Evaluation of Tobacco Germ Plasm for Resistance to the Tobacco Cyst Nematode, *Globodera tabacum solanacearum*

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ABSTRACT

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Twenty-four tobacco genotypes were evaluated in the greenhouse for resistance to a North Carolina isolate of the tobacco cyst nematode *Globodera tabacum* subsp. *solanacearum*. The experimental design was a split-plot with eight replications. The experiment was repeated three times. Genotypes were assigned to whole plots, and nematode infestation levels (0 or 10,000 eggs per 1,300 cm^3 of soil) were assigned to subplots. Fresh stalk and root weights of all plants were recorded. Two new variables were created based on these measurements: percent stalk weight [%SW = (SW of infected plant ÷ SW of uninfected plant) × 100] and percent root weight [%RW = (RW of infected plant ÷ RW of uninfected plant) × 100]. Nematode reproduction, measured as number of cysts and eggs per pot, and reproduction factor (final number of eggs ÷ initial number of eggs), was determined for each genotype. Significant differences ($P \leq 0.05$) were observed among tobacco genotypes for nematode reproduction. Low levels were detected in the cultivars Burley 21, PD 4, VA 81, NC 567, Speight G-80, Kutsaga Mammoth 10, Kutsaga 110, and in the flue-cured breeding lines Cyst 913 and 9025-1. Differences in vegetative growth among genotypes grown in nematode-infested soil were detected only between those with the highest and lowest %SW and %RW. Genotype shoot and root weight were not correlated with nematode reproduction. Nevertheless, results from these experiments indicate that nematode reproductive parameters may be used to evaluate tobacco germ plasm for resistance to *G. t.* subsp. *solanacearum* in the greenhouse.

Additional keywords: *Nicotiana tabacum*, screening

Cyst-forming nematodes are highly specialized pathogens with sedentary adult females that, upon death, develop into hardened cysts enclosing the eggs. The cyst constitutes a protective barrier for the progeny, which can remain viable for years in the absence of a host (1,3). The group known as the tobacco cyst nematodes, *Globodera tabacum* Lownsbery & Lownsbery (12), comprises three subspecies: *G. t.* subsp. *tabacum* Lownsbery & Lownsbery (12), *G. t.* subsp. *solanacearum* Miller & Gray (17), and *G. t.* subsp. *virginiae* Miller & Gray (16). These organisms are differentiated by host preference. *G. t.* subsp. *tabacum* parasitizes shade-grown cigar wrapper and field-grown broadleaf cigar tobaccos (*Nicotiana tabacum* L.) (13); *G. t.* subsp. *virginiae*, the horsenettle nematode, does not reproduce well in *Nicotiana* species (15); and *G. t.* subsp. *solanacearum* attacks flue-cured tobacco cultivars (10). In a 1983 report (10), tobacco yield loss due to *G. t.* subsp. *solanacearum* reached 15%, valued at $700,000.

Due to specificity between host and pathogen within the genus *Globodera*, the distribution of species of cyst nematodes is similar to that of their hosts (20). Thus, the geographical distribution of the tobacco cyst nematode is restricted to areas where tobacco and other solanaceous hosts are grown or occur naturally. In North America, *G. t.* subsp. *tabacum* was detected in Connecticut and Massachusetts, whereas *G. t.* subsp. *virginiae* and *G. t.* subsp. *solanacearum* were found in Virginia and North Carolina (14,18). Until recently, *G. t.* subsp. *solanacearum* was found only in southern Virginia, where it is widespread. This nematode was found in North Carolina for the first time in 1991 in flue-cured tobacco fields in Warren County adjoining the Virginia border (14). Concern is increasing about the spread of this pathogen to tobacco fields in North Carolina.

Although some authors have observed that certain tobacco cultivars support little tobacco cyst nematode reproduction (8,9,11), the availability of commercial resistant cultivars is limited. Due to this limitation, as well as to the spread of *G. t.* subsp. *solanacearum* (10), additional sources of resistance to North Carolina populations of this nematode need to be identified and described. Field screening of putative *G. t.* subsp. *solanacearum*–resistant tobacco genotypes requires uniform environments, which favor nematode development and reproduction. The non-uniform spatial distribution characteristic of field populations of nematodes constitutes a further limitation to field evaluation of germ plasm. Although uniform field distribution can be obtained with artificial soil infestation, success requires large amounts of inoculum and high nematode survival rates. Furthermore, the occurrence of pests or other confounding factors is more likely in the field than in controlled environments.

A reliable method for greenhouse evaluation of tobacco genotypes for tobacco cyst nematode resistance would circumvent some of the problems encountered in field screening. The objectives of our study were to develop a reliable method for evaluating tobacco germ plasm for resistance to *G. t.* subsp. *solanacearum* in a greenhouse and to determine the reactions of selected tobacco genotypes to a North Carolina population of *G. t.* subsp. *solanacearum*, based on nematode reproduction and tobacco vegetative growth.

MATERIALS AND METHODS

Genotypes. Twenty-four tobacco genotypes were evaluated for their reactions to the tobacco cyst nematode, *G. t.* subsp. *solanacearum*, in a greenhouse. Several genotypes were selected because they were reported resistant to *G. t.* subsp. *solanacearum* in field trials (6). Breeding lines of flue-cured tobacco (*N. tabacum*) 9021-1, 9021-2, 9021-3, 9021-4, 9023-1, 9025-1, 9025-2, 9028-1, 9029-2, 9030-1, 9032-1, and 9032-2 were obtained from the USDA-ARS, Crops Research Laboratory, Oxford, North Carolina (6). The advanced flue-cured tobacco breeding lines Cyst 482 ([PD 4 × Speight G-28] × VA 182), Cyst 904 ([Coker 319 × PD 4] × VA 182), and Cyst 913 ([NC 82 × PD 4] × PD 4) were developed at the Southern Piedmont Agricultural Research and Extension Center, Virginia Polytechnic Institute and State University, Blackstone, Virginia. Cultivars PD 4, VA 81, Speight G-80, and NC 567 have been reported to support low tobacco cyst nematode reproduction levels (5,8,9). Burley 21, Kutsaga Mammoth 10, and Kutsaga 110 were included because

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they possess a single gene that confers resistance to *Pseudomonas syringae* pv. *tabaci,* which is reported to be linked to the gene(s) controlling resistance to the tobacco cyst nematode (19). The flue-cured tobacco cultivar Jaraiz 1 was also tested, and Speight G-28 was used as a susceptible check.

Experimental design. The experimental design was a split-plot with a total of eight replications. Genotypes were assigned to whole plots, and nematode treatments (inoculated and noninoculated) were assigned to subplots. The experiment was repeated three times, with starting dates of December 1992, March 1993, and June 1993. Mean greenhouse temperatures for the duration of each experiment were 28, 30, and 32°C, respectively.

Plant inoculation. Seedlings of each tobacco genotype were grown in trays containing an artificial medium, Metromix (Grace-Sierra Horticultural Products Company, Milpitas, CA), for 1 month in a growth chamber at 25°C (16-h photoperiod and 400 ± 50 $\mu E \cdot m^{-2} \cdot s^{-1}$ light intensity). Seedling trays were transferred to the greenhouse for 1 week, at which time individual tobacco seedlings were transplanted into 15-cm-diameter autoclaved clay pots containing 1,300 cm^3 of sterile 1:1 (vol:vol) mix of sand and sandy loam soil (3% silt, 94% sand, 3% clay; pH 7.0).

An isolate from a North Carolina population of *G. t.* subsp. *solanacearum* maintained on the tobacco cultivar NC 95 in a greenhouse was used as inoculum. All equipment used for cyst extraction was sterilized for 20 min with a 0.05% sodium hypochlorite solution. Cysts were removed from roots and soil with pressurized water and collected on a 250-µm-pore sieve. Cysts were gently crushed with a ground glass Tenbroeck (hand-held) homogenizer (Fisher Scientific, Pittsburgh, PA) to release the eggs, which were collected on a 25-µm-pore sieve and washed into a beaker (2). The egg suspension was diluted to 200 eggs per ml, and 50 ml of the egg suspension was poured into the soil of each infested pot. Pots assigned to receive nematodes were infested 1 week after transplanting with a total of 10,000 eggs of *G. t.* subsp. *tabacum.*

Harvest. All plants were harvested 2 months after soil infestation. Tobacco stalks and roots were weighed. Cysts were collected from roots and soil of plants from infested pots as described above and processed by centrifugal flotation in a 1.8 M sucrose solution (2) to reduce debris. Cysts were counted under a dissecting microscope and then crushed to release the eggs as described for inoculum preparation. The eggs were diluted and stained with acid fuchsin for counting (4).

Numbers of cysts and eggs per pot were used to assess the differences in nematode reproduction among genotypes. A reproduction factor (RF) was calculated for each pot by dividing the final number of eggs per pot by the initial number (10,000 eggs). To assess differences in vegetative growth among inoculated genotypes, two variables were created based on shoot and root weights: percent stalk weight [%SW = (SW of infected plant ÷ SW of uninfected plant) × 100] and percent root weight [%RW = (RW of infected plant ÷ RW of uninfected plant) × 100]. Nematode data were transformed to $\log_{10}(x + 1)$ values. All data were subjected to analysis of variance. Comparisons among genotypes were made with the Waller-Duncan *k*-ratio *t* test ($k = 100$).

RESULTS

Significant differences ($P \leq 0.05$) among experiments were observed for number of cysts per pot and RF, but not for number of eggs per pot (Table 1). Several tobacco genotypes differed ($P \leq 0.01$) in their ability to support *G. t.* subsp. *solanacearum* reproduction as measured by number of cysts and eggs per pot, and RF. Genotype × experiment (G × E) interactions were significant ($P \leq 0.05$) for number of cysts per pot and RF. The significant interactions resulted primarily from changes in the magnitude of these two variables for each of the three experiments. Although variances were heterogeneous for number of cysts per pot and reproduction factors due to strong environmental effects, genotype rankings were consistent among the experiments; therefore, data from the three experiments were pooled for all variables.

Burley 21 supported the lowest number of cysts per pot (Fig. 1A). The mean number of cysts for this genotype was 60, followed by VA 81 (66 cysts), NC 567 (80), Cyst 913 (80), Kutsaga Mammoth 10 (85), 9025-1 (89), PD 4 (98), Speight G-80 (99), and Kutsaga 110 (102). The host suitability among these genotypes did not differ ($P \leq 0.05$). The most susceptible genotypes based on number of cysts per pot were 9030-1 (1,782), 9021-3 (1,302), and 9029-2 (1,224). The last two genotypes were significantly different from 9030-1.

Based on the number of eggs per pot (Fig. 1B), the most resistant genotype was again Burley 21 (2,383 eggs), followed by Speight G-80 (2,884), NC 567 (3,268), Kutsaga Mammoth 10 (3,696), Cyst 913 (4,314), 9025-1 (4,886), PD 4 (5,757), Kutsaga 110 (6,135), and VA 81 (6,909). Differences in egg numbers among these genotypes were not significant. Breeding line 9021-3 had the highest number of eggs per pot (116,144), but this was not different ($P \leq 0.05$) from either 9029-2 (90,956) or 9030-1 (88,023).

Tobacco genotypes with a reproduction factor less than 1 (Fig. 1C), indicating resistance, included Burley 21 (0.2), Speight G-80 (0.3), NC 567 (0.3), Kutsaga Mammoth 10 (0.4), Cyst 913 (0.4), 9025-1 (0.5), PD 4 (0.6), Kutsaga 110 (0.6), and VA 81 (0.7). However, these were not statistically different from 9023-1 or 9025-2, which had reproduction factors of 3.0 and 2.8, respectively. Based on RF, the most susceptible genotypes were 9021-3 (11.6), 9029-2 (9.1), and 9030-1 (8.8).

Experiment and genotype effects were significant for stalk weight, root weight, %SW, and %RW (Table 2). Furthermore, highly significant genotype × experiment (G × E) and experiment × inoculation (E × I) interactions were observed for stalk weight and root weight. Inoculated plants were significantly different from noninoculated plants for stalk weight, but not for root weight. Large standard errors were associated with root weight measurements.

Significant differences in stalk weights ($P \leq 0.05$) were observed between inoculated and noninoculated plants for Burley 21, 9029-2, 9021-4, Cyst 904, 9032-2, 9028-1, Jaraiz 1, Speight G-28, 9021-2,

Table 1. Analysis of variance in numbers of cysts and eggs, and reproduction factor (RF = final number eggs ÷ initial number eggs) of tobacco genotypes inoculated with the tobacco cyst nematode, *Globodera tabacum solanacearum,* in three different greenhouse experiments. Data were transformed to $\log_{10}(x + 1)$ values

Variable	Sources of variation	df	MSE	*F*	$P > F$[a]
Cysts	Experiment (E)[b]	2	14.4	4.5	0.02*
	Block (E)	21	3.2		
	Genotype (G)	23	6.3	23.0	0.01**
	G × E	46	0.4	1.4	0.04*
	Error	443	0.3		
Eggs	Experiment (E)[b]	2	27.2	2. 9	0.07
	Block (E)	21	9.2		
	Genotype (G)	23	9.8	12.6	0.01**
	G × E	46	0.8	1.1	0.01**
	Error	441	0.8		
RF	Experiment (E)[b]	2	3.6	4.8	0.02*
	Block (E)	21	0.8		
	Genotype (G)	23	1.7	21.9	0.01**
	G × E	46	0.2	2.1	0.01**
	Error	441	0.1		

[a] *, ** = Significantly different at $P = 0.05$ and $P = 0.01$, respectively.
[b] Experiments were tested using Block (E) as the error term.

NC 567, 9030-1, 9032-1, 9025-2, Cyst 482, and VA 81. Differences in root growth between inoculated and noninoculated treatments were significant ($P \leq 0.05$) for the following genotypes: 9021-4, Kutsaga 110, Speight G-28, 9021-2, and Cyst 482 (data not shown).

When stalk weights of the inoculated plants were calculated as a percentage of noninoculated treatments for a given genotype, 9021-4 (70%), Speight G-28 (70%), and Jaraiz 1 (71%) showed the lowest %SW. These genotypes were significantly different ($P \leq 0.05$) from 9021-3 (111%), Kutsaga 110 (108%), and 9025-1 (106%), which had the highest %SW. Speight G-28 (75%) and Cyst 482 (79%) showed the lowest %RW. These genotypes were statistically different ($P \leq 0.05$) only from those genotypes that showed the highest %RW: Kutsaga 110 (133%), 9023-1 (122%), and 9025-1 (120%).

DISCUSSION

The assay method we used detected differences in reproductive ability of *G. t.* subsp. *solanacearum* among tobacco genotypes in a fairly consistent manner. Although experiments were significantly different, these differences were due to the absolute degree of reproduction among the genotypes and not in their relative ranking. These results indicate that measuring reproductive parameters in the greenhouse can be used reliably to identify resistant germ plasm. Due to the correlation observed between the number of cysts and the number of eggs, counting the number of cysts may suffice in preliminary screening studies. This research confirmed previous field reports in which NC 567 (9), Speight G-80 (8), VA 81, and PD 4 (5) were found to support little reproduction of Virginia populations of *G. t.* subsp. *solanacearum*. The average RF for these cultivars across experiments and in each of the three individual experiments was less than one, indicating that nematode reproduction was suppressed.

Burley 21, the first tobacco cultivar with resistance to wildfire caused by *Pseudomonas syringae* pv. *tabaci* (7), supported the least nematode reproduction based on number of eggs and cysts per pot. Two other wildfire-resistant cultivars, Kutsaga Mammoth 10, and the paternal doubled-haploid, Kutsaga 110, also supported low cyst and egg numbers. The gene responsible for wildfire resistance is reported to be linked to the gene or genes that confer *G. t.* subsp. *solanacearum* resistance in tobacco (19). Our results are consistent with that hypothesis.

Genotypes with similar pedigrees varied in their resistance to *G. t.* subsp. *solanacearum*. Cyst 913 is an advanced breeding line developed from a three-way cross that included PD 4 (C. A. Wilkinson, *personal communication*). Cyst 913 had a mean RF lower than that of PD 4; however, RF values for Cyst 913 and PD 4 were not significantly different. Conversely, other lines with PD 4 in their pedigrees, such as Cyst 904 and Cyst 482, were found to support high numbers of cysts and eggs.

Except for 9025-1, which had an RF less than 1.0, the breeding lines obtained from Crops Research Laboratory (9021-3, 9029-2, 9030-1, 9028-1, 9032-1, 9021-2, 9032-2, 9021-4, 9021-1, 9023-1, and 9025-2) had RF values indicating susceptibility. Contrary to our observations, Gwynn et al. (6) found 9025-1 to be as susceptible as Coker 319 based on an estimate of the percentage of roots containing cysts. Furthermore, they reported no significant differences among 9029-2 and 9030-1 and the resistant check (VA 81). In our study, 9025-1 was found to be more resistant than PD 4, whereas 9029-2 and 9030-1 were two of the three most susceptible genotypes, based on the number of eggs and cysts per pot. The discrepancies in results may have been due to the different methods used to rate resistance. Visual estimation of percentage of infected root is less reliable than counting the number of cysts present in each plant (11).

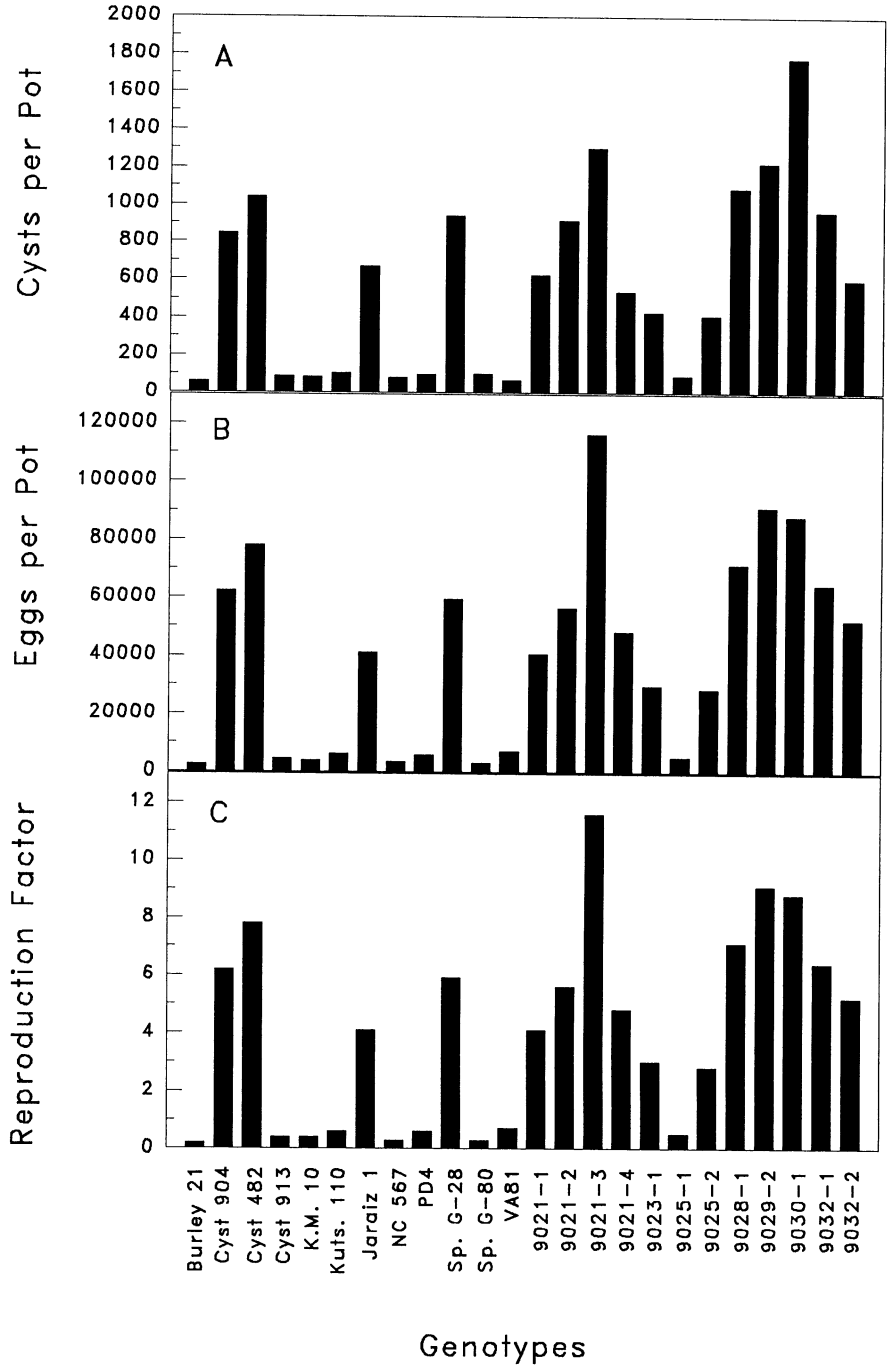

Fig. 1. Mean reaction of 24 tobacco genotypes 2 months after soil infestation (10,000 eggs per 1,300 cm^3 of soil) with *Globodera tabacum solanacearum*. **(A)** Number of cysts per pot. **(B)** Number of eggs per pot. **(C)** Reproduction factor (final number of eggs + initial number of eggs). Data represent means from three greenhouse experiments, each with eight replications.

Studies are necessary to determine the nature of inheritance of resistance in the genotypes included in our research. Cultivars PD 4 and VA 81 have not been genetically evaluated. LaMondia (11) indicated that resistance to *G. t.* subsp. *tabacum* in PD 4 and VA 81 was controlled by a single gene. Further evaluation of PD 4 and VA 81 is needed to confirm the mode of inheritance of resistance to *G. t.* subsp. *solanacearum* present in these cultivars.

Our attempt to detect resistance based on vegetative growth differences among genotypes was unsuccessful. Our failure to detect large growth differences among genotypes is most likely due to large standard errors for stalk and root weight measurements. Infection of some tobacco plants with tobacco mosaic virus (TMV) and with an unknown pathogenic agent (possibly *Erwinia* spp.) may have delayed their growth, thus contributing to the experimental error. Furthermore, the amount of time allowed for each experiment (60 days) may not have been enough to detect growth differences between susceptible and resistant genotypes in the greenhouse. We also observed differential loss of water among pots, which may have increased the variation within genotypes. Measurement of dry, rather than fresh, stalk and root weights may reduce the experimental error. Growth measurements proved too variable and thus may not be used as criteria for selecting resistant germ plasm in the greenhouse.

Our results indicate that resistance to *G. t.* subsp. *solanacearum* previously found in cultivars VA 81, PD 4, Speight G-80, and NC 567 is expressed when they are planted in soil infested with an isolate of this pathogen from North Carolina. Additional resistant cultivars, such as Burley 21, Kutsaga Mammoth 10, Kutsaga 110, and the breeding lines Cyst 913 and 9025-1, were identified. Field validation of these results may be necessary before any of these materials are used in a breeding program.

The Zimbabwean genotypes Kutsaga Mammoth 10 and Kutsaga 110 are not cultivated in United States; however, these cultivars support low *G. t.* subsp. *solanacearum* reproduction, and they are resistant to wildfire (C. A. Wilkinson, *personal communication*). The advanced breeding line Cyst 913 has not been released as a cultivar, but it performed well when tested in yield trials (C. A. Wilkinson, *personal communication*). The flue-cured tobacco cultivars VA 81, PD 4, NC 567, and Speight G-80 are commercially available to farmers, and they have been proved useful in an integrated pest management program (8,9) for controlling *G. t.* subsp. *solanacearum*.

Table 2. Analysis of variance for fresh stalk weight (SW), fresh root weight (RW), percent stalk weight (%SW), and percent root weight (%RW) of tobacco genotypes inoculated with the tobacco cyst nematode, *Globodera tabacum solanacearum*, in three different greenhouse experiments

Variable	Source of variation	df	MSE	*F*	$P > F^a$
SW	Experiment (E)	2	3,730,663.3	138.8	0.01**
	Error A[b]	21	26,880.3		
	Genotype (G)	23	8,122.2	6.1	0.01**
	G × E	46	3,627.8	2.7	0.01**
	Error B[b]	456	1,332.9		
	Inoculation (I)	1	168,257.2	131.2	0.01**
	Error C[b]	21	1,282.3		
	G × I	23	2,166.2	2.5	0.01**
	E × I	2	189,272.0	218.2	0.01**
	G × E × I	46	1,605.4	1.8	0.01**
	Error D[b]	457	867.5		
RW	Experiment (E)	2	292,792.2	87.6	0.01**
	Error A[b]	21	3,343.6		
	Genotype (G)	23	2,025.4	11.2	0.01**
	G × E	46	623.2	3.4	0.01**
	Error B[b]	456	181.2		
	Inoculation (I)	1	586 .2	2.9	0.10
	Error C[b]	21	202.2		
	G × I	23	361.1	2.2	0.01**
	E × I	2	10,752.3	65.3	0.01**
	G × E × I	46	170.1	1.0	0.42
	Error D[b]	453	164.6		
%SW[c]	Experiment (E)[d]	2	117,671.2	48.8	0.01**
	Block (E)	21	2,410.3		
	Genotype (G)	23	3,207.2	1.8	0.01*
	G × E	46	2,097.0	1.2	0.18
	Error	435	1,747.0		
%RW[c]	Experiment (E)[d]	2	104,182.1	41.6	0.01**
	Block (E)	21	2,501.5		
	Genotype (G)	23	4,805.5	1.9	0.01**
	G × E	46	2,576.0	1.0	0.45
	Error	431	2,532.6		

[a] *,** = Significantly different at $P = 0.05$ and $P = 0.01$, respectively.
[b] Error A = Block (E), Error B = G × Block (E), Error C = I × Block (E), Error D = G × I × Block (E).
[c] Weight (W) % = (Inoculated W + noninoculated W) × 100.
[d] Experiments were tested using Block (E) as the error term.

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