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Effect of fungal damage by *Fusarium* spp and *Diaporthe/Phomopsis* complex on protein quantity and quality of soybean seed

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Abstract: Seeds from three soybean genotypes were tested to evaluate the effect of fungal damage by *Fusarium* spp and *Diaporthe/Phomopsis* complex on protein quantity and quality. Fungus-infected seeds had higher protein contents than uninfected ones. A selective degradation of soluble proteins was detected in seeds infected with either fungus. Some of the storage proteins degraded were identified as α', α and β subfractions of the β-conglycinin, and A_3 subfraction of the glycinin. Furthermore, reductions in lipoxygenase and trypsin inhibitor activities were observed in fungus-infected seeds. Amino acid composition did not vary between infected and uninfected seed lots, so protein degradation should not affect amino acid structures.

Keywords: soybean; fungal damage; protein content; protein quality

INTRODUCTION

Soybean is an abundant and economical source of high-quality vegetable protein in many countries. Soybean genotypes cultivated in Argentina have 40% protein on average.[1–3] Protein quantity and quality of soybean seeds are determined by genotype and may vary depending on the environment. Furthermore, genotype and environment are known to play a critical role in fungal disease development.[4–6]

Fungal damage in soybeans usually implies poor seed quality.[7–10] Although seed quality may be interpreted in many ways, some of the changes produced by fungi can lead to both nutritional and functional deterioration, and ultimately to loss of commercial value. Among the changes caused by fungi is the one associated with seed proteins.

In recent years, on the central fringe of the Argentinean soybean growing area, due to favorable environmental conditions for development of *Fusarium* spp and *Diaporthe/Phomopsis* seed infection, a high incidence of these pathogens has been observed.[11]

This research was performed to evaluate the effect of fungal infection by pathogens from the *Diaporthe/Phomopsis* complex and *Fusarium* spp on seed protein quantity and quality of some selected soybean cultivars. A further aim of this study was to establish whether differences in total and soluble seed proteins could be explained by differences in amino acid composition.

MATERIALS AND METHODS

Plant material and experimental design

The experiment was conducted at Manfredi Experimental Station, INTA, Córdoba Province, Argentina. Three soybean genotypes (A 4100, Eureka 51 and FH 6686) were planted in a randomized block design with three replicates (30 plants each). Seeds of each cultivar/replicate combination were hand harvested separately, at maturity, and were tested for seed-borne fungi. The procedure used was a modification of that described by Kmetz *et al*.[12] For each cultivar/replicate combination, a 100 seed sample was randomly chosen and placed in 10 cm plastic culture plates containing potato–dextrose agar with four seeds per plate. Seeds were incubated under 12 h light and dark at room temperature. Seed-borne fungi were identified based on morphological characteristics at 14 days after planting.

Soybean seeds with and without characteristic symptoms induced by *Fusarium* spp or *Diaporthe/Phomopsis* complex were selected. These lots of symptomatic and asymptomatic seeds were surface-disinfected separately in 0.5% NaOCl for 5 min, then oven-dried (40 °C) under vacuum and stored until analysis.

Analytical methods

Total protein content was determined by the Kjeldahl method as $\%N \times 6.25$.[13] For soluble protein,

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lipoxygenase, trypsin inhibitor and amino acid analyses, 10 g of dried seed sample from each cultivar/replicate/treatment combination were pulverized in a standard mill, then delipified with *n*-hexano at room temperature, and finally stored at −10 °C in polyethylene bags.

Soluble proteins were extracted according to Zimmerman and Vick.[14] The extracted proteins were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) using a one-dimensional vertical slab gel containing 3% stacking gel and 10% separating gel. For comparison, proteins of known molecular weight, e.g. bovine serum albumin, pepsin, trypsinogen and lysozyme, were subjected to electrophoresis under identical conditions. Protein bands were also identified from the literature.[10,15] SDS-PAGE gels stained with Coomassie Brilliant Blue R-250 were scanned using an optical densitometry system according to Kim and Barbeau.[16]

Lipoxygenase activity was measured by spectrophotometric determination[17] and it was expressed as an optical density increase per mg protein per min. For trypsin inhibitor activity (TIA) determinations, the method of Liu and Markakis[18] was followed. TIA was expressed as TUI (trypsin units inhibited) per mg sample, where 1 TU is defined as 0.01 A_{410} under the assay conditions of the proposed method (pH 8.1, 37 °C). Lipoxygenase activity and TIA determinations were performed for each fungus as described previously.

For amino acid (AA) determinations, samples (50 mg) were hydrolyzed in 20 ml of 6 M HCl at 110 °C for 24 h. The mixture was then centrifugated, the supernatant was filtered and the pH was adjusted according to Alonso *et al*.[19] Extracts (1 ml) from each sample were derivatized and analyzed by HPLC according to the standard procedures described previously.[19] Tryptophan was not determined. Limiting AA determination was in agreement with the reference pattern established by the FAO/WHO.[20] Calculation of AA score was carried out by comparison of the AA composition of samples with that of the reference pattern (FAO/WHO):

$$\left(100 \times \frac{\begin{array}{c}\text{concentration of the most}\\ \text{limiting AA in sample (mg g}^{-1}\text{ protein)}\end{array}}{\begin{array}{c}\text{concentration of the AA in FAO/WHO}\\ \text{(pattern mg g}^{-1}\text{ protein)}\end{array}} \right)$$

Statistical analysis

Statistical differences between symptomatic and asymptomatic seed lots from each cultivar were estimated from an ANOVA test at the 5% level ($p = 0.05$) of significance. Whenever ANOVA indicated significant difference, a pair-wise comparison of means by least significant difference (LSD) was carried out.[21]

RESULTS

Damaged seeds were rarely infected by a single organism. An interaction between *Fusarium* spp and fungi of the *Diaporthe/Phomopsis* complex has been detected in many soybean growing areas from Argentina.[22] Those findings have been confirmed and, in addition, it was found that the fungi most frequently recovered were *Fusarium semitectum*, *Phomopsis longicolla* and *Diaporthe phaseolorum* var *sojae*. There were significant differences in field fungal incidence among the soybean genotypes studied. The mean percentages of fungal incidence were as follows: 31.3% for A 4100 cultivar; 40.3% for Eureka 51 cultivar; and 4.3% for FH 6686 cultivar.

Protein content differed significantly between infected and uninfected seed lots (Table 1). In all cultivars there was a consistent increase in protein content from seed lots infected with *Fusarium* spp and *Diaporthe/Phomopsis* complex. When all cultivars were considered, the fungus-infected seed lots showed an increase of 16.6% protein on an average.

The bulk of the proteins examined by SDS-PAGE (Fig 1) were storage proteins, which are the major constituents of seeds proteins.[23] The more intense bands which occurred in all cultivars belonged to this category. The patterns produced from extracts of different seed samples were compared visually on the basis of differences in relative intensity or presence/absence of specific bands. Since we used saline buffer solution for extraction of seed proteins, the majority of the bands were probably due to globulins. The 7 s (β-conglycinin) and 11S (glycinin) globulins constitute the main storage proteins of soybean seed.[15] The protein profile of infected seeds from A 4100 and Eureka 51 cultivars showed severe degradation of polypeptides at molecular weights of 58, 52 and 42 kDa which were tentatively identified as α', α and β subfractions of the β-conglycinin. In

Table 1. Protein contents (g kg^{-1}, dry basis), lipoxygenase (Lox, Δ OD mg^{-1} protein min^{-1}) and trypsin inhibitor (TIA, TUI, mg sample^{-1}) activities from asymptomatic (1) or symptomatic (2) seed lots with *Fusarium* spp and *Diaporthe/Phomopsis*

	Cultivar					
	A 4100		EUREKA 51		FH 6686	
Parameter	1	2	1	2	1	2
Protein content	$296.1^{a} \pm 6.2$	$354.4^{b} \pm 11.5$	$309.3^{a} \pm 13.1$	$338.6^{b} \pm 11.5$	$354.5^{a} \pm 7.0$	$428.7^{b} \pm 5.9$
Lox	$4.46^{a} \pm 0.6$	$1.32^{b} \pm 0.2$	$6.34^{a} \pm 0.7$	$0.94^{b} \pm 0.1$	$6.41^{a} \pm 0.2$	$1.15^{b} \pm 0.1$
TIA	$98.1^{a} \pm 6.2$	$43.4^{b} \pm 4.8$	$64.0^{a} \pm 11.0$	$64.7^{a} \pm 9.4$	$90.4^{a} \pm 3.7$	$72.3^{b} \pm 5.0$

Mean values ± standard deviations ($n = 3$). [ab] Significant difference ($p = 0.05$) between 1 and 2 for each cultivar.

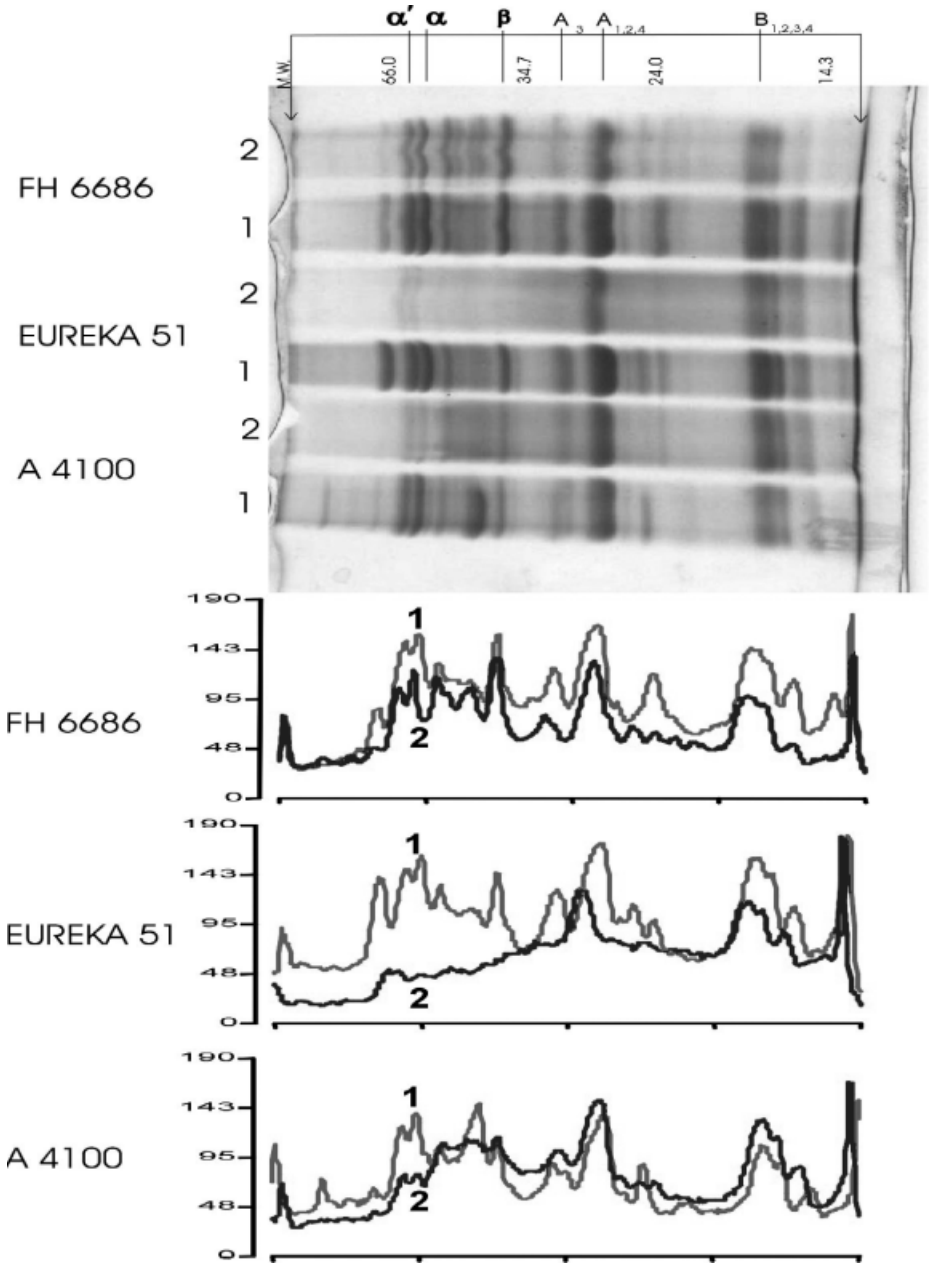

Figure 1. SDS-PAGE profiles and densitometer scans of soybean proteins from asymptomatic (1) or symptomatic (2) seed lots with *Fusarium* spp and *Diaporthe/Phomopsis*. MW, molecular weight markers (in kilodaltons): bovine serum albumin (MW 66.0), pepsin (MW 34.7), trypsinogen (MW 24.0) and lysozyme (MW 14.3).

addition, selective degradation of the A_3 subfraction (glycinin fraction) was observed in both A 4100 and Eureka 51 cultivars. There were minor differences in protein profiles between infected and uninfected seeds from FH 6686 cultivar. In a field trial,[22] this cultivar had reduced fungal incidence and severity. In this work, although the seed samples of symptomatic seed lots were chosen to obtain a 100% fungal incidence, it was noteworthy that fungal severity in the FH 6686 cultivar was lower than in the other two cultivars, resulting in a negligible degradation of the seed protein profile in the former cultivar.

In addition to storage proteins, other seed proteins also underwent changes as a result of fungal damage (Table 1). In all cultivars studied, extracts from infected seeds showed a significant decrease in lipoxygenase activity as compared with uninfected controls. Similarly, the lowest TIA values were observed in samples from infected seeds. Neither lipoxygenase activity nor TIA were detected in the mycelia of the fungi.

The AA profiles of the three soybean cultivars studied (Table 2) appeared to be very similar in composition. The following features were found to be common to seed samples of both treatments in all cultivars: (1) with the exception of tryptophan, all essential AA were found; (2) threonine and sulfur-containing AA (methionine and cysteine) were the most significant limiting AA; and (3) among the non-essential AA, the most abundant (>100 mg/g protein) were glutamic acid, alanine and proline.

Table 2. Amino acid composition (mg g^{-1} sample) and amino acid score from asymptomatic (1) or symptomatic (2) seed lots with *Fusarium* spp and *Diaporthe/Phomopsis*

	Cultivar					
	A 4100		EUREKA 51		FH 6686	
Amino acid	1	2	1	2	1	2
Essential						
Histidine	$27.4^{a} \pm 2.6$	$30.8^{a} \pm 2.6$	$29.5^{a} \pm 2.7$	$26.3^{a} \pm 1.1$	$27.7^{a} \pm 3.2$	$30.2^{a} \pm 1.5$
Threonine	$31.4^{a} \pm 1.4$	$32.0^{a} \pm 0.6$	$29.8^{a} \pm 0.6$	$30.7^{a} \pm 1.3$	$30.6^{a} \pm 0.8$	$30.3^{a} \pm 2.1$
Tyrosine	$25.9^{a} \pm 1.7$	$25.5^{a} \pm 2.8$	$27.6^{a} \pm 3.6$	$33.5^{a} \pm 2.4$	$29.6^{a} \pm 2.2$	$29.1^{a} \pm 4.6$
Valine	$55.1^{a} \pm 0.7$	$50.5^{a} \pm 1.9$	$53.9^{a} \pm 2.0$	$61.5^{b} \pm 3.3$	$52.8^{a} \pm 1.0$	$52.2^{a} \pm 1.3$
Methionine + cysteine	$16.9^{a} \pm 3.5$	$11.3^{a} \pm 2.8$	$26.4^{a} \pm 0.5$	$26.9^{a} \pm 0.9$	$25.5^{a} \pm 2.4$	$22.0^{a} \pm 4.4$
Isoleucine + leucine	$122.4^{a} \pm 6.2$	$122.4^{a} \pm 5.6$	$121.3^{a} \pm 4.2$	$125.0^{a} \pm 2.4$	$113.6^{a} \pm 6.3$	$114.6^{a} \pm 4.9$
Phenylalanine	$46.8^{a} \pm 7.2$	$47.6^{a} \pm 6.6$	$34.9^{a} \pm 3.8$	$37.7^{a} \pm 1.9$	$36.5^{a} \pm 1.5$	$43.3^{a} \pm 7.8$
Lysine	$97.2^{a} \pm 4.9$	$92.4^{a} \pm 12.4$	$81.5^{a} \pm 10.7$	$77.0^{a} \pm 10.2$	$114.5^{a} \pm 5.0$	$102.6^{a} \pm 10.8$
Non-essential						
Aspartic acid	$35.6^{a} \pm 4.8$	$38.0^{a} \pm 3.2$	$37.0^{a} \pm 7.8$	$20.4^{b} \pm 3.6$	$25.0^{a} \pm 7.2$	$28.0^{a} \pm 8.4$
Glutamic acid	$105.9^{a} \pm 3.2$	$109.9^{a} \pm 9.2$	$128.1^{a} \pm 0.6$	$81.6^{b} \pm 11.8$	$88.4^{a} \pm 10.4$	$83.0^{a} \pm 6.9$
Serine	$58.0^{a} \pm 1.5$	$60.0^{a} \pm 3.2$	$58.2^{a} \pm 2.7$	$55.0^{a} \pm 3.0$	$59.5^{a} \pm 2.6$	$58.4^{a} \pm 1.7$
Glycine	$89.8^{a} \pm 2.7$	$89.5^{a} \pm 2.1$	$97.1^{a} \pm 8.3$	$101.4^{a} \pm 3.2$	$93.3^{a} \pm 6.9$	$91.4^{a} \pm 6.4$
Arginine	$58.2^{a} \pm 2.7$	$59.4^{a} \pm 1.1$	$55.4^{a} \pm 1.1$	$57.1^{a} \pm 2.5$	$56.9^{a} \pm 1.5$	$56.2^{a} \pm 3.9$
Alanine	$125.4^{a} \pm 0.9$	$116.1^{a} \pm 8.7$	$124.3^{a} \pm 3.2$	$117.6^{a} \pm 4.0$	$118.2^{a} \pm 2.4$	$120.6^{a} \pm 1.1$
Proline	$108.0^{a} \pm 12.3$	$98.1^{a} \pm 10.4$	$119.3^{a} \pm 22.0$	$135.8^{a} \pm 3.1$	$110.4^{a} \pm 7.0$	$104.5^{a} \pm 4.9$
Amino acid score	$42.6^{a} \pm 3.6$	$28.9^{b} \pm 7.9$	$75.5^{a} \pm 1.4$	$76.6^{a} \pm 2.7$	$76.8^{a} \pm 0.9$	$56.4^{b} \pm 8.7$

Mean values ± standard deviations ($n = 3$). [ab] Significant difference ($p = 0.05$) between 1 and 2 for each cultivar.

The results obtained indicated that the effect of fungal damage on soybean seed AA composition was not statistically significant, except for valine, and aspactic and glutamic acids in the Eureka 51 cultivar. However, AA score showed a significant decrease in fungus-infected seeds from A 4100 and FH 6686 cultivars.

DISCUSSION AND CONCLUSIONS

The effect of fungal damage on soybean seed protein is not clear and discrepancies have occurred between data from laboratory and field tests. Wilson *et al*[9] postulated that soybean seeds present a linear increase in protein concentration in direct proportion to the level of fungal damage. On the other hand, Katsube[24] and Park *et al*[25] reported that soybean seeds severely infected with *Cercospora kikuchii* did not differ in protein content with respect to uninfected ones. Finally, Fábrega *et al*[10] showed that plants inoculated with *D phaseolorum* var *sojae* had significantly reduced protein content compared with those that were not inoculated. Our study using soybean seeds with 100% fungal incidence by *Fusarium* spp and fungi of the *Diaporthe/Phomopsis* complex showed that seed infection produced a remarkable increase in total protein content. However, this condition may be attributed to loss of seed carbohydrates.[8]

From the SDS-PAGE and densitometric analyses, it is apparent that the soybean proteins were hydrolyzed, resulting in general protein degradation. Furthermore, the densitometer scans of the fungus-infected seeds indicated that high-molecular-weight proteins were the most affected. The qualitative and quantitative changes in β-conglycinin and glycinin storage proteins of *Fusarium* spp and *Diaporthe/Phomopsis* infected seeds may affect the quality of soybean by-products. For example, it was observed that quantitative variations in glycinin affected textural characteristics, gelling time, sulfur and nitrogen contents, hardness of tofu gels and curd formation.[26]

The decrease in lipoxygenase activity of infected seeds could be a result of the degradation of this enzyme by effect of the fungi. Similar results were observed in seed coats of seeds infected with *C kikuchii*.[27]

At present, there are no published data about the effect of fungal damage on trypsin inhibitor activity. The results obtained in this work suggest, as proposed for lipoxygenase activity, some grade of degradation of these protease inhibitors.

Reductions in lipoxygenase activity in fungus-infected seeds could be interpreted as an apparent benefit since elimination or inactivation of lipoxygenase contribute to eliminate undesirable flavors in soybean protein preparations.[15,28,29] However, fungus-damaged soybeans are known to produce oils with lower oxidative stabilities and poor flavors,[9,10] because of which the significance of reduced lipoxygenase values is not clear.

The slight variability among treatments in individual AA contents indicates that protein degradation observed in SDS-PAGE gel could be due to a hydrolytic process in the presence of biologically active fungal components that do not affect AA structures. Although these facts should not affect the

AA bioavailability, the above-mentioned findings must be confirmed with *in vivo* or *in vitro* assays.

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