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Alternative Delivery Systems for the Inoculation of New Strains of Stylo

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Principal Investigator: Dr R.A. Date
CSIRO Tropical Agriculture
Cunningham Laboratory
306 Carmody Road
St Lucia Qld 4067

Telephone: 07 3214 2260
Fax: 07 3214 2288
E-mail: Dick.Date @tag.esiro.au

Project Objectives:

i) To release suitable strains of root-nodule bacteria (RNB) that demonstrate effective nodulation of *S.* sp.aff. *S. scabra* (now known as Caatinga stylo - *Stylosanthes seabrana.* Two cultivars, Primar and Unica have been released).

ii) To identify alternative delivery systems for establishing effective strains of RNB in soil prior to sowing S. sp.aff. *S. scabra.*

Project Summary:

This project is allied to CS079/CS153 concerning the selection of new cultivars of stylosanthes from the *Stylosanthes hamata* and *S. seabrana* germplasm. Selection of effective nitrogen fixing strains of rhizobia became essential when it was demonstrated that the performance of *S. seabrana* was declining because field grown plants were either not nodulating or not fixing nitrogen. New strains of RNB collected in Brazil in 1994 (MRC separate funding) proved to be effective and persistent over a three year period. Strain CB3481 was released to industry for the 1997/98 growing season and additional effective and field competent strains have been identified.

Dry-soil, near-surface sowing of small seeded legumes prior to the onset of seasonal rainfall is frequently the method of choice of producers in clay-soil arable land situations. Soil surface temperatures under these conditions frequently exceed 50 °C for 4-6 hr/day and are lethal to rhizobia introduced on the legume seed. Experiments assessing alternative methods of delivery were established at 5 sites in southern Queenlsand. The two major treatments were the introduction of the rhizobia to the soil with a cereal crop grown in the season preceding the sowing of the legume and deep placement of the inoculum at the time of sowing the legume. For Caatinga Stylo the introduction of the rhizobia by inoculation of wheat seed sown in May/June was better than placing the rhizobia 10 cm below the legume seed in the normal December/January sowing. Deep placement and seed inoculation were better treatments for Jaribu desmanthus.

The need to inoculate Caatinga stylo and the availability the selected strain of rhizobia have been publicised by newsletter and field day demonstrations.

Results:

Selection of strains of RNB.

Two series of field trials were established. One in January 1995 to assess the then available strains of RNB and one in January 1996 to assess new strains developed from glasshouse soil-pot assessment of rhizobia 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 and Brigalow soils) and in a red earth soil near Roma ("Holyrood"). The experiments failed to establish on the Narayen Brigalow soil, but were successfully established and managed over three years at the other sites. Yield (plant dry weight) data and proportion of nodules formed by the test strains (Tables 1, 2, 3 and 4) have identified several strains as highly effective and persistent. These strains produced 3-5 fold increases in plant dry weight, especially in the 2nd and 3rd growing seasons and they accounted for the majority of nodules formed. Based on the data from the 1995-sown trials (Tables 1 and 2), strain CB3481 was released to industry with the new cultivars Primar and Unica in 1997. As well as confirm the ability of CB3481 to satisfactorily nodulate *S. seabrana* over a 3 year period, the data from the 1996-sown trials (Tables 3 and 4), have identified several additional strains that could serve as replacement strains for CB3481, if this became necessary. Also, there was good evidence of the ability of these strains to spread to neighbouring areas as indicated in the proportions of strain CB3481, CB3488 and CB3495 found in uninoculated control plots (Table 5).

Alternative delivery of inoculum strains

Normal seed inoculation of *S. seabrana* is not a practical methodology for introducing these bacteria since the host plant is most often sown in mid-summer when soil is dry and near-surface temperatures exceed 40 and 50 °C for up to 8 hours per day. These temperatures are lethal to the RNB which survive only 1-3 days under such conditions. (Note: The experiments for selecting the new strains were completed using irrigation immediately after sowing to avoid this restriction).

Experiments established in January 1995 failed to provide satisfactory results since a non-field competent strain CB3480 was used (see Tables 1, 2, 3 and 4). A new series of experiments was initiated in which the success of rhizobia established in the soil prior to sowing the Caatinga Stylo was compared with that when the rhizobia were drilled in below the Caatinga Stylo at the time of sowing. Thus, wheat inoculated with CB3456 was sown in May/June (winter) to allow the rhizobia to establish in the soil, and the plot then oversown with uninoculated seed of Caatinga Stylo in December (summer). By this method it was anticipated that the RNB could establish in the soil and rhizosphere of the cereal crop and develop a sufficient population at a depth that would avoid the lethal temperatures of the summer period. In the second main treatment CB3546 was introduced by inoculation of polyethylene prills drilled-in to a depth of 10 cm under the oversown stylo seed in December. It was anticipated that soil temperature at a depth of 10 cm would be less adverse for the survival of the rhizobia. A cultivation or no cultivation treatment prior to oversowing the Caatinga Stylo was imposed on both delivery treatments (see details Table 6). Replicated trails were established at Narayen (Granite and Brigalow soils), Roma (red earth and 2 vertisol soils). Strain CB3546 is an antibiotic resistant variant of CB3481).

The growing seasons of the 1993 to 1999 period in southern Queensland were characterised by lower than expected and unevenly distributed rainfall. It was therefore not possible to harvest at all sites in the three years of the trials. The spaces in the Tables 7 and 8 indicate that no harvest for either nodules or plant dry weights was attempted because there had been little or no growth and or there were no obvious differences between treatments at the time of harvest. Specifically, there were no growth (plant dry weight) differences in samples taken in April/May 1996 nor were there many nodules. However, in April/May 1997, 1998 and 1999, there were nodules and dry weight differences (Tables 7 and 8). Strain treatments that were (visually) not better than the uninoculated control were not harvested. Plant dry weights have been expressed on a relative basis as a percentage of the best treatment within each site/year combination. Proportions of nodules formed by the original inoculum strain(s) was determined by serological typing of "squashed" nodule samples "stained" with specific antiserum, developed previously to each strain, and "counter-stained" with an FITC labelled goat anti-rabbit serum to visualise positive reactions via fluorescent microscopy.

Significant recovery of CB3546 was obtained at the Holyrood site but was less than significant at the other sites. At the Holyrood site, inoculation of the cereal crop resulted in higher plant yields and greater proportions of nodules due to CB3546. than were obtained where CB3546 was introduced either on plastic prills at 10 cm or on the surface-sown seed (Treatments 4,5 and 6). Treatment 6 represents the standard inoculation sowing regime used for sowing inoculated legume seed.

The failure to obtain responses at the Narayen Granite soil site cannot be explained. Visual rating of the plots in April/May 1996 indicated a growth difference in favour of Treatments 1 and 2, but this was not maintained in the 1996/97 season, when all plants were yellow and poorly grown. The failure of CB3546 to persist in this soil suggests that is not as field competent as CB3481 and was unable to persist and form nodules despite the absence of suitable native strains for Caatinga Stylo at this site.

A very significant observation was the relatively good proportions of CB3481 that were recovered in Treatment 3 (see Tables 6 and 7), despite the fact that it was applied to surface-sown seed. This information attests to the colonising and persistence of CB3481 and is additional support for retaining it as the recommended inoculum strain for Caatinga Stylo.

Soil temperature at the surface, 2, 5 and 10 cm depths were recorded during the experiments at Holyrood, Roma Research Station and at the two soil-type sites at the Narayen Research Station. Some typical soil temperature profiles, for the Holyrood site, are illustrated in Figure 1. These are temperatures for bare-soil and represent temperatures that might be experienced during summer when Caatinga Stylo is sown. Even at 10 cm soil temperature could be adverse to survival of seed-inoculated cells of rhizobia.

If Caatinga Stylo is dry-sown in summer conditions, this series of experiments suggests that until techniques for better survival of the inoculum on seed are available, inoculation of a preceding cereal crop affords a method of introducing CB3481 where Caatinga Stylo is to be grown for the first time. Standard inoculation and sowing is recommended where soil moisture is adequate for seed germination or if irrigation is available as in the trials reported herein.

A similar experiment with *Desmanthus virgatus* cv Jaribu was established at the Holyrood site. Treatments 1, 2, 5 and 6 (Table 6) were the same as used for Caatinga Stylo except that strain CB3547 (an antibiotic resistant selection of the recommended commercial inoculum strain CB3126) and Jartibu desmanthus replaced the CB3546/Caatinga Stylo combination. Results (Tables 7 and 8) were the reverse of those for Caatinga Stylo. In this instance the freshly inoculated and surface sown seed gave better relative yields and proportions of inoculum rhizobia (CB3126) than were obtained by inoculating the cereal sown in the preceding season. The faster-growing rhizobia for desmanthus may be less able to colonise and persist in the soil than the slower-growing rhizobia for Caatinga Stylo.

Other Comments:

Soil pot work indicated that Caatinga Stylo 1) is not nodulated effectively by native rhizobia, and 2) formation of nodules is very sensitive to available soil nitrogen. Nodulation of Caatinga Stylo is completely inhibited by available soil nitrogen. This observation explains Les Eyde's observations that plants were 'turning yellow" and were not nodulated. The implications for introducing new RNB are that they need survive not only the dry, high temperature establishment conditions but must survive 1 or 2 growing seasons as free living organisms until soil N levels decline. Data from the strain selection trials suggest that the strains have this capacity.

Papers, reports, media articles.

- Date, R.A., Eyde, L.A. and Liu, C.J. (1996) *Stylosanthes sp.aff. S. scabra* - a potential new forage plant for northern Australia. Tropical Grasslands 30, 133.
- Date, R. A. Demonstration of RNB strain selection plots and need for inoculation of *S.* sp.aff. *S. scabra.* CSIRO Tropical Agriculture Field Day, Lansdown Research Station, April 1997.
- Stylos for better beef. (1996) Partridge, I, Middleton, C and Shaw, K. QDPI, Brisbane.
- Progress reports in the Legumes for Clay Soils Newsletters.
- Three research papers based on MRC supported research are in preparation and will be submitted to the journal Tropical Grasslands by December 2000; their approximate titles are:

Date, R.A. and Eagles, D.A. (2001) Selection of effective bradyrhizobia for Caatinga Stylo (*Stylosanthes seabrana*): I. Isolation, authentication and soil-pot screening for effectiveness of nitrogen fixation.

Date, R.A. and Eagles, D.A. (2001) Selection of effective bradyrhizobia for Caatinga Stylo (*Stylosanthes seabrana*): II. Field assessment for persistence and effectiveness of nitrogen fixation.

Date, R.A. and Eagles, D.A (2001) An unusual rhizobia: acidic and micro-aerophyllic conditions required for isolation and maximum growth in artificial culture.

Table 1. Strain trial 1995. Proportion of nodules formed on Caatinga Stylo by inoculum strains

Holyrood	<------------------------%Nods-------------------------->			
	CB3053	CB3480	CB3481	Control
Apr-95	11	0	100	0
Apr-96	34	1	72	2 (2% CB3053)
Apr-97	0	0	75	0
Apr-98				

Narayen Granite	CB3053	CB3480	CB3481	Control
Apr-95	25	1	44	0
Jan-96	39	8	96	1
May-97	78	0	98	0
Mar-98	69		90	38

Lansdown	CB3053	CB3480	CB3481	Control
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)

Notes on Table 1.

1. Six strains CB2126, CB3053, CB3480, RAD969/4, RAD969/11, CB3481 were assessed in the 1995 Strain Trial. Strains CB2126, RAD969/4, RAD969/11 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 2. Strain trial 1995. Relative yield (as % best treatment in each year) for inoculated Caatinga Stylo

Holyrood	CB3053	CB3480	CB3481	Control
Apr-95	79	48	100	85
Apr-96	29	29	100	31
Apr-97			100	21
Apr-98				

Narayen Granite	CB3053	CB3480	CB3481	Control
Apr-95	←----------Lost to Wildlife------------->			
Jan-96	54	61	100	25
May-97	30	8	100	6
Mar-98	22		100	32

Lansdown	CB3053	CB3480	CB3481	Control
May-95	100	85	57	49
Jan-96	23	19	100	14
May-96		27	100	30
May-97			100	47
Mar-98	51		100	83

Notes on Table 2.

1. Data for Narayen Apr-95 unreliable due to grazing by wildlife prior to harvest.
2. Data for Lansdown May-95 unreliable due to accidental damage by wind drift herbicide.
3. Yield of controls for Narayen Mar-98, Lansdown May-97 and Mar-98 high due to contamination by effective N-fixing strains (see Notes on Table 1).

Table 3. Strain trial 1996. Summary proportion (%) nodules formed on Caatinga Stylo by inoculum strains

Strain	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					
CB3486						nd	nd			
CB3488		39	83	72		97	90			
CB3489		0	94	92	14	0	95		0.0	
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	6 (CB3481)	6 (CB3481)	0	5	4	0	0	25
			34 (CB3495)	42(CB3495)						
			30 (CB3488)	0 (CB3488)						

Notes on Table 3.

1. Eighteen (18) strains were selected for assessment in1996. Five (5) strains, CB2841, CB3487, CB3492, CB3493, CB3496 failed to respond at any site and were not harvested.
2. Holyrood 1996. Control tested only against antiserum for CB3481.
3. Holyrood 1997. Control tested against CB3481 (1.5%) and CB3495 (1.7%)
4. Holyrood 1998. Control versus CB3481 (6%), CB3495 (34%), CB3488 (30%) – mostly in 2 of the 3 replicates where control plots were near or adjacent to indicated strain plots.
5. Holyrood 1999. Control versus CB3481 (6%), CB3495 (42%), CB3488 (0%), CB3497 (0%).
6. nd = not determined – antisera for serological identification not available.

Table 4. Strain trial 1996. Summary Relative plant dry weights (% best for year) for inoculated Caatinga Stylo

	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 5. Strain trial 1996. *Ad hoc* evidence for spread of strains CB3481 and CB3495

Site	Year	Strain	Comment
Holyrood	1996	1.5% CB3481	Mostly in Rep 1, Control plot not adjacent to a inoculated CB3481 plot; no check for other strains
	1997	1.5% CB3481	Mostly in Rep 1, Control plot not adjacent to a inoculated CB3481 plot; no check for other strains
		1.7% CB3495	Mostly in Reps 1 and 2; Control plot not adjacent to an inoculated CB3495 plot
	1998	6% CB3481	Mostly in Rep2, Control plot 2 plots away from inoculated CB3481 plot
		34% CB3495	Mostly Reps 1 and 2, adjacent inoculated CB3495 plot in Rep 2
		30% CB3488	Mostly Reps 2 and 3, adjacent inoculated CB3488 in both Reps
	1999	6.4% CB3481	Mostly Rep 2. Control plot not near inoculated CB3481
		42% CB3495	Mostly Rep 1. Control plot immediately adjacent inoculated CB3495 plot
Lansdown	1998	56% CB3495	Mostly in Reps 2 and 3, Control plots not adjacent inoculated CB3495 but area under surface water due to cyclonic weather conditions in 1997

Table 6. Treatments used for the alternative delivery of inoculum rhizobia

Treatment number	Pretreatment	Cultivation	Inoculation
1	Wheat inoculated with CB3546, sown June 1996	No cultivation December 1996	Uninoculated Unica sown December 1996
2	Wheat inoculated with CB3546, sown June 1996	Cultivated at sowing December 1996	Uninoculated Unica sown December 1996
3	Wheat inoculated with CB3546, sown June 1996	No cultivation December 1996	Uninoculated Unica sown December 1996 and plastic prills inoculated with CB3481 drilled to depth of 10 cm in December 1996
4	Uninoculated wheat sown June 1996	No cultivation December 1996	Uninoculated Unica sown December 1996 and plastic prills inoculated with CB3546 drilled to depth of 10 cm in December 1996
5	Uninoculated wheat sown June 1996	Cultivated at sowing December 1996	Uninoculated Unica sown December 1996 and plastic prills inoculated with CB3546 drilled to 10 cm in December 1996
6	Uninoculated wheat sown June 1996	No cultivation December 1996	Unica inoculated with CB3546 sown December 1996

Table 7. Alternative delivery trial. Percentage recovery of inoculum rhizobia

Caatinga Stylo – rhizobial strain CB3546

Site	Year	Treatment Number						
		1	2	3	4	5	6	3(CB3481)
Holyrood	1997	58	48	30	27	37	16	37
	1998	23	53	13	29	16	7	17
	1999	5	32	0	1	1	3	51
Narayen Granite	1997	0	0	ns	1	11	ns	ns
	1998	0	1	ns	1	1	ns	ns
Narayen Brigalow	1997							
	1998	nn	0	0	20	0	nn	20
Roma Res. Stn	1997	6	4	7	5	2	3	0
	1998	4	4	7	5	3	4	17
Banoona	1997							
	1998	1	1	3	0	0	0	27
	1999	2		2	6		1	16

Jaribu Desmanthus - rhizobial strain CB3547

Site	Year	Treatments					
		1	2	3	4	5	6
Holyrood	1997	0	1	ns	ns	100	100
	1998	0	0	ns	ns	50	60
	1999	18	55	ns	ns	62	100

Notes on Table 7.

1. nn = no nodules; ns = not sown
2. In an adjacent area at the Banoona site, Dr T.J. Hall established a commercial 10 hectare area of each of Primar and Unica Caatinga Stylo using commercially prepared peat inoculant of CB3481. The two areas were sown later in 1996 than the alternative delivery experiment. Strain CB3481 was identified in random samples taken from Dr Hall's trial:

Sample time	Primar	Unica
May 1998	no nodules	3%
March 1999	70%	40%

Table 8. Alternative delivery trial. Relative plant dry weight yield (as % best treatment for each year).

Caatinga Stylo – rhizobial strain CB3546

Site	Year	Treatment					
		1	2	3	4	5	6
Holyrood	1997	63	92	100	66	88	73
	1998	89	100	70	64	68	58
	1999	54	100	64	43	61	46
Narayen Granite	1997	100	97	ns	55	64	ns
	1998	64	100	ns	20	21	ns
Narayen Brigalow	1997						
	1998	28	44	47	54	60	100
Roma Res. Stn	1997	68	81	66	100	66	67
	1998	77	49	53	100	54	76
Banoona	1997						
	1998	67	46	100	85	97	64

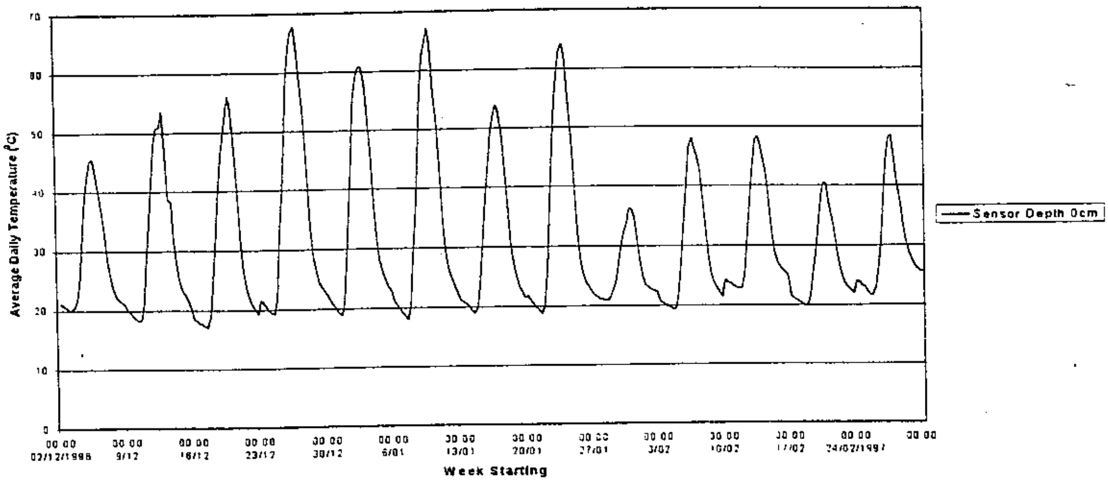
Jaribu Desmanthus – rhizobial strain CB3547

Site	Year	Treatments					
		1	2	3	4	5	6
Holyrood	1997	67	100	ns	ns	98	71
	1998	17	40	ns	ns	50	100

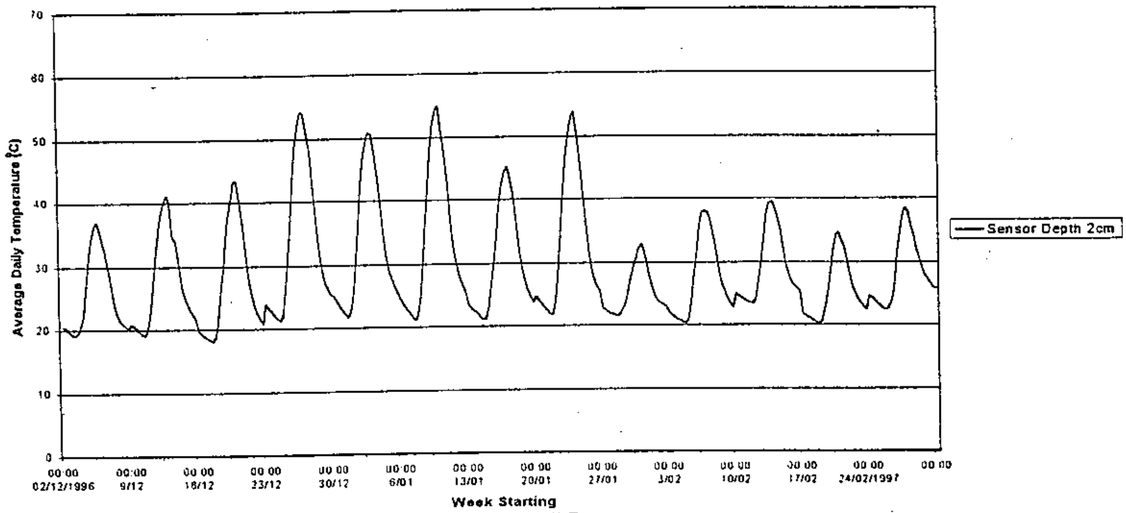
nn = no nodules; ns = not sown

Figure 1. Typical soil temperature profiles at 0, 2, 5 and 10 cm depth for bare soil at the Holyrood site.

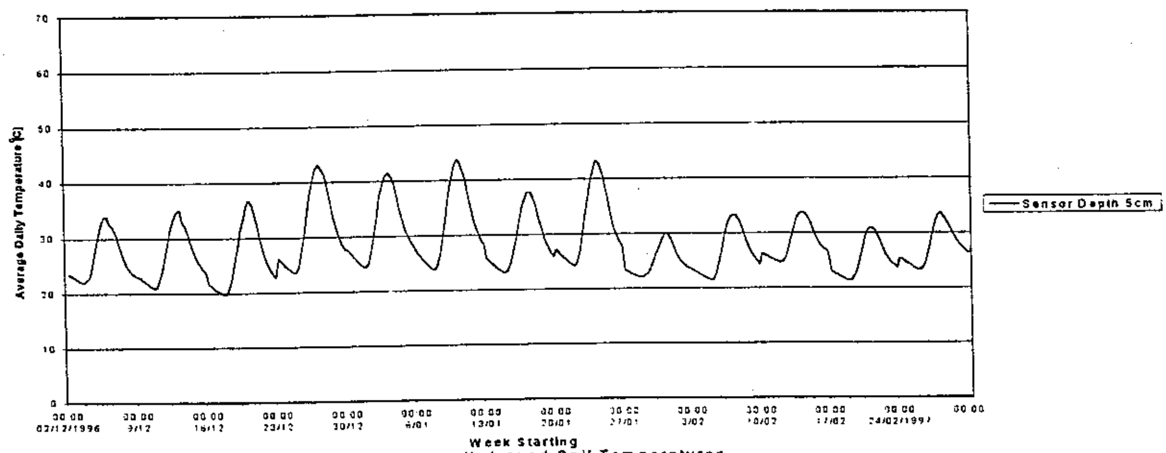
Holyrood Soil Temperatures



Holyrood Soil Temperatures



Holyrood Soil Temperatures



Holyrood Soil Temperatures