Lignin Content and Resistance to *Sclerotinia minor* in Peanut

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ABSTRACT

Lignin has been shown to be an important component for plant defense in several pathosystems, but the relationship between peanut stem lignin content and resistance in the field to Sclerotinia blight has not been investigated. Stem lignin was quantified from twenty runner, six virginia, and ten spanish genotypes grown in the greenhouse using the acetyl bromide method. Significant differences in lignin content were found within the runner and spanish entries, but not among the virginia genotypes. Disease data collected in the field over two to three years were used to test correlations between lignin content and Sclerotinia blight resistance for a subset of the runner and virginia entries. No significant correlations were found. Within the runner entries, the highest and lowest stem lignin content was found in entries with the most disease resistance. These results indicate that preformed stem lignin content is not a reliable predictor for resistance to Sclerotinia blight in peanut. In addition, commercial peanut cultivars appear to vary considerably in lignin content, and the genotypes with lower levels of stem lignin may be useful to producers who can use peanut haulm for animal feed. Southwest Runner, a cultivar with high resistance to Sclerotinia blight, had the lowest stem lignin content of the 36 peanut lines tested.

Key Words: *Arachis hypogaea* L., germplasm, Sclerotinia blight, *Sclerotinia minor* Jagger.

Lignins are complex phenolic macromolecules produced by plants that are composed of three phenylpropane alcohols: *p*-coumaryl, coniferyl, and sinapyl (Vance *et al*., 1980; Hatfield and Fukushima, 2005). In addition to providing structural support to tissues, lignin protects plants from herbivores and pathogens (Taiz and Zeiger, 1991). Most microorganisms, including many fungi, are unable to break down lignin, and lignin is known to be a critical component in multiple pathosystems for plant defense responses (Vance *et al*., 1980; Banniza *et al*., 2005; Bhuiyan *et al*., 2009; Xu *et al*., 2011; Eynck *et al*., 2012). Inhibition of lignin formation in resistant wheat genotypes resulted in increased susceptibility to *Puccinia gramins* f. sp. *tritici* (Moerschbacher *et al*., 1990). Lines of camelina resistant to *Sclerotinia sclerotiorum*, and cotton resistant to *Verticillium dahliae*, produced more lignin precursors upon infection than susceptible lines (Xu *et al.,* 2011; Eynck *et al*., 2012). In peas, preformed stem lignin content was negatively associated with disease severity of *Mycosphaerella pinodes* (Banniza *et al*., 2005). However, the association between lignin content on diseases in other crops was less favorable. Lignin levels in alfalfa lines did not appear to be related to alfalfa rust resistance (Webb *et al*., 1996), and higher lignin content in soybean was positively correlated with disease susceptibility to *Sclerotinia sclerotiorum* (Peltier et al., 2009).

Sclerotinia blight, caused by *S. minor* Jagger, is a major disease in the Virginia-Carolina and Southwest peanut production regions of the U.S. Progress in breeding disease-resistant peanuts may be accelerated if physical or biochemical traits associated with disease resistance were available, particularly if such screening methods were more efficient than conducting field trials. Few studies have examined the relationship between lignin and disease resistance in peanut. Godoy *et al*. (1985) found higher levels of lignin in pods in breeding lines that were more resistant to pod rot than in susceptible lines. Similarly, Liang and colleagues reported a negative correlation between lignin content in peanut seed and rate of infection by *Aspergillus flavus* (2006). While evaluating detached stem assays for resistance to Sclerotinia blight, Brenneman *et al*. (1988) observed that older stem tissue was less susceptible than younger tissue to Sclerotinia blight, and hypothesized *Sclerotinia minor* might be deterred by the greater lignin and/or decreased sugar content in older stems.

Few studies have quantified lignin in peanut stems and most reports are for perennial forage species such as *Arachis glabrata*, *A. pintoi*, and *A. repens* (Terrill *et al*., 1996; Gomes *et al*., 2011; Ferreira *et al*., 2012). In crops produced for forage or biofuels, cultivars with lower levels of lignin are

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generally more desirable than those with high levels (Hisano *et al*., 2009; Jung, 1989). Foster *et al*. (2012) evaluated the digestibility of peanut stover from three cultivars (C-99-R, Georgia 01-R, York) and also measured lignin content at growth stages 2 and 8 (Boote, 1982). As expected, more lignin was present in the dried, above-ground tissue at growth stage R8 (95-104 g/kg) than at R2 (42-71 g/kg). However, significant differences in lignin content among the three cultivars were not observed at either growth stage (Foster *et al*., 2012). Forage quality is currently not a priority for U.S. peanut breeding programs, but low-lignin haulm may be a valuable trait in developing countries.

The objectives of this study were to quantify lignin in multiple genotypes of peanut, and to test if preformed lignin content could be used as a predictor of susceptibility or resistance to Sclerotinia blight in genotypes of runner, spanish, and virginia peanuts.

Materials and Methods

A total of 35 peanut cultivars and advanced breeding lines, including twenty runner (*Arachis hypogaea* subsp. *hypogaea* var. *hypogaea*), ten spanish (*A. hypogaea* subsp. *fastigiata* var. *vulgaris*), and five Virginia (*A. hypogaea* subsp. *hypogaea* var. *hypogaea*) market types were evaluated for preformed lignin content (Table 1). The entries included genotypes known to vary in susceptibility to *Sclerotinia minor*. To avoid confounding effects from natural infections and because lignin content in greenhouse-grown corn was shown to be comparable to lignin from field-grown plants (Hatfield *et al*., 2008), peanuts were grown in the greenhouse. Four seeds of each genotype were planted in a 30.5-cm-diam bulb pan filled with a soil-less potting mix (Metro-Mix 350, Sun Gro Horticulture, Agawam, MA). Seedlings were culled to one plant per pot at the 2 to 3 true-leaf stage. Plants were grown in a greenhouse set at 22-32 C with 14 hrs of supplemental lighting from 600-watt LED lights (Grow Pro, LED Lighting for Less). Plants were fertilized with 100 ml of 0.2% ammonium nitrate biweekly, beginning at five weeks after planting. Three plants of each genotype were used per experiment, and genotypes were randomized within blocks by market type on greenhouse benches. The first experiment was planted on 18 Mar. 2014; the second experiment was planted on 15 Apr. 2014.

Plants were uprooted and stem samples were collected at 127 to 129 days after planting. Five-cm-long stem pieces were cut from the second pair of lateral branches from the bottom. The stem sections were cut so that the third node on the lateral branch (counting from the main stem) was in the center. The two stem pieces were dried for three to seven days in an envelope at 55C, and then stored in a closed plastic container containing desiccant at room temperature.

Lignin Extraction

The two stem pieces were ground together using a Wiley Mini-Mill (Thomas Scientific, Swedesboro, NJ) with a 0.425-mm screen mesh. The Mini-Mill was cleaned thoroughly between samples by vacuuming out remaining material and wiping down surfaces with 70% ethanol. The ground tissue, approximately 500 mg, was weighed into a 30-mL Oak Ridge centrifuge tube (Thermo Fisher Scientific, Waltham, MA). Cell wall components were isolated using a series of 14 extractions with 10 mL each of the following solutions: 50 mM NaCl buffer (1x), 80% ethanol (4x), acetone (2x), 2:1 chloroform:methanol (5x), and acetone (2x). The extractions removed compounds such as sugars, organic acids, fatty acids, flavenols, and pigments, which interfere with spectrophotometric analyses (Peltier et al., 2009). For the few samples that were significantly greater than 500 mg, an additional 2 mL of each solution was used for every additional 100 mg. After each solution was added, tubes were capped, placed horizontally, and shaken on an orbital shaker for 15 min. The tubes were then centrifuged for 10 min at 8360 x g, and the supernatant was removed with an aspirator. After the last acetone extraction, samples were allowed to air-dry in a fume hood, and were dried further at 55C overnight, at which point the samples remained at a constant weight.

The acetyl bromide method was used to estimate lignin content (Hatfield *et al*., 1999; Fukushima and Hatfield, 2004). A subsample (approximately 25 mg weighed accurately ±0.1mg) of dried sample was transferred to a 10-mL glass culture tube with screw-top Teflon-lined cap. Acetyl bromide reagent (2.5 mL) was added to the sample and the tube was incubated in a 50 C water bath for 2 hr. After cooling, the sample was mixed and a 1-mL subsample was transferred to a microcentrifuge tube. Non-dissolved proteins and carbohydrates were pelleted by centrifugation at 10000 x *g*, and 500 µL of the supernatant was mixed with 9.5 µL of glacial acetic acid (7.15 mL), 2 M NaOH (2 mL), and 0.5 M hydroxyl amine (0.35 mL) before measuring UV absorption spectrum (250 to 350 nm) in a scanning spectrophotometer. Concentrations of lignin were based upon the maximum absorbance reading at 280 nm, and controls for background absorbance were included. Two sub-

Table 1. Cultivars and breeding lines tested for lignin content, their susceptibility to *Sclerotinia minor* or *S. sclerotiorum*, and years evaluated in the field for Sclerotinia blight resistance.

Entry	Resistance	Reference	Years
Runner			
ARSOK-R35	High	(Bennett *et al*., 2016)	2013-15
ARSOK-R47	High	(Bennett and Chamberlin, 2015)	2014-15
ARSOK-R60B	High	(Bennett and Chamberlin, 2015)	2013-15
AT-120	Susceptible	(Damicone and Jackson, 2001)	-
AT-9899	Moderate	(Bennett and Chamberlin, 2014)	2014-15
Florida-07	Susceptible	(Gorbet and Tillman, 2009)	2014-15
Florunner	Susceptible	(Melouk *et al*., 1992; Goldman *et al*., 1995)	2014
Flavor Runner 458	Susceptible	(Bennett and Chamberlin, 2015)	2013-15
Georgia-03L	High	(Woodward *et al*., 2006; Bennett *et al*., 2016)	2014-15
Georgia-09B	Unknown	(Branch, 2010)	-
Georgia-06G	Unknown	(Branch, 2007)	-
McCloud	Unknown		2014-15
Okrun	Susceptible	(Woodward *et al*., 2006; Melouk *et al*., 2012)	2013-15
Red River Runner	Moderate	(Melouk *et al*., 2012)	2013-15
Southwest Runner	High	(Kirby *et al*., 1998)	2014-15
Tifguard	Susceptible	(Holbrook *et al*., 2008)	2014-15
Tamrun 96	Moderate	(Smith *et al*., 1998)	2014-15
Tamrun OL02	Moderate	(Simpson *et al*., 2006)	2014
Tamrun OL07	Moderate	(Baring *et al*., 2006b)	2013-15
Tamrun OL11	High	(Baring *et al*., 2013)	2014-15
Virginia			
ARSOK-V31	Moderate	(Bennett and Chamberlin, 2015)	2013-14
ARSOK-V41	Susceptible	(Bennett and Chamberlin, 2015)	2013-14
AT-07V	Susceptible	(Bennett and Chamberlin, 2015)	2013-14
Georgia-11J	Moderate	(Branch, 2012; Bennett and Chamberlin, 2014)	2013
Jupiter	Susceptible	(Bennett and Chamberlin, 2015)	2013-14
Spanish			
ARSOK-S1	High	(Chamberlin *et al*., 2015b)	-
ARSOK-S133-3	High		-
Chico	High	(Chappell *et al*., 1995)	-
F435	Unknown	(Norden *et al*., 1987)	-
OLé	Moderate	(Chamberlin *et al*., 2015a)	-
Olin	Moderate	(Simpson *et al*., 2003)	-
Pronto	Susceptible	(Goldman *et al*., 1995)	-
Spanco	Susceptible	(Goldman *et al*., 1995)	-
Tamnut OL06	Moderate	(Baring *et al*., 2006a)	-
Tamspan 90	High	(Smith *et al*., 1991; Woodward *et al*., 2006)	-

samples were analyzed for each genotype, and the mean of both subsamples was used in the data analyses. Alfalfa stem standards were included with each batch of samples in order to standardize acetyl bromide lignin responses. The extinction coefficient for alfalfa was used to determine the relative lignin content in each sample. Total lignin was measured as proportion of the total cell wall (mg/mg CW).

Field Data

Resistance to *Sclerotinia minor* in the field was evaluated in unrelated experiments in 2013 to 2015 for a subset of the runner entries, and in 2013 and 2014 for the virginia entries, (Bennett and Chamberlin, 2014, 2015, 2016). Entries were screened at the Caddo Research Station in Fort Cobb, OK, in naturally infested fields that are planted every other year in peanuts. Only data from runner and virginia entries evaluated for two or three years were used. Seed of the entries were planted in two 4.6-m-long rows with 0.9-m beds at a density of 14.4 seeds per meter. A randomized complete block design was used, with a minimum of three blocks/replications. Field plots were irrigated using a center pivot and managed for weeds. Foliar diseases and southern blight were managed over the three years with applications of the following fungicides: chlorothalonil, chlorothalonil + difenoconazole, cyproconazole, flutriafol, propiconazole, tebucanozole, and/or trifloxystrobin. Plots were not sprayed to control Sclerotinia blight. Disease ratings for Sclerotinia blight were taken approximately one to two weeks before harvest, by

counting the number of 15.2-cm-sections within each plot that had symptoms caused by *S. minor*.

Statistical Analyses

Mean stem lignin content among genotypes were compared within market types by one-way ANOVA using PROC GLIMMIX of SAS (SAS, ver. 9.3, SAS Institute, Cary, NC), with block and experiment as random factors. Mean incidence of Sclerotinia blight was analyzed similarly, but with year and block as random factors. The Type I error rate for pairwise comparisons of breeding lines and cultivars was controlled at $\alpha = 0.05$ using the ADJUST=TUKEY option. The relationship between mean stem lignin content and mean incidence of disease in the field was estimated using Pearson's correlation coefficient SAS with PROC CORR.

Results and Discussion

When the stems were harvested at 127-129 days after planting, the Spanish entries were approximately at the R8 to R9 growth stages, and the runner and most virginia entries were at the R8 stage (Boote, 1982). The virginia entry Georgia-11J was an exception by being at the R7 stage at harvest.

Lignin content

Significant differences in lignin content were found within the runner market types ($F = 2.65$; df $= 19, 94$; $P < 0.01$; Table 2). The runner entry with the numerically highest lignin content was the advanced breeding line ARSOK-R47 (162 g/kg). ARSOK-R47 did not differ significantly from the other entries in lignin content except AT-9899 ($P = 0.03$) and Southwest Runner ($P < 0.01$). Mean lignin content in the six Virginia entries varied from 162 g/kg (AT-08V) to 134 g/kg (Georgia-11J), but the differences were not statistically significant ($F = 1.34$; df $= 4, 24$; $P = 0.28$; Table 2). The Spanish entries differed in lignin content ($F = 3.39$; df $= 9, 48$; $P < 0.01$; Table 2), and Tamnut OL06 had the highest stem lignin (146 g/kg). Tamnut OL06 was statistically similar to all other entries in lignin content except Pronto ($P = 0.03$), Spanco ($P = 0.03$), and Chico ($P = 0.01$).

Field resistance to Sclerotinia blight and association with lignin content

Environmental conditions for Sclerotinia blight were moderately conducive in 2013, favorable in 2014, and not favorable in 2015. The mean incidence of Sclerotinia blight for the highly susceptible cultivar Flavor Runner 458 was 42.1% in 2013, 72% in 2014, and 13.5% in 2015. Significant differences among entries were found for both runner ($F = 9.09$; df $= 14, 152.1$; $P < 0.01$) and virginia ($F = 6.67$; df $= 3, 46$; $P < 0.01$) entries (Table 2). The most susceptible entries were Florida-07 (43.5% diseased), Flavor Runner 458 (43.0%), and Okrun (35%). ARSOK-R47, ARSOK-R60B, and Southwest Runner were most resistant to Sclerotinia blight with disease incidences of 12.5%, 9.6%, and 0.8%, respectively. Among the virginia entries, ARSOK-V31 was significantly more resistant than AT-07, Jupiter and ARSOK-V41 ($P < 0.01$). No significant correlations between lignin content and field resistance to Sclerotinia blight were found within the runner ($r = 0.15$; $F = 0.30$; $P = 0.59$) or virginia entries ($r = 0.84$; $F = 7.34$; $P = 0.07$; Table 2). Therefore, lignin content does not appear to be reliable predictor of Sclerotinia blight resistance.

The number of genotypes sampled for virginia and spanish peanuts were small and may not be a representative sample of either market type. A larger number ($n = 20$) of runner entries with a range of susceptibility to Sclerotinia blight were evaluated. While some of the more resistant entries, such as ARSOK lines R47 and R60B, had high levels of lignin, others did not. Southwest Runner, the cultivar most resistant to *S. minor* in the field trials, had the least stem lignin of all peanut entries. Thus, the negative correlation between lignin content and disease resistance found in other pathosystems (e.g. Banniza *et al*., 2005; Xu *et al*., 2011) does not seem to apply to peanuts and Sclerotinia blight. Others have also failed to find strong evidence supporting the role of preformed lignin in plant defense. Peltier *et al*. (2009) found soybean lines with greater stem lignin were actually more susceptible to *S. sclerotiorum*. Pea lines with high lignin content had slower lesion development but did not survive beyond two weeks, unlike the most resistant lines (Porter *et al*., 2009). However, this study only examined preformed lignin in peanut, and the quantity of lignin formed on-site in response to infection may be better correlated with disease resistance. In addition, further study is needed to determine if peanut lignin content is as consistent as it is in corn (Hatfield *et al*., 2008) when grown in different environments such as the field and greenhouse.

It was expected that more lignin would be present in spanish peanut stems to support the upright spanish plant architecture than in the prostrate runner and virginia lines. However, as a group, the spanish entries had lower mean lignin ($\bar{x} = 124$ g/kg; SD = 13.9) than virginias ($\bar{x} = 140$ g/kg; SD = 13.4) or runners ($\bar{x} = 145$ g/kg; SD = 11.5). Lignin content in plants generally increases with age (Buxton and Russell, 1988), but the Spanish market types were slightly more mature at harvest than the

Table 2. Mean stem lignin content in greenhouse-grown runner, Spanish, and Virginia peanut market types and Sclerotinia blight incidence in the field (runner and Virginia).

Entry	Mean Lignin (g/kg)[a]		Sclerotinia Blight (%)[b]		Correlation[c]
Runner					
ARSOK-R47	162	a	12.5	cd	0.15 n.s.
ARSOK-R60B	156	ab	9.6	cd	
AT-120	151	ab	-		
Tamrun OL02	151	ab	-		
Tamrun OL07	150	ab	28.4	a-c	
Okrun	150	ab	35.4	ab	
Georgia-03L	149	a-c	13.3	b-d	
Tifguard	147	a-c	29.0	b-c	
Florunner	145	a-c	-		
Tamrun OL11	139	a-c	16.0	b-d	
Tamrun 96	139	a-c	24.9	a-d	
ARSOK-R35	138	a-c	15.0	b-d	
Georgia-09B	138	a-c	-		
Flavor Runner 458	135	a-c	43.0	a	
Red River Runner	134	a-c	24.1	a-d	
Georgia-06G	132	a-c	-		
McCloud	132	a-c	34.6	a-c	
Florida-07	131	a-c	43.5	a	
AT-9899	118	bc	21.5	a-d	
Southwest Runner	104	c	0.8	d	
Virginia					
AT-07V	162	a	42.5	a	0.77 n.s.
Jupiter	151	a	43.3	a	
ARSOK-V41	144	a	41.1	a	
ARSOK-V31	136	a	18.9	b	
Georgia-11J	134	a	-		
Spanish					
Tamnut OL06	146	a	-		
OLé	138	ab	-		
F435	134	ab	-		
ARSOK-S1	132	ab	-		
Olin	125	ab	-		
ARSOK-S133	123	ab	-		
Tamspan 90	117	ab	-		
Pronto	109	b	-		
Spanco	108	b	-		
Chico	105	b	-		

[a]Mean kg lignin per kg cell wall fraction in stem. Standard errors for each market type ±: runner, 14.0; virginia, 10.0; spanish, 8.0. Numbers with the same lowercase letter within each market type are not significantly different ($\alpha = 0.05$).

[b]Field disease data for runners collected in 2013-2015; Virginia data from 2013 and 2014. Standard errors for runners ±11.7-12.6, Virginias ±4.0-4.7. Data not available (-). The following additional pairs of runner entries were significantly different in incidence of Sclerotinia blight: Red River Runner vs. Florida-07, Flavor Runner 458, and Southwest Runner; Okrun vs Tamrun OL11 and ARSOK-R35.

[c]Pearson's correlation coefficient between mean lignin content (mg) and incidence of Sclerotinia blight in the field for runner and Virginia peanuts. P values: runner, $P = 0.59$; virginia, $P = 0.23$.

runners and virginias. Lignin is not the only component within the cell wall that contributes to plant structural integrity, and the density of specific cell types, such as fiber, vascular, and pith cells, can have a major impact upon total lignin within a whole stem, regardless of growth stage. In addition, cultivated peanut is known to have low genetic variability (Cuc *et al*., 2008), and peanut breeders have crossed different market types to introgress desirable traits into currently available cultivars. For example, many high-oleic runner and virginia cultivars available today derive their high-oleic trait from F435, a spanish peanut (Norden *et al*. 1987). While none of the high-oleic runner and virginia cultivars grown today have the upright canopy characteristic of the spanish market type, plant

breeding may have affected lignin content among market types, if differences originally existed.

There are several methods for quantifying lignin (Hatfield and Fukushima, 2005), but most published studies of *Arachis* spp. use the acid detergent method (Van Soest, 1967). Foster and colleagues (2012), using the acid detergent method on three runner cultivars, found 95-104 g/kg lignin in a sample containing all aboveground tissue collected at the R8 stage. This study, using the acetyl bromide method on twenty runner entries at the same growth stage, obtained 104-162 g/kg lignin from stem sections of the second lowermost branch. Despite the popularity of the acid detergent protocol, several studies demonstrated that this method consistently underestimates lignin relative to other approaches such as the acetyl bromide method used here (Iiyama and Wallis, 1990; Hatfield et al., 1994; Jung et al., 1997; Fukushima and Hatfield, 2004). Results produced by different methods within and among crops are therefore difficult to compare, even before considering effects of the plant part analyzed or plant maturity. Fukushima and Hatfield (2004) compared several methods for quantifying lignin in several forage species including lower stems of alfalfa (in full bloom) and corn stalk (past anthesis). Mean estimates of alfalfa lignin were 92.5 g/kg with the acid detergent assay and 134.7 g/kg using acetyl bromide method. A greater difference between the two methods was found for the corn stalk: 24.8 g/kg for the acid detergent versus 91.9 g/kg for the acetyl bromide. From soybean stems collected at the R5 (beginning seed) stage, Peltier et al. (2009) obtained an average of 211 g of lignin/kg using the acetyl bromide method. Estimates of stem lignin content for the peanut genotypes in this study varied from 104 to 167 g/kg collected at the R8-R9 stage. Stem lignin levels in peanut thus appear to be lower than in soybean, and may be comparable to alfalfa. The new reduced-lignin cultivars of alfalfa, Hi-Gest (Alforex Seeds, Woodland, CA) and HarvXtra (Forage Genetics International, Nampa, ID), reportedly have 7 to 15% less lignin than conventional alfalfa (company websites). In this study, the spanish cultivar Chico had 15% less lignin (105 g/kg) compared to the mean for all spanish peanuts (124 g/kg), and cultivar Southwest Runner had 28% less lignin (104 g/kg) than the mean for the runner genotypes (145g/kg). Chico (Chappell *et al*., 1995) and Southwest Runner are also resistant to Sclerotinia blight. Both cultivars may be good options for low-input producers in developing countries, and organic producers in this country, seeking to add crop value by feeding peanut hay to livestock.

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