Genetic adaptation to frost tolerance in white clover

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Abstract

White clover (*Trifolium repens* L.) exhibits considerable variation for tolerance to sub-zero temperatures or frosting. Half-sib families selected for tolerance to frosting were compared for tolerance to artificial frosting with full-sib families from crosses between frost tolerant and Huia genotypes, collections of ecotypes from Wisconsin, USA and eastern Canada, and a range of cultivars of differing origin and morphology. Percentage leaf damage caused by frosting was less for frost tolerant selections than for New Zealand cultivars but similar to that of northern hemisphere medium and small leaved cultivars. Crosses between frost tolerant genotypes and Huia did not significantly improve frost tolerance compared with Huia. Frost tolerance was associated with small leaf size and to a lesser extent low cyanogenesis. The frost tolerant selections and crosses with Huia were compared with a range of cultivars for growth in grazed grass swards at Palmerston North and Moa Flats (Southland). No line had significantly better growth than Huia and more than 50% of the frost tolerant selections had significantly poorer growth than Huia. Frost tolerant lines had more depressed winter growth relative to their autumn growth than New Zealand and Australian cultivars. Selection for frost tolerance in white clover can result in progeny that are prostrate, small leaved and unproductive. However, this association is not complete and there appears to be some variation that may allow selection for both reasonable frost tolerance and good growth.

Additional key words: *breeding, cultivars, cyanogenic, ecotypes, growth, leaf size,* Trifolium repens

Introduction

White clover (*Trifolium repens* L.) is found from the sub-arctic to the subtropics and therefore exhibits considerable variation for tolerance to sub-zero temperatures or frosting (Caradus *et al.*, 1989). Comparison of populations from high latitude sites has shown that clovers from exposed sites with little snow cover were more frost tolerant than those from more sheltered sites (Ollerenshaw and Haycock, 1984). This study has also shown that few genotypes combined frost tolerance with an ability to grow at low temperatures.

A large percentage (≈ 80%) of the world trade of white clover seed consists of New Zealand cultivars. Much of this is sold into Europe where New Zealand cultivars can fail to persist because of their poor winter hardiness (Frame and Newbould, 1984). In part this is related to their relatively poor frost tolerance.

Heritability of frost tolerance in white clover is moderately high (h_N = 0.75 - 0.93) (Caradus *et al.*, 1990) suggesting that selection for this character and incorporation into agronomically suitable but frost-sensitive cultivars can be achieved. The present study aims to (a) highlight the extent of the variation for frost tolerance within white clover and (b) describe the field performance of frost tolerant selections and crosses between frost-tolerant and Huia genotypes.

Method

Artificial frosting studies

Plant material: In 2 trials, one conducted in 1990 and the other in 1991, 142 and 100 lines of white clover, respectively, were evaluated for frost tolerance. These lines could be grouped into 10 or 11 categories, described in Tables 1 and 2, and included cultivars, breeding lines selected for frost tolerance in previous screening trials (Caradus *et al.*, 1989) and ecotypes from regions subjected to cold winters. There were 21 lines common to both trials. The small and large leaf selections for frost tolerance, in trial 1 (Table 1) were characterised as having either smaller or larger leaves than Huia at time of selection. These lines were derived from polycrosses within the small and large leaf size groups. Pair crosses were used to combine frost tolerance and good growth characteristics exhibited in the field by genotypes of Huia, Crau, Nematode Resistant Pitau and northern European cultivars. In trial 2, frost tolerant selections (Table 2) were from polycrosses of genotypes selected for frost tolerance and a leaf size

Table 1. **Mean leaflet width and percentage of leaves damaged by -10°C frosting of white clover cultivars and breeding lines categorised by origin or breeding history in trial 1. Standard errors are given.**

Category	n	Leaflet width (mm)	Percentage leaves damaged by frost
Large leaf non-ladino	2	8.8 ± 0.37	79 ± 13.8
Ladino	2	9.7 ± 0.81	99 ± 0.9
Frost tolerant selection - large leaf	45	7.8 ± 0.10	70 ± 2.0
Frost tolerant selection - small leaf	38	7.1 ± 0.11	67 ± 3.2
Frost tolerant genotypes x Huia	12	7.6 ± 0.17	76 ± 7.2
Frost tolerant genotypes x Crau	4	7.3 ± 0.20	94 ± 2.6
Frost tolerant genotypes x Nematode Resistant Pitau	4	7.9 ± 0.64	79 ± 11.3
Frost tolerant genotypes x Northern Europe cultivar	6	7.9 ± 0.35	88 ± 3.6
Northern Hemisphere medium & small leaved cultivars	4	7.4 ± 0.66	75 ± 2.3
New Zealand cultivars	3	7.0 ± 0.43	83 ± 1.5
New Zealand - Huia		7.0	79
New Zealand high altitude ecotypes	1	5.8	37
Wisconsin, USA, ecotypes	14	7.0 ± 0.33	66 ± 5.8
Northern Europe x Huia crosses	5	8.8 ± 0.26	95 ± 5.4
Australian cultivars	2	9.9 ± 1.75	99 ± 1.3
Kruskal-Wallis analysis	ρ	***	***

larger than that of Huia.

Pre-germinated seed of lines to be examined was sown into trays (430 x 300 mm) of potting mix in early autumn. There were 4 seedlings of each line per row and 12 rows per tray. In trial 1, there were 4 replicate rows and in trial 2, 6 replicate rows of each line.

Seedlings were grown in a glasshouse for 4 weeks before being place outside to harden until early August when frost treatments began. Plants were trimmed with hand shears in late autumn. In trial 1, leaflet width of a second open leaf from stolon tip was measured on two random plots per row, in July. In trial 2, both leaflet width and cyanogenesis was measured on two random plants per row, in June. Cyanogenesis was determined using the picrate test (Corkill, 1942) with level of reaction visually scored from 0 (no reaction) through to 6 (strong reaction).

Conduct of artificial frosting studies: Plants were subjected to a single controlled frost of -10°C (Trial 1) or -12°C (Trial 2), as described by Greer and Warrington (1982). The temperature declined over a 6 h period from the day temperature of 12°C to the minimum temperature, was held at the minimum for 6 h, and then increased to 12°C over 4 h. The frost rooms are part of the controlled-environment facilities at DSIR Fruit and Trees, Palmerston North. After frosting, plants were placed outside and 2 days later the percentage of leaves damaged per row was recorded.

Field studies

Plant material: In 2 trials, one situated at Palmerston North (latitude 40°23') and the other at Moa Flat in Southland (latitude 45°43'), 111 lines of white clover were evaluated for agronomic performance. These included 29 half-sibs from genotypes selected as frost tolerant and with a leaf size larger than Huia, 44 half-sibs from genotypes selected as frost tolerant and with a leaf size smaller than Huia, 28 lines from pair crosses between a frost tolerant genotype and an agronomically

Table 2. Mean leaflet width, cyanogenesis score (0 - no reaction to 6 - intense reaction) and percentage of leaves damaged by -12°C frosting of white clover cultivars, ecotypes and breeding lines categorised by origin or breeding history in trial 2. Standard errors are given.

Category	n	Leaflet width (mm)	Cyanogenesis score	Percentage of leaves damaged by frost
Large leaf non-ladino	4	13.8 ± 0.31	4.6 ± 1.37	95 ± 0.5
Ladino	13	12.5 ± 0.31	0.3 ± 0.16	87 ± 1.9
Canadian ecotypes	25	9.8 ± 0.25	0.1 ± 0.03	63 ± 3.2
Frost tolerant selections	26	11.6 ± 0.14	0.1 ± 0.04	79 ± 1.7
North Hemisphere medium and small leaved cultivars	15	11.5 ± 0.24	0.2 ± 0.16	79 ± 3.1
New Zealand cultivars	4	11.0 ± 0.72	1.7 ± 0.42	92 ± 2.5
New Zealand - Huia		12.6	1.9	97
New Zealand high altitude ecotypes	2	8.3 ± 0.21	0.1 ± 0.09	48 ± 7.9
Wisconsin ecotypes	4	11.3 ± 0.74	0.0 ± 0.00	77 ± 4.8
Northern Europe x Huia crosses	5	12.0 ± 0.35	1.9 ± 0.43	81 ± 1.6
Australian cultivars	2	12.1 ± 0.83	2.2 ± 2.21	86 ± 5.4
Kruskal-Wallis analysis	ρ	***	***	***

elite Huia genotype with reciprocals bulked, and 10 control lines (4 from NZ - Huia, Tahora, Demand and Nelson lakes ecotype, 4 from Northern Europe - Undrom, Retor, Podkawa and S184 and 2 from Australia - Haifa and Clarence).

Experimental design and conduct: In spring 1989 glasshouse-grown seedlings were transplanted into 1m rows at 1m spacings in swards either sown 1 year previously with Grasslands Pacific (high endophyte) perennial ryegrass (*Lolium perenne* L.) at Palmerston North or consisting of a mixture of grass species at Moa Flat from which all volunteer white clover had been removed using the selective herbicide, dicamba. There were 10 seedlings planted per row, and rows were arranged in a randomised block design with 3 replicates.

At Palmerston North the trial was grazed 9 times per year to a height of 2-3 cm with intervals between grazing varying from 24 days in spring to 60 days in winter. At Moa Flat the trial was grazed 5 times per year to a height of 2-3 cm with intervals between grazings varying from 35 days in spring to 100 days in winter. Pathways between rows were kept free of clover using dicamba.

Growth was scored by visual estimation using a 0-10 scale prior to each grazing at Palmerston North and 0-5 scale 4 times per year (mid-season) at Moa Flat. Final growth scores were taken 2.5 years after planting.

Statistical analysis

Data were subjected to analysis of variance to compare lines. Residual distributions were not improved for leaflet width data by log-transformation, for cyanogenesis score data by square root transformation, or for proportion of leaves damaged by square root transformation.

Groups of lines contained unequal numbers of lines and were compared for significant differences using Kruskal-Wallis analysis, a non-parametric method of analyzing data using ranks. Standard errors were calculated for each group mean to determine where significant differences occurred.

Visual growth scores of field grown plants were averaged across seasons and analysed by analysis of variance. Once again residual distributions were not improved by log-transformation.

Results

Variation for tolerance to frost

New Zealand high altitude ecotypes, both collected from above the snowline, one in the Kaikoura Ranges and the other in Nelson Lakes National Park were among the most frost tolerant lines examined (Tables 1 and 2). Lines with comparable frost tolerance did however occur in other categories. In trial 1, lines with less than 40% leaf damage were in the large leaf frost tolerant selection (1 line, 3% of lines in that category), small leaf frost tolerant selection (6 lines, 16% of lines), frost tolerant x Huia genotype crosses (2 lines, 17% of lines), and Wisconsin ecotypes (3 lines, 21% of lines). In trial 2, lines with less than 55% leaf damage were in Canadian ecotypes (10 lines, 40% of lines), large leaf frost tolerant selection (1 line, 4% of lines), and the cultivar Undrom. This cultivar, bred in Sweden, was the most frost tolerant cultivar in both trials.

The most frost susceptible categories of lines in both trials were large leaf non-ladino, ladino, New Zealand cultivars, northern Europe x Huia crosses and Australian cultivars (Tables 1 and 2). These had predominantly large mean leaf sizes, except for the New Zealand cultivars. In trial 1, crosses of frost tolerant genotypes with either genotypes of Crau, Nematode resistant Pitau and Northern European cultivars (such as Donna, Gwenda, Sonja, Sabeda, Anna and Katrina) all gave frost susceptible progeny (Table 1). Crosses between frost tolerant genotypes and Huia did not significantly improve frost tolerance compared with Huia (Table 1). Percentage leaf damage on frost tolerant selections was less than that of the New Zealand cultivars and similar to that of northern Hemisphere medium and small leaved cultivars in both trials (Tables 1 and 2). There were 7 lines which had significantly ($p<0.05$) better frost tolerance but not significantly ($p>0.05$) poorer growth at Palmerston North than Huia. This was due to their better summer and autumn growth rather than better winter growth compared with other frost tolerant lines. These 7 lines, which are of special interest from a breeding perspective, came from the large leaf frost selections (1 line), small leaf frost selections (4 lines) and frost tolerant x Huia crosses (2 lines). None of the cultivars examined were in this category.

Frost tolerance was associated with small leaf size ($r = +0.70$, $p<0.001$, df=98 for correlation between leaflet width and percentage of leaves damaged by frost) and to a lesser extent low cyanogenesis ($r = +0.35$, $p<0.001$, df = 98 for correlation between cyanogenesis score and percentage of leaves damaged by frost, in trial 2. The relationship between leaf size and frost damage was also significant in trial 1, but the association was less pronounced ($r = +0.39$ $p<0.001$).

Repeatability of estimates for frost tolerance were calculated by comparing the reaction to frosting of the 21 lines common to both trials. Correlation between the 2 trials was significant for percentage of leaves damaged ($r = +0.46$, $p<0.05$) and for leaflet width ($r = +0.61$, $p<0.01$).

Growth in grazed swards at Palmerston North

No line or cultivar had a significantly ($P<0.05$) higher mean growth score than Huia; Grasslands Demand had the highest mean growth score. The majority of half sib families from genotypes selected for frost tolerance were significantly ($p>0.05$) poorer than Huia; 66% of large leaf and 73% of small leaf frost tolerant families. Similarly, 75% of northern European cultivars were significantly poorer than Huia. However, only 32% of families from crosses between frost tolerant genotypes and Huia genotypes were significantly poorer than Huia.

There was a significant ($p<0.001$) line x season interaction for visual growth score (Fig. 1a). This was largely due to low seasonal variation for growth of the 3 New Zealand cultivars compared with a significant reduction in growth from summer, through autumn into winter for all groups of lines containing frost tolerant germplasm. There was a significant ($p<0.05$) association between tolerance to artificial frosting and extent of depression in winter growth ($r = -0.78$, $p<0.05$, df=5 for correlation of damage due to frosting with depression in growth from autumn to winter relative to autumn growth). Relative depression in winter growth as a percentage (y) was related to degree of damage due to frosting (x) by the equation $y = 99 - 1.29x$. Thus when comparing a range of white clover lines it would be expected that for a 10% difference in leaf damage due to frosting between two lines there would be an associated 13% difference in relative depression in winter growth when grown in a cool temperate environment.

Growth in grazed swards at Moa Flat

The New Zealand cultivars had significantly ($P<0.05$) better growth than the small and large leaf frost tolerant selections, Nelson Lakes ecotypes and the Australian cultivars. However, at this site, with its colder winters, the New Zealand cultivars showed a reduction in growth from autumn to winter similar to that of groups of lines containing frost tolerant germplasm (Fig. 1b). Australian cultivars showed the least seasonal variation in growth. Again there was a significant ($P<0.001$) association between tolerance to artificial frosting and extent of depression in winter growth ($r = -0.96$, $P<0.001$, df = 5).

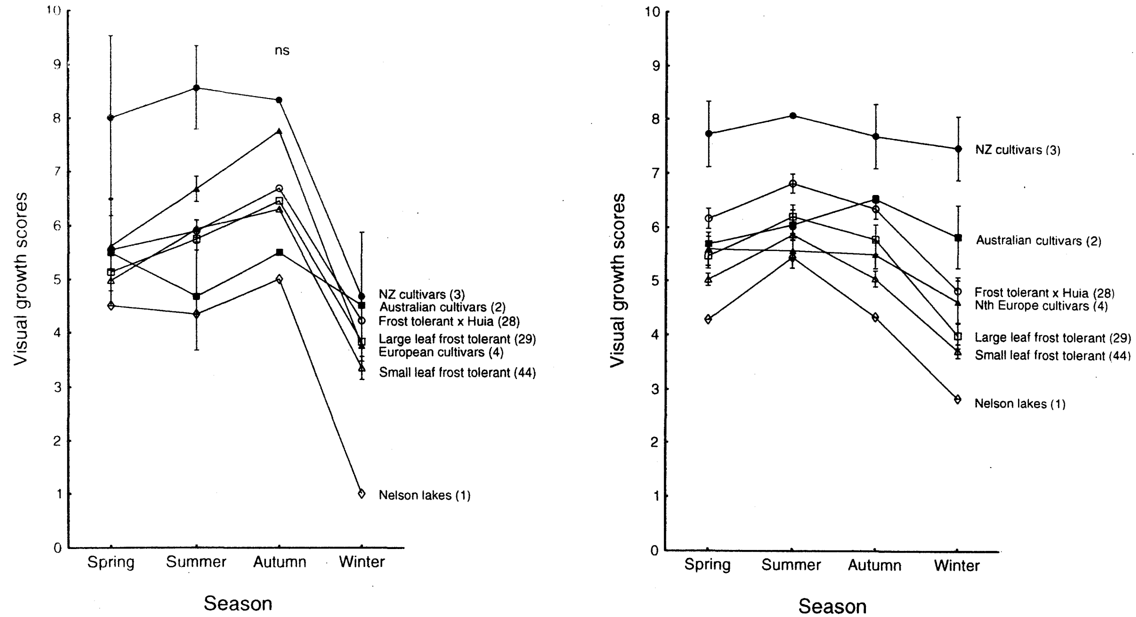

Figure 1. Mean seasonal growth scores of white clover cultivars and lines grown in grazed grass swards at (a) Palmerston North and (b) Moa Flat, Southland for 2.5 years. New Zealand cultivars indicated by •, Australian cultivars by ■, northern European cultivars by ▲, frost tolerant x Huia lines by o, large leaf frost tolerant lines by □, small leaf frost tolerant lines by Δ and the high altitude New Zealand ecotype 'Nelson lakes' by ◇.

Relative depression in winter growth as a percentage (y) was related to degree of damage due to frosting (x) by the equation $y = 116 - 0.96\,x$, such that a 10% difference in leaf damage due to frosting between 2 lines would be associated with a 10% difference in relative depression in winter growth when grown in a cold temperate environment.

Discussion

Natural selection has had a large impact on the frost tolerance of white clover germplasm such that ecotypes or cultivars from regions experiencing cold winters are more frost tolerant than those from areas with warmer winters. Ecotypes from Canada, Wisconsin USA and high altitude regions of New Zealand were more frost tolerant than cultivars bred in New Zealand or Australia (Tables 1 and 2). The Canadian ecotypes were from areas where winter temperatures drop to -40°C and where winter kill through prolonged periods of ice encasement is common (Fraser, 1989). Strong correlations between adaptation to frost and site of origin have been shown in other species. In a number of tree species frost tolerance has been associated with high altitude (Hawking *et al.*, 1991) and latitude (Ma, 1989). Ollerenshaw and Haycock (1984) were unable to relate frost tolerance of white clover with latitude of origin but did show that frost tolerance was related to exposure of the collection site to cold winds, such that ecotypes with high exposure ratings were the most frost tolerant.

A clinal relationship between cyanogenesis and mid-winter temperatures of place of origin has been shown for white clover (Daday, 1954), such that frequency of acyanogenic types increases as mid-winter temperature decreases. Similarly, Brighton and Horne (1977) suggested that temperature was the critical factor operating against cyanogenic forms with increasing altitude. Selection for frost tolerance has often resulted in selecting acyanogenic genotypes rather than cyanogenic genotypes (Caradus *et al.*, 1989). However, in the present study cyanogenesis was only weakly associated with frost tolerance, because of the overriding negative relationship between leaf size and frost tolerance. Hence the large leaved ladino types while acyanogenic were not necessarily frost tolerant (Table 2). Fraser (1989) found that while the Canadian ecotypes were predominantly acyanogenic the distribution of cyanogenic and acyanogenic genotypes did not appear to be related to mid-winter temperatures and concluded that acyanogenic plants were not necessarily more winter hardy than cyanogenic plants. There is, however, a good physiological basis for acyanogenic types being more predominant in frosted environments and relates to the inhibition of photosynthesis by the cyanide released when leaves are partly damaged by frosting (Foulds and Young, 1977).

Frost tolerance in white clover is associated with poor winter growth (Figure 1). Even among populations collected within the narrow latitude range of Norway there was evidence that the most frost tolerant populations from northern Norway had lower dry matter production than less frost tolerant populations from southern Norway (Junttila *et al.*, 1990a and b). In other species, such as lucerne (Smith, 1961), vetch (Acikgoz, 1982), perennial ryegrass and cocksfoot (Cooper, 1984), meadow fescue (Lebedeva, 1982), winter wheat (Nurpeisov and Urazeliev, 1986), rye (Klimov, 1987) and *Pinus armandii* (Ma, 1989) tolerance to frost has also been negatively associated with growth rate or plant height.

However, in some other studies winter growth and frost tolerance shows no association. For example, Ollerenshaw and Baker (1982) were unable to show that winter growth in the field was related to the winter temperature of place of origin of 13 white clover ecotypes used to demonstrate a clinal relationship between low temperature (5°C) growth in growth cabinets and winter temperature of place of origin (Ollerenshaw and Baker, 1981). Agronomic assessment as spaced plants indicated that winter and spring growth potential of 9 cultivars of Italian ryegrass (*Lolium multiflorum*) was unaffected by selection for cold tolerance in sub-zero temperatures (Hides, 1979). In chickpea, a subtropical grain legume, there was no significant correlation between cold tolerance to sub-zero temperatures in the field and growth habit (Singh *et al.*, 1989). It has also been shown in white clover that selection for low winter growth does not automatically result in enhanced frost tolerance (Caradus *et al.*, 1989). There does appear to be a slight chance that breeding a white clover cultivar that has good frost tolerance and acceptable all year round growth will be successful. In the present study genotypes were identified that combined these two characteristics relative to Huia. Ronningen (1953) while noting a negative correlation between winter injury and plant vigour of white clover at low temperature also discovered a number of genotypes which were exceptions to this trend. Ollerenshaw and Haycock (1984) also concluded that for white clover low temperature growth and frost tolerance is a rare combination in nature but sufficient variation exists to select genotypes that are both frost tolerant and able to grow at low positive temperatures. Fraser (1989) in her study of Canadian ecotypes identified a population with large leaves and long petioles for breeding an improved winter hardy white clover. Eagles and Othman (1981) postulated that cool season growth of Mediterranean types may be combined with winter hardiness by crossing with northern European types but expected that this would result in a compromise of the two characteristics. This indeed appears to be the case here for crosses between frost tolerant and Huia genotypes (Table 1 and Figure 1). Cooper (1964) warned that, for perennial ryegrass and cocksfoot, winter growth and frost tolerance may be physiologically interdependent and therefore difficult to separate.

Conclusion

There is considerable variation for frost tolerance in white clover which can be related to origin and morphology and to a lesser extent cyanogenesis. It is possible to successfully select for improved frost tolerance but growth, and in particular winter growth will be penalised. Only a few breeding lines developed in the present programme have been able to combine reasonable yields with good frost tolerance.

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