THE MAIZE TRUST

ANNUAL PROGRESS REPORT: OCTOBER 2008

A. IDENTIFICATION OF PROJECT AND PROJECT LEADER

1. Title of project:

Exploiting plant resistance to manage *Fusarium verticillioides* and fumonisins in maize in South Africa

2. Particulars of researcher:

Name and Surname: Altus Viljoen
Name of Institution: University of Stellenbosch
Contact address: Department of Plant Pathology
University of Stellenbosch
Private Bag X1
Matieland 7601
Tel: 021-808 4956
Fax: 021-808 4956
Email: altus@sun.ac.za

3. Aim of the project:

To develop, in collaboration with other research institutions, the required capacity, human resources and infrastructure to manage mycotoxin-producing *Fusarium* spp. on maize in South Africa. The contribution of the Department of Plant Pathology at Stellenbosch University will be to:

- Evaluate/develop molecular markers for the rapid and quantitative detection of toxin-producing *Fusarium* spp. in maize
- Investigate sources of resistance in local maize accessions to *F. verticillioides* and fumonisin contamination
- Enhance tolerance in existing maize cultivars to *F. verticillioides* and fumonisin contamination
- To establish infrastructure for the unconventional improvement of maize for resistance to Fusarium ear rot and fumonisin production

B. THE ACTIONS THAT HAVE BEEN TAKEN REGARDING THE PROJECT:

Rapid identification and quantitative detection of *Fusarium* spp.:

Rationale: Molecular markers that rapidly detect *Fusarium* spp. associated with maize in South Africa can substantially reduce the time and costs involved in plating out of maize kernels, accurately quantify toxin-producers in symptomless maize kernels, and assist in the development of prediction models for future disease outbreaks.

Progress: In order to successfully evaluate methods of disease management it is essential that the methods of disease assessment are accurate. An initial objective of this project, therefore, was to develop suitable methods of disease assessment. This includes molecular quantification of the fungi involved and analysis of the toxins they produce. A real-time PCR-based molecular technique (Taqman), using species-specific molecular markers and probes, is now in the process of being established at our facilities. This technique will be able to detect and quantify *Fusarium* spp. in maize kernels. As a first step we are using primers that specifically detect genes in *Fusarium* that are responsible for fumonisin production. The technique is successful, but the conditions required for optimal accuracy are currently being investigated. These conditions involve:

- High-throughput DNA extraction from maize samples: Three extraction methods have been compared that included manual DNA extraction, the use of DNA extraction kits, and the use of a mechanical robot for DNA extraction. The high-throughput robotic method would substantially reduce the time required for DNA extraction from maize sample, while costs would remain the same. Early results indicate that the robot extracted DNA as effectively as manual extraction and extraction kits.
- The use of multiplex DNA probes: The significance of a multiplex real-time PCR reaction is that an internal control can be added to the PCR reaction to ensure a successful reaction and to prevent false negative results. A method to use potato leaf roll virus (PLRV) DNA and a PLRV probe as an internal control in a multiplex format is currently being optimized.
- Quantification of toxin-producing *Fusarium* spp.: Several different fungal pathogens, including non-toxin producing *Fusarium* spp. can cause maize ear rot. Visual rating of Fusarium ear rot, therefore, can be inaccurate when predicting toxin contamination in maize. Maize cobs have, thus, been inoculated with a high fumonisin-producing isolate of *F. verticillioides* (MRC 826) in greenhouse trials. Our results showed that visual symptoms that developed compared well to real-time PCR results (Fig. 1). Our preliminary findings suggest that the technique will be successful at quantifying the fungal biomass.

Studies on the underlying mechanisms of resistance in local maize accessions to *F. verticillioides* and fumonisin contamination:

Rationale: Understanding defence responses underlying plant resistance is required for efficient and sustainable resistance to pathogens and their metabolites. Some of these responses can also serve as markers for rapid screening of plants in breeding programmes and for response to disease management strategies

Progress: - In this investigation, 25 maize breeding lines have been screened for resistance to *F. verticillioides* and fumonisin production in a field trial in Potchefstroom in the 2006/07 growing season. Symptom development and yield have been determined, and maize kernels were collected for studies on fungal colonization and toxin production. Natural infection by *Fusarium* spp., as determined by visual disease rating and the culture plating method, was insufficient to differentiate the resistance levels among the in-bred lines. Toxin analysis was carried out using the Enzyme Linked Immunosorbent Assay (ELISA) method, and our results will be verified using High Performance Liquid Chromatography (HPLC). The samples from this trial will be of use in the optimization of the real-time PCR quantification method. Final results of this trial will be available after HPLC and real-time PCR analyses (December 2008).

- The field trial in 2006/07 has been repeated in 2007/08 using 24 of the previous 25 breeding lines planted at two different locations. An artificial inoculation method has been used to better differentiate resistance levels. The 2007/08 trial has been harvested (June 2008) and visual rating of the trial is complete. In-bred lines showing potential resistance/tolerance and susceptibility have been found (Fig. 2). Maize samples from these trials have been harvested and will be analysed for toxin production and contamination with fumonisin-producing Fusaria using HPLC toxin analysis and real-time PCR, respectively, by December 2008. Twelve of the 24 in-bred lines from the field trials are currently being evaluated in the greenhouse. Resistant and susceptible maize lines could be used to study the mechanisms of resistance, or allow the development of a marker assisted breeding program.

- To study the histopathological basis of resistance, *F. verticillioides* has been genetically modified with Green Fluorescent Protein (GFP) *SGFP* gene, that is a variant of the *GFP* gene from the jellyfish *Aequoria victoria*. This means that *F. verticillioides* has the ability to express green fluorescence when viewed under a fluorescence microscope, and can thus be easily traced inside plant tissue.

To evaluate plant activators and other elicitors of induced resistance for reduction in Fusarium ear rot and mycotoxin production in maize

Objective: To determine whether plant activators, putative biocontrol organisms and soil management properties contribute to increased tolerance of maize to *F. verticillioides* and fumonisins, for inclusion in an integrated disease management strategy

Progress: - A number of plant activators that induce systemic acquired resistance in plants have been chosen for experimentation. These activators were selected for their ability to activate different metabolic pathways in plant defence systems, as well as their potential for commercial application.

- A field evaluation with plant activators was conducted in Potchefstroom during the 2006/07 season. The yield data, visual ratings of disease as well as ELISA toxin analysis have been completed. Natural infection levels in 2007, however, were insufficient to determine treatment effects. Toxin analysis must still be verified by HPLC. This field trial was repeated at two locations for the 2007/08 season. For these trials an artificial inoculation method was used to better determine treatment effects. These trials have been harvested and visually rated. From the visual rating it appears that there were low levels of symptoms development which makes it difficult to determine treatment effects. However we expect that there was a high level of infection which will be confirmed by HPLC and real-time PCR. The post-harvest processing is complete and the samples are due to undergo toxin analysis by HPLC. The samples will also be rated using real-time PCR.

- A hydroponics system for growing maize has been developed to conduct greenhouse trials with plant defence activators. A pilot trial was harvested in November 2007. The yield data as well as visual rating of disease and toxin analysis using ELISA have been carried out. The initial trial has yielded some interesting results but these must be confirmed by the subsequent trials. The trial has been repeated twice in 2008. Both of these trials have been harvested, and visual rating of disease has been completed (Fig. 3). It is difficult to interpret the visual results and, therefore, no conclusions about the treatment effects can be drawn yet. The samples have been processed and toxin analysis by HPLC as well as real-time PCR will be carried out.

Establishment of an unconventional maize and wheat improvement facility:

Rationale: Unconventional improvement has several advantages over classical breeding that include a reduction in time, labour and costs of plant improvement, and the use of existing and locally adopted material for plant improvement

Progress: - Candidate maize inbred lines for somaclonal variation have been received from the ARC-GCI. Twenty lines have been randomly selected and planted in the greenhouse in the middle of June. These candidate lines have developed very well and started shedding pollen. Hand pollinations will commence and take place in October, depending on the synchrony of plant pollen with silk emergence. Pollinations will be performed in duplicate on two consecutive days to ensure a good seedset. The primary maize ear of each line will be harvested 10-14 days after pollination and assessed for its ability to produce Type I callus, as not all maize lines are able to produce callus. A selection will be made from the lines that are able to produce callus for somaclonal variation and *Agrobacterium*-mediated transformation.

- Potential seedling assays for maize seedlings to determine resistance to *F. verticillioides* have been identified from the literature. These assays are currently being evaluated for their effectiveness in determining resistance to *F. verticillioides.* Preliminary seedling assays for resistance has not produced the required phenotypic reaction to the pathogen that would be needed to distinguish between susceptible and resistant mutants in mass screening. Several key parameters are currently being investigated in order to reproduce the seedling assays successfully and develop it into a robust and reproducible means of screening seedlings for resistance to *F. verticillioides*. Re-evaluation of our approach to the seedling assay has led us to two different approaches that seem to be mutually exclusive. These include (a) developing a seedling assay to determine potential resistance to the pathogen and (b) developing a seedling assay to determine whether a maize line may be sensitive or insensitive to fumonisin toxin FB1. The reason for this reassessment is due to the conflicting information from literature and the two schools of thought as to the role of fumonisins in either the pathogenicity or virulence of F*. verticillioides*.

- Preparations are underway for the transformation of *F. verticillioides* isolate MRC 826, a very aggressive fumonisin producer, for host-pathogen interaction studies. Commercial seed companies have been approached about contributing a known resistant line for these studies. Additionally, a literature review has revealed a few sources of resistance to *F. verticillioides* in Africa and the researchers have been approached about contributing these lines to this study.

- A facility capable of handling the radiation of maize kernels has been identified. This work will be performed at iThemba Laboratories in collaboration with Prof. Kobus Slabbert, the head of the Radiation Biophysics unit who has agreed to assist us with this pioneering study. An initial assay will be performed at the end of September 2008 to determine the optimal dosage (maximum radiation exposure for inducing mutations while not compromising on seed viability) for the maize kernels.

This will be done using iThemba's ^{60}Co gamma radiation and neutron facility in a parallel assay to determine a dose response curve so that a biological radiation effect for maize kernels can be determined. Previously, a stumbling block to the commencement of this assay was to determine an appropriate starting dose from which to develop a consequent dosage rate. Following the attendance of the International Symposium on Induced Mutation in Plants in Vienna, Austria in August and interactions with international researchers in this field, these stumbling blocks have been overcome and a comprehensive protocol has been established for the radiation of maize kernel. This protocol will be evaluated and optimized for mass radiation of maize kernels.

C. THE RESULTS THAT HAVE BEEN ACHIEVED:

See information in Section B.

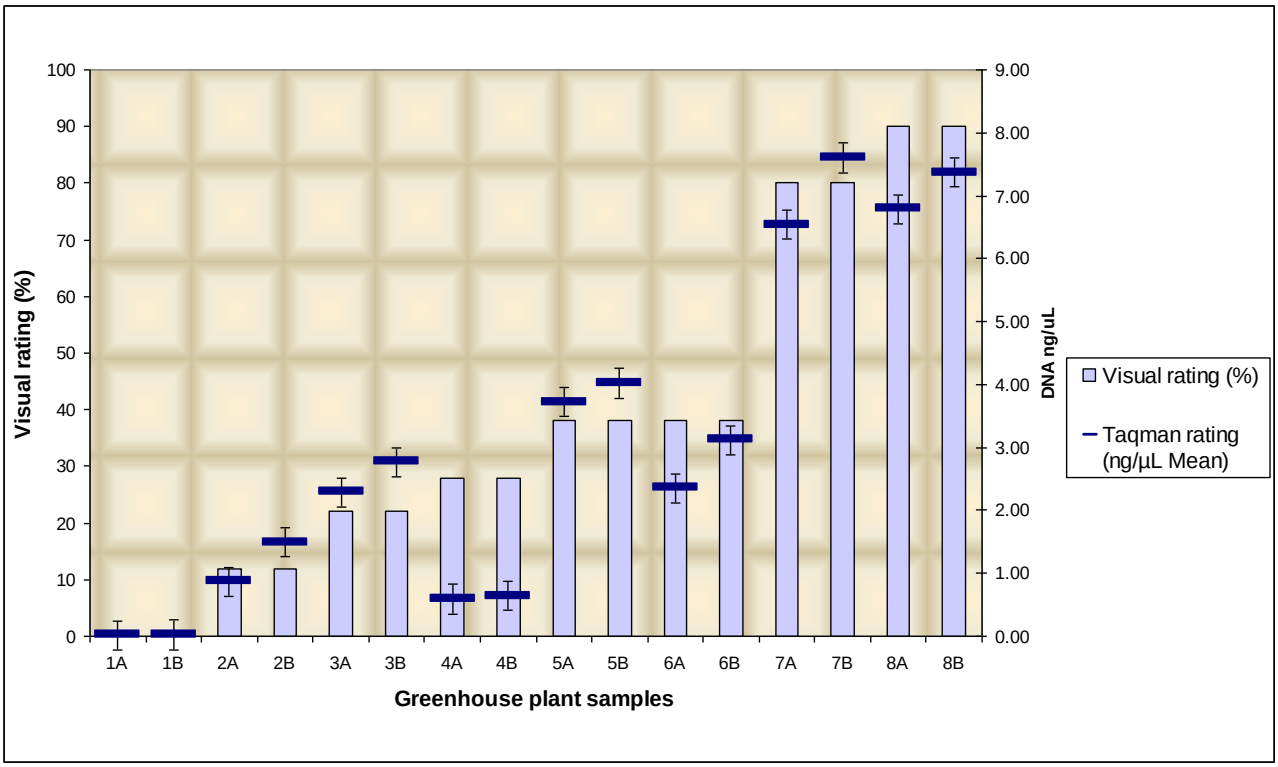

Fig. 1: Comparison of real-time PCR rating with visual rating of fungal infection in maize samples

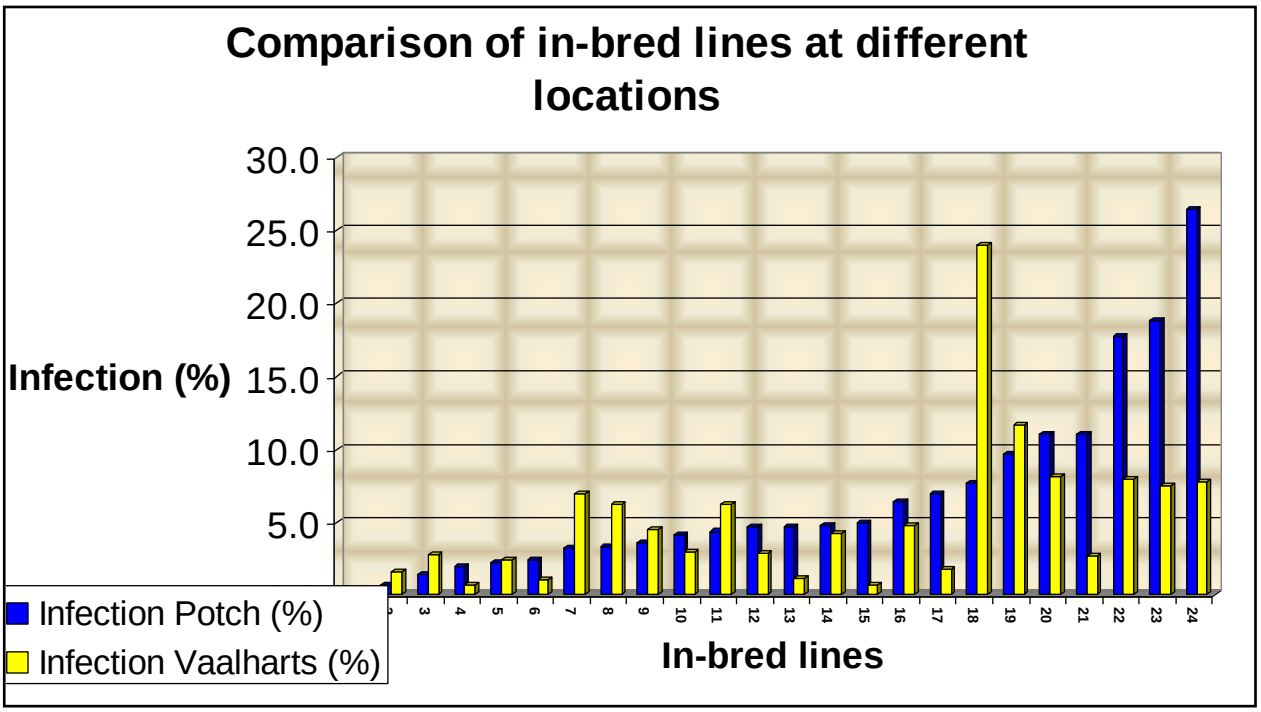

Figure 2: Comparison of in-bred line visual rating results from Potchefstroom and Vaalharts irrigation scheme

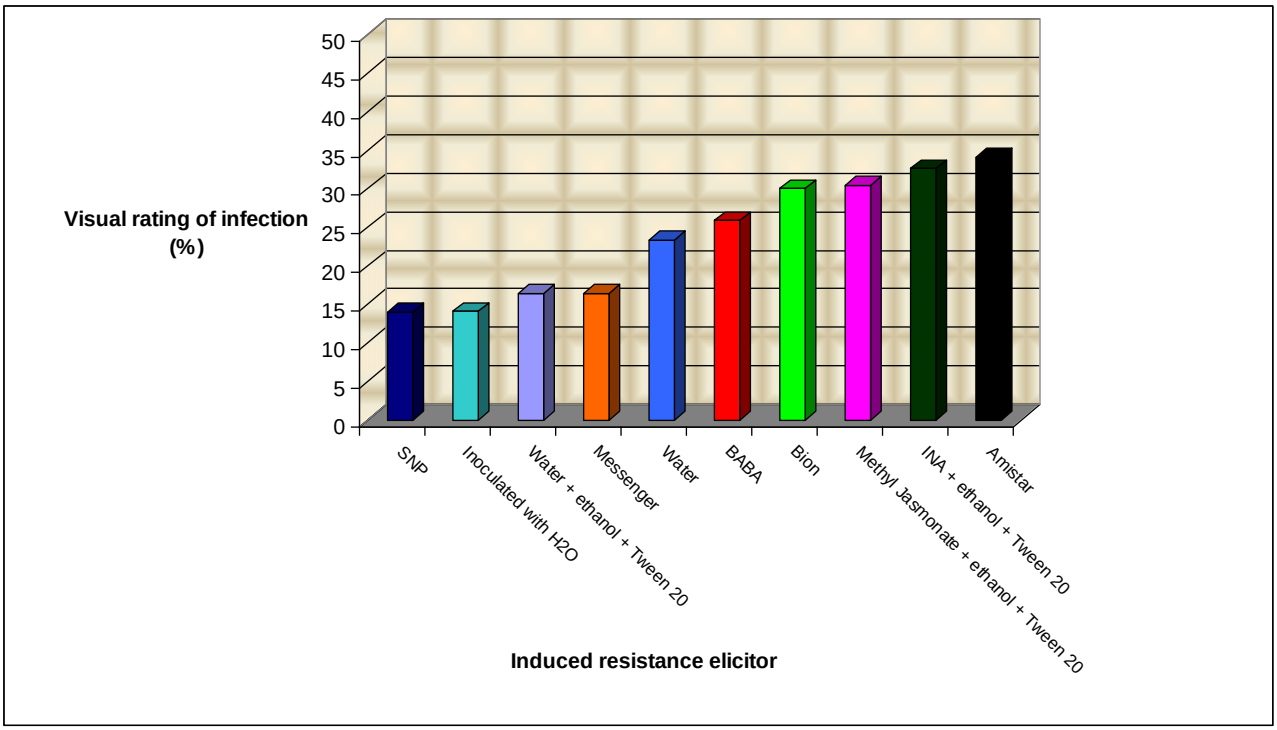

Figure 3: Visual rating of induced resistance greenhouse trial 1, 2007/8

D. PROBLEMS THAT HAVE BEEN ENCOUNTERED WITH THE PROJECT:

A low incidence of Fusarium infection was experienced during field trials in the 2006/7 season.

E. PROJECT OUTCOMES

Theses:

- 2008: Edson Ncube (MSc) – Mycotoxin levels in subsistence farming systems in South Africa

Conference presentations:

- Ncube, E., Flett, B. C. and Viljoen, A. 2007. Mycotoxin levels in subsistence farming systems in South Africa. Congress of the 45 Southern African Plant Pathology Society for Plant Pathology, Kopanong, South Africa, 21-24 January. *South African Journal of Science* 104.
- Ncube, E, Flett, B.C. and Viljoen, A. 2007. Agricultural decisions that may influence mycotoxin levels in subsistence farming systems in South Africa. Congress of the 45 Southern African Plant Pathology Society for Plant Pathology, Kopanong, South Africa, 21-24 January. *South African Journal of Science* 104.
- Ncube, E., Flett, B.C., Waalwijk, C. and Viljoen, A. 2008. Mycotoxin levels in subsistence farming systems in South Africa. Plant Production meeting, South Africa
- Ncube, E., Flett, B.C., Waalwijk, C. and Viljoen, A. 2008. Geographic distribution of *Fusarium* spp. and production of fumonisins in maize of subsistence farmers in South Africa. International Fusarium Workshop, Alghero, Sardinia, Italy, August 30-September 2

Overseas visits (2008):

- 9th International Congress of Plant Pathology, Torino, Italy, August 24-29 (Altus Viljoen)
- International Fusarium Workshop, Alghero, Sardinia, Italy, August 30-September 2 (Altus Viljoen)
- 3rd International Symposium on Fusarium Head Blight, Szeged, Hungary, 1-5 September (Altus Viljoen)
- International Symposium on Induced Mutations in Plants (ISIM), Vienna, Austria, 12th -15th August (Lindy Rose)
- ISM Workshop – training course: Detection techniques for mycotoxins and toxigenic fungi in the food chain, Bari, Italy, 29 September – 3 October (Ian Small)

F. MILESTONES THAT HAVE NOT BEEN ACHIEVED AND THE REASON FOR THAT:

The project is currently in its first year of execution, and already a great amount of work has been done and new technologies have been established.

G. AN ASSESSMENT OF THE ADEQUACY OF THE FUNDING TO COMPLETE THE EXECUTION OF THE PROJECT IN THE FORM OF AN EXPENDITURE STATEMENT

The funding for the current financial year was adequate for the execution of the research project. Our results rely to a great extend on the ability to reduce fungal colonization and toxin production in maize kernels. These variables, therefore, have to be tested throughout the project. Costs of both processes, both in terms of man hours and financial output, are relatively high. A third component that is quite costly, is travelling expenses, as all our trials are done in close collaboration with the ARC-GCI in Potchefstroom.

The following approaches were followed to reduce costs and time spent on fungal colonization and toxin analyses:

- Plating out of maize kernels is being replaced by quantitative real-time PCR, which is much less time consuming and more accurate, but the technique is costly. For each sample analysed, the price for isolation, identification and quantification is approximately R 50. We will do several thousand of these during the project.
- Toxin analysis is a vital part of all studies related to mycotoxin-producing fungi. The two most feasible methods to test for toxin content are ELISA and high performance liquid chromatography (HPLC). The choice of method is determined by the level of precision required as well as the quantity of toxin expected. The ELISA method is useful for routine testing of samples, but is limited by its narrow range of detection, making it inaccurate when high or low toxin levels are present. The test must also be repeated approximately three times to reduce variation. Cost per sample, therefore, often amount to as much as R 120 per sample. HPLC is very accurate for analysis over a wide range of toxin levels, and does not need to be repeated. However, the test is more expensive. HPLC toxin analysis at the Medical Research Council – PROMEC unit will cost us approximately R700 per sample. We are, therefore, optimising the technology at the Central Analytical Facilities (CAF) at the University of Stellenbosch, with a projected cost per sample of about R 100 per sample. This would also be preferable as it would enable the training of students in the use of HPLC for toxin analysis, while at the same time reducing the cost of the toxin analysis.

H. THE ESTIMATED DURATION OF THE PROJECT UNTIL COMPLETION

January 2008 – December 2012

To accelerate experimentation, the following technologies have been developed/implemented in our research:

- A greenhouse hydroponic system for growing maize has been developed to conduct evaluations. This allows two growing seasons data to be obtained during a period of one year. The additional “season” reduces the time required for completion of trials.
- To accelerate the identification of resistant maize genotypes, a small plant screening technique is being investigated. If successful this would allow the rapid screening of maize breeding material.
- Plating out of maize kernels is being replaced by quantitative real-time PCR, which is much less time consuming and more accurate, but the technique is costly.
- HPLC analysis at the CAF at the University of Stellenbosch will replace ELISA analysis in Potchefstroom and HPLC analysis at the MCR in Tygerberg.