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Research paper

Selection of effective strains of *Bradyrhizobium* for Caatinga stylo (*Stylosanthes seabrana*)

Selección de cepas efectivas de Bradyrhizobium *para* Stylosanthes seabrana

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Abstract

Caatinga stylo (*Stylosanthes seabrana*) is recommended as a forage legume for permanent and long-term ley pastures on clay soils of southern (cv. Primar CPI92838B) and central (cv. Unica CPI110361) Queensland. The release of the 2 cultivars was contingent on the availability of an effective and persistent strain of *Bradyrhizobium*, because suitable effective nitrogen-fixing strains do not occur naturally in the soils of the target regions. Effective strains of *Bradyrhizobium* (strains CB3480 and CB3481), suitable as inocula for Caatinga stylo, were selected from nodule material collected in Bahia, Brazil.

This paper documents soil-pot and field experiments that led to the selection of these persistent and effective strains of *Bradyrhizobium* and the eventual release of CPI92838B and CPI110361, respectively, as cvv. Primar and Unica.

Keywords: Inoculation, nitrogen fixation, nodulation, soil temperature, survival.

Resumen

Stylosanthes seabrana (Caatinga stylo) es una leguminosa forrajera recomendada para pasturas permanentes o, en rotación con cultivos, pasturas de larga duración en suelos arcillosos tanto en la zona sur (cv. Primar CPI92838B) como en la zona centro (cv. Unica CPI110361) de Queensland, Australia. La liberación de estos cultivares estuvo sujeta a la disponibilidad de una cepa efectiva y persistente de *Bradyrhizobium*, debido a que este tipo de cepas fijadoras de nitrógeno no ocurre en forma natural en los suelos de la región. A partir de material nodular recolectado en Bahia, Brasil, fueron seleccionadas como efectivas las cepas *Bradyrhizobium* CB3480 y CB3481, y adecuadas como inóculos para esta especie.

Este trabajo documenta la investigación realizada en materas y a nivel de campo que llevaron a la selección de estas cepas persistentes y efectivas de *Bradyrhizobium* y a la eventual liberación de *S. seabrana* CPI92838B y CPI110361, respectivamente, como cvs. Primar y Unica.

Palabras clave: Fijación de nitrógeno, inoculación, nodulación, supervivencia, temperatura del suelo.

Introduction

The selection and release of adapted accessions of Caatinga stylo [*Stylosanthes seabrana*; by the taxonomic database GRIN (www.ars-grin.gov), now considered to be *S. scabra*] for planting on clay soils and suitable effective and persistent strains of *Bradyrhizobium* arose from a series of experiments over several years. During field evaluation of accessions of *Stylosanthes*, at a range of sites in Queensland during the early 1990s (Edye 1994; Edye et al. 1998), it was observed that the accessions corresponding to Caatinga stylo were poorly and ineffectively nodulated and frequently were not nodulated at all. In addition, most accessions grew well for 1 or 2 years, then began to show classical signs of nitrogen deficiency [R.A. Date and (the Late) L.A. Edye unpublished data].

Strains of *Bradyrhizobium* isolated from some of the few nodules formed on plants from each of these sites were shown to be ineffective in nitrogen fixation, when tested in either aseptic tube culture or soil-pot assessments

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(R.A. Date unpublished data). Similar trials with strains of bradyrhizobia, isolated from other species of *Stylosanthes* but from the same geographical area (i.e. states of Bahia and Minas Gerais, Brazil) and already held in CSIRO's germplasm collection, similarly failed to form effective N-fixing associations with the exception of strains CB2126 and CB3053 (Date 2010). These failures led to the collection of new nodule material from Brazil in 1992 and 1994. The isolation and authentication of new strains from these nodules were completed in 1995 and preliminary effectiveness was demonstrated on a range of accessions of *S. seabrana* the following year (Date 2010).

In addition to the absence of suitable effective strains of bradyrhizobia, soil-pot experiments determined that plants, grown in soil from Edye's evaluation sites (Edye 1994; Edye et al. 1998), did not nodulate while soil nitrogen was available but subsequently nodulated effectively and grew satisfactorily, provided suitable *Bradyrhizobium* had been applied at the time of sowing. This observation indicated that, under field conditions, applied inoculum strains would need to persist for 1 or more growing seasons before plants would nodulate. Nitrogen inhibition of nodulation has been documented for a number of legumes (Streeter 1988; Carroll and Mathews 1990) and is demonstrated in the Caatinga stylo soil-pot experiments, where regrowth of uninoculated controls showed classical nitrogen-deficiency symptoms, whereas inoculated treatments did not (see plant dry weight differences between uninoculated and strain treatments in the ST series experiments below).

Strains CB3480 and CB3481 were isolated and selected from the nodule samples collected in Brazil in 1992. This success led to further collections of *S. seabrana* germplasm and nodule material in 1994. Morphological classification of the new germplasm (Date et al. 2010) and isolation and selection of additional strains of *Bradyrhizobium* have provided additional plant and bradyrhizobial lines for further selection work.

On these clay soils producers prefer to sow the small-seeded legumes on the surface (rolled or with shallow scarifying) prior to the onset of seasonal rainfall. Soil-surface temperatures under these conditions frequently exceed 50–60 °C for 4–6 h/d and are lethal to rhizobia on surface-sown inoculated seed where rhizobia may not survive more than 2–3 days (McInnes and Date 2005). An alternative method of introducing the inoculum to the system is required.

The work reported in this paper was completed over several years, as new germplasm of both *S. seabrana* and bradyrhizobia became available, with the aim of providing effectively nodulating cultivars of Caatinga stylo for the clay soil regions of southern and central Queensland.

Material and Methods

Bradyrhizobium

New strains of bradyrhizobia obtained from the 1992 and 1994 nodule collections in Brazil were authenticated as *Bradyrhizobium* by reinoculation of seedling plants of *S. seabrana* growing in aseptic tube culture (Norris and Date 1976), i.e. completing Koch's postulate. The dry weights of the same plants also served as an initial assessment of the nitrogen-fixation effectiveness of the isolates. From a bacteriological aspect, the new isolates (strains) were not typical of bradyrhizobia isolated from other species of *Stylosanthes*. They grew only on acidified nutrient agar and were very slow-growing (10–12 vs. 6–8 days) and then only to pin-head size (± 20% that of other bradyrhizobia; R.A. Date unpublished data).

Soil-pot technique

Air-dried soil from some of Edye's field sites (Table 1) was passed through a 5 mm sieve, then potted to within 15 mm of the top of 15 cm pots lined with plastic bags. The amount of dry soil varied between 1,800 and 1,950 g/pot depending on soil type. Pots were then watered to 95% field capacity and maintained at this level by being mounted on circular tables (10 pots per table) on an automatic watering machine (Andrew and Cowper 1973), which circulated the tables with pots on an endless chain belt around a glasshouse twice each 24 hours. There were 2 replicates of each strain x soil treatment. No fertilizer/nutrients were added to the potted soils.

Surface-sterilized and pre-germinated seed of *S. seabrana* CPI110361, later released as cv. Unica, was sown at 10 seeds per pot and later thinned to 6 plants, which were inoculated with 1 mL suspension of a peat culture of the test strains of bradyrhizobia. Plant tops were harvested at 6–8 weeks, dried at 60 °C for 48 hours and weighed. In instances where there were few differences between uninoculated controls and strain treatments, plants were allowed to regrow for a further 6–8 weeks and again plants tops were harvested and weighed. Dry weights of whole tops and leaf were used as indices of the effectiveness of the strain treatments. After harvest plant roots were washed out of soil and observations made on level of nodulation.

Table 1. Sites used by Edye (1995) for cultivar evaluation and soil used in soil-pot* and field† experiments (Northcote 1979; Edye 1995, 1997; Isbell 1996; Pengelly and Staples 1996; Edye et al. 1998).

Site	Lat (S)/Long (E) (decimal deg.)	Soil type (Isbell 1996) and Northcote (1979) classification
Banoona†	26.906/149.136	Vertisol
Brian Pastures*	25.650/151.750	Dark brown clay complex Ug5.24
Cardigan	20.228/146.644	Red Duplex
Gunalda	25.993/152.542	Red Podzolic Dr3.21
Hillgrove*	19.637/145.793	Euchrozem or Black Earth
Holyrood*†	26.817/148.750	Red Duplex Dy2.33
Lansdown†	19.648/146.823	Solodized Solonetz
Mt Garnet	17.717/145.150	Red Earth Duplex Dr2.51
Narayen brigalow*†	25.665/150.871	Self-mulching Brown Vertisol Ug5.32
Narayen granite*†	25.683/150.867	Mottled Yellow-red Podzolic Dy3.41
Roma Research Station†	26.578/148.765	Fertile Black Earth Ug5.22
Rostock	26.918/149.423	Self-mulching Grey Vertisol
Southedge	16.983/145.350	Red Earth Gn2.12

Experimental series

Four soil-pot experiments were completed:

- ST09 tested 8 strains of bradyrhizobia: 7 new strains from the nodule material collected in 1992 and strain CB3053 from the early screening work (Date 2010) plus an uninoculated control using soil from the Narayen granite, Narayen brigalow, Brian Pastures and Holyrood sites (Table 1).
- ST25 tested 23 new strains from nodule material collected in 1992, an uninoculated control and control with N added using soil from Narayen granite and Narayen brigalow sites (Table 1).
- ST50 tested 47 new strains from nodule material collected in 1994, 1 of the best strains (CB3480) from ST25, an uninoculated control and a control with N added using soil from the Narayen granite, Narayen brigalow, Hillgrove and Holyrood field sites (Table 1).
- ST130 tested a further 94 strains from nodule material collected in 1994, 12 existing strains, 1 'diagnostic strain' (Date and Norris 1979), 3 of the best strains from ST50 and 8 strains isolated from other species of *Stylosanthes*, plus uninoculated controls and controls with N added (1 of each on alternate tables of automatic watering machine) using soil from Narayen granite and Holyrood field sites (Table 1).

Data analysis and classification methods

For the soil-pot experiments data sets of plant top dry weights, leaf dry weights and nitrogen content were used as ratio attributes for each strain. These data were analyzed using simple ANOVA [strain x replication (x harvest time where appropriate)] and the strain means used as attributes in PATN (Belbin 1989) analysis. The means were range-standardized (TRND module) to provide the input data for ASO (with the Gower Metric option) to obtain symmetric matrices, which were classified by the hierarchical routine FUSE (UPGMA option). The routine GDEF was used to determine group composition and DEND (Dendrogram) to display group structure.

Field experiments

Two series of field trials were established: 1 in January 1995 to assess the strains of bradyrhizobia currently available; and another in January 1996 to assess new strains developed from glasshouse soil-pot assessment of bradyrhizobia arising from new material collected in Brazil in 1994. In each year experiments were established at the CSIRO field stations at Lansdown (solodic soil) and Narayen (granite soil) and in a red earth soil at Holyrood, near Roma (see Table 1). Field plots were comprised of a series of 5 m rows, each row representing a single strain of *Bradyrhizobium*. There were 3 replications of each strain treatment, randomized within replications. Each strain was prepared as a peat-based inoculum with more than 100 million cells per gram. Seed of cv. Unica was inoculated at the commercial rate of 250 g peat per 25 kg seed using a 5% solution of methyl cellulose, allowed to air dry and then sown the same day. Supplementary irrigation was used to ensure establishment of the Caatinga stylo. Plots were maintained weed-free in the establishment year only.

Harvests each year were made by taking 5 randomly selected 10 cm diameter core samples (to a depth of 15 cm) per row, recording the dry weight of plant tops from this area and washing out root samples to collect nodules for strain identification. Fluorescently labelled specific antibody typing (Somasegaran and Hoben 1985) was used to identify strains forming nodules.

Inoculum delivery

Experiments assessing alternative methods of delivery of the inoculum strains were established at 3 sites in southern Queensland. Treatments (Table 2), comparing surface vs. deep placement (8–10 cm) of the inoculum either on the seed of a preceding wheat crop or on inert plastic prills, were established at Roma Research Station, Holyrood and Banoona (see Table 1). *Bradyrhizobium* strain CB3546 was used to inoculate wheat seed (Treatments 1 and 2), plastic prills and Caatinga stylo seed in Treatments 3 and 4, respectively, and CB3481 to inoculate Caatinga stylo in Treatment 2 (see Table 2). The use of these 2 strains was to assess which method of inoculum introduction contributed most to the nodule population. Strain CB3546 is an antibiotic (rifamycin and streptomycin) -resistant variant of CB3481 and was equally effective in nitrogen fixation in sand-jar assay (6.2, 4.3, 5.5, 5.9 and 0.2 g dry weight/jar, respectively, for CB3481 mother culture, CB3481 commercial peat, CB3546 mother culture, CB3546 peat culture used in experiment and uninoculated control). It became obvious that strain CB3546 was serologically distinct from CB3481 and this method of distinguishing between the 2 strains proved simpler than attempting antibiotic-resistance assay of the strains forming the nodules.

In an adjacent area at the Banoona site, 2 commercial 10-ha areas (1 of cv. Primar and 1 of cv. Unica Caatinga stylo) were established by Queensland Department of Agriculture, Forestry and Fisheries (QDAFF) using commercially prepared peat inoculant of CB3481, 2 months later in 1996 than the alternative delivery experiment.

Results

More than 700 isolates, obtained from the nodules collected in 1992 and 1994, nodulated Caatinga stylo in the aseptic tube-culture authentication tests. In a sand-jar glasshouse nitrogen-fixation effectiveness assessment, based on plant dry weight as an index of nitrogen-fixation effectiveness (Date 2010), 154 of these were selected for further evaluation in the soil-pot experiments (Table 3).

ST09

Most strain treatments were better than uninoculated controls in the Narayen granite, Narayen brigalow and Holyrood soils and there were significant differences among strains within these 3 soils (Table 4). There were no significant differences between strain treatments and the uninoculated control plants growing in the Brian Pastures soil at the corresponding harvest time (Table 4), nor were there any differences after 4 successive regrowth periods. When soil was washed from plant roots after the fourth regrowth period, none of the plants was nodulated, suggesting that soil nitrogen remained at levels high enough for good plant growth and too high for plants to nodulate.

Table 2. Alternative delivery treatments for introducing *Bradyrhizobium* strains CB3546 and CB3481 for surface-sown Caatinga stylo (*Stylosanthes seabrana*) cv. Unica.

Treatment	Pretreatment June 1996	Inoculation December 1996
T1	Wheat inoculated with CB3546, sown 8–10 cm depth	Uninoculated Unica, surface-sown[1]
T2	Wheat inoculated with CB3546, sown 8–10 cm depth	Uninoculated Unica, surface-sown[1] 1996 over plastic prills inoculated with CB3481 and drilled to a depth of 8–10 cm
T3	Uninoculated wheat, sown 8–10 cm depth	Uninoculated Unica, surface-sown[1] over plastic prills inoculated with CB3546 and drilled to a depth of 8–10 cm
T4	Uninoculated wheat, sown 8–10 cm depth	Unica inoculated with CB3546, surface-sown[1]

[1]Surface-sown = broadcasting seed on surface, then raking of the top 0.5 cm soil and rolling to simulate commercial conditions.

Table 3. List of strains of *Bradyrhizobium*, host species of isolation and country of origin.

Strain	Host species	Country
Strains already in collection		
CB2126[1]	*Stylosanthes hamata*	Jamaica
CB2152[2]	*Stylosanthes hamata*	USA
CB2229	*Stylosanthes guianensis*	Costa Rica
CB2248	*Stylosanthes guianensis*	Costa Rica
CB2464	*Stylosanthes guianensis*	Brazil MG
CB2534	*Stylosanthes guianensis*	Australia
CB2797	*Macroptilium atropurpureum*	Brazil MG
CB2843	*Stylosanthes guianensis*	Australia
CB2851	*Stylosanthes guianensis*	Australia
CB3053[3]	*Stylosanthes hamata*	Antigua
Strains isolated from Edye field trials		
CB3451	*Stylosanthes seabrana*	Australia
CB3452	*Stylosanthes seabrana*	Australia
CB3453	*Stylosanthes seabrana*	Australia
CB3454	*Stylosanthes seabrana*	Australia
CB3455	*Stylosanthes seabrana*	Australia
CB3456	*Stylosanthes seabrana*	Australia
CB3486	*Stylosanthes seabrana*	Australia
CB3487[2]	*Stylosanthes seabrana*	Australia
CB3602	*Stylosanthes macrocephala*	Australia
CB3608	*Stylosanthes seabrana*	Australia
Strains isolated from 1992 and 1994 collections[4]		
CB3480[1]	*Stylosanthes seabrana*	Brazil
CB3481[1]	*Stylosanthes seabrana*	Brazil
CB3483[2]	*Stylosanthes seabrana*	Brazil
CB3484	*Stylosanthes seabrana*	Brazil
CB3485[2]	*Stylosanthes seabrana*	Brazil
CB3488[2]	*Stylosanthes seabrana*	Brazil
CB3489[2]	*Stylosanthes seabrana*	Brazil
CB3490[2]	*Stylosanthes seabrana*	Brazil
CB3491	*Stylosanthes seabrana*	Brazil
CB3492[2]	*Stylosanthes seabrana*	Brazil
CB3493[2]	*Stylosanthes seabrana*	Brazil
CB3494[2]	*Stylosanthes seabrana*	Brazil
CB3495[2]	*Stylosanthes seabrana*	Brazil
CB3496[2]	*Stylosanthes seabrana*	Brazil
CB3497[2]	*Stylosanthes seabrana*	Brazil
CB3581	*Stylosanthes* sp.[5]	Brazil
CB3582	*Stylosanthes* sp.[5]	Brazil
CB3583	*Stylosanthes seabrana*	Brazil
CB3584	*Stylosanthes* sp.[5]	Brazil
CB3585	*Stylosanthes* sp.[5]	Brazil
CB3586	*Stylosanthes* sp.[5]	Brazil
CB3587	*Stylosanthes seabrana*	Brazil
CB3589	*Stylosanthes* sp.[5]	Brazil
CB3590	*Stylosanthes seabrana*	Brazil
CB3591	*Stylosanthes* sp.[5]	Brazil
CB3592	*Stylosanthes* sp.[5]	Brazil
CB3593	*Stylosanthes seabrana*	Brazil
CB3594	*Stylosanthes seabrana*	Brazil
CB3595	*Stylosanthes seabrana*	Brazil
CB3596	*Stylosanthes* sp.[5]	Brazil
CB3597	*Stylosanthes* sp.[5]	Brazil
CB3598	*Stylosanthes seabrana*	Brazil
CB3599[3]	*Stylosanthes* sp.[5]	Brazil
CB3600[3]	*Stylosanthes seabrana*	Brazil
CB3601	*Stylosanthes* sp.[5]	Brazil
CB3603	*Stylosanthes seabrana*	Brazil
CB3604	*Stylosanthes seabrana*	Brazil
CB3605	*Stylosanthes seabrana*	Brazil
CB3606	*Stylosanthes seabrana*	Brazil
CB3607	*Stylosanthes seabrana*	Brazil
CB3609	*Stylosanthes seabrana*	Brazil
CB3610	*Stylosanthes seabrana*	Brazil
CB3611	*Stylosanthes seabrana*	Brazil
CB3612	*Stylosanthes seabrana*	Brazil
CB3613	*Stylosanthes seabrana*	Brazil
CB3614	*Stylosanthes seabrana*	Brazil
CB3615	*Stylosanthes seabrana*	Brazil
CB3616	*Stylosanthes seabrana*	Brazil
CB3617	*Stylosanthes seabrana*	Brazil
CB3618	*Stylosanthes seabrana*	Brazil
CB3619	*Stylosanthes seabrana*	Brazil
CB3620	*Stylosanthes seabrana*	Brazil
CB3621	*Stylosanthes seabrana*	Brazil
CB3622	*Stylosanthes seabrana*	Brazil
CB3623	*Stylosanthes seabrana*	Brazil
CB3624	*Stylosanthes seabrana*	Brazil
CB3625	*Stylosanthes seabrana*	Brazil
CB3626	*Stylosanthes seabrana*	Brazil
CB3627	*Stylosanthes seabrana*	Brazil
CB3628	*Stylosanthes seabrana*	Brazil
CB3629	*Stylosanthes seabrana*	Brazil
CB3630	*Stylosanthes seabrana*	Brazil
CB3631	*Stylosanthes seabrana*	Brazil
CB3632	*Stylosanthes seabrana*	Brazil
CB3633	*Stylosanthes seabrana*	Brazil
CB3634	*Stylosanthes seabrana*	Brazil
CB3635	*Stylosanthes seabrana*	Brazil
CB3636	*Stylosanthes seabrana*	Brazil
CB3637	*Stylosanthes seabrana*	Brazil
CB3638	*Stylosanthes seabrana*	Brazil
CB3639	*Stylosanthes seabrana*	Brazil
CB3639	*Stylosanthes seabrana*	Brazil
CB3640	*Stylosanthes seabrana*	Brazil
CB3641	*Stylosanthes seabrana*	Brazil
CB3642	*Stylosanthes seabrana*	Brazil
CB3643	*Stylosanthes seabrana*	Brazil
CB3644	*Stylosanthes seabrana*	Brazil
CB3645	*Stylosanthes seabrana*	Brazil
CB3646	*Stylosanthes seabrana*	Brazil
CB3648	*Stylosanthes seabrana*	Brazil
CB3649	*Stylosanthes seabrana*	Brazil
CB3650	*Stylosanthes seabrana*	Brazil
CB3651	*Stylosanthes seabrana*	Brazil
CB3652	*Stylosanthes seabrana*	Brazil
CB3653	*Stylosanthes seabrana*	Brazil
CB3654	*Stylosanthes seabrana*	Brazil
CB3655	*Stylosanthes seabrana*	Brazil

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Strain	Host species	Country
CB3656	*Stylosanthes seabrana*	Brazil
CB3657	*Stylosanthes seabrana*	Brazil
CB3658	*Stylosanthes seabrana*	Brazil
CB3659	*Stylosanthes seabrana*	Brazil
CB3660	*Stylosanthes seabrana*	Brazil
CB3661	*Stylosanthes seabrana*	Brazil
CB3662	*Stylosanthes seabrana*	Brazil
CB3663	*Stylosanthes seabrana*	Brazil
CB3664	*Stylosanthes seabrana*	Brazil
CB3665	*Stylosanthes seabrana*	Brazil
CB3666	*Stylosanthes seabrana*	Brazil
CB3667	*Stylosanthes seabrana*	Brazil
CB3668	*Stylosanthes seabrana*	Brazil
CB3669	*Stylosanthes seabrana*	Brazil
CB3670	*Stylosanthes seabrana*	Brazil
CB3671	*Stylosanthes seabrana*	Brazil
CB3672	*Stylosanthes seabrana*	Brazil
CB3673	*Stylosanthes seabrana*	Brazil
CB3674	*Stylosanthes seabrana*	Brazil
CB3675	*Stylosanthes seabrana*	Brazil
CB3676	*Stylosanthes seabrana*	Brazil
CB3677	*Stylosanthes seabrana*	Brazil
CB3678	*Stylosanthes seabrana*	Brazil
CB3679	*Stylosanthes seabrana*	Brazil
CB3680	*Stylosanthes seabrana*	Brazil
CB3681	*Stylosanthes seabrana*	Brazil
CB3682	*Stylosanthes seabrana*	Brazil
CB3683	*Stylosanthes seabrana*	Brazil
CB3684	*Stylosanthes seabrana*	Brazil
CB3685	*Stylosanthes seabrana*	Brazil
CB3686	*Stylosanthes seabrana*	Brazil
CB3687	*Stylosanthes seabrana*	Brazil
CB3688	*Stylosanthes seabrana*	Brazil
CB3689	*Stylosanthes seabrana*	Brazil
CB3690	*Stylosanthes seabrana*	Brazil
CB3691	*Stylosanthes seabrana*	Brazil
CB3692	*Stylosanthes seabrana*	Brazil
CB3693	*Stylosanthes seabrana*	Brazil
CB3694	*Stylosanthes seabrana*	Brazil
CB3696	*Stylosanthes seabrana*	Brazil
CB3697	*Stylosanthes seabrana*	Brazil
CB3698	*Stylosanthes seabrana*	Brazil
CB3699	*Stylosanthes seabrana*	Brazil
CB3700	*Stylosanthes seabrana*	Brazil
CB3701	*Stylosanthes seabrana*	Brazil
CB3702	*Stylosanthes seabrana*	Brazil
CB3703	*Stylosanthes seabrana*	Brazil
CB3704	*Stylosanthes macrocephala*	Brazil
CB3705	*Arachis* sp.	Brazil
CB3706	*Stylosanthes seabrana*	Brazil
CBX001	*Stylosanthes* sp.[5]	Brazil
CBX002	*Stylosanthes* sp.[5]	Brazil
CBX005	*Stylosanthes* sp.[5]	Brazil
CBX003	*Stylosanthes* sp.[5]	Brazil
CBX004	*Stylosanthes* sp.[5]	Brazil

Continued

[1]Strains used in 1995 and 1996 field trials.
[2]Strains used in 1996 field trials.
[3]Strains used in 1995 field trials.
[4]All strains isolated from nodule material collected in 1992 and 1994 from Bahia State, Brazil.
[5]Strains isolated from nodules formed on trap host *Macroptilium atropurpurem* inoculated with crushed nodules from *Stylosanthes* and authenticated by checking nodule-forming ability and effectiveness on *S. seabrana.*

Table 4. Plant top dry weights (g/pot) for Experiment ST09.

Strain	Soils (sites)			
	Brian Pastures	Narayen granite	Narayen brigalow	Holyrood
CB3053	8.1	11.0	5.6	11.1
CB3480	7.8	12.5	10.2	11.8
CB3581	8.2	10.3	4.7	9.4
CB3582	7.9	10.9	4.9	9.9
CB3584	8.0	9.4	3.5	7.9
CB3591	7.4	11.4	7.9	11.4
CB3592	8.0	11.5	8.5	11.0
CB3606	7.7	11.1	5.0	9.7
Uninoc control	7.6	9.6	3.7	7.6
LSD (5%)	0.6	0.6	0.6	0.6

ST25

At the 5-group level most strains formed effective nitrogen-fixing associations (Figure 1) but for some strains this was not obvious until after the regrowth harvest. Dry weight and leaf nitrogen data for a representative of each of the similarity groups are recorded in Table 5. Strains CB3480 and CB3586 were overall the most effective strains.

ST50

This group of strains showed some interaction responses between soils. Some differences were significant at the 1% level. Strains in Groups 1, 2, 3 and 4 were the most effective and had smaller dissimilarity values

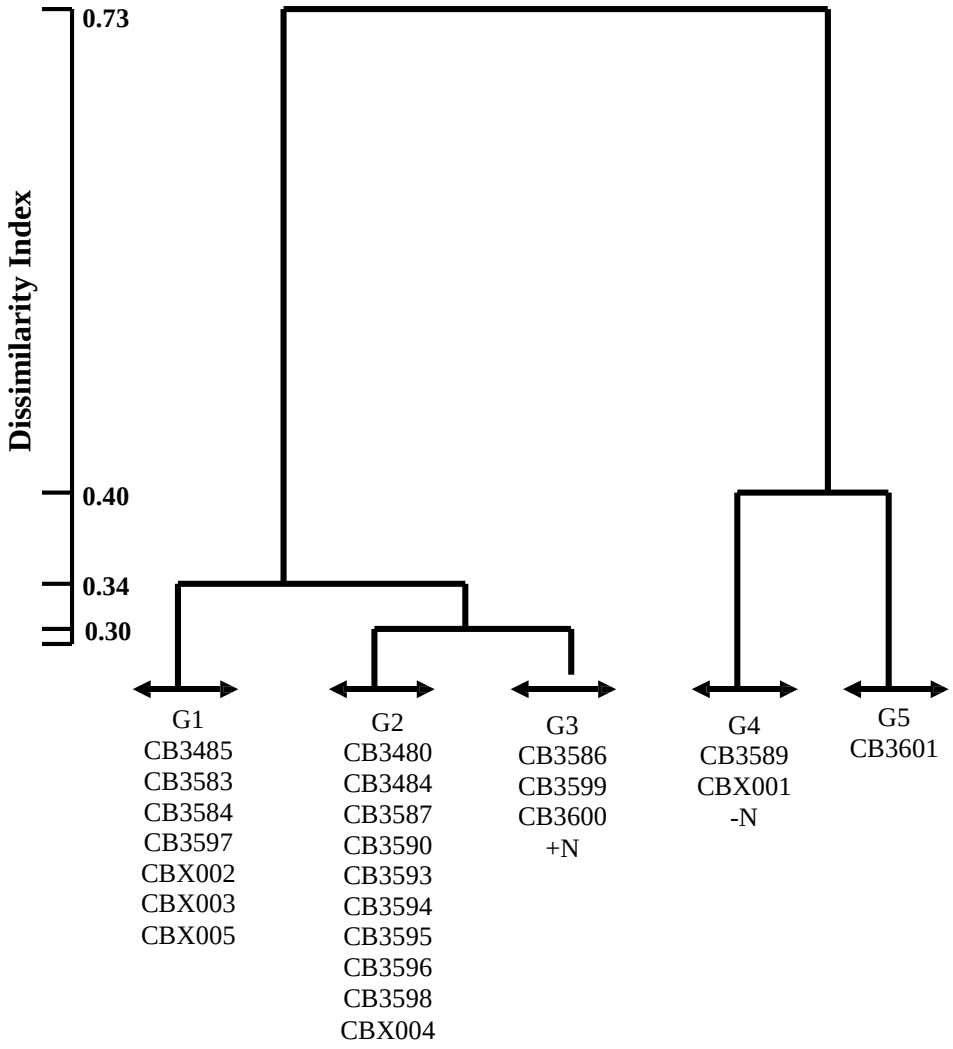

Figure 1. Similarity Groups G1–G5 of 23 strains of *Bradyrhizobium* effectiveness responses on Caatinga stylo (*Stylosanthes seabrana*) grown in Narayen granite and Narayen brigalow soils in soil-pot evaluations (ST25).

than the least effective strains in Group 5 (Figure 2 and Table 6). Strains CB3480, CB3489, CB3490, CB3491, CB3640, CB3673 and CB3684 were the best overall performers.

Group 5 strains had only 3–10 small (<0.5 mm) white nodules compared with many 0.5–1.5 mm nodules on plants in Groups 1, 2, 3 and 4. All nodules occurred at the root/lateral root/secondary root junctions.

ST130

As with Experiment ST50 there was a range of responses but most strains were effective (Figure 3 and Table 7). Dry weight data for the best-performing strains from the groups included those for strains CB3480, CB3481 and CB3489. There was a high level of dissimilarity between the effective strains in Groups 1, 2, 3 and 4 and those less-effective strains in Group 5.

Table 5. Top dry weight, leaf dry weight and leaf nitrogen content of Caatinga stylo grown in soil from Narayen granite (NG) and Narayen brigalow (NB) sites after inoculation with new strains of *Bradyrhizobium* collected in 1992 (ST25) with an unfertilized control and a N-fertilized control.

Group	Strain	Top dry weight (g)				Leaf dry weight (g)				Leaf nitrogen (mg)	
		NG		NB		NG		NB		NG	NB
		H1[1]	H2	H1	H2	H1	H2	H1	H2	H1	H1
G1	CB3583	3.2	3.1	17.3	3.4	2.6	1.5	8.2	1.6	53	131
G2	CB3480	5.0	2.4	19.1	4.0	2.1	1.2	10.0	1.8	54	229
G3	CB3586	4.3	2.7	20.3	3.6	2.3	1.3	10.8	1.7	57	238
G4	CB3589	2.1	3.1	14.5	2.8	2.3	1.5	7.7	1.3	33	95
G5	CB3601	2.2	2.1	16.4	1.6	1.8	1.1	8.2	0.7	21	93
Control	-N	2.2	2.5	12.1	1.4	2.4	1.3	6.5	0.7	28	79
Control	+N	3.5	2.6	20.8	3.4	2.4	1.3	11.1	1.6	41	206
	LSD (5%)	1.1	1.0	5.0	0.7	0.9	0.4	2.8	0.3	22	54

[1]H1 = Harvest 1; H2 = Harvest 2.

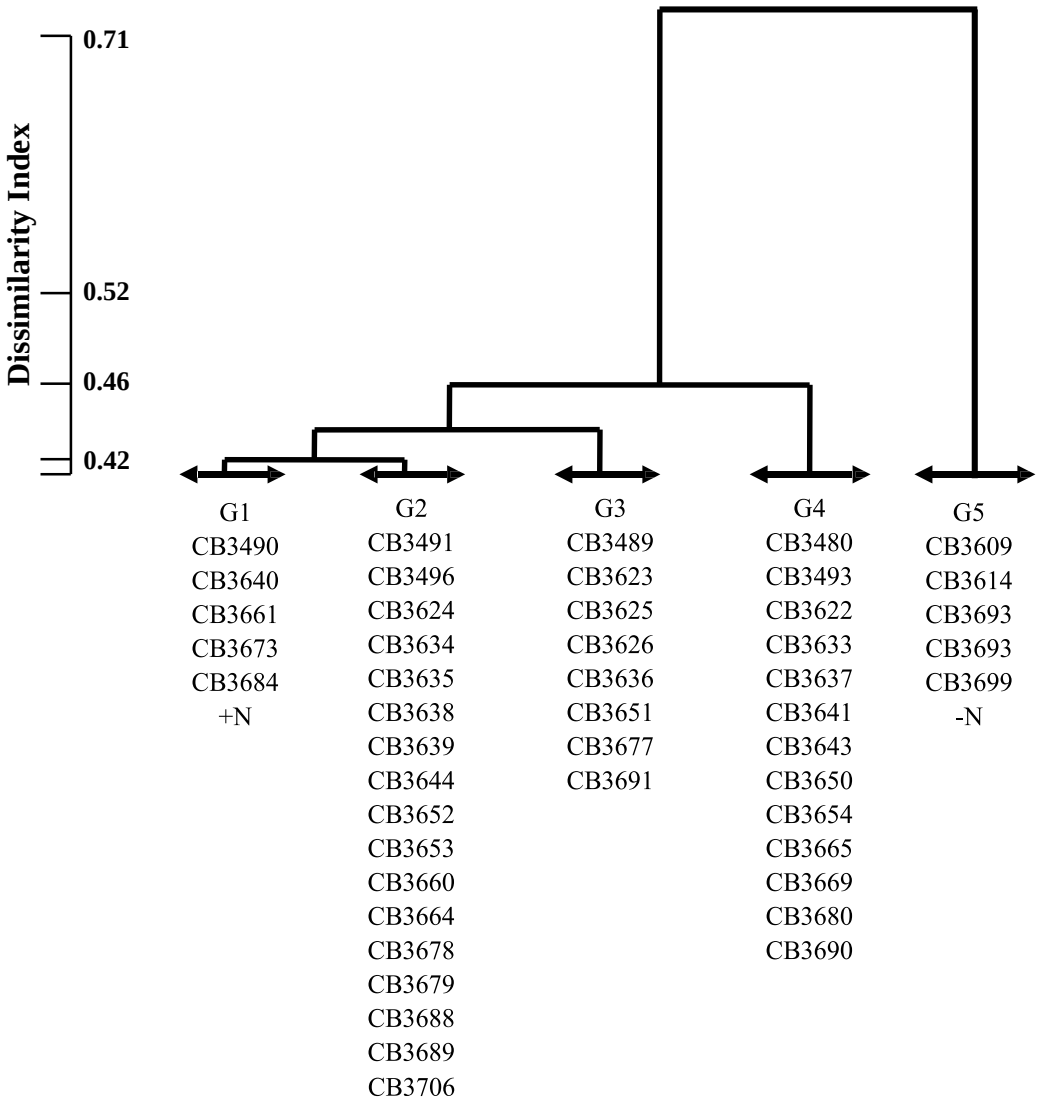

Figure 2. Similarity Groups G1–G5 of 47 new strains of *Bradyrhizobium* effectiveness responses on Caatinga stylo (*Stylosanthes seabrana*) grown in Narayen granite, Narayen brigalow, Holyrood and Hillgrove soils in soil-pot evaluations (ST50).

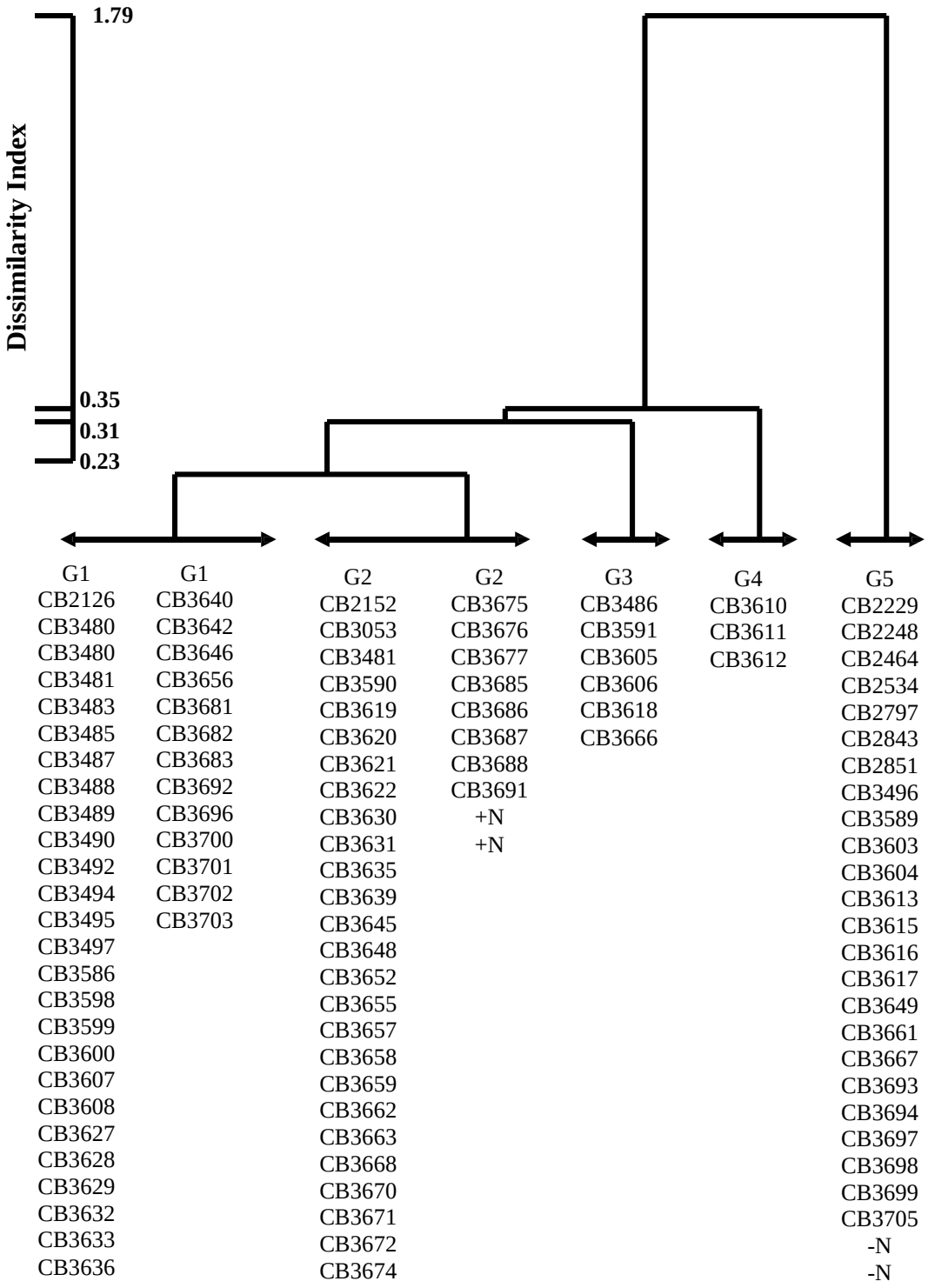

Figure 3. Similarity Groups G1-G5 of 94 new, 12 existing, 1 diagnostic and 3 of the best *Bradyrhizobium* strains from ST50 strains for effectiveness responses on Caatinga stylo (*Stylosanthes seabrana*) grown in Narayen granite, Narayen brigalow, Holyrood and Hillgrove soils in soil-pot evaluations (ST130).

Table 6. Plant-top dry weights for Caatinga stylo grown in soils from the Narayen granite (NG), Narayen brigalow (NB), Hillgrove and Holyrood sites inoculated with 47 new strains of *Bradyrhizobium* collected in 1994 (ST50) plus an unfertilized control and a N-fertilized control.

Group	Strain	Top dry weight (g/pot)							
		NG		NB		Hillgrove		Holyrood	
		H1[1]	H2	H1	H2	H1	H2	H1	H2
G1	CB3490	7.2	5.6	na[2]	6.9	7.7	6.5	5.8	6.2
G1	CB3640	7.6	5.5	na	7.4	8.3	7.5	6.3	6.6
G1	CB3661	3.6	3.2	na	3.6	7.9	4.8	4.4	5.8
G1	CB3673	6.2	5.3	na	6.2	7.9	5.4	5.5	5.5
G1	CB3684	6.3	5.4	na	8.3	7.9	6.5	5.7	5.7
G2	CB3491	7.4	5.1	na	6.8	8.0	7.3	6.8	7.6
G3	CB3489	8.1	6.2	na	7.5	7.1	6.1	6.3	6.8
G4	CB3480	7.7	5.3	na	8.7	8.6	7.1	6.5	6.8
G5	CB3699	2.2	0.7	na	3.3	7.8	5.1	4.4	4.0
Control	-N	2.1	0.8	na	2.9	9.2	6.8	3.9	4.1
Control	+N	4.7	9.0	na	9.3	7.9	9.5	5.7	7.6
	LSD (5%)	2.1	1.4		1.9	1.3	1.3	1.1	1.5
	LSD (1%)	2.8	1.9		2.6	1.7	1.7	1.5	2.0

[1]H1 = Harvest 1; H2 = Harvest 2.
[2]na = not available - many samples accidently destroyed. Of those recoverable top dry weight values ranged from 8.5 to 17.5 g/pot. Significantly there was no difference between samples for uninoculated and N-fertilized treatments.

Table 7. Plant top dry weights (g/pot) for best-performing strains of *Bradyrhizobium* on Caatinga stylo (*Stylosanthes seabrana*) grown in soils from Narayen granite and Holyrood sites inoculated with additional new strains of *Bradyrhizobium* collected in 1994 (ST130) plus an unfertilized control and a N-fertilized control.

Group	Strain	Whole tops				Leaves			
		Narayen granite		Holyrood		Narayen granite		Holyrood	
		H1[1]	H2	H1	H2	H1	H2	H1	H2
G1	CB3480	5.8	4.9	5.8	6.3	2.4	2.2	2.4	2.8
G1	CB3481	5.3	4.0	5.3	5.5	2.2	2.0	2.2	1.7
G1	CB3483	5.6	4.3	5.7	5.5	2.4	2.1	2.3	2.4
G1	CB3485	5.5	3.3	6.5	6.2	2.3	1.6	2.6	2.8
G1	CB3487	5.3	3.2	6.1	5.8	2.2	1.7	2.6	2.4
G1	CB3488	5.4	4.3	5.5	5.6	2.3	2.1	2.4	2.5
G1	CB3489	4.7	3.6	5.8	5.6	2.1	1.7	2.4	2.4
G1	CB3490	5.4	4.0	5.9	5.5	2.3	2.0	2.5	2.4
G1	CB3492	5.5	4.0	6.3	6.7	2.3	1.9	2.6	3.0
G1	CB3494	5.7	4.4	5.5	5.3	2.3	2.0	2.3	2.3
G1	CB3495	5.5	4.4	6.0	5.6	2.3	2.0	2.3	2.5
G1	CB3497	5.7	4.5	6.3	6.0	2.4	2.0	2.5	2.5
G2	CB3053	4.5	3.0	4.9	5.0	1.8	1.5	2.0	2.2
G2	CB3630	5.2	4.1	5.7	4.5	2.2	2.0	2.4	1.9
G3	CB3486	4.7	2.3	5.6	4.9	2.1	1.3	2.4	2.1
G3	CB3606	5.2	2.5	6.1	5.5	2.3	1.2	2.5	2.4
G4	CB3610	3.8	3.3	4.7	4.8	1.6	1.6	2.7	2.0
G5	CB3616	2.9	1.4	3.6	2.6	1.4	0.8	1.8	1.2
G5	CB3693	1.9	0.7	3.3	2.8	0.9	0.4	1.6	1.3
	-N	2.7	1.1	3.4	3.2	1.2	0.6	1.6	1.4
	+N	5.1	3.1	5.4	5.0	2.1	1.6	2.4	1.9
	LSD (5%)	0.6	0.9	0.7	1.2	0.3	0.4	0.4	0.6
	LSD (1%)	0.8	1.2	0.9	1.6	0.4	0.6	0.5	0.7

[1]H1 = Harvest 1; H2 = Harvest 2.

There were scattered small (<0.5 mm) nodules on the roots of plants in Group 5, whereas in Group 1 both tap and lateral roots were profusely populated with larger (1–1.5 mm) nodules. Similar but less numerous nodulation was recorded for plants in Groups 2, 3 and 4. Overall there were fewer nodules on plants grown in Narayen granite soil than in Holyrood soil.

Field experiments

In the trials sown in 1995 only strains CB3053, CB3480 and CB3481 had dry weight yields greater than the uninoculated controls. Strains CB2126, CB3599 and CB3600 failed to produce responses and were not harvested (Tables 8 and 9). Strains CB3053 and CB3481 formed the majority of nodules and only CB3481 maintained this high level in successive years. A significant level of 'contamination' of control plots by CB3481 was observed in later years (Tables 8 and 9).

As with the trials sown in 1995 only those plots in the trials sown in 1996 that demonstrated better growth responses than the uninoculated controls were harvested for plant dry weight yield and determination of the proportion of nodules formed by the inoculum strains (Tables 10 and 11). The proportional differences between inoculated and uninoculated treatments for plant dry weight yield increased in successive years and were greater at the Narayen granite and Lansdown sites than at Holyrood (Table 10). Strains CB3481, CB3494 and CB3495 formed a high proportion of the nodules at all sites and in all years (Table 11), although CB3494 failed to improve yield and form nodules in the year of sowing (1996). Strains CB3488 and CB3489 also formed a large proportion of the nodules at the Holyrood and Narayen granite sites but failed at Lansdown. There was significant 'contamination' of some control plots from adjacent inoculated rows in some replications (see ad hoc notes Table 12).

Table 8. Proportion of nodules formed by inoculum strains of *Bradyrhizobium* on Caatinga stylo (*Stylosanthes seabrana*) in trials sown in 1995. (See explanatory notes 1, 2 and 3).

Site	% nodules formed			
	CB3053[1]	CB3480	CB3481	Control[2]
Holyrood				
Apr-95	11	0	100	0
Apr-96	34	1	72	2 (2% CB3053)
Apr-97	0	0	75	0
Apr-98	-	-	-	-
Narayen granite				
Apr-95	25	1	44	0
Jan-96	39	8	96	1
May-97	78	0	98	0
Mar-98[3]	69		90	38
Lansdown				
May-95	31	0	47	0
Jan-96	81	25	86	0
May-96	-	34	81	0
May-97	-	-	59	31
Mar-98	11	-	67	13 (4% CB3053)

[1]Six strains CB2126, CB3053, CB3480, CB3481, CB3599 and CB3600 were assessed in the 1995 Strain Trial. Strains CB2126, CB3599 and CB3600 failed to respond and were not harvested.

[2]Values in control columns are for positive identification of CB3481. Values in brackets refer to the strain indicated.

[3]For Narayen Mar-98 and Lansdown May-97 and Mar-98, 2 of the 3 replicates of controls were adjacent to plots of CB3481.

Table 9. Relative yields (as % best treatment in each year) for inoculated Caatinga stylo (*Stylosanthes seabrana*) in strain trials sown in 1995. (See explanatory notes 1, 2 and 3).

Site	CB3053	CB3480	CB3481	Control[3]
Holyrood				
Apr-95	79	48	100	85
Apr-96	29	29	100	31
Apr-97	-	-	100	21
Apr-98	-	-	-	-
Narayen granite				
Apr-95	<----------Lost to wildlife[1]---------->			
Jan-96	54	61	100	25
May-97	30	8	100	6
Mar-98	22	-	100	32
Lansdown				
May-95	100	85	57[2]	49
Jan-96	23	19[2]	100	14
May-96	-	27	100	30
May-97	-	-	100	47
Mar-98	51	-	100	83

[1]Data for Narayen Apr-95 unreliable, due to grazing by wildlife prior to harvest.
[2]Data for CB3481 Lansdown May-95 and CB3480 in one replication may be unreliable due to accidental damage by wind drift herbicide.
[3]Yields of controls for Narayen Mar-98 and Lansdown May-97 and Mar-98 high due to contamination by effective N-fixing strains.

Table 10. Summary relative plant dry weights (% best for year) for inoculated Caatinga stylo (*Stylosanthes seabrana*) for strain trials sown in 1996.

Strain	Holyrood				Narayen granite			Lansdown		
	1996	1997	1998	1999	1996	1997	1998	1996	1997	1998
CB2152					35					
CB3480	100				49			59		
CB3481	88	76	97	88	30	34	44	74	63	47
CB3483		48	65	68		42	45		64	73
CB3485			45							
CB3486					100					
CB3488		54	75	53		42	90			
CB3489		74	81	87	75	49	79		58	
CB3490			67	100		34	68			100
CB3491									69	
CB3494		44	90	59		57	100			95
CB3495		100	99	91	87	100	86		100	90
CB3497		36	100							
Control	65	37	56	35	58	36	14	100	59	28

Table 11. Proportion (%) of nodules formed on Caatinga stylo (*Stylosanthes seabrana*) by inoculum strains for strain trials sown in 1996. (See explanatory notes 1 to 6).

Strain[1]	Holyrood				Narayen granite			Lansdown		
	1996	1997	1998	1999	1996	1997	1998	1996	1997	1998
CB2152	-	-	-	-	2	-	-	-	-	-
CB3480	15	-	-	-	0	-	-	0	-	-
CB3481	39	63	87	75	3	91	89	22	87	80
CB3483	-	0	0	0	-	0	0	-	3	5
CB3485	-	-	-	-	nd[6]	-	-	-	-	-
CB3486	-	-	-	-	-	nd	nd	-	-	-
CB3488	-	39	83	72		97	90			
CB3489	-	0	94	92	14	0	95	-	0	-
CB3490	-	-	85	91	-	80	94	-	-	83
CB3491	-	-	-	-	-	-	-	-	6	-
CB3494	-	96	93	100	-	96	97	-		100
CB3495	-	96	94	91	13	98	98		96	91
CB3497	-	51	19	-	-	-	-	-	-	-
Control	2[2]	2[3]	6[4] (CB3481) 34 (CB3495) 30 (CB3488)	6[5](CB3481) 42(CB3495) 0 (CB3488)	0	5	4	0	0	25

[1]Eighteen (18) strains were selected for assessment in 1996. Five (5) strains (CB2841, CB3487, CB3492, CB3493 and CB3496) failed to respond at any site and were not harvested.
[2]Holyrood 1996. Control tested against antiserum for only CB3481.
[3]Holyrood 1997. Control tested against only CB3481 (1.5%) and CB3495 (1.7%).
[4]Holyrood 1998. Control vs. CB3481 (6%), CB3495 (34%) and CB3488 (30%) – mostly in 2 of the 3 replicates where control plots were near or adjacent to indicated strain plots.
[5]Holyrood 1999. Control vs. CB3481 (6%), CB3495 (42%), CB3488 (0%) and CB3497 (0%).
[6]nd = not determined – antisera for serological identification not available.

Table 12. Ad hoc observational evidence for spread of strains CB3481 and CB3495 in strain trials sown in 1996.

Site	Year	Strain	Comment
Holyrood	1996	1.5% CB3481	Mostly in Rep 1, Control row not adjacent to an inoculated CB3481 row; no check for other strains
	1997	1.5% CB3481	Mostly in Rep 1, Control row not adjacent to an inoculated CB3481 row; no check for other strains
		1.7% CB3495	Mostly in Reps 1 and 2, Control row not adjacent to an inoculated CB3495 row
	1998	6% CB3481	Mostly in Rep 2, Control row 2 rows away from inoculated CB3481 row
		34% CB3495	Mostly in Reps 1 and 2, Control row adjacent to an inoculated CB3495 row in Rep 2
		30% CB3488	Mostly in Reps 2 and 3, Control row adjacent to an inoculated CB3488 row in both Reps
	1999	6.4% CB3481	Mostly in Rep 2, Control row not near an inoculated CB3481 row
		42% CB3495	Mostly in Rep 1, Control row immediately adjacent to an inoculated CB3495 row
Lansdown	1998	56% CB3495	Mostly in Reps 2 and 3, Control rows not adjacent to an inoculated CB3495 row but area under surface water due to cyclonic weather conditions in 1997

Inoculum delivery

For Caatinga stylo the introduction of the bradyrhizobia by inoculation of wheat seed sown in June (T1) was better than placing the bradyrhizobia 10 cm below the legume seed in the normal December-January sowing (T3) (Table 13). At the Holyrood site the combined total of nodules identifiable as CB3481 and CB3546 in T2 was similar to that for CB3546 alone in T1, but there was a trend for nodules from CB3481 to increase with time in T2 with a concomitant decline in those from CB3546. Nodule formation by strain CB3546 also declined in T1. There were similar but less marked declines at Roma Research Station and Banoona sites. Deep placement of the inoculum either on a preceding cereal crop (T1 and T2) or on inert plastic prills (beads) (T3) at the time of sowing the Caatinga stylo provided more nodules than when introduced as inoculum on the surface-sown legume seed (T4). The poor result in T4 confirms that inoculation of surface-sown seed for this legume is a risky practice (Table 13).

For farming systems for which these new cultivars have been selected, the preferred method of sowing the legume is as an associated crop with the cereal, often into dry soils and at high soil surface temperatures (>50 °C for 4–6 h/d; D.A. Eagles and R.A. Date unpublished data). Corresponding bare-soil temperatures at 2, 5 and 10 cm depths were, respectively, 40–50 °C for 6–8 h/d, 30–40 °C for 4–6 h/d, and frequently >30 °C for 8–10 h/d (D.A. Eagles and R.A. Date unpublished data). Temperature profiles at 0, 2, 5 and 10 cm depth for the Holyrood site are recorded in Figure 4 for the 1996-1997 season. Similar profiles were recorded for the 1997-1998 season and for the Narayen granite, Narayen brigalow and Roma Research Station sites in both seasons.

Discussion

This series of soil-pot assessments was aimed at selecting suitable strains of *Bradyrhizobium* for Caatinga stylo (*Stylosanthes seabrana*) in order to advance the release of the 2 new cultivars, Primar and Unica. Several strains, which showed particular promise, were selected and evaluated for their ability to survive in field situations and to form nodules in second and subsequent growing seasons, when soil nitrogen had been depleted. Twenty-four strains based on plant dry weight yield (used as an index of nitrogen-fixation effectiveness) were selected: 6 in trials sown in 1995 and 18 in 1996 at the Holyrood, Narayen granite and Lansdown sites.

These strains produced 2−7 fold increases in plant dry weight, especially in the 2nd, 3rd and 4th (for Holyrood) growing seasons, and accounted for the majority of nodules formed. Based on data from trials sown in 1995 (Tables 8 and 9), strain CB3481 was released to industry for the inoculation of the new cultivars, Primar and Unica, in 1997. As well as confirming the ability of CB3481 to satisfactorily nodulate *S. seabrana* over a 3-year period, the data from field trials sown in 1996 (Tables 10 and 11) have identified several additional strains that could serve as replacement strains for CB3481, if this became necessary. There was good evidence of the ability of these strains to spread to neighbouring areas as indicated by the proportions of strains CB3481, CB3488 and CB3495 found in uninoculated control plots (Tables 8, 10 and 12).

Only cv. Unica was used in these trials, due to limited availability of seed of cv. Primar seed at the time of experimentation; however, nitrogen-fixation effectiveness responses of the 2 cultivars in N-free glasshouse assessments (Date 2010) and in separate soil-pot experiments

Table 13. Percentage recovery of nodules containing inoculum strains CB3546 and CB3481 for alternative delivery trials at Holyrood, Roma Research Station and Banoona sites[1].

Site	Year	Treatment[2]				
		T1	T2/CB3546	T2/CB3481	T3	T4
Holyrood	1997	53	30	37	27	6
	1998	38	13	17	29	7
	1999	19	0	51	1	3
Roma Res. Stn	1997	5	7	0	5	3
	1998	4	7	17	5	4
Banoona	1997	-	-	-	-	-
	1998	1	3	27	0	0
	1999	2	2	16	6	1

[1]In random samples of nodules from adjacent QDAFF trials tested for strain CB3481 in May 1998, there were no nodules on Primar and 3% on Unica and in March 1999 values were 70 and 40%, respectively, for the 2 cultivars.
[2]Treatments T1, T2, T3 and T4 are described in Table 2.

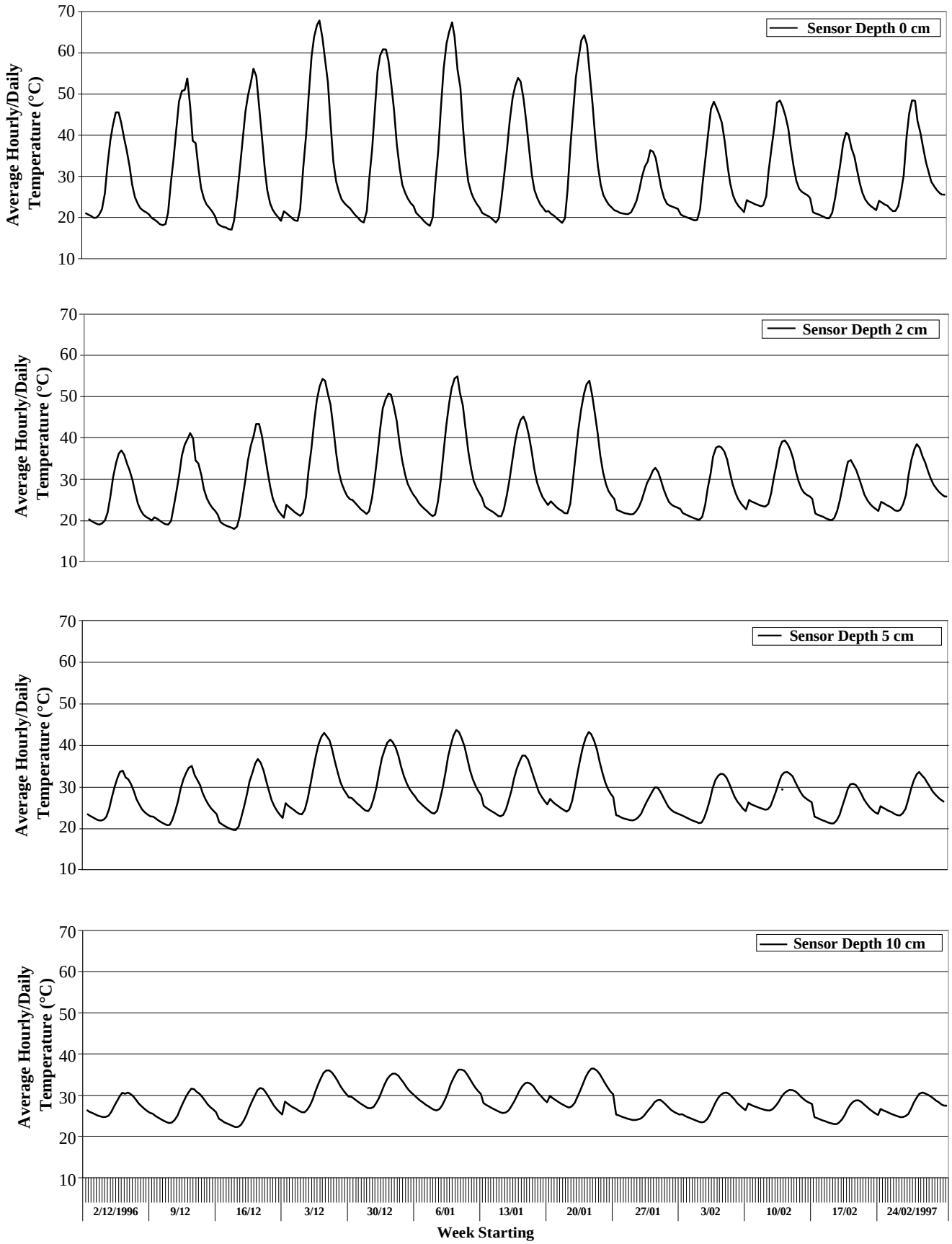

Figure 4. Holyrood site bare-soil temperature profiles 1996-1997 at 0, 2, 5 and 10 cm depth.

confirmed that both cultivars responded in the same way with the recommended strains of *Bradyrhizobium*. The good percentages of nodules identified as CB3481 on both cultivars in the commercial sowings (see Table 13) in the third growing season compared with the recoveries in the second growing season provide separate evidence of the inability of Caatinga stylo to nodulate, while soil nitrogen is available in the initial years, and that the recommended inoculum strain CB3481 persists into the second and third growing seasons.

The alternative delivery experiments suggest that deep placement of the inoculum, either by inoculation of a prior crop, e.g. wheat, or on an inert carrier, e.g. plastic prills, may confer an advantage over inoculation of surface-sown Caatinga stylo seed for producers in clay-soil cropping systems, where soil temperatures are detrimental to the inoculum *Bradyrhizobium*.

Commercially prepared inoculum (peat-based and freeze-dried forms) of strain CB3481 has been available since 1998. If new germplasm material is required for additional cultivar and strain selection and evaluation, the genetic resource collection of *S. seabrana* germplasm now resides with SARDI/Australian Pastures Genebank and the root-nodule bacteria (*Bradyrhizobium*) collection with the Centre for *Rhizobium* Studies (CRS), Murdoch University, Western Australia.

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