KPS2-1

Detection and removal of single mycotoxin contaminated maize grains following harvest

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Abstract

Grains highly contaminated by aflatoxin and fumonisin are unevenly distributed in a grain lot and may be concentrated in a very small percentage of the product. Near-infrared (NIR) reflectance spectra (500-1,700 nm) were analyzed to select the pair of absorbance bands (filters) giving the lowest classification error rate for removing whole yellow maize grains contaminated with aflatoxin (750 and 1,200 nm) or white maize grains contaminated with fumonisin (500 and 1200 nm) in a single pass through a commercial high speed sorter (@ 7,000 kg/hr). Our research also seeks to classify individual grains infected with different fungal species and to distinguish resistance and susceptibility reactions among corn varieties. Neural networks are being trained to classify grains by fungal species using principle components of the full reflectance spectra. Spectra of single maize grains can be measured automatically and grains with multiple symptoms and mycotoxins can be sorted into different fungal species categories at rates of about 1 per second using commercial instruments. Our initial work has shown that classification accuracies for severely discolored grains infected with *Aspergillus flavus*, *Stenocarpella maydis*, *Fusarium graminearum*, *Fusarium verticillioides*, and *Trichoderma viride* averaged 92.1 % and 94.8 % for two commercial corn hybrids. Protective endophytes, including mycoparasites that live asymptomatically in maize, are not readily distinguished from uninfected grains and represent confounding variables in maize variety trials for fungus-mycotoxin resistance.

Key words: aflatoxin, classification, corn, endophyte, endosperm, fumonisin, kernel-rot, maize pathogens, near-infrared, neural network, sorting.

Introduction

Fungi can reduce yield, quality, and nutritional value of the grain, while also contaminating it with fungal-derived chemicals, some of which are recognized as mycotoxins because of their deleterious biological effects in animals and humans (Richard and Payne, 2003). Aflatoxins produced by *Aspergillus flavus* Link and fumonisins produced by *Fusarium verticillioides* (Sacc.) Nirenb. are prominent among the mycotoxins associated with economic losses to maize (*Zea mays* L.) growers, grain handlers, livestock and poultry producers, and food and feed processors. No commercial corn hybrid is able to escape mycotoxin contamination when grown in environments conducive to outbreaks of aflatoxin or fumonisin. While conventional

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breeding has produced commercial maize hybrids with substantial resistance to *Fusarium graminearum* Schwabe, which produces the mycotoxins deoxynivalenol and zearalenone, efforts to produce hybrids with adequate resistance to *A. flavus* and *F. verticillioides* have proven more difficult, and effective practical control practices are lacking. As 'gatekeepers' to food safety, the food and feed industry must continue to rely on convenient, accurate and sensitive methods for detection of the major mycotoxins in grain. Seeds highly contaminated by aflatoxin and fumonisin are unevenly distributed in a seed lot and may be concentrated in a very small percentage of the product (Whitaker and Dickens, 1983; Desjardins et al., 1998; Whittaker et al., 1998; Pearson et al., 2001; Whittaker et al., 2001; Wicklow, 1994; Wicklow, 1999). Therefore, removing a small percentage of contaminated kernels, instead of discarding the entire lot is a reasonable approach for reducing aflatoxin or fumonisin contamination to satisfy statutory levels. Corn kernels infested with fungi are more friable and may have reduced densities (Shotwell et al., 1974). However, standard post harvest cleaning operations (aspiration, gravity table separation, grain scouring, and wet cleaning) have not been shown to be entirely effective for reducing aflatoxin or fumonisin levels in commercially harvested corn (Brekke et al., 1975; Pearson et al., 2004). Regional aflatoxin outbreaks are commonly accompanied by outbreaks in fumonisin (Mubatanhema et al., 2002) and therefore, aflatoxin and fumonisin can be present at unacceptable levels in the same grain samples at harvest (Chamberlain et al., 1993, Chu and Li, 1994, Yoshizawa et al., 1996, Shetty and Bhat, 1997, Ali et al., 1998, Medina-Martinez and Martinez, 2000, Ono et al., 2001).

Detection and sorting grain for food processors and industrial applications

Our research seeks to simultaneously eliminate both aflatoxin- and fumonisin-contaminated kernels in a single pass through a commercial optical sorter. High speed sorting equipment can process corn at rates of approximately 7,000 kg/hr. Most commercial sorting machines are able to only measure one spectral band of light while some machines can measure two bands. Only two-way sorts (i.e. "accept" or "reject") can be performed at high speed. In the past, red optical filters were used to separate mold-contaminated products using mono-chromatic sorters (Dickens and Whitaker, 1975). At present, bi-chromatic color sorters have near-infrared detecting capability in addition to visible light capability, which extend their usefulness for detecting mold-infected seeds based on both color and bio-chemical composition. Near-infrared transmittance (NIRT) and near-infrared reflectance (NIRR) spectroscopy have been used to evaluate internal quality on many whole nuts and grains. We have applied NIRR and NIT to optimizing the filter selection for bi-chromatic sorters and have shown that only a few absorbance bands in the visible and near infrared spectrum can detect whole yellow corn kernels highly contaminated in the field with aflatoxin (Pearson et al., 2001) and fumonisin (Dowell et al., 2002).

For high speed sorting operations, whole spectra cannot be acquired at throughput rates that are economically feasible. Near-infrared (NIR) reflectance spectra (500 - 1,700 nm) are analyzed to select the optimal pair of optical filters that can be used to detect and remove mycotoxin contaminated white or yellow maize grains through using high speed, high volume optical grain sorters. Sorter performance is verified with naturally fungus-infested grain samples from different hybrids, years and locations. The first phase involves inoculating corn ears in the field with either *A. flavus* or *F. verticillioides*, recording reflectance spectra of individual kernels, then chemically measuring aflatoxin or fumonisin in each kernel so that the most discriminating pair of absorbance bands could be chosen to separate between mycotoxin-contaminated and uncontaminated kernels (Dowell et al., 2002; Pearson et al., 2004).

Whole-kernel reflectance spectra from 500 to 1,700 nm are measured using a diode-array near-infrared spectrometer (DA7000, Perten Instruments, Springfield, IL). Kernels are manually placed on a bifurcated interactance probe attached to the spectrometer and light source (Figure 1). Spectra are first collected from all kernels oriented at the germ-down position (germ facing the optical fiber bundle), then a second set for kernels oriented germ-up. All spectra are stored on a hard disk for subsequent analysis.

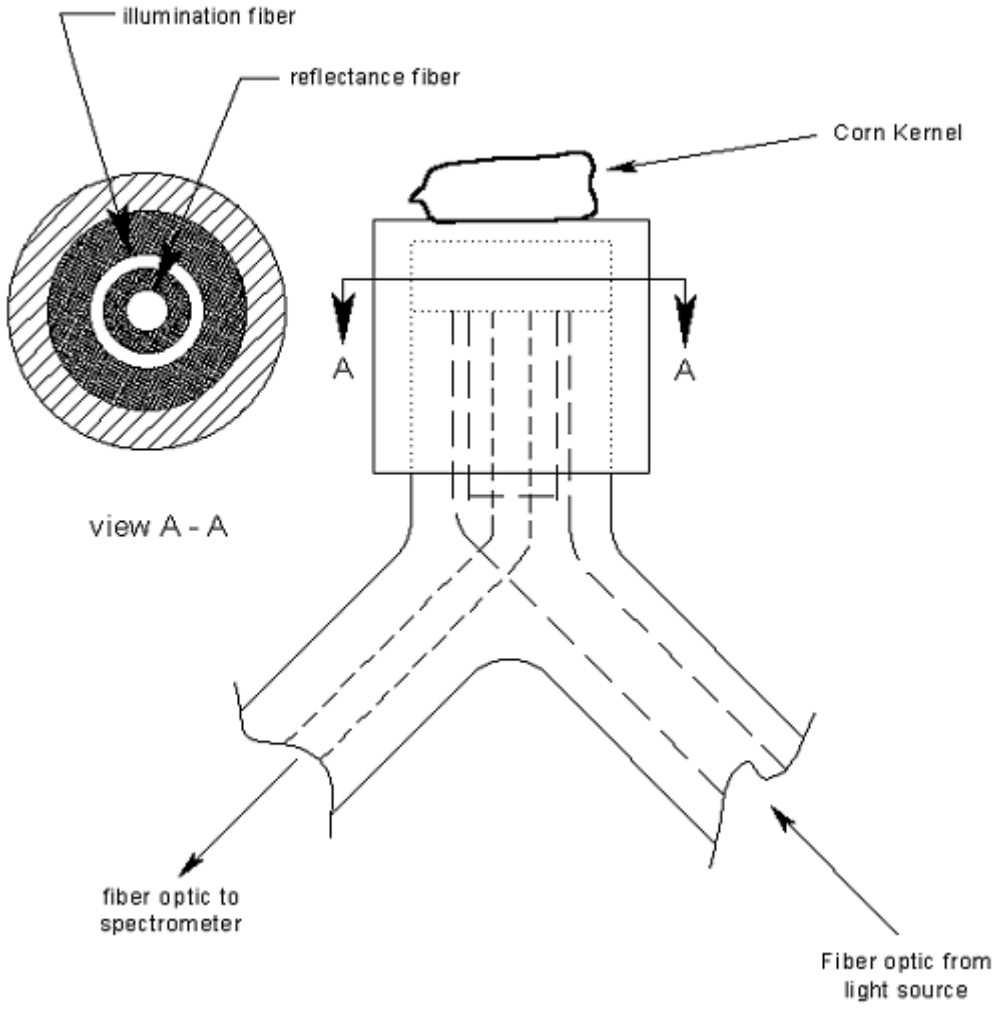

Figure 1. Bifurcated interactance probe attached to the spectrometer and light source to collect reflectance spectra of individual kernels. The viewing area was 17 mm in diameter and 10 mm above the termination of the illumination and reflectance fibers.

The next step is to select the optimal filters for discriminating contaminated or fungal infested kernels from non-contaminated or un-infested kernels. The procedure we developed tests all combinations of two spectral bands within the NIRR spectra collected from each kernel. The spectra from both sides of the kernel is taken into account independently, then linked back to the spectra from the opposite side of the kernel using binary logic. In other words, a kernel can be classified as contaminated if the spectra from only one side of the kernel indicates contamination or the decision rule can be set to classify kernels as contaminated only if the spectra from both sides of the kernel

indicate contamination. All sorting machines inspect at least two sides of kernels so this logic needs to be taken into account when selecting filters. The classification methods include both discriminant analysis and nearest neighbor schemes. Usually these two methods yield similar results, however, when the distribution of the data is abnormal, the nearest neighbor method tends to work better.

Single-kernel mycotoxin levels are determined after the reflectance spectra of all kernels have been measured. Individual kernels are then placed in an envelope of folded weighing paper, weighed, and crushed by striking with a hammer. The crushed kernels are extracted for either aflatoxin or fumonisin, appropriately scaled to weight of sample (Pearson et al., 2001; Dowell et al., 2002), following the manufacturer's instructions for the affinity chromatography procedures for Aflatest or Fumonitest (Vicam Inc., Watertown, MA).

The second phase involves application of the selected pair of absorbance bands in a commercial sorting machine (ScanMasterII 2000 DE, Satake-USA, Houston, TX) for separating aflatoxin- and fumonisin-contaminated corn at high speeds (Pearson et al., 2004). Here we use naturally infected grain that is commercially grown and harvested. After sorting, bulk samples from the 'accept' and 'reject' streams are chemically analyzed for aflatoxin and fumonisin to evaluate the sorter's performance for removing kernels contaminated with these mycotoxins. The pair of absorbance bands that obtained the lowest classification error rate (750 and 1,200 nm) were used to optimize a dual band high speed optical sorter for removing whole yellow corn kernels contaminated with aflatoxin and fumonisin (Pearson et al., 2004). This method was able to lower aflatoxin by an average of 81 % and fumonisin by 85 % for corn grown in Kansas yielding 'accepted grain' that would meet FDA guidelines for use in human food. Co-incidental removal of grains contaminated with fumonisin represents an added benefit when the primary objective of grain sorting is to remove "aflatoxin-contaminated" grains. Even so, the spectra of single kernels with aflatoxin below 100 ppb or fumonisin below 10 ppm have been found to be more similar to uncontaminated kernels (Pearson et al., 2001; Dowell et al., 2002). Further research, using additional sources of aflatoxin and fumonisin contaminated corn and guided by full spectrum neural network classification, is needed to improve sorting of corn with low levels of aflatoxin and fumonisin (Pearson et al., 2004).

Near infrared and reflectance spectra (500-1,700 nm) were analyzed to determine if they could be used to identify single whole white corn kernels contaminated with fumonisin in research requested and supported by the Texas Corn Producers Board (Lubbock, Texas). Kernels used for the study were obtained from grain processors in Illinois, Indiana, Kentucky, and Nebraska. Discriminate analysis was used to select the optimal pair of wavelengths to identify white corn kernels containing fumonisin. It was found that using the wavelength pair of 500 nm and 1,200 nm, approximately 77 % of the kernels having high levels of fumonisin (> 40 ppm) were correctly classified (Pearson and Wicklow, 2005). Additionally, approximately 96 % of the kernels having low levels of fumonisn (< 2 ppm) were correctly classified. In contrast, if only a single band is selected for distinguishing contaminated kernels, the accuracy for kernels having low fumonisin levels (< 2 ppm) drops to approximately 83 %. Thus, use of a dual band sorting machine for removal of white corn contaminated with fumonisin would result in 13 % less good product being removed than with a monochromatic sorter.

Previous work with yellow corn showed that approximately 85 % of the aflatoxin and fumonisin could be removed by high speed sorters using the spectral bands of 750 nm and 1,200 nm. It was hypothesized that the 750 nm band was detecting some color changes in fungal infested kernels while the 1200 nm band was responding to increased porosity of the degraded endosperm. Insect damaged kernels have low absorbance at 1,200 nm, due to feeding and fungal infestation, and would all be rejected. In the case of white corn, 500 nm was found to be more accurate than 750 nm for the visible spectral

band (Pearson and Wicklow, 2005). This may be due to the white corn germ and endosperm being of more uniform color than yellow corn kernels with a white germ. Because yellow corn absorbs more light at 500 nm, asymptomatic yellow corn kernels can be distinguished from white corn kernels.

Fungal-damaged kernels are of low quality and may have undesirable traits besides containing mycotoxins, and overall corn quality may be improved further by removing all fungal-damaged kernels through optical sorting. We wanted to determine if corn kernels infested with common fungi could be distinguished from un-infested kernels by imaging methods or high speed (~1,000 kernels/s) optical sorters. It was found that two NIR reflectance spectral bands centered at 715 nm and 965 nm can correctly identify 98.1 % of aymptomatic kernels and 96.6 % of kernels showing extensive discoloration and infected with *A. flavus*, *Aspergillus niger* v. Tieghem, *F. graminearum*, *F. verticillioides, Stenocarpella maydis* (Berk.) Sutton (syn. *Diplodia maydis* Berk.), or *Trichoderma zeae* Pers.: S.F.Gray (Pearson and Wicklow, 2006). These two spectral bands are easily implemented on high speed sorting machines for removal of fungal-damaged grain. High speed optical sorting machines are used to remove molded or discolored maize kernels from grain purchased for use in food products. Sorting machines can also be used to recover valuable asymptomatic grains in 'reject streams' from conventional grain cleaning operations.

Neural network classification of kernel symptom expression

Fungal infected maize kernels are classified by plant pathologists according to the type of disease symptoms produced, including kernel or ear rots, streaked or blotched kernels, etc. and their ecology (Wicklow et al., 1980; Samuels, 1984; Smith et al., 1988; Wicklow, 1995; White, 1999). Kernel symptom expression is a product of infecting fungal species, drought stress, and nutritional deficiencies (White, 1999). Reactions associated with maize varietal resistance or susceptibility can also contribute to the symptomology of infected grain (e.g. Wright and Billeter, 1974; Hart et al., 1984; Lambert and White, 1997; Walker and White, 2001; Naidoo et al., 2002; Clements et al., 2003). Seed color and form changes, detectable visually, are actually preceded by chemical changes in the grains caused by the fungus. For example, *A. flavus* initially infects the oil-rich germ using grain lipids for its growth and metabolism, and thus lipid hydrolysis takes place faster than the degradation of protein or starch in stored grain (Sauer and Christensen, 1969; Wacowicz, 1991; Pomeranz, 1992). Lipids are broken down by lipases to free fatty acids and glycerol; thus, the free fatty acid content of grain has been proposed as a sensitive index of incipient grain deterioration (Christensen and Kaufmann, 1969; Faraq et al., 1981; Richard-Molard, 1988; Pomeranz, 1992). Other more common species of kernel rotting fungi (e.g. *F. verticillioides*, *F. graminearum*, *Nigrospora oryzae* (Berk. & Br.) Petch, *Penicillium oxalicum* Currie, *S. maydis*, *T. viride*, etc.) may enter the seed proper based upon a different pathology and in earlier stages of kernel development, producing different symptoms of kernel infestation (Clayton, 1927; Johann, 1935; Koehler, 1942; Caldwell et al., 1981; Lawrence et al., 1981; Sutton, 1982; Bennett et al., 1988; Smart et al., 1990; Klapproth and Hawk, 1991; Munkvold et al., 1997). Breeders attempting to investigate maize varietal resistance to molds need rapid methods for identifying mold-infested kernels and ideally, the species of mold infecting each kernel. Full spectrum methods are needed to identify infecting fungal species so that the technology can potentially be used to automatically and rapidly detect fungal infested corn kernels.

Our research seeks to classify individual seeds infected with different fungal species and to distinguish resistance and susceptibility reactions among corn varieties (Pearson and Wicklow, 2006). A neural network was trained to identify infecting fungal species on single kernels using

principle components of the reflectance spectra as input features. The following procedures were used in this research: Full NIRR spectra are collected from individual grains removed from ears of commercial hybrids that we wound-inoculate with one of 11 commonly recorded fungi from corn kernels: *Acremonium zeae, A. flavus, A. niger, F. graminearum, F. verticillioides, S. maydis, N. oryzae, P. oxalicum, Penicillium funiculosum* Thom, *Penicillium pinophilum* Hedgcock, and *T. viride*. Principle components of the average germ-up and germ-down spectra were computed, and the first 20 principle components were fed as classifying features in a neural network (NeuralShell Classifier V2.01, Ward Systems Group Inc., Frederick, MD) to classify kernels by their infecting fungus. For this analysis, the spectra were mean centered then normalized by dividing each absorbance value by the average of the highest 5 % absorbance values in the entire spectra. Half of the data from asymptomatic and extensively discolored kernels were randomly assigned to a training set and the other half to a validation set. All of the kernels with minor discoloration were assigned to the validation set. The training set was used to compute the first 20 principle components (those explaining the most data set variance) and train the neural network with these. The eigenvectors computed for principle components of the training set were applied to the validation set and used to validate the neural network classification results. The neural network training used the genetic training algorithm, as this method is much less likely to over-fit the data (Lestander et al., 2003). The training was first started with all of the first 20 principle components. After training was completed, the software reported a relative importance of each principle component to the classification. The least-important principle component was removed and training started over again. This procedure was repeated until no further improvement was observed in the training set. If required, neural network classification accuracy can be improved with additional 'training sets' of fungal infested kernels. NIR screening requires periodic revalidation which may also be required for training the neural network. New hybrids infected with commonly recorded species of kernel infecting molds may also be added to the neural network.

Our initial work has shown that classification accuracies for severely discolored kernels infected with *A. flavus, F. graminearum, F. verticillioides,* S. *maydis,* and *T. viride* averaged 92.1 % for Pioneer 3394 corn and 94.8 % for Farm Service 7111 corn (Pearson and Wicklow, 2006). Classification accuracy for controls on these two calibrations was 100 %. However, accuracies for infested kernels with minor endosperm discoloration fell by more than half compared with extensively discolored kernels. Thus, the kernels need to show strong symptoms of infection in order for their species to be identified. These preliminary results suggest that full spectrum methods can be used to identify major infecting mold species accurately within a corn hybrid and reasonably well across different corn hybrids and harvest dates. Spectra of single corn kernels can be measured automatically and kernels sorted at low speed (~1 kernel/s) into different mold species categories using automated, full spectrum, commercial NIR machines such as those currently marketed by Brimrose Corporation (Baltimore, MD.), and Perten Instruments (Springfield, IL.). At low speed, the entire spectrum can be acquired and used as a basis for decision making. Multiple kernel symptoms can be separately classified and the kernels sorted into specific groups. This may be of use to breeders who need to rapidly screen samples for mold damage from different species. The ability to distinguish among resistant versus susceptible kernel reactions using commercial instruments could be used to guide a breeding program. We are presently using neural network classification in an effort to evaluate symptom expression in grain removed from *A. flavus* wound-inoculated ears of aflatoxin 'resistant' versus 'susceptible' maize lines as determined by a seed producing company. Post-harvest storage molds (e.g. *Aspergillus candidus* Link, *Chaetomium globosum* Kunze, *Eupenicillium*

cinnamopurpureum Scott & Stolk, *Eurotium* spp., *Monascus ruber* v. Tieghem, etc.) infest grain stored at different temperatures and humidities, sometimes killing and replacing pre-harvest fungal colonists infecting the grain (Wicklow, 1995; Wicklow et al., 1998). We also hope to distinguish among grain samples showing symptoms of mold damage resulting from different examples of improper storage.

Protective endophytes as confounding variables in maize resistance trials

In our effort to classify species of fungi infecting grain from both wound-inoculated and naturally infested maize ears, *A. zeae, F. verticillioides, P. funiculosum, and P. pinophilum* were commonly recorded from asymptomatic kernels. Neural network classification was unable to distinguish asymptomatic endophyte-infected kernels from asymptomatic kernels where no fungus was recorded. *Acremonium zeae* and *F. verticillioides*, have been shown to interfere with the growth of virulent fungal pathogens *F. graminearum* and *S. maydis*, as well as opportunistic saprotrotrophs such as *A. flavus* (Wicklow et al., 2005). *Penicillium funiculosum* and *P. pinophilum* are erroneously included in lists of kernel rotting molds when they are observed parasitizing kernel rotting fungi. Protective endophytes of corn should escape the negative effects of pathologist/breeder selection when their infections are symptomless and absent any noticeable impact on yield. These fungi either fail to elicit maize host defenses or they are not negatively impacted by the same suite of plant defenses that maize breeders have utilized successfully against destructive seed pathogens. Protective endophytes may also represent confounding variables when evaluating maize varietal resistance to bacterial or fungal diseases (Wicklow et al., 2005). Some of these endophytes are criticized as 'behaving badly' when rapidly responding to exclude other fungi from kernels damaged by ear and kernel feeding insects, or damage to kernels resulting from extensive drought or temperature stress during kernel filling, and also when endophytes are included among the 'usual suspects' implicated in maize stalk rot. The discovery of pyrrocidine antibiotic production by *A. zeae* (Wicklow et al., 2005) highlights the need for a greater overall understanding of the microbial interactions and bioactive metabolites in interference with mycotoxin contamination in corn prior to harvest. Microbial endophytes of cereals represent under explored sources of antifungal proteins and metabolites that can suppress fungal growth or silence genes critical to mycotoxin synthesis while also being adapted to function *in planta*.

Acknowledgement

D.T.W. thanks Dr. Vildes M. Scussel and the Executive Committee of the 9th IWCSPP for inviting his participation and providing support for his travel to the conference. 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 USDA.

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