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Seed inoculation with effective root-nodule bacteria enhances revegetation success

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Summary

1. Extensive clearing of native vegetation in Australia has contributed to major environmental problems, including land degradation, dryland salinity, soil erosion and loss of biodiversity. Re-establishing cover with deep-rooted perennial species is a major focus for conservation and sustainable land management, particularly with regard to hydrological control of recharge and saline discharge areas. However, considerable expense is involved in large-scale revegetation programmes and cost effectiveness is a real concern.

2. Low-cost revegetation approaches are needed that require little maintenance yet can substantially enhance reliable establishment and growth of native trees and shrubs. We evaluated results from direct-seeding field trials that examined the benefits of using native Australian *Acacia* species inoculated with effective strains of nitrogen-fixing root-nodule bacteria to revegetate degraded landscapes.

3. On average, inoculation led to a 118% increase in establishment of acacia seedlings, indicating that the use of elite strains of native bacteria can substantially reduce seed requirements. This is a major benefit given the expense of collecting sufficient native seed and the impacts of this activity on remnant population viability.

4. Particularly at sites experiencing harsher climatic conditions, subsequent survival of inoculated seedlings was significantly greater than for uninoculated controls. Moreover, inoculated acacias grew 10–58% faster than uninoculated controls during the critical early phase of establishment, although this varied among species and sites.

5. *Synthesis and applications*. Inoculation of *Acacia* species or other native leguminous shrubs and trees with elite strains of native rhizobia as part of direct-seeding techniques has the potential to increase the scope, rate and success of land restoration world-wide. Re-establishment of important plant–soil interactions in degraded soils can contribute significantly to the development of biodiverse self-regenerating native ecosystems in agricultural landscapes.

Key-words: *Acacia* spp., *Bradyrhizobium*, direct-seeding, inoculation, land restoration, legumes, nitrogen fixation, rhizobia, symbiosis

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Introduction

Symbiotic interactions between plants and mutualistic soil organisms are important components of all plant communities. There is considerable potential to use such organisms in re-establishing viable native plant communities in degraded landscapes (Smith, Charvat & Jacobson 1998; Requena *et al*. 2001; Caravaca *et al*. 2003) and in the conservation and reconstruction of endangered plant communities (Tlusty, Grossman & Graham 2004). For example, in Australia a large number of native shrubby legumes in the Fabales (e.g. *Acacia*, *Daviesia* and *Pultenaea*)

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nodulate and fix nitrogen, primarily with the rhizobial genus *Bradyrhizobium* (Lafay & Burdon 1998, 2001; Sprent 2001). These plant taxa, particularly *Acacia* spp., frequently dominate undisturbed ecosystems in abundance and overall biomass (Groves 1994).

Restoration of native vegetation and the regeneration of functioning ecosystems is now a major conservation focus in many parts of the world, where poor management practices have resulted in serious land-degradation problems (Perrow & Davy 2002). In Australia, the considerable investment in revegetation stems from the widely recognized relationship between the ongoing clearing and degradation of remnant vegetation and increases in dryland salinity, decreases in agricultural productivity and water quality and loss of biodiversity (National Land & Water Resources Audit 2001). It is generally acknowledged that in addition to its conservation value, the restoration of native vegetation can influence deep drainage, reduce soil erosion, counter and reverse increasing salinization of productive rural areas, and improve habitat for beneficial organisms. Thus, there is a need for low-cost, reliable approaches suited to catchment and subcatchment scale replanting with deep-rooted native perennial species (Farrington & Salama 1996; Knight, Beale & Dalton 1998).

In Australia, low fertility is characteristic of most soils, particularly with respect to nitrogen (N) and phosphorus (P) (Nix 1981). Plant–microbial associations that help circumvent low nutrient levels are therefore likely to be of significance in determining the species and structural diversity of plant communities, as well as in influencing their long-term viability (Selosse, Baudoin & Vandenkoornhuyse 2004). For example, in N-deficient environments the formation of effective N_2-fixing symbiotic associations between rhizobia and legumes assists seedling establishment, promotes rapid early seedling growth and increases plant survival. Effective nodulation thus represents an ecological advantage wherever competitive stress is a concern, especially in environments characterized by low fertility, weed competition and the threat of drought. Moreover, non-leguminous species also benefit from added N inputs to the soil (Khanna 1997), further enhancing our ability to establish biodiverse self-regenerating communities in agricultural landscapes (Requena *et al*. 2001).

As survival of rhizobia in soil is usually dependent on the presence of their hosts, native rhizobia are often undetectable in soils where native legumes have been cleared (Thrall *et al*. 2001). This means that in disturbed or degraded areas where revegetation is most crucial, sown legumes may require inoculation with effective strains of rhizobia to maximize establishment and growth. Although the importance of such interactions is well-recognized in agriculture and forestry, there has been little effort to use native plant–soil symbioses in large-scale direct-seeding approaches to the rehabilitation and restoration of natural systems.

Glasshouse inoculation trials with a range of *Acacia* species showed enormous variability among rhizobial isolates in their ability to form effective relationships with a given legume host and to influence plant growth (Burdon *et al*. 1999). When inoculated onto their host species of origin, the strains most effective in N_2-fixation were highly effective irrespective of host provenance. However, between *Acacia* species there was considerable specificity: strains that were effective on one *Acacia* species sometimes performed poorly on others (Thrall, Burdon & Woods 2000). These results highlight that host specificity among rhizobia is a common phenomenon and emphasize the practical need to identify effective strains for the suite of host species to be used for replanting.

The emphasis of our work is on the development of practical approaches to revegetation that require minimal input and maintenance yet can substantially enhance reliable establishment and subsequent growth of native trees and shrubs. This is particularly important given that seed for many native species is in short supply and is thus expensive to obtain. The current study focused on the ecological benefits of using elite strains of native rhizobia to inoculate the acacia component of diverse seed mixes used in restoration. In particular, we describe here results from large-scale field trials comparing the early establishment, growth and survival of inoculated and uninoculated acacias at a range of sites in south-eastern Australia.

Materials and methods

SITE CHARACTERISTICS

Eight sites were located in north-central Victoria and one in south-eastern New South Wales, Australia (Table 1). All sites had been largely cleared of native trees and shrubs in the late 1800s and early 1900s, and subjected to grazing at various intensities for many decades. Climates were similar at all sites, i.e. cool, moderately moist winters and hot, dry summers. The sites encompassed a range of soils that had never received fertilizer and were impoverished in nutrients. In particular, plant-available soil N was low, typically in the range 2–10 kg N ha^{-1} across each site (cf. Moore 1970; CSIRO Division of Soils 1983). At the time that the direct seeding trials were established in August–September 2002, south-eastern Australia was in severe drought (see 2002–03 rainfall compared with long-term averages in Table 1).

Soil samples were taken at all sites for measurements of pH and electrical conductivity (an index of soil salinity) and for enumeration of resident populations of rhizobia. Large rhizobial populations resident in soil represent the potential for intense competition that may exclude introduced inoculum from nodule formation on target hosts; smaller resident populations are less competitive (Brockwell, Holliday & Pilka 1988; Turk, Keyser & Singleton 1993). A minimum of 25 soil subsamples, each 10 cm in depth and 4 cm in diameter, were taken haphazardly across each site. Subsamples were combined into a single sample, air dried, thoroughly

Table 1. Geographic location and other details of the experimental revegetation sites. All sites are located in north-central Victoria, except for Hoskinstown which is in New South Wales. Annual rainfall data are in millimetres (Australian Bureau of Meteorology, www.bom.gov.au)

Site	Coordinates	Soil	Soil pH	EC* (dS m^{-1})	Weediness score†	Herbivory score‡	Average annual rainfall§	Total rainfall for 2002
Donald	36°22′S 142°59′E	Clay loam	6·51	4·1	1	1	388·6	227·2
St Arnaud	36°37′S 143°16′E	Clay loam	6·42	5·6	1	2	510·7	276·2
Newstead	37°07′S 144°03′E	Clay loam	5·72	9·9	3	2	613·4	461·6
Ravenswood	36°54′S 144°13′E	Clay loam	6·16	10·8	1	1	528·4	272·4
Shelbourne	36°51′S 144°01′E	Gravelly clay loam	4·74	17·5	3	3		
Timor West	36°56′S 143°45′E	Coarse sandy loam	4·58	1·6	0	1	532·4	302·2
Alma	37°02′S 143°41′E	Clay loam	6·68	21·5	1	1		
Bealiba	36°48′S 143°33′E	Coarse sandy loam	6·38	9·4	1	1		
Hoskinstown	35°25′S 149°27′E	Gravelly clay loam	4·58	3·5	2	2	737·4	465·0

*Soils sampled 0–10 cm for measurements of pH (determined in $CaCl_2$) and electrical conductivity (EC).
†Weediness scored as: 0, nil or inconsequential; 1, minor; 2, moderate; 3, severe.
‡Herbivory (as a result of feral, native and domestic animals) scored as: 1, minor; 2, moderate; 3, severe.
†§Geographically closest point for rainfall data for Alma and Bealiba is Timor West; closest for Shelbourne is Ravenswood.

mixed, passed through a sieve to remove stones and large pieces of undecomposed organic matter, and stored at room temperature. Soil pH_{Ca} and pH_{water} were measured in soil suspensions in 0·01 mol $CaCl_2$ L^{-1} (1 : 5) and in water that had been shaken for 30 min. Electrical conductivity (EC; dS m^{-1}) was measured on the same soil suspensions that had been used to measure pH_{water}.

INITIATION OF FIELD TRIALS

The sites were seeded with a range of native shrubs and trees, both legumes and non-legumes, including species of *Acacia*, *Allocasuarina*, *Calocephalus*, *Dillwynia*, *Dodonea*, *Eucalyptus*, *Hakea*, *Melaleuca* and *Solanum* (a roughly standard mix used in replanting projects in north-central Victoria, where the focus is on establishing biodiverse native communities). *Acacia mearnsii* De Wild. was sown at all sites, while *Acacia dealbata* Link. was sown at all sites but Donald and St Arnaud (Table 1). *Acacia acinacea* Lindl., *Acacia paradoxa* DC. and *Acacia pycnantha* Benth. were specific to the Victorian sites, while *Acacia rubida* A. Cunn. was only sown at the New South Wales site (Hoskinstown). Acacia seed provenances used at each site were local to that area, in accordance with standard practice.

Rhizobial strains from the CSIRO Plant Industry collection were chosen on the basis of prior evaluation of their effectiveness of N_2-fixation with regard to the acacia species used in the field trials (Brockwell *et al*. 1999). No attempt was made to match rhizobial strains with seed provenances, because evidence obtained by Burdon *et al*. (1999) and Thrall, Burdon & Woods (2000) indicated that N_2-fixing specificity between acacia hosts and strains of rhizobia does not generally extend down to the level of provenance within host species. Inocula were prepared in sterile peat (Roughley & Vincent 1967). Seeds were inoculated and lime-pelleted with the appropriate strains (*A. dealbata*, *A. mearnsii*, *A. acinacea*, *A. pycnantha*: strain CPBR2; *A. rubida*: CPBR11; *A. paradoxa*: CPBR1, CPBR2, CPBR3, CPBR6) using an enhancement of the method of Brockwell (1962). Inoculated seeds were stored at *c.* 15 °C until required. Populations of rhizobia on coated, inoculated seeds (in storage) were counted at 1-month intervals to assess viability.

Two weeks before seeding, 2-m wide strips of land were treated with glyphosate to provide a weed-free environment for germination and growth of young seedlings. Otherwise, there was no land preparation and no application of fertilizer. Acacia seed used in both inoculated and uninoculated treatments was treated with boiling water, soaked in smoked water for 1 h and air dried prior to inoculation and sowing. Direct seeding was carried out in a single operation using an enhanced modification of the seeder described by Dalton (1993). A disc on the seeder opened a wide, shallow furrow *c.* 5 cm deep. Acacia seed was delivered in a single row at 1 cm depth into the bottom of the furrow, sprayed with smoked water to assist in breaking dormancy and enhancing germination (Flematti *et al*. 2004), and the seed bed firmed with a press wheel.

Two acacia seed treatments were sown at each site: (i) coated, inoculated seed and (ii) uncoated, uninoculated seed. The direct seeder was calibrated between

treatments to ensure that sowing rates for coated and uncoated acacia seed were identical (average of 5 seeds m^{-1} species^{-1}). The uninoculated control was sown first at each site, followed by the inoculated treatment (seed of non-acacia species was sown simultaneously using a separate seed box). After sowing each site, the acacia seed box and the seeder delivery lines were disinfected with 95% ethanol as a precaution against inoculant carry-over between sites. Insofar as the area and topography of the land allowed, the two treatments were in parallel, adjacent rows *c.* 4 m apart. The total length of row for each treatment at each site varied from *c.* 0·6 to 1·5 km.

SEEDLING ESTABLISHMENT, GROWTH AND SURVIVAL

At each site, sections of planted rows were permanently marked into 25-m lengths (adjacent sections of inoculated and uninoculated rows were treated as blocks to minimize the effects of topographical, edaphic and microclimatic heterogeneity in statistical analyses). Site censuses were carried out in April and June 2003 and February 2004. At the time when the first observations were made on newly emerged seedlings, marked sections were mapped from pegged starting points using a tape measure to pinpoint the location of every plant of every species. This facilitated relocation of individuals for subsequent censuses and made it possible to follow individual plant development and survival, and to identify new seedlings that emerged subsequent to previous censuses.

Field observations were made on acacia seedling emergence, plant establishment, survival and plant height. Emergence was defined as the number of acacia seedlings visible at a time when no seedling exceeded 2 cm in height. Establishment was defined as the number of acacias m^{-1} found at each census. Survival was defined as (i) the proportion (%) of plants recorded at the first census that were still present at the second and third censuses, and (ii) the proportion of plants that appeared initially at the second census that were still present at the final census.

COUNTS OF RHIZOBIAL POPULATIONS

Rhizobia were counted (i) in soils from field trial sites, (ii) on coated, inoculated seeds and (iii) in seedling rhizospheres. Rhizobia on seed were counted using a spread-plate technique (Somasegaran & Hoben 1994). A serial dilution, plant infection, nodulation frequency test (Brockwell 1963) was used to estimate most probable numbers (MPN) in soils and rhizospheres. To enumerate populations of rhizosphere rhizobia, 12 acacia seedlings were collected haphazardly from each of three sites in December 2002 and from one site in August 2003. Seedling roots were prepared for counting as described by Herridge, Roughley & Brockwell (1984).

Test plants were *Macroptilium atropurpureum* (Mocino & Sesse ex DC.) Urban, *A. dealbata* and *Glycine clandestina* Wendl. *Macroptilium atropurpureum* nodulates promiscuously with virtually all strains of *Bradyrhizobium* spp.; *A. dealbata* is nodulated by rhizobia specific to acacias, which may include strains belonging to other rhizobial genera besides *Bradyrhizobium*; *G. clandestina* forms nodules with the rhizobia of native Australian *Glycine* spp., plants that occurred naturally at several field sites, as well as with the root-nodule bacteria of other native legumes. The lower limit of detection using this plant-infection test is 4 rhizobia g^{-1} soil. However, even this low value represents 6×10^9 rhizobia ha^{-1} (to a depth of 10 cm), a number that is usually sufficient to induce legume nodulation.

Test plants were grown on washed vermiculite, moistened with N-free McKnight's (1949) seedling nutrient solution, in 150 × 25 mm test tubes closed with plugs of flexible polyurethane foam. Each test was comprised of five 10-fold serial dilutions with three replicate test plants at each level of dilution. The tubes were placed in a glasshouse with a day/night temperature regime of *c.* 23/17 °C. Roots were examined for nodules 5–7 weeks after inoculation.

STATISTICAL ANALYSES

For rhizobial populations, values of MPN were calculated from the number of nodulated test plants at each dilution level by reference to frequency tables. Reliable limits of MPN were +(MPN × 3·8) and –(MPN × 0·26); that is, a difference of 1·16 $\log_{10}$ units represented a significant ($P = 0{\cdot}05$) difference (Brockwell 1980). For MPN on soil samples, the range of detection was between 4 and 240 000 rhizobia g^{-1}. Where replicate soil samples were enumerated, variation between the replicates was indicated by standard errors of geometric means. It should be noted, however, that the application of standard errors to MPN data should be treated with caution.

Seedlings of *Acacia acinacea*, *A. paradoxa* and *A. pycnantha* were readily distinguishable at an early stage, allowing for separate analyses by species at sites where sufficient germination had occurred. However, because young seedlings of *A. dealbata* and *A. mearnsii* were difficult to differentiate, at locations where both were sown (all but Donald and St Arnaud) data for these species were combined.

For each site, data relating to establishment (number of acacia seedlings m^{-1}) and growth [(i) actual height increase (cm) and (ii) growth increment (% increase) between censuses] were subjected to analysis of variance of untransformed values. Analyses of establishment treated paired 25-m sections of inoculated and uninoculated rows as blocks to minimize spatial effects (in almost all cases block effects were non-significant), and the main effects of inoculation treatment and block were assessed with two-way ANOVAs. Individual plant data were used for growth analyses, and plants that decreased in height between censuses were excluded as this was the result of herbivory. Although the level of herbivory varied across sites, no differences between inoculated and uninoculated treatments were observed.

Table 2. Most probable numbers (MPN) of resident rhizobia [$\log_{10}$(MPN + 1)] in soils at nine sites, detected by plant-infection tests using *Macroptilium atropurpureum* (2002; ± SE) and *M. atropurpureum*, *Glycine clandestina* and *Acacia dealbata* (2003) as test plants. Replicate counts: 2002, three or four soil samples per site; 2003, one sample per site. Between samples, a difference of 1·16 $\log_{10}$ units is significant ($P < 0{\cdot}05$)

	2002	2003		
Site	*Macroptilium*	*Macroptilium*	*Glycine*	*Acacia*
Donald	0·00 (0·00)	0·00	0·00	0·00
St Arnaud	1·39 (0·19)	0·00	0·00	0·00
Newstead	0·79 (0·05)	0·00	0·00	0·83
Ravenswood	0·95 (0·18)	0·00	0·00	0·00
Shelbourne	1·80 (0·43)	0·00	0·00	0·00
Timor West	0·23 (0·23)	0·00	0·00	0·00
Alma	3·98 (0·47)	0·70	0·70	0·00
Bealiba	3·29 (0·13)	0·00	0·00	0·00
Hoskinstown	0·18 (0·25)	0·70	0·70	1·38

Logistic regression was used to assess whether survival of acacia seedlings was significantly higher in inoculated treatments. For these analyses, only plants that had established by the first census (April 2003) were considered. All data were analysed separately for each trial site.

Results

Rainfall data for the trial site locations show the severity of the 2002–03 drought (Table 1). At all sites, soil moisture was suboptimal at the time of sowing and declined further during germination and establishment. Although not reflected in the rainfall data for the region, local moisture deficit was least at Ravenswood, as it was located in a low area that drained the surrounding hills. Although rainfall approached normality after April 2003, the mean deficit, averaged across all sites, for the 12-month period July 2002–June 2003 was 30%. One consequence of the drought was that, by the time acacia germination occurred, the benefits of herbicide application had disappeared and the young seedlings encountered intense competition from spring-growing weeds.

COUNTS OF RHIZOBIA IN SOIL, SEEDLING RHIZOSPHERES AND ON SEED

Soils for enumeration of naturally occurring acacia rhizobia were collected from all sites in 2002 immediately before sowing, and again in June 2003. At seven of the nine sites the numbers of rhizobia estimated in 2002 were very low (< 65 g^{-1} soil). The exceptions were Alma and Bealiba, where the estimates were > 1900 g^{-1}. Soil populations declined during the course of the study at all sites, and the numbers of rhizobia detected in 2003 were generally very small (Table 2). At four sites (St Arnaud, Shelbourne, Alma and Bealiba), the decline between 2002 and 2003 was significant. This was interpreted as a consequence of the prolonged period of soil moisture deficit. There were no significant differences in MPN values obtained using different test plant species.

Table 3. Rate of seed inoculation [$\log_{10}$(MPN + 1) rhizobia $seed^{-1}$] and survival in storage of rhizobia used to inoculate acacia seed (seed used in field trials were sown shortly after being inoculated). Standard errors of mean values for seed samples prepared and counted in August 2002 ranged from 0·07 to 0·12.

Preparation date	*Acacia* sp. (no. of provenances)	MPN rhizobia $seed^{-1}$ (months in storage)		
		0	1	3
August 2002 (sites 1–8)	*A. dealbata* (8)	5·08	3·88	3·20
	A. mearnsii (6)	5·40	4·45	2·78
	A. pycnantha (7)	5·26	4·05	2·90
	A. acinacea (7)	5·23	4·02	3·08
	A. paradoxa (7)	5·07	4·57	4·33
September 2002 (site 9)	*A. dealbata* (1)	5·74	4·83	
	A. mearnsii (1)	4·93	2·78	
	A. rubida (1)	5·83	4·88	

The numerical quality of peat cultures made from the inoculant strains was consistently high, ranging between $1{\cdot}94 \times 10^9$ and $3{\cdot}29 \times 10^9$ rhizobia g^{-1} peat. The numbers of rhizobia applied to acacia seeds were also consistently large (Table 3). Although there was some loss of viability in storage, seed populations after 3 months were never less than 600 rhizobia $seed^{-1}$ and, in some cases, exceeded 10 000 $seed^{-1}$.

In December 2002, counts at Ravenswood, Shelbourne and Hoskinstown indicated substantial numbers of rhizobia in the rhizospheres of both inoculated and uninoculated seedlings (Table 4) but no significant differences between treatments. It was apparent that the small populations of rhizobia resident in the soil were, nonetheless, sufficient in number to induce rhizosphere colonization and, presumably, nodulation of the acacia seedlings. In contrast, in September 2003 at Timor West, where substantial seedling germination did not occur until 9 months after sowing, very few rhizobia were detected in plant rhizospheres. No attempt was made to distinguish the identities of the strains that colonized the seedling rhizospheres.

Table 4. Most probable numbers (MPN) of rhizobia in seedling rhizospheres in 2002 from three sites (Ravenswood, Shelbourne and Hoskinstown) detected by plant-infection tests using *Macroptilium atropurpureum* (five replicate counts of three seedling roots per sample) and in 2003 from one site (Timor West) using *M. atropurpureum*, *Glycine clandestina* and *Acacia dealbata* (two replicate counts of 10 seedling roots per sample). No significant treatment differences were found for the 2002 data; statistical analyses were not feasible for the 2003 data

	Rhizobia rhizosphere^{-1} [$\log_{10}$(MPN + 1)]		Rhizobia per seedling root (back-transformed)	
Site	Inoculated	Uninoculated	Inoculated	Uninoculated
Ravenswood	1·36 (0·67)	2·28 (0·69)	22	190
Shelbourne	3·05 (0·55)	3·14 (0·51)	1121	1379
Hoskinstown	3·50 (0·21)	2·44 (0·45)	3161	274
Timor West (*Macroptilium*)	1·01 (NA)	0·55 (NA)	9	3
Timor West (*Glycine*)	0·00 (NA)	0·00 (NA)	0	0
Timor West (*Acacia*)	0·64 (NA)	0·37 (NA)	3	1

NA, not available.

Table 5. Establishment of acacia seedlings (all species combined) in relation to inoculation treatment at sites where seedling numbers were sufficient to allow statistical analyses. Results are from two-way ANOVAs treating paired 25-m sections of inoculated and uninoculated rows as blocks. At all sites, the mean number of seedlings m^{-1} was greater in the inoculated treatment. Significance: *$P < 0·05$; **$P < 0·01$; ***$P < 0·001$; ****$P < 0·0001$

	Census date April 2003		June 2003		February 2004	
Site (*n* blocks)	MS	*F*	MS	*F*	MS	*F*
Donald (24)						
Treatment	1·15	11·86**	1·72	17·46***	1·48	14·13***
Block	0·08	0·85	0·09	0·88	0·10	0·93
St Arnaud (23)						
Treatment	5·21	53·19****	6·46	55·38****	4·87	36·91****
Block	0·19	1·92	0·19	1·64	0·20	1·53
Newstead (21)						
Treatment	0·91	21·53***	0·97	19·78***	NA	NA
Block	0·04	0·93	0·05	1·01	NA	NA
Ravenswood (18)						
Treatment	1·34	7·82*	0·97	4·93*	1·77	7·16*
Block	0·57	3·32**	0·60	3·04*	0·48	1·93
Shelbourne (36)						
Treatment	0·09	9·26**	0·08	8·70**	NA	NA
Block	0·01	1·19	0·01	1·15	NA	NA
Alma (15)						
Treatment	NA	NA	0·30	5·73*	NA	NA
Block	NA	NA	0·07	1·36	NA	NA
Hoskinstown (33)						
Treatment	0·03	1·39	0·01	0·73	0·00	0·02
Block	0·06	2·52**	0·05	2·96**	0·07	3·79***

NA, not available.

SEEDLING EMERGENCE AND ESTABLISHMENT

The sites were sown in August–September 2002. Initial emergence immediately post-germination was measured in November 2002 and was highly variable across sites. The extremes were represented by Shelbourne (0·01 vs. 0·06 germinants m^{-1} in inoculated and uninoculated treatments, respectively), and Bealiba (2·55 vs. 2·61 germinants m^{-1}). No doubt this variation was largely the result of low soil moisture but other factors, e.g. variation in soil type and differential hardseededness between acacia provenances, were probably also involved. There was no evidence of any treatment effect (including the lime pellet that was part of the inoculation treatment) at this early post-germination stage. There was a second flush of germination at Timor West in June 2003, but these seedlings were not of sufficient stature (maximum height < 2 cm) to be classified as 'established', and virtually all of them had disappeared by February 2004 because of the lack of rain.

By April 2003, sufficient seedlings had become established at six sites to permit statistical analysis. At five of these sites, acacia establishment was significantly higher in the inoculated treatment (Table 5). Across sites the inoculated treatment was superior to the uninoculated control by an average ratio of 2·3 : 1·0 [range 1·3 : 1·0 (Ravenswood) to 3·2 : 1·0 (St Arnaud)]. Subsequent censuses following additional seedling recruitment

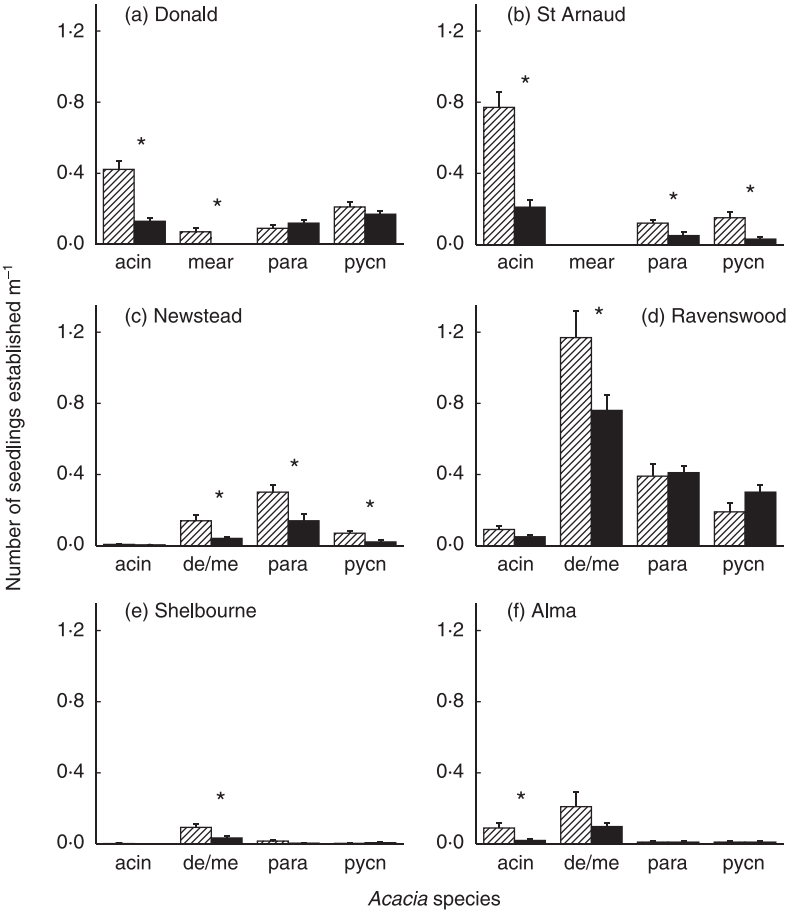

Fig. 1. Number of acacia seedlings established m^{-1} by June 2003 for sites where there was sufficient germination and where seedlings could be identified to species. *Acacia acinacea*, acin; *A. dealbata*/*A. mearnsii*, de/me (*A. dealbata* was not sown at Donald or St Arnaud); *A. paradoxa*, para; *A. pycnantha*, pycn. Hatched bars, inoculated treatments; solid black bars, uninoculated controls (means and standard errors shown). Asterisks denote significant treatment effects. *Acacia paradoxa*: St Arnaud, $F_{1,22} = 6{\cdot}61$, $P < 0{\cdot}05$; Newstead, $F_{1,20} = 7{\cdot}70$, $P < 0{\cdot}05$). *Acacia acinacea*: Donald, $F_{1,23} = 43{\cdot}80$, $P < 0{\cdot}0001$; St Arnaud, $F_{1,22} = 42{\cdot}50$, $P < 0{\cdot}0001$; Alma, $F_{1,14} = 6{\cdot}67$, $P < 0{\cdot}05$. *Acacia pycnantha*: St Arnaud, $F_{1,22} = 22{\cdot}54$, $P < 0{\cdot}0001$; Newstead, $F_{1,20} = 12{\cdot}52$, $P < 0{\cdot}01$). *Acacia dealbata*/*mearnsii*: Donald, $F_{1,23} = 18{\cdot}53$, $P < 0{\cdot}001$; Newstead, $F_{1,20} = 8{\cdot}71$, $P < 0{\cdot}01$; Ravenswood, $F_{1,17} = 10{\cdot}94$, $P < 0{\cdot}01$; Shelbourne, $F_{1,35} = 11{\cdot}82$, $P < 0{\cdot}01$.

produced similar results. Overall, acacia seedlings in inoculated rows continued to have better establishment than seedlings in uninoculated rows by ratios of 2·4 : 1·0 (June 2003) and 2·0 : 1·0 (February 2004). Generally, there was little indication of spatial variation in establishment within sites; block effects were only significant at Ravenswood and Hoskinstown (in the former case, this effect declined over time, while in the latter there was no evidence of a treatment effect; Table 5).

Individual acacia species varied considerably in establishment success among sites (Fig. 1). Thus, *A. acinacea* showed the highest establishment at the two westernmost sites (Donald and St Arnaud) but much lower numbers at the other sites, where one or more of the other species (*A. dealbata*/*mearnsii*, *A. paradoxa* and *A. pycnantha*) had relatively high seedling numbers. Analysis of establishment data for individual acacias from the June 2003 census showed that, in every case where there were significant treatment differences (11 of 23 comparisons), establishment was greater in inoculated treatments. For *A. paradoxa* these differences were fairly small (Fig. 1). Larger differences in establishment were observed at several sites for inoculated vs. uninoculated *A. acinacea*. Similarly large effects of inoculation were observed for *A. pycnantha* and *A. dealbata*/*mearnsii*. The maximum establishment differences in favour of inoculation for each species were 7·73 : 1·0 for *A. acinacea* (Alma), 2·17 : 1·0 for *A. paradoxa*, 4·43 : 1·0 for *A. pycnantha* (both at St Arnaud) and 3·38 : 1·0 for *A. dealbata*/*mearnsii* (Newstead).

SEEDLING SURVIVAL

Mapping the location of individual plants within rows made it possible to collect precise data relating to seedling survival at two time intervals (initial survival, April–June 2003; longer-term survival, June 2003–February 2004). Because the number of seedlings that did not appear until the second census was quite small, this aspect of the data did not lend itself to statistical analysis and results are only presented for seedlings present at the April 2003 census.

The percentage of established seedlings that survived until the final census varied considerably among sites;

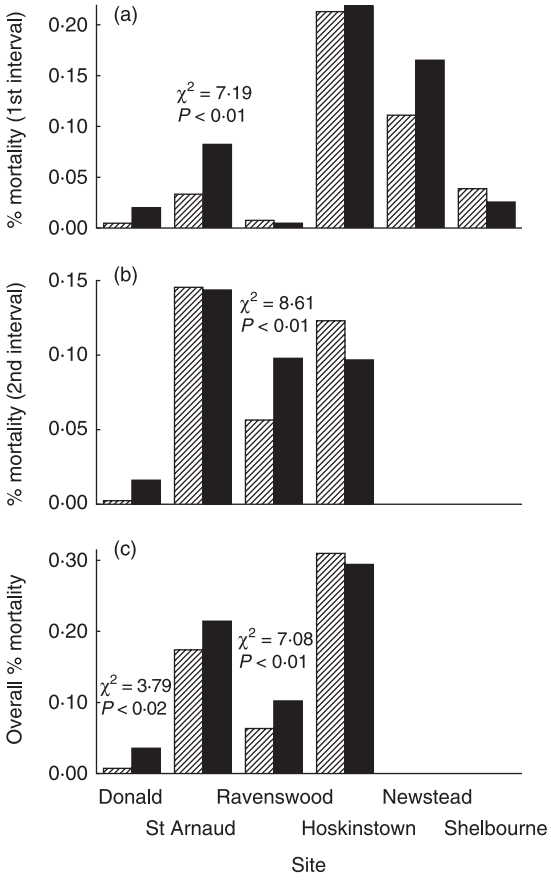

Fig. 2. Seedling mortality (all acacias combined): (a) between April and June 2003 (first time interval); (b) from June 2003 to February 2004 (second time interval); (c) from April 2003 to February 2004 (overall mortality) in relation to inoculation treatment. Hatched bars, inoculated treatments; solid black bars, uninoculated controls. Significant treatment differences are based on results from logistic regressions.

mortality was lowest at Donald (0·7% and 3·6% in inoculated and uninoculated treatments, respectively) and highest at Hoskinstown (31% and 29·4%). Survival was generally superior in the inoculated treatments, and the overall difference was significant for several comparisons between inoculated and uninoculated acacias (Fig. 2). There were also some significant treatment-related survival differences for individual species. Thus, *A. acinacea* survived better in inoculated treatments during the first time interval at St Arnaud ($\chi^2 = 3{\cdot}88$, $P < 0{\cdot}05$) and over the second time interval at Ravenswood ($\chi^2 = 5{\cdot}10$, $P < 0{\cdot}05$). *Acacia paradoxa* showed greater survival in inoculated treatments at Newstead (first time interval $\chi^2 = 5{\cdot}10$, $P < 0{\cdot}05$), and at Ravenswood (second time interval $\chi^2 = 7{\cdot}23$, $P < 0{\cdot}01$).

GROWTH OF *ACACIA* SEEDLINGS

Analyses of acacia seedling growth [(i) actual increase in height (cm) and (ii) percentage change from the previous census] in relation to inoculation treatment are presented for three sites (Donald, St Arnaud and Ravenswood) where it was feasible to collect data at all three censuses (April and June 2003 and February 2004) and where there was sufficient establishment to permit separate analyses for each species. At two other sites (Newstead and Shelbourne), analyses of growth data for the first two censuses showed no significant treatment effect for any species.

Although the average absolute height increases of inoculated acacias was 70% greater than the uninoculated controls (largely because of *A. pycnantha*) and the average incremental growth was 58% greater between the first two censuses, there was no consistent pattern of response to inoculation (Figs 3 and 4). However,

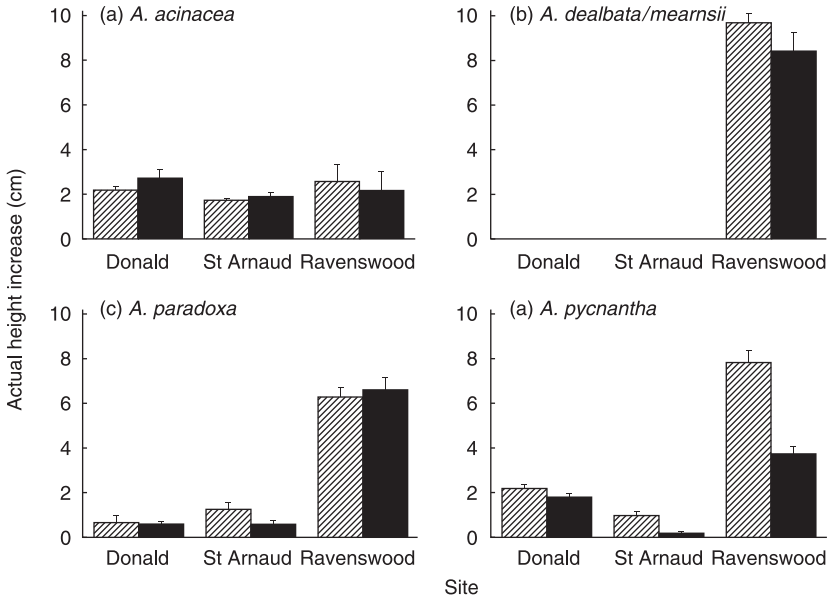

Fig. 3. Mean height increase (centimetre) from April to June 2003 for acacia seedlings established at the Donald, St Arnaud and Ravenswood sites. Hatched bars, inoculated treatments; solid black bars; uninoculated controls (standard errors from ANOVAs shown).

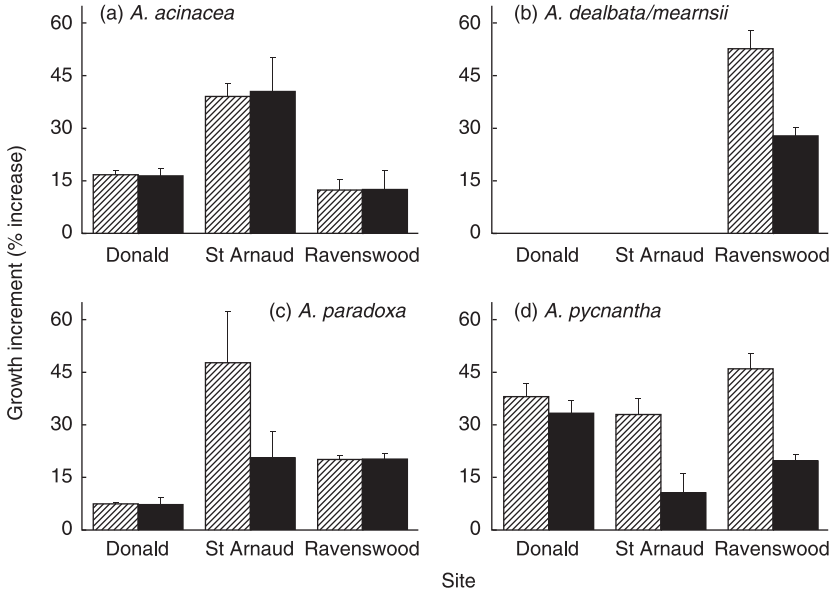

Fig. 4. Mean growth increment (% increase) from April to June 2003 for acacias established at the Donald, St Arnaud and Ravenswood sites. Hatched bars, inoculated treatments; solid black bars, uninoculated controls (standard errors from ANOVAs shown).

where treatment effects were significant the inoculated treatments always showed higher growth rates than the uninoculated controls. This was the case for *A. dealbata*/*mearnsii* (% increase, $F_{1,442} = 9{\cdot}24$, $P < 0{\cdot}01$) and *A. pycnantha* (height increase, $F_{1,126} = 45{\cdot}02$, $P < 0{\cdot}0001$; % increase, $F_{1,126} = 36{\cdot}26$, $P < 0{\cdot}0001$) at Ravenswood, and for *A. pycnantha* (height increase, $F_{1,57} = 4{\cdot}03$, $P < 0{\cdot}05$; % increase, $F_{1,57} = 5{\cdot}08$, $P < 0{\cdot}05$) at St Arnaud (Figs 3 and 4). Generally, treatment-related growth differences observed during the initial establishment phase (April–June 2003) had disappeared by February 2004.

We noted that the growth data at all sites were confounded by herbivory, the better plants being grazed preferentially, and this may have obscured treatment-related differences. At Donald and St Arnaud, the percentage of plants showing negative growth (because of herbivory) between the first and second census was 19% and 32%, respectively. Moderate to high rates of herbivory were also observed at other sites (Newstead, 26%; Hoskinstown, 52%). At Ravenswood, where the largest growth differences were observed, herbivory was relatively minor (16% of plants showed negative growth between the first and second censuses).

Discussion

Our direct-seeding trials were part of broader revegetation programmes in south-eastern Australia undertaken by catchment management authorities and landowners. The trials were sown and maintained under the same conditions as would normally apply to such replanting projects. They differed from typical agronomic experiments in that there was no supplementary watering to alleviate the effects of drought, no ongoing weed control and no fencing against herbivores. The primary objective was to evaluate the influence of rhizobial inoculation and symbiotic N_2-fixation on acacia establishment, growth and survival. Secondary aims were to consider interactions between the dynamics of the symbiosis and (i) soil moisture deficit, (ii) weediness and (iii) herbivory, insofar as they affected the relative success or failure of the trials.

INOCULANT AND NATURALLY OCCURRING RHIZOBIA

Immediately prior to establishing the trials, naturally occurring rhizobial populations were enumerated by plant-infection tests using *M. atropurpureum* (Table 2). As there are differences in the rhizobial affinities of *M. atropurpureum* (for *Bradyrhizobium* spp.) and *Acacia* spp. (for at least four rhizobial genera) (Sprent 2001), the possibility existed that these results may have been misleading with regard to the abundance of rhizobial strains effective on acacias. However, subsequent counts using *G. clandestina* (affinity for *Bradyrhizobium* spp.) and *A. dealbata* as test plants in addition to *M. atropurpureum* suggested that the latter species was suitable for enumerating acacia rhizobia and that the naturally occurring rhizobia belonged to the genus *Bradyrhizobium*.

At seven of the nine sites, the numbers of naturally occurring acacia rhizobia estimated immediately before sowing in 2002 were < 65 g^{-1} soil. Populations of this size would not be expected to compete with a persistent inoculant in nodule formation (Brockwell, Holliday & Pilka 1988; Thies, Singleton & Bohlool 1991; Turk, Keyser & Singleton 1993). In contrast, competition from large naturally occurring rhizobial populations at Alma and Bealiba (> 1900 rhizobia g^{-1}) made it unlikely that the inoculant would form more than a small proportion of nodules. Consistent with this observation, there was

little or no response to inoculation in terms of seedling establishment at either of these sites.

A primary aim of legume inoculation is to maximize inoculant survival during the periods between introduction to the soil, seed germination and the development of a rhizosphere that the inoculant rhizobia can colonize. One means of achieving this objective is to use heavy inoculation rates (Jenkins, Vincent & Waters 1954). In our trials, seed inoculant application rates were very high (*c.* 100 000 rhizobia seed^{-1}; Table 3). Although mortality would have been greater in the field than under the benign conditions of storage, it is likely that sufficient inoculant rhizobia would have persisted to colonize seedling rhizospheres, even where seed germination was delayed for several months. Indeed, the fact that survival of seedlings that did not germinate until after the first census was rather better in inoculated plots than in the uninoculated controls (data not shown) suggests that some inoculant persisted for at least 8 months after seeding.

Rhizobial colonization of legume rhizospheres is an indicator of nodulation potential (Herridge, Roughley & Brockwell 1984). At the three sites (Ravenswood, Shelbourne and Hoskinstown) where it was enumerated 4 months after sowing, rhizosphere colonization was extensive (Table 4). There were no substantial differences in colonization between inoculated and uninoculated treatments, indicating that even the very small naturally occurring rhizobial populations in uninoculated plots at those three sites were able to proliferate sufficiently to initiate the processes leading to nodulation. In contrast, at Timor West, the site worst affected by drought, rhizosphere numbers were negligible on acacia seedlings that had only recently emerged 12 months after seeding.

Legume nodulation depends on the establishment of a threshold population of rhizobia in the rhizosphere that is needed to initiate root infection (cf. Purchase & Nutman 1957). It is likely that attainment of that threshold occurred more quickly in inoculated rows, where large numbers of inoculant rhizobia were strategically delivered into the soil in close proximity to developing seedling rhizospheres, than in uninoculated rows, where small numbers of naturally occurring rhizobia were randomly distributed throughout the soil profile. It seems reasonable to assume, therefore, that the inoculated acacias would have nodulated and begun N_2-fixation sooner than uninoculated plants.

We did not test any of the strains of rhizobia that occurred naturally at the experimental sites for effectiveness of N_2-fixation in association with the target acacias. However, it is likely that they would have been less effective than the inoculant strains that were used in this investigation. The latter had been selected on the basis of replicated glasshouse effectiveness tests of a collection of several hundred diverse strains (Burdon *et al.* 1999; Thrall *et al.* 2000). Overall, our results strongly indicate that, wherever we observed a benefit from inoculation in our experiments, the response was the result of nodulation by populations of inoculant strains that were more effective in N_2-fixation than the naturally occurring strains at the experimental sites.

PLANT ESTABLISHMENT, SURVIVAL AND GROWTH

Direct-seeding is the only practical method of re-establishing native vegetation at the subcatchment and catchment scales necessary to ameliorate environmental problems caused by clearing of deep-rooted perennials. Currently, the difficulty and expense of collecting sufficient seed of native species for replanting represent substantial constraints on the ability of revegetation practitioners and natural resource managers to address large-scale environmental issues in Australia and elsewhere. Moreover, much of this seed is sourced from remnant bushland, national parks and nature reserves (Mortlock 1998), potentially representing significant negative impacts on population viability, particularly for species that are less common but still desirable in ecosystem reconstruction. The importance of enhanced establishment of native plants and the consequent reduction in seed quantity requirements cannot be overestimated.

The criteria used to judge inoculation success were initial establishment and survival of acacia seedlings and their absolute and incremental growth. Taken over all five *Acacia* spp., establishment in inoculated rows was superior to the uninoculated controls by a ratio of 2·3 : 1·0 despite the generally limiting soil moisture during most of the 8-month period prior to the first census in April 2003. Differences in establishment were enhanced, although not always significantly, by the fact that survival was generally better in inoculated than in uninoculated plots. Overall, the significantly greater establishment observed in inoculated treatments indicates that substantial reductions in seed usage are possible if beneficial soil symbionts are incorporated into replanting projects.

The legume symbiosis is profoundly influenced by environmental conditions (Vincent 1965). When soil moisture is non-limiting, acacia seedlings develop vigorous root systems that, irrespective of nodulation, are probably efficient scavengers of soil N. Under the soil-moisture deficiency of our experiments, acacia root development was restricted, no doubt reducing N-scavenging ability. In these circumstances, young seedlings would have been more dependent on atmospheric N, via the symbioses generated by inoculation, than might otherwise have been the case. Under optimal soil moisture conditions the response in acacia seedling establishment to inoculation with elite rhizobial strains may possibly have been of lesser magnitude than shown here.

At the same time, suboptimal soil moisture was probably also implicated in the generally non-significant growth responses to inoculation. However effective a strain of rhizobia might be, it cannot be expected to express its full capacity for N_2-fixation in the presence of a major limiting factor such as the ongoing soil moisture deficiencies that occurred during our study.

Under optimal soil moisture conditions, it is anticipated that acacia seedling N_2-fixation and growth responses to inoculation with elite rhizobia would be substantial. This is supported by growth responses at Ravenswood, the least dry of the experimental sites, where average heights were several times those seen for other sites.

INCREASING DIRECT-SEEDING SUCCESS IN REVEGETATION

Independent of inoculation, several factors contributed to the relative success of site restoration. Competition from weeds and grazing by animals had a major influence on acacia establishment and growth. Where herbicide treatment is only partially effective, it may be sensible to sow in late autumn or early winter to avoid competition from spring-growing weeds. It was also clear that it is essential to exercise rigorous control over feral grazing animals, especially rabbits. However, despite its widespread nature, the 2002–03 drought *per se* was not a major factor in restoration failure except at Timor West, where there was substantial germination but no following rain. This is encouraging because much of the land scheduled for replanting in south-eastern Australia is subject to periodic drought or has shallow soils where soil moisture deficiencies are a common occurrence.

Our results indicate that inoculation of *Acacia* spp. and other native leguminous shrubs and trees with elite rhizobial strains as part of direct-seeding techniques has the potential to increase the scope, rate and success of land-restoration efforts world-wide. For example, vast areas of degraded, infertile *Imperata* grasslands in Indonesia are scheduled for reforestation with *Acacia mangium* Willd. (Turnbull, Midgley & Cossalter 1998). There is scope for using legumes to revegetate degraded landscapes in Chile (Arredondo *et al*. 1998) and the Atlantic lowlands of Cost Rica (Tilki & Fisher 1998). Legumes are also useful for reclaiming old mine sites (Franco & de Faria 1997), including spoil from open-cut operations (Langkamp, Swinden & Dalling 1979). The success of such initiatives is likely to be significantly enhanced by inoculation of legume seed with elite N_2-fixing strains of rhizobia. Not only will this increase adoption and uptake of direct-seeding by land managers, but greater cost effectiveness and reduced impacts on native plant communities can be achieved through better seed usage.

Further significant increases in the establishment of key native plant species, especially on impoverished soils, may be achieved by incorporating other beneficial soil symbionts such as mycorrhizal fungi (Requena *et al*. 2001). Several studies suggest that the synergistic benefits of dual inoculation with rhizobia and mycorrhizae may be considerable (Hatimi 1999; Marques, Pagano & Scotti 2001; Andre, Neyra & Duponnois 2003). Other plant–soil interactions (e.g. N_2-fixing actinomycetes in the genus *Frankia* and host plants in the Casuarinaceae) are also likely to be important in land reclamation. A number of casuarinas are tolerant of saline and/or water-logged soils (Diem & Dommergues 1990) and there may also be synergies between *Frankia* and mycorrhizal fungi (Vasanthakrishna, Bagyaraj & Nirmalnath 1994; Schwencke & Carú 2001). Clearly, we are only in the embryonic stages of effectively utilizing soil organisms in the restoration of functioning native ecosystems.

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