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Assessment of the potential for horizontal gene flow from transgenic bananas to rhizosphere inhabiting microorganisms

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ABSTRACT

Recent advances in plant biotechnology, especially genetic engineering have heightened promises for mitigating biotic and abiotic constraints. The use of biotechnology to mitigate biotic constraints has raised several biosafety concerns mainly gene flow. Of interest in crop cultivation is gene flow to rhizosphere microorganisms. In this study, transgenic banana plantlets having the hygromycin resistance (*hpt*) gene as selection marker and beta-glucuronidase (*gus*A) gene as a reporter marker were used to investigate gene flow to *Agrobacterium tumefaciens, Escherichia coli* (DH5α) and natural soil bacteria. The plants were potted in soil in which the microorganisms were inoculated and later isolated from the rhizosphere on selective medium supplemented with hygromycin. DNA was extracted from bacterial organisms that established on selective medium and subsequently amplified using *hpt* and *gus*A specific primers to confirm putative gene flow. Highly significant differences (P=0.001) were observed among the means of the CFUs of *E. coli* and natural soil bacteria that were re-isolated on normal and selective media however, plant genotype effects on the re-isolation of the test organisms were not observed (P≥0.05). PCR screens for the marker genes revealed no uptake of the transgenes by the microorganisms suggesting therefore a very low probability of gene flow from banana. Given the differences in physiology and root exudation patterns among different crops, and their ability to induce competence among microbial organisms, its worthwhile pursuing these studies on a case by case basis with respect to different crops and antibiotic markers.

Keywords: *Biosafety, biotechnology, biotic constraints, Musa* sp*, Uganda*

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Introduction

Rhizosphere microorganisms are those that live within a volume of soil that is in direct contact with plant roots and thereby influenced. They comprise fungal, bacterial and protozoans amongst other microbial groups and in crops like bananas, often reach upto very high densities per gram of soil due to the highly diverse habitat provided (Siciliano *et al*., 1998). Bacteria constitute an important component of these microorganisms and they have developed associations with plants which range from parasitic, symbiotic, epiphytic to endophytic associations. The diversity and development of these microorganisms is to a large extent stimulated by root exudates and other factors such as root exudation patterns, root structure, duration of the season/ growth, soil type, crop stage, cropping practices and other environmental factors (Smalla *et al*., 2001). Furthermore, species diversity of plants affects the amounts of root exudates and rhizo-deposition in the different root zones. This significantly affects the structural and functional diversity of the rhizosphere microorganisms (Dunfield and Germida, 2004). Therefore, the influence of plants on soil microbes is greatest in the rhizosphere and the magnitude of this influence is determined by the extent of microbial interactions and the plant (Dunfield and Germida, 2004; Kowalchuk *et al.*, 2003).

Changes in plants due to genetic modifications such as the use of biotechnological approaches to develop transgenic plants could have alterations on these effects through horizontal geneflow, a process which involves the uptake and eventual expression of plant DNA by competent microorganisms (Cooper and Sweet, 2001; Nielsen, 2003). The use of biotechnology approaches to develop transgenic plants has followed the promise of the technology in the control of emergent pest and disease problems over other strategies

such as cultural, chemical, biological and conventional breeding that have various associated constraints which make them inefficient at providing the requisite control (Okori, 2004; Tripathi *et al*., 2004; Vuylsteke *et al*., 1995).

In bananas, which are a major food source in East Africa and especially in Uganda where they are an important source of food security and household incomes for the local farming communities, biotechnological approaches have been employed in the mitigation of some of these constraints. For example the use of anti-microbial proteins and chitinases in AAB plantains and grande naine cultivars to induce resistance to black sigatoka (*Mycosphaerella fijiensis*) and; the use of maganins in rasthali cultivar (AAB) to induce resistance to *Fusarium oxysporum* f.sp. *cubense* and *Mycosphaerella musicola* (Atkinson *et al*., 2003). In Uganda, research has been undertaken on the use of chitinases from rice and papaya in the induction of resistance in bananas to black sigatoka (*Mycosphaerella fijiensis*) (Swennen and Sagi, 1996); while there are ongoing efforts on the use of *hrap* genes in bananas to control banana bacterial wilt. These research efforts should consider potential gene flow concerns.

The occurrence of horizontal gene flow to rhizosphere microbial communities can have potential negative effects on their major biological and ecological functions (Dröge *et al*., 1999). Moreover, if the genes taken up have the ability to improve the fitness of the recipient microorganisms, directional selection may ultimately lead 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 (Cooper and Sweet, 2001). The objective of this study therefore was to investigate horizontal gene flow from transgenic bananas to rhizobacteria.

Materials and methods

Study materials. The materials used in the study included; transgenic banana plantlets from local cultivars (Mbwazirume and Mpologoma) transformed with *ß-glucuronidase* (*gus*A) reporter gene under the control of *CaMV35S* promoter and terminated by a nos sequence with a hygromycin resistance gene used as the selectable marker. The microorganisms tested included: *Agrobacterium tumefaciens* (EHA 105) resistant to the gentamycin sulphate antibiotic, *Escherichia coli* (DH5α) and naturally occurring soil bacteria obtained from the rhizosphere of banana plants.

Experimental design. The experiment was set up with treatments that comprised of transgenic banana plantlets containing the *gus*A and *hpt* genes and non transgenic banana plants that were used as the control. The plantlets' rhizospheres were innoculated with either *A. tumefaciens* or *E. coli*. The experiments were run for a period of 13-16 weeks during which normal agronomic practices were done to ensure proper growth. Each experiment had 5 transgenic plants and 2 controls. The experiments were repeated three times. Re-isolation of the bacterial organisms from the rhizosphere region was done three times.

Media preparation. For culture of *E. coli* and natural soil bacterial isolates, Luria Bertani (LB) media (Cools *et al*., 2001) was used which was prepared by autoclaving constituted media for 15 minutes at 121 ^{o}C and cooled prior to addition of a fungicide, cyclohexamide (150 µg/ml) (VWR International, England). The media was subsequently dispensed into petri-plates (90 mm). Selective LB media was prepared by adding an antibiotic, hygromycin (50 µg/ml) (Duchefa Biochemicals, Netherlands) and a fungicide, cyclohexamide (150 µg/ml) (VWR International, England) to autoclaved and cooled media. The selective media was dispensed into petri-plates (90mm). Normal and selective media for *A. tumefaciens* re-isolation was prepared as for *E. coli* except that gentamycin sulphate (50 µg/ml) (Duchefa Biochemicals, Netherlands) was added as a selective antibiotic.

Re-isolation of inoculated experimental organisms

Inoculum dosage optimisation experiments

The bacterial innocula used comprised of *A. tumefaciens* and *E.coli*. Dosage optimisation was initially done to establish the right amounts of bacteria to apply in the soil that would be easily re-isolated. The optimisation process involved the use of tissue culture banana plantlets grown in plastic pots (400 ml volume) in sterile soil for a period of 2 months. Some of the plants were inoculated with an overnight culture of *E. coli* using either 1 ml, 2 ml, 3 ml or 4 ml. The other plants were inoculated with a 48 hour old culture of *A. tumefaciens* using the same volumes that were used with *E. coli*. The inoculation process was done by pouring the bacterial culture solution concentrically around the stem base region of the plants. The experiment was left to stand for 10 days prior to the re-isolation of the bacteria. The 3ml volume gave optimal results for *A. tumefaciens* and was therefore adopted for the study while in *E. coli*; the 2ml inoculum volume was adopted.

Inoculum re-isolation optimization

Plantlets inoculated with the 2 ml and 3 ml bacterial innoculum dosages were initially used during the re-isolation of *A. tumefaciens* and *E. coli*, respectively. One gram soil samples were derived from each of the pots from the stem base region of the plant and vortexed for 2 minutes in 2 ml of sterile water in sterile 50 ml centrifuge tubes. The soil sample was subsequently serially diluted to 10^{-2}, 10^{-4} and 10^{-6}. This procedure was followed for the rhizosphere soil samples that were respectively inoculated with *E. coli* and *A. tumefaciens*. Diluted solutions (100 µl) were spread on LB media (Cools *et al*., 2001). The plates spread with *E. coli* were incubated overnight at 37^{o}C while the *A. tumefaciens* culture plates were incubated at 28^{o}C for two days. Colony forming units (CFUs) were compared across the different plates for each of *E. coli* and *A. tumefaciens* to establish the optimum dilution for usage in the experiments. In *A. tumefaciens* and *E.coli* respectively, the 10^{-2} and 10^{-4} dilutions gave the most distinct and countable re-isolated colonies and were therefore adopted for use in the experiments.

Gene flow analysis to the experimental organisms

Agrobacterium tumefaciens and *E. coli* were inoculated into the rhizospheres of the respective experimental plants using optimised innoculum volumes. This was followed with the re-isolation of the bacteria from the rhizospheres of transgenic and non-transgenic banana plantlets for the study of geneflow. Two 1 g soil samples collected three times at intervals of 10 days from the rhizosphere of each plant were used for re-isolation purposes. The intervals used were adopted to enable acclimatisation of the microorganisms to the banana rhizosphere and also allow for adequate exposure to root exudates. The soil samples derived from the rhizosphere of plantlets inoculated with *A. tumefaciens* were suspended in sterile water and diluted to 10^{-2} CFU. Of this solution, 100 µl was inoculated into LB plates containing selective media (2 LB plates containing 50 µg/ml hygromycin) and a control (without the antibiotic). A similar procedure was used for soil samples derived from the rhizospheres of transgenic plantlets inoculated with *E. coli*. The exception was the initial dilution of the bacterial suspension to 10^{-4} CFU. Both *A. tumefaciens* and other bacterial cultures were incubated at 28^{0}C for 48 hours while the *E. coli* cultures were incubated at 37^{0}C for 24 hours. Colony counts were taken and used to compute the CFUs to enable working with the actual amounts of bacterial organisms that were in the sample of soil taken from the rhizosphere region of the experimental plants.

Agrobacterium tumefaciens colonies that grew on selective media were subjected to a second round of selection on plates containing hygromycin (50 µg/ml) (Duchefa Biochemicals, Netherlands) antibiotics to confirm their survival. A similar procedure was used for *E. coli* and the natural soil bacterial isolates that grew on selective media. The selection plates used for the re-isolation of *E. coli* and the natural soil bacteria isolates had hygromycin (50 µg/ml) (Duchefa Biochemicals, Netherlands) as the selection antibiotic. In order to culture sufficient quantities of bacteria for DNA isolation, re-isolated colonies of *A. tumefaciens*, *E. coli* and natural soil bacteria were cultured in LB broth media. A 25 mls solution of LB broth in 100 ml erlenmeyer flasks containing only hygromycin (for *E. coli* and natural soil bacteria) or gentamycin sulphate and hygromycin (for *A. tumefaciens*) was used to culture the test isolates. The flasks containing *E. coli* were incubated overnight at 37^{0}C while those that had *A. tumefaciens* and natural soil bacterial isolates were incubated at 28^{0}C for 48 hours. Optical densities were taken at the end of the incubation period for all the bacterial cultures. The bacterial cells in solution were harvested by centrifugation (MIKRRO 250, Berlin Germany) at 6000 rpm for 15 minutes at 4^{0}C in sterile 50 ml centrifugation tubes.

Genomic DNA extraction and PCR screens. Genomic DNA was extracted from the harvested bacterial cells that grew on selection media using a protocol by Mahuku (2004). The bacterial cells were crushed using sterile sand and 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 using a gel documentation system. The PCR was performed using primers specific to the gusA and hpt genes. The gusA primers used were; primer 1- 5' TTTAACTATGCCGGGATCCATCGC 3' and primer 2- 5' CCAGTCGAGCATCTCTTCAGCGTA 3'. The PCR involved a 2 min initial denaturation step at 94^{o}C and 30 cycles consisting of 1 min denaturation at 94^{o}C, 1 min primer annealing at 60^{o}C and a 1 min extension at 72^{o}C followed by a 10 min extension step at 72^{o}C. The amplification products were size fractionated by agarose gel, electrophoresed in TAE buffer (Maniatis *et al.*, 1989) at 80 volts for one hour. 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 bacterial colonies that grew on media were expressed as CFUs using the formula below;

$$CFUs = \frac{\text{No. of colonies}}{\text{Dilution factor}} \; X \; \text{volume of sterile water used to dilute the soil sample}$$

The data collected were subjected to t-tests for comparison of means. The analysis for geneflow to rhizo-bacteria data were subjected to ANOVA using the GenStat Discovery Edition 3 (Lawes Experimental Trust Rothamstead Experimental Station UK). Where significant differences were found between treatment means, these were compared using Fishers Protected Least Significant Difference Test (Steel *et al*., 1997).

Results

Re-isolation of rhizosphere inoculated bacteria

Inoculum dosage optimisation. A comparison of best dilution volumes revealed significant differences at 5% level (P<2.35, t = 1.19) in *A. tumefaciens*. Distinct colonies were obtained with the 3 ml volume (13 x 10^4 CFUs as compared to the 8.60 x 10^4 CFUs, 16 x 10^4 CFUs and 26 x 10^4 CFUs obtained with the 1ml, 2 ml and 4 ml volumes, respectively. In *E. coli*, the inoculum dosage used influenced the bacterial colony counts at re-isolation at the 5% level (P < 2.35, t= 1.24) with the highest colony count observed where the 2 ml dosage volume was used and the least in the 3 ml inoculum volume.

Inoculum re-isolation optimisation. In *A. tumefaciens,* significantly more colonies established with the 10^{-2} dilution compared to other dilution factors (P< 6.31, t=4.30). About 83 colonies were obtained with the 10^{-2} dilution whereas 1 colony and 0 colonies where observed for the 10^{-4} and 10^{-6} dilution factors, respectively. Results from the re-isolations in *E. coli* revealed that all the dilutions gave similar results and none were significantly different (P ≥ 0.05). The 10^{-4} dilution plate however, had more distinct colonies as compared to the 10^{-2} and 10^{-6} dilutions.

Analysis of geneflow to the experimental organisms. On plates, a total 1089 colonies (853 on normal media and 236

on selection media) were obtained from *E. coli* while in *A. tumefaciens* and natural soil bacterial isolates, 12503 colonies (12457 on normal media and 46 on selection media) and 615 colonies (564 on normal media and 51 on selection media) respectively were observed to grow. Generally more isolates from *E. coli* grew on selection media than from *A. tumefaciens* and natural soil bacteria. The lowest count of isolates that grew on selection was observed in *A. tumefaciens*. The total CFUs that grew on selective media were 8.0×10^6 for natural soil bacterial isolates, 4.26×10^7 for *E. coli* and 5.80×10^4 for *A. tumefaciens*. Highly significant differences (P=0.001) were observed amongst the means of the CFUs of *E. coli* that were re-isolated on both normal and selective media (Table 1). Marked differences (P=0.001) were similarly observed amongst the means of the CFUs derived from the different plants. However significant plant genotype effects on the re-isolation of *E. coli* were not observed (P≥0.05) (Table 1). In general more bacteria were re-isolated from non- selective media (Table 2). Interestingly, plants that supported high numbers of bacteria on non selective media similarly generated high numbers on selective media.

Table 1: Analysis of variance for the means of the CFUs of the experimental microorganisms that established on normal and selective media

Source of variation	Degrees of freedom	Mean square	F. pr
E.coli			
Replicate	2	2480289	
Plant	5	1465884	<.001
Treatment	1	3206770	<.001
Plant.Treatment	5	3206770	0.911
Error	22	146660	
Total	35		
Natural Soil bacterial isolates			
Replicate	2	207515	
Plant	8	195282	0.748
Treatment	1	30380866	<.001
Plant.Treatment	8	252260	0.597
Error	34	310619	
Total	53		
A.tumefaciens			
Replicate	2	914930	
Plant	4	194385	0.583
Treatment	1	1840816	0.017
Plant.Treatment	4	192720	0.587
Error	18	266403	
Total	29		

Data were transformed using the square root transformation to normalize the variances (Sokal and Rolf, 1995)

Table 2. Mean colony forming units of experimental organisms that established on both normal and selective media

Plant	Treatments	
	Normal media	Selective media
Escherichia coli		
Transgenic plants		
EP2	1753	1240
EP3_1	1491	1054
EP5	2507	1772
EP6	1644	1162
Non-transgenic (controls)		
EPN2	1854	1311
LSD		264.7
CV%		26.1
Natural soil bacterial isolates		
Transgenic plants		
EP1	1886	404
EP2	1594	364
EP3_1	1965	316
EP3_2	1846	574
EP4	1512	428
EP6	2676	499
EP7	2095	236
Non transgenic controls		
EPN1	1703	779
EPN2	2141	316
LSD		308.3
CV (%)		47
Agrobacterium tumefaciens		
Transgenic plants		
EP1	982	45.0
EP3_2	199	79.0
EP4	469	15.0
EP7	827	47.0
Control		
EPN1	202	15
LSD		396
CV (%)		105

Data were transformed using the square root transformation to normalize the variances (Sokal and Rolf, 1995)

In the naturally occurring re-isolated rhizobacteria, highly significant (P=0.001) differences were observed amongst the treatments though the re-isolates from the plants were not markedly different (P≥0.05). Plant genotype did not influence (P≥ 0.05) bacterial re-isolation (Table 1). The means among bacteria re-isolated from the rhizosphere of the transgenic plants were not significantly different although there were

some differences between reactions of the isolates (Table 2). With *A. tumefaciens*, no significant differences (P≥0.05) were observed among the means of the CFUs of the isolates derived from the plants used in the experiments though differences (P≤0.05) in treatments used were noticeable. The re-isolation of *A. tumefaciens* was not influenced (P≥0.05) by the genotype of the plants (Table 1). Among the means of the CFUs of *A. tumefaciens* from the rhizospheres of transgenic plants and the non transgenic plants (controls), no significant differences (P ≥ 0.05) were similarly observed though some re-isolates reacted differently (Table 2).

Molecular analysis and PCR screens for *gus*A and *hpt* genes. A total 36 bacterial re-isolates (8 from *E. coli*, 9 from *A. tumefaciens* and 22 isolates from natural soil bacteria) were used for DNA extraction. The PCR was performed using *gus*A and *hpt* specific primers and, the amplicons were electrophoresed on a 1% agarose gel. Initial amplification of the DNA extracted from the experimental plants with *gus*A and *hpt* specific primers revealed amplicons corresponding to *hpt* and *gus*A genes respectively in plates 1A and 1B. An amplified fragment of about 500bp corresponding to the internal fragment of *gus*A 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 bacteria re-isolated from the rhizosphere of the experimental plants (Plate 1C and 1D).

Discussion. The results from this study suggested that no horizontal gene flow occurred to the bacteria tested. The non influence of the genotype of the plants used in the study on the re-isolation of the bacterial organisms suggests all re-isolated bacteria from the rhizosphere of transgenic plants reacted in a similar manner to the bactericide and were thus non-transformed. Interestingly for all bacteria re-isolated, some survived on selective media suggesting uptake of the transgene. However, the absence of PCR amplicons of the *gus*A and *hpt* genes in all the re-isolated bacteria surviving on selective media provides support for absence of gene flow and for the theory that the bacteria used in this study possess endogenous capacity to degrade antibiotics. Indeed, other studies on *E. coli* have revealed the presence of a plasmid encoded antibiotic resistance gene to hygromycin (Rao *et al.*, 1983). Elsewhere studies have demonstrated the occurrence of antibiotic resistance genes on plasmids (Riesenfeld *et al.*, 2004). However, it should be noted that in respect of horizontal gene flow, natural events have been detected in *E. coli*. Studies by Doolittle *et al*. (1990) revealed that *E. coli* integrated a second glyceraldehdye-3-phosphate dehydrogenase gene from a eukaryotic host. In case of *A. tumefaciens*, a similar response was found on both selective and non-selective media suggesting absence of geneflow from bananas. Similar reports have been made in other studies involving *A. tumefaciens* (Broer *et al.*, 1996).

The absence of gene flow could be attributed to various reasons. Firstly, transformation of bacteria requires that cells be competent (able to take up exogenous DNA). The ability to naturally develop competent cells has been reported among bacteria (Palmen and Hellingwerf, 1997). However, this capacity may be compounded by the physiological state of the cell and the influence of environmental factors (Lorenz and Wackernagel, 1992). In other bacteria, competence "state" development is influenced by absence or presence of certain amino acids and glucose availability which modulate DNA-binding and uptake machinery (Palmen and Hellingwerf, 1997). Availability of amino acids and glucose are inadvertently influenced by rhizosphere conditions especially enzyme activities that may enhance bio- degradation of both DNA and these ingredients (Bertolla and Simonet, 1999; Nielsen *et al*., 1998). The bio-degradation process may also degrade DNA reducing dosages for adsorption and ultimately uptake (Bertolla and Simonet, 1999). In this study, bacteria

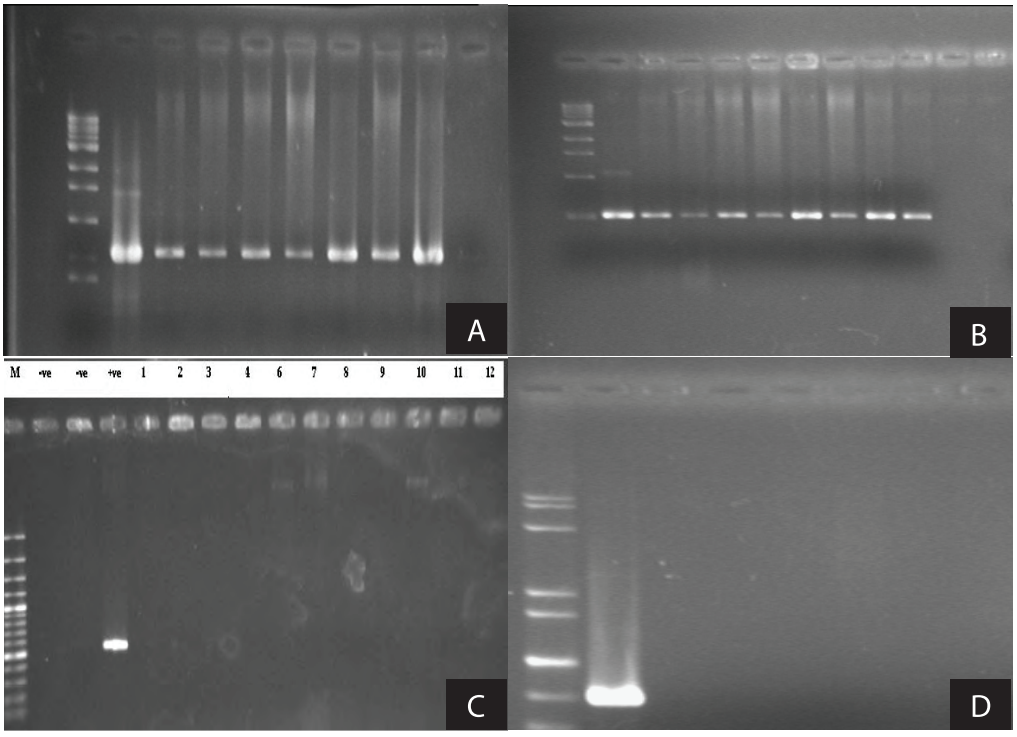

Plate 1. Electropherogram showing the presence of the hygromycin hpt (A) and β–glucuronidase (B) genes in the genetically engineered banana plants; and the absence of *β–glucuronidase* (C) and the hpt genes (D) in the microbial DNA

were inoculated into the rhizosphere and grown in soil for 13 weeks. The failure to uptake DNA from plants (banana) could thus be attributed to inavailability of transgenic DNA or more importantly, the lack of competent state limited gene transfer. Furthermore, eukaryotic DNA molecules are usually associated with proteins such as histones which condense DNA and could therefore drastically interfere with the uptake and recombination mechanisms of bacteria (Bertolla and Simonet, 1999). These studies on Agrobacterium and other soil inhabiting bacteria suggest a very low probability of gene flow from banana.

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