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In vitro selection of maize rhizobacteria to study potential biological control of *Aspergillus* section *Flavi* and aflatoxin production

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Abstract

The aims of this study were to select bacterial isolates from the non-rhizophere of maize soil and to examine their antagonistic activity against *Aspergillus* section *Flavi* strains. The first selection was made through ecophysiological responses of bacterial isolates to water activity (a_w) and temperature stress. Subsequently, an Index of Dominance test (I_D), ecological similarity and inhibition of the lag phase prior to growth, growth rate and aflatoxin B_1 accumulation were used as criteria. From the first assay nine bacterial strains were selected. They grew well at 25 and 30 °C, with growth optima between 0.982 and 0.955 a_W using 48 h of incubation. There was ecological similarity between the bacterial strains *Bacillus subtilis* (RCB 3, RCB 6), *Pseudomonas solanacearum* RCB 5, *Amphibacillus xylanus* RCB 27 and aflatoxigenic *Aspergillus* section *Flavi* strains at 0.982 at 25 °C. The predominant interaction between all selected bacteria and fungi in dual culture was mutual intermingling at 0.982. Mutual inhibition on contact and mutual inhibition at a distance was observed at 0.955 a_w, between only four bacteria and some *Aspergillus* strains. *Bacillus subtilis* RCB 55 showed antifungal activity against *Aspergillus* section *Flavi* strains. *Amphibacillus xylanus* RCB 27, *B. subtilis* RCB 90 and *Sporolactobacillus inulinus* RCB 196 increased the lag phase prior to growth and decreased the growth rate of *Aspergillus* section *Flavi* strains. *Bacillus subtilis* strains (RCB 6, RCB 55, RCB 90) and *P. solanacearum* RCB 110 inhibited aflatoxin accumulation. *Bacillus subtilis* RCB 90 completely inhibited aflatoxin B_1 accumulation at 0.982 a_W. These results show that the bacterial strains selected have potential for controlling *Aspergillus* section *Flavi* over a wide range of relevant environmental conditions in the stored maize ecosystem.

Introduction

Aflatoxins are toxic secondary metabolites produced during growth of *Aspergillus flavus*, *A. parasiticus*, and *A. nomius* (Diener et al., 1987; Kurtzman et al., 1987; CAST, 1989). These fungi have the ability to invade various agricultural commodities during maturation in the field or after harvest and contaminate them with aflatoxins. Colonization of maize by *Aspergillus* section *Flavi* has often resulted in aflatoxin contamination at harvest (Chulze et al., 1989; Torres et al., 1997). In storage, depending on environmental conditions, aflatoxins can accumulate in the grain due to *Aspergillus* section *Flavi* colonization. Mycelia and spores of aflatoxigenic fungi in stored products can also significantly decrease quality and economic value of the harvested grain.

Microbiological colonization of stored products is controlled by environmental conditions, such us water activity (a_w), temperature, pH, and intergranular gas composition (Lacey and Magan, 1991). However, in the storage agroecosystem complex interactions occur between biotic and

abiotic factors. Interspecific interactions between microbial species can occur, and the outcome is dependent on the prevailing environmental conditions. Inhibition of growth of *Aspergillus* section *Flavi* by specific bacteria can influence aflatoxin production. For example, lactic acid bacteria and *Bacillus subtilis* were tested for their ability to control aflatoxigenic fungi (Kimura and Hirano, 1988; Gourama and Bullerman, 1995). Bacterial isolates from the maize agroecosystem could be useful biocontrol agents in strategies for post-harvest control.

To select a potential biocontrol agent it is important to take account of the relationship between biological interactions and environmental stress factors. Interactions between aflatoxigenic strains of *A. flavus* and other colonizers of maize modify fungal behaviour during storage. It is important to use criteria to determine the outcome of several interactions. For example, an Index of Dominance (I_D) can compare the competitive ability of microbial species to dominate under a particular set of environmental conditions. It has been observed that several interactions were influenced by water activity, temperature and substrate (Lacey and Magan, 1991). Changes in environmental factors cause an impact that can be decisive in determining the co-existence level or dominance of species in a particular ecological niche (Marín et al., 1995, 1991). Reduction in growth, caused by the presence of specific bacterial species can influence mycotoxin production. It is important that any potential biocontrol agent must have the ability to decrease germination or growth as well as aflatoxin production.

To obtain information on the potential of antagonists, studies were carried out to control *Aspergillus* section *Flavi* and aflatoxin production using the following objectives:

(a) Evaluation of bacteria from the non-rhizosphere soil of a maize field; (b) determination of the ecophysiological responses of bacterial isolates to different water activity and temperature stresses, relevant to the pathogen; (c) determine the ability of the bacterial isolates to compete with *Aspergillus* section *Flavi* strains using an Index of dominance (I_D) under different environmental conditions; (d) establish the Niche Overlap Indices (NOI) and antibiosis assay between bacterial isolates and *Aspergillus* section *Flavi*; (e) determine the effect of bacterial isolates on lag phase prior to growth and growth rate of *Aspergillus* section *Flavi*; (f) examine the effect antifungal metabolites produced by the bacterial isolates on aflatoxin B_1 production.

Materials and methods

Fungal isolates

Eight isolates belonging to *Aspergillus* section *Flavi*, five of *A. flavus* (RCT 23, RCT 22, RCT 104, RCD 65 and RCI 105) and three of *A. parasiticus* (RCT 9, RCT 20 and RCD 106), were used in these experiments. These isolates were all recovered from maize agroecosystems (Nesci and Etcheverry, 2002). The isolates were maintained at 4 °C on slants of malt extract agar (MEA) and in 15% glycerol at –80 °C.

Bacterial isolates

Commercial fields of maize in Río Cuarto, Córdoba, Argentina were selected. Soil samples were collected during the periods September (pre-planting), December (growing maize) and May (post-harvest). Non-rhizosphere soil samples were collected from a commercial field of maize during pre-planting, maize-growing and post-harvest periods. Fifteen samples were collected during each of these periods. Each of the samples consisted of a mixture of 10 soil samples (5–10 g each) taken from the top 3 cm of soil at different points within the field on a diagonal transect at 100 m intervals. Samples weighing 100 g were thoroughly mixed, passed through a testing sieve (2 mm mesh size) and the soil separated from the debris. Soil samples were stored at 5 °C. Soil samples were suspended in 0.1% peptone water solution. Populations were determined using serial dilutions of 10^{-1} to 10^{-9} from each sample and 0.1 ml aliquots of three-fold dilutions were plated on: nutrient agar (NA), 10% tryptic soy broth (TSB), plus 2% agar for heterotrophic bacteria (TSBA), cetrimide agar for pseudomonads and yeast extract mannitol agar (YEMA): mannitol 10 g, PO_4H_2K 0.5 g, yeast extract 1 g, SO_4Mg 7 H_2O 0.2 g, NaCl

0.1 g, agar 15 g, distilled water 1000 ml. Plates were incubated at 28°C for 24–48 h. Total counts and counts per colony type was made from each medium. One colony per colony type was isolate and purified on TSBA. Colonization of soil samples was assessed as colony forming units (CFUs) g^{-1}. Bacterial identification was performed according to Bergey's Manual of Systematic Bacteriology (Holt et al., 1994). Isolates were maintained on NA slants at 4 °C. Bacterial isolates from the genera *Bacillus*, *Sporolactobacillus*, *Agromyces*, *Amphibacillus* and pseudomonads were selected to determine the range of growth at different water activities and temperatures.

Ecophysiological responses of antagonistics bacteria isolates in relation to water activity and temperature stress

Fifty-two bacterial isolates from non-rhizosphere soil were screened for water activity and temperature stress. The basic medium was TSB. The water activity (a_w) of the basic medium was 0.999; this water activity was modified with glycerol to 0.982, 0.955, 0.937 and 0.907 (Dallyn and Fox, 1980). The experiments were carried out by inoculation with 1 ml of 1×10^9 cfu ml^{-1} suspension of each bacterial isolate. Each isolate was grown in 9 ml TSB and incubated in a shaker (Incubator Shaker-Series 25 New Brunswick-Scientific Co., Inc. U.S.A) at 20, 25 and 30 °C for periods of 24 and 48 h. The optical density (OD) at 620 nm of each treatment was determined with a spectrophotometer (Genesys 5-Spectronic Instruments). All experiments were carried out with three replicates, per treatment. From this study nine bacterial isolates were selected for the next studies namely NOI, I_D, antifungal activity, growth studies and aflatoxin control in co-culture with *Aspergillus* section *Flavi* strains. Table 1 shows the list of bacterial isolates used.

Niche Overlap Index

Experiments were carried out on agar plates to determine the C-sources utilized by the fungi and the bacteria. The basic medium used was water agar containing 1.5% agar supplement with different C-compounds at 10 mM concentration. We considered the carbon sources present in grains of maize: D-fructose, D-galactose, α-D-glucose, D-rafinose, sucrose, aspartic L-acid, glutamic L-acid, L-histidine, L-phenylalanine, L-leucine, L-proline, L-treonine, dextrine, D-melobiose, L-alanine, D-serine, L-serine, L-arginine. The a_w of the basic medium was 0.999 and this was modified with glycerol to 0.982, 0.955 and 0.937 (Dallyn and Fox, 1980). Spores of each species of *Aspergillus* obtained from 1.5% MEA were suspended in 0.2% water agar and inoculated onto each plate containing the different carbon source. Suspensions of bacteria were grown for 24 h in TSB; each isolate was inoculated onto each plate with the different carbon source treatments. The plates were incubated at 25 °C for up to 10 days prior to examination for utilization of carbon sources. Three replicates were used per treatment.

Table 1. Bacterial isolates selected

Number	Bacterial isolate
RCB 3	*Bacillus subtilis*
RCB 5	*Pseudomonas solanacearum*
RCB 6	*Bacillus subtilis*
RCB 27	*Amphibacillus xylanus*
RCB 55	*Bacillus subtilis*
RCB 90	*Bacillus subtilis*
RCB 93	*Sporolactobacillus inulinus*
RCB 110	*Pseudomonas solanacearum*
RCB 196	*Sporolactobacillus inulinus*

The Niche Overlap Index (NOI) was defined as the number of carbon sources utilized by the antagonist and pathogen as a proportion of the total number of carbon sources utilized by those in question (Wilson and Lindow, 1994a, b). The NOI was determined as the number of carbon sources in common utilized by both strains (bacterial strain-*Aspergillus* strain) as a proportion of total number of carbon sources utilized by the aflatoxigenic strain. The NOIs were estimated for each strain in a pair. NOI values of >0.9 represent occupation or the same niche while scores of <0.9 represent occupation of separate niches (Wilson and Lindow, 1994a, b).

Index of Dominance(I_D)

The basic medium used was maize meal extract agar (MMEA) containing 3% maize meal and 1.5% agar. The water activity of the basic medium (0.999) was adjusted to 0.982, 0.955 and 0.937 by the addition of known amounts of the non-ionic solute, glycerol, according to Dallyn and Fox

(1980). A streak of bacterial suspension grown for 24 h in TSB was inoculated in the middle of each Petri plate. Spores of each species of *Aspergillus* obtained from MEA were suspended in 0.2% water agar. Petri plates were inoculated with a single culture of *Aspergillus* at two points equidistant from the centre and edge of the plate. The interaction of each dual culture was examined macroscopically, the type of interaction determined, and I_D numerical scores assigned. The scores were 1/1: mutual intermingling, 2/2: mutual inhibition on contact, 3/3: mutual inhibition at a distance, 4/0: dominance of one species on contact, 5/0: dominance at a distance (Magan and Lacey, 1984). All experiments were carried out in triplicate.

Antifungal activities of bacteria on aflatoxigenic Aspergillus *section* Flavi

Culture medium at different water activity levels was prepared following the procedure used for the I_D studies. Petri dishes containing MMEA were inoculated with 10 µl of 10^7 spores ml^{-1} of each *Aspergillus* section *Flavi* isolate in the centre of each plate respectively. Ten microlitres of the different bacterial antagonists at a concentration of 10^9 cells ml^{-1} were inoculated into wells of 2.5 mm diam around the fungal inoculum. Treatments were incubated at 25°C for up to 7 days in polyethylene bags. This assessment was carried out with three separate replicates per treatment. The inhibitory effects of bacterial strains on the linear growth of the *Aspergillus* strains were determined. The growth of the cultures containing bacteria-*Aspergillus* strain was compared with the control cultures (*Aspergillus*). The differences between antagonistic isolates in relation to antifungal activity over *Aspergillus* section *Flavi* were determined by Duncan's Multiple Range Test.

Effect of interaction between species on growth

The inhibitory activity on lag phase and growth rate of screened bacteria against *A. flavus* and *A. parasiticus* were tested. Each bacterial suspension with 10^9 cfu ml^{-1} was pour-plated in 20 ml of MMEA and after solidification, *Aspergillus* species were spot inoculated with spores suspended in semi-solid agar (Pitt, 1979). Petri plates of the same a_w were sealed in polyethylene bags. The inoculated plates were incubated at 25 °C. The colony radius was measured daily. For each colony, two radii, measured at right angles to one another, were averaged to find the mean radius for that colony. All colony radii were determined by using three replicates for each test interaction. The radial growth rate (mm d^{-1}) was subsequently calculated by linear regression of the linear phase for growth and the time at which the line intercepted the *x*-axis was used to calculate the lag phase in relation to fungal strain, bacteria and water activity. After the incubation period, controls and treatments were frozen for later extraction and aflatoxin B_1 quantification.

Aflatoxin B_1 analysis

The agar medium and biomass (1 cm × 1 cm) was taken from co-inoculated cultures (aflatoxin producers + bacterial antagonist) incubated at 25 °C for 11 days, transferred to an eppendorf tube and 500 µl of chloroform added. The mixture was shaken at 850 g for 20 min. The chloroform extract was dried under nitrogen gas. The residue was redissolved in 10 µl of chloroform for screening by TLC (Geisen, 1996). Samples were quantitatively determined by HPLC following the methodology of detection of Trucksess et al. (1994). An aliquot (200 µl) was derivatized with 700 µl of trifluoroacetic acid:acetic acid:water (20:10:70). The aflatoxin derivatized (50 µl solution) was analyzed using a reversed-phase HPLC/florescence detection system. The HPLC system consisted of an HP 1100 pump (Hewlett Packard, Palo Alto, CA, USA) connected to an HP 1046A programmable fluorescence detector, and the quantification was done by a Hewlett Packard workstation. Chromatographic separations were performed on a stainless steel, C_{18} reverse phase column (150 mm × 4.6 mm id, 5 µm particle size; Luna-Phenomenex, Torrance, CA, USA). The limit of detection of the analytical method was 1 ng g^{-1}.

Results

Bacteria and fungi isolated from non-rhizospheric soil

Analyses of bacterial and fungal populations from 45 non-rhizosphere soil samples demonstrated

significant differences between the incidence in the pre-planting, growing and post-harvest periods (Figure 1). The values were in a range of 5.4–11.2 (log cfu) for the bacterial population and 1.5–5.6 (log cfu) for the fungal population. In the pre-planting period, the main bacterial group isolated was *Bacillus*, while during the growing period the dominant genera were *Arthrobacter spp., Curtobacterium spp*., Gram (–) bacilli and *Bacillus* spp. Post-harvest, the major components were from the genera *Bacillus, Listeria* and pseudomonads (Table 2).

Table 2. Incidence of bacteria isolated from non-rhizospheric soil during different sampling period

Sampling period	Bacteria isolated	Percentage of isolation
1	*Bacillus*	100
2	*Arthrobacter*	24
	Gram (–) bacilli	16*
	Curtobacterium	16*
	Bacillus	14
	Listeria	7
	Amphibacillus	3.5
	Agromyces	2
	Others	10.5
3	*Bacillus*	32
	Listeria	10
	Pseudomonas	9
	Arthrobacter	7
	Sporolactobacillus	3
	Agromyces	1
	Others	4

1. Pre-planting, 2. Maize growing, 3. Post-harvest.
*Percentages are not significantly different ($P < 0.05$).

Ecophysiological responses of bacterial isolates to water activity and temperature stress

Absorbance (OD) changes were an indication of bacterial growth at different a_w levels and temperatures. These are shown in Figure 2. Cell growth was obtained at all temperature assays with OD increasing with increasing temperature. None of the isolates was able to growth at 0.939 and 0.90 a_w (data not shown). Optimum growth was obtained after 48 h incubation. Grow was higher at 25 °C at 0.982 and 0.955 a_w. Growth generally decreased with all a_w treatments at all temperatures.

Niche Overlap Indices

Table 3 shows the levels of niche overlap and niche exclusion between the *A. flavus*, *A. parasiticus* strains and bacterial strains at 0.982 a_w based on the ability to utilize the 18 carbon sources tested

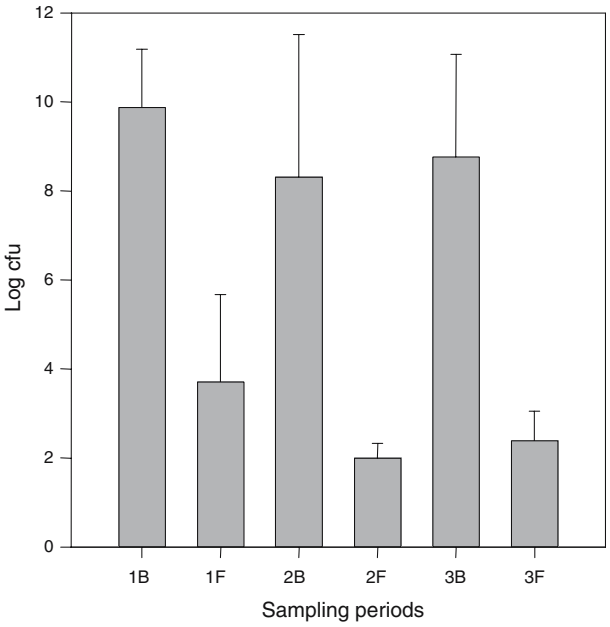

Figure 1. Incidence (CFU) of bacteria and fungi isolated from non-rhizospheric soil during different sampling periods, 1B (bacteria)/1F (fungi): Pre-planting, 2B (bacteria)/2F (fungi): Maize growing, and 3B (bacteria)/3F (fungi): Post-harvest. Bars indicate means and standard deviation.

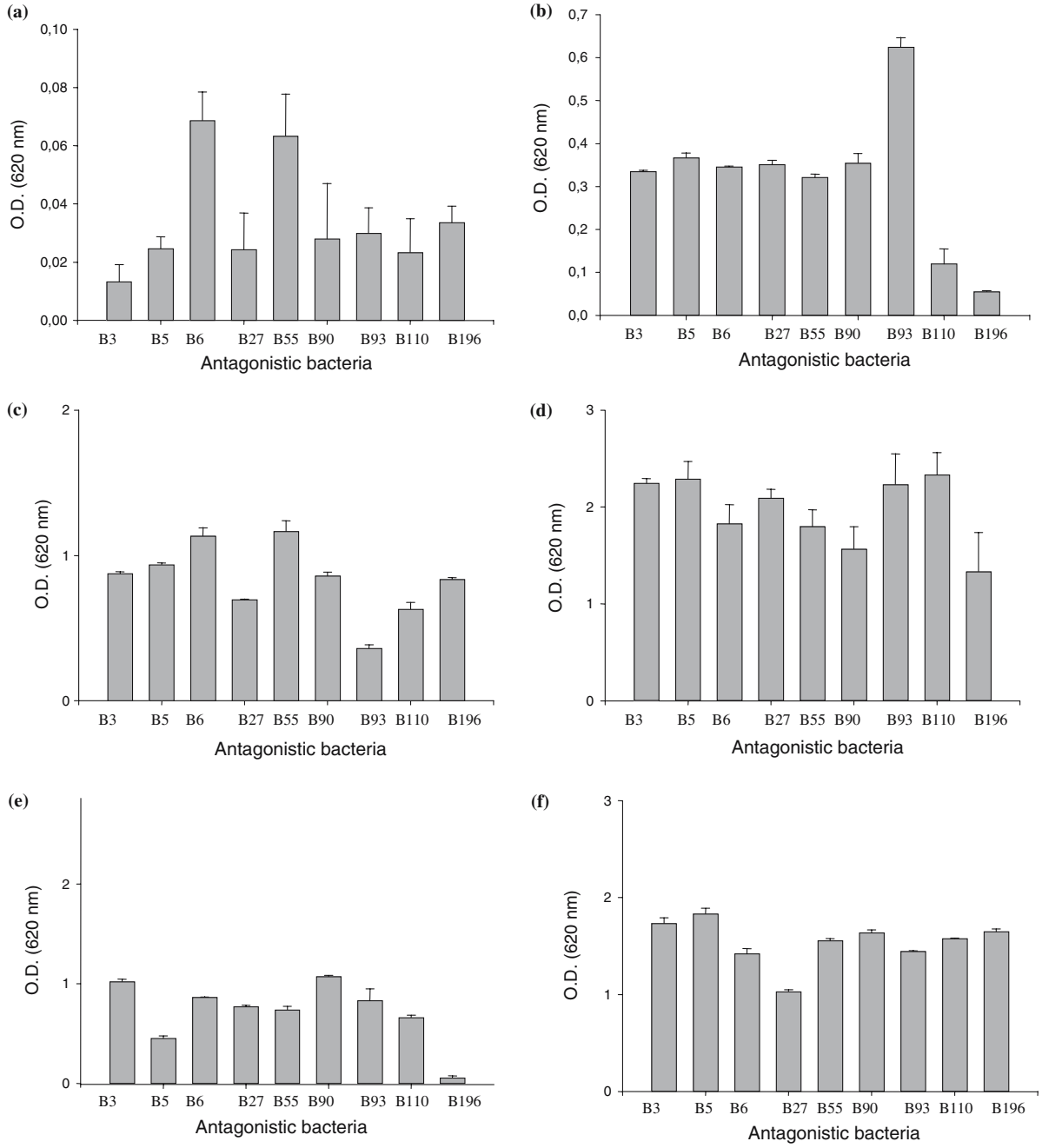

Figure 2. Comparison of growth (OD 620 nm) of bacterial isolates in relation to water activity and temperatures: (a) 0.955 and (b) 0.982 at 20 °C; (c) 0.955 and (d) 0.982 at 25 °C; (e) 0.955 and (f) 0.982 at 30 °C all after 48 h. Bars indicate means and standard deviation.

(niche size). At the lowest a_w levels examined (0.901, 0.809 and 0.747) none of the bacterial strains was able to utilize the carbon sources used (data not shown). All the bacterial strains had lowest NOI_s at 0.955 a_w (data not shown). The NOI values were always <0.8, indicative of occupation of different niches. However, when water availability was increased to 0.982 all the bacteria and *Aspergillus* assayed were able to use more carbon compounds. The NOI_s varied for interacting

Table 3. NOI for bacteria paired with different *A. flavus* and *A. parasiticus* strains derived from C-sources utilization at 25 °C and 0.982 a_w

Bacterial isolates	Size of niche	NOI*Asp*$_a$/mo	NOI*Asp*$_b$/mo	NOI*Asp*$_c$/mo	NOI*Asp*$_d$/mo	NOI*Asp*$_e$/mo	NOI*Asp*$_f$/mo	NOI*Asp*$_g$/mo	NOI*Asp*$_h$/mo
RCB 3	18	1/0.94	1/1	1/0.88	1/0.94	1/0.94	1/1	1/0.88	1/1
RCB 5	18	1/0.94	1/1	1/0.88	1/0.94	1/0.94	1/1	1/0.88	1/1
RCB 6	18	1/0.94	1/1	1/0.88	1/0.94	1/0.94	1/1	1/0.88	1/1
RCB 27	18	1/0.94	1/1	1/0.88	1/0.94	1/0.94	1/1	1/0.88	1/1
RCB 55	17	0.94/0.94	0.94/1	0.93/0.88	0.94/0.94	0.94/0.94	0.94/0.94	0.94/0.88	0.94/0.94
RCB 90	17	0.94/0.94	0.94/1	0.93/0.88	0.94/0.94	0.94/0.94	0.94/1	0.94/0.88	0.94/1
RCB 93	14	0.76/0.93	0.77/1	0.75/0.85	0.76/0.93	0.76/0.93	0.77/1	0.75/0.86	0.77/1
RCB 110	17	0.94/0.94	0.94/1	0.93/0.88	0.94/0.94	0.94/0.94	0.94/1	0.94/0.88	0.94/1
RCB 196	12	0.70/1	0.66/1	0.62/0.83	0.70/1	0.70/1	0.66/1	0.75/1	0.66/1

Maximum C-sources utilized: 18.
[a]NOI obtained by pairing the competing strain with RCT 9 strain of *A. parasiticus*.
[b]NOI obtained by pairing the competing strain with RCT 20 strain of *A. parasiticus*.
[c]NOI obtained by pairing the competing strain with RCD 106 strain of *A. parasiticus*.
[d]NOI obtained by pairing the competing strain with RCT 23 strain of *A. flavus*.
[e]NOI obtained by pairing the competing strain with RCT 22 strain of *A. flavus*.
[f]NOI obtained by pairing the competing strain with RCT 104 strain of *A. flavus*.
[g]NOI obtained by pairing the competing strain with RCD 65 strain of *A. flavus*.
[h]NOI obtained by pairing the competing strain with RCI 105 strain of *A. flavus*.

species. The NOI of the bacterial strains *Bacillus subtilis* RCB 3, *Pseudomonas solanacearum* RCB 5, *Bacillus subtilis* RCB 6 and *Amphibacillus xylanus* RCB 27 were >0.9 when paired with *Aspergillus* strains RCT 9, RCT 22, RCT 20, RCD 106, RCT 104 and RCI 105. NOI_s values > 0.9 were indicative of the coexistence with the pathogens. It was notice that *A. flavus* and *A. parasiticus* had similar NOI_s when paired with bacterial strains. All isolates showed NOI values <0.8 when paired with one *A. flavus* strain (RCD 65).

Interactions of Aspergillus *section* Flavi *with bacteria isolates*

Table 4 shows that the antagonistic activity was more important at 0.955 a_w. Almost 90% of bacterial strains showed inhibitory activity to the *Aspergillus* strains. At this a_w, *Sporolactobacillus inulinus* RCB 93 showed inhibition of 50% of the *Aspergillus* strains. At 0.982 a_w 75% of the fungi analysed were inhibited by *Bacillus subtilis* RCB 55. Strains *Bacillus subtilis* RCB 3, *Amphibacillus xylanus* RCB 27 and *Sporolactobacillus inulinus* RCB 196 had no inhibitory activity against any of the *Aspergillus* strains at 0.982 a_w. Similar results were obtained with *Bacillus subtilis* RCB 55 at the lowest a_w level tested (0.955).

The I_D between the bacterial strains and *Aspergillus* section *Flavi* are shown in Table 5. The predominant interaction between the bacterial antagonists and fungi in dual culture was mutual intermingling ($I_D = 1/1$). This was clearer at 0.982 a_w, except between bacterial isolate RCB 55 and six of the *Aspergillus* section *Flavi* strains where they were inhibited on contact ($I_D = 4/0$). Mutual inhibition on contact ($I_D = 2/2$) and mutual inhibition at a distance ($I_D = 3/3$) always

Table 4. Percentage of antifungal capacity of bacteria isolates on *Aspergillus* section *Flavi* strains at different water activities and 25 °C

Bacterial isolates	Percentage inhibited strains	
	Water activity	
	0.982	0.955
RCB 3	0a	25c
RCB 5	12.5b	25c
RCB 6	12.5b	25c
RCB 27	0a	12.5b
RCB 55	75c	0a
RCB 90	12.5b	25c
RCB 93	12.5b	50d
RCB 110	12.5b	25c
RCB 196	0a	37.5e

Means in a column with a letter in common are not significantly different (Duncan's multiple range test at $P < 0.05$).

Table 5. Indices of Dominance (I_D) between bacterial strains and different *Aspergillus* section *Flavi* strains on maize meal extract agar

Bacterial isolates		Strain of *Aspergillus* section *Flavi* (RC)							
	a_w	T9	T104	D106	D65	T20	I105	T23	T22
RCB 3	0.982	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1
	0.955	1/1	1/1	3/3	0/4*	3/3	0/4*	0/4*	0/4*
RCB 5	0.982	1/1	1/1	1/1	1/1	4/0	1/1	1/1	1/1
	0.955	0/4*	1/1	3/3	0/4*	3/3	1/1	0/5*	0/4*
RCB 6	0.982	1/1	1/1	1/1	4/0	1/1	1/1	1/1	1/1
	0.955	1/1	1/1	2/2	0/4*	3/3	1/1	0/4*	1/1
RCB 27	0.982	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1
	0.955	1/1	0/4*	1/1	1/1	4/0	0/4*	1/1	0/4*
RCB 55	0.982	4/0	4/0	4/0	4/0	4/0	1/1	4/0	1/1
	0.955	0/4*	1/1	1/1	1/1	1/1	0/4*	0/5*	0/4*
RCB 90	0.982	4/0	1/1	1/1	1/1	1/1	1/1	1/1	1/1
	0.955	4/0	1/1	1/1	1/1	3/3	1/1	1/1	0/4*
RCB 93	0.982	1/1	1/1	1/1	1/1	4/0	1/1	1/1	1/1
	0.955	1/1	1/1	3/3	2/2	3/3	2/2	0/4*	1/1
RCB 110	0.982	1/1	1/1	1/1	4/0	1/1	1/1	1/1	1/1
	0.955	0/4*	1/1	5/0	1/1	3/3	0/4*	0/4*	1/1
RCB 196	0.982	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1
	0.955	2/2	0/4*	4/0	1/1	3/3	1/1	0/4*	0/4*

Index of Dominance I_D:
1/1 Mutual intermingling.
2/2 Mutual inhibition on contact.
3/3 Mutual inhibition at a distance.
4/0 Dominance of one species on contact.
5/0 Dominance at a distance.
I_D: *Aspergillus* section *Flavi*/bacterial isolates.
*I_D: bacterial isolates/*Aspergillus* section *Flavi*.

occurred at 0.955 a_w. Dominance at a distance (I_D = 5/0) only occurred between four bacterial strains and some *Aspergillus* section *Flavi* strains at 0.955 a_w. At the lowest a_w (0.955) some of the bacterial strains were inhibited by the *Aspergillus* strains (I_D = 0/4, I_D = 0/5). The I_D for the bacterial isolate RCB 55 against *A. flavus* and *A. parasiticus* reflects their dominance at 0.982 a_w. In contrast, *Sporolactobacillus inulinus* RCB 196 was the most competitive at 0.955 a_w.

The lag phase of *A. flavus* and *A. parasiticus* of paired cultures at 0.982 are shown in Table 6. According to Duncan's multiple range test the lag phases of *A. flavus* and *A. parasiticus* were increased by interaction with *Amphibacillus xylanus* RCB 27 at 0.982 a_w, the values ranged between 5.5 and 7.1 days. At 0.955 a_w none of the bacterial strains increased the lag phase of *Aspergillus* strains (data not shown). Control cultures of *Aspergillus* section *Flavi* strains grew faster at 0.982 a_w than at 0.955 a_w, with the values ranging from 4.5–5.5 mm h^{-1} to 1.8–2.2 mm h^{-1}, respectively. Table 7 shows that at 0.982 a_w, 30% of the bacterial strains in this study had significant inhibitory effects on mycelial growth of eight *Aspergillus* species strains assayed according to Duncan's multiple range test. However, none of bacterial strains completely inhibited growth of *Aspergillus* section *Flavi*. *Amphibacillus xylanus* RCB 27 significantly reduced growth rates of the five *Aspergillus* section *Flavi* strains assayed. *Bacillus subtilis* RCB 90 and *Sporolactobacillus inulinus* RCB 196 showed the ability to decrease growth rates of paired cultures. At 0.955 a_w none of bacterial strains inhibited the growth rate of *A. flavus* and *A. parasiticus* strains (data not shown). This study showed that *Amphibacillus xylanus* RCB 27, *Bacillus subtilis* RCB 90 and *Sporolactobacillus inulinus* RCB 196 significantly reduced the growth rate and increased the lag phase of *Aspergillus* strains at 0.982 a_w.

We studied the aflatoxin B_1 accumulation in paired cultures between eight strains of *Aspergillus* section *Flavi* in interaction with bacterial strains

Table 6. Effect of bacteria-*Aspergillus* interactions on the lag phase at 0.982 a_w

Bacterial isolates	Lag phases							
	Aspergillus strains (RC)							
	T9	T104	D106	D65	T20	I105	T23	T22
Control	2.9a	2.9a	3.1a	2.9a	2.8a	2.8a	2.9a	2.8a
RCB 3	–	–	3.2a	3.1a	3.2c	–	–	–
RCB 6	3.2b	–	3.2a	3.7d	3.4e	3.5c	3.2b	3.5c
RCB 27	5.6e	5.8d	7.1d	6.7f	6g	5.5g	5.6f	5.7f
RCB 55	–	–	–	–	2.9a	–	–	–
RCB 65	–	–	–	–	–	3b	–	–
RCB 90	4.7c	4.5c	4.3b	3.4c	3.3d	4.2d	3.9d	4.6d
RCB 93	3.2b	3a	–	3.2b	3.4e	4.2e	3.3c	3.1b
RCB 110	3a	3b	–	–	3.1b	2.9a	3a	2.9a
RCB 196	5d	4.5c	5.2c	6e	4.8f	4.7f	5e	4.8e

(–) No increase of lag phase.
Means in a column with a letter in common are not significantly different (Duncan's multiple range test at $P < 0.05$).

(RCB 3, RCB 5, RCB 6, RCB 27, RCB 55, RCB 90, RCB 93, RCB 110 and RCB 196) in MMEA at 0.982 and 0.955 a_w (Table 8). At 0.982 a_w all *Aspergillus* strains accumulated aflatoxin B_1 with the exception of isolate RCD 106. However, at 0.955 a_w only one strain of *Aspergillus* (RCT 23) accumulated aflatoxin B_1. At 0.982 a_w we observed that strains RCB 6, RCB 55, RCB 90 of *Bacillus subtilis* and strain RCB 110 of *Pseudomonas solanaceanrum* inhibited the aflatoxin B_1 accumulation of 87, 87, 100 and 75% of the *Aspergillus* strains assayed respectively. At 0.982 a_w complete inhibition of aflatoxin B_1 was achieved by interaction of the bacterial isolate RCB 90 with all the *Aspergillus* strains assayed.

Discussion

This study has shown the selection steps for the identification of a possible biological control agent of aflatoxin-producing fungi, by taking a range of important environmental and ecological parameters relevant to the agroecosystem into account. In nature, aflatoxin-producing fungi share the same habitat with other microorganisms that can influence aflatoxin production (Munimbazi and Bullerman, 1998). The bacterial isolates from the maize agroecosystem assayed showed good growth at the water activities and temperatures that are appropriate for aflatoxin production by *Aspergillus* section *Flavi* (Gqaleni et al., 1996). The manipulation of several environmental factors can change synergistically the nature and the extension of the growth of fungi in the ecosystem of the stored grain so that there is a decrease or prevention of spoilage and mycotoxin production (Lacey and Magan, 1991). Ecological similarity and coexistence obtained with the NOI between microorganisms have been studied in biological control agents against pathogens on plant surfaces (Wilson and Lindow, 1994a, b). However, in these studies the impact of changing temperature and water availability on NOI has not been considered. These environmental factors have been demonstrated to change the NOI between maize-colonizing fungi by Marín et al. (1998). They suggested that the number and type of carbon sources used between interacting species are dependent on environmental conditions. These studies concentrated on competitiveness between spoilage fungi and the influence of changing water availability on NOI. The relationship between non-rhizobacterial antagonists paired with aflatoxigenic species and the impact of changing water activity on the NOI not has previously been considered.

In the present study, we observed that the niche size and NOI were markedly influenced by different water activities at 25 °C. This implies that the amount of niche overlap changes with environmental conditions. The utilization patterns of C-sources were the same for *Aspergillus* strains and some bacterial strains (RCB 3, RCB 5, RCB 6 and RCB 27) in this study. Thus, ecological similarity suggests the capacity to compete with aflatoxigenic strains at 0.982 and 25 °C.

Table 7. Bacterial influence on growth rate of *Aspergillus flavus* and *A. parasiticus* strains at 0.982 a_w

Isolates	Growth rate means (mm d^{-1})	Isolates	Growth rate means (mm d^{-1})
RCT 9(control)	5.3a	RCT 20(control)	5a
RCB 3	3.1e	RCB 3	3.1f
RCB 6	3.9d	RCB 6	3.8d
RCB 27	1.8h	RCB 27	1.8h
RCB 55	5.3a	RCB 55	4.3c
RCB 65	4.8b	RCB 65	4.8b
RCB 90	2g	RCB 90	3.4e
RCB 93	3.9d	RCB 93	3.7d
RCB 110	4.4c	RCB 110	4.4c
RCB 196	2.2f	RCB 196	2.2g
RCT 104(control)	4.5[a]	RCI 105(control)	5a
RCB 3	3.7c	RCB 3	3.8e
RCB 6	3.6c	RCB 6	4.1d
RCB 27	1.8e	RCB 27	1.8h
RCB 55	4.5a	RCB 55	5a
RCB 65	4.5a	RCB 65	4.8b
RCB 90	2.1d	RCB 90	2.2gh
RCB 93	4.2b	RCB 93	2.8f
RCB 110	4.3b	RCB 110	4.5c
RCB 196	2.2d	RCB 196	2.2g
RCD 106(control)	4.5a	RCT 23(control)	5a
RCB 3	3.2c	RCB 3	3.5f
RCB 6	4.3b	RCB 6	4.5c
RCB 27	1.5f	RCB 27	2j
RCB 55	4.5a	RCB 55	5a
RCB 65	4.5a	RCB 65	4.9b
RCB 90	1.8e	RCB 90	2.7h
RCB 93	4.5[a]	RCB 93	3.6e
RCB 110	4.2b	RCB 110	4.4d
RCB 196	2.1d	RCB 196	2.5g
RCD 65(control)	4.6a	RCT 22(control)	5.5a
RCB 3	3.2d	RCB 3	5.1b
RCB 6	4.4b	RCB 6	3.9e
RCB 27	1.7f	RCB 27	1.9h
RCB 55	4.6a	RCB 55	5.5a
RCB 65	4.6a	RCB 65	4.7c
RCB 90	2.2e	RCB 90	1.7i
RCB 93	3.8c	RCB 93	3.6f
RCB 110	4.5a	RCB 110	4.6d
RCB196	1.6g	RCB196	2.3g

Means in a column with a letter in common are not significantly different (Duncan's multiple range test at $P = 0.05$).

In this study, we used both NOI and I_D to examined interspecific interactions between *Aspergillus* section *Flavi* and bacterial strains. At 0.982 a_w the results showed that the predominant interaction was mutual intermingling between all bacterial and *Aspergillus* strains. In this study, *Bacillus subtilis* RCB 55 showed antifungal activities against *Aspergillus* section *Flavi* strains by the detection of zones of fungal inhibition in MMEA. In contrast, *Amphibacillus xylanus* RCB 27, *Bacillus subtilis* RCB 90 and *Sporolactobacillus inulinus* RCB 196 were able to increase the lag phase and inhibit growth of eight *Aspergillus* section *Flavi* strains.

Munimbazi and Bullerman (1998) showed that six *Bacillus pumilus* strains inhibited aflatoxin production and mycelial growth of *A. parasiticus* NRRL 2999 when both organisms were grown simultaneously in liquid medium. Kimura and Hirano (1988) reported on isolates of *Bacillus subtilis* inhibiting growth and production of aflatoxins by *A. flavus* NRRL 3357 in corn and by

Table 8. Bacterial influence on aflatoxin B_1 production of *Aspergillus* section *Flavi* at 0.982 and 0.955 a_w

Bacterial isolates	*Aspergillus* strains (RC)								
	a_w	T9	T104	D106	D65	T20	I105	T23	T22
Control	0.98	107.14	4.28	nd	28.5	7.14	29.63	3.6	17.14
	0.95	nd	nd	nd	nd	nd	nd	15.15	nd
RCB 3	0.98	17(84)	18.9	9.6	14.3(50)	18.3	nd(100)	14.7	18.3(6.7)
	0.95	85	nd	nd	6.2	59.3	nd	172.5	59.3
RCB 6	0.98	31.8(70)	nd(100)	nd	nd(100)	nd(100)	nd(100)	nd(100)	nd(100)
	0.95	16.12	nd	nd	3.16	48.8	nd	37	48.8
RCB 27	0.98	70(35)	70.4	1.53	92	52.4	64.4	53.4	52.4
	0.95	63	34	59.4	0.86	46.5	49.5	52.5	46.5
RCB 55	0.98	16.4(85)	nd(100)	nd	nd(100)	nd(100)	nd(100)	nd(100)	nd(100)
	0.95	12.8	2.78	nd	nd	10.4	nd	32	10.4
RCB 65	0.98	26.2(75)	nd(100)	nd	5.76(80)	3.7(48)	4.6(84)	21.5	3.7(78)
	0.95	nd	nd	nd	nd	10.9	nd	14(7.2)	10.9
RCB 90	0.98	nd(100)	nd(100)	nd	nd(100)	nd(100)	nd(100)	nd(100)	nd(100)
	0.95	nd	nd	nd	nd	83	nd	119.6	83
RCB 93	0.98	nd(100)	18.9	9.6	14.3(50)	18.3	nd(100)	14.7	18.3
	0.95	nd	nd	nd	nd	nd	nd	nd(100)	nd
RCB 110	0.98	nd(100)	1.8(56)	nd	nd(100)	nd(100)	3.6(88)	nd(100)	nd(100)
	0.95	3.29	Nd	3.6	nd	3.25	nd	nd(100)	3.25
RCB 196	0.98	nd(100)	nd(100)	6.09	3.26(89)	47.8	3.4(88)	4.17	47.8
	0.95	20.1	2.85	nd	nd	nd	nd	53.3	nd

( ) aflatoxin B_1 inhibition percentage.
Aflatoxin B_1 values were in ng g^{-1} medium.

A. parasiticus NRRL 2999 in peanuts. However, the important effects of a_w were not examined. Other studies have found that aflatoxin production is inhibited by lactic acid bacteria (Gourama and Bullerman, 1995) and *Bacillus subtilis* (Kimura and Hirano, 1988) strain ECCT-501, and a member of the *Enterobacteriaceae* family was used to suppress *Pythium ultimum*, cause of damping-off of many important vegetables (Nelson et al., 1986). Experiments *in vitro* and on grain with *A. flavus* and other maize fungi have demonstrated the influence of these interactions on aflatoxin production (Wicklow et al., 1980). Treatment of field peanut soil with specific, non-toxigenic strains of *A. flavus* and *A. parasiticus*, each lacking the ability to produce aflatoxins, cyclopiazonic acid, and known biosynthetic precursors of aflatoxins (Dorner et al., 1998), significantly reduced preharvest aflatoxin contamination (Dorner and Cole, 2002). Cotty and Bhatnagar (1994) showed that one atoxigenic *A. flavus* strain which produced many of the enzymatic activities present in the aflatoxin biosynthesis pathway but did not produce aflatoxins, was the most effective atoxigenic strain for reducing contamination in greenhouse tests in cotton bolls. The problem of the use of non-toxigenic strains of *A. flavus* for out-competing toxigenic strains, is that certain atoxigenic strains of *A. flavus* are known to be unstable and can convert to a highly toxigenic phenotype (Schindler et al., 1980; Clevstrom and Ljunggren, 1985). The stability of the non-aflatoxin producing phenotype may be an important consideration in selecting strains for use in strategies to prevent aflatoxin contamination through intraspecific competition (Cole and Cotty, 1990; Brown et al., 1991). The impact of changing a_w on the relationship between non-rhizosphere antagonistic bacteria and aflatoxin B_1 production by *Aspergillus* section *Flavi* strains has not been previously examined. Non-rhizosphere strains of *Bacillus subtilis* RCB 6, RCB 55, RCB 90 and *Pseudomonas solanacearum* RCB 110 effectively inhibited aflatoxin B_1 accumulation *in vitro*.

The screening procedure used in the present work reveals that different non-rhizobacteria may be considered good candidates for biological control in the ecosystem of stored maize. Enhancement of the efficacy of biocontrol agents may still be required for successful use. Studies are now in progress to determine the effect of manipulation of the growth of biocontrol non-rhizobacteria, by

changing nutrient status and water stress, because this may affect intracellular accumulation of compatible solutes. The effect of combinations of these bacteria to enhance multiple mechanisms of action for application in the stored grain ecosystem for maize is also being studied. The tolerance of biocontrol non-rhizobacteria to such modifications could increase their biological control potential.

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