Food and Nutrition Sciences, 2014, 5, 1843-1859
Published Online October 2014 in SciRes. http://www.scirp.org/journal/fns
http://dx.doi.org/10.4236/fns.2014.519198

Scientific Research

Soybean Seed Phenolics, Sugars, and Minerals Are Altered by Charcoal Rot Infection in MG III Soybean Cultivars*

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Received 17 August 2014; revised 4 September 2014; accepted 11 September 2014

Abstract

Soybean seed is a major source of phytochemical compounds that impact human health nutrition and livestock meal. Charcoal rot is a disease caused by the fungus *Macrophomina phaseolina* (Tassi) Goid, and thought to infect the plants through roots by a toxin-mediated mechanism, resulting in yield loss and poor seed quality, especially under drought conditions. Limited information is available on the effect of charcoal rot on seed phytochemical compounds and mineral nutrition in soybean. Therefore, the objective of this research was to investigate the effect of charcoal rot infection on seed phenol, seed coat lignin, isoflavones, and minerals using susceptible (S) (DK 3964) and moderately resistant (MR) (AG 3905) maturity group (MG)III soybean cultivars to charcoal rot. A two-year field experiment was conducted, and infested soil with charcoal rot (infested soil conditions, INF) or control (non-infested soil conditions, NINF) was used. The results showed that the moderately resistant genotype had higher concentrations of seed phenolics, total isoflavones, and seed coat lignin under infested and non-infested conditions and under irrigated or non-irrigated conditions compared with the susceptible genotype. The same general trend was found for seed K, Ca, P, Mn, Zn, B, and Cu concentrations in the moderately resistant genotype compared with the susceptible genotype. Our research demonstrated that these seed phytochemical constituents may explain the differences between susceptible and moderately resistant cultivars and may play an important role in the resistance to charcoal rot.

*Mention of trade names or commercial products in this publication is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the US Department of Agriculture.

How to cite this paper: Bellaloui, N., *et al.* (2014) Soybean Seed Phenolics, Sugars, and Minerals Are Altered by Charcoal Rot Infection in MG III Soybean Cultivars. *Food and Nutrition Sciences*, **5**, 1843-1859.
http://dx.doi.org/10.4236/fns.2014.519198

Keywords

Charcoal Rot, Isoflavones, Lignin, Minerals, Phenolics

1. Introduction

Soybean is a major crop in the world, and soybean seeds are major oil and soymeal sources in the world for human and animal feed. Soybean seeds contain protein, oil, minerals, sugars, and phenolics. Phenolics are secondary metabolites and have human health benefits because of their antioxidant properties [1]-[4] and contribute to the defense in plants against biotic (fungus, bacteria, virus, and insect infection) or abiotic stresses such as drought and high heat. Phenolics are natural chemical compounds containing a hydroxyl group (−OH) bonded to an aromatic hydrocarbon, and possess an aromatic ring bearing phenol or polyphenol hydroxyl substituents such as esters, methyl ethers, and glycosides [5]. Phenolics are produced via the shikimate-phenyl-propanoids-flavonoids pathways [6] [7], and they can be C6 (simple phenol, benzoquinones), C6-C1 (phenolic acid), C6-C3-C6 (flavonoids, isoflavonoids), (C6)n, or (C6-C3-C6)n (lignins) [8] [9].

Charcoal rot is a major soybean disease, leading to loss in production [10] [11], seed quality [12] [13] (**Figure 1**), and phytosanitary problems for soybean export [14]. Charcoal rot is caused by the fungus *Macrophomina phaseolina* (Tassi) Goid which infects plant roots from the soil by a toxin-mediated mechanism. *Macrophomina phaseolina* secretes two toxins (**Figure 2**), phaseolinone [15] [16] and (-)-botryodiplodin [17]. Although the mechanisms of charcoal rot infection via toxins are not well understood, infection of crop through the roots is

Figure 1. Charcoal rot infestation in the field. Top left = irrigation minimized charcoal rot effects on seed-fill; Top right = soybean grown in non-irrigated field with significant effects of charcoal rot infestation on seed-fill; effect of irrigated (bottom left, soybean on the right) and non-irrigated (bottom left, soybean on the left) on yield loss; Bottom right = Charcoal rot infestation in the field.

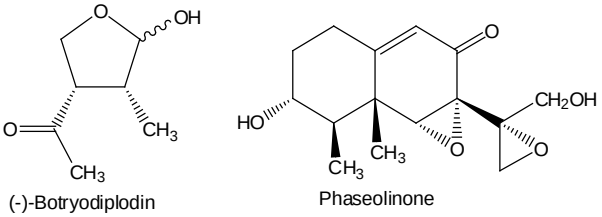

Figure 2. Charcoal rot toxins [67].

thought to occur by two mechanisms [18]. The first mechanism is through physical penetration of tissue, and the second through secretion of toxins, killing local plant tissue, allowing the fungus to enter through the necrotic area created by the toxin. The toxin-mediated infection of plants occurs 1) by secretion of hydrolytic enzymes or toxins that induce activation of endogenous hydrolytic enzymes; and 2) by secretion of toxins to divide cells, targeting the meristematic tissue at root tips and providing convenient access of the fungus to the vascular system of the infected plant.

Phenolics, including phenol, lignin, and flavonoids are effective inhibitors of hydrolytic enzymes, leading to fungal resistance to toxin-mediated hydrolytic enzymatic infection. Although phenolics were found to be genetically controlled and affected by biotic and abiotic stress factors, the mechanisms of how these factors influence the content and the biosynthesis of phenolics are still not understood. So far there are no commercial soybean cultivars that are fully resistant to charcoal rot disease, and development of soybean genotypes resistant to charcoal rot infection is crucial to manage the disease [19] [20]. For example, a moderately resistant germplasm line DT97-4290 was developed and field-tested and released [21] at Stoneville, MS, USA. The line DT97-4290 was further evaluated for seed protein, oil, and fatty acids [22] and quality [23] in the field under irrigation and non-irrigation and with and without charcoal rot infestation. The moderately resistant (DT97-4290) (maturity group, MG, IV) and AS 3905 (MG III) and susceptible lines (Egyptian and DK 3964) were evaluated under field conditions and with and without irrigation and with and without charcoal rot infestation [11].

Isoflavones are a form of flavonoid in soybean seeds that have multiple chemical functions, including antioxidative actions and health benefits [24]. Isoflavones are categorized based on their functional groups into four subgroups, including aglycons, glucosides, malonylglucosides, and acetylglucosides. Isoflavones occur in storage form as 6-*O″*-malonyl-*β*-glucosides of the respective aglycones genistein, daidzein, and glycitein. The concentration of isoflavoneaglycones is low while significant levels of their simple *β*-glucoside (*i.e*. glucose not conjugated with acetyl or malonyl groups) exist [25]. It was reported that isoflavones prevent certain forms of cancer and reduce risks of cardiovascular diseases [26]. Two forms of isoflavones, genistein and daidzein, have been found to have the ability to control or inhibit the growth of human breast cancer cell lines in culture, and this is may be due to the strong antioxidative properties of genistein [27] [28].

Mechanisms of how phenolics such as lignin and isoflavones contribute to the plant defense against charcoal rot have not been established. It was reported that lignin content in the plants is altered by biotic and abiotic stress factors, indicating complex genetic and physiological control [29]. They reported that abiotic stress factors such as mineral deficiency, drought stress, temperatures, and biotic stress factors such as plant genotype, infection by fungi, bacteria, and viruses can also alter the biosynthesis of lignin in plants [29]. The contribution of lignin to the plant defense may be due to its composition and structure. Lignins are complex racemic aromatic heteropolymersderived fromhydroxycinnamyl alcohol monomers with different methoxylation. These monomers are *p*-coumaryl, coniferyl, and sinapylalcohols [30]. Lignin occupies the spaces in the cell wall between cellulose, hemicellulose, and pectin, especially in xylemtracheids and vessel elements, creating crosslinkings of polysaccharides, supporting the cell wall [31] [32]. The cell wall polysaccharides are highly hydrophilic, but lignin is more hydrophobic, making vascular tissue of the plants water efficient conductors [32]. Soybean seeds contain phenolic compounds, including chlorogenic acid, caffeic acid, ferulic acid, and *p*-coumaric acid, and the content of these phenolic acids range from 28% to 72% of the total phenol. These monolignols produce *p*-hydroxyphenyl, guaiacyl, and syringylphenylpropanoid units when incorporated into the lignin polymer. The content and composition of lignins vary among cell types and individual cell wall layers, and are influenced by environmental stress factors [33]. Lignin constitutes a major component of cell wall, giving rigidity, structural support and impermeability to water [33] and is important for mechanical support, water transport and resistance against pathogens in vascular plants [33] [34]. Previous research showed that lignin deposition is related with mechanical resistance, cell wall protection and microorganism resistance [35], and mechanical damage was related to lignin content and peroxidases activity [36].

Adequate mineral nutrition is crucial to plant defense. It is well established that resistance to pathogens is genetically controlled; however, mineral nutrition can play a major role in resistance or tolerance to pathogens [37] [38]. It was reported that the effect of mineral nutrition is substantial in moderately susceptible, but lower in highly susceptible or resistant to diseases [37]. Previous research showed that minerals such B, Mn, and Cu play a major role in phenolics and lignin synthesis [37] [39] and in cell wall and membrane integrity. Deficiencies of K and Zn resulted in high cell wall leakage of sugar and amino acid to leaf apoplast, allowing the pathogen to penetrate the cell [38] [40]; B deficiency led to higher fungal infection [41]; low Ca content in plant tissue re-

sulted in cell wall leakage of sugars and amino acids from cytoplasm to apoplast; and Cu deficiency led to impairment of defense compound synthesis, accumulation of soluble carbohydrates, and reduced lignin synthesis, resulting in lower disease resistance [38].

Based on the above discussion, it is clear that the plant defense against pathogens is complex, mechanisms explaining the role of phenolics and mineral nutrition are not well understood, and further research in this area is needed. Therefore, the objective of this research chapter was to further investigate the effects of charcoal rot infection on seed phenolics and mineral nutrition in susceptible and moderately resistant cultivars. This research will also review current research in the area of plant resistance and roles of phenolics and mineral nutrition. Since seed sugars were reported to possibly play a significant role under drought conditions, seed sugars (sucrose, raffinose, and stachyose) were also evaluated.

2. Materials and Methods

Field trials were conducted in 2004 and 2005 at Delta Research and Extension Center Stoneville, MS (33˚26'N), MS, USA. Irrigated and non-irrigated treatments were imposed. Two soybean maturity group (MG) III cultivars were selected: AG 3905 (moderately resistant) and DK 3964 cultivar (susceptible). Seeds treatment was performed as previously described in [11] [22]. The seeds were treated with mefenoxam (R)-2-{2,6-(dimethylphenyl)-methoxyacetylamino}-propionic acid methyl ester fungicide prior to planting as a precaution against stand loss due to *Pythium* spp. Fumigation with methyl bromide in combination with chloropicrin was conducted two weeks prior to planting as described in details in [11]. The plots were either infested with *M. phaseolina* or not infested. Planting was 4 May in 2004 and 6 May in 2005. Plants were harvested in mid-September. The treatments were: irrigated-infested and irrigated-noninfested; nonirrigated-infested and nonirrigated-noninfested. The experiment was furrow irrigated, and irrigation was applied whenever water potential reached −50 kPa at 30 cm depth. Soil water potential was monitored using tensiometers according to others [22] [42] [43]. Irrigation was applied as described elsewhere [11]. Seeds from each replicate and each treatment were harvested at harvest maturity (R8) for seed composition and phenolics analyses.

2.1. Disease Assessment and Scoring

Disease was measured by the intensity of discoloration on a scale of 1 - 5 as described elsewhere [11], in which 1 = no discoloration and 5 = highly discolored. The scale for root and stem disease severity was based on those of [21], and it was divided into four classes as follows: resistant (values of 1), moderately resistant (values > 1 and ≤2), moderately susceptible (values > 2 and <3), and susceptible (values 3 - 5). In addition to the discoloration scale method, disease assessment was also made in this experiment using colony forming unit (CFU) according to [23]. In the CFU method, assessment of host tissue colonization by *M. phaseolina* was conducted on three replications by destructively sampling 10 plants at the R7 growth stage. Samples included both roots and aboveground portions of the plant. Samples were taken from the lower stem and root, including lateral and fibrous roots as described elsewhere [23]. The total counts of CFU were taken from each plot, converted to CFU per gram (CFU count × 200) of ground root and stem tissue, and expressed for each genotype [23].

2.2. Lignin Determination in Seed Coat

Lignin in the seed coat of matured seed was determined as previously reported by others [44]-[46]. Briefly, seeds were soaked for 12 h, then the seed coat was removed, and dried in an oven for 16 h at 105˚C. A sample of 250 mg of dry seed coat was homogenized in 50 mM potassium phosphate buffer (7 mL at pH 7.0) with a mortar and pestle. The centrifugation (1400 g for 10 minutes) of the mixture was conducted according to others [47] [48]. The pellet resulting from the centrifugation was washed and centrifuged twice with 7 mL phosphate buffer at pH 7.0; 3× with 7 ml 1% (v/v) Triton X-100 at pH 7.0 buffer; 2× with 7 ml 1 M NaCl at pH 7.0 buffer; 2× with 7 ml of distilled water; and 2× with 5 ml acetone. The pellet was then dried in an oven at 60˚C for 24 h, and placed in a centrifuge tube containing a reaction mixture of 1.2 ml thioglycolic acid with 6 mL 2 M HCl. After heating and cooling, the mixture was centrifuged at 1400 g for 5 min, and the supernatant discarded. The pellet containing the lignin-thioglycolic acid (LTGA) complex was washed 3× with 7 ml distilled water and then extracted by shaking at 30˚C for 18 h at 115 oscillations min^{-1} in 6 ml of 0.5 M NaOH, centrifuged at 1400 g for 5 min, and the supernatant was stored at −20˚C. The chemical washing of the combined pellet and supernatant was con-

ducted according to [18]. The concentration of lignin was measured by reading the supernatant at absorbance at 280 nm using a Beckman Coulter DU 800 spectrophotometer (Fullerton, CA). The concentration of lignin was expressed as mg LTGA/g dry weight.

2.3. Determination of Total Phenol Concentration

The concentration of total phenol was measured in mature seeds using the Folin-Ciocalteu assay and gallic acid as standard base according to others [49] with modification [50]. Briefly, seeds were ground and passed through a 60-mesh sieve, and a sample of 0.5 was extracted twice with 10 ml acetone/water (50:50, v/v). An aliquot of 200 µl of seed extract and 1 ml Folin-Ciocalteu reagent were mixed. Then 1.0 ml of 20% Na_2CO_3 was added with distilled water to make a 5 mL final volume. The color development occurred after 90 min of incubation at room temperature. The concentration of total phenol was measured spectrophotometrically by reading the samples at 765 nm using a Beckman Coulter DU 800 spectrophotometer (Fullerton, California). The concentration of total phenol was expressed as mg GAE (gallic acid equivalent) per 100 g of sample (mg GAE/100g).

2.4. Analysis of Isoflavones

The concentration of the total isoflavones (daidzein, genistein, and glycitein) was measured in mature seeds. About 25 g of seed were ground using a Laboratory Mill 3600 (Perten, Springfield, IL). Isoflavones were measured according to others [18] [51] [52] using a near-infrared reflectance (NIR) diode array feed analyzer (Perten, Spring Field, IL). The analysis was conducted on a dry matter basis. Total isoflavones were considered the sum of the major three individual isoflavones daidzein, genistein, and glycitein.

2.5. Determination of Seed Sucrose, Raffinose, and Stachyose Concentrations

Concentration of seed sucrose, raffinose, and stachyose was measured in R8 seed according to others [53] [54] using an AD 7200 diode array feed analyzer (Perten, Springfield, IL). Briefly, about 25 g of seed were ground using a Laboratory Mill 3600 (Perten, Springfield, IL). Initial calibration equations were developed by the Department of Agronomy and Plant Genetics, University of Minnesota St. Paul, MN using Thermo Galactic Grams PLS IQ software, developed by Pertencompany (Perten, Springfield, IL). Analyses of sugars were performed based on a seed dry matter basis [53].

2.6. Seed Glucose Determination

The concentration of glucose in mature seeds was measured according to the enzymatic reaction using Glucose (HK) Assay Kit from Sigma, USA, Product Code GAHK-20 [55]. In this reaction, glucose is phosphorylated by adenosine triphosphate (ATP) in a reaction catalyzed by hexokinase. Glucose-6-phosphate (G6P) resulting is then oxidized to form 6-phosphogluconate in the presence of oxidized nicotinamide adenine dinucleotide (NAD) in a reaction catalyzed by glucose-6-phosphate dehydrogenase (G6PDH). An equimolar amount of NAD is reduced to NADH during this oxidation, and the increase in absorbance at 340 nm is directly proportional to glucose concentration in the sample. Mature seed samples were ground using a Laboratory Mill 3600 (Perten, Springfield, IL) to obtain uniform particles. A dry, ground sample of 0.1 mg was extracted with deionized water. The extraction procedure of glucose from seeds was conducted as described elsewhere [56], and as instructed by Glucose (HK) Assay Kit from Sigma. To measure the concentration of glucose, the absorbance of the samples was read at 340 nm using a Beckman Coulter DU 800 spectrophotometer (Fullerton, CA). The concentration of glucose was expressed as $mg \cdot g \cdot dwt^{-1}$.

2.7. Seed Fructose Determination

The concentration of fructose in mature seeds was determined enzymatically according to Fructose Assay Kit from Sigma, USA, Product Code FA-20 [57]. In this reaction, fructose is phosphorylated by ATP in a reaction catalyzed by hexokinase, and the resulting fructose 6-phosphate is then converted to G6P by phosphoglucose isomerase (PGI). Then, oxidation of G6P to 6-phosphogluconate takes place in the presence of NAD in the reaction catalyzed by glucose-6-phosphate dehydrogenase (G6PDH). An equimolar amount of NAD is reduced to NADH, and the consequent increase in absorbance at 340 nm is directly proportional to fructose concentration in a sample. A sample of 0.1 mg was extracted according to Fructose Assay Kit from Sigma, and is detailed else-

where [56] as instructed by Fructose Assay Kit from Sigma. The concentration of fructose in samples was determined spectrophotometrically by reading the samples at absorbance of 340 nm using a Beckman Coulter DU 800 spectrophotometer (Fullerton, CA). The concentration of fructose was expressed as $mg \cdot g \cdot dwt^{-1}$.

2.8. Boron Determination

Total boron in mature seeds at R8 was measured according to others [49] [58] [59] using the Azomethine-H method. Briefly, one g of dry, ground sample was ashed at 500˚C for 8 h, the samples were extracted with 20 ml 2 M HCl at 90˚C for 10 min, and the extract was filtered. A sample of 2 ml was added to 4 ml buffer solution (containing 25% ammonium acetate, 1.5% EDTA, and 12.5% acetic acid) and 4 ml azomethine-H solution containing 0.45% azomethine-H and 1% ascorbic acid [60]. Boron concentration was measured by reading the samples at 420 nm using a Beckman Coulter DU 800 spectrophotometer (Fullerton, CA).

2.9. Iron Measurement

The concentration of iron in seeds was measured after acid (60% m/m $HClO_4$ + 70% m/m HNO_3) wet digestion, extraction, and reaction of the reduced ferrous Fe with 1, 10-phenanthroline according to others [61] [62]. The seed samples were acid digested. Detailed extraction procedures and measurements were detailed in [63]. Briefly, 2 g of dried ground seed was acid digested, then the acids were removed by volatilization, and the soluble constituents were dissolved in 2 M of HCl. To prepare the standard curve, iron standard solutions were prepared in 0.4 M HCl, ranging from 0.0 to 4 $\mu g \cdot ml^{-1}$ of Fe(II). A phenanthroline solution of 0.25% (w/v) was prepared in 25% (v/v) ethanol. Quinol solution of 1% w/v was used as reagent. A mixture of 4 ml of a sample in 1 ml of 0.4 M HCl and 1 ml of quinol solution were added and mixed, then 3 ml of the phenanthroline solution and 5 ml of the tri-sodium citrate solution (8% w/v) were added. The solution was diluted to 25 ml with distilled water and incubated at room temperature for 4 h. The concentration of Fe in samples was measured spectrophotometrically by reading at 510 nm using a Beckman Coulter DU 800 spectrophotometer.

2.10. Phosphorus Measurement

The concentration of phosphorus in seeds was measured spectrophotometrically as the yellow phosphor-vanado-molybdate complex according to [64] and detailed in previous reports [63]. Briefly, a dried ground seed sample of 2 g was ashed, 10 ml of 6 M HCl was added, and then the sample was evaporated to dryness using a water bath. Two milliliters of 36% v/v HCl was added to the sample under heat until boiled. The extraction, filtration, and measurement using 5 M HCl and 5 ml of ammonium molybdate-ammonium metavanadate reagent were detailed elsewhere [63]. Phosphorus concentration in samples was determined using a Beckman Coulter DU 800 spectrophotometer at 400 nm. Dihydrogen orthophosphates was used to produce a standard curve of phosphorus standard solutions (0 - 50 $\mu g \cdot ml^{-1}$ of P).

2.11. Seed Mineral Analyses (N, Ca, Mg, S, and Zn)

Mature seeds at R8 were collected, and seed mineral concentrations were determined. Seed samples were ground to pass through a 1 mm sieve using a Laboratory Mill 3600 (Perten, Springfield, IL). Seed N, S, Ca, Mg, and Zn concentrations were analyzed using inductively coupled plasma (ICP) spectrometry as described by others [63]. Nitrogen and S were determined using an elemental analyzer (LECO CNS-2000, LECO Corporation, MI) [63].

2.12. Experimental Design and Statistical Analysis

The design was a split plot with irrigation as the main plot, infestation as subplot, and genotype as sub-subplot. Proc GLM in SAS was used for data analysis [65]. Three replicates were used. Fisher's Least Significant Difference test with 5% as the level of significance was used to separate means.

3. Results

3.1. Soil Infestation and Plant Infection

Charcoal rot infection to S and MR cultivars, expressed as colony forming unit per gram (CFU/g), showed that the S cultivar DK 3964 was significantly ($P \leq 0.05$) infected with charcoal rot compared with the MR cultivar

AG 3905 under IRR or NIRR conditions (**Figure 3** and **Figure 4**). The level of infection in NIRR and INF was the highest and the level of magnitude of infection between S and MR cultivars was extremely large. Therefore, infested soil with charcoal rot resulted in significant infection to S cultivar compared with MR cultivar, and the differences between S and MR cultivars in seed phenolics, sugars, and minerals can be explained in terms of infection as well as infestation.

3.2. Analysis of Variance

Analysis of variance showed that year (Y), infestation treatment (T), cultivar (Cv), and irrigation (Ir) had significant effects on total phenol, lignin, isoflavones, and specific minerals in seeds (**Table 1**). There were significant differences in the interaction among these factors for phenol, lignin, isoflavones, and minerals, indicating that the infestation effects depended on cultivar, irrigation, and year. Because of these interactions, the results were presented by each year and by each irrigation treatment. Analysis of variance showed that year, infestation treatment, cultivar, and irrigation had significant effects on sugars (glucose, fructose, sucrose, raffinose, and stachyose) in seeds (**Table 2**). There were significant differences in the interaction among these factors for sugars, indicating that the infestation effects depended on cultivar, irrigation, and year. Because of the significant effects of the interactions between the main factors, the results were presented by year and irrigation treatment.

3.3. Effect of Charcoal Rot Infestation on Seed Phenol, Lignin, Total Isoflavones, and Minerals under Irrigated Conditions

Seed phenol, lignin, and total isoflavone concentrations were higher in MR cultivar than in S cultivar under infestation (INF) than under non-infestation (NINF) conditions (**Table 3**). Seed phenol, lignin, and isoflavones in

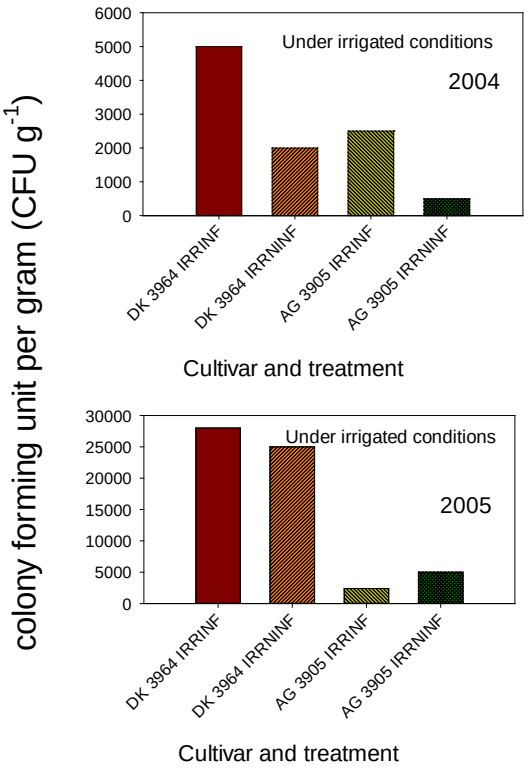

Figure 3. Charcoal rot levels (colonyforming unit per gram (CFU g^{-1}) in susceptible cultivar (DK 3964) underirrigate-dinfested (DK 3964 IRRINF) and irrigated non-infested (DK 3964 IRRNINF) and in moderatelyresistant cultivar (AG 3905) underirrigatedinfested (AG 3905 IRRINF) and irrigated non-infested (AG 3905 IRRNINF) in 2004 and 2005.

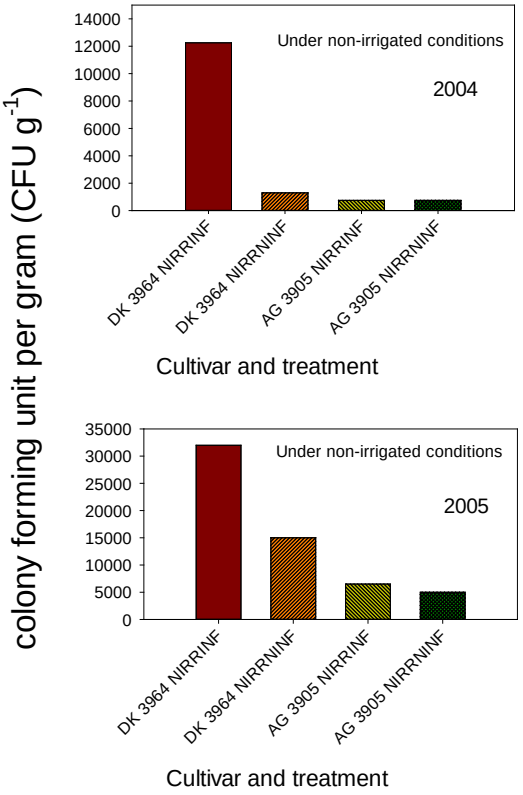

Figure 4. Charcoal rot levels (colonyforming unit per gram (CFU g^{-1}) in susceptible cultivar (DK 3964) under non-irrigate-dinfested (DK 3964 NIRRINF) and non-irrigated non-infested (DK 3964 NIRRNINF) and in moderatelyresistant cultivar (AG 3905) under non-irrigatedinfested (AG 3905 NIRRINF) and non-irrigated non-infested (AG 3905 NIRRNINF) in 2004 and 2005.

Table 1. Analysis of variance of the effects of year (Y), charcoal rot infestation treatment (T), cultivar (Cv), and irrigation (Ir) on seed total phenol, seed coat lignin, total isoflavones, nitrogen, and minerals in susceptible (DK 3964) and moderately resistant (AG 3905) soybean cultivars to charcoal rot infestation.

Source of variability	Phenol	Lignin	Total isoflavones	N	P	K	Ca	Mg	Mn	B	Zn	Fe	Cu
Year (Y)	***	*	**	*	*	*	*	**	*	**	*	*	*
Treatment (T)	*	*	*	*	*	**	**	***	*	*	*	*	*
Cultivar (Cv) Irrigation (Ir)	*	*	*	**	*	*	*	*	*	*	NS	*	*
Y × T	**	*	***	**	*	*	*	**	**	**	*	*	*
Y × Cv	NS	*	**	NS	**	*	NS	NS	**	*	*	*	*
Y × Ir	*	**	***	**	*	**	**	*	*	***	**	*	*
T × Cv	*	*	*	NS	*	**	**	NS	NS	*	**	*	*
T × Ir	**	*	***	NS	*	**	**	NS	NS	**	*	*	**
Cv × Ir	*	*	**	*	*	**	**	*	*	**	*	**	**
Y × T × Cv	**	**	***	*	*	*	*	*	*	*	**	*	*
Y × T × Ir	*	*	***	**	**	*	*	*	*	*	*	*	**
T × Cv × Ir	*	**	**	*	*	**	**	**	*	**	*	*	**
Y × Cv × Ir	*	*	***	*	*	*	*	*	*	**	*	*	*
Y × T × Cv × Ir	*	*	***	**	**	*	*	*	*	***	**	*	*

*: Significance at $P \leq 0.05$; **: Significance at $P \leq 0.01$; ***: Significance at $P \leq 0.001$.

Table 2. Analysis of variance of the effects of year (Y), charcoal rot infestation treatment (T), cultivar (Cv), and irrigation (Ir) on seed sugars (mg/g) in susceptible (DK 3964) and moderately resistant (AG 3905) soybean cultivars to charcoal rot infestation.

Source of variability	Glucose	Fructose	Sucrose	Raffinose	Stachyose
Year (Y)	**	*	***	*	***
Treatment (T)	**	*	***	*	**
Cultivar (Cv)	*	*	*	*	*
Irrigation (Ir)	**	*	*	*	**
Y × T	**	*	**	*	*
Y × Cv	NS	*	**	NS	*
Y × Ir	*	*	**	**	*
T × Cv	*	*	*	NS	*
T × Ir	**	NS	**	NS	NS
Cv × Ir	*	*	*	*	*
Y × T × Cv	**	**	**	*	*
Y × T × Ir	*	NS	**	NS	NS
T × Cv × Ir	*	*	*	*	*
Y × Cv × Ir	*	*	*	*	*
Y × T × Cv × Ir	*	*	*	*	*

*: Significance at $P \leq 0.05$; **: Significance at $P \leq 0.01$; ***: Significance at $P \leq 0.001$.

Table 3. Effect of infestation by the charcoal rot fungus, *Macrophomina phaseolina*, on total phenol (g/100g), lignin (mg GAE/g), total isoflavones (µg/mg), N, P, K, Ca, Mg (%), Mn, B, Zn, Fe, and Cu (mg/kg) in seeds of irrigated soybean cultivars that are sensitive (DK 3964) or moderately resistant (AG 3905) to charcoal rot infestation in 2004 and 2005.

2004, Irrigated														
Source of variability	Infestation	Phenol	Lignin	Total isoflavones	N	P	K	Ca	Mg	Mn	B	Zn	Fe	Cu
DK 3964	NINF	4.3c	7.4d	1732c	5.4a	0.38c	1.1a	0.21c	0.32b	15.3c	33.2c	27.5b	89.5a	3.6c
AG 3905		5.8b	8.6c	1666d	4.3b	0.43b	1.2a	0.36b	0.38a	31.5b	39.5b	29.5b	84.3a	3.9c
DK 3964	INF	5.7b	9.5b	1845b	5.6a	0.39c	1.5a	0.23c	0.36a	17.5c	35.7c	28.5b	104a	4.3b
AG 3905		8.4a	13.6a	2343a	4.8b	0.65a	1.7a	0.47a	0.35a	38.3a	43.6a	38.6a	98.6a	7.8a
2005, Irrigated														
Source of variability	Infestation	Phenol	Lignin[b]	Total isoflavones	N	P	K	Ca	Mg	Mn	B	Zn	Fe	Cu
DK 3964	NINF	4.4c	6.5d	1453c	4.6a	0.31c	1.3c	0.25c	0.23b	17.5c	31.6b	25.4b	74.7c	4.6c
AG 3905		5.9b	7.1c	1542b	4.8a	0.46b	1.7b	0.34b	0.31a	31.6b	38.9b	24.8b	94.2a	5.3a
DK 3964	INF	5.3b	8.5b	1543b	4.9a	0.45b	1.9b	0.21c	0.26b	15.4c	38.5b	24.3b	80.5b	5.2b
AG 3905		7.6a	11.5a	1965a	5.1a	0.62a	2.4a	0.43a	0.32a	38.4a	53.5a	41.6a	91.6a	8.5a

The experiment was conducted at Jamie Whitten Delta States Research Center, Stoneville, MS. Means within a column separately followed by the same letter are not significantly different at the 5% level using Fisher's Least. Lignin was measured in the seed coat. NINF = noninfested; INF = infested.

both MR and S cultivars increased, but MR cultivars showed higher increase. For example, in S cultivar the increase of phenol, lignin, and isoflavone concentrations was 32.6%, 6.5%, and 28.4%, respectively, while the increase of phenol, lignin, and isoflavone concentrations in MR cultivar was 44.8%, 40.6%, and 58%, respectively. There were significant differences in phenol, lignin, and isoflavone concentrations between cultivars (**Table 3**). This pattern was shown in 2004 and 2005. However, in 2005 (**Table 3**), the concentration of isoflavones was lower than in 2004 due to higher temperature as described before by Mengistu *et al*. [23]. Seed minerals (K, P, Ca, Mn, B, Zn, and Cu) were higher in MR cultivar than in S cultivar under INF conditions. Seed N and Mg did not show differences between MR cultivar and S cultivar under INF conditions (**Table 3**). Mineral concentrations in seed were different between cultivars.

3.4. Effect of Charcoal Rot Infestation on Seed Phenol, Lignin, Total Isoflavones, and Minerals under Non-Irrigated Conditions

The concentrations of phenol, lignin, and isoflavones were higher in MR cultivar than in S cultivar (**Table 4**), although the concentration of isoflavones was significantly lower under non-irrigated conditions in both cultivars under NINF and INF conditions (**Table 4**). Both cultivars showed higher concentrations of phenol, lignin, and isoflavones under INF than under NINF conditions, although the response of MR cultivar to INF was significantly greater. For example, in S cultivar under INF conditions the concentrations of phenol, lignin, and isoflavones were 25%, 15%, 6.6%, respectively. However, the concentrations of phenol, lignin, and isoflavones in MR cultivar under INF were 66%, 72%, and 21.7%, respectively. Seed K, P, Ca, Mn, B, Zn, and Cu were higher in MR cultivar than in S cultivar in INF. Seed N, P, B, Zn, and Cu decreased in S cultivar in INF compared with NINF. This observation was observed only under non-irrigated conditions in both years. In MR cultivar, however, the concentrations of N and P in seed were maintained, and the concentrations of B, Zn, and Cu increased in MR cultivar in INF (**Table 4**). Generally, the level of minerals was lower in non-irrigated than in irrigated (**Table 3** and **Table 4**).

3.5. Effect of Charcoal Rot Infestation on Seed Sugars under Irrigated and Non-Irrigated Conditions

Seed glucose and fructose concentrations were higher in both MR and S cultivars in response to charcoal rot

Table 4. Effect of infestation by the charcoal rot fungus, *Macrophomina phaseolina*, on total phenol (g/100g), lignin (mg GAE/g), total isoflavones (µg/mg), N, P, K, Ca, Mg (%), Mn, B, Zn, Fe, and Cu (mg/kg) in seeds of non-irrigated soybean genotypes that are sensitive (DK 3964, S) or moderately resistant (AG 3905, MR) to charcoal rot infestation in 2004 and 2005.

2004, Non-irrigated														
Source of variability	Infestation	Phenol	Lignin	Total isoflavones	N	P	K	Ca	Mg	Mn	B	Zn	Fe	Cu
DK 3964	NINF	5.4d	7.3c	1364c	4.3a	0.21c	1.1c	0.30c	0.28b	13.5c	32.6c	19.5b	68.5a	2.5b
AG 3905		7.4b	7.9b	1432b	4.9a	0.34b	1.5b	0.37b	0.35a	29.7b	37.1b	20.6b	65.4a	2.9b
DK 3964	INF	6.8c	8.4b	1454b	3.1b	0.12d	0.78d	0.36b	0.21b	12.5c	21.5d	13.7c	71.5a	2.7b
AG 3905		12.3a	13.6a	1743a	4.7a	0.47a	2.0a	0.53a	0.37a	35.0a	46.3a	32.6a	69.5a	5.8a
2005, Non-irrigated														
Source of variability	Infestation	Phenol	Lignin[b]	Total isoflavones	N	P	K	Ca	Mg	Mn	B	Zn	Fe	Cu
DK 3964	NINF	4.6c	5.7c	1124c	4.0b	0.25b	0.87b	0.24c	0.21c	13.2c	33.2a	18.5b	56.4b	2.0b
AG 3905		5.4b	6.8b	1254b	4.8a	0.36a	1.1a	0.32b	0.34a	30.1b	27.4b	21.4b	62.4a	3.1a
DK 3964	INF	5.4b	4.2d	1025d	3.1b	0.13c	0.73c	0.21c	0.27b	10.1c	21.1c	11.4c	47.5c	1.1c
AG 3905		7.8a	8.6a	1543a	4.7a	0.39a	1.5a	0.49a	0.36a	35.6a	35.4a	27.6a	59.4a	3.5a

The experiment was conducted at Jamie Whitten Delta States Research Center, Stoneville, MS. Means within a column separately followed by the same letter are not significantly different at the 5% level using Fisher's Least. Lignin was measured in the seed coat. NINF = noninfested; INF = infested.

infestation (**Table 5**). Seed sucrose concentration was lower in INF in both MR and S cultivars, although the concentration in MR cultivar was higher than in S cultivar. Seed raffinose and stachyose were higher in INF than in NINF in both cultivars, opposing the trend of sucrose. Glucose, fructose, sucrose, raffinose concentrations were different between cultivars under INF or NINF conditions, but this difference depended on year (**Table 5**). This general trend was shown in 2004 and 2005 under INF or NINF. However, glucose and fructose concentrations were higher in INF than NINF, especially under non-irrigated conditions in 2004 and 2005 (**Table 6**), opposing sucrose trend. Raffinose and stachyose concentrations were higher in INF than NINF as opposed to sucrose.

4. Discussion

4.1. Phenolics and Resistance to Charcoal Rot

The higher concentration of phenolics (phenol, lignin, and isoflavones) in the MR cultivar than in the S cultivar in NINF and INF under irrigated and non-irrigated indicated that phenolics may have a possible role in resistance against charcoal rot. Previous research showed that lignin content can be altered by biotic (fungi, bacteria, and virus) and abiotic stress (heat, drought, and mineral deficiencies) [29]. The increase of phenolics in MR cultivar and its ability to maintain higher levels of phenolics under INF and NINF may reflect a possible mechanism to provide cell wall and seed coat with structural support and integrity against disease infection and drought stress. It was reported that phenolics, including lignin, are major compounds of cell wall and cell integrity, giving rigidity and impermeability properties [33].

The effects of charcoal rot infestation on seed composition and seed phenolics were investigated, and found that there were no significant differences in seed protein levels when MR genotype DT97-4290 was grown under infested or non-infested conditions under irrigated and non-irrigated [22]. On the other hand, Pharoah and Egyptian (S genotypes) did not maintain seed protein and linolenic fatty acid levels under infested conditions compared with non-infested conditions [22] [66]. Also, charcoal rot moderately resistant genotypes DT97-4290 and AG 3905, and their maturity equivalent susceptible genotypes Egyptian and DK 3964 were evaluated, and found that MR genotype had the lowest charcoal rot infection compared with the other genotypes, resulting in better seed quality [22] [65] and production [23] [65]. Our results support the previous research in that lignin could be related with mechanical support, water transport, and defense against pathogens [33] [34], and lignin deposition in the seed coat tissue provides mechanical resistance and cell wall protection against microorganisms.

Table 5. Effect of infestation by the charcoal rot fungus, *Macrophomina phaseolina*, on sugars (mg/g) in seeds of irrigated soybean genotypes that are sensitive (DK 3964) or moderately resistant (AG 3905) to charcoal rot infestation in 2004 and 2005.

2004, Irrigated						
Source of variability	Infestation	Glucose	Fructose	Sucrose	Raffinose	Stachyose
DK 3964	NINF	2.5c	0.78c	43.1b	5.4c	46.3b
AG 3905		3.6b	0.62d	55.7a	4.6d	38.5c
DK 3964	INF	3.7b	1.2b	34.6c	6.5b	57.4a
AG 3905		4.6a	1.7a	46.1b	7.8a	58.5a
2005, Irrigated						
Source of variability	Infestation	Glucose	Fructose	Sucrose	Raffinose	Stachyose
DK 3964	NINF	2.4b	0.87c	42.5b	5.4c	46.3b
AG 3905		1.3c	0.79d	51.3a	4.6d	38.5c
DK 3964	INF	3.5a	1.3b	31.2d	6.5b	42.5b
AG 3905		3.2a	2.1a	39.7c	7.8a	56.3a

[a]The experiment was conducted at Jamie Whitten Delta States Research Center, Stoneville, MS. Means within a column separately followed by the same letter are not significantly different at the 5% level using Fisher's Least. NINF = noninfested; INF = infested.

Table 6. Effect of infestation by the charcoal rot fungus, *Macrophomina phaseolina*, on sugars (mg/g) in seeds of non-irrigated soybean genotypes that are sensitive (DK 3964) or moderately resistant (AG 3905) to charcoal rot in 2004 and 2005.

2004, Non-irrigated						
Source of variability	Infestation	Glucose	Fructose	Sucrose	Raffinose	Stachyose
DK 3964	NINF	1.5d	1.1a	45.3b	4.5d	30.6c
AG 3905		2.5c	0.92b	57.3a	5.2c	42.6b
DK 3964	INF	3.4b	1.7a	37.6c	6.4b	47.8b
AG 3905		4.7a	1.5a	48.4b	7.3a	56.3a
2005, Non-irrigated						
Source of variability	Infestation	Glucose	Fructose	Sucrose	Raffinose	Stachyose
DK 3964	NINF	3.4d	0.94b	36.5c	4.6c	41.6b
AG 3905		4.2c	0.82c	49.7a	3.6d	38.6c
DK 3964	INF	5.7b	1.5a	32.5c	5.8b	47.6b
AG 3905		6.6a	1.9a	43.5b	6.6a	51.5a

The experiment was conducted at Jamie Whitten Delta States Research Center, Stoneville, MS. Means within a column separately followed by the same letter are not significantly different at the 5% level using Fisher's Least. NINF = noninfested; INF = infested.

When six soybean cultivars were investigated, it was found that the cultivars Doko and Parana had higher content of lignin and peroxidase activities compared with cultivars Savana, Paranagoiana, FT-10, and Santa Rosa, indicating that phenolics, including phenol, lignin, and isoflavones, are associated with defense against charcoal rot [35] [36].

The higher concentrations of total isoflavones in MR cultivar than in S cultivar in NINF and INF under irrigated and non-irrigated indicated also possible association between charcoal rot resistance and isoflavones levels. Our findings are supported by our previous research on charcoal rot and phomopsis seed decay where soybean genotypes resistant to *Phomopsis* or moderately resistant to charcoal rot showed higher phenol, lignin, and total isoflavones compared with susceptible genotypes [18] [67]. This is also in agreement with previous research, indicating that isoflavones act as a repellant against pathogens [68]. It was reported that the phytoalexins, a class to which isoflavones belong to, are synthesized in plant cells as a response to plant infection by the fungus and a result of the interaction between the host and the pathogen [69]. This interaction between the fungus and the host leads to phytohormones production and *de novo* expression of the enzymes involved in their biosynthetic pathway [69]. It was shown that after infection, a rapid accumulation of phytoalexins was observed in response to interaction with *Phytophthora megasperma* [39]. Daidzeinisoflavone, a phytoalexin, was an immediate precursor of the glyceollins and conjugates of daidzein, and was rapidly hydrolyzed to free daidzein during the interactions between soybean and *Phytophthora megasperma* f. sp. *glycinea*. Our previous research indicated that individual isoflavones (daidzein, genistein, and glycitein) were higher in genotypes resistant to *Phomospsis* or moderately resistant to charcoal rot compared with susceptible genotypes [18] [67]. It is still not clear how phytoalexins, including isoflavones are involved in the defense in response to the pathogen attack. Although higher accumulation of isoflavones was observed in MR genotype than in S genotypes in the current research, the mechanisms by which isoflavones are involved in the defense mechanism remain unclear, and further research is needed.

4.2. Minerals and Resistance to Charcoal Rot

The higher levels of K, P, Ca, Mn, B, Zn, and Cu in NINF and INF under irrigated and nonirrigated conditions in MR cultivar indicated a possible role of these specific minerals in disease resistance. Although resistance is genetically controlled, mineral nutrition can play a major role in resistance or tolerance to pathogens [37] [38]. It was reported that the effect of mineral nutrition is substantial in moderately susceptible cultivars, but lower in highly susceptible or resistant ones [37]. This may be because some minerals such B, Mn, and Cu play a major

role in phenolics and lignin synthesis [37] [39] and in cell wall and membrane integrity. It was found that K and Zn deficiencies resulted in high cell wall leakage of sugar and amino acid concentrations to leaf apoplast, allowing the pathogen to penetrate the cell [38] [40]. Also, it was found that B deficiency led to higher fungal infection and low Ca content in plant tissue, resulting in cell wall leakage of sugars and amino acids from cytoplasm to apoplast [41]. Calcium has a role in stability of cell wall through polygalacturonates and in the activity of enzymes like polygalacturonase which dissolves the middle lamella, and both of these two processes were found to be inhibited by Ca deficiency. Copper was found to inhibit diseases, as Cu deficiency led to impairment of defense compound synthesis, accumulation of soluble carbohydrates, and reduced lignin synthesis, resulting in lower disease resistance [38].

The mechanisms of minerals or phenolics involvement in the disease resistance against the fungus or toxin is not known, but possible explanations can be put forward as follows. Minerals such as Ca, B, Mn, Cu, and phenolics are involved in cell wall integrity and cell membrane permeability and integrity [37]. Biotic or abiotic stress factors that alter the balance of mineral uptake and translocation, phenolics metabolism and lignin synthesis can change the membrane phospholipids and affect cell wall and cell membrane integrity, enhancing the fungal penetration [37]. For example, the higher level of B in MR cultivar than in S cultivar suggests that B may have a role in resistance because of its role in cell membrane and cell wall integrity [18] [67] [70] [71] and its indirect effects on phenol metabolism and lignin biosynthesis [37]. Previous research showed that there is an association between B and phenolics metabolism, affecting disease resistance [18] [37] [67]. Boron has structural and metabolic role [37] and is involved in growth [72], seed quality [73], sugar transport and carbohydrates metabolism, cell wall synthesis, lignification, cell wall structure, RNA metabolism, respiration, indole acetic acid metabolism, phenolics metabolism, membrane integrity [74], ascorbate metabolism [75] and oxygen activation [37]. Previous research indicated that B is involved in formation of boron-pectin complexes [42] [71] [76] and plasma membrane integrity [37] [74]. It is suggested that plant resistance to pathogens occurs when adequate mineral nutrition and regular biosynthesis of phenolics are satisfied. Under these conditions, when a pathogen interacts with the resistant plant, the defense mechanism and signaling system are turned on to respond to the possible pathogen infection. The defense mechanism is achieved at two levels: mechanical at the cell wall level; and biochemical and molecular through a signaling system and gene expression. At the cell wall level, the plant deploys reactive oxygen species (such as superoxide and hydrogen peroxide) and mechanical strength of cell wall (such as lignin) to stop and prevent the pathogen from spreading. If the pathogen succeeds in penetrating the cell, using its toxin or toxin-containing enzymes to degrade the cell wall, then another level of resistance involves a signaling system that is capable of degrading pathogen toxin and toxin-containing enzymes.

4.3. Sugars and Resistance to Charcoal Rot

The higher levels of glucose and fructose in both R and S cultivars in INF indicated that these monosaccharides accumulated as a response to disease stress. The decrease of sucrose in INF in both cultivars may be due to the accumulation of glucose and fructose as these monosaccharides are the building block of sucrose. The increase of raffinose and stachyose in INF and under non-irrigated conditions suggested that both raffinose and stachyose may have a role in disease resistance and drought stress tolerance [77]. Although the biological functions of raffinose and stachyose are not well understood [78], oligosaccharides (sucrose, raffinose, and stachyose) contribute to seed quality [79], the acquisition of desiccation tolerance during seed development and maturation and protection of seeds against damage during seed dehydration and aging. Soybean seed raffinose and stachyose are undesirable constituents because they have negative effects on the nutritive value of soymeal and are indigestible by humans and animals, causing flatulence or diarrhea in nonruminants [80]. The soybean seed industry is interested in soybean seed with low raffinose and stachyose levels [81], and a high level of seed glucose, fructose, and sucrose is desirable because it improves the taste and flavor of soy-based products such as tofu, soymilk, and natto [82].

5. Conclusion

Our research demonstrated that phenolics, including phenol, lignin, and isoflavones, and specific minerals, including K, Ca, Mn, B, Zn, and Cu, may significantly contribute to plant defense by preventing pathogen entrance at the cell wall level through cell wall lignin-pectin complex and cell wall mechanical resistance. The increase of glucose and fructose in INF under irrigated and non-irrigated indicated that these sugars accumulated

due to the lack of conversion to sucrose. The increase of raffinose and stachyose in INF under irrigated and non-irrigated may indicate a significant role of these sugars in disease resistance and drought tolerance during seed maturation. Further research is needed for charcoal rot resistance selection, and to develop a germplasm of near-isogenic lines differing in levels of resistance to charcoal rot, but with similar genetic background to avoid genotype confounding. The negative effects of charcoal rot infection and drought on phenolics, minerals, and sugars in susceptible cultivars present a seed quality challenge.

Acknowledgements

We thank Sandra Mosley for lab assistance. This research was funded by United States Department of Agriculture, Agricultural Research Service project number 6402-21220-012-00D. The US Department of Agriculture (USDA) is an equal opportunity provider and employer. Mention of trade names or commercial products in this publication is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the US Department of Agriculture.

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