Greenhouse Testing of Transgenic Peanut for Resistance to *Sclerotinia minor*

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ABSTRACT

Fungal diseases of peanut, such as Sclerotinia blight caused by *Sclerotinia minor* Jagger, are responsible for increased production costs and yield losses of up to 50% for peanut producers in the Southwest, North Carolina, and Virginia. A few cultivars with moderate disease resistance, such as Southwest Runner, have been developed through traditional breeding practices. An urgent need exists for developing peanut cultivars that are resistant to the broad spectrum of fungal pathogens that pose a recurring threat to peanut health. Transgenic peanut plant lines containing anti-fungal genes have been produced from somatic embryos of the susceptible cultivar Okrun and tested under greenhouse conditions for resistance to *S. minor* by inoculation with a mycelial plug. Disease symptoms of lesion length and vascular collapse were recorded for 30 transgenic peanut lines, non-transgenic Okrun, and Southwest Runner. The reaction of the majority of transgenic peanut lines to *S. minor* infection was indistinguishable from that of the susceptible cultivar Okrun. However, three transgenic lines had a significant increase in resistance to *S. minor* as compared to Okrun, and one line demonstrated levels of resistance comparable to the moderately resistant cultivar Southwest Runner.

Key Words: Disease resistance, transformation.

Sclerotinia blight of peanut (*Arachis hypogaea* L.) continues to persist as a major problem limiting peanut production in Virginia (22), North Carolina (21), Oklahoma (25), and Texas (27). The disease, caused by the soilborne fungal pathogen *Sclerotinia minor* Jagger, was first found in Virginia in 1971 (20). Symptoms of the disease include flagging, wilting, and necrosis of one or more stems (18). Sclerotia of *S. minor* can persist in the field for 4 to 5 yr in the absence of peanut. Sclerotinia blight can cause yield losses of 10 to 50% depending upon severity of infection (18). Such losses have resulted in efforts to develop efficient and effective methods for disease management. Repeated application of fungicides during the growing season is most often used as a means of disease control. In addition to environmental risk considerations, this method of disease management is costly to the producer and may eventually result in the selection of fungicide-resistant strains of *S. minor*.

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Host plant resistance offers an economically efficient and environmentally safe solution for control of Sclerotinia blight. Efforts have been made to effectively screen peanut germplasm for resistance to *S. minor* (4, 7, 17) under field and greenhouse conditions. Melouk *et al.* (17) developed a detached shoot technique to evaluate the reaction of peanut germplasm to *S. minor* under greenhouse conditions. Goldman *et al.* (7) modified this technique for use with whole plants and reported a high correlation of greenhouse and field testing results for *S. minor* resistance using this technique. Thus, greenhouse screening for *S. minor* resistance has been shown to be effective in eliminating highly susceptible peanut germplasm before field-testing. Moderate resistance to *S. minor* infection has been reported for the peanut cultivars Tamspan 90 (23) and Southwest Runner (11). However, few cultivars with suitable levels of *S. minor* resistance have been developed and released for commercial use through traditional breeding practices (11, 23, 24), possibly due to the complex nature of the endogenous resistance trait.

Genetic engineering of peanut for fungal resistance offers another possible solution for the control of *S. minor*. Recently, the discovery of anti-fungal genes has facilitated the development of transgenic plants with increased resistance to a broad range of fungal pathogens (2, 5, 12-14). These genes often encode host defense response proteins such as hydrolases that degrade chitins and glucans, which are major components of many fungal cell walls (26). Previously, we reported the production and initial evaluation of transgenic peanut lines containing such hydrolase genes (6). Levels of hydrolase activity observed in some transgenic peanut lines were comparable to that reported for plants with elevated fungal resistance (6, 14). In this report, transgenic peanut lines containing hydrolase gene(s) were evaluated under greenhouse conditions to estimate levels of resistance to *S. minor* infection.

Materials and Methods

Thirty transgenic peanut lines containing a chitinase from rice (*Oryza sativa* L.) and/or a β-1,3 glucanase from alfalfa (*Medicago sativa* L.) were evaluated for resistance to *S. minor* infection. All transgenic plant lines were generated from the cultivar Okrun and tested for the presence of the transgene(s) as previously reported (6). Individual plants confirmed positive for the transgene(s) via PCR (6) prior to testing for *S. minor* resistance. Susceptible (Okrun) and moderately resistant (Southwest Runner) control plants were included in each experiment. Greenhouse evaluations for *S. minor* resistance were performed as reported by Goldman *et al.* (7). Ten random plants from each control and transgenic plant line were grown in individual pots (10-cm diam.) in a mixture of sand, soil, and shredded peat. Side branches were

trimmed from the main stem of 6 wk old plants 2 d prior to inoculation with a 4-mm diam. mycelial plug from a 2-d-old culture of *S. minor* (isolate Mel #3) grown on potato dextrose agar. Mycelial plugs were placed at the axil between the main stem and leaf petiole at the first node. Inoculated plants were incubated in 100% relative humidity chambers and maintained at 23 ± 5 C. Disease reaction was monitored by measuring lesion length in cm at 2, 3, and 4 d post-inoculation (dpi). Lesion expansion data among plant lines were analyzed using PROC MIXED in PC SAS Version 8.2 (2001, SAS Institute, Cary, NC). A repeated measures analysis was conducted to assess the response of plant line differences over time. Differences in lesion growth in the plant lines as compared to Okrun were evaluated with an LSMEANS statement with DIFF and SLICE options. Negative differences indicate lines with lesions smaller than Okrun, whereas positive differences indicate larger lesions. Differences were considered significant when $P < 0.05$. Phenotype of lesions was recorded as previously described (17). Lesions were also classified into three categories according to severity: type 1 = lesion at inoculation point only; type 2 = unilateral stem lesion resulting in wilting; type 3 = girdled stem lesion resulting in vascular collapse.

Results

Average lesion lengths recorded at 2, 3, and 4 dpi for all 30 transgenic plant lines tested for resistance to *S. minor* as well as for the susceptible (Okrun) and resistant (Southwest Runner) controls are shown in Table 1. Differences among lesion lengths were not statistically significant ($P \leq 0.05$) until 4 dpi. Fifteen of the 30 transgenic lines tested averaged smaller lesions than Okrun. Among these 15 lines, 11 contain a rice chitinase transgene, two contain an alfalfa glucanase transgene, and two contain both transgenes. Reduction in lesion size for transgenic lines compared to Okrun ranged from 1 to 22%. None of the transgenic lines had an average lesion size smaller than Southwest Runner.

The statistical analysis for all peanut plant lines tested in comparison to Okrun at 4 dpi. is shown in Table 2. Differences in lesion lengths are indicative of lesion size. Of the 30 transgenic lines tested, only nine lines were statistically different from Okrun at $P \leq 0.05$. Only transgenic line 133 consistently averaged smaller lesions than Okrun. Eight lines (nos. 23, 74, 87, 90, 135, 146, 412, and 561) averaged larger lesions. Transgenic line 133 (difference = -15.78) showed the highest level of resistance to *S. minor* infection, which was similar to the moderately resistant control Southwest Runner (difference = -15.10). Figure 1 illustrates lesions typical of *S. minor* infection of peanut stems at 4 dpi. Figure lA shows resistant and susceptible controls along with two representative transgenic lines (nos. 487 and 133). In general, lesion type was consistent with lesion difference analysis. Plant line 133 was significantly ($P \leq 0.05$) more resistant to infection than Okrun for lesion differences and exhibited type 1 lesions typical of the resistant cultivar Southwest Runner. Moreover, all but three of the plant lines significantly more susceptible than Okrun exhibited type 3 lesions (Table 1). Type 1 *S. minor* lesions can be seen in Figure 1B and 1D (Southwest Runner and line 133, respectively) and a type 3 lesion is shown in Figure lC (Okrun).

Table 1. Average lesion length[a] and type on stems of peanut lines following inoculation with *S. minor*.

Plant line	Days post inoculation 2	3	4	Lesion type[d]
	cm	cm	cm	
Okrun	.17	.40	.78	3
SW Runner	.13	.32	.60	1
23	.26	.44	.80	3
24	.12	.35	.84	2
33	.18	.36	.80	2
35	.21	.42	.79	2
51	.16	.48	.76	2
74	.25	.44	.74	3
81	.17	.43	.69	2
87	.22	.42	.79	3
90	.22	.42	.80	2
133	.19	.42	.61	1
135	.26	.46	.90	3
139	.18	.42	.77	3
145	.13	.40	.73	2
146	.26	.50	.87	2
157	.19	.46	.79	3
412	.30	.48	.80	3
421	.12	.41	.69	3
461	.17	.43	.78	2
487[c]	.12	.39	.69	1
503[b]	.18	.48	.83	2
505[b]	.13	.39	.72	3
511	.18	.37	.79	3
514	.16	.37	.62	1
517	.13	.31	.68	3
531	.17	.39	.77	3
535	.18	.36	.71	3
540[c]	.16	.43	.80	2
542[b]	.22	.36	.62	1
561	.20	.36	.85	2
654[c]	.17	.37	.61	3

[a]Calculated from three separate tests, each using 10 plants per line. Numbered plants contain a chitinase transgene unless otherwise designated.

[b]Plant lines contain glucanase transgene.

[c]Plant lines contain glucanase and chitinase transgene.

[d]Typical lesion for more than 50% of inoculated plants per plant line where 1 = lesion at inoculation point only, 2 = unilateral stem lesion resulting in wilting, and 3 = girdled stem lesion resulting in vascular collapse.

Discussion

Peanuts are susceptible to many types of pathogens, with the most widespread damage in most peanut production regions being caused by fungi (18). Soilborne fungi cause diseases that adversely affect peanut health and productivity throughout most production areas of the U.S. Fungal diseases such as pod rot (*Rhizoctonia solani* Kühn, *Pythium myriotylum* Drechs), crown rot (*Aspergillus niger* Tiegh), southern stem rot (*Sclerotiorum rolfsii* Sacc) occur in all U.S. peanut-producing areas, while others such as Sclerotinia blight are more limited in distribution.

Most fungi contain chitin, a homopolymer of β-1,4-linked N-acetyl-glucosamine, as a major component of

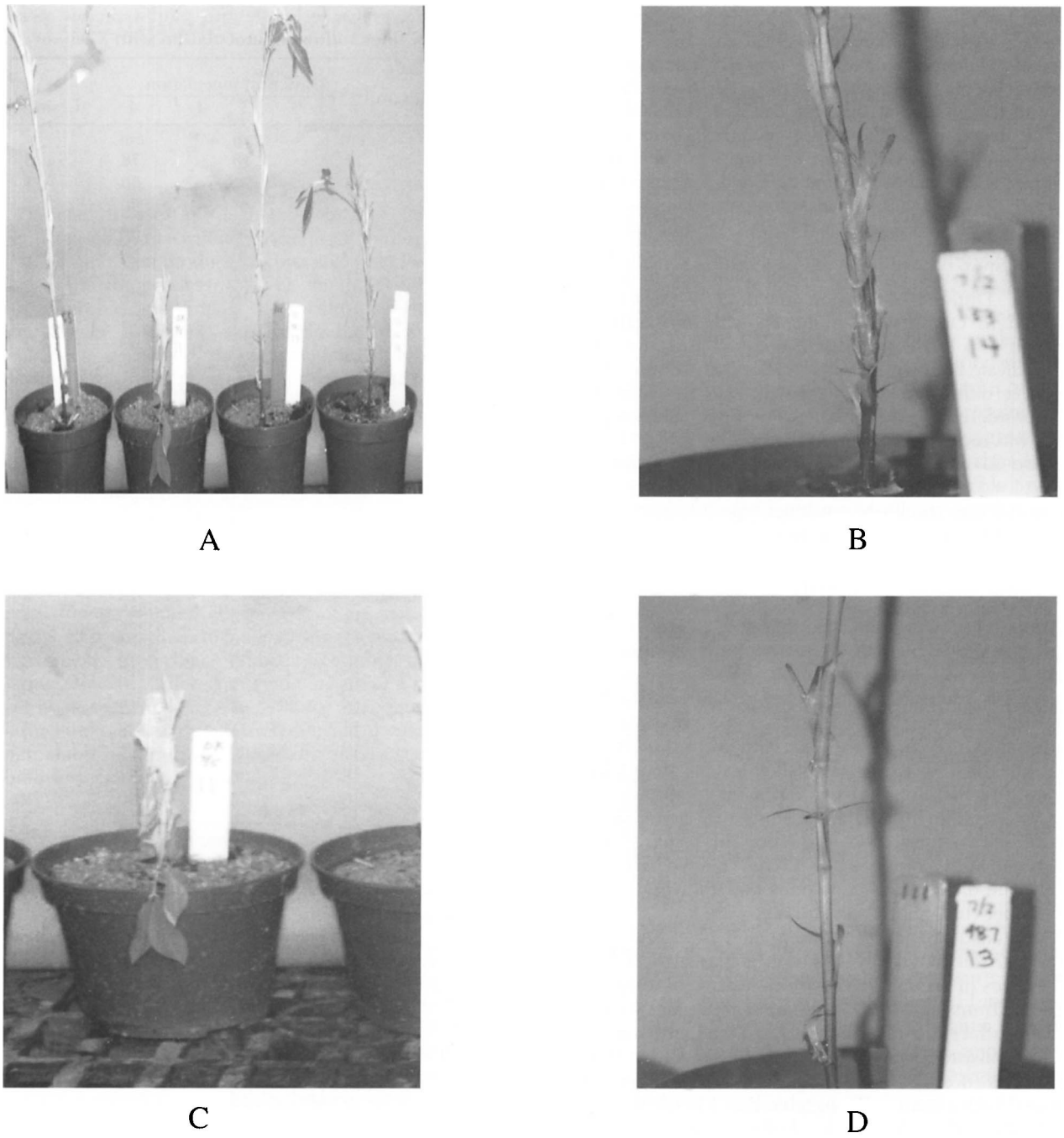

Fig. 1. (A) *Sclerotinia minor* lesions at 4 dpi. on (left to right) Southwest Runner, Okrun, line 487 and line 133. (B) Type 1 lesion on resistant cultivar Southwest Runner (C) Type 3 lesion on susceptible cultivar Okrun (D) Type 1 lesion on plant line 133.

their cell walls (26). All organisms that contain chitin also produce chitinases which are hydrolases that degrade the polymer by breaking its β-1,4 linkages, presumably for morphogenesis of cell walls and exoskeletons (8). Although plants do not produce chitins, many plants have been shown to produce chitinases as a defense response to chitin-containing pathogens (1, 3, 9, 10). Another hydrolase, β-l,3-glucanase, has also been suggested as a certain plant defense system against fungal infection (14-16, 19). The activities of hydrolases such as chitinases and glucanases make them attractive candidates for over-expression in transgenic plants to increase fungal resistance. This study evaluated 30 transgenic lines containing chitinase and/or glucanase transgenes for response to *S. minor* infection under greenhouse conditions, which have proven useful in evaluating peanut germplasm for this pathogen (4, 7, 17).

Although the majority of transgenic lines evaluated in this study were not significantly different from Okrun in their reaction to *S. minor* infection, nine of the plant lines had a significant deviation from the susceptible control. In particular, transgenic plant line 133 consistently had smaller lesions than Okrun, and was statistically indistinguishable from the moderately resistant control

Table 2. Differences in lesion lengths on stems of transgenic peanut lines from those of non-transformed krun control at 4 dpi. P-values are for tests of differences equal 0.

Plant line[a]	Difference	P value
SW Runner	-15.10	.0020
23	15.77	.0271
24	4.62	.1793
33	13.10	.0656
35	9.67	.0694
51	-2.40	.6878
74	11.19	.0182
81	-9.00	.0897
87	14.89	.0182
90	16.60	.0007
133	-15.78	.0034
135	24.10	.0001
139	-0.77	.8826
145	-4.75	.4538
146	22.10	.0006
157	1.67	.8090
412	17.10	.0041
421	8.77	.2166
461	1.50	.6451
487[c]	-8.40	.1615
503[b]	5.00	.4688
505[b]	-5.05	.1323
511	1.00	.8847
514	-0.10	.9834
517	1.45	.6545
531	-0.50	.9495
535	10.10	.1549
540[c]	2.00	.7717
542[b]	3.10	.6615
561	13.10	.0273
654[c]	-0.65	.8410

[a]Numbered plants contain a chitinase transgene unless otherwise designated.

[b]Plant lines contain glucanase transgene.

[c]Plant lines contain glucanase and chitinase transgene.

Southwest Runner. No direct correlation between transgene expression levels (6) and resistance to *S. minor* infection were made, but the current study indicates that inclusion of the gene does not guarantee effective expression to the target pathogen. Plant line 133 demonstrated statistically significant levels of resistance to *S. minor* infection and contains the rice chitinase transgene, possibly suggesting its utility for increasing fungal resistance (at least for *S. minor*) over the alfalfa glucanase transgene. Future experiments will ascertain if a correlation exists between transgene expression levels (6) and resistance to *S. minor* infection.

The results from this study are encouraging regarding the levels of resistance to *S. minor* infection expected when these transgenic plant lines are tested under field conditions. Goldman *et al.* (7) also reported a high correlation between greenhouse and field testing results with the methodology explained in the currrent study. Further, they indicated that Southwest Runner demonstrated levels of resistance to *S. minor* up to three times higher in the field than levels seen in greenhouse testing (7). Although Southwest Runner is moderately resistant to *S. minor* infection in the field and greenhouse, it unfortunately produces relatively small seeds which are undesirable by the peanut industry. Thus, Southwest Runner has limited production in the U.S. (11, pers. commun., Oklahoma State Univ. Coop. Ext. Ser.).

The transgenic peanut lines evaluated in this study were generated from Okrun, a popular peanut cultivar that produces desirable seed but is highly susceptible to Sclerotinia blight. Transgenic line 133 produces seed which shells and grades similar to that of the parent genotype Okrun (data not shown), while demonstrating moderate resistance levels to *S. minor*. The production and commercial release of such transgenic lines with high quality seed, high yield, and increased fungal resistance would be extremely beneficial to the peanut industry.

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