Performance of nematode-resistant white clover in field trials

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Abstract

Breeding for improved resistance and tolerance to the clover root-knot nematode, *Meloidogyne trifoliophila,* and the clover cyst nematode, *Heterodera trifolii*, has been successful in white clover, *Trifolium repens*. White clover lines developed from three independent breeding programmes were established in field trials at Lincoln, Palmerston North, Cambridge and Kerikeri. Plants were established in areas either untreated or treated with nematicide, so as to have contrasting pest loads on the plant material and evaluated for up to 4 years. Plant vigour was scored before each grazing at each site. Clover cyst nematode cysts were counted twice in Palmerston North and all nematodes were counted in stained roots on four occasions in Cambridge.

Resistant lines from the clover cyst nematode programme performed better than susceptible lines and as well as most cultivars reflecting the high level of resistance developed in this glasshouse-based programme. This resistance was also reflected in the generally lower number of cysts counted under resistant lines from Palmerston North. The root-knot nematode resistant material performed better than the susceptible and as well as most cultivars. The tolerance selections, developed under field conditions, performed as well as, or better than the cultivars. The selections from the breeding programmes have exhibited strong agronomic potential across locations and years and the best material has been crossed, with progeny being assessed in current field trials.

Keywords: *Heterodera trifolii*, *Meloidogyne trifoliophila*, nematode, pasture, resistance, tolerance, white clover

Introduction

Pests and diseases adversely affect white clover (*Trifolium repens*) performance in New Zealand pastures (Gaynor & Skipp 1987). The rapid spread of the clover root weevil (*Sitona lepidus*) has clearly highlighted this, but has also reduced the awareness of the significant impact that other economic pests such as soil-borne nematodes have on clover performance. Single factors such as nematodes rarely remove a plant species from the sward, rather it is the additive affect of abiotic and biotic stresses that kill a plant (Woodfield & Caradus 1996). These pest impacts are intensified in grazed pastures where plant competition is intense and the balance between ryegrass and white clover is particularly sensitive to changes in available soil N (Brock & Hay 2001).

The major biotic constraints to white clover in NZ are clover root weevil (Eerens *et al.* 2005) and the root-infecting, clover root-knot nematode (*Meloidogyne trifoliophila* -CRKN) and clover cyst nematode (*Heterodera trifolii* -CCN). Other constraints include viruses (Guy & Forster 1996), several insect pests (Gaynor & Skipp 1987), and fungi (Skipp & Hampton 1996). Reductions in any component of the total stress load on white clover will increase plant survival and maintain viable populations.

Nematode numbers in NZ pastures can be reduced by nematicides (but impractical and costly), biological control and crop rotation (Watson & Mercer 2000), and by growing plants bred for either resistance or tolerance (Mercer & Watson 1996). Resistance reduces the number of pest individuals through abiosis whereas tolerance allows plants to endure the presence of the pests.

Recurrent selection has identified and improved resistance to CRKN and CCN. Seven generations of selection for resistance to the CRKN lowered galls/plant in resistant material to 19% of that in susceptible material (Mercer *et al.* 2000). However, this level of resistance was approximately halved when resistant lines were crossed with elite cultivars to improve their agronomic characteristics. The difficulty of delivering this CRKN resistance is its apparently recessive genetic control (Barrett *et al.* 2005).

In contrast to CRKN, four generations of breeding resistance to the CCN reduced the mean number of cysts per plant on resistant material to 4% of that observed on susceptible material. Furthermore, CRKN resistance held up in the first cross with cultivars (to improve their agronomic characteristics) with only a modest increase in cyst numbers on resistant material to 20% of that on the crosses with susceptible lines (Mercer *et al.* 1999a). This paper reports on the multi-site field evaluations of the white clover lines from the recurrent selection programmes for resistance and tolerance to these two pests.

Material and methods

Plant material

A recurrent selection programme for resistance to CRKN and CCN in white clover was conducted in glasshouses at AgResearch, Grasslands Research Centre, Palmerston North. Plants supporting fewest nematodes (resistant) and most nematodes (susceptible) were polycrossed and the progeny subjected to further cycles of crossing and selection. The most resistant progeny were finally crossed with commercial cultivars to improve general adaptation to NZ farming systems (Mercer *et al.* 2000; Mercer *et al.* 1999b). The CRKN and CCN lines evaluated were bulks of four to seven half-sib families from crosses with five elite cultivars (Sustain, Demand, Prestige, Kopu and Huia). In the tables, data from back cross (BC) families are combined and referred to as BC_1 or BC_2.

A concurrent selection programme for tolerance to nematodes was conducted at AgResearch, Ruakura Research Centre, Hamilton. The best-performing lines from nematode-infested trays were grown in the field under plastic mulch. Plants were scored for vigour and the best plants were poly crossed to provide seed for the next cycle of selection (Watson *et al.* 1989). The tolerant lines evaluated were the result of four cycles of selection.

The commercial white clover cultivars, Kopu, Sustain, Demand and Prestige were included in the trials as benchmarks for agronomic performance and level of nematode susceptibility.

Field trials

CCN programme

Twenty resistant and four susceptible lines were evaluated at sites at Lincoln, Palmerston North and Cambridge from 1997 to 2000. Plants were first grown in trays of potting mix and transplanted at 12 weeks old. At each site, plant material was inserted into plots, blocks of which were either untreated (U) or treated (T) with nematicide. There were four replicates. The nematicide, oxamyl was applied as a foliar spray (3 kg ai/ha) and followed a month later by fenamiphos (5 kg ai/ha), both before rain or irrigation. The nematicide-treated areas were treated again each spring and autumn to prevent nematode populations recovering. The plants destined for the untreated (U) plots were inoculated in trays at 5 weeks old with CCN. Ten plants of each line were planted into 1 m rows, 1 m apart, into a grass sward previously cleared of clovers by herbicide applications (Dicamba at 2 l/ha applied 4 months before the trial started). The integrity of the plots was maintained with Dicamba applications in between plots to prevent plots intermingling. Clover root weevil was controlled where necessary by regular spraying with the synthetic pyrethroid decamethrin.

CCN programme

Sixteen resistant and four susceptible lines were evaluated at sites at Lincoln, Palmerston North and Cambridge from 1998 to 2000. Plant raising, site preparation, replication, nematicide treatments, and plot size were as above except that plants for the U plots were inoculated with CRKN at 5 weeks old.

Tolerance programme

Seven tolerant lines, four CRKN-resistant lines and five CCN-resistant lines were evaluated at sites at Palmerston North, Cambridge and Kerikeri from 1998 to 2000. Plant raising, site preparation, nematicide treatments, and plot size were as above except that plants for the U plots were inoculated with CCN and CRKN at 5 weeks old.

The material from the breeding programmes are referred to as "lines" and the commercial controls as "cultivars". When referring to the line/cultivar effects and the interaction between sites and lines/cultivars in ANOVA results, the term "entries" includes lines and cultivars.

Field trials grazing and plant vigour assessments

Trials at Kerikeri, Palmerston North and Lincoln were grazed by sheep and at Cambridge by dairy cattle. Plant vigour was scored visually on a 1-9 scale before each grazing. The response to nematicide treatment of the plots was calculated by dividing the mean vigour in the treated plots (T) by the values for the untreated plots (U) on each date. Lower values indicate less response to nematicide due to resistance and higher values indicate the greater response of susceptible material.

Field trials nematode counts

In Palmerston North, three matching pairs of lines, i.e. resistant and susceptible lines from the same generation of the breeding programme, were chosen from the CCN programme. The resistant lines were the best performing ones in the spring of 1999 that also had a matching and poor performing susceptible line. In late-December 1999 and in early-January 2000, four 25 x 75 mm cores per plot were removed and bulked. Cores were taken at random but adjacent to clover plants and distributed along the plot. Total stolon length was measured and cysts were extracted and counted in two categories, new (full of eggs) and old (empty). Results from the two samplings were combined.

In four Cambridge trials, selected lines were sampled on three occasions adjacent to clover plants (two 50 x 50 mm cores per plot). Roots were washed out and stained and the numbers of nematodes per g of stained root were calculated.

Statistical analysis

Analysis of variance was performed on the vigour scores, response to nematicide and counts of nematodes, with log transformation where necessary. Pair-wise comparisons were also made using Duncan's multiple range tests. For the data from the trials of the tolerance programme selections, residual maximum likelihood was used to compare means across sites and years because of uneven replication.

Results

Plant vigour assessments and responses to nematicide

CCN programme

Site (P<0.001) and entry (P<0.001) effects were significant. The absence of a significant interaction between site and entry (P = 0.12) suggested the relative performance of the entries was similar across sites. The resistant lines had significantly (P<0.05) higher mean vigour scores than their respective susceptible lines (Table 1). The best performing resistant lines did not have significantly higher mean vigour than the top-performing cultivars (Table 1). The T/U values differed between sites (P< 0.001) and entries (P< 0.05) but there was no interaction between site and entry (P = 0.6). The line with the highest T/U ratio was susceptible BC_2, with an average ratio of 1.62, significantly higher than the resistant lines (Table 1). However, resistant lines did not differ in T/U values from cultivars.

CRKN programme

Site (P<0.001) and entry (P<0.001) effects were significant but there was no evidence of interaction between site and entry. The resistant lines outperformed the susceptible lines (Table 2). Sustain had the highest overall vigour, however, the resistant lines were as good as the other commercial cultivars. The T/U values differed between sites (P<0.01) but there was no significant interaction between site and entry. The T/U value for the resistant lines was significantly lower than for the cultivars Demand and Kopu but was not different to other cultivars and lines (Table 2).

Tolerance programme

The site (P<0.001) and entry (P<0.001) effects were significant, as was the interaction between site and entry (P<0.001) indicating inconsistent performance across sites. The medium-leaf tolerant line had a significantly

Table 1 Mean vigour scores and responses to nematicide treatment (T/U) in a three-site[1] field comparison of white clover lines from the CCN resistance breeding programme. Data are overall means of 71 assessments from 1997 to 2000. Vigour data are from plots not treated with nematicide.

Line	Number of lines	Vigour relative to Demand = 100	T/U (log values)
Resistant BC_1	10	88	1.27 (0.241)
Susceptible BC_1	2	73	1.29 (0.255)
Resistant BC_2	10	92	1.25 (0.220)
Susceptible BC_2	2	73	1.62 (0.481)
Demand	1	100	1.18 (0.169)
Sustain	1	100	1.22 (0.197)
Prestige	1	99	1.06 (0.060)
Huia	1	83	1.42 (0.348)
Kopu	1	80	1.47 (0.387)
LSD 5% for comparisons of breeding lines	-	5	(0.117)
LSD 5% for comparisons of cultivars	-	12	(0.262)
LSD 5% for comparisons of breeding lines and cultivars	-	9	(0.203)

[1]Sites were Lincoln, Palmerston North, and Cambridge.

Table 2 Mean vigour scores and responses to nematicide treatment (T/U) in a three-site[1] field comparison of white clover lines from the CRKN resistance breeding programme. Data are overall means of 33 assessments from 1998 to 2000. Vigour data are from plots not treated with nematicide.

Line	Number of lines	Vigour relative to Demand = 100	T/U (log values)
Resistant BC_1	16	101	1.19 (0.175)
Susceptible BC_1	4	87	1.35 (0.297)
Sustain	1	117	1.17 (0.158)
Prestige	1	103	1.09 (0.082
Demand	1	100	1.51 (0.411)
Kopu	1	96	1.52 (0.416)
LSD 5% for comparisons of breeding lines	-	8	(0.143)
LSD 5% for comparisons of cultivars	-	16	(0.286)
LSD 5% for comparisons of breeding lines and cultivars	-	12	(0.226)

[1]Sites were Lincoln, Palmerston North, and Cambridge.

Table 3 Mean vigour scores and responses to nematicide treatment (T/U) in a three-site[1] field comparison of white clover lines from the tolerance breeding programme. Data are overall means of 43 assessments from 1998 to 2000. Vigour data are from plots not treated with nematicide.

Line	Number of lines	Vigour relative to Demand = 100	T/U (log values)
Tolerant small leaf	3	112	1.20 (0.184)
Tolerant medium leaf	2	123	1.18 (0.168)
Tolerant large leaf	2	113	1.25 (0.225)
RKN resistant BC_1	4	98	1.23 (0.206)
CCN resistant BC_1	5	111	1.29 (0.252)
Prestige	1	115	1.24 (0.212)
Kopu	1	107	1.20 (0.180)
Sustain	1	106	1.27 (0.236)
Demand	1	100	1.45 (0.375)
LSD (5%) for comparisons of breeding lines	-	7	(0.126)
LSD (5%) for comparisons of cultivars	-	15	(0.264)
LSD (5%) for comparisons of breeding lines and cultivars	-	12	(0.203)

[1]Sites were Palmerston North, Cambridge, and Kerikeri.

Table 4 Numbers of new clover cyst nematode cysts and of new and old cysts per mm of stolon in a bulk of four cores/plot in a grazed field trial in Palmerston North. Means are for 16 bulks of four cores (two samplings and eight reps).

	Line	New cysts/ 100cc soil[1]	New cysts/mm of stolon	Old cysts/mm of stolon
Pair one	Resistant $BC2F_1$	14 a	0.114 a	0.101 a
	Susceptible $BC2F_1$	24 a	0.276 b	0.236 b
Pair two	Resistant BC_1	18 a	0.236 a	0.182 a
	Susceptible BC_1	24 a	0.301 a	0.372 a
Pair three	Resistant BC_2	16 a	0.146 a	0.122 a
	Susceptible BC_2	16 a	0.213 a	0.244 a

[1]Within a pair and within a column, means with a letter in common do not differ (P < 0.05).

(P<0.05) higher mean than all other lines and the three poorer-performing cultivars (Table 3). There were no significant differences between the cultivars, even though Prestige had 10-15% higher vigour than the others. The T/U values differed between sites (P< 0.01) but not entries (P = 0.55) and there was no interaction between site and entry (P = 0.76) (Table 3).

Nematode counts

At Palmerston North, cyst numbers were highly variable. Resistant lines tended to have fewer new and old cysts than their susceptible counterparts, however this was only significant (P<0.05) for two of the nine comparisons (Table 4). For pair one, the number of new cysts per mm of stolon length under the resistant line was 41% of that of the susceptible line and the number of old cysts/mm under the resistant line was 43% of that under the susceptible line. There were no significant differences at the Cambridge site.

Discussion

Field evaluations of CCN- and CRKN-resistant selections from the glasshouse-based breeding programme and of tolerant selections from the field-based programme, have exhibited strong agronomic potential across locations and years. Selection for resistance under controlled environmental conditions in a glasshouse (Mercer *et al.* 2000) produced germplasm with better agronomic performance in the field than their susceptible counterparts.

This is the first report internationally of field tests of white clover breeding lines resistant to parasitism by CRKN and CCN. There have been several previous reports of white clover with resistance to a number of root-knot nematode species under glasshouse conditions (Kouame *et al.* 1998; Pederson & Quesenberry 1995; Windham & Pederson 1992). However, there are no published reports of field evaluations of these resistant populations. The same is true for CCN with Cook & Mizen (1989) having reported resistance but with no field evaluation to date.

The resistant and tolerant selections also performed favourably in comparison to the commercial cultivars. In particular, the CCN-resistant lines and the medium-leaved tolerant selection performed as well as the control(s) and warrant inclusion in commercial breeding programmes. In comparison, the field performance of the CRKN-resistant selections was slightly poorer than the best cultivar. This poorer performance reflects a balance between (a) the positive effects from the resistance/cultivar's contribution, and (b) the negative effects of the years of breeding solely in an artificial environment. The resistant and tolerant material with good agronomic performance in these trials has now been backcrossed with elite cultivars and further testing for resistance and field performance is occurring currently. We expect the nematode resistance to have a positive effect when incorporated in cultivars with better adaptation to NZ grazing systems.

It is difficult to correlate the dynamics of nematode populations with variations in plant performance. Ideally, seasonal or monthly data on plant performance and nematode parasitism would have been collected on a site-by-site basis to facilitate a better understanding of the epidemiology of the pest/host interaction. Clearly, the best time to test resistance is during high pest population pressures but these cannot be predicted in place and time. Our strategy of using four sites over 4 years incorporated some pest outbreaks but ideally we would have identified these with regular sampling of roots. We expected a bigger site-by-line interaction reflecting the contrasting importance of nematodes at the sites but the patchy nature of outbreaks was masked in the averaging of the data. The field evaluations also served as a performance test and the best material has been sequestered.

Nematode counts at Palmerston North reflected the resistance in the white clover but statistical significance was achieved in only two of nine comparisons.Factors contributing to the high levels of variation included resistance to CCN being under polygenic control and the material planted being untested progeny (for resistance to CCN) from polycrosses – therefore each plot contained a range of resistance levels. Another factor is the patchy distribution of nematodes in soil and roots reflecting the reproductive success of the previous generation, the laying of eggs in batches (in CCN cysts), and the discrete distribution of clover roots in the soil.

The overall poor response to applied nematicides explains why the T/U values did not completely reflect the differences in mean vigour between the best and worst performing entries. Nematicide application was technically challenging, required rain or irrigation, and sometimes grazing had to be delayed. Such comparisons between blocks of U and T plots are no longer used in our field assessments of germplasm.

Overall, the resistant and tolerant nematode material looks promising and the best material has been crossed to combine the germplasm from the three programmes. The progeny are currently being assessed in a further round of field evaluations on the path to eventual commercial release.

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