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Investigation of fungal development in maize kernels using NIR hyperspectral imaging and multivariate data analysis

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ABSTRACT

Near infrared (NIR) hyperspectral imaging and hyperspectral image analysis were evaluated for their potential to track changes in fungal contamination on and fungal activity immediately under the surface of whole maize kernels (*Zea mays* L.) infected with *Fusarium verticillioides*. Hyperspectral images of clean and infected kernels were acquired using a SisuChema hyperspectral pushbroom imaging system with a spectral range of 1000–2498 nm at predetermined time intervals after infection. Background, bad pixels and shading of acquired absorbance images were removed using exploratory principal component analysis (PCA). When plotting PC4 against PC5, with percentage sum of squares (%SS) 0.49% and 0.34%, three distinct clusters were apparent in the score plot and this was associated with degree of infection. Loading line plots, with prominent peaks at 1900 nm and 2136 nm, confirmed that the source of variation was due to changes in starch and protein. Partial least squares (PLS) regression models, with time as the *Y* variable, were calculated and also indicated that changes over time were apparent. Variable importance plots (VIP) confirmed the peaks observed in the PCA loading line plots. More systematic future experiments are needed to confirm this, but it can already be concluded that early detection of fungal contamination and activity is possible.

1. Introduction

Maize (*Zea mays* L.) is a vital source of energy and is one of the most important dietary staple foods in the world (Schierbaum, 2007). It is used for human consumption in diverse forms, from specialised foods in developed countries, to staple food in undeveloped countries (Malvar et al., 2008). In addition to being used for human consumption, maize is also used for animal feed.

Fusarium verticillioides, a pathogen of maize, is a concern primarily since it produces secondary metabolites toxic to animals and humans (Bacon et al., 2008; Duncan and Howard, 2010; Munkvold, 2003). As it infects maize, which represents a significant component in the human food supply chain, the need for understanding the biology of host–pathogen interaction exists (Duncan and Howard, 2010). *F. verticillioides* is an endophyte since its hyphae occur systemically in the leaves, stems, roots and cobs of the maize plant (Bacon et al., 1992; Bacon and Hinton, 1996; Fandohan et al., 2003; Munkvold and Desjardins, 1997; Schulthess et al., 2002). *F. verticillioides*, however, is capable of causing asymptomatic as well as symptomatic infections (Bacon et al., 1992; Bacon and Hinton, 1996; Fandohan et al., 2003; Munkvold and Desjardins, 1997). Thus, if the fungal contamination of maize plants and kernels is not detected early on, contamination in the form of mycotoxins can enter the food chain. *Fusarium* mycotoxins are a relevant problem in the cereal supply chain and are known to be carcinogenic to humans and animals. Many methods have been utilised to determine fungal contamination and the presence of fungi on cereals. Traditional methods include microbiological techniques (diagnostic media and microscopy) (Bacon et al., 1992; Medina-Martinez and Martinez, 2000; Muthomi et al., 2008) or immunological methods for detection of toxin (Castells et al., 2008; Paepens et al., 2004; Sydenham et al., 1996). These techniques are known to be time consuming, labour

Abbreviations: CNS, control non-sterilised; CNSD, control non-sterilised germ down; CNSU, control non-sterilised germ up; CS, control sterilised; CSD, control sterilised down; CSU, control sterilised up; D, germ down; dH_2O, distilled water; HgCdTe, mercury cadmium telluride; NaOCl, sodium hypochlorite; NIR, near infrared; NS, non-sterilised; NSD, non-sterilised germ down; NSU, non-sterilised germ up; PC, principal component; PCA, principal component analysis; PDA, potato dextrose agar; PLS, partial least squares; Q^2, coefficient of determination for prediction; RMSEP, root mean square error of prediction; S, sterilised; SD, sterilised germ down; SNV, standard normal variate; SS, sum of squares; SU, sterilised germ up; SVM, support vector machines; SWIR, short wave infrared; U, germ up; VIP, variable importance plot.

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intensive and to produce harmful by-products. They are also usually expensive.

Near infrared (NIR) hyperspectral imaging is an imaging technique in which spectral and spatial information are combined to obtain NIR hyperspectral images (Geladi et al., 2004, 2007; Gowen et al., 2007; Koehler et al., 2002). NIR hyperspectral images are three-dimensional arrays of the form, $\mathbf{X}$ ($m \times k \times \lambda$), where m and k are the spatial axes information and the λ axis represents the spectral information. The 3-dimensional structure of the hypercube requires reorganisation to a 2-dimensional matrix, adapting the image for further pre-treatments. Statistical treatments such as principal component analysis (PCA), an unsupervised classification or dimensionality reduction technique (Cowe and McNicol, 1985), can be applied to the data. It reduces the data to a much smaller number of principal components (PCs) and can also be used as an exploratory technique. Principal component (PC) score images and score plots are used interactively to investigate sample images for special features or irregularities in samples. If anomalies are observed, during interpretation of cleaned images (irrelevant pixels have been removed), they will most likely be due to chemical variation. This observed variation can be explained by studying the accompanying loading line plots. Partial least squares regression (PLS) is a powerful regression technique that uses the latent variable approach to find the fundamental relations between two matrices ($\mathbf{X}$ and $\mathbf{Y}$) (Liu and Rayens, 2007; Martens, 2001; Wold et al., 2001). PLS uses the y-data structure to decompose $\mathbf{X}$ so that the outcome constitutes an optimal regression vector.

NIR hyperspectral imaging is fast becoming an important non-destructive technique for the investigation of fungal contamination on cereals. It has been applied to maize kernels for the indirect detection of *F. verticillioides* with accuracies up to 86% (Williams et al., 2010). Using the visible-near infrared spectral range (400–1000 nm), hyperspectral imaging was investigated for the early detection of toxigenic fungi on maize (Del Fiore et al., 2010). In another study, the application of multispectral imaging (720–940 nm) was studied to predict the fumonisin content of milled maize (Firrao et al., 2010). Similar studies have been done on wheat, where a hyperspectral imaging system in the range of 400–1000 nm has been used to detect early infection by *Fusarium* (Bauriegel et al., 2011). NIR hyperspectral imaging and support vector machines (SVM) have been evaluated for the classification of fungal infected wheat kernels with classification rates of 92.9% for *Aspergillus niger*; 87.2% for *A. glaucus* and 99.3% and 100% for two *Penicillium* species, respectively (Zhang et al., 2007). In a similar study two-class and four-class classification models yielded classification rates of 97.8% and 95%, respectively, to distinguish between infected and non-infected wheat kernels.

The aim of this work was to detect fungal contamination of maize kernels prior to the appearance of visual symptoms and to monitor chemical changes associated with fungal activity using NIR hyperspectral imaging and multivariate data analysis techniques.

2. Material and methods

2.1. Maize kernel sterilisation

White maize kernels of intermediate hardness were used for the study. Prior to any treatment, the kernels were randomly split into two groups, a) kernels to be sterilised (S) and (b) kernels without sterilisation, i.e. non-sterilised (NS). To completely remove both surface and internally borne fungi from the kernels, they were first surfaced sterilised by rinsing in a combination of 70% ethanol and 1% NaOCl solution followed by rinsing with sterile distilled water (dH_2O). Thereafter the sterilised kernels were imbibed in sterile dH_2O for 4 h, kept in a water bath at 60 °C for 5 min (Bacon et al., 1994) and left to dry in a laminar flow for 1 h. The kernels were subsequently considered sterile and ready for inoculation. From each of the groups (S and NS), a subset was taken and used as controls (CS and CNS).

2.2. Fungal spore suspension and inoculation

Fungal spore suspensions were prepared from petri-dishes cultured with *F. verticillioides* (MRC 0826) kindly supplied by the Department of Plant Pathology, Stellenbosch University. Prior to spore preparation, the culture was transferred onto potato dextrose agar (PDA) (Merck) and incubated at 28 °C. After 7 days, sterile water with Tween 20 (6 drops L^{-1}) was used to wash spores from the agar surface. The spore suspension was poured through two layers of sterile cheesecloth to remove mycelium, thereafter the suspension was adjusted to 10^6 spores mL^{-1} using a haemocytometer (Boeco, Germany). Finally, the kernels (from groups S and NS) were inoculated by dipping into the spore suspension for 30 s and allowed to dry at room temperature. Thereafter 40 kernels (two sets of 20 each) randomly selected from each group were placed on two respective petri-dishes (20 kernels per petri-dish). On one petri-dish, the kernels were orientated germ up (U) while on the other, the kernels were oriented germ down (D). This was done for each treatment (SU, SD, NSU, NSD, CSU, CSD, CNSU and CNSD) after which the petri-dishes were incubated at 28 °C. Each treatment therefore consisted of 2 petri-dishes; each with 20 kernels orientated either germ up or germ down. After the images were collected from all petri-dishes, one kernel was removed from the second petri-dish at each time interval. These were used to test for the presence of the fungus by placing the kernel onto PDA and incubating at 28 °C for three days. This resulted in the second petri-dish of T8 only having 11 kernels available for validation.

2.3. NIR hyperspectral system and imaging

Hyperspectral images were acquired with the SisuChema short wave infrared (SWIR) camera (Specim, Spectral Imaging Ltd, Oulu, Finland). The camera comprised an imaging spectrograph coupled to a 2-D array Mercury-cadmium-telluride (HgCdTe) detector. Individual images were acquired within a spectral range of 1000–2500 nm at 10 nm resolution, spectral sampling per pixel of 6.3 nm and a field of view of 100 mm × 100 mm. Images of the entire petri-dish, without removing the lid, were collected to prevent contamination and were taken at predetermined time intervals after inoculation. Nine images (T0-T8) were collected at nine specified time intervals (0, 17, 20, 23, 26, 40, 43, 69, and 90 h). The time intervals indicate periods of time after inoculation. It would have been ideal to ensure the intervals to be evenly spread; due to technical issues (power failure) it was not possible. The power failure caused a problem with wavelength accuracy, therefore the 40 h image, could not be used. White and dark references were captured prior to each sample image and were subsequently used for image correction and calibration. For the white reference, a 100% standard was used and for the dark reference, the shutter was closed.

2.4. Hyperspectral image analysis

Images were analysed using the Evince v.2.5.0 (UmBio AB, Umeå, Sweden) hyperspectral image analysis software package and MATLAB v 7.10 (The MathWorks, Massachusetts, USA). The image