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Screening Subterranean Clover (*Trifolium* spp.) Germplasm for Resistance to *Meloidogyne* Species[1]

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Abstract: This study was conducted to identify lines of subterranean clover (*Trifolium* spp.) with resistance to *Meloidogyne arenaria* (Neal, 1989) Chitwood, 1949, race 1; *M. incognita* (Kofoid and White, 1919) Chitwood, 1949, race 3; and *M. javanica* (Treub, 1885) Chitwood, 1949. A collection of 134 subterranean clover lines was evaluated and all had intermediate to high susceptibility. Root galling was negatively correlated with both seed and dry matter yields. Soil fumigation significantly reduced the nematode population in the field. Results indicate there is limited genetic resistance to root-knot nematodes among subterranean clover lines. Alternative sources of variation for this trait should be investigated.

Key words: egg mass, legume, *Meloidogyne arenaria, M. incognita, M. javanica,* root galling, root-knot nematode, soil fumigation, subterranean clover, susceptibility, *Trifolium* spp.

A need for low input, high quality forage crops in Florida has resulted in increased use of forage legumes. Ruelke and Prine (12) evaluated several forage legumes in the genus *Trifolium* in Gainesville and concluded that subterranean (sub) clover has promising agronomic features for north central Florida. Sub clover includes *Trifolium subterraneum* L., *T. brachycalycinum* Katzn. and Morley, and *T. yanninicum* Katzn. and Morley (10). Germplasm collections of more than 600 lines from local accessions and plant introductions have previously been evaluated for adaptation in Florida (Prine, unpubl.). Because root-knot nematodes (*Meloidogyne* spp.) may cause significant losses to *Trifolium* species in Florida (1,4,9,11,13), identification and development of cultivars with high resistance to root-knot nematodes is important to the forage industry. The purpose of this study was to screen agronomically desirable sub clover lines for resistance to *Meloidogyne arenaria* (Neal, 1889) Chitwood, 1949; *M. incognita* (Kofoid and White, 1919) Chitwood, 1949; and *M. javanica* (Treub, 1885) Chitwood, 1949.

Materials and Methods

Three greenhouse experiments and one field test were conducted to investigate resistance in sub clover.

Greenhouse: In greenhouse experiment 1, conducted from 19 June 1984 to 14 August 1984, 134 sub clover lines selected for their vigor were tested in a split-plot design with three replications for response to *Meloidogyne arenaria* race 1, *M. javanica,* and *M. incognita* races 1 and 3. In experiment 2, 34 sub clover entries selected from experiment 1 were examined from 16 July 1985 to 14 September 1985 for consistency of their responses to the three *Meloidogyne* species. Three lines of alfalfa (*Medicago sativa* L.) with known resistance levels were included in experiment 2. The 10 best sub clover lines from experiment 2 and five newly introduced lines were further tested from 11 December 1986 to 5 February 1987 in experiment 3 using the same design. In all experiments, the plant genotypes constituted the subplot treatments and the nematode species were the main plots. A single plant in each of three replications was used. Race 1 of *M. incognita* was not included in experiments 2 and 3, but a combination of the three nema-

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tode species in equal proportion was used as the fourth main-plot treatment. This mixed treatment exerted the combined effect of the nematodes that may occur in field conditions.

Seed were germinated on filter paper in petri dishes and transplanted singly into 150-cm^3 methyl bromide fumigated Arredondo fine sand—loamy, silicious, hyperthermic, Grossarenic Paleudult (92% sand, 5% silt, 2% clay, 1% organic matter; pH 6.1). The plants were grown with adequate moisture, light, and fertility in a greenhouse. They were inoculated with the sub clover group *Rhizobium* (*R. trifolii*) (6). Eighteen days after transplanting, the plants were inoculated with 1,500 nematode eggs per container. In each subplot, an uninoculated plant served as a control treatment. Nematode inocula were collected by the method of Hussey and Barker (7). Eggs were diluted in water to the appropriate concentration and kept in suspension with a magnetic stirrer. A 5-ml aliquot was injected into the seedling rhizosphere with an automatic syringe.

Plants were removed from the containers 60 days after inoculation and their root systems were washed gently. The roots then were immersed in an aqueous solution of phloxine B (15 mg/liter water) for 15–20 minutes to stain the egg masses (3,5). Root galls and (or) egg masses were closely examined and each plant was rated for both galls and egg masses on a 0 to 5 scale: 0 = no galls or egg masses; 1 = 1–2; 2 = 3–10; 3 = 11–30; 4 = 31–100; 5 = more than 100 galls or egg masses, or dead plant (15). A mean score was calculated for each plant (mean score = [gall score + egg mass score] × 0.50). Data were subjected to an analysis of variance, and treatment means were separated using Duncan's multiple-range test. Correlations between gall and egg mass ratings were determined. All analyses were carried out according to the Statistical Analysis System (14).

Field: The 10 sub clover lines selected from experiment 2 were evaluated in the field in 1986–87. A 40 × 20-m area at the University of Florida agronomy farm in Gainesville, Florida, was tilled and divided into eight plots. The area was known to be infested by the three *Meloidogyne* species in the greenhouse experiments. Average density for the three nematode species combined was 950 nematodes/100 cc soil. One of two adjacent plots was fumigated using 1,3-D (1,3-dichloropropene, formulated as Telone II, at 93.5 liter/ha); the other was not fumigated. The experiment was replicated four times in a split-plot design with fumigation as main plot treatment and sub clover lines as subplots. In each main plot, the sub clover entries were planted 2 g seed/row at random in 2-m-long rows on 26 November 1986. The Arredondo fine sandy soil (92% sand, 5% silt, 2% clay, 1% organic matter; pH 6.1) was fertilized with 886 kg/ha of 0-10-20 (N-P_2O_5-K_2O). The seeds were inoculated with *R. trifolii* at planting. The plots were irrigated when necessary to maintain adequate soil moisture. On 21 May 1987 half of each subplot (1-m-long row) was selected at random and the plants were removed to measure forage yield and to score root galling as described for the greenhouse experiments. The remaining plants were scored for root galling as already described and seed was hand harvested on 10 June 1987. The data were subjected to an analysis of variance, and treatment means were separated using Duncan's multiple-range test. Correlations between root galling and dry matter and seed weight were calculated. All analyses were performed using the Statistical Analysis System (14).

Results and Discussion

Greenhouse: When combined over nematode species, all sub clover lines tested were susceptible to root-knot nematode. Significant interaction between the nematode species and the plant genotypes was observed in all experiments. High correlation was found between egg mass scores and gall scores in all experiments (R^2 = 0.76–0.81; $P < 0.001$), indicating that either of these parameters could have been used to determine the susceptibility of sub clover to *Meloidogyne* spp. In experiment 1, mean

gall scores varied from 2.7 to 4.6 when scores from all four nematode treatments were averaged. The cultivar Mt. Barker had the highest score (4.5) and was selected as a susceptible check for the next experiments. Cultivars Nungarin and Yarloop had the lowest scores (2.7 and 3.4, respectively). *Meloidogyne incognita* race 3 had the highest mean gall score of the four species tested.

In experiment 2, mean gall and egg mass scores of individual plants averaged 4+, 82% of the time, indicating that the germplasm tested was largely susceptible (Table 1). There were, however, significant differences among the lines. Cultivars Nungarin and Yarloop had moderate gall and egg mass scores in almost all nematode treatments, which was consistent with experiment 1. Although these cultivars are not well adapted to Florida conditions, they might be useful in breeding sub clover lines agronomically adapted to Florida. These two cultivars are among the few sub clover genotypes that have been systematically characterized. Cultivar Yarloop is in the species *Trifolium yanninicum* and cultivar Nungarin belongs to the species *T. subterraneum*, as does Mt. Barker. No definite conclusion can be drawn about the comparative resistance of the three sub clover species to root-knot nematodes. Unlike the sub clover entries, the three alfalfa cultivars were highly resistant to at least one of the root-knot nematode species. Cultivar Moapa 69 was the most resistant. This is in accordance with the stated level of resistance of these three cultivars (16). *Meloidogyne arenaria* and *M. incognita* race 3 produced the most egg masses and galls. The mixture of the three nematode species also had high average scores, which suggests that the combined effect of the nematodes on a susceptible plant is about the same as the effect of a single species.

In experiment 3, the mean response ranged from 2.3 to 4.1 (Table 2). All nematode species produced the same level of galls. The overall mean response was 3.3 for *Meloidogyne incognita*, 3.3 for *M. arenaria*, and 3.2 for *M. javanica*. Variation in the ranking of the nematodes through the three experiments may be due to the fact that the majority of these sub clover entries were selected for reaction to the most severe nematode treatments in experiment 1 and were closely grouped. The selected entries were then similar in reaction to the different nematodes. Reaction to the combined effect of the nematodes ranged from 2.7 to 3.7. Of the 10 entries selected from

TABLE 1. Mean scores† of 34 subterranean clover cultivars and lines and three alfalfa cultivars inoculated with three species of *Meloidogyne* in greenhouse experiment 2.

Cultivars and lines	*M. arenaria*	*M. incognita*	*M. javanica*	Mixed‡
Moapa	0.0 a	0.3 a	1.0 ab	0.0 a
Caliverde	0.7 ab	3.0 b	2.0 bc	2.3 b
Lahontan	1.3 b	4.0 b–e	0.0 a	2.7 bc
Yarloop	4.0 cd	4.7 de	3.0 cd	3.7 cde
Nungarin	4.0 cd	3.7 bcd	3.7 def	3.3 bcd
Meteora	4.3 cd	4.3 cde	4.3 efg	4.3 def
Mt. Barker	4.3 cd	4.7 de	4.7 fg	4.3 def
Woogenellup	4.7 cd	4.7 de	4.3 efg	4.7 ef
Tallarook	4.7 cd	4.7 de	4.0 d–g	4.7 ef
Tier-6	3.7 c	4.0 b–e	3.7 def	4.3 def
PI 384718	3.7 c	4.0 b–e	4.7 fg	4.0 def
PI 233870	3.7 c	4.3 cde	4.3 efg	4.3 def
47294B	4.0 cd	4.0 b–e	4.0 d–g	3.7 cde
109	4.0 cd	3.7 bcd	4.0 d–g	4.3 def
Casterton	4.0 cd	4.3 cde	4.3 efg	4.0 def
PI 233869	4.0 cd	4.0 b–e	3.3 de	4.0 def
Seaton Park	4.0 cd	4.0 b–e	3.7 def	3.3 bcd
V-48	4.0 cd	4.3 cde	4.0 d–g	4.3 def
W	4.0 cd	4.0 b–e	4.0 d–g	4.3 def
PI 277431	4.3 cd	4.7 de	4.0 d–g	4.3 def
PI 239909	4.3 cd	4.0 b–e	4.3 efg	5.0 f
Composite	4.3 cd	4.0 b–e	4.3 efg	4.0 def
I-47	4.3 cd	4.3 cde	4.0 d–g	4.0 def
PI 291871	4.3 cd	3.3 bc	4.0 d–g	4.0 def
B13	4.3 cd	4.3 cde	4.3 efg	4.0 def
PI 277437	4.3 cd	4.7 de	4.7 fg	5.0 f
PI 233871	4.3 cd	3.7 bcd	4.0 d–g	4.3 def
Mississippi	4.3 cd	4.3 cde	4.7 fg	4.3 def
B30	4.3 cd	5.0 e	4.3 efg	5.0 f
PI 385031	4.3 cd	4.0 b–e	5.0 g	4.3 def
Portugal	4.7 cd	4.0 b–e	4.0 d–g	4.3 def
PI 241461	4.7 cd	4.7 de	4.3 efg	4.7 ef
30908B	4.7 cd	4.3 cde	3.3 de	4.3 def
47306	4.7 cd	4.7 de	4.0 d–g	4.7 ef
PI 401564	4.7 cd	4.3 cde	4.7 fg	5.0 f
IV-21	5.0 d	4.3 cde	4.3 efg	4.0 def
PI 190572	5.0 d	4.0 b–e	4.0 d–g	4.3 def

Means followed by the same letter in the same column are not significantly different ($P < 0.05$) according to Duncan's multiple-range test.

† Mean score = (gall score + egg score) × 0.5.

‡ All three species of *Meloidogyne* mixed together.

TABLE 2. Mean scores† of 15 subterranean clover cultivars and lines inoculated with three species of *Meloidogyne* in experiment 3.

Cultivars and lines	*M. arenaria*	*M. incognita*	*M. javanica*	Mixed‡
PI 190572	2.7 a	3.1 abc	3.7 cd	3.2 a–d
PI 190567	2.7 a	3.0 abc	4.1 d	3.3 b–e
W	2.8 ab	2.7 a	3.6 cd	3.1 abc
47306	3.0 abc	2.7 a	3.4 bcd	3.0 abc
IV-21	3.1 a–d	2.7 a	2.3 a	2.7 a
PI 233869	3.1 a–d	2.9 ab	2.8 abc	3.2 b–e
PI 233873	3.3 a–e	4.0 c	3.2 bcd	2.9 ab
Casterton	3.4 a–e	2.8 ab	2.6 ab	3.2 b–e
PI 277431	3.4 a–e	4.0 c	3.4 bcd	3.3 b–e
99476	3.5 b–e	3.7 abc	3.6 cd	3.4 cde
PI 233871	3.5 b–e	3.5 abc	3.5 bcd	3.4 cde
PI 274430	3.6 cde	4.0 c	3.4 bcd	3.2 b–e
PI 239909	3.6 cde	3.2 abc	3.5 bcd	3.4 cde
PI 206389	3.7 de	3.9 bc	3.9 d	3.7 e
Mt. Barker	3.9 e	3.4 abc	3.5 bcd	3.6 de

Means followed by the same letter in the same column are not significantly different ($P < 0.05$) according to Duncan's multiple-range test.

† Mean score = (gall score + egg score) × 0.5.

‡ All three species of *Meloidogyne* mixed together.

experiment 2, Mt. Barker was the most susceptible cultivar at 3.6. Selection IV-21 exhibited the best resistance level with an average mean score of 2.7, and 47306, W, and PI 190572 were slightly less resistant with average mean scores of 3.0, 3.1, and 3.2, respectively. One of the five new accessions, PI 233873, also had an acceptable level of resistance, ranking second of the 15 lines tested with a mean score of 2.9 (Table 2).

On the whole, the responses of the sub clover lines in experiment 3 were lower than those in experiments 1 and 2. The variation in the aggressiveness of the nematode species over the three experiments may be related to external factors. The susceptible check, Mt. Barker, also had limited gall response in experiment 3. High temperature is known to increase the vulnerability of some plants to nematode attack (2). This may explain the severity of the first two screenings which were conducted during the summer period.

Field: Fumigated plots had significantly lower galling than nonfumigated plots ($P < 0.0001$). Very little galling was found on some entries on fumigated plots. A nematode assay of the field at the time of dry matter harvest shows that *Meloidogyne* spp. population densities on nonfumigated plots were 300–400 times greater than those of fumigated plots. There were also differences among the sub clover lines ($P < 0.018$) in their reaction to nematode infection in the field. Entries PI 233869, Casterton, PI 277431, PI 239909, and IV-21 had significantly fewer galls than Mt. Barker on nonfumigated plots (Table 3).

Soil fumigation did not significantly affect the plant dry weight and seed yield. It has been reported that legumes infected with *Meloidogyne* spp. formed more lateral roots than uninfected plants (8). This response may explain the similarity of the

TABLE 3. Gall scores, dry matter, and seed yield of 10 subterranean clover cultivars and lines evaluated in the field at Gainesville, Florida, in 1986–87.

Cultivars and lines	Gall score		Dry matter (g/plant)		Seed yield (g/plant)	
	(1)†	(2)†	(1)	(2)	(1)	(2)
PI 233869	0.0 a	1.8 a	31.7 ab	30.6 ab	1.0 bc	1.4 a
Casterton	0.0 a	1.8 a	24.4 b	17.1 b	1.9 abc	0.5 a
PI 277431	0.0 a	1.7 a	61.4 a	22.6 ab	0.5 c	0.8 a
PI 239909	0.0 a	1.8 a	24.8 b	37.2 ab	1.1 bc	0.5 a
IV-21	0.5 a	1.5 a	38.9 ab	34.7 ab	2.8 a	1.7 a
PI 190572	0.0 a	2.3 ab	36.5 ab	44.2 ab	2.2 ab	1.4 a
PI 233871	0.3 a	2.3 ab	18.8 b	34.4 ab	1.6 abc	1.3 a
Mt. Barker	0.5 a	2.8 ab	21.4 b	46.1 ab	1.2 bc	1.3 a
W	0.3 a	3.3 b	30.2 ab	53.6 a	2.5 ab	2.2 a
47306	0.3 a	3.3 b	29.6 ab	35.3 ab	1.1 bc	1.6 a

Means followed by the same letter in the same column are not significantly different ($P < 0.05$) according to Duncan's multiple-range test.

† (1) = fumigated plot; (2) = nonfumigated plot.

forage yields on the two main plot treatments. It should be noted, however, that the fumigated plots were very slow to establish. The 8-day period allowed between the application of the fumigant and the sowing date could have been too short to avoid nematicide reduction of *R. trifolii* nodulation and delay of plant growth on fumigated plots. Significant differences in seed and dry matter production were found among the lines, but there were no significant differences in seed yield on nonfumigated plots. Negative correlations were found between root galling and dry matter yield ($R = -0.30$, $P = 0.05$) and between root galling and seed yield ($R = -0.26$, $P = 0.02$).

The results of the study indicated that heavy galling in the field will reduce the potential yield of sub clover. A need to evaluate additional sub clover germplasm sources, especially germplasm from areas with known root-knot nematode problems, is necessary to determine if resistance to *Meloidogyne* spp. occurs in these species. Most other *Trifolium* spp. evaluated in Florida do not have root-knot nematode resistance (1,11).

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