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Does horizontal gene flow occur in transgenic banana/ *Fusarium oxysporum* (V5W2-9 NH3) associations?

D. Kabuye[a], L. Tripathi[b], E. Niyibigira[c], J. Tripathi[b] and P. Okori[a*]

[a]*Department of Agricultural Production, Makerere University P.O. Box 7062 Kampala, Uganda.*

[b]*International Institute of Tropical Agriculture, P.O.Box 7878 Kampala Uganda.*

[c]*Office of the Prime Minister. P.O. Box 341 Kampala, Uganda.*

**Corresponding author : email, pokori@agric.mak.ac.ug*

ABSTRACT

Fusarium oxysporum (V5W2-9 NH3) a fungal non-pathogenic *Fusarium* species is useful in the bio-control of nematodes in bananas. With the advent of the usage of biotechnological approaches to mitigate crop production constraints, several biosafety concerns have emerged. Of interest is geneflow to phyllosphere microorganisms. In this study, transgenic banana plantlets having the hygromycin resistance (*hpt*) gene as a selection marker and ß-glucuronidase (*gus*A) gene as reporter marker were used to investigate gene flow to *F. oxysporum* (V5W2-9 NH3). The plantlets were inoculated with *F. oxysporum* (V5W2-9 NH3) and transferred into 10 litre buckets containing sterile soil. After colonisation, the endophytes were re-isolated from the plants on selective medium. DNA was extracted from the re-isolates and amplified using hpt and *gusA* specific primers to confirm putative gene flow. Results indicated that although plant colonisation was significantly affected by media composition (P≤0.0001), there was no interaction between media composition and the banana genomic structure (P=0.31). PCR screens also showed that the microorganisms were not able to take up the transgenes from the plants suggesting therefore that the cultivation of transgenic bananas will not be a threat to the fungal communities with which they are associated.

Keywords: *biosafety, Fusarium species, endophyte, non-pathogenic fungus.*

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Introduction

Endophytes generally refer to fungi inhabiting plant tissues, though there are species of bacteria that have been observed to live endophytically within the plant tissues where they play crucial roles (Stoltzfus *et al.*, 1997). Fungal endophytes play important roles in the growth of plants through mutualistic associations. There are various types of endophytes, some occurring in monocots, while others in dicot plant species. The most important genera that have been isolated from bananas are *Fusarium* and *Acremonium*. Others include; *Penicillium*, *Aspergillus*, *Gongromella* and *Trichorderma*. *Fusarium* is the most dominant genus with *F. oxysporum* being the most widely spread species (Hallman and Sikora, 1994; Niere *et al*., 2002). There are various strains of *Fusarium oxysporum* some of which are pathogenic mainly causing vascular wilts. Some *fusarium* species such as *Fusarium oxysporum* (V5W2-9 NH3) are non-pathogenic and play important roles in improving plant growth through inhibitory effects on nematodes and weevil development (Hallman and Sikora, 1994; Niere *et al*., 1999; Paparu, 2008). With respect to the environment, *Fusaria* unlike other biological control agents reduce the risk of side-effects on non target organisms including crops and humans since they live inside plant tissues (Niere *et al*., 2002).

Due to free lying DNA within plant tissues, endophytes by virtue of their location are predisposed to this DNA and ultimately take it up through horizontal gene flow using mechanisms such as homologous and illegitimate recombination (Magee *et al*., 2003; Zhang *et al*., 2005). Use of biotechnological approaches in the mitigation of biotic constraints in bananas for example the use of chitinases from

rice and papaya in the induction of resistance in bananas to black sigatoka (*Mycosphaerella fijiensis*) (Swennen and Sagi, 1996) and maganins to induce resistance to *Fusarium oxysporum* f.sp. *cubense* and *Mycosphaerella musicola* in rasthali cultivar (AAB) (Atkinson *et al*., 2003), raises biosafety concerns especially gene flow. The occurrence of horizontal gene flow to these microbial communities can have potential negative effects on their major biological and ecological functions (Dröge *et al.*, 1999). Due to the fact that fungi release large amounts of DNA in the environment during their life cycles in addition to the occurrence of conjugatory gene transfers between fungi and bacterial communities (Paul *et al.*, 1987; Dunn-Coleman and Wang, 1998), uptaken DNA will be shared with other microorganisms in the environment thereby leading to the emergence of new populations that may even be pathogenic to the landraces and other plants and disrupt the biological diversity in the environment (Teycheney and Tepfer, 1999; Cooper and Sweet, 2001). This study therefore sought to investigate the occurrence of horizontal gene transfer of a *ß- glucuronidase* and *hpt* gene in transgenic bananas to an associated fungal endophyte, *Fusarium oxyporum* (V5W2-9 NH3).

Materials and methods

The materials used in the study included; transgenic banana plantlets from local cultivars (Mbwazirume and Mpologoma) transformed with *ß-glucuronidase* (gusA) reporter gene under the control of CaMV35S promoter and a hygromycin resistance gene as the selectable marker and terminated by a *nos* sequence. The microorganisms tested were *Fusarium oxysporum* (V5W2-9 NH3).

Experimental design. The experiment was set up following a Complete Randomised Design (CRD). The treatments comprised of transgenic banana plantlets containing *gus*A and *hpt* genes and non transgenic control banana plants. These plantlets were innoculated with *F. oxysporum* (V5W2-9 NH3). The experiment was run for a period of 13-16 weeks during which normal agronomic practices were done to ensure proper growth. Each replicate had 5 transgenic plants and 2 controls. The experiment was repeated three times. Re-isolation of the fungal organisms was done by destructive sampling of the roots. The re-isolations were done three times.

Media preparation. The fungal organisms were cultured on half strength Potato Dextrose Agar (PDA) which was prepared by autoclaving constituted media for 15 minutes at 121°C and cooled prior to the addition of streptomycin sulphate (Duchefa Biochemicals, Netherlands), penicillin (Duchefa Biochemicals, Netherlands) and chlorotetracyclin (Duchefa Biochemicals, Netherlands) antibiotics as well as potassium chlorate (Lab Express Inc New Jersey, USA). The media was subsequently dispensed into petri-plates (90mm). Normal half strength PDA comprised of the antibiotics above and potassium chlorate (Lab Express Inc New Jersey, USA). Selective half strength PDA contained all the above antibiotics, potassium chlorate (Lab Express Inc New Jersey, USA) as well as hygromycin (50 µg/ml) (Duchefa Biochemicals, Netherlands).

Inoculation and re-isolation of experimental organisms

The transgenic plantlets including the controls were established in a nutrient solution for 2 months for adequate root mass development (Fig. 1). At inoculation stage, pure *Fusarium oxysporum* (V5W2-9 NH3) cultures (Paparu, 2008) raised on half strength potato dextrose agar (Duchefa Biochemicals, Netherlands) were used as inoculum. Spore counts were standardised using a haemocytometer. The banana plantlets were exposed to the fungal inoculum by immersion in ritar jar flasks containing 1.5 x 10^6 spores/ml for four hours. Inoculated plantlets were later transferred to 10 L buckets having sterile soil which had been steam sterilised at 100°C using moist heat over a water bath in a metallic cylindrical drum for a period of up to 3-4 days.

After the first eight weeks, two root samples were taken from each of the plants. The root samples were washed and sterilised in 57% sodium hypochlorite (Reckit Bectinson, East Africa Limited Nairobi, Kenya) for 30 seconds, 75% ethanol for one minute and finally rinsed in sterile water for 1 minute. Each root was cut into 12 small cylindrical discs of which 6 root discs were plated on normal half strength PDA (Duchefa Biochemicals, Netherlands). The remaining 6 root discs were plated on selective half strength PDA. This procedure was repeated for each of the 2 roots excised from all the experimental plants. The plates were incubated for 7-10 days at room temperature. A total 1584 banana root cylindrical discs were assessed for *Fusarium oxysporum* (V5W2-9 NH3) (Paparu, 2008) colonisation with major emphasis on its microscopic characteristics.

Molecular analyses to establish evidence of gene flow. Fungal colonies growing on selection media were sub-cultured onto fresh selective half strength PDA plates and incubated for 7-10 days at room temperature for genomic DNA extraction. This was done to confirm the ability of the

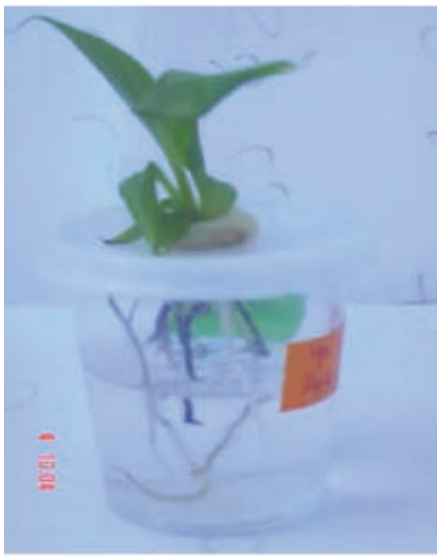

Fig. 1: A transgenic plantlet establishing in nutrient solution prior inoculation.

fungal colonies to re-establish on selection media. Where wild fungal isolates were observed, they were subjected to the same procedures as above.

Genomic DNA extraction and PCR screens. Thirty six (36) fungal isolates (including 30 *Fusarium oxysporum* and some wild fungal isolates, and 6 re-isolates from the controls) were used for genomic DNA extraction according to Mahuku (2004). The mycelia of the fungal colonies were crushed in sterile sand using miniature pestles. The DNA extraction buffer (Tris EDTA SDS (TES) contained: 0.2 M Tris-HCl [pH 8], 10 mM EDTA [pH 8], 0.5 M NaCl, 1% SDS. The quality of the extracted DNA was assessed by running 5 µl of the DNA and 2 µl of loading dye in a 1% agarose gel in TAE buffer. The gel was stained in 1% ethidium bromide for 20 minutes and visualized under an ultra violet trans-illuminator. The bands on the gel were documented in a gel documentation system. PCR was performed using primers specific to the gusA and hpt genes. The primers used were; primer 1- 5' TTTAACTATGCCGGGATCCATCGC 3' and primer 2- 5' CCAGTCGAGCATCTCTTCAGCGTA 3'. PCR involved a 2 minute initial denaturation step at 94°C and 30 cycles consisting of 1 minute denaturation at 94°C, 1 minute primer annealing at 60°C and a 1 minute extension at 72°C followed by a 10 min extension step at 72°C. The amplification products were size fractionated by agarose gel, electrophoresed in TAE buffer at 80 volts for one hour (Maniatis *et al*., 1989). The gels were stained with 1% ethidium bromide in an aqueous solution and were examined for amplification of the *gus*A and *hpt* genes.

Statistical analysis of the results. The data were subjected to analysis of variance (ANOVA) using the Statistical Analysis Systems (SAS) (version 9.1) (SAS Institute Inc, North Carolina, USA). Isolation frequencies of *F. oxysporum* (V5W2-9 NH3) among the plant roots were analysed using categorical logistic regression. Likelihood ratio tests were performed to investigate differences within factors (*F. oxysporum* (V5W2-9 NH3) and plant roots). In cases where significant differences ($P \leq 0.05$) were detected, the Dunn-sidak correction factor was used as a posterion testing tool (Sokal and Rolf, 1995) using the SAS system (SAS, 1989).

Results

Gene flow analysis from transgenic plants to *F. oxysporum* (V5W2-9 NH3). Plant colonisation was low with only 40 root segments colonised by the fungus. A total 36 fungi grew on normal media of which 20 where from the transgenic plants and 16 from the controls. Four (4) fungi established on selection media of which 3 were from the controls and only one established from the transgenic plants. Overall, *F. oxysporum* colonisation averaged 27.3% in transgenic plants and 20.5% in the non-transgenic plants. Plant colonisation by *F. oxysporum* (V5W2-9 NH3) was significantly influenced by the banana genotype ($P \leq 0.034$) (Table 1). Although there was no effect of sampling time on colonisation ($P = 0.076$) among plants of the same genotype, plant colonisation was significantly affected by the media composition ($P \leq 0.0001$), with higher percentage colonisation observed for roots that were plated on non-selective media compared to those that were plated on selective media ($P \leq 0.025$) (Table 1). There was no interaction between media composition and banana genomic structure ($P = 0.31$). Some wild fungal isolates were similarly observed to grow especially on selective media as well as even on the non-selective media.

Table 1. Logistic regression analysis for Fusarium oxysporum (V5W2-9 NH3)

Source of variation	Degrees of freedom	Sums of squares	Probability
Media	1	23.16	<0.0001
Treatment	1	4.48	0.034
Weeks	2	5.14	0.076
Interactions			
Media * treatment	1	1.04	0.308
Media * weeks	2	4.61	0.0997
Treatment*weeks	2	0.79	0.675

Molecular analysis and PCR screens for gusA and hpt gene. PCR was performed using *gusA* and *hpt* specific primers. The amplicons were electrophoresed on a 1% agarose gel. An amplified fragment of about 500bp corresponding to the internal fragment of *gusA* gene was observed in the positive control (plasmid DNA (pCAMBIA1201)) (Plate 1C) and an amplified fragment corresponding to the *hpt* gene was also observed in the positive control (Plate 1D). No amplified fragment was observed with DNA extracted from the fungi re-isolated from the phyllosphere of the experimental plants (Plate 1C and 1D).

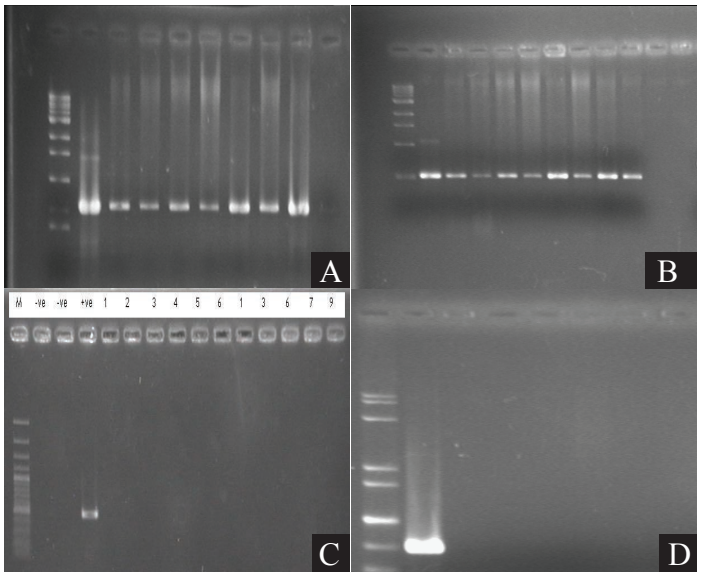

Plate 1. Electropherogram showing the presence of the hygromycin hpt (A) and β–glucuronidase (B) genes in the genetically engineered banana plants; and the absence of β–glucuronidase (C) and the hpt genes (D) in the microbial DNA.

Discussion.

In the study, the fungal re-isolates from transgenic banana phyllospheres were not transformed with the *gus*A and *hpt* genes. The test fungi were able to survive on selective media suggesting endogenous capacity to degrade the antibiotics. However, attempts to amplify the transgene were negative with no amplicons affirming that *F. oxysporum* (V5W2-9 NH3) re-isolates were indeed not transgenic. Among other fungi however, horizontal gene flow has been demonstrated. Whereas gene flow may occur through homologous and illegitimate recombination (Magee *et al*., 2003; Ruiz-Díez, 2002), up taken DNA are rapidly destroyed (Hoffman *et al*., 1994). This phenomenon may explain in part the survival of re-isolates on selective media and failure to detect the *gus*A and *hpt* gene. Nevertheless, the survival of re-isolates even prior to exposure to transgenic banana suggests these fungi (*F. oxysporum* (V5W2-9 NH3)) possess mechanisms to degrade hygromycin. This study thus found no evidence for gene flow. These results agree with earlier studies (Zhang *et al*., 2005) which failed to show any gene flow of the *Streptomyces hygroscopicus* bar gene under the control of a *Cochliobolous heterostrophus* glyceraldehyde-3-phosphate dehydrogenase (GPD) promoter in transgenic poplar to *Amanita muscaria* ectomycorrhizas. However, other studies have detected horizontal gene flow events to *Aspergillus niger* in soil microcosms after co-cultivation with transgenic plants of *Datura innoxia* expressing the hygromycin B gene under the control of the 35S promoter (Hoffman *et al*., 1994). This is interestingly one of the few reports of horizontal gene flow to fungal organisms. Taken together, this study found no empirical evidence of the occurrence of horizontal gene flow from transgenic banana to root endophytic *Fusarium oxysporum* (V5W2-9 NH3) suggesting therefore that cultivation of such transgenic bananas may not be a threat to the beneficial fungal communities with which they are associated. However, the differences in physiology in plants and their interactions with micro-organisms would warrant pursuing these studies on case by case basis.

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