Evaluation of Detached Shoot and Leaflet Inoculation Techniques to Screen Peanut Genotypes for Resistance to Rhizoctonia Limb Rot

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ABSTRACT

Identifying peanut genotypes with resistance to Rhizoctonia limb rot in the field is slow and costly. The objectives of this study were to compare detached leaflet and shoot inoculations as methods of screening for resistance to Rhizoctonia limb rot and to assess the relationship between genotype reactions *in vitro* and in the field. Eleven peanut germplasm selections and four cultivars, which exhibited a range of susceptibility to limb rot in previous field experiments in 1996 and 1997, were selected for evaluation. Detached lower reproductive limbs were suspended in Hoagland's solution and inoculated using a mixture of soil and oat seeds infested with *Rhizoctonia solani*. Detached leaflets on moistened filter paper in petri dishes were inoculated with hyphal plugs. Georgia Green and selections 512 and 283 were the most susceptible of all the genotypes to leaflet infections although Georgia Green is partially resistant to limb rot in the field. Disease levels for all genotypes were higher in the detached shoot inoculation study than in previous field experiments and symptoms observed were typical of those seen in the field. However, there was no correlation in relative susceptibility of the genotypes between the detached leaflet, shoot, or previous field experiments. Susceptibility of genotypes in the field may be determined by the combined resistance of the different plant parts or by factors not evaluated in this study. The detached shoot inoculation technique did not reproduce the same range of disease reactions on diverse peanut genotypes that were observed in the field experiments, but it did prove to be a good method of generating high levels of disease *in vitro*. The technique could probably be adapted to evaluate other factors such as relative fungicide performance or effects of environment on disease.

Key Words: *Arachis hypogaea*, foliar blight, groundnut.

Rhizoctonia solani Kühn AG-4 is one of the most common soil-borne pathogens infecting peanut in the U.S. (Melouk and Shokes, 1995). *Rhizoctonia solani* causes seed decay, pre-emergence and postemergence damping-off of seedlings, hypocotyl and root necrosis, peg rot, pod rot, limb rot, and foliar blight of mature plants (Bell and Sumner, 1984). Rhizoctonia limb rot first became a significant problem in peanut in Georgia in the mid-1980s (Thompson, 1982), particularly in irrigated peanuts. Heavily watered fields with excessive vegetative growth are most susceptible, and stems and leaves of lower branches are often extensively decayed by the pathogen. Since the disease attacks the plant at or near the soil surface, it is difficult to quantify damage until plants are inverted. However, one indicator of severe disease is the extensive necrosis of foliage in the lower plant canopy. The role of leaf infections in the epidemiology of the disease is not known. Pods are frequently decayed by the fungus and typically the youngest pods formed at the distal end of the limb terminals suffer the heaviest damage under heavy disease pressure, but older pods near the taproot may be affected also (Brenneman, 1997). The growers in Georgia have lost an average of $12 million annually from limb rot infections over the past 10 yr based on the Univ. of Georgia Coop. Ext. Serv. estimates between 1990 and 1999.

Management of limb rot is best achieved by using an integrated approach that includes crop rotations, proper fertilization, irrigation management, fungicide sprays,

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and cultivar resistance. Only two cultivars grown in the Southeastern U.S., Georgia Browne and Georgia Green, are reported to have partial resistance to limb rot (Branch and Brenneman, 1993; Franke *et al.*, 1999). Unfortunately, Georgia Browne is not currently grown due to its small seed size. Additional cultivars with limb rot resistance are needed.

Resistance equal to that found in Georgia Browne has been identified in several genotypes from the U.S. peanut germplasm collection (Franke *et al.*, 1999). However, only a small portion of the germplasm collection has been evaluated because of the exorbitant amount of time and resources needed to screen all 7432 accessions. It is more difficult to screen cultivars for resistance to limb rot than for resistance to hypocotyl rot because severity of limb rot is assessed at harvest, compared to early season assessments for hypocotyl rot. Several greenhouse techniques involving inoculation of attached limbs with various sources of inoculum have been used to screen genotypes for limb rot resistance with varying degress of success (Barnes *et al.*, 1990; Franke and Brenneman, unpub. data). These techniques were not very efficient in generating high levels of disease and they require large amounts of space and time, allowing only a small number of genotypes to be evaluated.

A more efficient method of screening genotypes for resistance to limb rot is needed that would not require large amounts of space and could be done relatively quickly. Several studies have demonstrated the effectiveness of detached shoot or leaf methods for identifying potential sources of disease resistance in peanut to foliar and soil-borne diseases (Melouk and Banks, 1978; Brenneman *et al.*, 1988; Melouk *et al.*, 1992). These techniques were successful in identifying sources of resistance to Sclerotinia blight (*Sclerotinia minor* Jagger) and early leaf spot (*Cercospora arachidicola* Hori) in small, controlled-environment studies using minimal amounts of inoculum and plants. The relative susceptibility of peanut leaves from different cultivars to infections by *R. solani* is not known, nor is it known whether there is any correlation between susceptibility of leaves and limbs to infections by *R. solani*.

The objectives of this study were to (a) evaluate selected accessions and cultivars previously screened in field experiments for resistance to limb rot using detached shoots, (b) evaluate the same genotypes for resistance to foliar blight using detached leaflets, and (c) to assess the relationship between genotype reactions *in vitro* and in previous field experiments.

Materials and Methods

Plant Material. Eleven peanut accessions and the cultivars Florunner, Southern Runner, Georgia Green, and Georgia Browne were chosen to conduct the research based on their wide range of susceptibility to limb rot based on field experiments in 1996 and 1997 (Franke *et al.*, 1999). Florunner and Southern Runner are both susceptible to limb rot whereas Georgia Browne and Georgia Green have partial resistance, and Georgia Green is currently the most widely grown cultivar in the Southeastern United States. Seeds treated with carboxin, pentachloronitrobenzene, and captan (Vitavax PC, 113.6 g/45.4 kg of seed) (Gustafson, McKinney, TX) and Hi Stick *Bradyrhizobium* peanut inoculant (198.8 g/90.8 kg of seed) (Microbio, Hemel, Hempstead, U.K.) were incubated in moist paper towels at 28 C for 36 to 48 hr. Three germinated seeds per genotype were planted in each of 10 7.6-L plastic pots filled with a mixture (1:2 v/v) of pasteurized soil (fine-loamy, siliceous, thermic Plinthic Kandiudults) and a commercial growing medium (Promix®). After emergence, plants were thinned to two per pot. Fourteen days after planting, limeplaster ($CaCO_3$ $MgCO_3$) was applied (500 mg/kg of soil), and 60 d after planting ammonium nitrate (NH_4NO_3) (20 mg/kg of soil) was applied to maintain vegetative growth and relieve mild nitrogen deficiency symptoms. Acephate (Orthene 75S, 0.84kg/ha) and imidacloprid (Merit 75WSP, 0.3g/L) were applied as needed to control thrips and whiteflies, respectively.

Inoculum Production. Oat seeds were soaked in water for 24 hr and then autoclaved in plastic Nalgene containers at 121 C for 90 min on 2 consecutive d. Autoclaved oat seeds were inoculated with five mycelial plugs per container of R. *solani* AG-4 (JY-1) recovered from an infected peanut seedling in 1997. Inoculum was incubated at 27 C and containers were shaken periodically to insure uniform growth of the pathogen. After 14 d, the inoculum was placed in shallow plastic pans and dried in an oven at 40 C for 48 hr. Inoculum was stored at room temperature in polyethylene bags until use.

Shoot Inoculations. Lateral reproductive branches (15 to 30 cm long) were excised from 15-wk-old plants, and leaves from the basal 9.0 cm were removed. The cut end of shoots were immersed immediately in Hoagland's solution (Hoagland and Arnon, 1950) in 1.5 × 9.0 cm tubes. Tubes were plugged with cotton to support shoots, leaving about 10 to 20 cm of the stem with leaves attached extending above the cotton plugs. Fifteen tubes, one per genotype, were placed in 38.1-cm-long wooden racks for each replicate. The racks were placed in shallow plastic pans (28 cm × 42 cm) and a layer (approx. 7 cm deep) of mixed pasteurized field soil and growing medium as previously described was placed in the bottom of the plastic pan. The racks were tilted at approximately a 40° angle so that each detached shoot rested on the pasteurized soil mixture. Infested whole oat seed was mixed with the growing medium to attain approximately 140 viable propagules per 100 g medium. One hundred thirteen grams of infested medium was placed on top of the detached shoots. The medium was moistened with water from a spray bottle and trays were placed in growth chambers maintained at 23 C and a 12-hr photoperiod (149μE/m²/s). Near 100% relative humidity was maintained using humidifiers. The experiment was a randomized complete block design with six replications and the test was repeated once. The lesion lengths (mm), numbers of lesions, girdling lesions (lesions that completely girdled the limb), and lesions longer than 2.54 cm were determined for each limb 14 d after inoculation. Data were analyzed using analysis of variance. Means were separated using Fisher's Protected Least Significant Difference (LSD) (P = 0.05). Correlation coefficients were calculated to evaluate the relationship between the results from the field screening project completed in 1997 (Franke *et al.*, 1999) and the detached shoot technique to assess the similarity of the two screening methods. Symptomatic shoots were placed on potato dextrose agar (PDA) containing chloramphenicol and chlortetracycline (100 μg/L of each) to verify that R. *solani* was the causal organism.

Leaflet Inoculations. For each genotype, 10 leaflets from 15-wk-old plants were removed from the last fully expanded leaf at the apex of each mainstem. Leaflets were washed with deionized water prior to inoculation. Whatman #2 filter paper (9-cm-diam.) was placed in petri plates (10-cm-diam.) and moistened with sterile deionized water. Leaflets (one per plate) were placed on the filter paper and a 4-mm-diam. mycelial plug from the edge of 2-d-old cultures on PDA was placed on adaxial surface of each leaflet adjacent to the petiole. The plates were covered, placed in a plastic container, and incubated on a lab bench with an average temperature of 24 C and 95% relative humidity. The experiment was a randomized complete block design. There was one inoculated leaflet per genotype for each replication and there were 10 replications of each genotype in the experiment, which was repeated once.

Disease severity, the percentage of leaflet area with disease symptoms, was visually estimated 8 d after inoculation. The mean disease severity for each genotype was calculated. Disease incidence, the percentage of symptomatic leaflets per genotype, was determined in each experiment. Data were analyzed using analysis of variance and means for each genotype were separated using Fisher's Protected LSD (P = 0.05). Correlation coefficients were calculated among disease responses for the detached leaflet and shoot assays, and data from the field experiment that was completed in 1997 (Franke *et al.*, 1999) to determine if there was a correlation between *in vitro* and field experiments. Symptomatic leaflets were cultured on PDA containing 100 µg/L of each chloramphenicol and chlortetracycline to verify that *R. solani* was the causal organism.

Results

The sunken, eliptical lesions with concentric rings that formed on the detached shoots were identical to those observed in the field. *Rhizoctonia solani* was reisolated from arbitrarily cultured infected shoot tissue. In the detached limb studies, data for all variables from the two experiments were combined due to a lack of significant differences between experiments (P > 0.05) (Table 1). There was a fourfold difference in the number of lesions per stem between selection 458, the most susceptible, and Georgia Browne, the most resistant entry in the study. Except for Southern Runner, the cultivars were equally susceptible to limb infections, but significantly (P < 0.05) less susceptible than selections 513, 512, 458, 302, and 234. The numbers of girdling lesions and lesions longer than 2.54 cm per stem were not significantly different among genotypes. Lesion lengths ranged from 17 mm for core selection 197 to 7 mm for core selection 287 (Table 1).

Lesions with concentric rings resulting in a target-like appearance developed on the inoculated leaflets and are typical symptoms of foliar blight. *Rhizoctonia solani* was reisolated from all the infected leaflet tissue that was cultured. No significant difference was observed between experiments (P > 0.05); therefore, data were combined. Severity of foliar blight over the two experiments ranged from 2 to 73% , with selection 287 having the lowest disease severity and Georgia Green the highest (Table 1). Of the selections evaluated, entries 283 and 512 had the highest level of disease severity with 52 and 61% of leaflet area exhibiting symptoms, respectively. Among the cultivars, Georgia Browne, Florunner, and Southern Runner had significantly (P < 0.05) lower disease severity ratings than Georgia Green. Florunner and Georgia Browne were equally susceptible to leaflet infection, but only Florunner had significantly smaller leaf lesions than Southern Runner.

Table 1. Reaction of peanut lines to inoculation with *Rhizoctonia solani* under growth chamber conditions.[a]

Peanut genotypes	PI	Detached shoot: Lesions/ stem	Detached shoot: Lesion length	Detached leaflet[b]: Severity[c]	Detached leaflet[b]: Incidence
	no.	no.	mm	%	%
458	268996	2.6	12.5	23.0	65
512	240567	2.0	14.2	61.3	85
234	159664	1.8	9.7	24.7	60
513	288126	1.7	16.5	35.2	75
302	372336	1.6	15.2	21.3	50
335	153323	1.5	10.3	35.9	70
366	268968	1.5	10.8	12.8	60
287	355271	1.4	7.4	1.6	35
208	274193	1.3	13.2	31.2	75
South. Runner	--	1.3	8.4	32.9	90
283	196712	1.2	11.8	51.5	75
197	331326	0.9	16.6	44.8	100
Florunner	--	0.8	9.2	5.5	30
Georgia Green	--	0.7	11.3	73.1	100
Georgia Browne	--	0.6	9.3	14.5	60
LSD (P < 0.05)	--	0.9	NS	21.8	38

[a]Values are means of 12 limbs and 20 leaflets per genotype.

[a]Percent of necrotic area on leaflet.

[b]Percentages were calculated from two separate tests, each using 10 leaflets (replications) per genotype.

The selections and cultivars also differed in the percentage of leaflets that were infected, which ranged from 100% for selection 197 and Georgia Green to 30% for Florunner (Table 1). Four selections, Georgia Browne, and Florunner had lower percentages of infected leaflets than Georgia Green or selection 197.

The mean numbers of lesions, girdling lesions, and lesions longer than 2.54 cm per stem were calculated for each genotype. Correlation analysis of these data with the field results and leaflet infections demonstrated no significant relationships among these variables (Table 2). The only significant (P < 0.05) correlations were between lesion length and girdling lesions per stem in the field and lesions longer than 2.54 cm from the growth chamber study.

Discussion

The detached shoot technique produced a large number of lesions that were identical to those observed in the field. There were higher levels of disease in the growth chamber experiment compared to the field experiment. Inoculum density can influence disease severity but, based on our calculations, the inoculum density in the growth chamber experiment was slightly lower than was used in the field experiment. Studies of seedling disease

Table 2. Correlations between components of resistance to Rhizoctonia limb rot and foliar blight in the field and growth chambers (GC).[a]

Variables	Correlation coefficient	P > r
Field lesions/stem vs. GC lesions/stem	-0.093	0.7415
Field gird. lesions/stem vs. GC gird. lesions/stem	0.075	0.7907
Field lesions > 2.54 cm vs. GC lesions > 2.54 cm	-0.156	0.5800
Percentage leaflet infection vs. field lesions/stem	-0.378	0.1646
Percentage leaflet infection vs. field gird. lesions/stem	-0.299	0.2788
Percentage leaflet infection vs. field lesions > 2.54 cm	-0.141	0.6162
Percentage leaflet infection vs. GC lesions/stem	-0.059	0.8334
Percentage leaflet infection vs. GC gird. lesions/stem	0.240	0.3885
Percentage leaflet infection vs. GC lesions > 2.54 cm	0.061	0.8300
GC lesion length vs. field lesions/stem	-0.216	0.4394
GC lesion length vs. field gird. lesions/stem	-0.022	0.9380
GC lesion length vs. field lesions > 2.54 cm	-0.223	0.4242
GC lesion length vs. GC lesions/stem	0.216	0.4383
GC lesion length vs. GC gird. lesions/stem	0.741	0.0016
GC lesion length vs. GC lesions > 2.54 cm	0.780	0.0006

[a]Fifteen observations were made for each variable.

and pod rot caused by *R. solani* have reported inoculum densities ranging from 22 to 256 CFU per 100 g of soil (Woodard and Jones, 1982; Filonow *et al.*, 1988). The amount of inoculum used in both the detached shoot and field experiments was within the range used for the other experiments. Therefore, the increase in the number of lesions per stem was probably a result of more favorable environmental conditions in the growth chambers compared to those in the field. In fact, preliminary experiments with several different inoculation procedures indicated that sustained periods of very high humidity are required to achieve consistent disease levels.

Observations during the growing season have shown that fields with a high level of Rhizoctonia limb rot also have high levels of foliar blight caused by *R. solani*. Although necrotic leaves are often found on stems with no other apparent symptoms, these high levels of foliar blight have been suggested to cause increases in limb rot incidence. Detached leaflet inoculations can provide additional information regarding the contribution of foliar blight to increased limb rot.

Other studies have determined the correlation of results from detached leaf inoculations with disease in the field (Melouk and Banks, 1978; Mehan *et al.*, 1994); however, in the present study no correlation was found between susceptibility of peanut leaflet tissue and peanut limbs to *R. solani* infection. Results did indicate that there are major genotypic differences in the susceptibility of peanut leaflets to foliar blight *in vitro*. Some entries had very low disease severity ratings whereas others had as much as 73% of the leaflet infected (Table 1.) Results from previous field experiments conducted in 1996 and 1997 showed that Florunner was more susceptible to limb infections than Southern Runner, and that Georgia Green had a level of susceptibility comparable to Georgia Browne (Franke *et al.*, 1999). In the leaflet study, Southern Runner and Georgia Browne were equally susceptible to leaflet infections, but Georgia Green was the most susceptible of the commercial cultivars. These results describe real genotypic differences; however, they suggest that inoculation of peanut leaflets would not be a reliable method of identifying sources of resistance to limb infections. Similar work by Amand and Wehner (1995) demonstrated that a detached leaf inoculation technique was not a good method of identifying sources of resistance in cucumber (*Cucumis sativus* L.) to gummy stem blight caused by *Didymella bryoniae*.

Both Georgia Browne and Georgia Green have smaller plant canopies that create less favorable environmental conditions for disease development, and semi-erect bunch growth habits that reduce limb contact with the soil and inoculum. It has been speculated that, because of these characteristics, the resistance observed in the field is a result of disease escape due to plant architecture. Overall, there was no correlation in the range of susceptibility of tissue between the field data and the detached shoot data from the growth chamber. However, the data from the detached shoot technique demonstrated that the level of resistance seen in Georgia Browne and Georgia Green was similar to that observed in previous field experiments and could be the result of a true resistance mechanism, rather than an example of disease escape.

Rhizoctonia limb rot has been difficult to reproduce *in vitro*. In an earlier study, Barnes *et al.* (1990) evaluated 18 peanut genotypes in the greenhouse for resistance to limb rot infections and showed that wounding was necessary for large lesions to develop. Based on results from preliminary work conducted in our lab, wounding also appeared to be critical for the infection to become established. Interestingly, the detached shoot inoculation technique developed here reliably produced lesions that were very similar to those seen in the field without wounding. Apparently, wounding is not necessary for infections when environmental and nutritional conditions are optimal, and it is preferable to use nonwounded tissue for germplasm evaluation.

Disease development in the field is a complex relationship involving specific environmental conditions and relative susceptibility of host tissue that is often difficult to create when conducting field experiments. Thus, the primary reason for evaluating these techniques was to determine if they could be used as fast, reliable early-screening methods for evaluating large numbers of genotypes for resistance to Rhizoctonia limb rot in controlled climates. Genotypes that passed the early screen then could be evaluated in field experiments. Neither technique we used was successful in reproducing the same range of disease reactions observed in the field experiments, but the detached shoot technique successfully produced disease *in vitro*. With further evaluation this *in vitro* technique might be adapted to evaluate other factors such as relative fungicide performance, or effects of environment on disease development.

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