Detection of Asymptomatic Fungal Infections of Soybean Seeds by Ultrasound Analysis

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ABSTRACT

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Different levels of asymptomatic, seed-borne infection by storage fungi (*Aspergillus* and *Penicillium* spp.) or *Phomopsis* seed decay (PSD) (*Phomopsis longicolla*, *Diaporthe phaseolorum* var. *sojae*, and *D. phaseolorum* var. *caulivora*) were induced in sub-lots of separate soybean seed lots by incubation of seeds or pods, respectively, for different times at 25°C and at a relative humidity >95%. Seeds were then air-dried to a constant moisture content in the laboratory atmosphere, and each sub-lot was tested for incidence of infection, germination, and moisture content. Individual seeds in each sub-lot also were dropped 10 cm onto a transducer in an ultrasound analyzer. The average peak value of the ultrasound signals for each sub-lot, which indicates the weight of seeds, decreased linearly as the incidence of seed infection by storage fungi ($r^2 = 0.85$) or PSD ($r^2 = 0.82$) increased. The slope and width of the signal, which indicates seed softness, increased as seed infection increased for both groups of fungi, although coefficients of determination were lower (r^2 ranged from 0.42 to 0.59). Germination values, which decreased as seed infection for both pathogens increased, showed similar but inverse relationships to ultrasound parameters. Peak values of ultrasound signals decreased, and slope and width increased, as seed moisture content increased for sub-lots of soybeans at three levels of infection by *Phomopsis* seed decay. The potential for ultrasound technology to identify soybean seeds with asymptomatic infections of seed-borne pathogens was thus established.

Additional keywords: acoustic waves, seed pathology, seed processing, seed quality

Seed conditioning (or seed processing) is a practice used to size and grade seeds and to remove foreign matter, such as soil, weed, or crop parts from harvested seed lots. It can also remove propagules of plant pathogens or diseased seeds. Examples include separation of sclerotia of *Sclerotinia sclerotiorum* and *Phomopsis*-infected seeds and plant debris from soybean seed lots passed over air-screen cleaners and gravity tables (12). Paulsen (11) also utilized a computer vision system to detect purple-stained soybean seed infected with *Cercospora kikuchii* with a 91% degree of accuracy. Seed conditioning equipment can identify and eliminate these materials from a seed lot because it is sensitive to particular physical attributes such as size, shape, color, density, or texture of the seeds (3). Conventional equipment cannot

remove diseased seeds that have no clear-cut physical changes. These asymptomatic seeds, however, can still have adverse effects on seed germination and vigor or allow diseases to be transmitted to the crops grown from these seeds.

A recently developed ultrasound analyzer system (10,13) can assess soybean seed quality based on parameters of the ultrasound signal generated by a seed dropped onto a transducer. The peak of the sound wave indicates weight of the seed; whereas the slope and width indicate softness. Seed weight and softness are physical characteristics that can be affected by plant pathogens. Storage fungi infection, caused by *Aspergillus* and *Penicillium* spp., and *Phomopsis* seed decay (PSD), caused by *Phomopsis longicolla* T.W. Hobbs, *Diaporthe phaseolorum* (Cooke & Ellis) Sacc. var. *sojae* (S.G. Lehman) Wehmeyer, and *D. phaseolorum* var. *caulivora* (K.L. Athow & R.M. Caldwell), are economically important diseases of soybean seeds that can cause asymptomatic infection of seeds and can seriously impact germination and vigor (4,7,8). The objective of this study was to investigate the ability of ultrasound analysis to detect asymptomatic infection of soybean seed infection by these pathogens and thus to determine its potential as a means of removing asymptomatic seeds from seed lots during conditioning.

MATERIALS AND METHODS

Production of infected seeds. Different levels of infection by storage fungi were generated in sub-lots of a soybean seed lot (cultivar A2358) that had a low incidence (<1%) of seeds with visible blemishes and a standard warm germination value of 93.5% (1). Five 500-g sub-lots of seeds were placed on enamel trays and stacked at equal spacing in an airtight germination cart in which the relative humidity was maintained at 95% by the presence of five trays with paper wadding (Kimpac, Kimberly-Clark, Roswell, GA) saturated with a 4:1 (vol/vol) water-glycerol mixture. The cart was kept in a dark room maintained at 25°C, and one sub-lot was removed after 0, 10, 20, 30, and 40 days. As each sub-lot was removed, the seeds were air-dried on paper towels until the seed moisture content equilibrated to the ambient relative humidity (40 to 60%) of the laboratory. Seed moisture content of each sub-lot was measured on 15-g samples by a standard oven method (6).

The incidence of storage fungi infection generated in each incubation treatment was determined by surface sterilizing 100 seeds from each sub-lot in 5% sodium hypochlorite solution for 1 min, rinsing in sterile water for 10 s, and incubating in the dark at 25°C for 14 days on malt salt agar. Seeds from which colonies of storage fungi grew were counted. A standard warm germination test (1) was carried out on another 400 seeds from each sub-lot.

To obtain seed lots with different levels of infection by PSD, a field plot (10 × 30 m) was planted with the soybean cultivar H9220 in 1994 near Ames, Iowa. The incidence of pod infection by *Phomopsis*-*Diaporthe* spp. in the plot was estimated to be 45% in a pod test (9) carried out on 100 pods sampled at random from the planting at growth stage R6 (pods containing green seed that fills the pod cavity at four of the uppermost nodes on the main stem) (5). At harvest maturity, approximately 50 to 75 kg of soybean plants were collected at random from the planting. Pods were carefully removed from the branches to avoid splitting and stored in paper bags at 10°C and 50% relative humidity. Seed infection by PSD was then induced, according to the method of Balducchi and McGee (2), by placing 15 sets of 250 pods on a wire grid 2 cm above Kimpac saturated with 400 ml of water in a transparent plastic box (26.5

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× 20 × 9.5 cm). The boxes were sealed in transparent plastic bags and placed in an incubator maintained at 25°C under continuous light. At 0, 3, 5, 7, and 9 days of incubation, three randomly selected boxes were removed. Seeds within the pods from these boxes were extracted by hand, taking care to avoid mechanical damage, and were combined to make one sub-lot. These were laid on paper towels and allowed to air-dry until the seed moisture content equilibrated to the ambient relative humidity (40 to 60%) of the laboratory. Seed moisture content of each sub-lot was measured on 15-g samples by a standard oven method (6).

To determine the level of infection by PSD, 100 seeds from each sub-lot were surface-disinfested in 0.5% sodium hypochlorite for 1 min and rinsed in sterilized distilled water for 10 s. These seeds were plated on potato dextrose agar adjusted to pH 4.5 with lactic acid and incubated at 25°C in the dark for 14 days, and the numbers of seeds from which PSD colonies developed were counted. A standard warm germination test (1) was carried out on another 400 seeds from each sub-lot.

A second set of sub-lots of seeds with similar infection levels was independently prepared for each pathogen by the same procedure.

The ultrasound analyzer. The ultrasound analyzer, developed by Shyy and Misra (13), is a multicomponent system that includes a piezoelectric transducer, a Kiethly 194 high-speed voltage digitizer, a personal computer, an IEEE-488 interface board, and an ASSYST engineering package. Individual seeds are dropped approximately 10 cm onto a piezoelectric transducer tilted at a 30° angle. The seed is then released, undamaged, into a collecting pan. A low-frequency sound wave is generated by the transducer on impact and is converted into an electrical impulse signal, amplified, and passed on to a voltage digitizer. The digitizer converts the electrical signal from analog to digital form, which is transferred to a personal computer where the data acquisition program measures the magnitude of the electrical impulse signal at time intervals. The data are plotted to a bell curve and quantified as numerical

values for the peak, the width, and the slope parameters (Fig. 1). The peak value increases with weight of the seed; whereas the slope decreases and the width increases with the degree of softness of the seed (13).

Measurement of parameters of ultrasound signals from seeds. Ultrasound analysis was carried out on samples of seeds from the sub-lots in which different levels of infection by storage fungi or PSD had been induced. For seeds infected with storage fungi, the experiment was performed separately on each of the two seed lots in which the infection levels had been generated independently. In experiment 1, 500 seeds from each sub-lot were dropped singly onto the transducer of the ultrasound analyzer in five replications of 100 seeds; whereas in experiment 2, 1,000 seeds were analyzed in five replications of 200. For seeds infected with PSD, duplicate experiments were also performed, and in both, 1,000 seeds from each seed lot were analyzed in five replications of 200. In all experiments, the peak, slope, and width parameters of the ultrasound wave were recorded for each soybean seed.

Measurement of parameters of ultrasound signals for seeds at different infection levels with PSD and seed moisture content. Sub-lots of soybean seeds were generated with 1, 7, and 62.25% infection rates by PSD with the protocol described above. Moisture content levels were adjusted within each sub-lot by exposing four samples of 200 g of seeds to different times in plastic boxes at >95% relative humidity and 25°C, as described above. For the three sub-lots, seed moisture contents at each incubation period were 6.1 to 6.4%, 7.5 to 8.3%, 9.2 to 9.7%, and 11.2 to 12.2% for incubation periods of 0, 6, 12, or 24 h, respectively. Immediately after sampling of each sub-lot, seed mois-

ture was measured as described above, and ultrasound analysis was performed on 150 seeds.

Statistical analysis. The average of all individual seed values for the peak, slope, or width for each parameter of the ultrasound signal was used as the unit of measurement for each replicate in all experiments. Regression analyses (SAS Institute, Cary, NC) were then performed on the data to relate seed infection to incubation period and infection levels to germination. In a similar manner, regression analyses were used to relate the levels of PSD and storage fungi infection to their corresponding ultrasound parameter values.

RESULTS

Induced seed infection levels. Different incidences of infection by storage fungi and PSD were successfully induced by the incubation techniques in both sets of experiments (Table 1). Increased incidence of storage fungi infection was negatively correlated with seed germination, with r^2 values of 0.92 for experiment 1 and 0.89 for experiment 2. The same relationships were evident between PSD infection and germination with r^2 values of 0.84 in both experiments 1 and 2. No symptoms of infection by either pathogen group were evident in any of the sub-lots. Moisture contents of the sub-lots following exposure to high relative humidity and subsequent air-drying to the ambient relative humidity of the laboratory were fairly constant, ranging from 6.0 to 6.19% across incubation treatments and experiments.

Relationship of seed infection by storage fungi and PSD to parameters of ultrasound signals. Peak values of ultrasound signals, averaged across 100 seeds in a sub-lot in experiment 1, decreased linearly ($r^2 = 0.85$) as the incidence of seed infected with storage fungi increased

Fig. 1. Representation of an electrical impulse signal generated by one seed, indicating where peak, slope, and width are measured.

Table 1. The incidences of storage fungi and Phomopsis seed decay (PSD) infection, germination value, and moisture content after dry-down in soybean seeds after incubation for different times at relative humidities >95% and 25°C

Incubation ^w	Experiment 1			Experiment 2		
	Infection ^x (%)	Germination ^y (%)	Moisture ^z (%)	Infection (%)	Germination (%)	Moisture (%)
Storage fungi						
0	10	93.50	6.03	24	98.50	6.03
10	33	83.80	6.01	51	97.00	6.05
20	70	78.90	6.02	68	95.00	6.04
30	100	71.50	6.03	79	93.25	6.08
40	100	65.00	6.12	100	87.75	6.09
PSD						
0	1	96.00	6.05	3	98.50	6.08
3	7	60.00	6.05	10	92.75	6.13
5	38	55.50	6.02	35	70.00	6.13
7	48	33.50	6.03	56	53.00	6.16
9	62	17.25	6.09	56	18.00	6.19

^w Days of incubation.

^x Storage fungi infection induced by incubating at >95% relative humidity and 25°C; PSD infection induced by exposing infected soybean pods to the same conditions. Seeds surface-sterilized and plated on malt-salt agar (storage fungi) and potato dextrose agar (PSD).

^y Seeds germinated on Kimpac for 7 days at 25°C.

^z Determined by oven test.

(Fig. 2A). Slope and width of the signal increased as seed infection increased, but the coefficient of determinations between each parameter and seed infection were lower, with r^2 values of 0.50 and 0.59 for slope and width, respectively (Fig. 2B and C). Germination values, already shown to de-

crease as seed infection by storage fungi increased (Table 1), had an inverse relationship to the ultrasound signals found for storage fungi infection, in that peak values increased and slope and width decreased as germination values increased. Again, the coefficient of determination obtained for

the peak values, 0.81, was higher than that for slope and width, 0.48 and 0.53, respectively (Fig. 2D to F). In experiment 2, coefficients of determination for peak, slope, and width values of 0.83, 0.70, and 0.47, respectively, in relation to seed infection, and 0.65, 0.69, and 0.43, respec-

Fig. 2. Relationship between levels of storage fungi infection (A–C) and resulting warm germination percentages (D–F) and the peak, slope, and width acoustic parameters of soybean seeds.

tively, in relation to germination, were similar to those obtained in experiment 1. This was also true for the slopes and intercepts of the regression lines.

Comparable results were obtained for seeds infected by PSD. Again, peak values of ultrasound signals, averaged across 100 seeds in a sub-lot, decreased linearly ($r^2 = 0.83$) as the incidence of seed infection with PSD increased (Fig. 3A). Also, slope and width of the signal increased as seed

infection increased, but the coefficient of determinations between each parameter and seed infection were lower, with r^2 values of 0.42 and 0.48 for slope and width, respectively (Fig. 3B and C). Germination values showed the same inverse relationships with ultrasound signals of PSD-infected seeds, with an increase in peak values and decreased slope and width as germination increased values (Fig. 3D to F). Coefficient of determinations were, how-

ever, substantially lower than those for seed infection, with r^2 values of 0.42, 0.25, and 0.36 for peak, slope, and width of ultrasound signals, respectively. Results obtained in the repeat of this experiment showed generally lower coefficient of determinations than for those obtained in experiment 1. Peak, slope, and width values in relation to seed infection were 0.45, 0.23, and 0.41, respectively, and 0.17, 0.09, and 0.36, respectively, in relation to germination.

Fig. 3. Relationship between level of Phomopsis seed decay infection (A–C) and resulting warm germination percentages (D–F) on the peak, slope, and width acoustic parameters of soybean seeds.

Effects of seed moisture content on parameters of ultrasound signals for seeds with different infection levels with PSD. Peak values decreased and slope and width increased as seed moisture content increased for sub-lots at all three levels of infection by PSD

(Fig. 4). High coefficients of determination levels (r^2 values of 0.87 to 0.99) occurred between values for each parameter and seed moisture for the three sub-lots. Slopes of the regression lines also were similar for all sub-lots for each parameter.

Fig. 4. Effects of soybean seed moisture content on the mean peak (A) mean slope (B), and mean width (C) parameter values of seeds infected with 1, 7, and 62% Phomopsis seed decay.

DISCUSSION

This study has demonstrated that parameters of an ultrasound signal generated by the impact of soybean seeds dropped onto a sensor can be related to the incidence of seed infection by storage fungi and PSD. Values for the peak of the ultrasound signal, indicative of seed weight in soybeans (10), showed the highest correlations with seed infection for both fungi. Because reduced size is a recognized symptom of seeds infected by storage fungi and PSD (8), the ultrasound signal presumably detected changes in seed weight that may occur in infected but asymptomatic seeds. The slope and width values of the signal, indicators of seed softness (10,13), were moderately well-correlated with infection level by storage fungi, but were poorly correlated to PSD infection. The differences in responses for the two types of pathogens may be due to extent of invasion of the seed tissues and the seed parts infected. Storage fungi will rapidly colonize seed coats and cotyledons, and invade embryos in seeds exposed to 95% relative humidity at 25°C (4). Invasion and digestion of internal tissues of seeds by storage fungi would likely increase the softness of the seed tissues. On the other hand, seed softness may be less affected by PSD, because the causal fungi mainly colonize the seed coats rather than the cotyledons or embryo in asymptomatic seeds. The reduction in germination values of PSD-infected soybean seeds results from infection of germinating seedlings by inoculum of PSD located in seed-coat tissues (15).

Because the seeds tested in this study showed no obvious physical change as a result of infection by storage fungi or PSD, the results suggest that the ultrasound signal could identify diseased, asymptomatic seeds. Adaptation of the technology to identify and eliminate these seeds in a seed conditioning line will require considerable developmental work. This study only showed the relationships to seed infection and germination of parameters of ultrasound signals averaged across a group of seeds, using laboratory-scale equipment. Practical versions of the technology will require defined cutoff values for the parameters that ensure identification and elimination of individual infected seeds from seed-lots within certain tolerance levels. In a preliminary attempt to define cutoff values for storage fungi infection of soybeans, Walcott (14) found that 50 seeds from a 25-g lot with peak and width values of <500 mV and >3 ms, respectively contained 64% of seeds infected by storage fungi; whereas 50 seeds from the same lot with values of >600 mV and <2 ms contained 32% of seeds infected. These peak-width values were arbitrarily selected and need to be further adjusted to more effectively separate infected from healthy seeds, but the experiment did demonstrate the

feasibility of establishing appropriate cutoff values for the ultrasound parameters. Seed moisture content will be another important consideration in establishing cutoff values. But the strong linear relationship determined between seed moisture content and peak, width, or slope values of ultrasound signals for three seed sub-lots with different infection levels of PSD indicate that the relative change in the signal parameters due to seed moisture content was similar for seed lots with different infection levels. Cutoff values, therefore, could probably be adjusted to compensate for the moisture content of lots passing through ultrasound equipment. Other considerations in equipment design will include the need to deal with seeds that are not spherical in shape, fast handling of high volumes of seeds, and minimal mechanical damage to seeds during the dropping phase.

This study has demonstrated the potential of ultrasound technology as a means of identifying asymptomatic seeds infected by fungi. It is entirely likely that seed infected by bacteria and viruses could also be identified. But the fact that ultrasound signals were less effective in detecting asymptomatic soybean seeds infected by PSD than

by storage fungi, two pathogen groups that infect soybean seeds in different ways, suggests that this technology may be of value only for particular pathogens. A better understanding will have to be gained of internal changes in seeds that are detectable by ultrasound signals and of the types of pathogens that cause such changes before the full potential for this technology can be exploited.

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