chemical coatings to seed, their aptness for use with live rhizobia is uncertain. Investigations into the Flexicoil® seeder, found that excessive force dislodged some of the rhizobial inoculant from the seed while passing through seeding lines (Gary Bullard, pers. comm.). Air inlets when located near hot motors also present a problem since high air temperatures are detrimental to rhizobial survival.

Most pasture legume seed is coated using a rotating drum, due to its relatively low cost and simplicity (Scott et al., 1997). Large dough or cement mixers are often used by custom and pre-inoculators for lime-pelleting of seed. Inoculant is applied to the seed coat with an adhesive and a final coat of superfine limestone or some other particulate material is then added to the drum and may be over-sprayed with adhesive. In cases where the coated seeds are prepared prior to sowing, they are air-dried in shallow layers or by using forced air (approx. 25 °C) on an airbed.

The time used for mixing and the amounts of adhesive and pelleting material all affect the physical properties of the pelleted seed. Investigations of lime-pelleting found a good pellet should have an even coat of limestone and appear dry without loose limestone on the surface (Roughley et al., 1966; Roughley, 1970). If the coated seeds have a glossy appearance they have probably been mixed too long or without enough limestone. Lime-pelleted seed should be firm enough to withstand light impact (such as dropping on the floor and lightly rolling in the fingers). Application of fluidised-bed technology to coating legume seeds is less common (Mullett et al., 1973). This process allows finer control by suspending the seeds on a cushion of pressurised air and spraying inoculant from above. Herridge and Roughley (1974) compared seed pelleted using a fluidised-bed with the conventional rotating drum technique. The fluidised-bed produced a firm pellet but the survival of the inoculum was low, possibly due to the air temperature occasionally reaching 35 °C. The integrity of the pellet is important to ensure powder does not dislodge during sowing causing blockages in seed-drilling lines. However, the conditions required to obtain pellets of high integrity may compromise viability of the rhizobial inoculant. New materials and methods should be tested that allow the production of high quality pellets without exposing cells to harsh conditions.

3. Survival of rhizobia on seed

3.1. Evidence of poor survival on seed and its impact on legume yield

Death of all species of rhizobia on inoculated seed occurs rapidly, particularly when environmental conditions are unfavourable (Bowen and Kennedy, 1959; Marshall, 1964; Diatloff, 1967; Brockwell et al., 1987). Early in the 20th century, researchers recognised the problem of poor survival of rhizobia on legume seed, and its partial amelioration through low temperature storage and the use of additives (Fred et al., 1932). Inoculation techniques were usually assessed in terms of resulting nodulation from 'grow-out' tests, and an increase in nodulation would often be attributed to improved survival (J.A. Thompson, unpub. PhD thesis, University of Sydney, 1964).

Survival on seed directly affects the resulting legume yield. Roughley et al. (1993) identified a large mortality factor on lupin seed between inoculation and sowing. When inoculation provided $\log_{10}5.15$ viable bradyrhizobia per seed only 4.8% ($\log_{10}3.83$) of the inoculum was still viable at sowing (3.75 h after inoculation) and only 0.83% ($\log_{10}3.07$) after 22.5 h in the soil. Furthermore, increases in the rate of inoculation from $\log_{10}4.27$ to 6.27 and 7.28 significantly increased root colonization, early nodulation and nodule mass. The recommended rate of application of lupin inoculant provides $\log_{10}5.55$ rhizobia per seed.

They concluded these observations emphasise the need for care in handling of inoculated seed to reduce death rates of rhizobia and the need for new, higher standards for commercial inoculants. Elegba and Rennie (1984) found soybean yield increased when 10^6 rhizobia were applied per seed rather than the recommended number of 10^5. Similarly, Hume and Blair (1992) reported an increase in soybean grain yield of 15–25% with an increase in the rate of inoculation from $\log_{10}5.0$ to 6.0. It is clear that improved survival of the inoculant on seed would directly affect nodulation and subsequent yield of the plant. All these results call into question the current standards set for inoculants and that there should be an increase in the number of viable rhizobia applied per seed.

3.2. Factors affecting survival of rhizobia on seed

Death of rhizobia is common to all known inoculation procedures and has been attributed to three main sources: desiccation, the toxic nature of soluble seed coat exudates and unfavourable storage temperatures (Date, 1968; Taylor and Lloyd, 1968; Thompson et al., 1975; Vincent et al., 1962).

Desiccation is a major contributing factor to poor survival on seed (Vincent et al., 1962; Thompson, 1964). A study of the survival of *R. leguminosarum* bv. *trifolii* on glass beads under dry conditions indicated that there were two distinct phases of death (Vincent et al., 1962). After an initial rapid decline in cell numbers between 0 and 24 h that coincided with a rapid loss of water, there followed a period during which water loss and death rate declined. Cells survived best at 100% relative humidity and no viable cells were detected after 27 h at relative humidities below 60%. Waters demonstrated a direct relationship between water loss in peat and survival of rhizobia (unpublished data, cited in Vincent (1958)). Further research indicated that tolerance to desiccation varies between strains of rhizobia. Bushby and Marshall (1977b) found that slow-growing strains of rhizobia survived better than fast-growing strains after desiccation in sandy soil. In their experiments, air-dried soil (10 g) was inoculated with 4 ml of either fast- or slow-growing rhizobia to provide 10^6 cells g^{-1} of soil. After drying in a 28 °C forced-draught oven overnight, only 10^2 fast-growing rhizobia survived compared with 10^5 slow-growing rhizobia g^{-1} of soil.

Atmospheric relative humidity impacted differentially on strains of *S. meliloti* and *Bradyrhizobium japonicum* immobilised on cellulose filters. The rate at which cells dried was an important determinant of their survival, which was favoured by slow drying (Mary et al., 1994, 1985). $\text{Log}_{10}2.0$ viable cells of *S. meliloti* survived after storage for 100 d at relative humidities of 22, 43.6 and 67.8% and no viable cells were detected after storage at 3 and 83.5%. Numbers of viable cells of *B. japonicum* steadily declined to zero by 100 d at all relative humidities. Slow rehydration, when dried cells were equilibrated at intermediate water activities before full rehydration, resulted in better survival than when bacteria were rapidly rehydrated (Kosanke et al., 1992).

The stress of desiccation is further complicated by the toxic nature of O_2 (reviewed in H.V.A. Bushby, unpub. PhD thesis, University of Tasmania, 1974). Two stages of desiccation were identified; partial dehydration when the relative humidities were still high followed by dehydration where relative humidities are below 70% and where O_2 becomes toxic. The targets of O_2 damage are proteins, membranes and nucleic acids (reviewed by Potts (1994)). Protein damage through metal-catalysed (Fe^{3+}) oxidation reactions and lipid peroxidation leads to a loss of membrane semi-permeability and ultimately cell lysis. Accumulation of breaks in the DNA molecule occurred during exposure of bacterial cells to the superoxide anion radical (O_2^-) and hydrogen peroxide (H_2O_2). O_2 has a detrimental effect on the survival of freeze-dried bacteria (Williams, 1954; Mellor, 1978). As a result freeze-dried cultures survive better stored under vacuum or N_2 than when stored in air (Mellor, 1978).

Water-soluble substances from the seedcoat may be inhibitory to rhizobia. One unpublished study noted more clover rhizobia died on seeds than on glass beads, possibly due to inhibitory seedcoat diffusates (R.W. McLeod, cited in Vincent (1958)). Vincent et al. (1962) repeated these experiments using seeds of *T. subterraneum* cv. Woogenellup. Variable death rates indicated that the drying stage (0–27 h) could be sub-divided into two stages, 0–5 h and 5–27 h. Higher death rates in the first 5 h suggested that inhibitory substances were most active when the seed contained readily available moisture.

Exudates from legume seeds of different genera (J.A. Thompson, unpub. PhD thesis, University of Sydney, 1964) and cultivars of arrowleaf clover (Materon and Weaver, 1984a,b) varied greatly in toxicity. Bowen (1961) demonstrated high toxicity seed exudates from *Centrosema pubescens* and low toxicity of those from *Medicago sativa* cv. Hunter River.

The active compounds were analogous to polyphenolic compounds with the tri-hydroxilic grouping on the phenolic B-ring such as delphinidin, quercetin, myricetin and tannins (Fottrell et al., 1964; Thompson, 1964; Masterson, 1965). Young and Paterson (1980) found seeds of *M. sativa* contained cyanidin, which was not toxic to rhizobia, and suggested that selection of non-toxic genotypes could be useful to assist survival during seed inoculation. Materon and Weaver (1984a) tested this hypothesis using non-toxic seeds of arrowleaf clover. They found that the non-toxic seeds maintained a larger rhizobial population than a toxic variety. Thompson (1964) found that toxicity of water extracts of clover seeds was higher after extraction for 6–8 h than 24 h. He speculated that the disappearance of the inhibitory substances over time may be due to enzymic inactivation or masking by non-inhibitory substances produced in the later hours. Thompson (1960), Bowen

(1961) and Masterson (1962) agreed seed coat toxins were not altered by autoclaving.

Storing inoculated seed at a low temperature (5 °C) benefits survival, but is not practical (Date, 1968). Vincent (1958) demonstrated death rates of clover rhizobia on glass beads increased when storage temperatures were raised from 5 to 26 and 37 °C. Bowen and Kennedy (1959) demonstrated the susceptibility of many rhizobia to temperatures of 40 °C. Greatest stress from temperature is experienced when sowing onto the surface of dry soil. This is of particular concern in tropical areas where soil surface temperatures reach maxima of 70 °C in soils around Roma, Queensland (McInnes and Date, 1999), and 62 °C in sandy soils of Western Australia (Chatel and Parker, 1973). Sowing tropical pasture species such as *Desmanthus* and *Stylosanthes* into these soils severely compromises the viability of their associated rhizobia (McInnes and Date, 1999).

The effects on survival of desiccation and temperature are not mutually exclusive. Rhizobia are more sensitive to moist heat than dry heat (Vincent et al., 1962; Wilkins, 1967). Wilkins (1967) found that nodule bacteria for *Medicago* spp. were capable of surviving for 32 h in dry soil at 60 °C but did not survive for 5 h in moist soil at 55 °C. Lyophilised rhizobia survive better at high temperatures than peat cultures (Kremer and Peterson, 1982, 1983; McInnes and Date, 1999) but the cells must be kept dry to maintain tolerance to high temperatures.

There is some evidence that strains may be selected with high temperature tolerance. Philpotts (1977) found a range of isolates (*R. leguminosarum* bv. *trifolii*) from white clover (*T. repens*) growing on the far north coast of NSW survived better on seed held at 35 °C than the commercial inoculant strain, TA1 which was originally isolated in Tasmania. AbdelGadir and Alexander (1997) found that starving cells of *R. leguminosarum* bv. *phaseoli* in phosphate buffer enhanced their heat resistance enabling 10^6 to survive for 250 h whereas less than 10^2 of the non-starved cells survived the period at 40 °C. However, nodules produced on kidney beans were small and light brown compared to large, dark brown nodules of non-heat tolerant strains indicating a possible loss in effectiveness.

In summary, survival of rhizobia on legume seed is influenced by environmental factors leading to desiccation, exposure of macromolecules to toxic levels of O_2 and high temperatures, and mobilisation of inhibitory substances from the seedcoat. These factors interact to create a complex environment in which survival responses are difficult to interpret.

3.3. Inoculant formulation and survival

There have been several reviews of inoculant formulation in the literature (Date and Roughley, 1977; Burton, 1979, 1981; Brockwell and Bottomley, 1995; Stephens and Rask, 2000). The common forms of inoculant include agar cultures, peat, liquid or broth, freeze-dried and granular. Date found survival of rhizobia on seed applied using peat culture was superior to that using agar culture (R.A. Date, unpub. PhD thesis, University of Sydney, 1959). Only the survival of rhizobia from agar culture could be improved by the addition of sucrose at a concentration of 10%. No further improvement was observed with peat cultures indicating that peat already afforded some protection of cells on seed. Cells from both forms of culture survived better at 18 °C rather than 25 °C, thus confirming earlier observations that storage at lower temperatures and the use of additives could improve survival on seed. Two main functions of carrier materials have been identified namely; they must support the growth of the organism and also support high viable numbers over an acceptable period of time. Due to the scarcity of peat in many countries, several materials have been tested for these properties. They include soils, clays, charcoal, plant byproducts such as bagasse, lucerne powder and corn compost, perlite, rock phosphate and talc (Stephens and Rask, 2000). Despite awareness of the need to improve survival of rhizobia on inoculated seed, development of inoculant carriers remains focussed on maximising numbers of rhizobia to meet quality control standards, convenience and affordability. Unlike peat, there is little information about the ability of these alternative carriers to improve the subsequent survival of rhizobia on legume seed. Physiological and morphological changes during the maturation of the peat inoculant have been shown to affect survival (Dart et al., 1969; Materon and Weaver, 1985; Feng et al., 2002). Examination of peat particles by electron microscopy reveals a highly irregular structure providing many crevices that may act to protect the rhizobia on seed. Changes to the rhizobial cell such as thickening of the cell wall, the disappearance of polyhydroxybutyrate (PHB) granules and appearance of heat shock proteins have been observed (Feng et al., 2002). There is an apparent correlation between these changes and improved survival but the mechanisms are unclear. Poor survival on seed may be overcome by applying liquid and solid inoculants directly to soil. However, larger quantities of inoculant are generally required when soil inoculation is used thereby increasing costs (Burton, 1981).

Freeze-dried cells have also been considered as alternative inoculants (Appleman and Sears, 1944; McLeod and Roughley, 1961; Kremer and Peterson, 1982; McInnes and Date, 1999). McLeod and Roughley (1961) tested the suitability of commercially produced freeze-dried cultures of clover and medic rhizobia as inoculants. Their results from glasshouse and field trials indicated that they were equally as effective as peat cultures at producing nodules. They could also be stored at temperatures up to 37 °C without a significant loss of viability over 6 months. However, Vincent (1965) found freeze-dried cells had poor survival when re-wetted on seeds. Vegetable oils have been used in conjunction with freeze-dried cells.

Kremer and Peterson (1982) found that suspension of freeze-dried cells in oil provided better survival on seed especially in the first few days. Improved survival was related to reduced absorption of water by the dried cells.

Polymers used as alternative inoculant carriers may allow for better control of the rhizobial microenvironment. Polyacrylamide (PAM), a synthetic polymer, was used as an alternative carrier to peat (Dommergues and Hoang, 1979). PAM entrapped rhizobia survived better than peat and liquid cultures after storage at 30 °C. In pots, soybean roots collected viable rhizobia as they passed through PAM blocks and nodules were formed after emergence. However, mobility of rhizobia was decreased and viability was only maintained when the block was kept moist.

PAM could be replaced by the biopolymers xanthan and carob gum, and alginate (Jung et al., 1982). Cells could be encapsulated by these polymers, dried or semi-dried and included in the seed coating. Xanthan gum is extracted from cultures of *Xanthomonas* spp. and this was found to be successful when mixed with carob gum, an extract from locust bean (*Ceratonia siliqua*) consisting of β-1,4 galactomannan. After 100 d of storage at 28 °C, viable cells of *B. japonicum* entrapped and dried in alginate and a xanthan–carob gum mixture were reduced from $\log_{10}7.6$ to $\log_{10}6.8$ and $\log_{10}7.8–8.0$ to $\log_{10}6.0–7.3$ ml^{-1} of broth, respectively. The resulting nodulation after 15 d old dried xanthan–carob gum entrapped rhizobia was stored on seeds for 48 d was not significantly different to seeds freshly inoculated with peat cultures. The protective nature of biopolymers was attributed to their ability to limit heat transfer, good rheological properties and high water activities (Mugnier and Jung, 1985). Survival of *B. japonicum* entrapped in dried xanthan–carob gum was dependent on the relative humidity during storage. After 8 d of storage at 28 °C survival was best at 6% relative humidity ($>\log_{10}10.0$ g^{-1} dry polymer) and decreased as relative humidity increased to 75% ($<\log_{10}4.0$ g^{-1} dry polymer). The addition of mannitol to the gum altered the moisture characteristics and improved survival. The results were explained in terms of the point of mobilisation of solutes. This is the point where free water appears in the polymer matrix allowing certain solutes to mobilise that lead to cell death. These results emphasise the importance of the solvent properties of water in biological systems.

Recent work has focussed on the encapsulation of bacteria in synthetic and semisynthetic polymers (Amiet-Charpenter et al., 1998). Survival of *Pseudomonas fluorescens/putida* in microparticles of methacrylic polymer was better than in ethylcellulose or modified starch and a moisture content of 25% in the particles after spray drying was optimal for survival. The addition of silica at a rate of 4.5%, modifying water loss from the particles, further improved survival. Concentrations of silica above and below this rate did not improve survival indicating that the optimum moisture content for survival is not necessarily a maximum or minimum.

Despite the relative success of polymer encapsulated cells in improving survival, the technology has not been adopted by the inoculant industry because of the high cost of increased technical handling (Fages, 1992; Bashan et al., 2002). This highlights the need for new technologies to be affordable so that the low cost of inoculants is maintained and well suited to production within current facilities if they are to be readily adopted by manufacturers.

4. Physiological tolerance to desiccation

4.1. The production of compatible solutes or osmoprotectants

Under osmotic stress, a balance between internal and external water potentials can be reached if the cells accumulate compatible solutes or osmoprotectants. These include potassium ions, glutamate, glutamine, proline, quaternary amines (glycine betaine) and the sugars trehalose, sucrose and glucosylglycerol. Compatible solutes help maintain the stability of proteins during osmotic stress via a ‘preferential exclusion mechanism’. Here the solute is held at a finite distance from the protein allowing the surface to be preferentially hydrated (for an extensive review of desiccation tolerance of prokaryotes, see Potts (1994)).

If the relative humidity of the environment permits metabolic activity then the cells may achieve water balance through the de novo synthesis of compatible solutes. When no water is available in the immediate environment as in air-drying or desiccation, the cells cannot rely on ‘preferential exclusion mechanisms’. To account for the maintenance of biological integrity during desiccation, a ‘water replacement hypothesis’ was developed by Crowe and Crowe (1986). Some desiccation-tolerant cells accumulate large amounts of the disaccharides trehalose and sucrose (reviewed by Crowe et al. (2001)). In systems where carbohydrates are dried in the presence of proteins, carbohydrates lose the capacity to form intermolecular hydrogen bonds. Instead, hydrogen bonding occurs between the carbohydrate and protein preserving its structure (Tsevtkov et al., 1989). Similarly, hydrogen bonding occurs between trehalose and phospholipids in membranes. Some models suggest a mechanism whereby trehalose fits between phosphates of adjacent phospholipids replacing four hydrating water molecules (Gaber et al., 1986). Recent models indicate that the mechanism is simply related to the ability of the solute to physically decrease the force exerted on membranes as they approach one another under desiccation, decreasing membrane fluid-to-gel phase transitions (Bryant et al., 2001). Several applications for trehalose have been reported including the stabilisation of vaccines and liposomes (Crowe et al., 2001). It is produced in response to stress by yeasts (Mansure et al., 1997). However, for trehalose to stabilise cells it must be on either side of the membrane (Crowe et al., 1998).

Trehalose may accumulate by transport from the environment or through de novo synthesis. Streeter (1985) found all species of *Rhizobium* accumulated trehalose to varying degrees and in most slow-growing strains, trehalose accounted for greater than 80% of the total mono- and disaccharides. There was an apparent relationship between the high accumulation of trehalose and survival in water culture. *B. japonicum* survived better in water culture than the fast-growing strains. None of the slow-growing strains tested grew with trehalose as the sole C source indicating that these strains may be deficient in trehalose-uptake systems and that accumulation is by de novo synthesis. A membrane-transport protein for trehalose was identified in yeast (*Saccharomyces cerevisiae*) and *Thermococcus litoralis* (Eleutherio et al., 1993; Han et al., 1995; Diez et al., 2001). Crowe et al. (2001) suggested that incorporation of the genes regulating its expression into cells without a transport protein might be a useful way of introducing trehalose into cells. An increase in the trehalose content of rhizobial cells may result in improved desiccation tolerance.

4.2. The role of exopolysaccharides (EPS)

Many bacteria produce and often live within a matrix of exopolysaccharide (EPS) in their natural environment, for example in soil (Foster, 1981) and lungs (Roberson and Firestone, 1992). EPS functions to anchor cells to substrata, protect against phagocytosis, mask antibody recognition and prevent lysis by other bacteria (Tease and Walker, 1987). Their protective role against desiccation stress is unclear and there are several conflicting reports. Large amounts of water-holding EPS were produced by *Pseudomonas* spp. in response to desiccation (Roberson and Firestone, 1992). The EPS contained several times its weight in water. However, Hartel and Alexander (1986) found that non-mucoid strains of *Bradyrhizobium* were more resistant to drying in a sandy soil than mucoid strains. Elsewhere, EPS collected from *Azotobacter* by centrifugation did not improve survival of *R. leguminosarum* bv. *trifolii* on seed when compared to YMA grown rhizobia (R.A. Date, pers. comm.).

S. meliloti produces two types of acidic EPS, succinoglycan (EPS I) and galactoglucan (EPS II). Recent studies have shown the low molecular weight form of EPS I is implicated in nodule formation or symbiotically active (Battisti et al., 1992; Reuber and Walker, 1993), whereas EPS II is only symbiotically active in the absence of EPS I (Glazebrook and Walker, 1989; Leigh and Walker, 1994). More EPS II is produced in low phosphate environments and this may be an adaptation to the soil environment which is relatively low in phosphate compared with nodules (Mendrygal and Gonzalez, 2000). This environmentally mediated production of EPS II suggests it may have a role as a stress response mechanism.

A better understanding of the physiological tolerance mechanisms of rhizobia in response to the various environmental stresses that compromise survival will assist in the selection of robust strains more compatible with seed coating applications. Conditioning cells to induce the production of osmoprotectants such as trehalose or protective EPS may be a useful way to increase the tolerance of cells to desiccating conditions experienced on seed surfaces.

5. Additives to improve rhizobial survival on seed

5.1. Sugars, amino acids and sugar alcohols

In early studies on the freeze-drying of bacteria, the nature of the suspending media was identified as an important aid to survival (Heller, 1941; Annear, 1956, 1962; Vincent, 1958). Extensive research has been carried out on the use of bacterial nutrients as suspending agents for freeze-drying and storage of cells (Heller, 1941; Appleman and Sears, 1944; Annear, 1956, 1962; Redway and Lapage, 1974; Dye, 1982). Heller (1941) investigated the protective effects of crystalline compounds and colloids during desiccation of *Streptococcus pyogenes* C203 and *Escherichia coli* (*communior*). Sucrose proved to be a superior suspending agent to glucose, xylose, tryptophane, salicin, saline and water for both species. Heller concluded that survival was related to the assimilability and solubility of the compound. Furthermore, Vincent (1958) reported 24–44% of cells suspended in a 10% sucrose solution survived primary drying whereas only 0.1% survived when suspended in water. Sucrose was a superior suspending agent to sorbitol, mannitol, lysine, amino acid mixtures, milk and yeast mannitol broth.

The poor survival of rhizobia on seeds and beads was improved by the addition of sucrose. McLeod found that the incorporation of 10% sucrose into yeast mannitol broth improved the survival on glass beads compared with unamended broth (cited in Vincent (1958)). Elsewhere, Vincent et al. (1962) reported survival of broth cultures of *R. leguminosarum* bv. *trifolii* on glass beads was much higher after suspension in a solution of maltose than when suspended in other sugars, sorbitol and sodium chloride. The improved survival with maltose could not be attributed to concentration or properties previously suggested to have an effect, in particular, assimilability, presence or absence of a carbonyl group, osmotic pressure exerted, molecular size and solubility (Heller, 1941; Scott, 1960). Nor was the better performance of maltose attributable to a decrease in the rate of dehydration of the cells as improved survival over 48 h was not paralleled by a decrease in the rate of water loss (Vincent et al., 1962). They suggested the difference might rest in the particular molecular configuration of maltose affecting its interaction with biological surfaces. This explanation resembles Crowe and Crowe's water replacement hypothesis (discussed in Section 4.1) that places the beneficial effect of polyhydroxyl sugars at the surfaces of

macromolecules. Addition of maltose to the suspending medium ameliorated poor survival at lower inoculation rates. It improved survival over a range of relative humidities and allowed cells to grow at 100% RH. However, addition of maltose could not negate the effects of inhibitory substances from the seed coat.

5.2. Colloids

In Heller's (1941) investigation of the protective effect of colloids, *S. pyogenes* C203 survived best in peptone (1 and 10% w/v) followed by gastric mucin, gum tragacanth, starch and aluminium hydroxide. Survival was positively correlated with solubility, hydrophilicity and gold number (Table 2). The gold number relates to the protective effect one colloid has on another (colloidal gold in this case) against the precipitating action of salt. Improved survival in this context is analogous to the protective colloid effect where the bacteria represent one colloid and the suspension the other. This protective effect is known as colloidal stabilisation.

5.3. Adhesives

The adhesives used in current agricultural practice for the inoculation of legumes are essentially polymeric in nature (i.e. high molecular weight compounds). Examples include gum arabic, methylcellulose (MC), polyvinylpyrollidone (PVP), caseinate salts and polyvinylacetate (PVA). Much of the research on adhesives has focussed on their ability to maintain the viability of rhizobia on the legume seed (Scott, 1989). However, very little progress has been made in identifying the exact mechanism by which survival is improved by these polymers.

Gum arabic is a complex carbohydrate extracted from *Acacia*. It enhances rhizobial survival and is widely used as an adhesive in inoculation of legume seeds. Vincent et al. (1962) found that gum arabic not only protected cells against desiccation on beads but resulted in better survival on seeds than maltose suggesting some protection against toxic seedcoat factors. However, the difference in adhesion to seed of these two additives was not considered. Variable quality, availability, cost and the need for high concentrations (15–40% w/v) has limited the use of gum arabic as an inoculant adhesive. Methyl cellulose, a non-ionic water-soluble cellulose ether, is a more widely used adhesive. It is readily available, its quality is relatively consistent as it is a semi-synthetic polymer and it is relatively low-cost due to the application of low concentrations (1.5% w/v) (Scott, 1989). However, there are variable reports on the protection of rhizobia by methyl cellulose when compared with gum arabic and it is generally considered to be less effective. Date (1968, 1970) found that the commercial methyl cellulose products Methofas®, Cellofas® and Methocel® did not provide the same degree of protection as gum arabic on lime pelleted seed, Elegba and Rennie (1984) found no difference between survival, nodule mass and plant yield when rhizobia was applied to soybeans with methyl cellulose and gum arabic. However, the results are difficult to interpret due to the disparate nature and different concentrations of the two polymers. This is further confounded by comparisons between different *Rhizobium* and legume species.

Water-soluble polyvinylpyrrolidone (PVP) was patented as a seed-coating agent by Lloyd (1983). PVP is a synthetic vinyl polymer produced by free radical vinyl polymerisation of the monomer vinylpyrrolidone. Hale and Mathers (1977) demonstrated the commercial product Polyclar AT (a water insoluble, high molecular weight form of PVP defined as polyvinyl polypyrrolidone, PVPP) adsorbed toxic seed exudates from clover seeds. They concluded PVPP used at the rate of 10% (w/w) of the seed, would not be cost effective and the advantage, based on survival data, insignificant. Improved survival could be achieved if the soluble form of PVP was included in the inoculant slurry (Lloyd, 1983). Interestingly, their earlier United Kingdom patent specification (No. 1465979) had described a method whereby seeds were coated with PVP followed by the inoculant slurry. The PVP coat could absorb the moisture from the slurry producing free-flowing seeds without a drying step. However, the inventors speculated that toxic seed exudates were released from the PVP layer upon re-wetting with the slurry and this proved detrimental to the survival of rhizobia. The success of PVP has been

Table 2
Summary of the average percentage reductions in viable cells per day of *Streptococcus pyogenes* C203 in various colloids in the desiccated and fluid states

Suspending medium	Physical property	Gold number	Average percent reduction per day	
			Desiccated	Fluid control
$Al(OH)_3$	Insoluble, hydrophobic	–	25.7	83.5
Starch	Very slightly soluble	5.0–25.0	20.3	66.9
Gum tragacanth	Slightly soluble, more hydrophilic than starch	2.0	13.2	54.9
Gastric mucin	Slightly soluble, more hydrophilic than gum tragacanth	0.05	10.0	31.3
Peptone (1%)	Very soluble, very hydrophilic	0.001	0.9	17.4
Peptone (10%)	Very soluble, very hydrophilic	0.001	0.9	3.4

Reproduced from Heller (1941). Gold number is calculated as the number of mg of colloid necessary to protect a fixed quantity of colloidal gold (0.6 mg in 10 ml) against the precipitating action of NaCl (1 ml of 10% solution).

recognised and included in co-polymer formulations found in the patent literature. Vinyl pyrrolidone-vinyl acetate and vinyl pyrrolidone-unsubstituted styrene co-polymers were patented by Williams (1992) and Williams and Day (1994) (Agricultural Genetics Co. Ltd, UK). The poly(vinylpyrrolidone-vinyl acetate) co-polymer was used by mixing a 10% solution with peat cultures of *B. japonicum* and *R. leguminosarum* bv. *trifolii* and applying these to soybean seeds. The inventors claim that the seeds were free-flowing, maintained high numbers of viable rhizobial cells for greater than 3 months and had no adverse effect on germination. The poly(vinylpyrrolidone-styrene) co-polymer was injected directly into peat followed by injection of rhizobial broth. The peat cultures were cured for 7 d and the product showed good adhesion to soybean seed. Bushby and Marshall (1977b) found the addition of PVP to soil provided better protection of both fast- and slow-growing strains of rhizobia whereas polyethylene glycol 6000 and montmorillonite clay only protected fast-growing strains. These results indicate that PVP provides some protection from desiccation as well as from inhibitory seedcoat substances.

Prior to PVP, Lloyd (1979) patented the use of a caseinate salt as a seed coating. Although the precise action of this adhesive was unknown, he speculated that improved survival of rhizobia could be attributed to a reduction in the toxic effect of the seed coat. Both sodium and potassium caseinate salts were effective. However the potassium salt was preferred as it was thought to assist in the growth of the rhizobia. It is produced by neutralising casein with alkali. The water-soluble product had good adhesive properties, could be used at low concentrations and formed a flexible film.

Evidently, the various additives applied during seed inoculation have provided some protection to rhizobia. However, due to the complexity of the coated seed interpretation of results is often speculative in relation to the precise mechanisms involved. There is a need for a more systematic approach to the use of additives that benefit rhizobial survival on seed.

6. Properties of polymers required to improve survival of rhizobia on seed

Certain properties of polymers can be identified as having a beneficial effect on survival. Polymers should logically be non-toxic and free from preservatives that may be harmful to bacteria. A complex chemical nature would be advantageous so faster-growing antagonists in the soil could not rapidly utilise the polymeric coating and out-compete the rhizobia. The polymer should also be dispersable in water to allow release of rhizobia from the polymer matrix upon wetting and their subsequent multiplication in the rhizosphere.

Not only should the polymer be water dispersable but the nature of water within the polymer matrix is critical to survival. Availability of water to act as a solvent is of great importance in biological systems (Mugnier and Jung, 1985). The solvent properties of water in biopolymers have been described in terms of moisture sorption isotherm (MSI) theory which depicts the relationship between equilibrium moisture content and water activity at constant temperature. Generally, at low water activities, water in the matrix is tightly bound to specific sorption sites. At high water activities it is free to act as a solvent and participate in chemical reactions. The moisture sorption isotherm varies depending on the nature of the solid material. Bushby and Marshall (1977a) measured moisture sorption isotherms of fast- and slow-growing strains of rhizobia and Ca-montmorillonite. They found that the poorer-surviving fast-growing strains had a higher affinity for water than the slow-growing strains. Cells of fast-growing strains contained approximately 2% water at 10% relative humidity and reached 10–11% water at 65% relative humidity. Above 65% there is a rapid adsorption of water to moisture contents between 24 and 34% at 85% relative humidity. The water content of the slow-growing strains increased from approximately 1–4% over the range of 10–65% relative humidity. At relative humidities above 65% the moisture sorption was much more rapid reaching between 12 and 24% moisture content at 85% relative humidity. Interestingly, three of the four slow-growing strains tested had a final moisture content similar to three of the four fast-growing strains indicating that adsorption at the higher relative humidities (>65%) was faster for these slow-growing strains than for the fast-growing strains. The affinity of Ca-montmorillonite for water was higher than that of the bacteria. Bushby and Marshall (1977a) suggested that it may therefore afford protection of fast-growing species by reducing the internal water content.

The importance of colloidal stabilisation on the survival of dried bacteria has not been investigated since Heller (1941). He demonstrated that the protective colloid properties of the suspending agent were positively correlated to survival. Steric stabilisation is thought to be widespread in biological systems due to the consistent occurrence of cells bathed in solutions of high ionic strength and the abundance of water-soluble polymers (Napper, 1983). Colloids may be stabilised by several mechanisms: electrostatic, steric, depletion or electrosteric (for a detailed account of colloidal stabilisation, see Napper (1983)). Steric stabilisation occurs when amphipathic polymers, having hydrophobic and hydrophilic parts, are attached to the surfaces of the colloidal particles. If the particles to be stabilised are hydrophobic in aqueous solution, then the hydrophobic part of the polymer binds to the particles and the hydrophilic ends repel one another thus maintaining the particles in suspension. One of the most important stabilisers in aqueous dispersions is polyvinyl alcohol (PVA). This is prepared by the hydrolysis of polyvinyl acetate (PVAc). Partial hydrolysis essentially results in an amphipathic PVA, PVAc co-polymer. As concentrations of salt increase in the cell

environment under desiccation stress, stabilising polymers may be useful in reducing the extent of protein precipitation or the coagulation of cells. Maintenance of macromolecular structure may improve biological integrity thus leading to improved survival.

Cells in the desiccated state are susceptible to oxidative damage (Potts, 1984), hence the O_2 permeability of polymers may be critical to cell survival. The permeability of polymers to O_2 is affected by the addition of plasticisers (Pauly, 1989). Amiet-Charpenter et al. (1998) speculated that the addition of a plasticiser to microparticles of ethyl cellulose might have adversely affected survival of micro-encapsulated bacteria by increasing O_2 permeability. Lien found a relationship between increased O_2 permeability of PVA films and increased water sorption (L. Lien, unpub. Hons. thesis, University of Sydney, 2000). Permeability of a polymer also varies according to physical factors such as density, crystallinity and orientation (Gontard et al., 1996).

Interaction with some seed exposes rhizobia to inhibitory substances from the seed coat. These substances have been identified as polyphenols and their inhibitory effect can be inactivated by the addition of materials capable of phenolic adsorption. All phenols take part in hydrogen bonding and the hydrogen bond formed between phenols and N-substituted amides are the strongest (Loomis and Battaile, 1966). Both soluble and insoluble forms of PVP can form insoluble complexes with phenols and the amounts bound were from 31 to 44% of the dry weight of polymer. Phenolic binding was also observed in proteins such as casein and synthetic polyamides. PVP is a strong proton acceptor and therefore has a high capacity for binding phenolics. Hale and Mathers (1977) demonstrated phenolic adsorption by several materials. Antibiotic activity of toxic seed diffusates was suppressed by insoluble PVP, activated charcoal and skim milk powder.

It is clear that several polymer properties affect survival of rhizobia on legume seed. Polymer development in this field requires a systematic approach, perhaps through mathematical modelling in order to achieve optimum quality for seed coating and survival of rhizobial inoculants.

7. Conclusions

The currently known methods of inoculation limit the benefits of high quality legume inoculants produced in Australia. Research undertaken to date has identified factors that affect survival of rhizobia on legume seed and observed improvements in survival when various additives were used. However, the interpretation of data from this research has been difficult and often speculative due to the complex nature of the coated seed environment and the disparate nature of the additives being compared. There is an increasing demand for pre-inoculated seed and a clear indication that an increase in the number of viable rhizobia delivered to the rhizosphere on seed would improve yield, therefore there is a need to further clarify the factors affecting rhizobial survival on legume seed and to understand the physiological mechanisms of desiccation tolerance. Such an understanding may identify new selection criteria to screen *Rhizobium* strains for tolerance to stress factors encountered on drying seedcoats. Clear benefits to survival have also been demonstrated after selection of non-toxic seed cultivars. In terms of additives, focus on specific properties of polymeric adhesives that improve survival would be advantageous. Polymers need to be selected carefully so that individual properties can be isolated and specific affects attributed to them. Desirable properties may then be optimised contributing to an overall positive effect on survival.

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