

Survival of rhizobia on seed of *Desmanthus virgatus* stored at different temperatures

AGUSTIN C. BECERRA STIEFEL¹,
R.A. DATE² AND N.J. BRANDON²

¹Department of Plant Production, University of Queensland, St Lucia, Queensland, Australia
Present address: W. Paunera 153, 5000 Cordoba, Argentina

²CSIRO Tropical Agriculture, St Lucia, Queensland, Australia

Abstract

The survival of *Rhizobium* strain CB3126 on inoculated seed of *Desmanthus virgatus* cv. Bayamo was determined after 3, 7, 14, 28 and 39 days storage at 25, 35, 45 and 55°C. Survival was assessed by observing the presence or absence of nodules on aseptically growing desmanthus inoculated with material washed from the stored seed. Plant colour, height and dry weight were compared with those of plants grown from seed freshly inoculated with CB3126 and plants grown with fertiliser N.

Temperatures above 45°C significantly reduced bacterial survival after only 3–7 d of storage. At 35°C, nodulation was reduced significantly when the inoculated seed was stored for 14 d or longer. There was no effect of storage time on final nodulation when seed was stored at 25°C. However, early chlorosis of plants indicated that nodulation was delayed by the longer storage times, even at 25 and 35°C. The dry weight of desmanthus inoculated with freshly prepared peat was significantly higher than that following storage in all but the 25°C-3 d storage treatment.

The implications of these observations for field sowings of inoculated seed of *Desmanthus virgatus* are discussed in relation to seedling nodulation and establishment.

Correspondence: Dr R.A. Date, CSIRO Tropical Agriculture, Cunningham Laboratory, 306 Carmody Rd, St Lucia, Qld 4067, Australia. e-mail: dick.date@tag.csiro.au

Introduction

The association of legumes with their appropriate root-nodule bacteria (*Rhizobium* or *Bradyrhizobium*) determines their ability to fix atmospheric N and is therefore important in determining nutritive value, production and sustainability of legumes in pastures (Date 1970; Norman 1982). When native rhizobial strains are either absent or ineffective, it is necessary to introduce effective strains to ensure adequate nitrogen fixation. The most common method of introducing rhizobia into soil is by inoculation of the legume seed prior to sowing (Brockwell 1962; Danso *et al.* 1990). The introduced rhizobia must compete with indigenous strains and, in some instances, survive under harsh chemical, physical and biological soil conditions (Dart 1974). In some agricultural systems, inoculated seed of legumes is sown into moist soil favourable for rhizobial survival. In others, it is necessary to sow the seed into dry soils where inoculum must survive until sufficient rainfall occurs to allow germination. These conditions can diminish significantly the survival of rhizobia on seed, leading to poor or delayed seedling nodulation and, therefore, poor seedling growth in the field (Brockwell 1962; Brockwell and Phillips 1970).

The shrub legume *Desmanthus virgatus* (desmanthus) has been released commercially as a forage plant in the tropics and subtropics, including clay soil areas of semi-arid Queensland (Cook *et al.* 1993). Recent glasshouse and field experiments have demonstrated the need to inoculate seed of *Desmanthus* to obtain effective nodulation when sown into some Australian soils (Date 1991; Bahnisch *et al.* 1998; Brandon *et al.* 1998). However, the seed is small and, therefore, needs to be sown shallowly (Brandon and Jones 1998). Therefore, seed and inoculum are exposed to high temperature and moisture stress after sowing and there is a high risk of death of the

inoculum and nodulation failure. Consequently, we studied the survival of the commercially-recommended strain of *Rhizobium* CB3126 (Date 1991) on seed of *Desmanthus virgatus* stored at different temperatures for different periods of time.

Materials and methods

Seed and inoculum

Seed of *Desmanthus virgatus* cv. Bayamo was inoculated using peat culture of *Rhizobium* strain CB3126 and a methyl cellulose-based adhesive (80 g seed + 8 g peat + 2.5 mL 1.5% w/v adhesive) following the technique of Roughley *et al.* (1966). The inoculant provided c. 2000 rhizobia per seed. The minimum standard required by the AIRCS (Australian Inoculant Research and Control Service) for commercial inoculants is 1000 per seed at the time of sowing.

Seed storage treatments

Immediately after inoculation, the seed was stored at 25, 35, 45 and 55°C for periods of 3, 7, 14, 28 and 39 days. Twenty gram samples of the freshly inoculated seed were placed in small containers inside a larger plastic vessel containing silica gel to maintain constant dry conditions throughout the experiment.

Tests for survival of inoculum on stored seed

Triplicate samples of 10 seeds from each temperature treatment were obtained after each period of storage. Each sample of seed was soaked for 30 minutes in 10 mL sterile water and then shaken, with glass beads, for 30 seconds on a vibrator. The presence or absence of viable rhizobia was detected in a grow-out test in which the 10 mL suspension from the seed washing was used to inoculate 4 seedlings of *D. virgatus* growing aseptically in N-free sand-culture (Norris and Date 1976) maintained in a glasshouse at the CSIRO Cunningham Laboratory (27°37'S, 153°19'E) from April–July 1994. There were 3 additional control treatments: uninoculated seed; seed inoculated with freshly prepared peat; and nitrogen-fertilised plants that received 10 mL of KNO₃ solution (8.79 g/L) at 26 days and a further 20 mL at 38 days.

Plant height was recorded at 0, 4, 10, 19, 30 and 41 d following inoculation and leaf colour was recorded daily. A scale of 1 to 12 (yellow to dark green, respectively) was derived from the standard colour chart No. 144 (Royal Horticultural Society of London Colour Chart). The delay (number of days) between inoculation and onset of fixation (green colouration in plant) *vis-a-vis* the three controls, was used as a rating to indicate relative number of rhizobia in the samples.

At 41 d after inoculation, plants were removed from sand-jars and the roots were washed. Presence or absence of nodules was recorded for each plant in each sand-jar and used as an index of rhizobial survival. Whole plants were then dried in a forced-air oven for 48 h at 70°C and weighed to determine dry matter accumulation for each sand-jar (4 plants/sand-jar).

Statistical analysis

Since storage times finished at different times, conditions in the glasshouse were not identical throughout the test. Data are therefore presented as relative yield, standardised on the 'plus N' control. Relative dry weight (%) was analysed using analysis of variance for a randomised complete block design. Plants with nodules were assigned a value of 1 and plants without nodules a value of 0. Proportions of plants that had nodulated were then subjected to analysis of variance using plants as sub-samples, without the need for a transformation.

Results

Plant inoculation responses

Increased storage time and temperature decreased the proportion of plants effectively nodulated by the inoculum (Table 1). There was a significant temperature × storage time interaction ($P < 0.05$) due to the greater effects of storage time in reducing nodulation at the higher temperatures.

The percentage of plants nodulated declined significantly after 3, 7 and 14 days at 55, 45 and 35°C, respectively (Table 1). No plants nodulated following storage of inoculated seed at 55°C for 7 d or 45°C for 14 d. Less than 50% of plants nodulated following storage for more than 14 d at 35°C. Storage at 25°C did not reduce nodulation (Table 1).

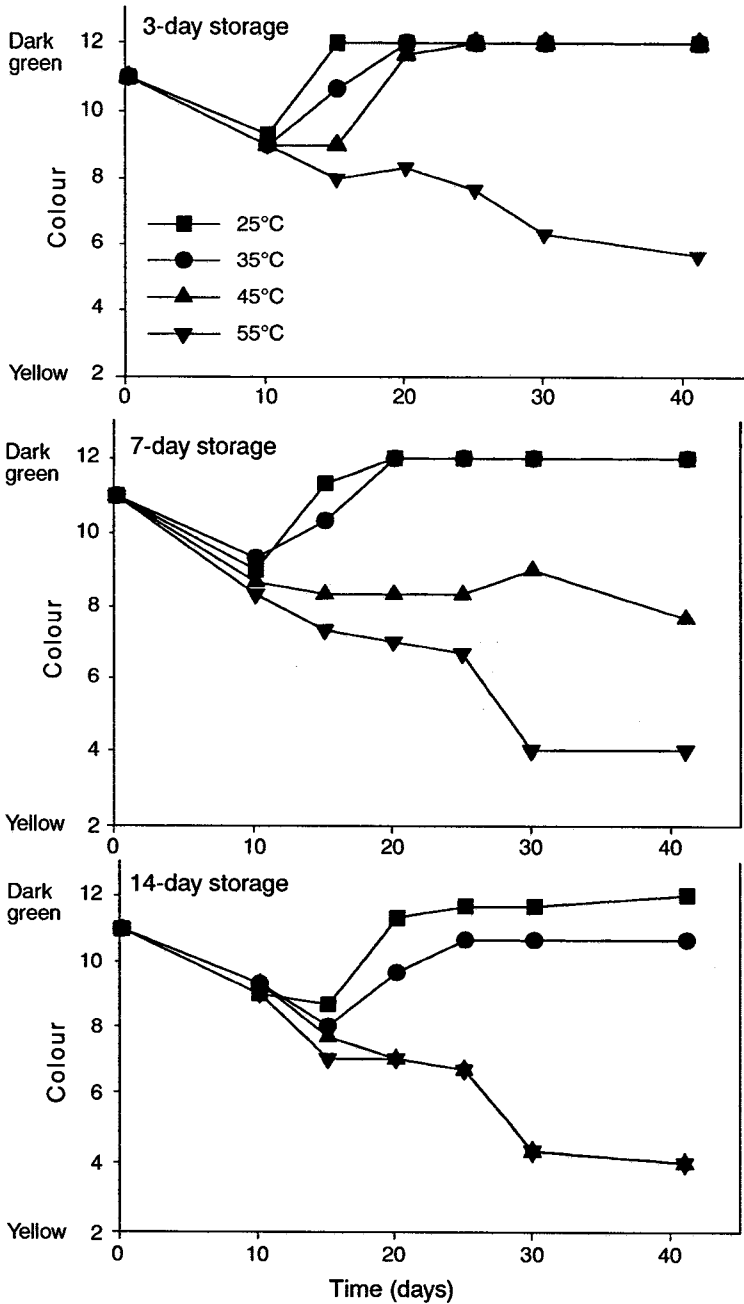


Figure 1. Colour changes of *Desmanthus virgatus* seedlings after inoculation with inoculum solution from seed stored at 25, 35, 45 and 55°C.

Table 1. Percentage of plants nodulated from rhizobia surviving on seed stored for periods up to 39 days at 4 temperatures.

Time (days)	Temperatures (°C)			
	25	35	45	55
3	100	100	100	8
7	100	100	25	0
14	100	67	0	0
28	83	42	0	0
39	100	17	0	0

LSD ($P < 0.05$): Time \times temperature = 29%.

Plant colour changes

Rhizobial numbers declined with both increasing temperature and time of storage. There was no yellowing in the +N and fresh peat treatments (± 11 on the colour scale). Plants inoculated with the washings from seed stored at temperatures above 25°C for 28 and 39 days remained yellow. In all cases, *desmanthus* seedlings in the grow-out tests showed a colour change from green to pale green during the first 10 days after inoculation (Figure 1). Later, colour either reverted to green–dark green or progressed to yellow. These colour changes reflected the time to onset of effective N fixation. Thus, for example, grow-out

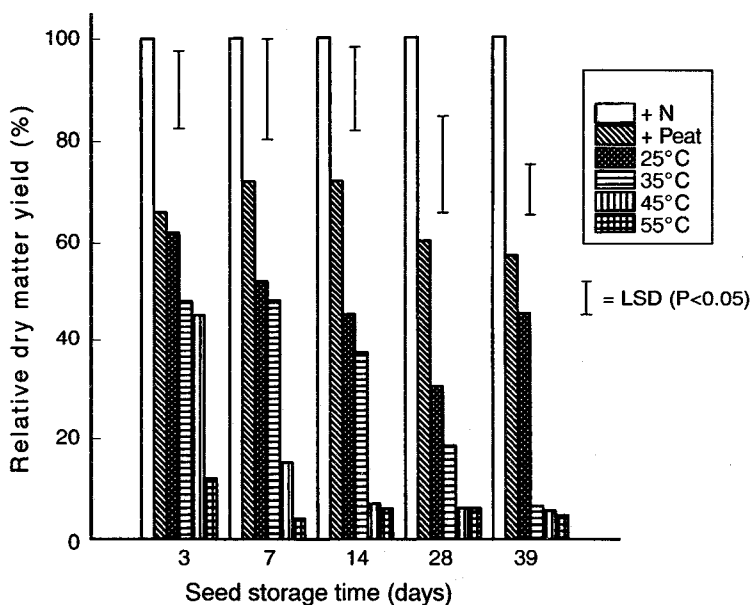
test plants became green sooner, when inoculated with material washed from seed stored for 3 days at 25°C, than when inoculated with material washed from seed stored at 35 or 45°C. Plants inoculated with the material washed from seed stored at 55°C continued to change to yellow. The delays in onset of greening of plants inoculated with the washings from the seed stored at 25, 35 and 45°C reflected fewer surviving rhizobia as temperature increased. Similar interpretations can be made for the 7- and 14-day storage periods (Figure 1).

Plant height changes

The height of all nodulated grow-out test plants was unaffected by treatment and ranged between 12–16 cm (data not shown). Plants not forming nodules and uninoculated controls achieved a maximum height of 5 cm.

Plant dry matter accumulation

At each sampling time, +N treatments produced a higher dry matter yield 41 days after seedling inoculation than the other treatments at all sampling times (Figure 2). For each storage time,

**Figure 2.** Relative dry matter accumulation of *Desmanthus* seedlings 41 days after inoculation with inoculum solution from seed stored at different temperatures and times.

yield declined as the temperature of seed storage increased. Even short periods of storage at the lower temperatures were sufficient to reduce significantly dry matter yield relative to plants inoculated with freshly prepared inoculum. The only exception was when seed was stored for 3 days at 25°C.

Discussion and conclusion

The most important fact confirmed by this investigation was the high vulnerability of rhizobia on stored, inoculated seed under hot, dry conditions. Even short periods of storage (7 days) at low temperature significantly reduced final dry weight of plants relative to fresh, inoculated seed. Unfortunately, because *desmanthus* is summer-growing and small-seeded, it must be sown shallowly, usually into dry soil, where soil temperatures above 50°C have been recorded (Marshall 1963; Brockwell and Phillips 1970; Hacker 1989) for 6–8 h/day (R.A. Date, unpublished data). Although tropical and subtropical Queensland receives predominantly summer rainfall, high rainfall variability means that seed will usually have at least a week and usually a longer period, when daily soil temperature maxima are >50°C. Temperatures above 40°C can reduce significantly rhizobial survival on inoculated seed (Brockwell and Phillips 1965; Day *et al.* 1978). The results of our experiment were consistent with these previous studies and confirmed the poor survival of inoculum at 45 and 55°C.

Death of inoculum can occur also at temperatures <40°C when seed is stored for longer periods. For example, in the current experiment, a storage temperature of 35°C for more than 7 days gradually reduced rhizobial survival to a point where only 17% of plants were nodulated effectively when stored for 39 days. Inoculum survived longer at lower temperatures, with all plants nodulating following storage at 25°C. This is consistent with findings of Herridge and Roughley (1974), who found that temperatures up to 25°C had little or no effect on rhizobial survival on seed. However, long periods of storage (14–39 d), even at low temperature, delayed nodulation as measured by change in plant colour. This is attributed to a reduction in bacterial numbers on the stored seed.

Our results showed that effective nodulation, plant colour, plant height and plant dry matter

were affected similarly by the temperature treatments. Nodulation failure severely limited *desmanthus* growth. Although expected in sand culture, similar results were found in soil by Bahnisch *et al.* (1998), who reported that growth of *desmanthus* was reduced significantly in clay soils devoid of effective native rhizobia when compared with soils with effective native strains or inoculated with CB3126. Although these soils were reasonably fertile, nodulation failure reduced N concentration in leaves. Similar results were found in 2 of 4 soils in the field by Brandon and Date (1998).

Kremer and Peterson (1983) reported a clear correlation between plant dry weight of cowpea and peanut and numbers of rhizobia per seed at planting. In our experiment, the lower dry matter production of *desmanthus* inoculated with stored inoculum compared with fresh inoculum is attributed to the decreasing number of viable rhizobia remaining on the stored seed. We believe that rhizobial numbers in the inoculum solution from stored seed were insufficient to produce a rapid colonisation of the rhizosphere. At the lower temperature of 25°C, the surviving bacteria were sufficient to ensure nodulation of most plants. However, higher temperatures and longer storage times resulted in nodulation failure.

The grow-out test should be used only as an indicator of presence or absence of surviving rhizobia. Under glasshouse conditions, it is highly likely that as few as 1–3 rhizobia/seed would be sufficient to nodulate plants. However, such a low inoculum potential would be unlikely to do so under field conditions, where experience suggests that a minimum of at least 1000 rhizobia/seed is required. Therefore, it would be inadvisable to sow seed stored at 25°C for 39 d and expect prompt nodulation.

Our results show that there is likelihood of poor survival of *Rhizobium* when dry-sowing *Desmanthus*. Kremer *et al.* (1982) and Kremer and Peterson (1983) suggested that some oil-based carriers may provide protection for rhizobia against extremely hot and dry environments. Similarly, Brockwell and Phillips (1965; 1970) reported that rhizobial survival on seed sown into hot, dry soils was consistently better in those cases in which seed was inoculated and pelleted with CaCO₃. The development of new methods of sowing, new inoculation techniques using bacterial protective agents, and the selection of rhizobial strains more tolerant of high

temperatures are major challenges for researchers dealing with the introduction of legume species to tropical and subtropical environments.

Acknowledgement

We acknowledge the help of Dr H.M. Shelton in the design of the experiment.

References

- BAHNISCH, G.A., DATE, R.A., BRANDON, N.J. and PITTAWAY, P. (1998) Growth responses of *Desmanthus virgatus* to inoculation with *Rhizobium* strain CB3126. I. A pot trial using 8 clay soils from central and southern Queensland. *Tropical Grasslands*, **32**, 13–19.
- BRANDON, N.J., DATE, R.A., CLEM, R.L., ROBERTSON, B.A. and GRAHAM, T.W.G. (1998) Growth responses of *Desmanthus virgatus* to inoculation with *Rhizobium* strain CB3126. II. A field trial at 4 sites in south-east Queensland. *Tropical Grasslands*, **32**, 20–27.
- BRANDON, N.J. and JONES, R.M. (1998) The effect of sowing depth and duration of watering on emergence of tropical legumes in clay soil under controlled temperature. *Tropical Grasslands*, **32**, (in press).
- BROCKWELL, J. (1962) Studies on seed pelleting as an aid to legume seed inoculation. I. Coating materials, adhesives, and methods of inoculation. *Australian Journal of Agricultural Research*, **13**, 638–649.
- BROCKWELL, J. and PHILLIPS, L.J. (1965) Survival at high temperatures of *Rhizobium meliloti* in peat inoculant on legume seed. *Australian Journal of Science*, **27**, 332–333.
- BROCKWELL, J. and PHILLIPS, L.J. (1970) Studies on seed pelleting as an aid to legume seed inoculation. 3. Survival of *Rhizobium* applied to seed sown into dry, hot soil. *Australian Journal of Experimental Agriculture and Animal Husbandry*, **10**, 739–744.
- COOK, B.G., GRAHAM, T.W.G., CLEM, R.L., HALL, T.J. and QUIRK, M.F. (1993) Evaluation and development of *Desmanthus virgatus* on medium to heavy textured soils in Queensland, Australia. *Proceedings of the XVII International Grassland Congress, Palmerston North and Rockhampton, 1993*. pp. 2148–2149.
- DANSO, S.K.A., KAPUYA, J. and HARDERSON, G. (1990) Nitrogen fixation and growth of soybean as influenced by varying methods of inoculation with *Bradyrhizobium japonicum*. *Plant and Soil*, **125**, 81–86.
- DART, P.J. (1974) Development of root-nodule symbiosis. In: Quispel, A. (ed.) *The Biology of Nitrogen Fixation*. pp. 381–429. (North-Holland Publishing Company: Amsterdam).
- DATE, R.A. (1970) Microbiological problems in the inoculation and nodulation of legumes. *Plant and Soil*, **32**, 703–725.
- DATE, R.A. (1991) Nitrogen fixation in *Desmanthus*: strain specificity of *Rhizobium* and responses to inoculation in acidic and alkaline soil. *Tropical Grasslands*, **25**, 47–55.
- DAY, J.M., ROUGHLEY, R.J., EAGLESHAM, A.R.J., DYE, M. and WHITE, S.P. (1978) Effect of high soil temperature on nodulation of cowpea, *Vigna unguiculata*. *Proceedings of the Association of Applied Biologists*, **88**, 476–480.
- HACKER, J.B. (1989) The potential for buffel grass renewal from seed in 16-year-old buffel grass-Siratro pastures in south-east Queensland. *Journal of Applied Ecology*, **26**, 213–222.
- HERRIDGE, D.F. and ROUGHLEY, R.J. (1974) Survival of some slow growing *Rhizobium* on inoculated legume seed. *Plant and Soil*, **40**, 441–444.
- KREMER, R.J., POLO, J. and PETERSON, H.L. (1982) Effect of suspending agent and temperature on survival of *Rhizobium* in fertilisers. *Soil Science*, **46**, 539–541.
- KREMER, R.J. and PETERSON, H.L. (1983) Effect of inoculant carrier on survival of *Rhizobium* on inoculated seed. *Soil Science*, **134**, 117–125.
- MARSHALL, K.C. (1963) Survival of root nodule bacteria in dry soils exposed to high temperatures. *Australian Journal of Agricultural Research*, **15**, 273–281.
- NORMAN, M.J.T. (1982) A role for legumes in tropical agriculture. In: Graham, P.H. and Harris, S.C. (eds) *Biological Nitrogen Fixation Technology for Tropical Agriculture*. pp. 9–26. (Centro Internacional de Agricultura Tropical: Cali, Colombia).
- NORRIS, D.O. and DATE, R.A. (1976) Legume bacteriology. In: Shaw, N.H. and Bryan, W.W. (eds) *Tropical Pasture Research; Principles and Methods*. pp. 171–174. *CAB Bulletin No. 51*. (Commonwealth Bureau of Pastures and Field Crops: Hurley, England).
- ROUGHLEY, R.J., DATE, R.A. and WALKER, M.H. (1966) Inoculating and lime pelleting seed. *Agricultural Gazette New South Wales*, **77**, 142–146.

(Received for publication January 17, 1997; accepted August 5, 1997)