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Response of *Trifolium repens* Clones to Infection by *Meloidogyne incognita* and Peanut Stunt Virus[1]

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Abstract: The responses of selected clones of white clover (*Trifolium repens*) to simultaneous infection by the southern root-knot nematode (*Meloidogyne incognita*) and peanut stunt virus (PSV) were determined. Two white clover clones, which were resistant (NC-R) or sensitive (NC-S) to ozone injury, were evaluated. Plant growth and *M. incognita* reproduction were measured. Root, stolon, and top growth were reduced by PSV infection, which affected NC-R more than NC-S. Both clones were tolerant of *M. incognita,* but NC-R had less root galling and less nematode reproduction than NC-S, and thus was less susceptible to *M. incognita.* Reductions in root growth of plants infected with both *M. incognita* and PSV were greater than in plants infected by either pathogen alone. Nematode reproduction tended to be lower on PSV-infected plants.

Key words: Meloidogyne incognita, nematode, ozone, peanut stunt virus, root-knot nematode, *Trifolium repens,* white clover.

White clover (*Trifolium repens*) is an important forage legume grown on an estimated 22.5 million ha in the temperate United States (3). It is grown extensively in southeastern states and is the predominant perennial legume in pastures in this region (15). In southeastern pastures, white clover stands commonly decline within 2 to 3 years after establishment (4). This decline and lack of persistence have been attributed to one or more factors (19) including diseases caused by viruses (1), nematodes (2), and ozone (8,11). Among the viruses that infect white clover in the southeastern states, peanut stunt virus (PSV) is the most widespread and common (16,17) and causes the greatest crop losses (6,7). The southern root-knot nematode (*Meloidogyne incognita*) is considered the most important nematode associated with white clover in this region (18,24).

White clover clones were recently selected for resistance (NC-R) or sensitivity (NC-S) to ozone (10). Vegetative growth of NC-S was reduced more than that of NC-R when both clones were chronically exposed to successively higher levels of ozone (9). This differential response formed the basis for a proposal to use the clones as biological indicators for ozone concentrations. Both NC-R and NC-S are susceptible to infection by PSV, which reduced vegetative growth of both clones but had a greater effect on NC-R than NC-S (9). The reactions of NC-R and NC-S to *M. incognita* alone or in combination with PSV were not known. Therefore selected aspects of the resistance or susceptibility (ability to support nematode reproduction) and tolerance or intolerance (host growth in the presence of the nematode) of these clones to infection by *M. incognita* were investigated. Specific objectives of the present study were: i) to determine the effects of *M. incognita* and PSV on NC-R and NC-S; ii) to characterize the resistance–susceptibility and tolerance–intolerance of these clones to *M. incognita;* and iii) to determine the effects of PSV infection on reproduction of *M. incognita.*

Materials and Methods

Environmental conditions: Experiments were conducted in a greenhouse at Mississippi State, with natural light and day length from 14 January to 20 March and 26 March to 10 June, 1991. Air temperature was 25 ± 5 C. Plants were watered as needed to prevent wilting.

White clover plants: Plants of white clover

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clones NC-R and NC-S (10) were propagated by stolon cuttings rooted and grown in Jiffy Mix (CASSCO, Montgomery, AL) (containing equal parts peat moss and vermiculite) in 15-cm-d clay pots. Virus-free and PSV-infected plants of both clones were started from stolon cuttings of healthy and infected plants, respectively, and infections were verified by ELISA (9). Healthy and infected plants were maintained separately in adjacent insect-proof cages before the experiment. The experiment was run twice, and plants used in each run were started from stolon tip cuttings made at the beginning of each run. Stolon tips were cut midway between the third and fourth nodes proximal to the growing point. Expanded leaves were removed and the length of each stolon piece was recorded. Stolon pieces were inoculated with *Rhizobium leguminosarum* biovar *trifolii* by dipping each into peat-based inoculum (Nitragin Co., Milwaukee, WI). Stolon pieces were then transplanted to 15-cm-d clay pots containing a methyl bromide-sterilized mixture of soil and sand (80% sand, 6% clay, and 14% silt).

Nematode inoculations: A race 4 population of *M. incognita* was increased on tomato (*Lycopersicon esculentum* cv. Floradel) in the greenhouse. After 8–10 weeks, nematode eggs were collected from tomato roots with NaOCl (13). A water suspension containing 6,000 eggs was transferred by pipette into each pot. Rooted stolon cuttings in the first and second experiments were inoculated 11 and 21 days, respectively, after transplanting.

White clover growth: Plants in the two experiments were removed from pots at 66 and 77 days after inoculation, respectively, and the soil was carefully washed from the roots. Primary stolon length (mm), number of secondary stolons, and the numbers of rooted nodes on secondary stolons were recorded. Roots were removed, weighed, and held overnight at 5 C in plastic bags pending nematode reproduction measurements. Plant tops (stolons and leaves) were oven dried at 65 C and weighed.

Nematode reproduction: Egg masses on white clover roots were stained with phloxine B (5,12). Root galling was assessed by visual estimation of the percentage of the root system galled (PRSG). The PRSG rating scale of 0–5 was as follows: 0 = no galling, 1 = 1–10%, 2 = 11–25%, 3 = 26–75%, 4 = 76–90%, and 5 = 91–100% of the root system galled. Root systems were also evaluated for nematode reproduction based on counts of egg masses. An egg mass index on a scale of 0–5 was used, with 0 = 0, 1 = 1 or 2, 2 = 3–10, 3 = 11–30, 4 = 31–100, and 5 = >100 egg masses present. Subsequently, eggs were collected from each root system with NaOCl (13) and counted. Eggs per plant, eggs per gram of root, and reproduction factor (RF = final number of eggs/initial number of eggs) were calculated for each plant and treatment.

Experimental design and data analysis: A complete factorial set of eight treatments was arranged in six randomized complete blocks. Treatments comprised all combinations of two clover clones (NC-R or NC-S), two levels of PSV (present or absent), and two levels of *M. incognita* (present or absent). Data from the two runs were pooled and subjected to analysis of variance (ANOVA) with a general linear models procedure (21) to test the main effects of clone, nematode, and virus. Virus treatment effects on nematode reproduction were determined by analysis of a subset of data from nematode-inoculated treatments only. Egg counts were transformed to log (x + 1) for ANOVA. Main treatment effects and factor interactions were compared by ANOVA F tests with one degree of freedom (only differences with $P \leq 0.05$ were reported).

Results

Many two- and three-way interactions involving the test were found in ANOVA of pooled data from tests 1 and 2; therefore, data from tests 1 and 2 were analyzed and reported separately. Significant variance in clover growth occurred due to main effects of clover clone (genotype), vi-

rus (±PSV), and root-knot nematode (±*M. incognita*) (Table 1).

Differences between clover clones were not apparent in test 1 but were detected in test 2. In general, NC-R had greater growth than NC-S (Table 2) and was affected more by PSV (Fig. 1). Virus infection significantly reduced clover growth in both tests (Tables 1, 2). Effects of *M. incognita* infection were detected only in root growth (Table 1). Mean top dry weight values (from pooled data of both tests) were 1.9, 2.2, 1.1, and 1.1 plant, respectively, for healthy control, *M. incognita*, PSV, and *M. incognita* + PSV treatments of NC-R, and 1.3, 1.3, 1.2, and 0.9 g/plant, respectively, for the same treatments of NC-S. Interactions occurred in test 2 where the interaction of virus and genotype affected primary stolon growth (Fig. 1A) and top dry weight (Fig. 1B) and the interaction between virus and nematode affected root fresh weight (Fig. 2A) and number of rooting nodes on secondary stolons (Fig. 2B).

Genotype had a significant effect on reproduction of *M. incognita* in both tests

TABLE 2. Main effects treatment means for growth per plant in tests of two white clover genotypes (NC-R and NC-S) with and without peanut stunt virus (PVS) and root-knot nematodes, *Meloidogyne incognita (Mi)*.

Main effect	Primary stolon growth (mm)	Root fresh weight (g)	Rooting nodes on secondary stolons (no.)	Top dry weight (g)
Genotype				
Test 1				
NC-R	125	4.5	1.9	1.1
NC-S	133	4.4	1.6	1.0
Test 2				
NC-R	219	7.1	15.2	2.0
NC-S	220	5.3	5.7	1.3
Virus				
Test 1				
−PSV	149	5.3	2.1	1.3
+PSV	109	3.7	1.4	0.8
Test 2				
−PSV	266	7.1	13.5	2.0
+PSV	171	5.3	7.6	1.3
Nematode				
Test 1				
−Mi	137	5.3	2.4	1.2
+Mi	122	3.7	1.1	0.9
Test 2				
−Mi	230	6.0	7.5	1.6
+Mi	208	6.2	13.0	1.8

TABLE 1. Mean squares from analysis of variance of factorial experiment measuring growth of two white clover genotypes (Gen) in response to infection by *Meloidogyne incognita* (Mi) and peanut stunt virus (PVS).

Source of variation	df†	Primary stolon growth (mm)	Root fresh weight (g)	Rooting nodes on secondary stolons (no.)	Top dry weight (g)
		Test 1			
Replication	5	2071	11	11	0.5
Genotype	1	667	<1	3	0.1
PSV	1	19240**‡	33*	6	2.9**
Mi	1	2775	31*	15	1.1
Gen × PSV	1	5225	13	42	0.8
Gen × Mi	1	124	<1	20	0.3
PSV × Mi	1	1036	2	29	0.4
Gen × PSV × Mi	1	4200	4	<1	0.5
Error	35	1855	6	16	0.3
		Test 2			
Replication	5	1180	3	56	0.2
Genotype	1	17	66**	1002**	6.4**
PSV	1	99494***	43*	447*	6.6**
Mi	1	6459	<1	405*	0.7
Gen × PSV	1	18531*	26	273	3.3*
Gen × Mi	1	94	23	172	1.4
PSV × Mi	1	39	34*	886*	2.4
Gen × PSV × Mi	1	5574	11	31	0.7
Error	35	2933	8	86	0.6

† df = degrees of freedom.
‡ ANOVA *F* test significant: * = ($P \leq 0.05$); ** = ($P \leq 0.01$); *** = ($P \leq 0.001$).

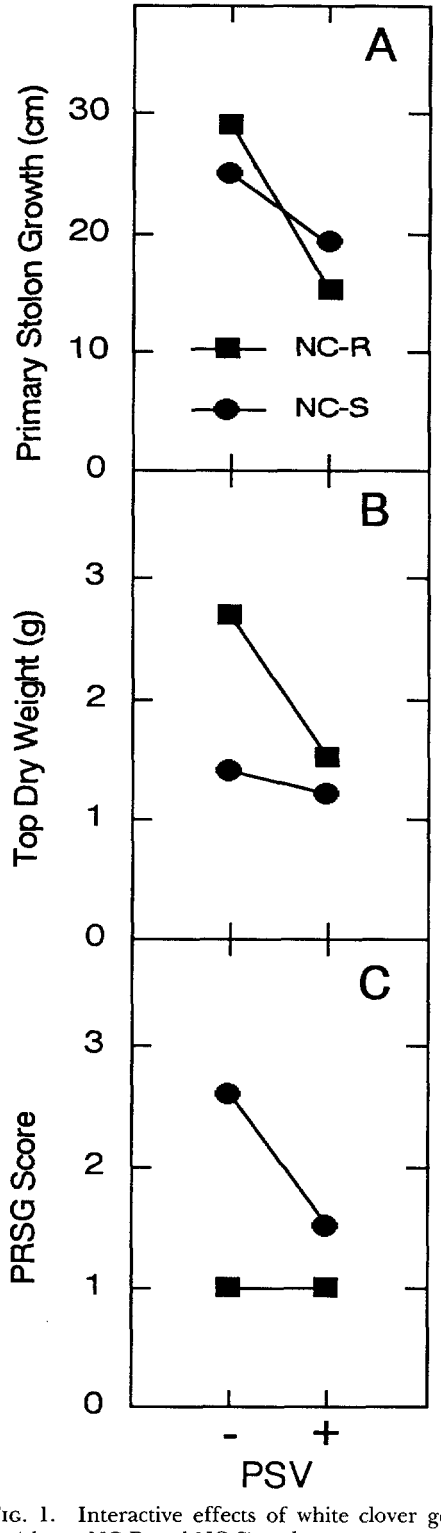

FIG. 1. Interactive effects of white clover genotype (clones NC-R and NC-S) and peanut stunt virus (PSV) infection on (A) primary stolon growth in test 2, (B) top (leaves and stolons) dry weight production in test 2, and (C) percent root system galling (PRSG) score by *Meloidogyne incognita* in test 1.

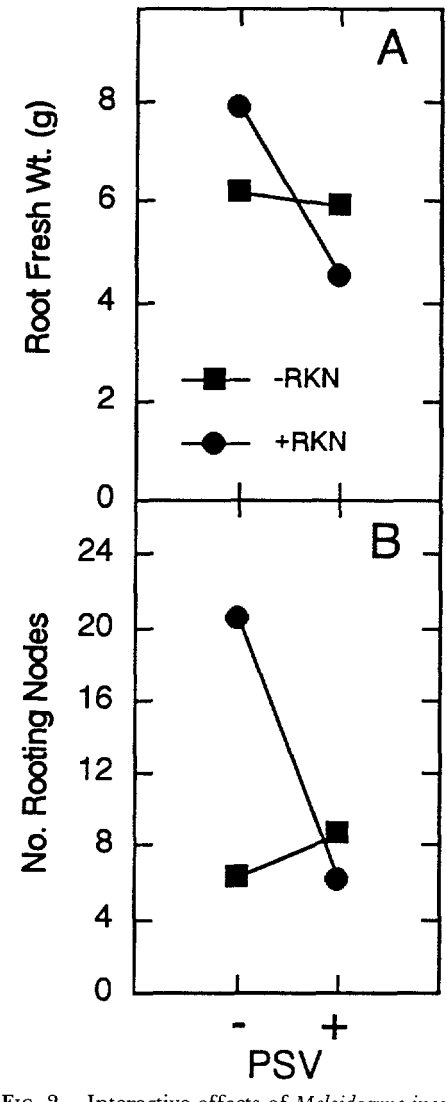

FIG. 2. Interactive effects of *Meloidogyne incognita* and peanut stunt virus (PSV) infection on (A) fresh weight of white clover roots in test 2 and (B) the number of rooting nodes on secondary stolons of white clover in test 2.

(Table 3). Scores for reproductive parameters were generally higher for NC-S than NC-R (Table 4). Only one significant interaction occurred in the reproductive parameters measured: in test 1, virus infection affected the amount of root system galling by *M. incognita* more in NC-S than in NC-R (Fig. 1C). Virus infection tended to reduce scores of reproduction parameters in both tests (Table 4), but most differences were not significant (Table 3).

TABLE 3. Mean squares from analysis of variance of factorial experiment measuring reproduction of *Meloidogyne incognita* on two white clover genotypes (Gen) with and without infection by peanut stunt virus (PSV).

Source of variation	df†	Percent root system galled (PRSG)‡	Egg mass index (EI)§	Log (x + 1) eggs per g of root	Log (x + 1) eggs	RF‖
			Test 1			
Replication	5	0.24	0.20	0.39	0.19	30
Genotype	1	7.04***¶	2.67**	7.66***	10.71***	496***
PSV	1	2.04**	1.50**	0.92	0.01	77
Gen × PSV	1	2.04**	0.17	1.20	0.78	63
Error	15	0.24	0.18	0.43	0.19	25
			Test 2			
Replication	5	0.10	0.19	0.63	0.19	32
Genotype	1	19.38***	1.51*	0.17	1.15*	25
PSV	1	0.60	0.13	0.14	1.23*	6
Gen × PSV	1	0.80	0.22	0.43	0.01	48
Error	15	0.04	0.12	0.34	0.22	30

† df = degrees of freedom.
‡ PRSG (percentage of the root system galled): 0 = no galling, 1 = 1–10%, 2 = 11–25%, 3 = 26–75%, 4 = 76–90%, and 5 = 91–100%.
§ EI (rated on a scale of 0–5): 0 = 0, 1 = 1 or 2, 2 = 3–10, 3 = 11–30, 4 = 31–100, and 5 = >100 egg masses present.
‖ RF (reproduction factor) = (final number of eggs/initial number of eggs).
¶ ANOVA *F* test significant: * = ($P \leq 0.05$); ** = ($P \leq 0.01$); *** = ($P \leq 0.001$).

DISCUSSION

The susceptibility of white clover to *M. incognita* and PSV has been documented (1,2,18,24). Stands of white clover infected with either one of these pathogens are unproductive and eventually disappear. The present study is the first to determine the combined effects of *M. incognita* and PSV on white clover. Generally, growth of both clover clones studied here was reduced more when they were infected with both pathogens than when infected by either alone. The effects of mixed infections of *M. incognita* and PSV on white clover growth were especially evident in the root system, where root fresh weight and the number of rooting nodes on secondary stolons was reduced more by mixed infection than would have been expected from the combined separate effects (Fig. 2).

TABLE 4. Effects of white clover genotype and peanut stunt virus (PSV) infection on reproduction of *Meloidogyne incognita* on white clover.

	Mean per plant				
Variable	Percent root system galled (PRSG)†	Egg mass index (EI)‡	Log (x + 1) eggs per g of root	Log (x + 1) eggs	RF§
Genotype			Test 1		
NC-R	1.0	4.2	8.6	9.9	3.6
NC-S	2.1	4.8	10.0	11.0	12.7
			Test 2		
NC-R	1.0	4.5	9.0	10.9	11.0
NC-S	2.9	5.0	9.5	10.8	9.2
Virus			Test 1		
−PSV	1.9	4.8	9.3	10.6	9.9
+PSV	1.3	4.3	9.3	10.2	6.3
			Test 2		
−PSV	2.0	4.8	9.0	10.9	10.4
+PSV	1.9	4.6	9.5	10.8	10.0

† PRSG (percentage of the root system galled): 0 = no galling, 1 = 1–10%, 2 = 11–25%, 3 = 26–75%, 4 = 76–90%, and 5 = 91–100%.
‡ EI (rated on a scale of 0–5): 0 = 0, 1 = 1 or 2, 2 = 3–10, 3 = 11–30, 4 = 31–100, and 5 = >100 egg masses present.
§ RF (reproduction factor) = (final number of eggs/initial number of eggs).

Windham and Pederson (23) rated white clover grown in containers in the greenhouse for levels of tolerance to *M. incognita* based on percent top dry weight reduction of nematode-inoculated plants compared with uninoculated plants. They defined tolerance as "high" if inoculated plants had 4% or less reduction in top dry weight. By their criterion, clones NC-R and NC-S were both highly tolerant to *M. incognita* infection at the inoculum level used in our studies. The apparent stimulatory effect of *M. incognita* on root initiation observed in test 2 of our study (Table 2) has also been

observed with other *Meloidogyne* species on some soybean cultivars (14,22).

Infection of white clover by PSV is especially damaging in its effects on growth parameters known to be important for persistence, including primary stolon growth, root growth, and production of secondary stolons. Reductions reported here are consistent with earlier work (7,20), and document for the first time the adverse effects of PSV infection on growth, productivity, and potential for persistence of white clover clones NC-R and NC-S. Our results showed a greater effect of PSV infection on growth of NC-R than NC-S and confirmed those of Heagle et al. (9). Differences in response to PSV infection have also been reported for clones of the white clover cultivar Tillman (20). Future use of NC-R and NC-S as ozone indicators in the field will require knowledge of the level of infection by PSV (and possibly other viruses and pathogens) within the indicator plant population.

We conclude that growth reductions in these greenhouse-grown white clover clones were due primarily to PSV. Clone NC-R was shown to be more susceptible to damage from PSV than clone NC-S, whereas NC-S supported more *M. incognita* reproduction and had a higher level of root galling than NC-R. Although infection with PSV produced few significant effects on *M. incognita* reproduction during the relatively short duration of these evaluations, *M. incognita* reproduction tended to be lower on PSV-infected plants. Both clones were excellent hosts for *M. incognita,* but nematode infection had little effect on clover growth. According to these results, NC-S and NC-R were characterized as susceptible to and tolerant of *M. incognita.* Factors that may have influenced this result were rooting of stolon cuttings prior to nematode inoculation and the level of inoculum used. Infestation of the soil with nematodes prior to or at the same time that stolon cuttings were transplanted, use of higher levels of inoculum, and longer duration of tests would be expected to increase the effects of nematodes on clover growth. Other plant stresses, such as drought (which occurs commonly in the field), may also accentuate damage by *M. incognita.* Future studies should investigate the long-term effects of PSV and *M. incognita* infections on growth and persistence of white clover under field conditions, and should incorporate resistance to PSV, *M. incognita,* and ozone into a multiple-disease-resistant white clover germplasm.

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