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Reaction of *Trifolium semipilosum* to four species of root-knot nematode (*Meloidogyne* spp)

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Abstract. The clover root-knot nematode, *Meloidogyne trifoliophila* (*Mt*), debilitates white clover (*Trifolium repens*) in New Zealand pastures. Genetic resistance (R) to *Mt* in white clover is complexly inherited and difficult to utilise in breeding programmes. Single-gene, dominant, and complete R to *Mt* has been identified at the *TRKR* locus in *T. semipilosum*. The current study aimed to characterise the *T. semipilosum* reaction to three additional root-knot nematode species, viz. *M. hapla, M. javanica,* and *M. incognita* and if resistant, to determine if *TRKR* was the R source. Tests were conducted in peat-based potting mix in a temperature-controlled glasshouse using a subset of *Mt*-resistant and *Mt*-susceptible plants from the full-sib *T. semipilosum* population used to map *TRKR*. *Mt* resistant and susceptible white clover plants were challenged with *Mt*, *M. hapla*, *M. incognita*, and *M. javanica* for comparison. Experiments confirmed that *T. semipilosum* was able to host the four *Meloidogyne* species. Some *T. semipilosum* plants exhibited R to *M. javanica* and *M. incognita* whereas all were uniformly susceptible to *M. hapla*. There were no correlated reactions among the full-sib plants to challenge by the four nematode species, suggesting the genetic factor(s) conferring R to *M. javanica* and *M. incognita* are independent of the *TRKR* locus. Furthermore, the R to *Mt* is complete, with no galls formed on challenged plants whereas the observed R to *M. javanica* and *M. incognita* is apparently partial. White clover plants either resistant or susceptible to *Mt* were uniformly susceptible to *M. hapla* and *M. javanica*. These experiments suggest that *T. semipilosum* is a source of multiple nematode R loci. We plan to characterise further nematode resistances at loci in *Trifolium* genomes, and to investigate transfer of R alleles from *T. semipilosum* to white clover and other economic species.

Introduction

Root-knot nematodes are globally distributed pathogens, causing substantial economic losses in most genera of economic plants (Manzanilla-Lopez *et al.* 2004), including forages (Cook and Yeates 1993). These losses are either direct effects, or the parasite's contribution to formation of disease complexes (Khan 1993). Temperate pastoral agriculture benefits from white clover (*Trifolium repens*), a forage legume which contributes to nitrogen fixation, nutritive value and enhanced intake, while complementing seasonal growth patterns of forage grasses in mixed swards (Caradus *et al.* 1995). The clover root-knot nematode, *Meloidogyne trifoliophila* (*Mt*), seriously debilitates white clover in New Zealand pastures, constraining herbage yield and nitrogen fixation (Mercer and Watson 1996). Preferred *Mt* control strategies are durable host plant resistance (R), and/or high levels of tolerance, in elite white clover varieties. A recurrent selection programme has identified R to *Mt* in white clover, apparently conferred by multiple recessive genes (Mercer *et al.* 2000), which has proven difficult to utilise in white clover breeding programmes due to the strong outbreeding nature of this species (Mercer *et al.* 2005).

Introgression of single-locus nematode R from secondary (inter-specific) gene pools has been a successful alternative when simply inherited R is unavailable in the

primary (intra-specific) gene pool (Starr *et al.* 2002). A survey of the white clover primary, secondary, and tertiary gene pools has identified complete R to *Mt* in an African clover, *T. semipilosum* conferred by a dominant allele at the *TRKR* locus on linkage group D (Barrett *et al.* 2005).

In addition to forage legumes, *Meloidogyne* nematode species cause significant losses in cereal, legume, fibre, and horticultural crops. For many crops, genetic solutions are complex, and only partial R is available. For example, the only characterised genetic R to *M. incognita* in soybean is partial and multigenic (Li *et al.* 2001, Silva *et al.* 2001), suggesting there is opportunity for identification of single-gene R sources with a high level of efficacy. It is preferred that R gene(s) with efficacy against multiple nematode species are used, as R to a single species may simply shift the balance of nematode species in the rhizosphere toward those species for which no R is deployed (Barker 1989).

Ongoing research is examining the range of efficacy of nematode R in *T. semipilosum*, including temperature dependence (Mercer 2005) and challenge by other *Meloidogyne* species (this study). Understanding the scope of the R will help prioritise options to utilise this genetic R source.

The objectives of the research described in this paper were to determine whether *T. semipilosum* is a compatible host to three root-knot nematode species, viz. *M. hapla, M. javanica,* and *M. incognita* and to determine whether *T. semipilosum* exhibits genetic R to any of these species. For comparative purposes, a survey of white clover germplasm exhibiting differential reaction to *Mt* was included.

Materials and Methods

Expt. 1: *M. trifoliophila versus T. semipilosum* and *T. repens*

Four *T. semipilosum* genotypes (Table 1) were cloned, being the resistant and susceptible parents, and one resistant and one susceptible full-sib offspring from the mapping population segregating for R to *Mt* at the *TRKR* locus (Barrett *et al*. 2005). Clonal plant copies were generated by layering stolons into neighbouring empty pots and cutting them free from the parent plant only once rooted in the new pot. Plants were maintained and inoculated in 110-mm-diameter planter pots. A fraction of each root system, being root masses under two adjacent nodes of the stolon, was inoculated with 5,000 *Mt* eggs extracted in NaOCl (Hussey and Barker 1973) from a culture on *T. repens* roots.

Eight *T. repens* genotypes (Table 1) were cloned: six had exhibited a partial resistance reaction to *Mt* and two were susceptible (Mercer *et al.* 2000). To generate a population of clones, stolon tips were rooted in pasteurised peat-based potting mix in a glasshouse maintained at 20–25°C. Four week-old *T. repens* cuttings were replanted into fresh mix in 60-mm-diameter pots, one per pot, and a suspension of 2,000 *Mt* eggs poured into holes around the roots, which were then closed. Plants were maintained in a greenhouse at 20–25°C.

For both clover species, roots were washed free of mix 4 weeks post-inoculation. Galls and NaOCl-extracted eggs were counted.

Expt. 2: M. hapla *versus T. semipilosum* and *T. repens*

Procedures were as above, except that *M. hapla* inoculum was raised on tomato (*Lycopersicon esculentum*) roots.

Expt. 3: *M. javanica versus T. semipilosum* and *T. repens*

Stolons with uninfected and unchallenged roots from each of the four *T. semipilosum* genotypes used in Expt. 1 were rinsed, divided and laid out on the surface of the potting mix in a half-filled 100-mm-diameter pot and 50,000 eggs poured over. Pots were filled with fresh mix and maintained as above for six weeks until harvest. Galls and egg masses were counted. Twenty-two additional *TRKR* segregants were assessed for reaction to *M. javanica* as rooted layers, one layer per genotype. Subsequently, copies were inoculated at 5,000 eggs per 60-mm-diameter pot and galls counted six weeks later (data not shown). From this second set, clean copies of 12 genotypes were re-inoculated with *M. javanica*, the 12 were comprised of six resistant and six susceptible to *Mt* with each of these six comprised of four putative resistant genotypes and two putative susceptible genotypes to *M. javanica*. Plants were grown in 100-mm-diameter plastic planter bags and inoculum was added to the separate nodal root systems, which were treated as replicates in ANOVA. Roots were washed and galls counted 4 weeks after inoculation.

Methods were the same as Expt. 1 for *T. repens*, except that inoculum was raised on tomato roots, and only galls were counted.

Expt. 4: *M. incognita* versus *T. semipilosum*

Three *T. semipilosum* genotypes (Table 3) from the full-sib population were cloned: two had exhibited a partial resistance reaction to *Mt* and one was susceptible. Methods were the same as for Expt. 1, except copies were inoculated in 60-mm-diameter pots at 2,000 eggs per copy, and 8 weeks later roots were washed, galls and egg masses counted, and percentage of root system galled was recorded. Subsequently 11 *T. semipilosum* genotypes (Table 3) were cloned and inoculated with 400 eggs each in 60-mm-diameter pots, and 8 weeks later roots were washed, galls and egg masses counted, and percentage galling assessed. A subset of the second set (Table 4) was cloned, inoculated as above, and after 7 weeks, roots were washed, galls counted, and gall size and egg mass visibility were recorded.

Identifications and Statistical Analyses

Identity and purity of *M. javanica* and *M. incognita* cultures used for inoculum were confirmed by inspection of perineal patterns. To minimise pot positional effects within trays and glasshouses, pots were arranged at random within blocks by nematode species. Trays of plants were turned end-for-end and rotated around the greenhouse weekly. Data were subjected to ANOVA or Chi-square analysis as appropriate, with transformation as necessary.

Results

Expt. 1:

For both *T. semipilosum* and white clover, the *Mt* response phenotype of all plant material confirmed results of previous experiments which indicated partial R in *T. repens* and complete R in *T. semipilosum* conferred by *TRKR*.

Expt. 2:

Both *T. semipilosum* and white clover were able to host *M. hapla* throughout its lifecycle. Gall and egg mass numbers did not significantly vary among individuals within species, suggesting the germplasm surveyed is uniformly susceptible to *M. hapla* under these experimental conditions (Table 1).

Expt. 3:
Both *T. semipilosum* and *T. repens* were able to host *M. javanica* throughout its lifecycle. *M. javanica* gall numbers and egg mass numbers varied significantly ($P<0.05$) among replicated copies of *T. semipilosum* parent and progeny genotypes (Table 1). The *M. javanica* R is conferred by the same parent plant that contributed the *Mt* R allele at the TRKR locus, and the loci conferring R to *Mt* and *M. javanica* are either loosely linked or independently inherited (Tables 1 and 2). *M. javanica* gall numbers did not vary significantly among white clover genotypes (Table 1).
Expt. 4:
T. semipilosum was able to host *M. incognita* throughout its lifecycle. The reaction to *M. incognita* was similar to the reaction observed to *M. javanica*, in that partial resistance to *M incognita* is apparent in this population (Table 3). The five genotypes which were tested in a replicated trial indicated a consistent reaction phenotype, and exhibited significant differences in reaction phenotype among the subset of the full-sib population (Table 4). The loci conferring R to *Mt* and *M. incognita* are either loosely linked or independently inherited (Tables 3 and 4).

Discussion
Development of genetic solutions to plant-parasitic nematodes will deliver substantial benefits to the New Zealand pastoral industry and may also benefit other crop species. The current experiments have shown *T. semipilosum* to be a source of R to multiple root-knot nematode species. This survey of one full-sib family has confirmed R to *Mt*, and has identified R to two of the three additional root-knot nematode species tested. These resistances appear to be independent of the *TRKR* locus, which is consistent with the gene-for-gene hypothesis. While it will be more complicated to transfer multiple loci to white clover or other species, a multi-locus R package in a forage legume may enhance the durability of the genetic protection against root-knot nematodes.

The two distinct classes of reaction phenotypes of *T. semipilosum* to *M. javanica* reflect the selection of extreme phenotypes among progeny that included individuals with intermediate phenotypes (data not shown). Preliminary analysis of replicated data suggests that the *M. javanica* reaction may be caused by one major genetic factor conferred by the *Mt* resistant parent, modified by minor quantitative genetic effects. The genetic factor(s) confer a 10 – 100X reduction in level of infection by *M. javanica* (Table 2). A similar case was observed in the *T. semipilosum* reaction to *M. incognita* with the possible segregation of a major genetic factor conferring R, modified by minor genetic effects. The parents of the full-sib population were unavailable for testing, so it is unknown which parent confers R to *M. incognita*.

The observed uniformly susceptible reaction of *T. semipilosum* to *M. hapla* may be the result of the narrow germplasm base used in this study, or the lack of natural habitat overlap. A further survey of *T. semipilosum* germplasm is warranted to determine if R to *M. hapla* does exist.

The white clover genotypes exhibiting partial R to *Mt* did not exhibit R to *M. hapla* or *M. javanica* (Table 1) indicating an extensive germplasm survey, recurrent selection programmes, and/or transfer from secondary gene pools will be necessary to develop elite white clover with R to these pathogens. MSNR4, a North American *T. repens* line resistant to three species of root-knot nematode has been reported (Pederson and Quesenberry 1995). When tested against a New Zealand strain of *Mt*, it did not exhibit significant levels of R, indicating it may be of use in developing a multiple gene

R package but is of little utility on its own in New Zealand pastures which are primarily infected with *Mt* (Mercer *et al.* 2000).

The data suggest that both *M. javanica* and *M. incognita* R reactions are under genetic control independent of *TRKR* (Tables 2 and 3). Constraints prevented the testing of the genetic relationship between the *M. javanica* and *M. incognita* resistance source(s) in this sample of the population, an area of research that is warranted by the global impact of these two plant pathogens.

Work is underway to confirm a suitable genetic bridge between *T. semipilosum* and *T. repens* allowing transfer of the *TRKR* locus into white clover and the possible transfer of other R loci with activity against plant-parasitic nematodes. Further investigation of *T. semipilosum* germplasm for R to other pathogens is warranted, as is further investigation of the efficacy of the R to *Mt*, *M. javanica* and *M. incognita*. Evaluation of the R against a range of *Mt*, *M. javanica* and *M. incognita* isolates is also warranted to further characterise the extent of the R.

This experiment demonstrated the value of the secondary gene pool in *Trifolium* as a source of novel R alleles, and highlights opportunities to explore and utilise this global resource for the improvement of forage legumes and other economic plant species.

Table 1. Mean numbers of *Meloidogyne* root-knot nematode species galls and eggs per plant on *Trifolium repens* and *T. semipilosum* genotypes resistant or susceptible to *Mt*.

Host Species	Confirmed host reaction to *Mt*	Host genotype identification[a]	*M. hapla* galls per plant[b]	*M. hapla* eggs per plant	*M. javanica* galls per plant	*M. javanica* egg masses per plant
Trifolium repens	Susceptible	1	45a	260a	12a	-
	Susceptible	2	73a	350a	8a	-
	Resistant	3	36a	320a	38a	-
	Resistant	4	39a	490a	10a	-
	Resistant	5	23a	-	4a	-
	Resistant	6	43a	450a	11a	-
	Resistant	7	19a	20a	11a	-
	Resistant	8	32a	35a	14a	-
T. semipilosum	Susceptible	Parent 10-7	70a	300a	57b	14b
	Susceptible	TSM 004	35a	20a	10a	3ab
	Resistant	Parent 6-8	32a	250a	3a	1a
	Resistant	TSM 019	7a	65a	20a	8ab

[a]Susceptible *T. repens* genotypes are progeny of Barblanca x Crusader; resistant genotypes are progeny of C17515-2 x C17513-2.

[b]Within each nematode x host combination, values with a letter in common do not differ at $P<0.05$.

Table 2. Confirmation of partial resistance to *M. javanica* in *T. semipilosum.* Mean numbers of *M. javanica* galls per nodal root on *T. semipilosum* genotypes resistant or susceptible to *Mt*.

Host reaction to *Mt*	Previous host reaction to *M. javanica*	Host genotype	Number of nodal roots	Galls per nodal root[a]
Resistant	Resistant	TSM 225	7	0.14a
Resistant	Resistant	TSM 227	4	0a
Resistant	Resistant	TSM 228	13	1.4a
Resistant	Resistant	TSM 231	3	7.3a
Resistant	Susceptible	TSM 212	7	87b
Resistant	Susceptible	TSM 218	5	75b
Susceptible	Resistant	TSM 214	4	1.3a
Susceptible	Resistant	TSM 220	5	0.4a
Susceptible	Resistant	TSM 223	4	1.8a
Susceptible	Resistant	TSM 229	6	4.2a
Susceptible	Susceptible	TSM 232	3	95b
Susceptible	Susceptible	TSM 235	4	82b

[a]Means with a letter in common do not differ at P<0.001.

Table 3. Mean numbers of *M. incognita* galls, egg masses and percentage of root system galled on *T. semipilosum* genotypes resistant or susceptible to *Mt*.

Host reaction to *Mt*	Host genotype	Number of copies	Galls per copy[a] (range)	Egg masses per copy (range)	% root system galled (range)
12 December 2001 inoculation at 2,000 eggs per copy					
Susceptible	TSM 136	4	110b (30-200)	52b (8-100)	46b (5-90)
Resistant	TSM 070	6	51b (18-100)	14ab (0-20)	18b (8-50)
Resistant	TSM 131	6	0.7a (0-2)	0.7a (0-2)	0.3c (0-1)
12 May 2004 inoculation at 400 eggs per copy					
Susceptible	TSM 208	2	0.5 (0-1)	0.5 (0-1)	< 1
Susceptible	TSM 209	2	5.5 (2-9)	2 (1-3)	< 1
Susceptible	TSM 210	2	15 (12-18)	8.5 (8-9)	< 1
Susceptible	TSM 211	2	14.5 (11-18)	13.5 (7-20)	< 1
Resistant	TSM 201	2	0	0	< 1
Resistant	TSM 202	2	24 (20-28)	17 (13-21)	< 1
Resistant	TSM 203	1	1	1	< 1
Resistant	TSM 204	1	6	4	< 1
Resistant	TSM 205	1	24	30	< 1
Resistant	TSM 206	1	2.5 (1-4)	2.5 (1-4)	< 1
Resistant	TSM 207	1	1	1	< 1

Copies were inoculated in 60-mm-diam. pots 8 weeks previously for both dates.
[a] Within a column for the 2001 experiment, means with a letter in common do not differ at P<0.05.

Table 4. Confirmation of partial resistance to *M. incognita* in *T. semipilosum.* Mean numbers of *M. incognita* galls, range of gall size, and presence of egg masses on *T. semipilosum* genotypes resistant or susceptible to *Mt*.

Host reaction to *Mt*	Host genotype	Number of copies	Galls per copy[a]	Gall size	Egg masses readily seen
Susceptible	TSM 208	7	7a	All small	No
Susceptible	TSM 210	4	230b	Full range	Yes
Susceptible	TSM 211	2	250b	Full range	Yes
Resistant	TSM 201	6	10a	All small	No
Resistant	TSM 203	6	39a	Full range	Yes

[a]Means with a letter in common do not differ at P<0.001.
Copies were inoculated in 100-mm-diam. pots 7 weeks previously.

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