final report

Project code: P.PSH.0164

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Date published: April 2014

PUBLISHED BY

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Locked Bag 991
NORTH SYDNEY NSW 2059

Drought tolerant white clover cultivar for dry margins

Meat & Livestock Australia acknowledges the matching funds provided by the Australian Government to support the research and development detailed in this publication.

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NSW DEPARTMENT OF
PRIMARY INDUSTRIES

Abstract

A breeding project using conventional breeding has developed white clover (*Trifolium repens* L.) cultivars for ‘dry margins’ environments. This is the third breeding cycle of the national program that has previously developed cultivars with tolerance of summer moisture-stress to provide reliably persistent cultivars for the existing white clover zone (850–1,000 mm AAR). This breeding cycle has developed three experimental varieties with ‘local adaptation’ to low rainfall (700–850 mm AAR) conditions. The breeding strategy comprised (i) selecting superior genotypes, (ii) crossing elite germplasm and (iii) *in situ* progeny testing of breeding lines for the expression of key traits. The selection criteria were stolon survival through hot/dry summers (and strong autumn recovery), high herbage yield (including high warm-season growth and high winter activity) and reliable persistence for at least four years. Parental selection was also applied for seed yield, uniformity of leaf size and flowering pattern, and freedom from disease and virus symptoms. When commercialised, cultivars developed from this set of experimental varieties will contribute a new perennial legume option for livestock producers in ‘dry margins’ environments, favourable benefits for the pastoral landscape and increased security of food and fibre for domestic and export markets.

Executive Summary

A breeding project was undertaken between April 2005 and June 2009 by the NSWDPI/AgResearch 'White Clover Improvement Alliance' to develop drought tolerant white clover cultivars for the low rainfall 'dry margins' of eastern Australia.

This project (P164) is the third cycle of white clover breeding for Australian dryland pastures. It follows a national program of breeding that developed i) a broad adaptation cultivar (NuSiral) and ii) cultivars with tolerance of summer moisture-stress (Trophy and Saracen) for the existing white clover zone (850–1,000 mm AAR). The broad aim of the national program is to develop 'locally adapted' cultivars that are reliable and provide stable legume-based pastures for improved productivity and sustainability of the pasture resource.

The aim of the P164 'dry margins' breeding cycle was to '… to breed a white clover cultivar for beef and sheep pastures in dry margins environments of Australia' to extend white clover into low rainfall environments (700–850 mm AAR). Component activities of P164 were:

- To progeny test breeding lines at 2 evaluation sites in over 3 growth cycles
- To determine seed production potential of elite breeding lines
- To produce nucleus seed of a pre-release cultivar for commercialisation.

Candidate lines were progressed from the TR041 cycle and crossed by AgResearch using additional elite material from the AgResearch and NSWDPI germplasm collections, evaluation of the breeding lines was undertaken by NSWDPI at two 'dry margins' sites in northern NSW, and final screening for cultivar development was undertaken by AgResearch in preparation for a subsequent commercialisation phase. The primary selection criteria were:

- 'Herbage yield' and 'presence' longitudinal profiles exceeding the industry standard Haifa (and all comparator cultivars)
- Favourable combination of high stolon thickness x high stolon density for high stolon survival (through summer moisture-stress) and rapid autumn regrowth
- Desirable plant characteristics including medium/large leaf size, early vigour and mid/late flowering maturity.

The project has developed three experimental varieties (P164–39, P164–50, P164–70) that exhibit sound agronomic performance under the low rainfall (700-850 mm AAR) conditions of North-West NSW. All three are medium/large in leaf size (with exceptional leaf size stability), mid-season in flowering maturity and high in stolon density. The three experimental varieties have superior colonising ability and early vigour (compared with the industry standard Haifa), and consistently and substantially out-perform Haifa (and all other check cultivars) in seasonal herbage yield. All three experimental varieties maintain high presence (> 80% cover) for at least three years and two of the experimental cultivars (P164–39, P164–70) are exceptional in retaining a large component of their stolon structure following seasonal drought that is indicative of long-term persistence.

Also, it is evident that the three cycles (DAN045, TR041, P164) of breeding and selection has aggregated genes and isolated breeding lines that form a valuable set of locally adapted and persistent germplasm. The evaluation phase in P164 identified 16 lines that persisted into the long-term (> four years) and exhibited yield profiles comparable with the best performing cultivar (Tribute). Eight of these were exceptional in outperforming Tribute. These highly persistent 'derived lines' provide a valuable set of 'locally adapted' germplasm for progressing

into new breeding cycles to develop ‘new generation’ cultivars for the hot and dry conditions expected with climate change.

The goal of breeding white clover for beef and sheep pastures in dry margins environments of Australia is close to realisation with the nomination of these three ‘experimental varieties’ for subsequent progressing to cultivar status and commercialisation. This will extend the use of white clover into the ‘dry margins’: the North-West Slopes, Southern and Monaro Tablelands of NSW, homoclimes in the other States, and elsewhere in low rainfall environments internationally.

With successful commercialisation of Trophy for the high rainfall existing white clover zone, and the P164 cultivar for the dry margins, the potential benefits are to:

- Maintain 50 per cent of the high rainfall zone under perennial clover-based pastures within 10 years of the commercialisation of Trophy
- Expand the use of perennial clover-based pastures into 25 per cent of the dry margins within 10 years of the commercialisation the P164 cultivar.

For livestock producers, these locally- cultivars will provide valuable options:

- Enhance the feed-year by increasing the feed supply, mitigating the winter feed-gap and increasing the supply of high quality leguminous forage over summer/autumn – leading to increased capability of producing products to market specifications
- Increase carrying capacity – with favourable consequences for financial viability
- Reduce fluctuations in the season-to-season and year-to-year feed supply – with benefits for cash flow and reduction in risk
- Increase soil fertility through the N-fixing properties of pasture legumes, reduce the use of N fertiliser and decrease the ‘carbon footprint’ of the grazing industries
- Provide a perennial legume for problem soils (eg acidic soils not suited to lucerne)
- Rehabilitate ‘run-down’ pastures and degraded landscapes through increasing groundcover and improving water balance in the soil profile.

For the wider pastoral landscape:

- Perennialisation of pastures will provide stable groundcover, prevent soil erosion, suppress invasive weeds and retain moisture for improved water-use
- Increased grazing productivity and more uniform financial performance of grazing enterprises will improve gross production from the grazing sector and reduce the social welfare expenditure of government on graziers in times of market down-turn and drought

There will also be significant social benefits that include:

- ‘Trickle-down’ benefits to seed retailers and associated agri-business that provides commercial and support-services to the grazing industries and strengthens rural communities
- Increase capability of the grazing industries to provide consumers with security of food and fibre and exporters with reliable supply of products.

A 10-year plan has also been developed for the continuation of breeding, evaluation and commercialisation of a new generation of white clover cultivars for ‘climate change’. This will more fully exploit the potential of these derived breeding lines (and the established capability of the Alliance) to develop robust white clover cultivars with tolerance of ‘heat & dry’ for this new era.

Contents

1.1 The Breeding Program

Under Australian conditions of extensive grazing, legume-based pastures are the most important feed resource supporting the grazing industries because of their contribution to feed quality and to the sustainability of soil fertility, water resources and crop production where ley farming is practiced (Archer 1995). White clover is the main perennial pasture legume in high rainfall temperate regions (Pearson *et al*. 1997) due to its broad adaptation. The white clover zone in Australia encompasses approximately 8 million hectares (Clements 1987) with potential to extend to 16 million hectares (Ayres and Caradus 2002).

Although pasture improvement based on white clover and superphosphate in Australia during the 1950's and 1960's was very successful (Ayres and Davies 2000), by the early 1980's poor clover persistence and high herbage yield fluctuations emerged as major problems, especially in summer-rainfall environments (McDonald 1988). This lack of reliability, particularly in marginal environments with commercially available white clover cultivars is attributed to poor persistence (Robinson and Lazenby 1976; Kenny and Reed 1984; Gillard *et al*. 1989). Sensitivity to summer moisture-stress is identified as the major factor limiting persistence in dryland environments (McCaskill and Blair 1988; Archer and Robinson 1989; Hutchinson *et al.* 1995). Stolon survival through periods of soil moisture-stress is a pre-requisite for persistence because white clover in the sward is slow to recover full production from seedling recruitment (Archer and Robinson 1989).

In 1987, a national white clover improvement program commenced at Glen Innes with the establishment of a Germplasm Resource Centre comprising a world sourced white clover collection, seeds laboratory and glasshouse/nursery complex. The team initially undertook germplasm collection, conservation and characterisation work to develop an 800 line working collection and to define breeding objectives. This early work developed local knowledge of the adaptative characteristics, mechanisms of regeneration and limitations of contemporary white clover cultivars (Lane *et al.* 1997, 2000), together with heritability estimates and analysis of G x E interactions (Jahufer *et al.* 2002). This information provided 'science-based' breeding objectives and a sound breeding strategy for white clover improvement in Australia.

Breeding in conjunction with AgResearch, and with strong industry and commercial support, commenced from 1994. The program set out to develop 3 dry-land variety types:

- A broad adaptation variety for the heartland white clover zone (850 - 1,000mm AAR)
- A variety with tolerance of summer-moisture stress to improve the reliability of white clover
- A 'dry margins' (700 – 850 mm AAR) variety to expand the potential zone of adaptation of white clover to some 16 million ha.

1.2 Past Achievements – Current Directions

Key variety products from the collaboration to date include:

- A broad adaptation cultivar – NuSiral (released in 1999) – developed as a high rainfall cultivar for summer-rainfall and winter-rainfall environments (Ayres, Lane *et al*. 2002)
- A cultivar with tolerance of summer moisture-stress – DAN85/TR041 cycles developed Trophy (released in 2008) and Saracen (for international release) – these cultivars were developed for high rainfall environments (850–1,000 mm AAR) where summer moisture-stress limits persistence (Ayres, Caradus *et al*. 2007).

The current P164 cycle was initiated in December 2004 to develop a 'dry margins' cultivar for the 650–850 mm AAR zone.

1.3 Co-funding Stakeholders

NSW DPI, AgResearch (NZ), Grasslanz Technology, Agricom, and Meat and Livestock Australia have a long-standing Alliance directed at the development of white clover varieties for Australia. Currently, the breeders are NSW DPI and AgResearch Ltd, the co-funders are Grasslanz and Meat and Livestock Australia, and the commercial partner is Agricom (recently amalgamated into PGG Wrightsons). A joint funding arrangement seeks to ensure that industry and commercial priorities are at the forefront of the planning and implementation of the research, and that the cultivars arising from the project are effectively commercialised and adopted. A considerable strength of this collaborative breeding program has been the seamless integration of breeding with commercialisation (both domestically and internationally) through the active involvement of the commercial partner through the course of the breeding project.

The project is administered under a 'Research Agreement' (MLA, NSW DPI, AgResearch), a 'Partner Agreement' (MLA, Agricom, Grasslanz) and a 'Commercialisation Agreement' (AgResearch, Grasslanz, NSW DPI, Agricom). With the advent of the current P164 breeding cycle, funding has been provided partially through the commercial partner and partially through the Australian Government 'Partnerships in Innovation' program coordinated by the MLA donor company with co-funding from Grasslanz commercial connections. NSW DPIs contributes an 'in kind' commitment that represents approximately 50 per cent of the total project budget.

While project management is provided by the donor company, management of P164 activities is provided by the P164 Management Committee comprising five members: NSW DPI (Dr John Ayres, chairperson), AgResearch (Dr Zulfi Jahufer), MLA (Cameron Allan), Grasslanz (Bruce Belgrave) and PGG-Wrightsons (Richard Green). The committee routinely receives significant contributions of ideas from 'observers': Dr Derek Woodfield (AgResearch); Leah Lane, John Ryan and Russell Martin (NSW DPI); and Dr Bob Murison (University of New England). The P164 Management Committee meets 6-monthly to oversight the conduct of the project.

Prior investment has included partial funding through the following projects:

1. *DAN 104 White Clover Improvement - National Field Testing of the Australian White Clover Collection (Ayres JF, Norton MR, Jahufer MZZ, FitzGerald RD, 1991/92 - 1994/95, Wool Research & Development Corporation, Agricultural Research & Advisory Station, Glen Innes, NSW)*
2. *DAN 085 'National Temperate Perennial Legume Improvement Program - white clover breeding for dryland sheep and cattle pastures' (Ayres JF, Caradus JR, 1994/95 - 1996/97, Meat Research Corporation, Glen Innes Agricultural Research & Advisory Station)*
3. *'Growth and development of white clover (Trifolium repens) under moisture stress' (Jacobs BC, Ayres JF 1995/96 - 1996/97, University of Sydney Research Grants Scheme).*
4. *'Reselection within Siral - a white clover cultivar with broad adaptation to NSW dryland environments' (Ayres JF, Caradus JR, 1995/96 - 1997/98, Agricom Commercial Funds).*

5. 'TR 041 Australasian Perennial Legume Improvement Program - white clover breeding for dryland pastures' (JF Ayres & JR Caradus, 1997/98 - 2000/2001, Meat and Livestock Australia)

2 Project Objectives

2.1 Objectives of the Breeding Program

A fundamental platform of the white clover improvement program has been the recognition that imported white clover cultivars are not adapted to the environmental stresses of dryland pastures in eastern Australia. Imported cultivars are especially vulnerable to the seasonal droughts that are characteristic of Australian dryland pasture environments, and the increasing incidence of the severe episodic droughts. The associated lack of year-to-year reliability of imported cultivars impacts negatively on i) the level of clover content, ii) the long-term persistence of clover in pastures, iii) the grazing value of improved pastures (and livestock production), and iv) the profitability from cattle and sheep enterprises.

<u>The broad aim of the program</u> is to develop white clover cultivars that are reliable and provide stable legume-based pastures for improved productivity and sustainability of the pasture resource.

<u>The broad aim of the P164 cycle</u> is '… to breed a white clover cultivar for beef and sheep pastures in dry margins environments of Australia, or as otherwise directed by the P164 Management Group'. These new varieties will have two significant applications: a) to improve the reliability of white clover in the current white clover zone, and b) to extend white clover into the dry margins.

<u>Specific objectives of the P164 cycle</u> are:

- To progeny test breeding lines at 2 evaluation sites over 3 growth cycles
- To determine seed production potential of elite breeding lines
- To produce nucleus seed of a pre-release cultivar for commercialisation.

2.2 Breeding Objectives and Selection Traits

Extensive survey work throughout the Australian white clover zone has determined that the key environmental challenge to the agronomic performance of imported white clover cultivars is summer moisture-stress. Accordingly, improvement of stolon survival through periodic moisture-stress, especially in summer-autumn is the primary breeding objective for white clover improvement for Australian dryland pastures (Ayres *et al.* 1996).

In situ selection for tolerance of moisture stress and competitive vigour under grazing is the essence of the plant improvement strategy (Ayres, Caradus *et al.* 1996), and innovative design and analysis elements increase the effectiveness of the evaluation phase (Murison, Ayres *et al.* 2006). Emphasis is placed on improving stolon characteristics that enhance vegetative perennation rather than pursuit of the 'annual habit'. Naturalised ecotypes of white clover are known to readily regenerate through the annual habit (flowering and seeling recruitment) but they typically contribute little to the feed supply (Godwin 1981). By contrast, improving the stolon structure provides colonizing ability, longevity of the original population and persistence of the clover stand through improved survival of stolons through moisture stress.

Accordingly, the breeding strategy used in P164 placed emphasis on stolon characteristics for stolon survival and vegetative perennation, high herbage yield (including spring growth, summer-autumn regrowth and winter activity) and high seed production capability. This strategy, based on accessing, breeding and *in situ* evaluation of medium-large leaf and mid-season maturity germplasm from Mediterranean environments develops breeding lines likely to possess tolerance of moisture stress combined with cool season growth potential.

Selection Characteristics

Primary Characteristics:

- Seasonal presence and yield profiles > Haifa by 100%

Associated Traits:

- High stolon thickness (similar to Haifa) plus high stolon density (40% > than Haifa)
- 80% stolon survival through summer moisture stress (compared with 20% for Haifa)
- High autumn regrowth (100% > Haifa)

Secondary Traits:

- Leaf size … medium to medium/large
- Flowering maturity … mid to late
- Early vigour … rapid spread x high early growth
- HCN status … low to moderate

3 Methodology

The progeny testing phase has involved the development and evaluation of 95 breeding lines comprising elite material selected for drought tolerance (and progressed by crossing processes) from the earlier projects (DAN85, TR041), as well as lines developed within the AgResearch pre-breeding programs and NSW DPI lines (‘drought selections’, ecotypes, high stolon density ‘correlation breakers’).

3.1 The Germplasm

The germplasm entries (Appendix 1) include lines from the following sources:

1. Breeding lines being progressed from the previous (TR041) breeding cycle
2. Selections from the AgResearch/Agricom gene pool including:
   - Lines developed in south-east USA under high summer temperatures and summer moisture stress
   - Lines developed under dryland conditions in Eastern New Zealand
   - Lines developed in South America
   - New lines developed from crosses among selected lines from TR041 and crosses between TR041 germplasm and elite cultivars
   - Germplasm containing unique traits such as nematode resistance
   - Hybrids between white clover and Caucasian clover developed by AgResearch and Agricom with potential to survive in drier environments.
3. Elite lines from the NSW DPI working collection including:-
   - Drought selections (populations based on > 600 genotypes collected over 15 years following droughts and severe moisture stress events)
   - ‘Correlation–breakers’ (lines combining medium/large leaf x high stolon density)
   - Promising ecotypes (lines from 2 NSW ecotype collections short-listed for superior persistence and yield performance)
   - Lines possessing morphological characteristics associated with persistence.

3.2 Location of the Component Studies

3.2.1 Characterisation glasshouse in New Zealand

The glasshouse characterisation was based at the AgResearch Grasslands Research Centre in Palmerston North. During morphological Characterisation of the breeding lines the temperature in the glasshouse was maintained at 23^{o}C to 26^{o}C (day) and 15^{o}C to 17^{o}C (night). The distribution of light during the day was even across the glasshouse. Irrigation was applied using capillary matting on which the pots containing the plants, grown in potting mix, were placed. This ensured a continuous supply of water.

3.2.2 Evaluation nurseries in NSW

The breeding lines were evaluated concurrently at 2 sites: a field nursery at Glen Innes Agricultural Research & Advisory Station ('Glen Innes') on the Northern Tablelands of NSW, and a field nursery on Macintyre Station ('Inverell') on the North-west Slopes of NSW.

Figure 1 The evaluation sites in NSW; Macintyre Station, Inverell (left) and Glen Innes Agricultural Research & Advisory Station, Glen Innes (right)

The Northern Tablelands is a high rainfall (775–1,000 mm AAR), elevated, hilly landscape (750–1,400 metres altitude); the North-west Slopes receives less rainfall (600–775 mm AAR) and the heat factor is greater (Tables 1, 2).

The climate of the Northern Tablelands is characterised by high rainfall with marked summer incidence, a long frosting interval and cold winter conditions (Table 2).

Conditions on the adjacent North-West Slopes follow a similar pattern of summer incidence but winters are milder and moisture-stress over summer/autumn is greater.

Pasture growth periodicity in both regions is limited by high evaporation in summer and low temperature in winter. However, the winter-cold effects are greater on the Northern Tablelands and moisture-stress over summer is greater on the North-West Slopes.

Table 1 Key features of the field sites

	Glen Innes[‡]	Inverell[†]
Latitude/longitude	29°42'S/151°42'E	29°78'S/151°08'E
Elevation (m)	1057	664
Average annual rainfall (mm)	847	803
Temperature range[a]	(25.0/0.6)	(29.3/3.5)
Daylength[b]	14 h	14 h
Frosts (number per annum)	104	34
Soil pH (1:5 $CaCl_2$)	5.1	5.9
Soil phosphorus (mg/kg, Bray-1)	3.4	15
Companion grass	Tall fescue	Tall fescue

[†]Climatic data from Meteorological Bureau Station 056013 at Glen Innes
[‡]Climatic data from Meteorological Bureau Station 056018 at Inverell
[a] Mean monthly maximum (mid-summer)/mean monthly minimum (mid-winter)
[b] Duration (hours) of daylight on the summer solstice (21 December)

Table 2 Climate summary for the field nurseries (Glen Innes Meteorological Station 056013, Pindari Dam Meteorological Station 05410)

	Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec	Total
							a) Glen Innes						
Mean daily max. temp (^oC)	25.2	24.4	23.1	19.8	16.2	13.1	12.4	13.9	16.8	19.8	22.1	24.4	
Mean daily min. temp (^oC)	13.5	13.3	11.4	7.9	4.9	1.8	0.7	1.2	4.1	7.1	9.8	12.0	
Mean monthly rainfall (mm)	106	94	72	41	50	43	57	49	54	78	88	108	848
Mean daily evaporation (mm)	171	134	133	93	62	48	53	75	105	137	153	174	1336
Number of frosts	0	0	1	4	11	17	21	20	12	5	1	1	92
							b) Pindari Dam[A]						
Mean daily max. temp (^oC)	31.2	30.5	28.9	25.5	21.5	17.9	17.1	18.8	22.1	25.1	27.5	30.2	
Mean daily min. temp (^oC)	17.5	17.2	14.8	10.8	7	3.2	2.1	2.9	6.2	10.1	13.5	16.1	
Mean monthly rainfall (mm)	99	85	66	47	47	33	47	38	47	72	91	88	758
Mean daily evaporation (mm)	233	193	183	129	84	60	65	93	129	171	195	233	1788
Number of frosts	0	0	0	0	4	13	18	15	6	1	0	0	57

[A] Pindari Dam Meteorological Station is 15 km north-east of the Macintyre station site

The soil at the Glen Innes site is a highly buffered black soil, mildly acidic and with a low available P status. Fertiliser history shows annual applications of 150 kg/ha single superphosphate during 1988 to 1998. From 1998, the paddock was set aside for pasture trials and received only one application (Spring 2003) of 175 kg/ha Granulock 15. Because the phosphate status was low, the fertiliser program for the evaluation study included annual applications of Super M fertiliser at 130 kg/ha plus 100 kg/ha muriate of potash.

The soil at the Inverell site is a red-brown alluvial day-loam, mildly acidic with medium P status and low S level. The fertiliser program for the evaluation study included a compound (N, P, S) fertiliser (100kg/ha 600S) when establishing the grass followed by annual topdressing with SF45.

Large fluctuations in seasonal rainfall and severe moisture-stress were pervading features of the three years under which the breeding lines were evaluated.

Notably, there were 5 instances at the Glen Innes site when soil moisture declined to a dry profile (Figure 1). During the 2007/08 summer into the autumn and winter of 2008, rainfall remained well below the average leading to a level of protracted soil moisture deficit that was acutely detrimental to the survival of white clover.

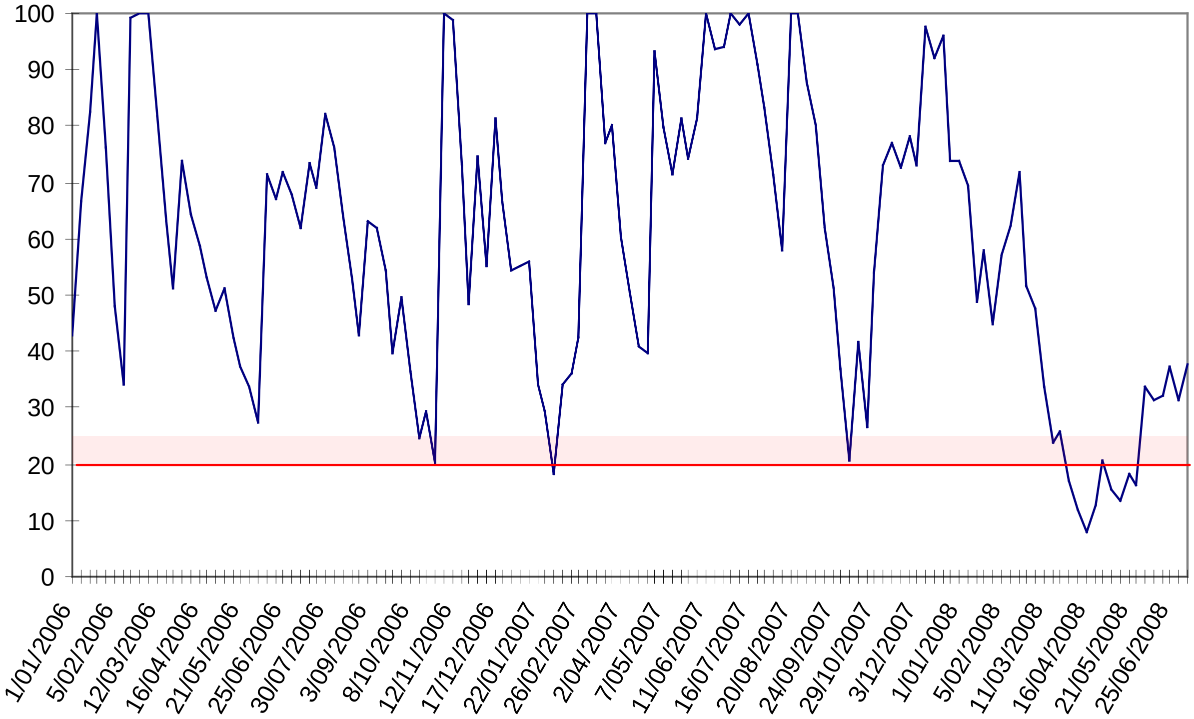


Figure 1 Relative soil moisture balance (%) at Glen Innes, 2006-2008

Note: Relative soil moisture is an estimate of relative available soil moisture as a percentage of water-holding capacity calculated after the method of Smith and Johns (1975).

At the Inverell site, although no soil moisture balance data are available, it is clear that the added stress of a high heat load in summer imposed a greater moisture stress challenge for the white clover populations.

With the Inverell site located centrally in the ‘dry margins and the Glen Innes site located on the dry fringe of the high rainfall zone and together with both sites subjected to an adverse run of dry conditions, this provided a strong ‘dry margins’ challenge for persistence of the breeding lines.

3.2.3 Merit testing sites

Palmerston North, New Zealand

The germplasm screening nursery and merit testing site are both situated at the AgResearch Grasslands Research Centre (latitude 40° 22' 58S, longitude 175° 36' 47E, altitude 37 m). The soil type for both field trials was a fine silty over sandy mixed, mesic, Dystric Fluventic Eutrochrept (Cowie, 1978). The long term mean annual rainfall at Palmerston North is 965 mm.

Glenormiston in Victoria, Australia

The merit testing site in Australia is based at the experimental farm of the South West TAFE campus at Glenormiston, Victoria (latitude 38° 8' 60S, longitude 142° 58' 60E, altitude 114 m). The soil type at the experimental site is a dark brown sandy clay loam over clay, classified as a Brown Chromosol (Isbell, 2002). The soil represents typical duplex soils of the region. Inherent soil fertility is low and surface horizons are acidic. The soil has good drainage**.** The long term mean annual rainfall at Glenormiston is 648 mm.

3.3 Evaluation of the breeding lines

3.3.1 Design and analysis

Three blocks of the 95 breeding lines and 5 check cultivars were evaluated in plots of 2x1 metres. The plots were laid out in a 15 row by 23 column design so that each experiment occupied an area of 15 x 46 metres. The role of the check plots (12 reps) was to estimate changes across the field site that were not due to genetic differences (between the breeding lines), but rather to inherent or ephemeral spatial variation across the field site. Check cultivars were spaced approximately 7 plots apart and were used to estimate the spatial heterogeneity of the field due to local influences of paddock history, moisture drying order or fertility gradients.

Figure 3 One of the evaluation sites illustrating plot layout

3.3.2 Measurement protocols

The agronomic and morphological characters were measured as follows:

Row spread
Row spread measurements were undertaken in the 1st growth cycle at 6-weekly intervals to estimate capacity to spread and colonise intra-row spaces. Row spread was estimated by measuring the lateral spread (from stolon extension and branching) from the central row at three fixed points (50 cm, 100 cm, 150 cm) along the row using a 75 cm x 200 cm quadrat frame. Longitudinal data was ‘splined’ to illustrate the rapidity and extent of spread in the 1st growth cycle. Results are indicative of *early vigour*.

Clover presence
From the 2nd growth cycle, clover presence (proportion of the plot occupied by clover) estimates were undertaken (2 observers) 6-weekly on a 0-9 rating system (1=10%,

2=20%...9=90% or greater) for each of 4 quadrants of the plot. Longitudinal data were 'splined' to illustrate the changing presence of clover content of each line. Results in later growth cycles (especially the 4th growth cycle) are indicative of *persistence*.

Clover yield
Clover yield was estimated 6-weekly in conjunction with either row spread (growth cycle 1) or clover presence (growth cycles 2, 3) on a 0 - 9 linear scale where 1 = trace clover herbage mass and 9 = maximum clover herbage mass. Estimates were obtained from 2 independent observers, each providing rating values for 4 fixed quadrats along the centre of each plot. At each measurement occasion, rating estimates were also applied to 20 calibration points (identified with numbered pegs); these (20 x 10 cm quadrat) were harvested for weight determinations (fresh weight, dry weight), and regression analysis used to calibrate the ratings data against herbage mass data. Data were 'splined' to provide results indicative of *herbage yield*.

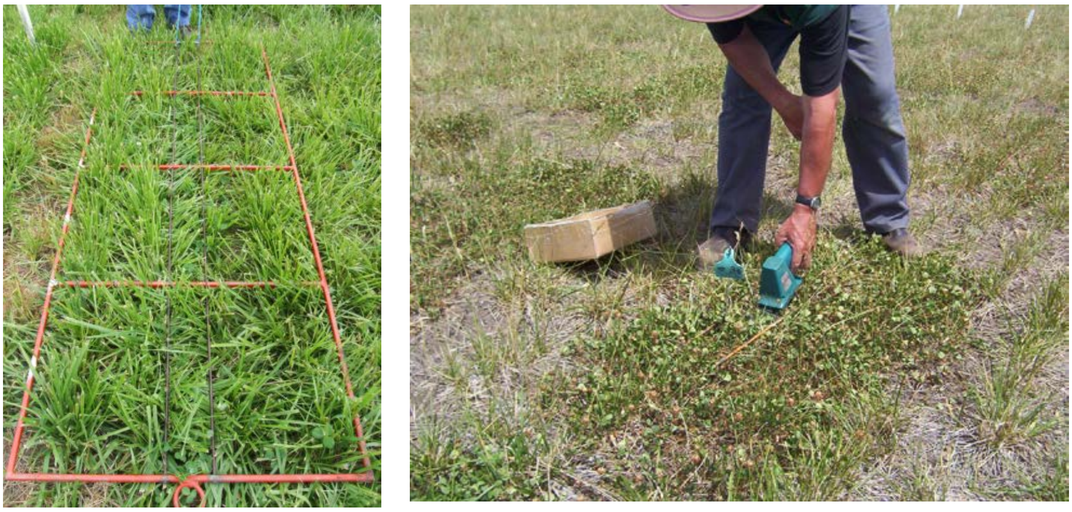


Figure 3 Measuring clover yield and sampling for calibrating the visual scores

Leaf size stability
Leaf size was rated 6-weekly as *small*, *medium, large* or intermediate categories (small-medium, medium-large) in conjunction with measurement of spread, presence and yield. The longitudinal patterns of leaf size provide an indication of the degree of *leaf size stability* under varying environmental and stress conditions.

Stolon survival
Stolon survival was estimated by measuring *stolon number* at the beginning (December) and end (April) of summer moisture-stress over the summers of the second growth cycle (2006/07) and the third growth cycle (2007/08). *Stolon number* was measured by counting the number of stolons intercepting a 25 cm line segment (4 segments per plot) placed along the centre of each plot. Stolon survival was expressed as the difference between *stolon number* before and after 5 months of summer moisture-stress.

Flowering intensity
Flowering intensity was assessed at full bloom stage of flowering (early-mid October in years 2 and 3) by counting flowers in a 10 cm x 20 cm quadrat placed along the centre of each plot.

Characterisation:
Breeding lines and cultivar standards were also characterised for morphological characters (leaf size, stolon thickness, petiole length), leaf markings, HCN frequency and quercetin content. This was undertaken on plants in pot culture under glasshouse conditions – this work is detailed in 4.1.1.

3.3.3 The data

At each of the two sites (Glen Innes, Inverell), the comparative performance of the breeding lines was assessed as i) longitudinal profiles of agronomic performance (clover yield, clover persistence) or as ii) the expression of relevant plant traits (leaf size stability, stolon survival, early vigour, flowering intensity) under the presence of typical stresses (grass competition, grazing pressure, climatic stress).

Statistical models were used to account for these spatial effects to enhance the accuracy of comparisons among the breeding lines so that interpretation of the results was objective, accurate and reliable. The statistical model to analyse the responses is a mixed model with differences amongst the checks represented as ‘fixed effects’ and ‘random effects’ were assumed to be due to differences amongst the breeding lines and spatial effects due to plot position.

4 Results

The project successfully completed the field evaluation of the 95 breeding lines and 5 cultivar checks and identified 3 outstanding synthetics that are superior in agronomic performance to both Haifa and the other white clover cultivars under test. Results to the 4th growth cycle show that:

- Haifa performed poorly and Tribute was the best performing check cultivar
- Three lines (39, 50, 70) performed substantially better than Tribute in yield and persistence
- A further 25 lines were identified as having outstanding persistence – these represent a pool of locally adapted germplasm for progressing to a future breeding cycle.

The following provides a summary of the characteristics of these 3 ‘experimental varieties’.

4.1 Evaluation of the Breeding Lines

4.1.1 Glasshouse characterisation

The three experimental varieties 39, 50 and 70 are generally medium/large in leaf size (with exceptional leaf size stability), mid-season in flowering maturity and high in stolon density. In comparison with Haifa which has relatively high leaf size stability, the experimental varieties are similar/if not better (lines 50 and 70 show even greater leaf size stability), higher in stolon density and all three are substantially later in flowering maturity (Table 3).

The three experimental varieties are mid-season in flowering – the order of maturity is 39 (early mid-season), 70 (mid-season) and 50 (late mid-season).

The HCN status of line 39 and line 70 are similar to Haifa, whereas line 50 is relatively lower in frequency of cyanogenic leaves.

P164 line 39

- Medium/large leaf size, high leaf size stability with moderate incidence (73%) of leaves exhibiting a full leaf crescent and low (17 %) incidence of leaves with red flecking
- Medium stolon thickness and high stolon density
- Mid-season in flowering maturity (about 2 weeks later than Haifa).

P164 line 50

- Medium/large leaf size, very high leaf size stability and high incidence (90%) of leaves showing a full leaf crescent but without red flecking
- Medium stolon thickness and very high stolon density
- Mid-season in flowering maturity (about 4 weeks later than Haifa).

P164 line 70

- Medium/large in leaf size, very high leaf size stability and moderate incidence (86%) of leaves showing a full crescent leaf mark and low (17%) incidence of red flecking
- Medium stolon thickness and high stolon density
- Mid-season in flowering maturity (about 3 weeks later than Haifa).

Table 3 Morphological characteristics of the experimental varieties

	Haifa	Huia	Trophy	Nomad	Tribute	39	50	70
Glasshouse morphological characteristics								
Stolon thickness (mm)	2.28	1.74	2.13	1.58	2.07	2.14	2.26	2.12
Leaf width (mm)	19.6	12.6	17.9	13.6	18.3	18.0	18.6	19.4
Leaf length (mm)	21.0	17.3	18.1	13.7	21.0	17.6	19.2	20.0
Petiole length (mm)	250	148	184	175	193	182	166	203
Leaf size stability (frequency of reversion to a smaller leaf size under field conditions in NSW)								
(%)	23	69	38	46	39	23	15	15
Flower prolificacy and flowering maturity (under field conditions in northern NSW)								
Glen Innes Flowers/m^2 (se)	279 (58)	273 (60)	294 (60)	267 (60)	207 (46)	206 (58)	162 (43)	220 (69)
Glen Innes Maturity (days)	Early (35)	Mid (49)	Mid (43)	Late (52)	Late (50)	Mid (41)	Mid (48)	Mid (44)
Inverell Flowers/m^2 (se)	380 (88)	398 (93)	383 (88)	281 (73)	326 (86)	417 (135)	277 (83)	288 (87)
Inverell Maturity (days)	Early (9)	Mid (35)	Mid (32)	Late (44)	Late (42)	Mid (37)	Mid (35)	Mid (40)
Stolon number, No/25 cm transect (se)								
Glen Innes Pre-summer	4.73 (1.31)	6.71 (1.93)	5.82 (1.66)	6.53 (1.72)	6.01 (1.60)	5.62 (1.72)	5.11 (1.56)	4.29 (1.46)
Glen Innes Post-summer	3.81 (1.18)	4.05 (1.32)	4.10 (1.32)	3.77 (1.16)	4.70 (1.54)	3.90 (1.35)	2.24 (0.81)	3.51 (1.22)
Inverell Pre-summer count	5.96 (1.41)	7.07 (1.76)	7.06 (1.70)	8.33 (1.89)	7.67 (1.80)	7.22 (1.97)	8.07 (2.20)	7.23 (1.97)
Inverell Post-summer count	0.75 (0.41)	0.42 (0.24)	0.53 (0.29)	3.60 (2.27)	2.02 (1.29)	0.90 (0.61)	2.92 (1.74)	1.89 (0.94)
Cyanogenic Frequency (%)	90	81	97	30	93	93	73	90

4.1.2 Field evaluation – early growth-vigour

Figure 3 illustrates the colonising ability (spread) and early growth vigour (yield score) of the full set of test lines, with reference to Haifa (positioned where the coordinates intersect).

The experimental varieties expressed both better colonising ability and early vigour than Haifa at either one or other of the two field sites, demonstrating their potentially high early growth-vigour. Line 39 expressed exceptionally high colonising ability and growth-vigour at the Glen Innes site, and line 50 expressed very high colonising ability at the Inverell site.

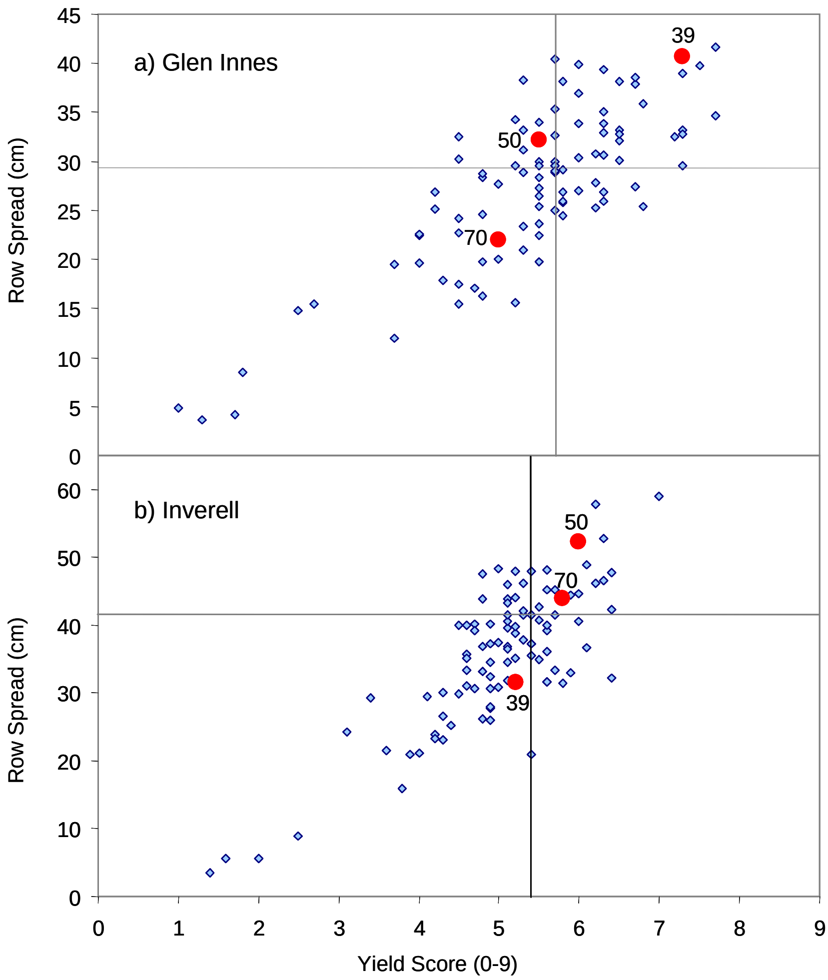


Figure 3 Scatter diagrams or 'row spread X 'yield score' at the Glen Innes and Inverell sites in January 2006 – planting occurred in October 2005

4.1.3 Field evaluation – herbage yield

Longitudinal profiles of the herbage yield of the experimental varieties (in comparison with Haifa) at the two sites are presented in Figures 4 and 5. Profiles for all lines are presented in Appendix 10.2.

At the Glen Innes site (which experienced 6 instances of severe moisture stress during the evaluation), the 3 experimental varieties 39, 50 and 70 consistently outperformed Haifa (and all other check cultivars) in herbage yield up to and including commencement of the 4th growth cycle (Figure 4). At the Inverell site (where climatic conditions were hot and dry during each summer/autumn), all lines expired by the end of the 2nd growth cycle – during the first two growth cycles at the Inverell site, the herbage yield of 50 and 70 was consistently greater than Haifa, while 39 was similar to Haifa (Figure 5).

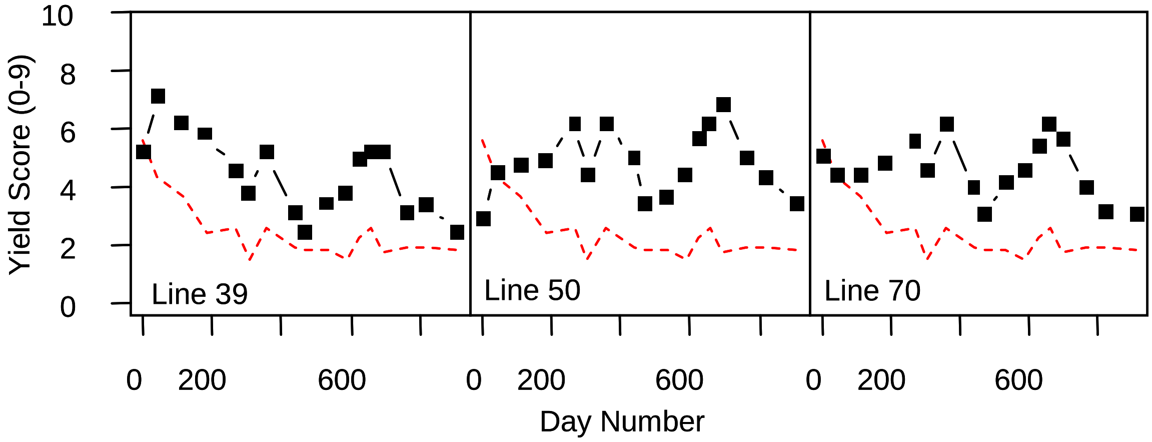


Figure 4 Longitudinal profiles of herbage yield of the experimental varieties (■) compared with Haifa (– – –) at the Glen Innes site

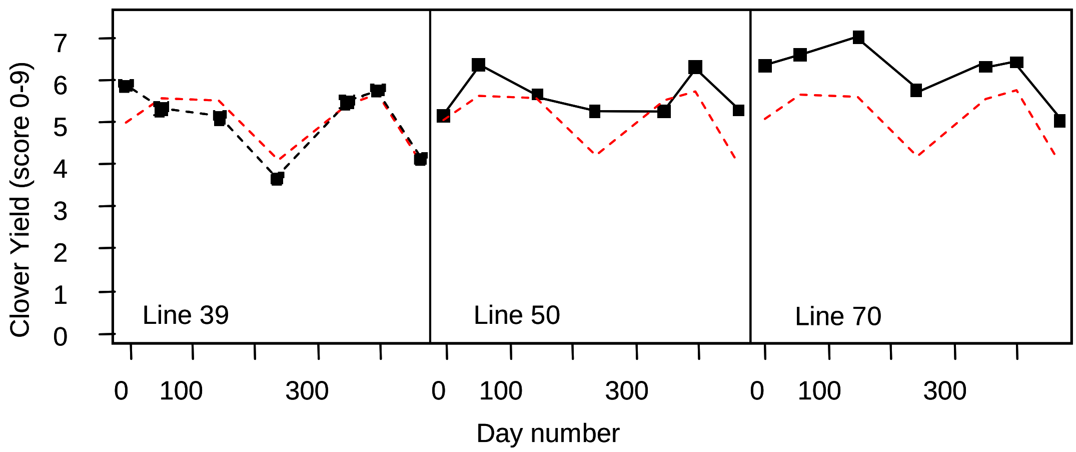


Figure 5 Longitudinal profiles of herbage yield of the experimental varieties (■) compared with Haifa (– – –) at the Inverell site

4.1.4 Field evaluation – clover presence, persistence

Longitudinal profiles of clover presence of the experimental varieties (in comparison with Haifa) at the two sites are presented in Figures 6 and 7. Profiles for all lines are presented in Appendix 10.3.

At Glen Innes, the experimental varieties 50 and 70 maintained exceptionally high presence (> 80% cover) for the first three growth cycles. Following severely adverse summer/autumn moisture stress in the third summer/autumn, retained strong persistence (70% cover) into the 4th growth cycle. At the Inverell site, the experimental varieties declined to only trace presence (< 20% cover) by commencement of the 3rd growth cycle – as did Haifa and all other lines.

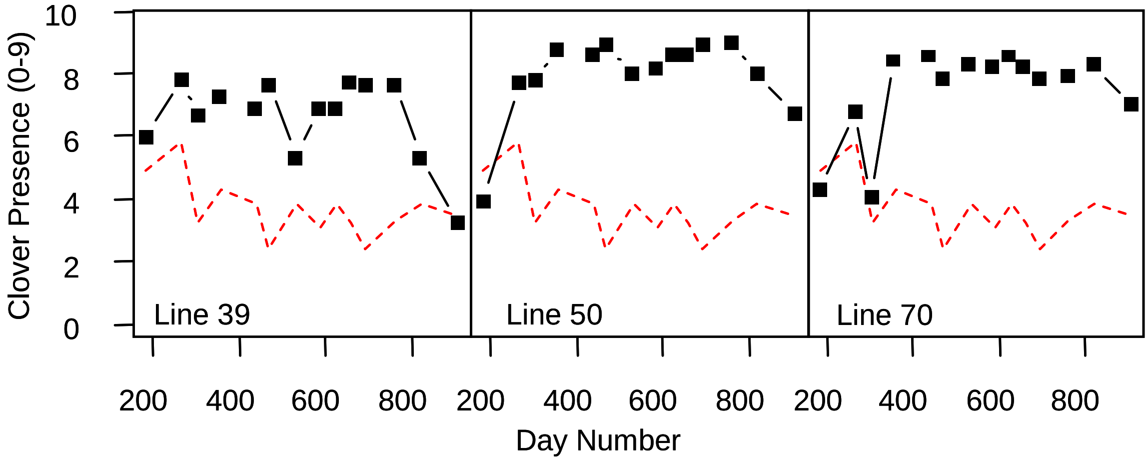


Figure 6 Longitudinal profiles of clover presence of the experimental varieties (■) compared with Haifa (– – –) at the Glen Innes site

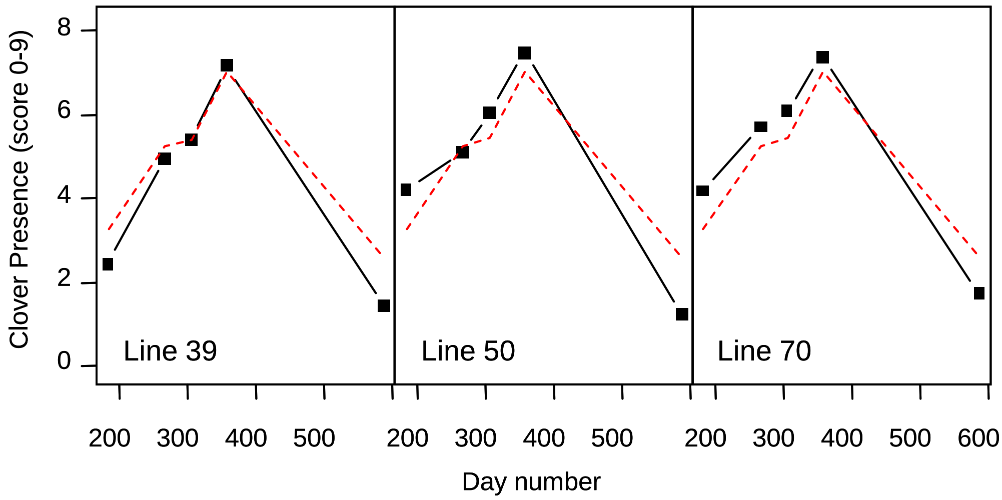


Figure 7 Longitudinal profiles of clover presence of the experimental varieties (■) compared with Haifa (– – –) at the Inverell site

4.1.5 Field evaluation – stolon survival

Stolon survival results for the experimental varieties (in comparison with Haifa) at the two sites are presented in Figure 8. Data are presented for two summers at the Glen Innes site and for one summer at the Inverell site.

At Glen Innes in 2006/07 (where typical summer moisture-stress conditions prevailed), line 70 was exceptional in expanding its stolon structure through summer/autumn while lines 39 and 50 retained better stolon survival than Haifa. In 2007/08 (which included an extended and severe period of severe seasonal moisture stress), 50 and 70 retained a *medium* level (> 2 stolons/25 cm) of stolon survival while Haifa and 30 declined to *low* levels.

At Inverell, the combination of hot and dry conditions was adverse for the survival of stolons of Haifa and all white clover populations, except for 50 and 70 which maintained a *medium* level of stolon survival.

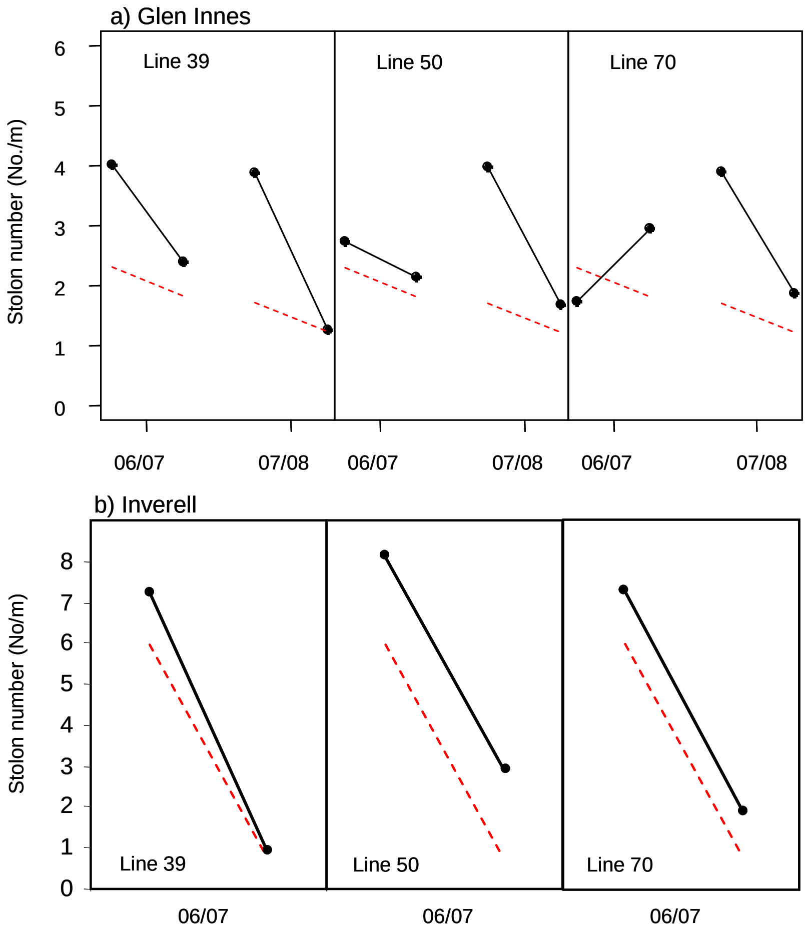


Figure 8 Stolon survival (through summer/autumn moisture stress) of the experimental varieties (■) compared with Haifa (– – –) at a) Glen Innes and b) Inverell

4.1.6 Field evaluation – seedling regeneration

With demise of the original populations of all lines at the Inverell site during the 2nd summer/autumn, opportunity was taken to measure clover presence from seedling regeneration during the subsequent year. Results for the experimental varieties, in comparison with Haifa, are presented in Figure 9.

All check cultivars (Haifa, Huia, Trophy, Tribute, Nomad) showed strong regeneration but suffered total demise during the following summer/autumn (Milestone 7 Report). Of the experimental varieties, 70 exhibited best regeneration capability similar to Haifa which reputedly has high regeneration capability. However, 70 along with all cultivars and lines suffered total demise with the onset of heat and dry associated of summer moisture stress in the subsequent summer/autumn.

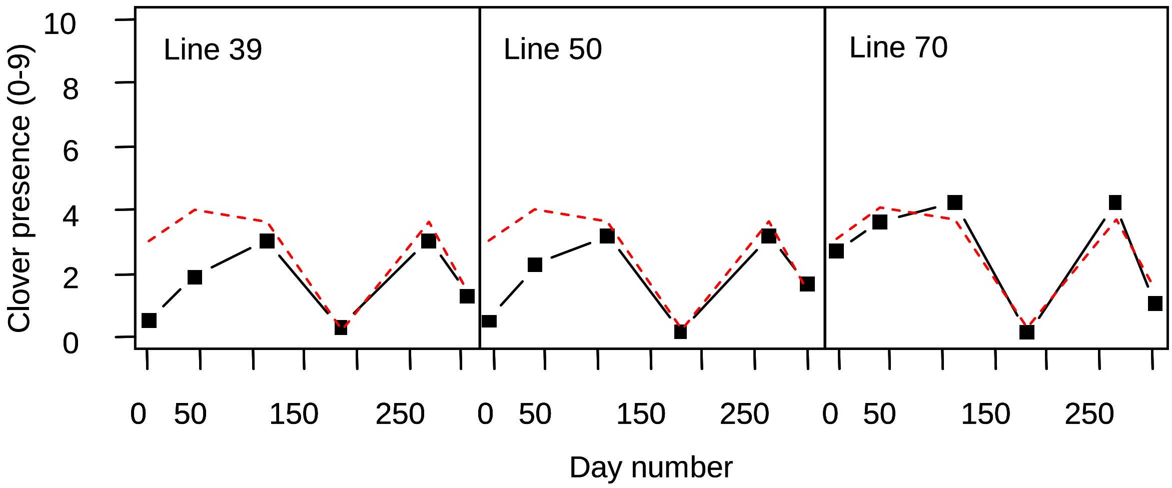


Figure 9 Seedling regeneration of the experimental varieties (■) compared with Haifa (– – –) at the Inverell site

4.2 Final Screening and Cultivar Development

Seed Production Screening of P164 elite lines – 2007/08

Initial selection of the P164 breeding lines resulted in identifying lines 39, 47, 50, 51, 69 and 70. During the summer 2007/2008 period, the seed production potential of the lines 39, 47 and 51 was evaluated. The objective of this evaluation is to identify and select parental plants with good seed yield capacity and a compact flowering pattern. The selected genotypes would then be polycrossed to generate a synthetic I population.

Table 4. Characteristics of the lead candidate lines based on performance to the end of year 2 at the 2 evaluation sites in NSW (Glen Innes, Macintyre). *** > best check cultivar; ** = best check cultivar; * > Haifa and < best check cultivar

Candidate lines	Presence	Yield	Stolon survival GI Mac	Stolon thickness	Leaf size	Early vigour	HCN rating	Flowering intensity	Adaptation
39 (7x12)	***	***	** **	Medium	Medium	***	93	**	Tablelands
47 (9x19)	**	**	* *	Medium	M/Large	***	100	**	Tablelands & slopes
50 (11xTribute)	**	*	* ***	Thick	Medium	***	73	*	Tablelands & slopes
51 (11x19)	**	**	** **	Medium	Large	***	100	*	Tablelands
69 (DS-M)	***	***	- *	Medium	M/Small	**	92	**	Tablelands
70 (DS-ML	***	***	* **	Medium	Medium	**	90	*	Slopes

Parental Selection

(a) Line P164-51

The original line 51 had a high proportion of low seed yielding plants, however, there were sufficient plants with reasonable seed yield to make a viable selection (Table 5). The variability in peak flowering date (Figure 10) is of more concern in that it would be possible to pick out an early-flowering and a late-flowering selection. It was decided that an earlier-flowering type be selected due to this being potentially better for seed production in Australia. The early-selection has parents with 68% higher seed yield potential than the base population (Table 5). It is a medium to medium-large leaved line rather than a large-leaved line as suggested from NSW field trial data.

Table 5. Seed production traits of P164-51 at Lincoln 2007/08

P164-51	**Inflorescences no./plt**	**Seed yield g/plt**	**Leaf size (1-10)**	**Peduncle Height cm**
Original	112.1	13.91	5.8	2.72
Selection	111.4	23.44	5.7	2.70
	-0.6%	+68%	-1.8%	-0.8%

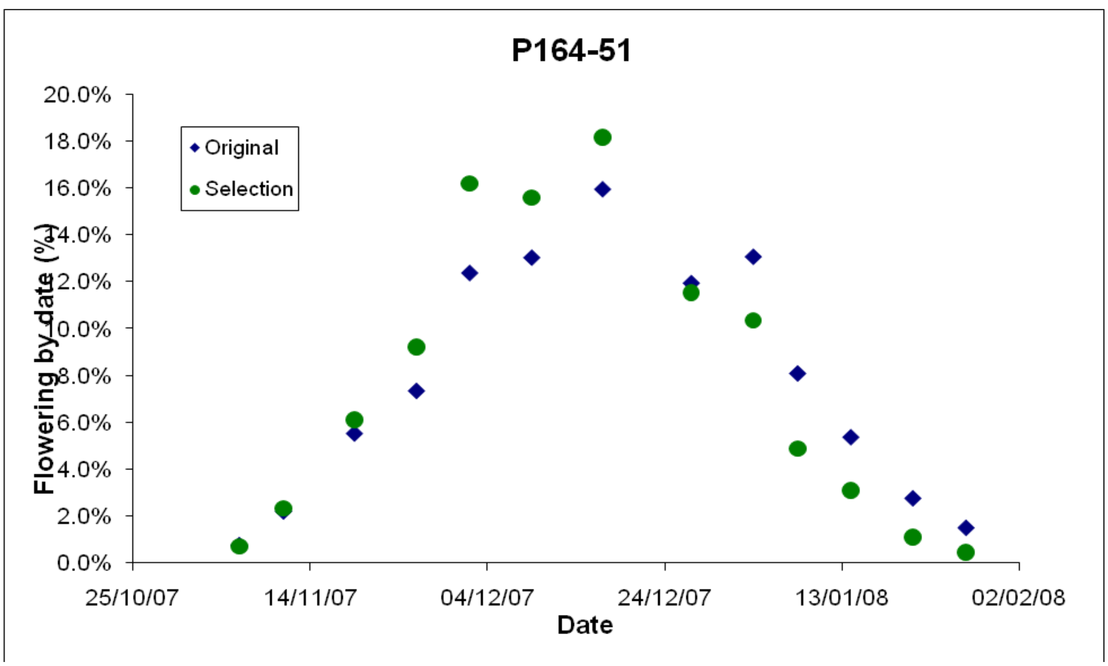


Figure 10. Flowering pattern of P164-51 at Lincoln 2007/08

(b) Line P164-47

Line 47 had a good consistent flowering pattern and was fairly early-flowering peaking in mid-December (Figure 11). The base seed yield of this population is definitely better than for line 51.

The selected parents had similar flower numbers but much better seed yield due to better seed yield per inflorescence (Table 6). Line 47 is a medium to medium-large leaved line.

Table 6: Seed production traits of P164-47 at Lincoln 2007/08

P164-47	**Inflorescences no./plt**	**Seed yield g/plt**	**Leaf size (1-10)**	**Peduncle Height cm**
Original	110	18.6	6.2	2.2
Selection	104	25.3	6.4	2.3
	-4.8%	+36%	+3%	+4%

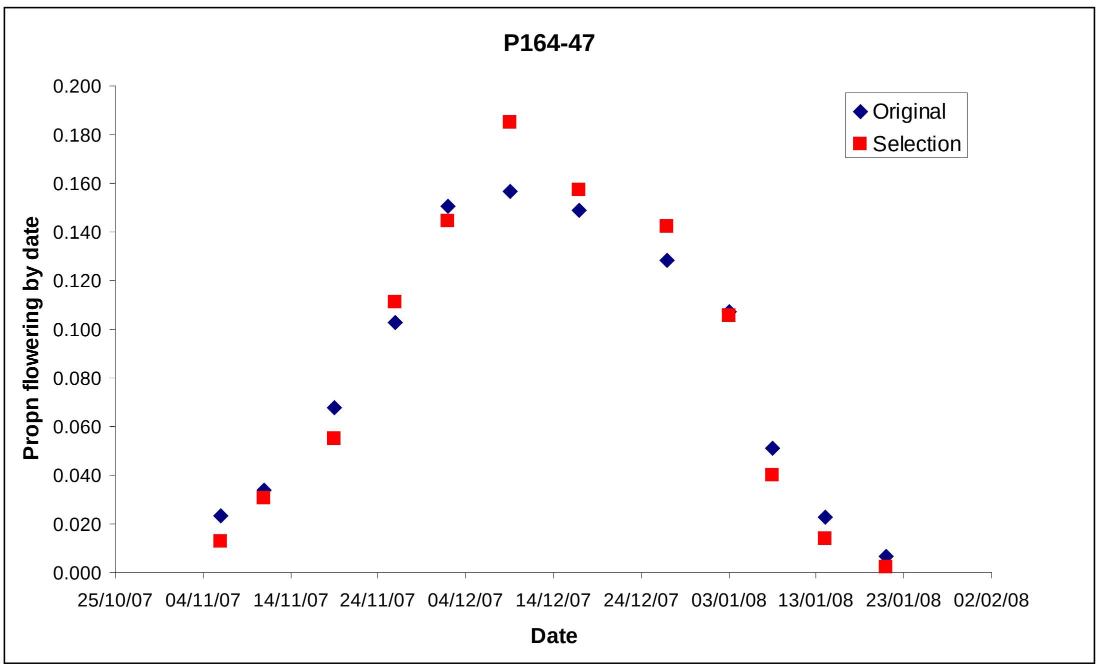


Figure 11. Flowering pattern of P164-47 at Lincoln 2007/08

(C) Line P164-39

Line 39 had a consistent flowering pattern peaking in mid-December (Figure 12). There was a hint of bimodal flowering with a secondary peak in early January. The selected parents have much better seed yield potential than the original population through a lift in seed/inflorescence and inflorescences per plant (Table 7). Line 39 is a medium to medium-large leaved line.

Table 7: Seed production traits of P164-39 at Lincoln 2007/08

P164-47	Inflorescences no./plt	Seed yield g/plt	Leaf size (1-10)	Peduncle height cm
Original	157.5	19.0	6.5	3.4
Selection	170.6	27.0	6.3	3.5
	+8%	+42%	-3%	+3%

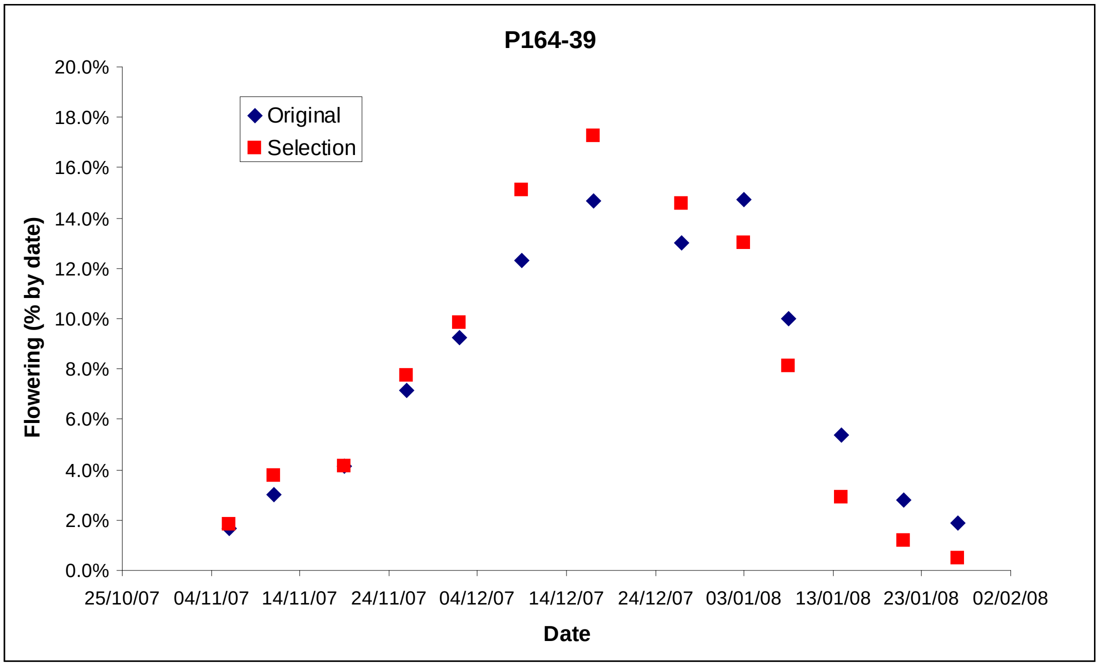


Figure 12: Flowering pattern of P164-39 at Lincoln 2007/08

Seed Production Screening of P164 elite lines – 2008/09

Lines P164-50, P164-69 and P164-70 are currently being assessed for their seed production potential at Lincoln over the summer period 2009/09.

The final assessment of P164 lines based on agronomic performance resulted in lines P164-39, P164-50 and P164-70 being selected. A further selection among these 3 lines will be carried out on their seed yield potential which will identify the best line for commercialization. Genotypes of this line with superior seed yielding potential will form the parental plants that will be polycrossed to produce the experimental synthetic I seed. This seed will be provided to Grasslanz for large scale seed increase. Synthetic I seed from the other elite selections will be available for evaluation in the new breeding program.

4.3 Commercialisation Plan

The final and crucial phase in the cultivar development process is to maximize seed yield and seed quality of the new experimental synthetics, to evaluate their regional and international performance, and to develop a sound marketing strategy. This will ensure reliable delivery of the "new forage technology" to farmers with the optimal technology support.

Grasslands Innovation Limited is a 50:50 joint venture between Grasslanz^{TM} and PGG Wrightson. This combines the forage seed production and seed technology skill base of Grasslanz^{TM} and the global marketing network of PGG Wrightson. Currently, PGG Wrightson Seeds holds two thirds of the forage seed market in Australasia. The international market proprietary share of Grassland Innovation products is growing rapidly.

PGG Wrightson Seeds aims to deliver the new persistent and productive white clover cultivars, developed from the breeding program, to farmers. This is currently focused on the Australian region, but also may have application to other overseas markets.

A key priority of all the parties involved in P164 is to supply the Australian grazing industries with new persistent and productive white clover cultivars that will enhance animal production and enhance the sustainability of pasture ecosystems.

In terms of commercialization of P164 bred material, the following steps are in place:

a) P164 elite selections are currently being entered into regional testing in Australia,
   – Victoria: Glenormiston (GIL trial), Ballarat (GIL trial)
   – NSW: 3 sites in stage 2, contained within the proposed 10-year breeding plan.
b) Following at least 2 years agronomic performance data, the best P164 line will be submitted for commercialisation. Once a line has been approved, then commercial multiplication will commence and the development of a marketing plan will (marketing brochures, on-farm demonstrations and commercial seed multiplication) begin.
c) The regional evaluation trials will be inspected by PGG Wrightson staff in Australia responsible for marketing forage products. This will primarily involve Rob Salmon (Product Development manager) and Peter Young (Australian General Manager) who will be a key part of the stage II review to commercialise products.

4.4 Role of Quercetin

In a constantly changing environment there are many abiotic adverse stress conditions such as heat, cold, drought, salinity and UV-B, which influence plant growth and crop production. Unlike animals, higher plants, which are sessile, cannot escape from their surroundings, but adapt themselves to changing environments by inducing a series of molecular responses to cope with these problems. Treutter (2005) reviewed the roles of flavonoids in plant defence against pathogens, herbivores, and environmental stress. He stated that the induction of defence-related flavonoids is modified by other determining factors and competition between growth and secondary metabolism may exist. In an evolutionary context, stress-related oxidative pressure may have been a major trigger for the distribution and abundance of flavonoids. Resistant genotypes in general all show significant increases in flavone or flavonol synthesis which accumulate rapidly in epidermal vacuoles and infrequently also in epicuticular waxes, and act as a "sunscreen". Sinapate esters can also act as protective pigments against UV light. Photoprotection seems to be a predominant role of flavonoids which is afforded by the high Quercetin:Kaempferol ratio in Petunia leaves (Ryan 2002; Ryan 2001; Mahdavian 2008). Also, studies at AgResearch identified a link between moisture stress tolerance and the presence of the flavonol, quercetin (Hoffman *et al*. 2003). These findings provided the basis for screening the breeding lines and control cultivars evaluated in project P164. The objective was to investigate the association of flavonol expression and plant persistence/herbage production in white clover.

5 Discussion

5.1 The experimental varieties

The project has developed three experimental varieties (P164–39, P164–50, P164–70) by conventional breeding processes (selecting superior genotypes, crossing elite germplasm, progeny testing derived breeding lines) that show sound agronomic performance under the low rainfall (700-850 mm AAR) conditions of North-West NSW. Progressing these through parental selection will likely result in the development of a ‘locally adapted’ white clover cultivar for the ‘dry margins’ (700–850 mm AAR) of eastern Australia. This has potential to greatly expand the perennial legume zone.

The primary selection criteria were:

- ‘Herbage yield’ and ‘presence’ longitudinal profiles exceeding the industry standard Haifa (and all comparator cultivars)
- Favourable combination of high stolon thickness x high stolon density for high stolon survival (through summer moisture-stress) and rapid autumn regrowth
- Desirable plant characteristics including medium/large leaf size, early vigour and mid/late flowering maturity.

The three experimental varieties P164–39, P164–50 and P164–70 are medium/large in leaf size (with exceptional leaf size stability), mid-season in flowering maturity and high in stolon density. The HCN status of line 39 and line 70 are similar to Haifa, whereas line 50 is relatively lower in frequency of cyanogenic leaves. Specifically:

P164–39

- Medium/large leaf size, high leaf size stability with moderate incidence of leaves with leaf crescent and low level red flecking
- Medium stolon thickness x high stolon density
- Mid-season maturity.

P164–50

- Medium/large leaf size, very high leaf size stability and high incidence of leaves with leaf crescent but without red flecking
- Medium stolon thickness x very high stolon density
- Late maturity.

P164–70

- Medium/large in leaf size, very high leaf size stability and moderate incidence of leaves with crescent leaf mark and low level red flecking

- Medium stolon thickness x high stolon density
- Mid-late maturity.

The 3 experimental varieties have superior colonising ability and early vigour compared with Haifa, and consistently and substantially out-perform Haifa (and all other check cultivars) in seasonal herbage yield. All three experimental varieties maintain high presence (> 80% cover) for at least 3 years and both P164–39 and P164–70 are exceptional in retaining a large component of their stolon structures following seasonal drought that is indicative of potentially long-term persistence.

5.2 Elite 'locally adapted' lines

In addition to the experimental varieties 39, 50 and 70 that maintained exceptional performance into year 4, three cultivars (Goliath, Durana, Will) and 16 lines persisted (Figure 13) and showed yield profiles (Figure 14) comparable with Tribute – the best performing check cultivar.

Of these, 8 lines (23, 42, 53, 56, 64, 69, 72, 91) were exceptional in outperforming Tribute. These 8 lines, together with experimental varieties 50 and 70 and cv. Tribute and Will form a valuable set of 'locally adapted' germplasm for progressing into new breeding cycles to develop 'new generation' cultivars for the hot and dry conditions expected with climate change.

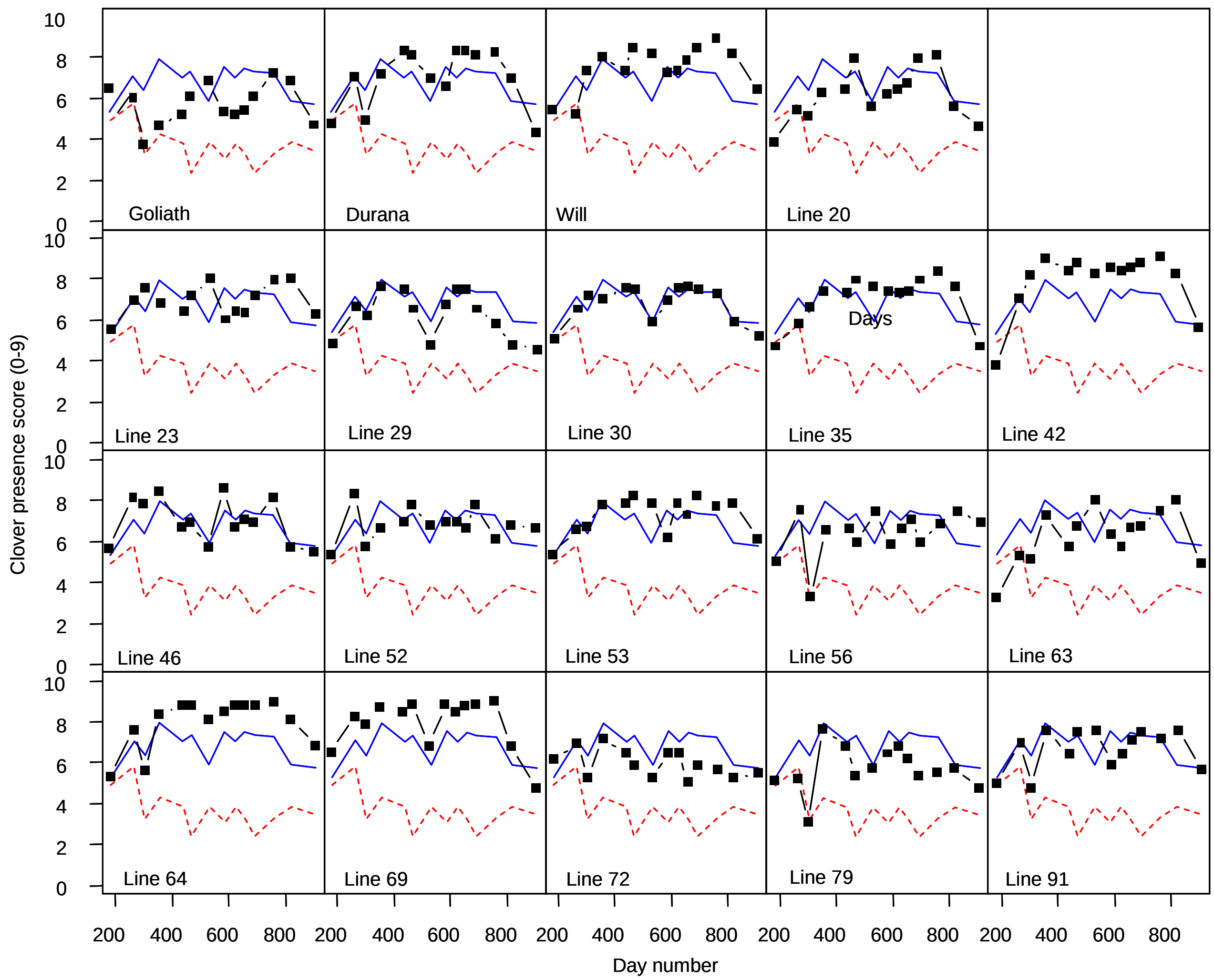


Figure 13 Longitudinal profiles of clover presence of lines showing persistence (■) to year 4 at Glen Innes compared with Haifa (– – –) and Tribute (–––)

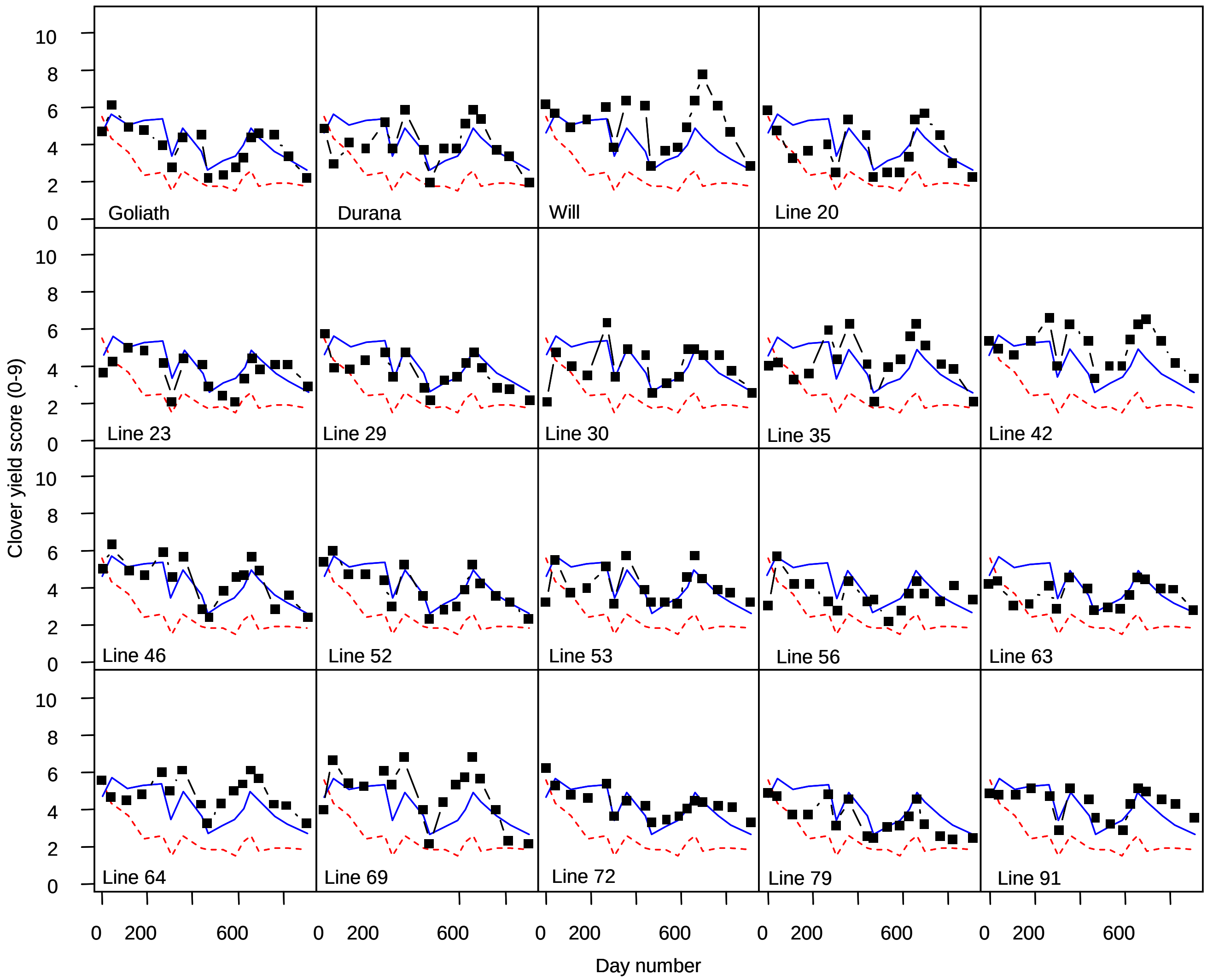


Figure 14 Longitudinal profiles of herbage yield of lines showing persistence (■) to year 4 at Glen Innes compared with Haifa (– – –) and Tribute (–––)

5.3 Quercetin profile in breeding lines

The profiling of quercetin expression in the breeding lines and commercial controls was carried out in two stages; a) preliminary Characterisation of all breeding lines and controls, and b) Characterisation of superior and poor performing lines post summer (2006/2007) moisture stress.

a) Preliminary characterisation of breeding lines and control cultivars for Flavonoid expression profiles

Material and methods

A random sample of 10 genotypes from each of the 100 breeding lines and cultivars, characterised for plant morphology was taken in September 2005 for flavonoid expression testing. Each breeding line/control cultivar was represented by a bulk sample consisting of 2 to 3 trifoliate leaves from each of the 10 genotypes. Each bulk foliar sample was freeze-dried immediately after the leaves were picked.

Fifty mg of freeze-dried, ground leaf material were weighed into 2 ml screw-cap tubes and extracted with 1 ml 80% MeOH/ 0.1% HOAc (30 min, RT, shaking). Extracts were centrifuged (13,000 g, 15 min), supernatants transferred into glass vials and analysed by HPLC using UV and fluorescence detection (Column: Phenomenex Luna RP C18 5u, 150 mm 4.6 mm; flowrate: 1 ml/min); Detector A: fluorescence (Exc. 250, Em. 418, Response time 1.5, Range 1.6, Sensitivity Low); Detector B: UV-PDA (PhotoDiodeArray), UV-maxplot; Solvent A: 99.9% H_2O, 0.1% formic acid; Solvent B: 99.9% Acetonitrile, 0.1% Formic acid; Gradient: 0 min B 5%, 6 min B 5%, 11 min B 10%, 26 min B 17%, 31 min B 23%, 41 min B 30%, 45min B 50%, 52min B 50%, 55 min B 97%, 59 min B 5%, 70 min B 5%. Compounds were quantified based on calibrations with known standards.

Data analysis

The results from the flavonoid expression assessment were analysed using pattern analysis (Watson *et al*. 1995; Kroonenberg 1994; and Gabriel 1971). Some of the morphological characterisation and plant HCN results, measured earlier, were also compared with the flavonoid concentration results.

Results and discussion

The TR41 breeding lines showed high expression profiles for quercetin as well as flavonol (Table 8). The AgResearch drought selections were also high in quercetin and flavonol expression having higher levels than the rest of the breeding lines and cultivars. The top 10 and lower 10 breeding lines for quercetin expression are listed in Table 9. Seven of these lines are TR41 selections while a further two lines, 87 and 69 are NSW drought selections. Line 5, Lincoln dryland survivors were also high in quercetin. There was only one TR41 selection, line 26, among the bottom 10 lines based on quercetin (Table 9).

Table 8. Means of quercetin and flavonol expression based on the source of the lines and cultivars.

Entries	Quercetin (mg/g DW)	Flavonols (mg/g DW)	Total flavonoids (mg/g DW)
AgResearch drought selections	2.18	3.81	5.99
NSW drought selections	1.52	2.88	4.40
TR41 lines	**2.31**	**4.12**	**6.43**
US and South American cultivars	2.02	3.38	5.40
Control cultivars	1.85	3.55	5.40
Other	1.88	3.40	5.28

Table 9. The top and lower 10 breeding lines for Quercetin expression

Line No.	Description	Quercetin (mg/g DW)
	Top 10 lines	
16	*TR041 Line 12 x Tribute*	4.930
13	*TR041 Line 12 x Line 23*	4.451
11	*TR041 Line 8 x Line 12*	3.965
38	*TR041 Line 13 x Nu Siral*	3.916
5	Lincoln dryland survivors	3.574
69	Medium leaf survivors ex Glen Innes	3.571
18	*TR041 Line 23 x Emerald*	3.526
32	*TR041 Line 11 x Tribute*	3.479
87	C9008 - Italy	3.466
5	*TR041 Line 12 x Emerald*	3.316
	Bottom 10 lines	
83	Gwenda	1.029
79	Medium leaf/relatively high stolon density	1.007
100	Medium small leaf ex Tas Dept.Agr	0.966
88	C9021 - Portugal	0.934
86	C9002 - Italy	0.878
90	C9038 - Spain	0.855
26	*TR041 Line 8 x Line 25*	0.844
98	C10537 - Azerbaijan	0.671
77	Linfield ecotype	0.605
95	CPI 32017	0.582

Figures 15 and 16 indicated the association of plant quercetin expression and the two traits leaf length and HCN concentration, respectively. Breeding lines 11, 13, 16 and 18 had relatively high quercetin expression in comparison to the control cultivars. The leaf sizes of these lines were in the larger category (Figure 15). The TR41 lines identified in Figure 15 appear to have high HCN levels (Figure 16). Two NSW selections lines 87 and 89 had a combination of high quercetin and low HCN expression.

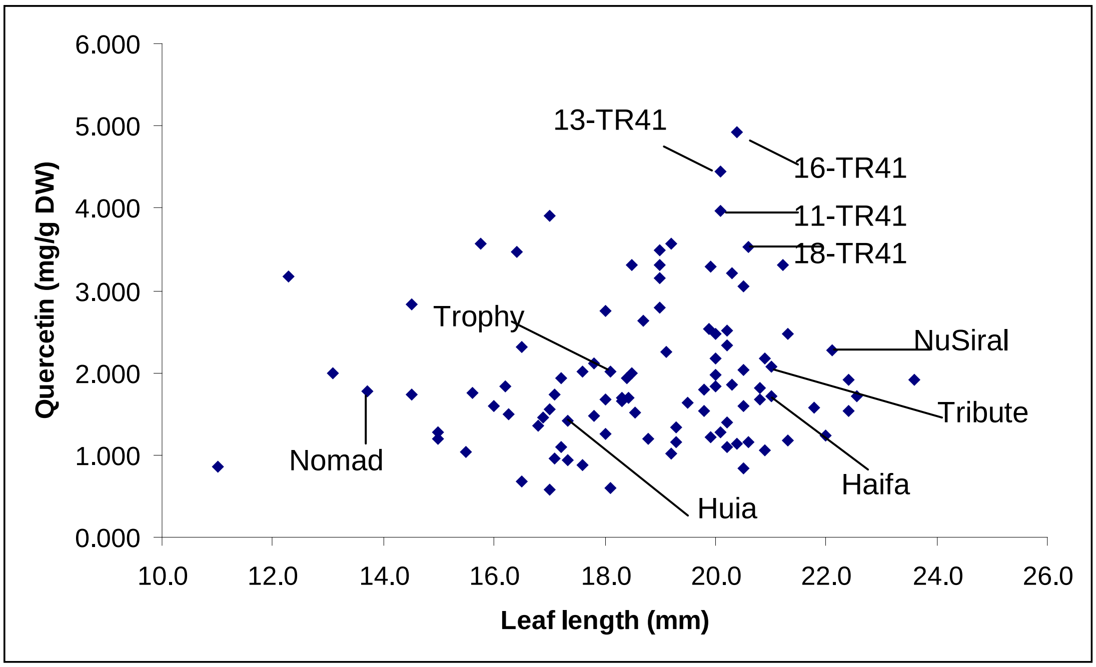


Figure 15. Association of leaf length (mm) with plant quercetin (mg/g DW) concentration in the breeding lines and cultivars. The positions of the control cultivars are indicated.

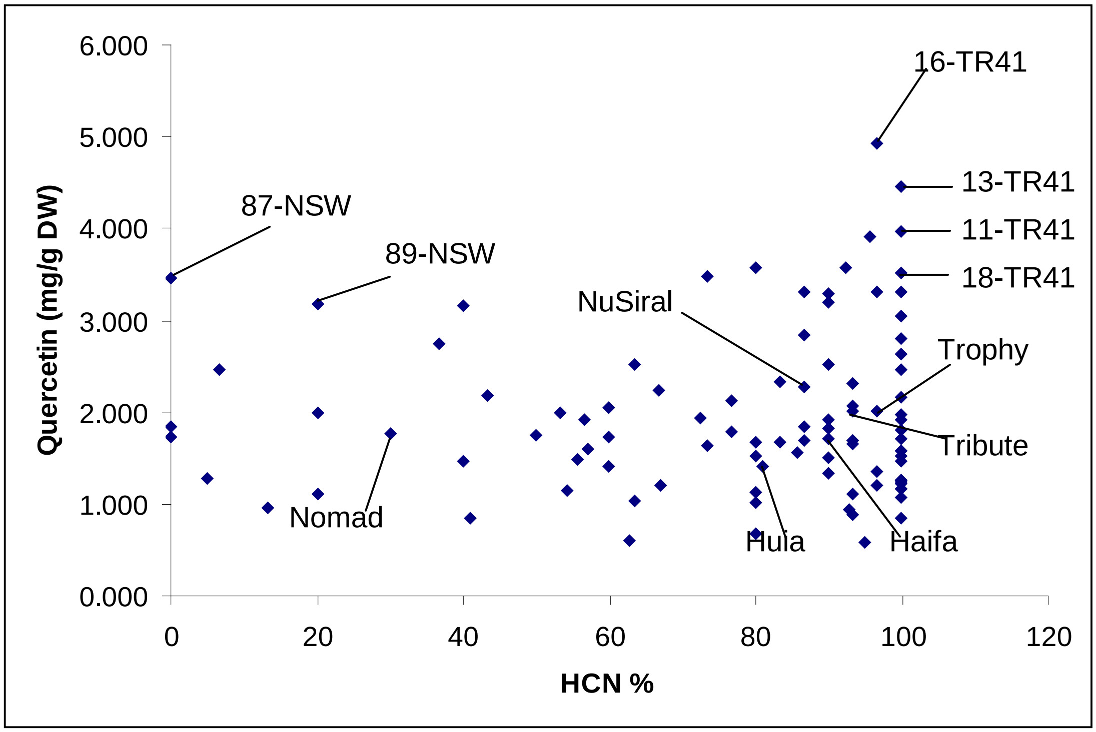


Figure 16. Association of HCN (%) and plant quercetin (mg/g DW) concentration in the breeding lines and cultivars.

The biplot in Figure 17 generated from pattern analysis indicated 4 distinct groups of lines relative to the 6 directional vectors; Ca, Cafferic acid; Cg, Cyanidin; Fl, Flavonols; Fg, Formononetin; Ka, Kaempferol; Qu, Quercetin. Group 3 consisted of 17 lines, that were all TR41 selections. The members of group 3 were characterized by a higher expression flavonols, kaempferol, quercetin and cyanidin. These flavonoids also showed a strong positive correlation.

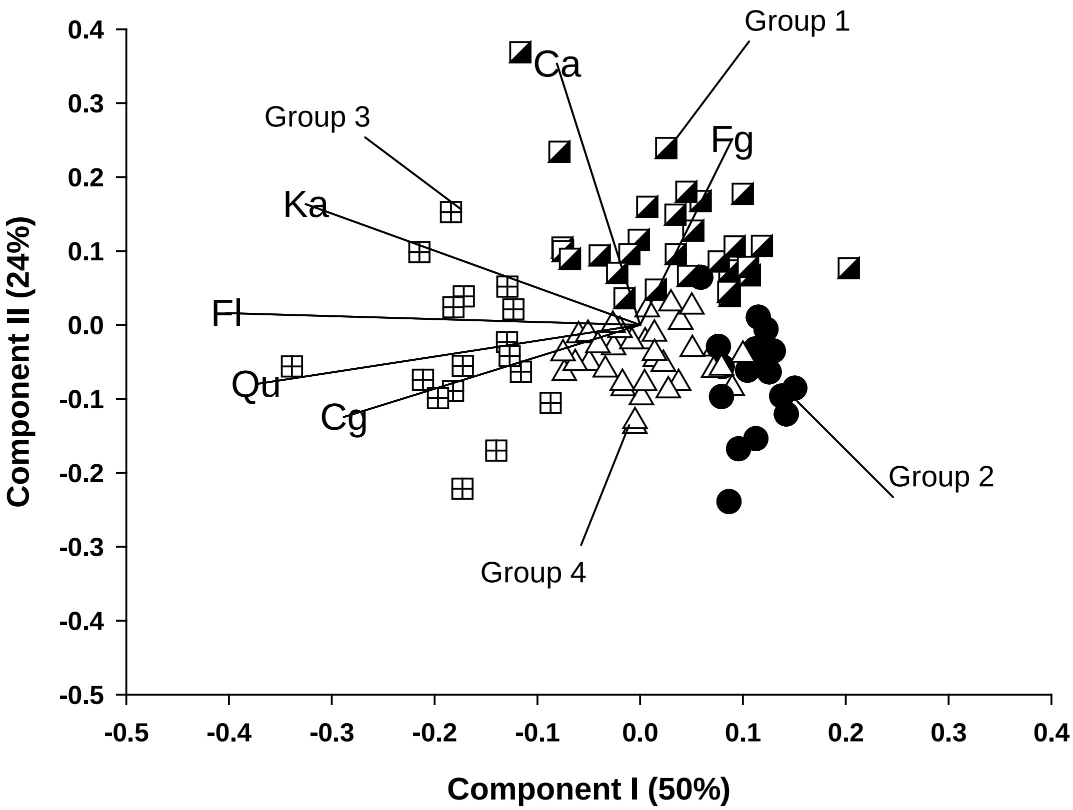


Figure 17. Biplot from principal component analysis showing the variation among the population of 98 breeding lines and cultivars relative to the directional vectors that represent: Ca, Cafferic acid; Cg, Cyanidin; Fl, Flavonols; Fg, Formononetin; Ka, Kaempferol; Qu, Quercetin. The symbols represent the 4 breeding line/cultivar groups that were based on flavonoid expression.

Outcomes

The preliminary screening of the breeding lines and cultivars indicated a range of expression for quercetin as well as the other flavonoids that was greater than expected. The spring harvest should have seen flavonoid levels at there lowest, however our results found high levels with the highest levels occurring in lines that had previously been selected for drought and/or heat tolerance.

Some large leaved breeding lines exhibited high quercetin expression and had a high HCN concentration. There were two NSW selections that were relatively high in quercetin but had low HCN expression. All the lines with high flavonoid expression were TR41 selections.

b) Characterisation of superior and poor performing lines post summer (2006/2007) moisture stress.

Analysis of the flavonoid expression levels in breeding lines with superior drought tolerance in NSW at the end of the 2006/2007 summer will be helpful in determining whether quercetin expression and plant performance are correlated under moisture stress. This will also indicate the potential to use quercetin expression as a predictor of tolerance to moisture stress.

The preliminary Characterisation of the expression profile of the flavonoid quercetin across the total set of 100 breeding lines and controls indicated significant (*P<0.05*) variation among the lines. The next step was to test quercetin expression levels, post 2006/2007 summer moisture stress, of the top 10% and bottom 5% lines relative to the control cultivars evaluated at the trials in Macintyre and Glen Innes.

Unfortunately, the severe drought conditions during the 2006/2007 summer period resulted in the death of most plants of all lines in the trial at Macintyre. In February 2007, at the Glen Innes site, leaf samples were taken from the 5 controls and 10 breeding lines which had a relatively good leaf cover. It was not possible to sample the poor performing lines as there were no leaves.

The data on quercetin expression from the best 10 lines and controls at Glen Innes at the end of the 2006/2007 summer period was analysed.

Materials & Methods

Leaf sampling:

In mid February 2007, leaf samples from 10 breeding lines and 5 control cultivars, that had sufficient foliage to make up 1g to 2g per sample each, were taken from the 3 replicates of the trial at Glen Innes. Each sample was stored in a plastic bag that was placed in a thermal box containing ice blocks. The samples were transported to Tamworth, NSW, where they were freeze-dried. The freeze-dried samples were then sent to the University of Lincoln in New Zealand, where they were analysed for flavonoids by Dr. Rainer Hoffman.

Chemical analysis:

The white clover laminae were ground to a fine powder in liquid nitrogen and 25 mg of the powder was extracted in darkness for 24 h in 3 mL MeOH-H_2O-HOAc (79:20:1). After filtration and centrifugation, 25 µL was used for HPLC analysis, with chromatography performed on a Waters HPLC with photodiode array (PDA) monitoring.

The HPLC column was a Merck Supersphere Lichrocart 125-4 RP-18 endcapped column. The gradient solvent system comprised solvent A [1.5% H3PO4] and solvent B [HOAc : CH3CN : H3PO4 : H2O (20 : 24 : 1.5 : 54.5)], mixed using a linear gradient starting with 80% A, decreasing to 33% A at 30 min, 10% A at 33 min and 0% at 39.3 min. Injection volume was 25 uL and flow rate 0.8 ml/min. Flavonol peaks were measured at 352 nm and quantified using a rutin standard curve. Quercetin and kaempferol derivative peaks were identified on the basis of the on-line spectrum recorded for each identifiable peak.

Chromatograms showed a characteristic pattern of quercetin and kaempferol derivative peaks for white clover, with the main quercetin peak representing quercetin-3-O-β-D-xylopyranosyl-(1→2)-β-D-galactopyranoside. Similarly, a kaempferol-3-O-β-D-xylopyranosyl-(1→2)-β-D-galactopyranoside peak was present in the samples.

Statistical analysis:

The analysis of the flavonoid expression data was conducted using the variance component analysis procedure, REML option, in GenStat 9.1 (2006).

Results & Discussion

The analysis of variance indicated no significant (*P>0.05*) differences among the 10 breeding lines and 5 controls. Figures 18 and 19 show the comparison of results of quercetin expression of the 10 breeding lines and 5 controls in this study and their analysis in 2005 conducted at Palmerston North, reported in Milestone P164-4. Line 69 had a high expression of quercetin in both environments. This was followed by lines 52 and 51 (Figure 18). Cultivar Trophy showed relatively high expression of quercetin in both environments (Figure 19).

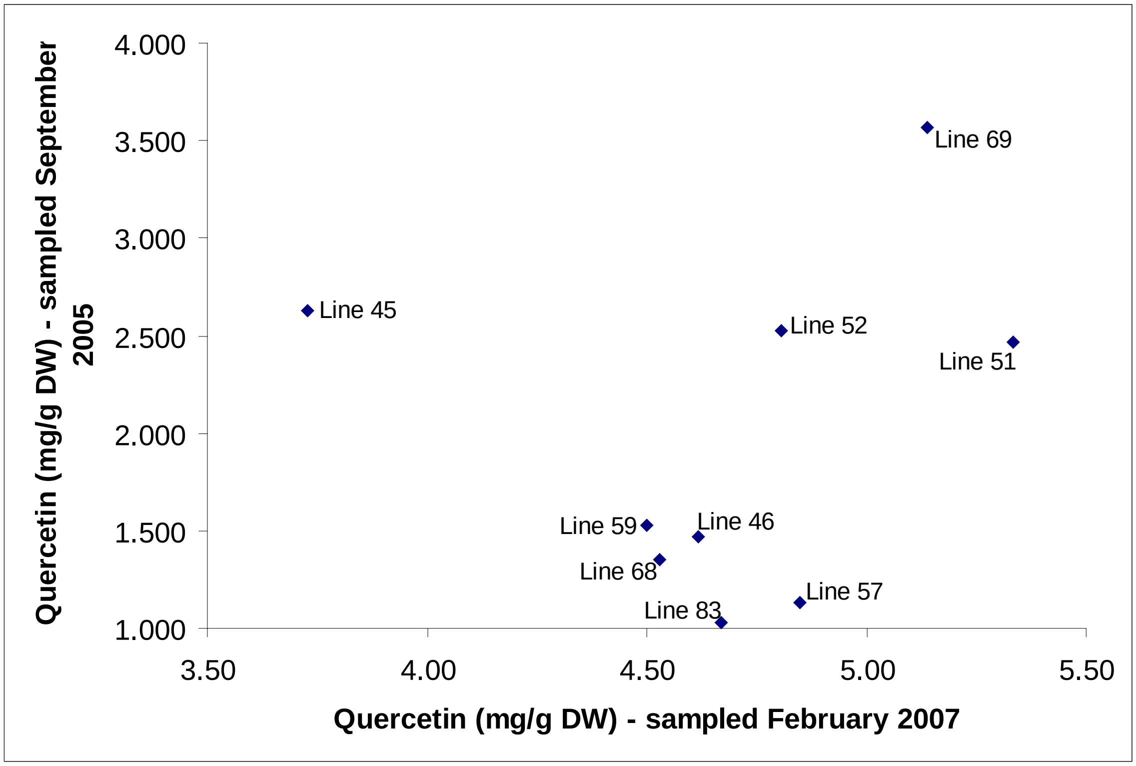


Figure 18. Comparison of quercetin expression (mg/g DW) of the top 10 breeding lines sampled from the Glen Innes field site in February 2007 and their results in 2005 conducted at Palmerston North.

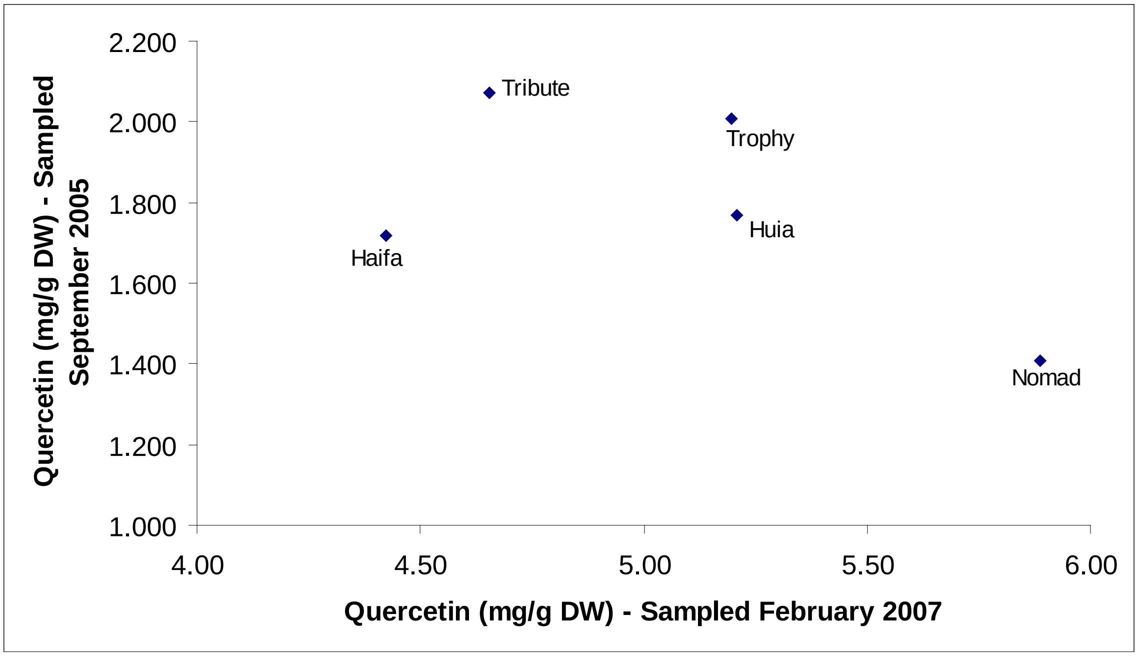


Figure 19. Comparison of quercetin expression (mg/g DW) of the 5 control cultivars sampled from the Glen Innes field site in February 2007 and their results in 2005 conducted at Palmerston North.

It is unfortunate that the poorly performing lines at Glen Innes were not sampled due to their poor or nil leaf production. This could have provided discrimination among the high and low performing lines for quercetin expression. However, the results have indicated that line 69, a key recommended line for persistence and performance, showed high quercetin expression.

Quercetin expression in selected elite lines, commercial controls and less productive breeding lines, post summer 2007/2008.

On the 25th of March 2008, leaf samples from 6 elite selections, 9 poor performing breeding lines and 5 control cultivars, were taken from 3 replicates of the field trial at Glen Innes. Each sample was stored in a plastic bag that was placed in a thermal box containing ice blocks. The samples were transported to Tamworth, NSW, where they were freeze-dried. The freeze-dried samples were then sent to the University of Lincoln in New Zealand, where they were analysed for flavonoids by Dr. Rainer Hoffman. The chemical analysis conducted was similar to that described above.

Results & Discussion

The analysis of variance indicated significant ($P<0.05$) differences among the 15 breeding lines and 5 commercial controls. Elite line 50 had significantly ($P<0.05$) higher quercetin expression than the controls Nomad, Tribute and Huia (Figure 20). Although, other elite lines such as 39, 69 and 70, were not significantly ($P>0.05$) different to the controls, there was a trend of higher quercetin expression in these lines relative to the controls Haifa, Nomad, Tribute and Huia.

The association of quercetin expression and plant persistence of white clover under higher temperatures and low rainfall needs to be further investigated by conducting an experiment in an environment such as Macintyre.

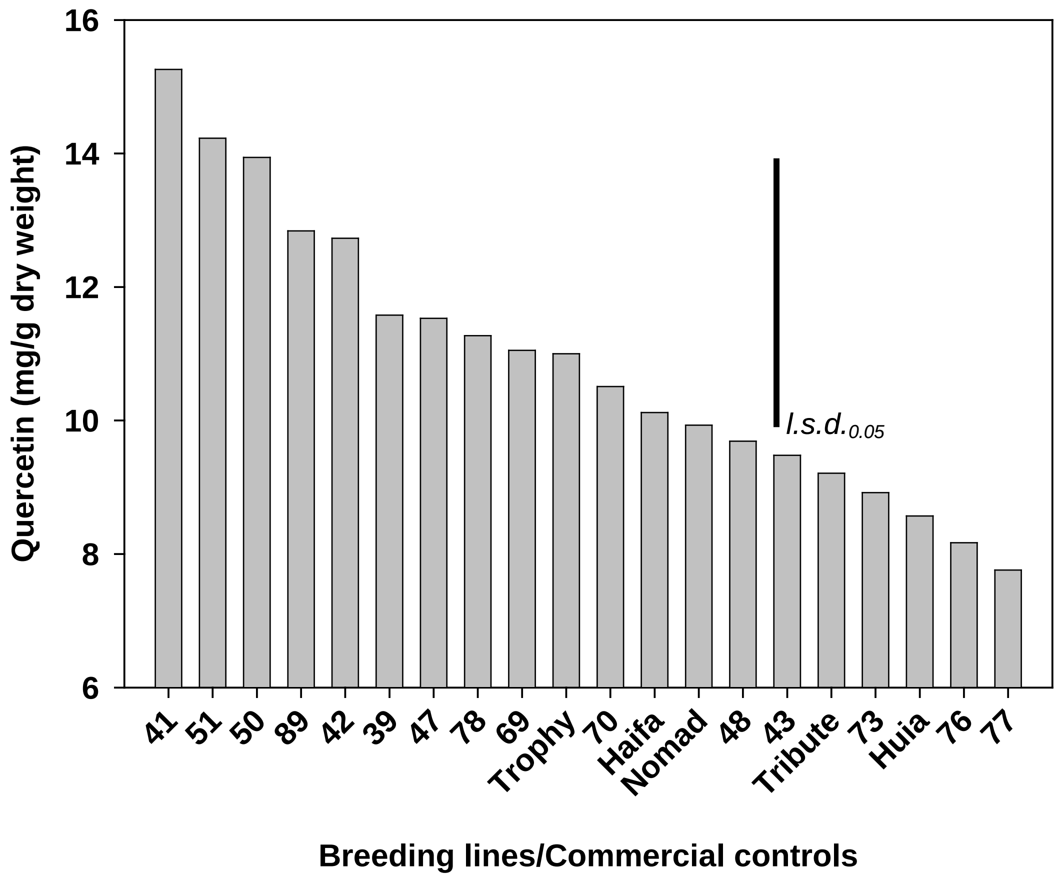


Figure 20. Quercetin expression (mg/g DW) of the sampled 15 breeding lines and 5 commercial controls from the Glen Innes field site in April 2008.

References

Cowie JD. 1978. Soils and agriculture of Kairanga County, North Island, New Zealand. New Zealand Soil Bureau Bull. 33.

Gabriel KR. 1971. The biplot graphical display of matrices with application to principal component analysis. *Biometrika* 58, 453-67.

GenStat. 2003 GenStat for Windows. Release 7.1. Sixth Edition. VSN International Ltd., Oxford.

Hofmann RW, Campbell BD, Swinny EE, Bloor SJ, Markham KR, Fountain DW. 2003. White clover sensitivity to UV-B radiation – biochemical relationships and interaction with drought. *Proceedings of the New Zealand Grassland Association 65*: 273-277.

Isabell RF. 2002. In: The Australian Soil Classification. CSIRO Publishing.

Kroonenberg PMK.1994. The TUCKALS line: A suite of programs for three-way data analysis. *Computational Statistics and Data Analysis 18*, 73-96.

Mahdavian K. Ghorbanli M, Kalantari KM. 2008. The effects of ultraviolet radiation on the contents of chlorophyll, flavonoid, anthocyanin and proline in Capsicum annuum L. *Turkish Journal of Botany, 32*(1), 25-33.

Ryan KG,, Swinny EE, Markham KR, Winefield C. (2002). Flavonoid gene expression and UV photoprotection in transgenic and mutant Petunia leaves. *Phytochemistry, 59*(1), 23-32.

Ryan KG, Swinny EE, Winefield C. and Markham KR. 2001. Flavonoids and UV photoprotection in Arabidopsis mutants. *Zeitschrift Fur Naturforschung C-a Journal of Biosciences, 56*(9-10), 745-754.

Treutter D. 2005. Significance of flavonoids in plant resistance and enhancement of their biosynthesis. *Plant Biology, 7*(6), 581-591.

Watson SL. DeLacy IH, Podlich DW. Basford KE. 1995. GEBEI An analysis package using agglomerative hierarchical classificatory and SVD ordination procedures for genotype x environment data. *Centre for Statistics Research Report*, Department of Agriculture, The University of Queensland, Australia.

5.4 Future Directions

There are two broad opportunities in dryland pasture environments in Australia for white clover cultivars that possess traits for adaptation to heat and dry:

1. To extend the benefits of white clover technology into marginal environments
2. To improve industry readiness to accommodate the future impacts of climate change.

The NSWDPI/AgResearch Alliance has not fully exploited the potential to develop 'dry margins' varieties. This is an important national target that the Alliance is well equipped to pursue through additional cycles of breeding. This will fully utilise the pool of derived germplasm (progressing from DAN85, TR41, P164) to develop 'locally-adapted' white clover cultivars for challenging environmental conditions.

Moreover, the development of white clover cultivars for the 'dry margins' and 'heat & dry' applications is of major significance globally. Pastoral systems are being pushed into more marginal environments world-wide, so there will be international applications of cultivars developed from the germplasm pool progressing from P164.

Climate change in particular represents a challenging new influence. Breeding objectives need to be revised to accommodate this new and adverse influence on the adaptation and persistence of perennial pastures. In the era of global warming and increased greenhouse gases, the primary new challenges for pastures will include:-

- Increase in climate variability *… less reliable seasonal conditions*
- Increase in ambient temperature …. *increased heat factor*
- Decline in rainfall …. *reduced soil moisture*

Further cycles of white clover breeding from the Alliance, with a focus on traits for adaptation to 'heat & dry' are necessary to consolidate extending the zone of adaptation of white clover into the dry margins, and also to increase preparedness for the adverse effects of climate change across the white clover zone. In this context, the future goal for further white clover breeding is to develop a suite of white clover cultivars that are locally-adapted to the key targets:

- *The existing white clover zone* – permanent pastures in the high rainfall (750-1,000 mm AAR) Perennial Grass/Perennial Legume Zone where encroaching climate change will require adaptation to 'heat and dry'
- *The dry margins* – native/naturalised pastures in the medium rainfall (650-750 mm AAR) Perennial Grass/Annual Legume Zone where low rainfall combined with a high heat factor combine to constitute greater moisture stress

It is proposed that the next phase of the breeding program will consist of the following 3 stages (Figure 1):

- Stage 1: Breeding and selection for climate change
- Stage 2: Regional evaluation to determine and demonstrate the agronomic merit and genetic gain achieved with variety products of the program

Stage 3: Development and commercialization

A 10-year plan has been developed by the Alliance for planning future directions of the program.

6 Success in Achieving Objectives

The project has successfully met milestone targets with timeliness and within budget.

The conduct of project activities closely followed the planned milestones which were structured on 6-monthly steps. The completion of milestone activities was accompanied by milestone reports that were tabled for consideration and endorsement by a management committee prior to submitting to MLA. All milestone reports were submitted by the target deadlines and were formally accepted by MLA.

The project maintained a productive association between the stakeholders and project workers, and produced valuable variety products supported by a 'value-adding' knowledge base of scientific publications that will greatly enhance commercialisation.

The P164 Management Committee provided an oversighting body that regularly audited progress with the project, provided guidance and was a decision-making authority for directing the project. The committee had representation from each stakeholder – the Agencies (NSW DPI, AgResearch), the co-funders (MLA, Grasslanz) and the licensed commercial partner (PGG Seeds); and, received regular contributions from key collaborators (UNE biometrician, NSWDPI and AgResearch support staff).

In terms of <u>specific objectives</u>, the project has completed the progeny testing of the 95 breeding lines at the two sites. The project set out to undertake this testing over three growth cycles (years) at a site where 'dry margins' conditions are moderate (Glen Innes) and at a site where 'dry margins' conditions are severe (Inverell). The evaluation at Glen Innes was completely successful in developing full data-sets for three consecutive years that included a sequence of challenging moisture-stress events. At the Inverell site, the typically adverse influences of 'heat & dry' resulted in achieving only two years of data because of demise of all original populations following exposure to the moisture-stress of two summers. However, advantage was taken to both i) sample surviving plants to provide a collection of some 650 genotypes for use in a subsequent breeding cycle and ii) to study regeneration from the soil seedbank. These unplanned outcomes will be important for future directions of the breeding program – a 10-year plan has been developed to plan these new directions.

In terms of the <u>broad aim</u> of the project, namely "…*to breed a white clover cultivar for beef and sheep pastures in dry margins environments of Australia*", this aim is close to realisation with the nomination of three 'experimental varieties' for subsequent progressing to cultivar status and commercialisation.

In terms of the seed production potential of the selected elite breeding lines, line 39 was evaluated together with two other agronomically promising lines 47 and 51, at Lincoln during the summer period 2007/2008. Line 39 is medium to medium-large leaved with a higher seed yield than lines 47 and 51. Currently, lines 50 and 70 are being evaluated at Lincoln for their seed yield potential. The final decision on selecting one of the three lines; 39, 50 or 70, for commercialization will be made in February 2009. Pre-nucleus seed production will occur in 2009/2010. Nucleus seed will be produced in 2010/2011. This will be followed by a decision on commercialization of the selected line in 2011.

7 Impact on Meat and Livestock Industry

Under Australian conditions of extensive grazing, legume-based pastures are an important feed resource because of the contribution of the legume component to feed quality and to the sustainability of soil fertility, water resources and crop production (especially where ley farming is practised). White clover is the main perennial legume for high rainfall temperate regions due to widespread industry awareness of these strengths combined with its broad adaptation to soil conditions and grazing applications.

The development of the locally-adapted cultivars Trophy and Saracen (and Tribute in Victoria) represents important progress in providing farmers with a reliable white clover option for the high rainfall zone, specifically the Northern Tablelands and more favoured districts in central and southern NSW.

It is expected that a cultivar arising from one of the short-listed lines from P164 will extend the use of white clover into the dry margins (North-West Slopes, Southern and Monaro Tablelands).

Desk-top analysis indicates that this will maintain an existing zone of adaptation of 6 million hectares, and moreover, open up potential to expand the potential zone of adaptation of white clover-based pastures to some 16 million hectares. Prior survey work indicates that the potential adoption rate for new pasture cultivars in ‘safe’ country (viz. high rainfall) is of the order of 50 per cent while the corresponding rate in ‘marginal’ country (viz. dry margins) is 25 per cent.

For the industry, the potential of the program is to:

- Maintain 50 per cent of the high rainfall zone under perennial clover-based pastures within 10 years of the commercialisation of locally-adapted white clover cultivars that persist through vegetative regeneration
- Expand the use of perennial clover-based pastures into 25 per cent of the dry margins within 10 years of the commercialisation of robust drought-tolerant white clover cultivars that persist through seedling regeneration.

For livestock producers, locally-adapted white clover cultivars will provide valuable options:

- Enhance the feed-year by increasing spring feed supply, mitigating the winter feed-gap and increasing the supply of high quality leguminous forage over summer – with benefits to providing increased capability for producing products to market specifications
- Increase carrying capacity – with favourable consequences for financial viability
- Reduce fluctuations in the season-to-season and year-to-year feed supply – with benefits for cash flow and reduction in risk
- Increase soil fertility through the N-fixing properties of pasture legumes, reduce the use of N fertiliser and decrease the ‘carbon footprint’ of the grazing industries
- Provide a perennial legume for problem soils (eg acidic soils not suited to lucerne)
- Rehabilitate ‘run-down’ pastures and degraded landscapes through increasing groundcover and improving water balance in the soil profile.

For the wider public, productive and sustainable clover–based pastures arising from the use of these ‘locally-adapted’ white clover cultivars will also provide important ‘public-good’ benefits:

- Perennialisation of the agricultural landscape with associated benefits – stable groundcover, prevention of soil erosion, suppression of invasive weeds, moisture retention for better water-use
- Increased productivity and more uniform financial performance of grazing enterprises that will improve gross production from the grazing sector and reduce the social welfare expenditure of government on graziers in times of market down-turn and drought
- ‘Trickle-down’ benefits to seed retailers and associated agri-business that provides commercial and support-services to the grazing industries and strengthens rural communities
- Increase capability of the grazing industries to provide consumers with security of food and fibre and exporters with reliable supply of products.

8 Conclusions and Recommendations

The project has developed Australia’s first locally adapted white clover cultivar for the ‘dry margins’. This cultivar builds on the achievements of the national program (NSWDPI/AgResearch Alliance) in developing a broad adaptation cultivar (NuSiral released in 1999) and cultivars with tolerance of summer moisture-stress (Trophy released in 2008 and Saracen for subsequent release internationally).

Together with Tribute (developed by AgResearch in Victoria), NuSiral and Trophy for the first time provide locally adapted cultivars that can confidently be expected to be both more productive, persistent and reliable than imported white clover cultivars for dryland pastures in the high rainfall (850–1,000 mm AAR) zone. The P164 cultivar(s) adds to this achievement by providing potential to extend white clover into the ‘dry margins’ – the 700 to 850 mm AAR zone that includes the North-West Slopes, Central Tablelands, Southern/Monaro Tablelands of NSW and homoclimes in the other States and elsewhere internationally.

Successful completion of this P164 project represents completion of the ‘blueprint’ for the National White Clover Improvement Program that was planned at a national workshop in 1987. This workshop foreshadowed a program that would develop i) a broad adaptation cultivar to replace the public cultivar Haifa (that has progressively deteriorated in genetic integrity, plant type and agronomic performance), ii) a cultivar with tolerance of summer moisture-stress (the predominant environmental stress that challenges the reliability of imported cultivars), and iii) a ‘dry-margins’ cultivar that provides potential to double the size of the Australian white clover zone by extending white clover use into lower rainfall environments.

With the merits of the P164 cultivar(s) proven at the two NSW ‘dry margins’ evaluation sites, it is recommended that they be progressed without delay through cultivar development and commercialisation. The ‘dry margins’ target zone in Australia for the P164 cultivar(s) is sharply different and additional to the current white clover zone so a ‘dry margins’ cultivar will be readily differentiated for a new target region, target applications and target market. These targets are large, both in Australia and internationally.

Despite these achievements of Australia now having a suite of locally adapted cultivars for both the existing white clover zone and for the dry margins, the prospect of encroaching ‘heat & dry’ in the

Australian pastoral zone with the onset of climate change is a new and immediate threat to the sustainability of perennial legume-based pastures.

It is fortuitous that the three cycles (DAN045, TR041, P164) of breeding and selection within the Alliance's program has aggregated genes and isolated some 16 breeding lines that now form a valuable germplasm set for future breeding for tolerance of more frequent 'seasonal drought' and more intensive 'heat & dry' that are expected to accompany encroaching climate change.

A 10-year plan has been developed for the continuation of breeding, evaluation and commercialisation of a new generation of white clover cultivars for climate change. This will more fully exploit the potential of these derived breeding lines, and the established capability of the Alliance, to develop robust white clover cultivars with tolerance of 'heat & dry' for this new era.

9 Bibliography

Archer KA. (1995). The New South Wales pasture base. *Proceedings of the Tenth Annual Conference of the Grassland Society of NSW .* (Eds. JF Ayres, DL Michalk, H Lloyd Davies,) pp 10-13.(Grassland Society of NSW Incc.:Orange).

Archer KA, Robinson GG. (1989). The role of stolons and seedlings in the persistence and production of white clover *(Trifolium repens* L. cv. Huia) in temperate pastures on the northern tablelands, New South Wales. *Australian Journal of Agricultural Research* **40**, 605–16.

Ayres JF, Caradus JR, Lane LA, Murison RD (1996) White clover breeding for dryland sheep and cattle pastures in Australia. In 'White clover: New Zealand's competitive edge'. (Ed. D.R. Woodfield) pp. 155–158. (Agronomy Society of New Zealand: Christchurch)

Ayres JF, Lloyd Davies H (2000) Temperate pastures for grazing livestock. *Asian-Australasian Journal of Animal Science,* **13** Supplement July 2000 B, 1-12.

Ayres JF, Lane LA, Caradus JR (2002) Grasslands NuSiral – a new white clover (*Trifolium repens.* L.) cultivar for drylands pastures. *Australian Journal of Experimental Agriculture* **42**, 1023-1025*.*

Ayres JF, Caradus JR, Murison RD, Lane LA, Woodfield DR (2007) Grasslands Trophy – a new white clover (*Trifolium repens* L.) cultivar with tolerance of summer moisture stress. *Australian Journal of Experimental Agriculture* **47 (1)**:110-115.

Clements RJ (1987). An Australian white clover breeding program: justification, objective, timing and resources needed. *In* 'Proceedings of a Specialist Workshop on National White Clover Improvement'. University of New England, Armidale, 18–19 August 1987. (Ed. M. L. Curll) pp. 5.1–5. (University of New England: Armidale.)

Gillard P, Bishop A, Reid R (1989) Introduction and evaluation of pasture legumes in high rainfall north-western Tasmania. In 'Proceedings of the 5th Australian agronomy conference'. Perth. (Ed. G.P. Ayling) p. 521. (Australian Society of Agronomy: Parkville, Vic.)

Godwin DG (1981) Uptake and efficiency of utilisation of phosphorus by white clover. CM.Rur.Sc. Thesis, University of New England, Armidale.

Hutchinson KJ, King KL, Wilkinson DR (1995) Effects of rainfall, moisture stress, and stocking rate on the persistence of white clover over 30 years. *Australian Journal of Experimental Agriculture* **35**, 1039–1047.

Jahufer, M.Z.Z., Cooper, M., Ayres, J.F. and Bray, R.A. (2002). Identification of research to improve the efficiency of conventional breeding strategies for white clover in Australia – A review. *Australian Journal of Agricultural Research* **53**, 239-257.

Kenny PT, Reed KFM (1984) Effects of pasture type on the growth and wool production of weaner sheep during summer and autumn. *Australian Journal of Experimental Agriculture and Animal Husbandry* **24**, 322–331.

Lane LA, Ayres JF, Lovett JV (1997a) A review of the introduction and use of white clover *(Trifolium repens* L.) in Australia — significance for breeding objectives. *Australian Journal of Experimental Agriculture* **37**, 831-839.

Lane, L.A., Ayres, J.F. and Lovett, J.V. (2000). A white clover (*Trifolium repens* L.) ecotype collection from northern New South Wales, Australia. *Plant Genetic Resources Newsletter* No. 121: 10 – 12.

Lane, L.A., Ayres, J.F. and Lovett, J.V. (2000). The pastoral significance, adaptive characteristics, and grazing value of white clover (*Trifolium repens* L.) for dryland environments in Australia: a review. *Australian Journal of Experimental Agriculture* **40,** 1033 – 1046.

McCaskill MR, Blair GJ (1988) Medium-term climatic variation of the Northern Tablelands. *Mathematics and Computers in Simulation* **30**, 159–164.

McDonald W (1988) Developments with perennial legumes. In ‘Proceedings of third annual conference of Grasslands Society of NSW’. (Eds R. Ison, A. Andrews, J. Read, D. Wright) pp. 10–15. (Grassland Society of NSW Inc.: Orange, NSW)

Murison R, Ayres J, Lane L, Woodfield D (2006) Statistical methods to address spatial variation in pasture evaluation trials. In 'Thirteenth Australasian Plant Breeding Conference' pp 339-346 (Ed. C.F. Mercer) (New Zealand Grassland Association: Dunedin, New Zealand)

Pearson CJ, Brown R, Collins WJ, Archer KA, Wood MS, Petersen G, Bootle B (1997) An Australian temperate pastures database. *Australian Journal of Agricultural Research* **48**, 453–65.

Robinson GG, Lazenby A (1976) Effect of superphosphate, white clover and stocking rate on the productivity of natural pastures, Northern Tablelands, New South Wales. *Australian Journal of Experimental Agriculture and Animal Husbandry* **16**, 209–217.

10 Appendices

10.1 White clover germplasm entries

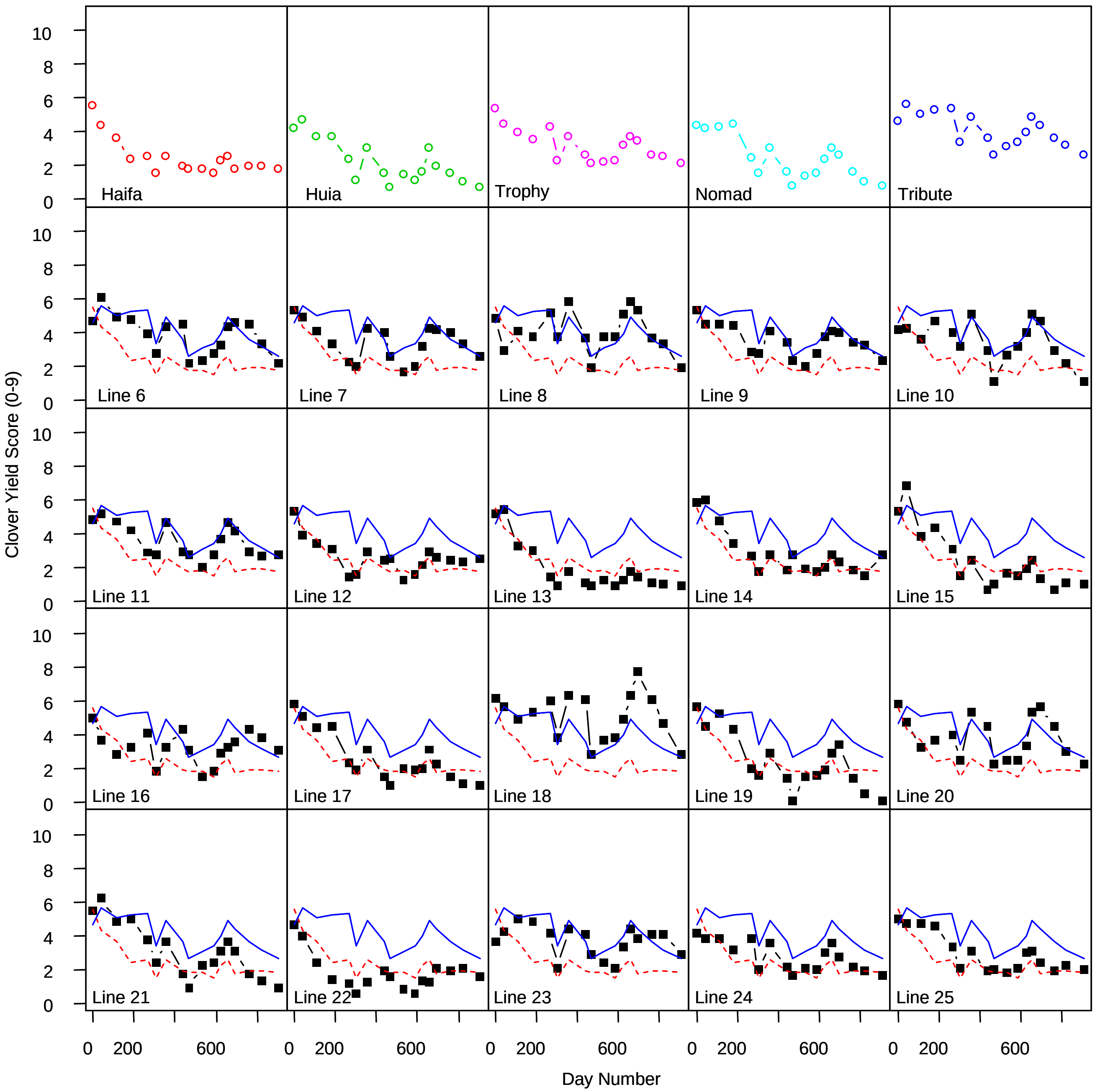
Line	Access-ion	Cate-gory	Description	Line	Accession	Cate-gory	Description
1	C22141	A	TIF x Demand	51	C22988	F	Durana
2	C21162	A	(ETN x Sustain) x SRVR	52	C22939	F	Patriot
3	C23725	A	Patriot x Crusader	53	C23783	F	Resolute (Tallapoosa)
4	C5940	B	DC40 dryland w.clover coll'n	54	C22986	F	GC132 (ETN x Sustain)
5	C24326	B	Lincoln dryland survivors	55	C22985	F	GC133 (TIF x SRVR)
6	C23741	B	Nomad x Crusader	56	C23759	F	Grasslands Mainstay (Dryland x Morgantown)
7	C23743	B	Syrian x Nomad	57	C22194	F	Grasslands Nomad
8	AB514	C	Interspecific hybrid	58	C19736	F	Grasslands NuSiral
9	C24325	C	Intraspecific F1 hybrid	59	C21905	F	Grasslands Tribute
10	C24177	C	Nematode resistant selection	60	C23513	F	Grasslands Trophy
11	C23802	D	Line 8 x Line 12	61	C23514	F	Grasslands Saracen
12	C23801	D	Line 8 x Line 23	62	C13173	F	Haifa
13	C23818	D	Line 12 x Line 23	63	C22936	F	Quest (Gene Pool F)
14	C23803	D	Line 8 x Crusader	64	C21906	F	Grasslands Emerald
15	C23804	D	Line 8 x Patriot	65	C13341	F	Will
16	C23817	D	Line 12 x Tribute	66	C7544	F	Grasslands Huia
17	C23816	D	Line 12 x Crusader	67	ATRC 804	G	Small leaf survivors Glen Innes (1990-2000)
18	C23828	D	Line 23 x Emerald	68	ATRC 805	G	Med/Small leaf survivors Glen Innes ('90-'00)
19	C23827	D	Line 23 x NuSiral	69	ATRC 806	G	Medium leaf survivors Glen Innes ('90-'00)
20	C23797	D	Line 7 x Line 11	70	ATRC 807	G	Med/large leaf survivors Glen Innes ('90-'00)
21	C23796	D	Line 7 x Line 12	71	ATRC 808	G	Large leaf survivors Glen Innes ('90-'00)
22	C23795	D	Line7 x Line 13	72	C21311	H	TR41 line 9
23	C23798	D	Line 7 x Line 25	73	C21317	H	TR41 line 11
24	C23794	D	Line 7 x Crusader	74	C21321	H	TR41 line 13
25	C23800	D	Line 8 x Line 11	75	C21305	H	TR41 line 25
26	C23799	D	Line 8 x Line 25	76	ATRC 738	I	Box Hills ecotype - medium leaf
27	C23808	D	Line 9 x Line 12	77	ATRC 764	I	Linfield ecotype - medium leaf
28	C23807	D	Line 9 x Line 13	78	ATRC 770	I	Eungai South ecotype - medium leaf
29	C23806	D	Line 9 x Line 19	79	ATRC 068	J	Medium leaf/relatively high stolon density
30	C23805	D	Line 9 x Emerald	80	ATRC 131	J	CPI 45885 (Turkey) - med leaf/high stolon dens
31	C23809	D	Line 11 x NuSiral	81	ATRC 172	J	Medium leaf/stolon density ex NZ commercial
32	C23810	D	Line 11 x Tribute	82	ATRC 051	K	USDA 294546 - France
33	C23811	D	Line 11 x Line 19	83	ATRC 192	K	cv. Gwenda
34	C23812	D	Line 11 x Line 23	84	ATRC 235	K	C7572 - Portugal
35	C23815	D	Line 12 x Emerald	85	ATRC 242	K	C8989 - Portugal
36	C23814	D	Line 12 x Line 25	86	ATRC 256	K	C9002 - Italy
37	C23813	D	Line 12 x Patriot	87	ATRC 262	K	C9008 - Portugal
38	C23819	D	Line 13 x NuSiral	88	ATRC 272	K	C7649 - Portugal
39	C23820	D	Line 13 x Patriot	89	ATRC 274	K	C9038 - Spain
40	C23821	D	Line 13 x Line 19	90	ATRC 287	K	C9229 - Armidale

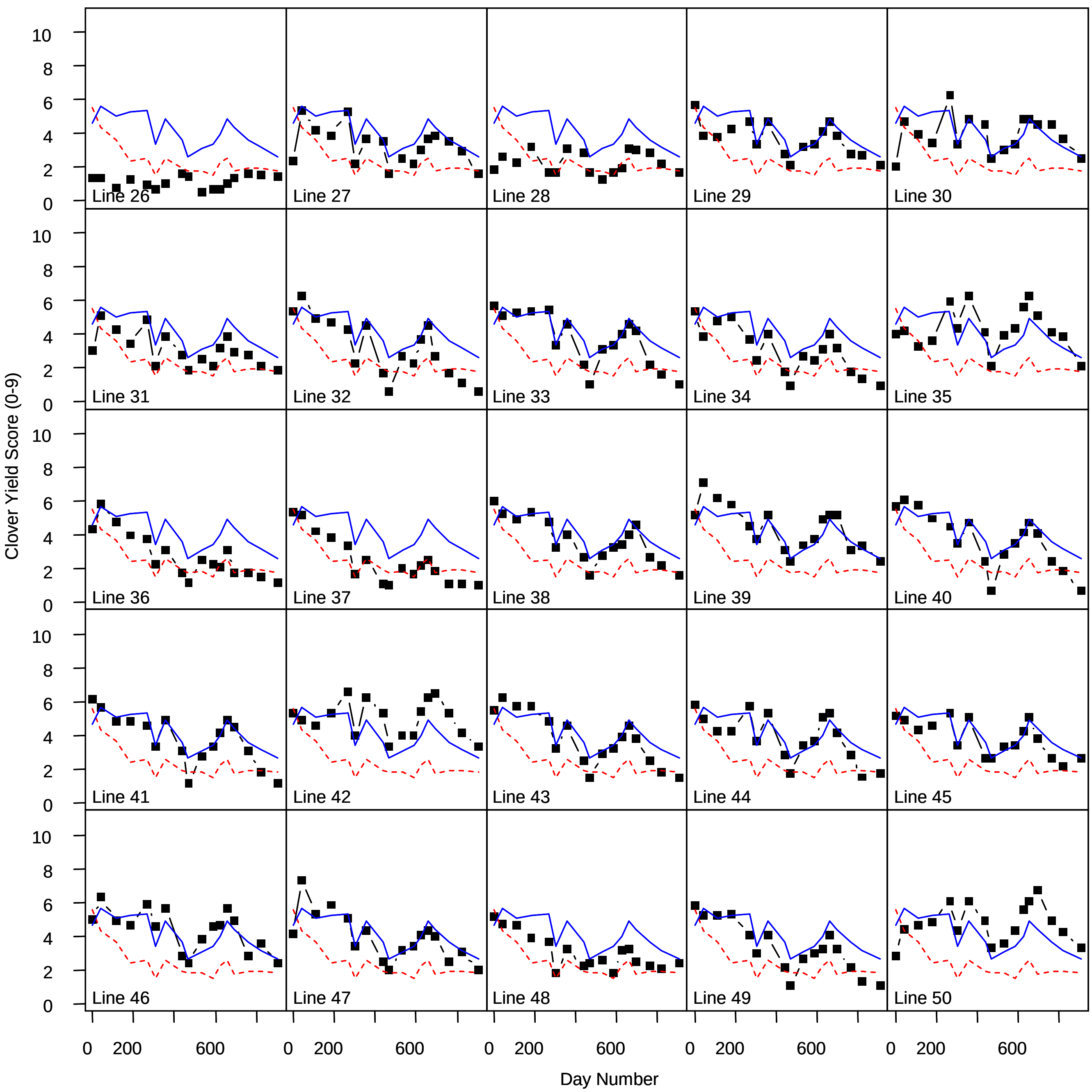
41	C23822	D	Line 19 x Patriot	91	ATRC 309	K	SA 12273 - Turkey
42	C23823	D	Line 19 x NuSiral	92	ATRC 350	K	C9498 - Spain
43	C23824	D	Line 19 x Line 25	93	ATRC 396	K	C9489 - Portugal
44	C23826	D	Line 23 x Line 25	94	ATRC 401	K	C9031 - Spain
45	C23825	D	Line 23 x Tribute	95	ATRC 475	K	CPI 32017 - France
46	C23829	D	Line 23 x Patriot	96	ATRC 499	K	CPI 53569 - New Zealand
47	C24321	E	Syn 8 sel: persist. Argentina	97	ATRC 523	K	Italy - small leaf
48	C23793	E	GC126 (Rio Nogoya)	98	ATRC 537	K	C10537 - Azerbaijan
49	C23256	F	Goliath (GC124)	99	ATRC 558	K	SA 35845 - Russia
50	C23792	F	GC125 (Ladino x S.Am eco)	100	ATRC 687	K	crossed line from Tas Dept.Ag

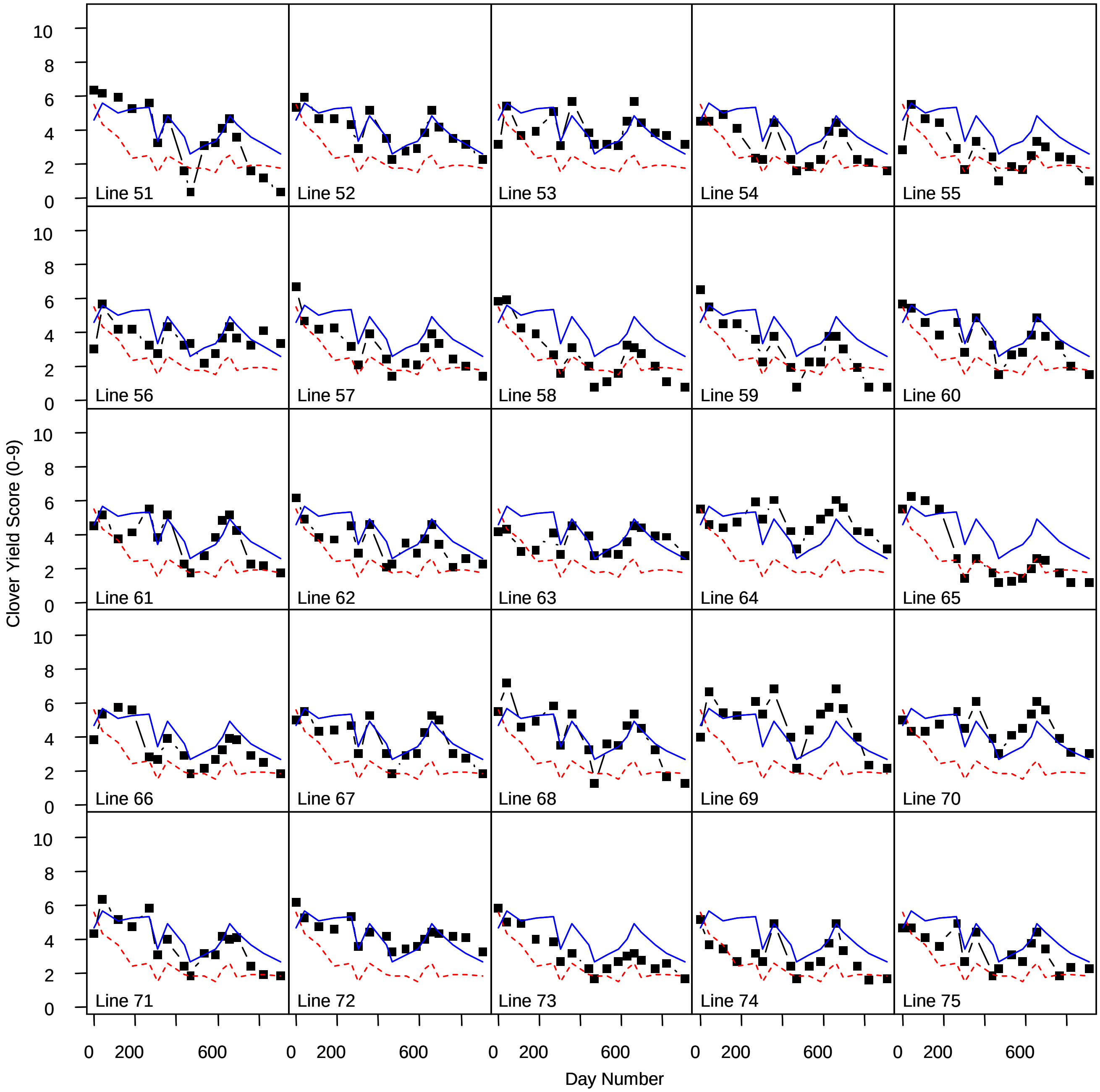
Germplasm entry categories:	
A	USA drought and heat tolerant selections - developed in south-east USA under high summer temperatures and and summer moisture stress
B	NZ Dryland selections - developed under dryland conditions in Eastern New Zealand
C	Novel AgResearch selections - germplasm containing unique traits such as nematode resistance, high floral condensed tannins, interspecific hybrids
D	TR041 elite crosses
E	Elite South American selections
F	Cultivars
G	NSW drought selections (surviving genotypes selected at Glen Innes and crossed within leaf size categories)
H	Lines progressed from Project TR41 showing exceptional persistence in northern NSW)
I	Lines from Northern NSW ecotype work (refer Lane, et.al, 2000, AJAR 51: 985-997)
J	Lines nominated from pattern analysis on field characterisation data as "dry margins" types
K	Lines nominated from DPI glasshouse "characterisation " as possessing indicative persistence characters (taproots, high stolon density/longer leaf)

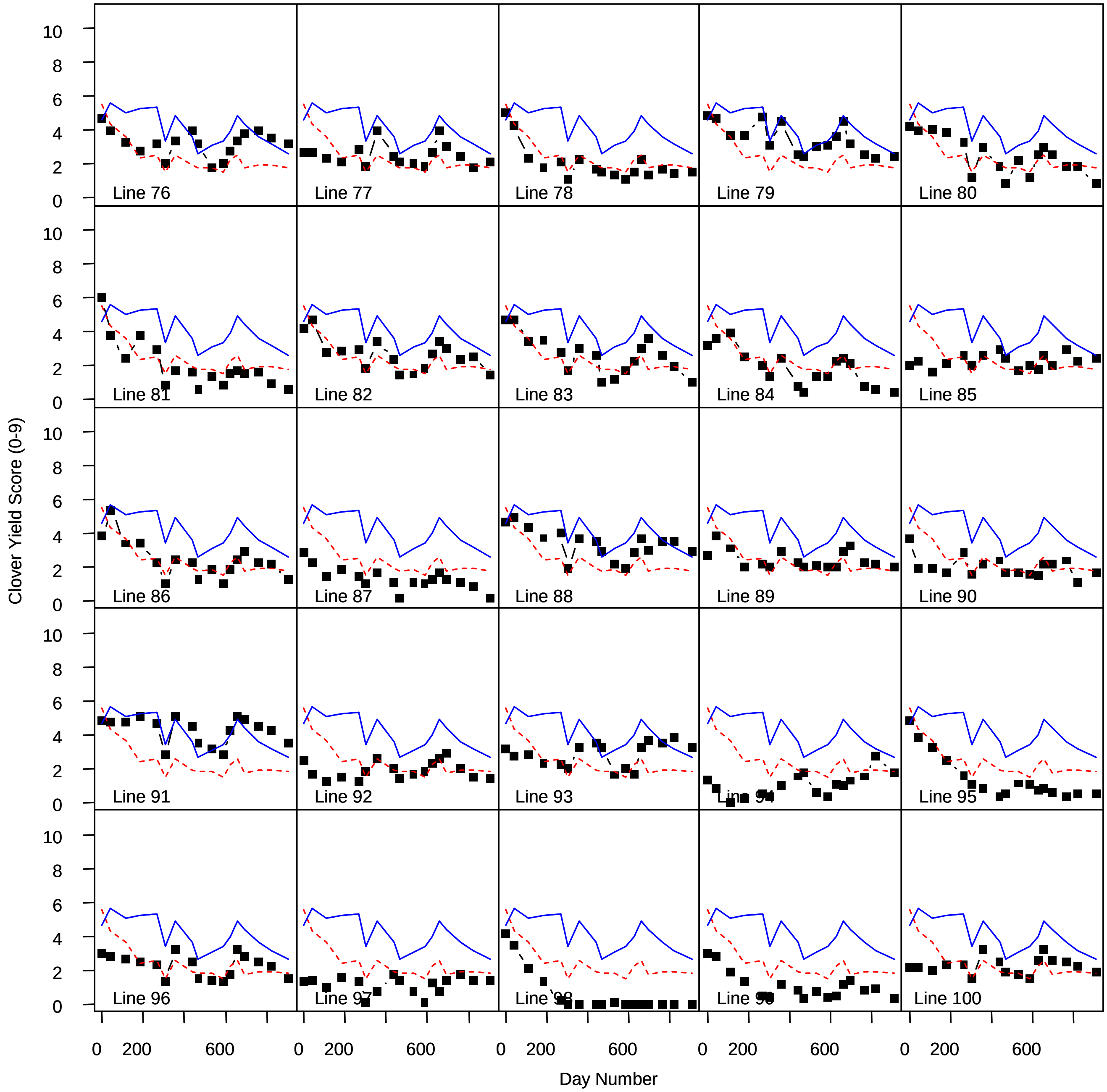
10.2 Longitudinal profiles of herbage yield at Glen Innes

Splines of the 5 cultivars Haifa, Huia, Trophy, Nomad, Tribute and 95 breeding lines (■) showing comparisons with Haifa (– – –) and Tribute —)



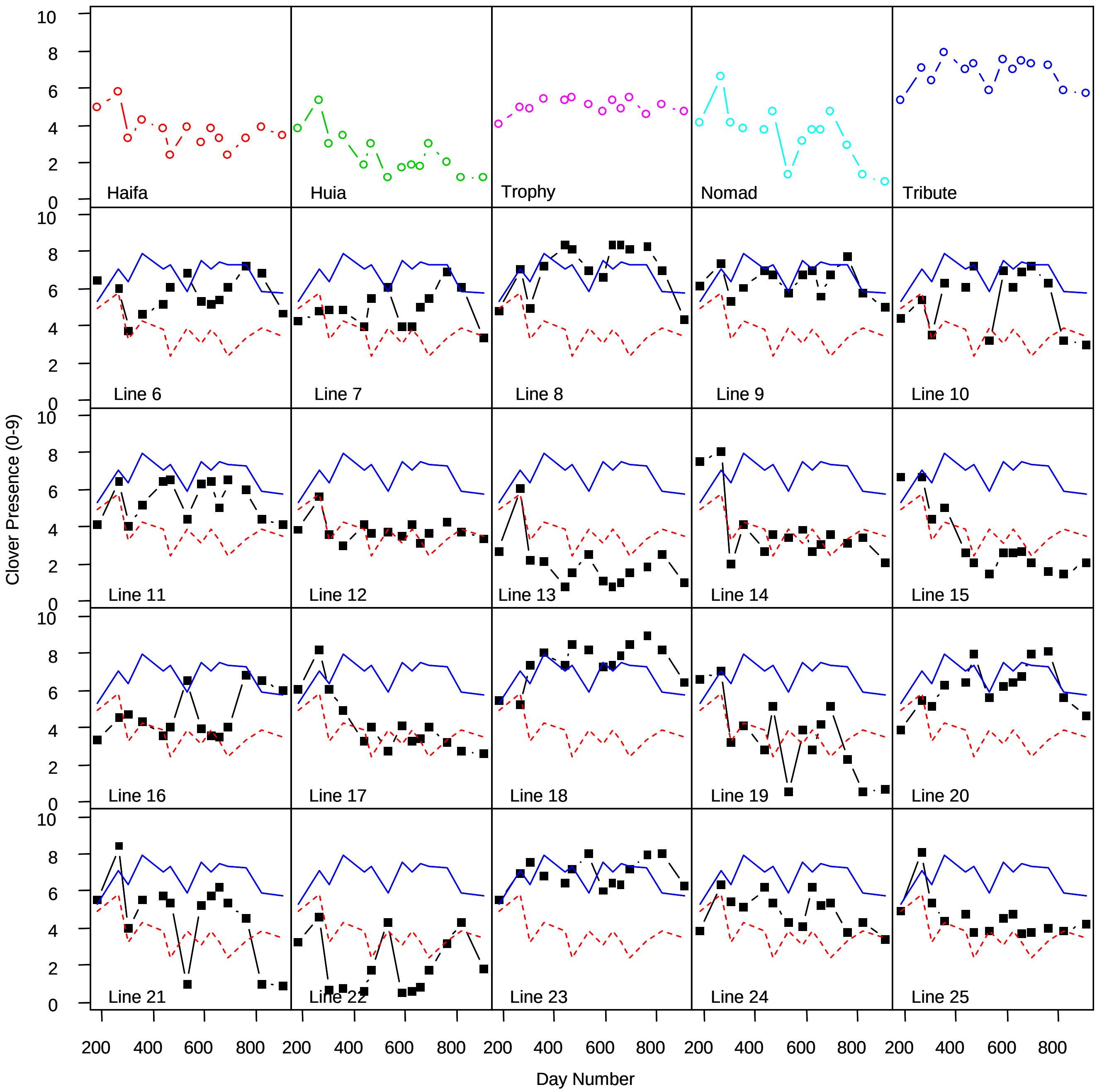

Clover Yield Score (0-9)
Day Number
Line 26
Line 27
Line 28
Line 29
Line 30
Line 31
Line 32
Line 33
Line 34
Line 35
Line 36
Line 37
Line 38
Line 39
Line 40
Line 41
Line 42
Line 43
Line 44
Line 45
Line 46
Line 47
Line 48
Line 49
Line 50
0 2 4 6 8 10
0 200 600

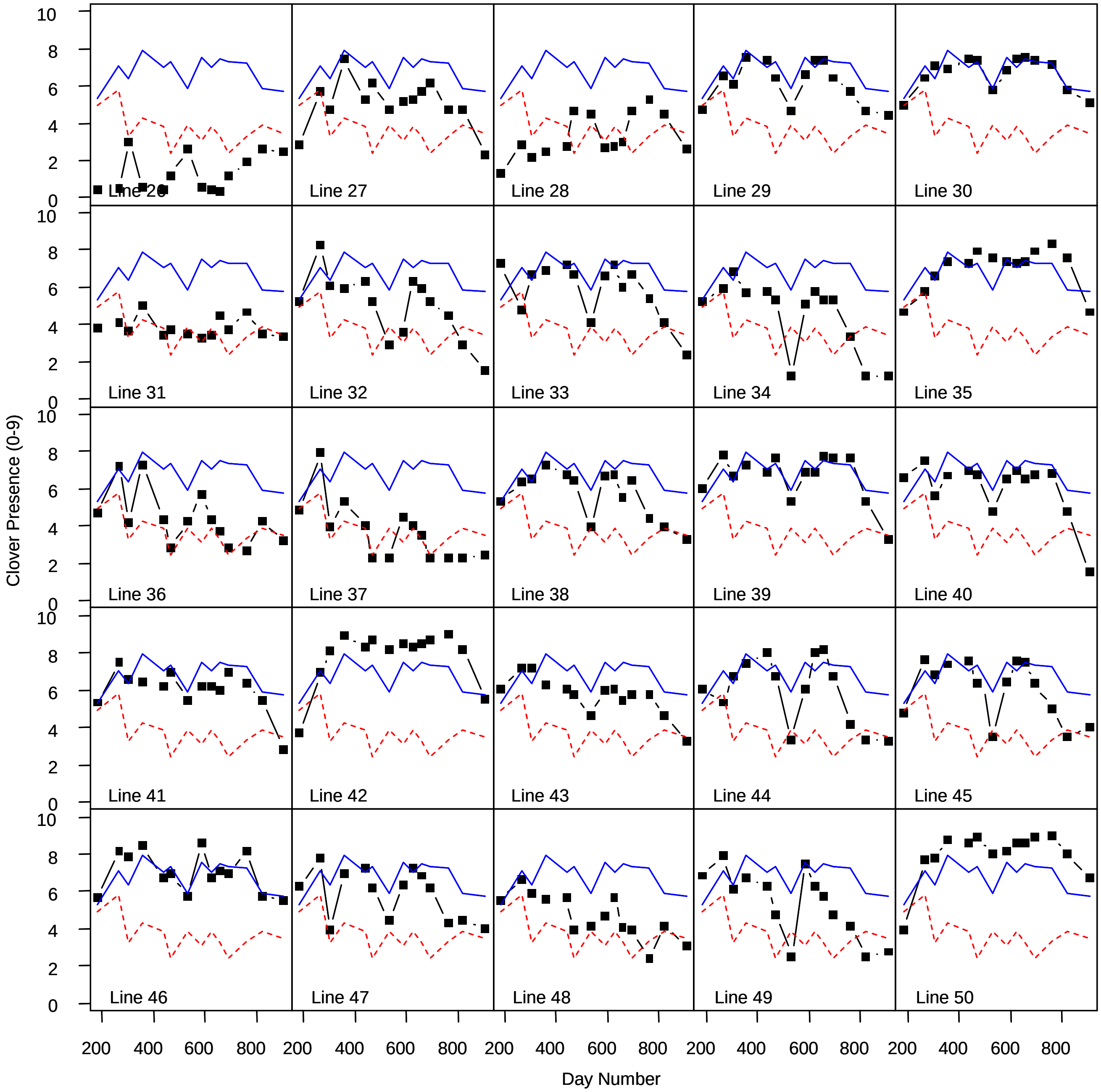

Clover Yield Score (0-9)
Day Number
Line 51
Line 52
Line 53
Line 54
Line 55
Line 56
Line 57
Line 58
Line 59
Line 60
Line 61
Line 62
Line 63
Line 64
Line 65
Line 66
Line 67
Line 68
Line 69
Line 70
Line 71
Line 72
Line 73
Line 74
Line 75

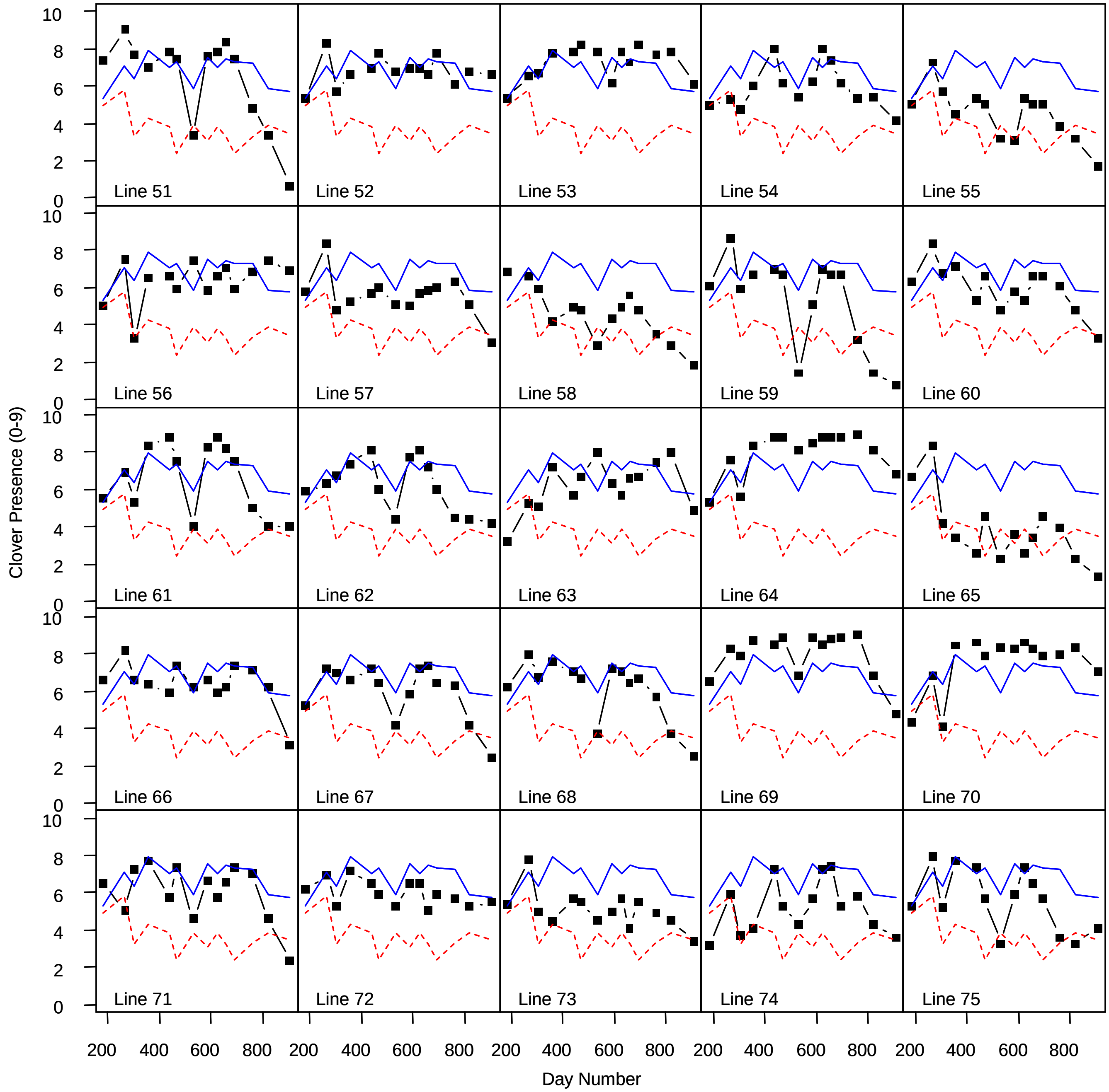

Clover Yield Score (0-9)
Day Number
Line 76
Line 77
Line 78
Line 79
Line 80
Line 81
Line 82
Line 83
Line 84
Line 85
Line 86
Line 87
Line 88
Line 89
Line 90
Line 91
Line 92
Line 93
Line 94
Line 95
Line 96
Line 97
Line 98
Line 99
Line 100
0 2 4 6 8 10
0 200 600

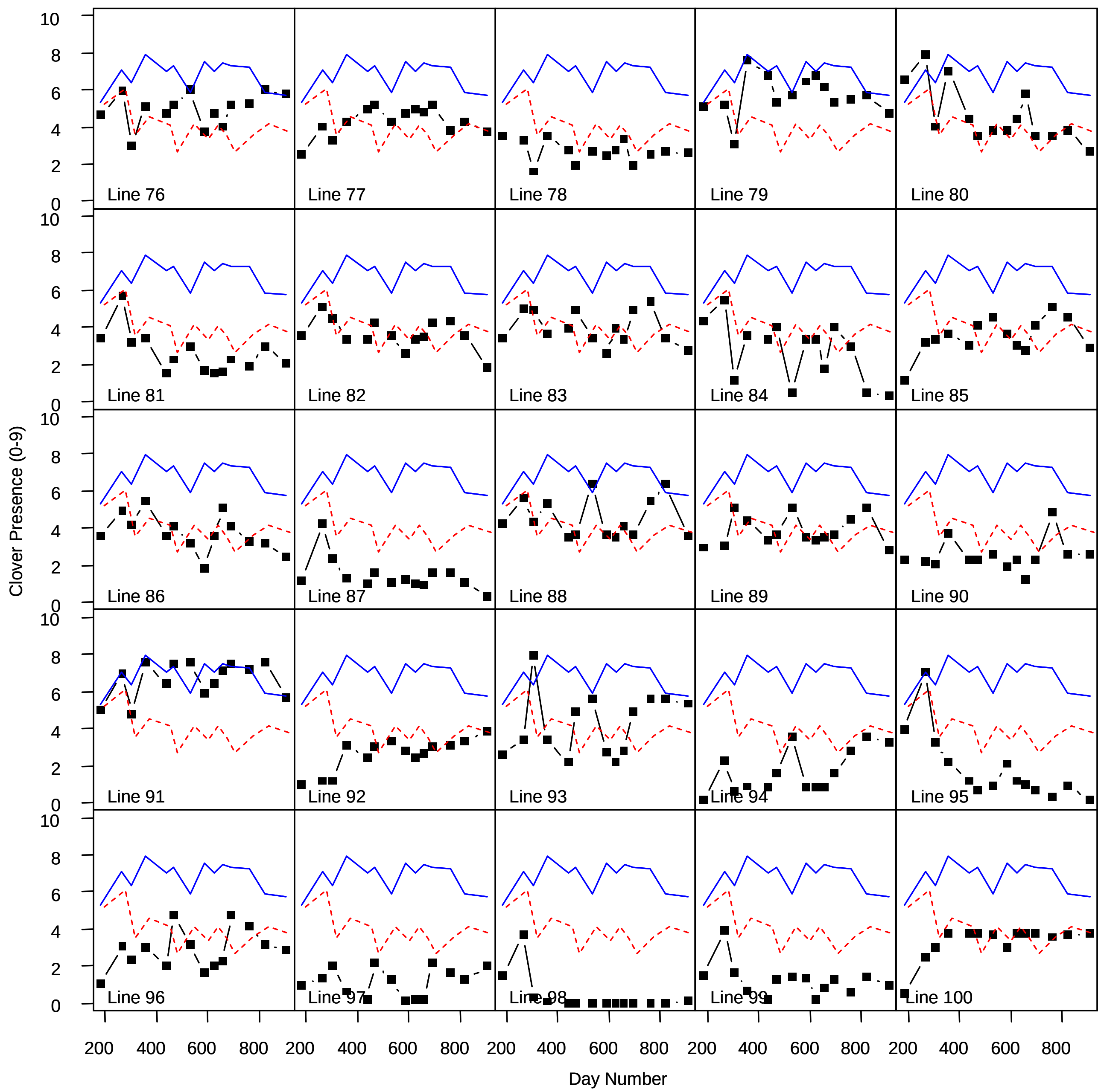
10.3 Longitudinal profiles of clover presence at Glen Innes

Splines of the 5 cultivars Haifa, Huia, Trophy, Nomad, Tribute and 95 breeding lines (■) showing comparisons with Haifa (– – –) and Tribute —)




Clover Presence (0-9)
Day Number
Line 26
Line 27
Line 28
Line 29
Line 30
Line 31
Line 32
Line 33
Line 34
Line 35
Line 36
Line 37
Line 38
Line 39
Line 40
Line 41
Line 42
Line 43
Line 44
Line 45
Line 46
Line 47
Line 48
Line 49
Line 50


Clover Presence (0-9)
Day Number
Line 51
Line 52
Line 53
Line 54
Line 55
Line 56
Line 57
Line 58
Line 59
Line 60
Line 61
Line 62
Line 63
Line 64
Line 65
Line 66
Line 67
Line 68
Line 69
Line 70
Line 71
Line 72
Line 73
Line 74
Line 75


Clover Presence (0-9)
0
2
4
6
8
10
Line 76
Line 77
Line 78
Line 79
Line 80
Line 81
Line 82
Line 83
Line 84
Line 85
Line 86
Line 87
Line 88
Line 89
Line 90
Line 91
Line 92
Line 93
Line 94
Line 95
Line 96
Line 97
Line 98
Line 99
Line 100
200
400
600
800
Day Number

10.4 Publications

Scientific papers

Woodfield DR, Caradus JR, Widdup KH, Ayres JF, Bouton I (2002) Global breeding and marketing of white clover. 'Plant Breeding for the 12th Millennium' JA McComb (Ed.) Proceedings of the Twelth Australasian Plant Breeding Conference, 15-20 September 2002 (Australasian Plant Breeding Assoc Inc: Perth Western Australia).

Jahufer MZZ, Woodfield DR, Ford JL, Widdup KH, Ayres JF, Lane LA (2005) Evaluation of white clover breeding lines in the Australasian region. In 'Proceedings of the XX International Grasslands Congress: Offered papers, Dublin, Ireland'. (Eds FP O'Mara, RJ Wilkins, L't Mannetfe, DK Lovett, Pam Rogers, TM Boland) p. 81. (Wageningen Academic Publishers: Dordrecht).

Murison R, Ayres J, Lane L, Woodfield D (2006) Statistical methods to address spatial variation in pasture evaluation trials. In 'Thirteenth Australasian Plant Breeding Conference' pp 339-346 (Ed. C.F. Mercer) (New Zealand Grassland Association: Dunedin, New Zealand)

Jahufer, M.Z.Z., Woodfield, D.R., Ford, J.L., Widdup, K.H., Ayres, J.F. and Lane, L.A. (2005) Evaluation of white clover breeding lines in the Australasian region. In 'XX International Grassland Congress: Offered papers.' (Eds. F.P. O'Mara, R.J. Wilkins, L. 't Mannetje, D.K. Lovett, P.A.M. Rogers, T.M. Boland), pp 81. (Wagenningen Academic Publishers: The Netherlands).

Ayres JF, Caradus JR, Murison RD, Lane LA, Woodfield DR (2007) Grasslands Trophy – a new white clover (*Trifolium repens* L.) cultivar with tolerance of summer moisture stress. *Australian Journal of Experimental Agriculture* **47 (1)**:110-115.

Ayres JF, Caradus JR, Murison RD, Lane LA, Woodfield DR (2008) Trophy – a locally adapted white clover cultivar for dry-land pastures. Proceedings of the 23rd Annual Conference of the Grassland Society of NSW , 'Pastures at the Cutting Edge' (Eds SP Boschma, LM Serafin, JF Ayres) pp 116 –119. (Grassland Society of NSW, Orange, NSW)

Murison RD, Ayres JF, Lane LA, Jahufer MZZ (2008) Design and analysis for spatial effects in pasture trials. Proceedings of the 23rd Annual Conference of the Grassland Society of NSW , 'Pastures at the Cutting Edge' (Eds SP Boschma, LM Serafin, JF Ayres) pp 111 – 113. (Grassland Society of NSW, Orange, NSW)

Feature articles

Ayres JF, Lane LA, Woodfield DW (2003) Update on white clover breeding. *Grassland Society of NSW Newsletter* **18**(4): 12-14.

Ayres JF (2004) White clover. *NSW Agriculture Agnote* DPI-270, third edition, April 2004.

Ayres JF (2005) Drought tolerant white clover for dry margins environments. NSW Department of Primary Industries 'Projects on the Web' (http://wwwi.agric.nsw.gov.au/projectswebsearch)

Ayres JF (2005) New drought tolerant white clover. NSW Agriculture Today September issue

Ayres JF (2005) Extending white clover into the dry margins. MLA 'Feedback' 2005

Ayres JF (2005) New white clover for low rainfall areas. Feedback – the Meat & Livestock Industry Journal August 2005 issue p 8.

Ayres JF, Lane LA, Woodfield DR, Murison RA (2005) Extending white clover into the dry margins. *Grassland Society of NSW Newsletter* **20** (4): 10-12.

Ayres JF, Lane LA, Jahufer MZZ, Woodfield DR, Murison RA (2007) White clover breeding for Australia. *Grassland Society of NSW Newsletter* **22** (3): 10-14.

Ayres JF, Lane LA (2008) 'Trophy white clover – a new cultivar for dryland pastures. NSW Department of Primary Industries Primefact 821, ISBN 1832-6668, NSW Department of Primary Industries: Orange, NSW