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Potential of Grape Epiphytic Antagonists to Biocontrol *Aspergillus* Transmission and Accumulation of Aflatoxin B1 and Ochratoxin A in Post-Harvest Taify Table Grape

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Abstract

Taify grape is one of the important summer fruits in the Taif area in Saudi Arabia. A part of grape spoiled because by fungi and their toxins, influencing intersection processing, and export, alongside the quality of superior general welfare risk of exposure. The aim of this work was to check the ability of live cells and crude cell-free extracts of the grape epiphytic antagonists *Pseudomonas aeruginosa, Bacillus vallismortis* and *B. amyloliquefaciens* to control the transmission of *Aspergillus niger, A. parasiticus* and *A. tubingensis* to post-harvest Taify table grape berries (*Vitis vinifera L.*). Gathering of aflatoxin B1 (AFB1) and ochratoxin A (OTA) was also evaluated. Also, the activity of peroxidase enzyme, total phenol, and lipid peroxidation evaluated. The healthy and injured grape berry inoculated or suspended in the live bacterium cell suspension or bacteria free extract were challenged by the above fungi and stored at 5°C and 20°C for 28 and 50 days, individually. The treatments were effective for lessening the parasites misuse, spoil advance, and decay rate, and AFB1 and OTA aggregation. A pre-dousing of the in-place grape berries was prevalent. The peroxidase enzyme and the total phenol expanded further, slightness the lipid peroxidation, all reinforce the antagonists. The bacteria, their cell-free extracts, the incited peroxidase enzyme, total phenol and the brought down lipid peroxidation, made the medium ominous to the fungal establishment and further developing, and after that developing aggregate AFB1 and OTA ceased. Additional work expected to recognize the bacterial causal variable as well as the active metabolites.

Keywords: *Aspergillus* sp.; Antagonistic bacteria; Mycotoxins; Peroxidase enzyme; Total phenols; Lipid peroxidation

Introduction

Grapevine (*Vitis vinifera L.*) is one of the most important fruit crops worldwide. It has reached all countries but is successfully cultivated only in temperate climate regions with sufficient rain, warm and dry summers and relatively mild winters [1]. Genetic relationships between wild and domesticated grapevine distributed from Middle East countries to European rural areas was studied by many workers [2]. In the Taif area in Saudi Arabia, the grape taken as the unity of the important summer fruits. The significant portion of the grape is lost due to over-ripening, physical injuries, disorders, and diseases caused by fungi, bacteria and/or viruses during pre and/or post-harvest periods, affecting production, processing, and export, along with fruit quality [3,4]. If efficient steps are not followed to prevent the spread of these microorganisms and their toxin [5], subsequent processes infected and affectedness a public health hazard [6]. Contamination of grapes with fungi was studied [7,8]. The main ochratoxingenic and/or aflatoxigenic fungi are much concerned with some members of black Aspergilli and Penicilli contaminating grape [8-11]. Mycotoxins were produced by the genera Aspergillus, Fusarium, and Penicillium, during crop growth, harvest or storage [12]. Among the most important and harmful mycotoxin in the world is ochratoxin A (OTA) [13]. High levels of OTA contamination were reported on dried vine fruits (e.g. Raisins) worldwide, showing frequently around 100% of the samples contaminated [14]. Damaging effects of chemical control to post-harvest fruit pathogens, mycotoxins, and the establishment of fruit pathogen resistance to fungicides encouraged scientists to look for alternative safe control methods. In this context, much success achieved [8,15-23]. Progress was attained from the reduction of the post-harvest diseases and the tale of their toxins by stress from natural flora by utilizing the epiphytic and endophytic microorganisms existing in the yield [8,24,25]. Ponsone and his research team [26] demonstrated the efficacy of two yeast strains of *Kluyveromyces thermotolerant* for reducing OTA accumulation (from 3% to 100%) and the growth rate of ochratoxigenic fungi (from 11% to 82.5%), in the field. Prevention and control terminal of black Aspergilli and to reduce mycotoxin hazard were reviewed by Sanzania and his coworkers [11]. OTA removal was also possible by using both dead and alive yeast strains [26-28]. Grapevine metabolites are extremely regarded by environmental or pathogen approach [1]. In this respect, plant resistance induction due to pathogens, the abiotic and biotic agents were described [29,30]. It was reported that various defense reactions such as hypersensitivity, the product of phytoalexins, antimicrobial proteins, and strengthen the cell walls, all initiated in plants when they infected with various pathogens [31]. Increased activeness of phenols, peroxidase, and lipid peroxidation, in response to infection by the pathogens, also reported [29-33]. Induced resistance enhances the plants to mobilize appropriate cellular defines responses before or during pathogen attack [34]. It is generally systemic and triggered by non-pathogenic bacteria [31,35]. Induced systemic resistance (ISR) varies in their effect against non-pathogenic and pathogenic organisms [30,31,34,35]. In plants exhibiting induced systemic resistance, beyond infection sites, deposition of

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callose, lignin, phenolics, and calcium reported [31] and activities of hydrolytic enzyme increased [32,36]. In view of the above, the objective of our study was to assess the ability of certain isolates of epiphytic and antagonistic *Pseudomonas* and *Bacillus* sp., isolated from grape berries, to prevent the transmission of the mycotoxigenic *Aspergillus sp.* to post-harvest Taify table grape berries (*Vitis vinifera L.*). Subsequently, accumulation of the aflatoxin B1 (AFB1) and the ochratoxin A (OTA) in the grape berries. The measurements of activities of the peroxidase enzyme, total phenols, and lipid peroxidation, in reaction to the fungal challenge and bacterial treatment, were also taken in this work.

Materials and Methods

Samples

Thirty Taify table grape berry samples (*Vitis vinifera L.*) were collected in 2016 from six private farms in Taif city, Saudi Arabia. Samples were harvested in July during the source of the grape harvest. Grape bunches (each roughly 1 kg) were gathered and invested in previously sterilized cardboard boxes and kept at 4°C until analysis.

Microorganisms used

Aspergillus niger BAVSH1, *A. parasiticus* BAVSH4, and *A. tubingensis* BAVSH5, previously isolated from contaminated grape berries and identified to the molecular level by El-Shanshoury and his coworkers [8] used for challenges in Taify table grape and to check OTA and AFB1 production. The antagonistic grape epiphytic bacteria *Pseudomonas aeruginosa* EBVHSH17, *Bacillus vallismortis* EBHVSH28 and *B. amyloliquefaciens* EBHVSH29, previously isolated and identified to the molecular level by El-Shanshoury and his coworkers [8] employed to prevent the *Aspergillus* transmission, control *Aspergillus* rot and aflatoxin B1, and ochratoxin A gathering in post-harvest Taify Table grape.

Detection and determination of aflatoxin B1 and ochratoxin A in *Aspergillus* sp.

Fungal isolates belonging to the genus *Aspergillus* were grown on Sabouraud's agar medium [8] for five days. Agar plugs (5 mm diameter) taken from a growing edge of a 5-day-old test fungal colony and transferred to 500 ml Erlenmeyer flask containing sterile 100 ml Sabouraud's liquid media. Erlenmeyer flasks containing inoculated media incubated for 10 days at 28°C in the dark. The content of each flask filtered through Whatman No. 4 filter paper and extracted in a separating funnel with chloroform (three times; each extraction, 5 ml). The organic extracts containing Aflatoxin B1 (AFB1) and Ochratoxin A (OTA) combined, evaporated to dryness and suspended in 1 ml of chloroform and dried with steam bath (50°C). The dried extract kept in the refrigerator at 5°C for further analysis. Separation and detection of AFB1 and OTA in the sample extract carried out using thin layer chromatography (TLC) on pre-coated plastic sheets of silica gel 60 without fluorescence indicator (20 × 20 cm, layer thickness 0.2 mm, Merck) according to Makun et al. [37]. TLC performed on all samples by using chloroform: acetone (9:1, v/v) for AFB1 or benzene: Glacial acetic acid (9:1 v/v) for OTA as development solvents. The plates were examined under long wave UV light (365 nm). Blue fluorescence indicates that AFB1 is present, while the bluish-green fluorescence indicates the presence of OTA. For confirmation, a TLC plate sprayed with a fine mist of H_2SO_4, 50% (v/v) solution in water for AFB1, while spraying with an alcoholic solution of sodium bicarbonate (6.0 g of $NaHCO_3$ in 100 ml H_2O+20 ml ethanol) for OTA and let dry. Under longwave UV light, the blue fluorescence of AFB1 turns to yellow while the blue-green fluorescence of OTA turns to blue and becomes more intense. The quantities of AFB1 and OTA determined by reading the silica gel plates quantitatively by fluoro-densitometer (TLD-100 Vita-Torn) after the methods described elsewhere [38-42].

Grape berries, antagonists, and fungal pathogens

Taify table grape berries (*Vitis vinifera* L.) harvested at maturity and those having uniformity in size and ripeness and lack of any clear injuries or infection selected. Berries samples were surface-disinfected with sodium hypochlorite at 0.1% (v/v) for 1 min, rinsed with sterile tap water for three times, and allowed to air dry at room temperature (20°C). Inoculants suspensions and concentrations of the bacteria *P. aeruginosa*, *B. vallismortis* and *B. amyloliquefaciens* prepared and adjusted to 10^8 cells/ml colony forming Unit (CFU). Spore suspensions and concentrations of the pathogens *A. niger*, *A. parasiticus* and *A. tubingensis* were prepared and adjusted to 10^4 spores/ml CFU as before described by El-Shanshoury and his coworkers [8].

In vivo control of *Aspergillus* transmission and rot disease

For postharvest *Aspergillus* transmission and disease assays, the surface-disinfected grape berries injured in the middle with a sterile cylindrical tool (about 2 mm diameter and 3 mm deep) and the cut tissue removed. Into each wound, 30 µl of sterile water or living cell suspension in sterile distilled water by the antagonistic bacteria *P. aeruginosa*, *B. vallismortis* and *B. amyloliquefaciens*, adjusted at 1×10^8 cells/ml pipetted and inoculated. In place grape berries immersed and shaken with either the living bacterial cell suspension at 1×10^8 cells/ml or with crude cell-free extracts from freeze-dried and thawed bacterial cell suspension at 1×10^8 cells/ml, for 20 min at 120 rpm. After the suspensions were absorbed, the berries injured after 72 hours. After the proper interval, berries challenged with the pathogens *A. niger*, *A. parasiticus* or *A. tubingensis*. Berries injured and 1×10^4 spores/ml CFU were introduced in 30 µl. Injuries treated with sterile distilled water and with fungi spore suspension served as the inoculated positive disease controls. In each experiment, injuries treated with either sterile distilled water or antagonistic bacteria suspension, without pathogen inoculation used as negative controls. For all experiments, negative controls did not show disease symptoms. After air drying, the treated berries stored at 20 and 5°C in pre-disinfected, covered plastic containers to keep up a high relative humidity. The lesion diameters of the infected fruits examined daily and recorded every fourth day after inoculation, started after four and ten days for those incubated at 20°C and 5°C, respectively. Inhibition (%)=[(average lesion diameter of infected wounds in the control average lesion diameter of infected injuries in the treatment)/average lesion diameter of infected injuries in the control] × 100. Each experiment consisted of three replicates per treatment.

Analysis of the aflatoxin B1 and the ochratoxin A in fungi infected grape berries

Grape berries showed fungal infection freeze-dried, ground and subjected to aflatoxin B1 (AFB1) and ochratoxin A (OTA) determination. 5 grams of milled sample extracted according to the method of Golinski and Grabarkiewicz-Szezesna [43], using chloroform (three times; each extraction, 5 ml). The organic extracts containing AFB1 and OTA joined, evaporated to dryness and suspended in 1 ml of chloroform and dried with steam bath (50°C). The dried extract was kept in the refrigerator at 5°C for further analysis. Identification and estimation of AFB1 and OTA in the sample extract carried out as described above.

Grape berries, peroxidase, lipid peroxidation, and total phenols

The surface-disinfected grape berries injured and inoculated with

living cells of antagonists. Intact fruits suspended in living bacterial cells or in cell-free extract and injured after 72 h. Injured fruits were then fugally challenged and stored in sterile covered plastic containers at 20°C for different time intervals, up to 36 h. Treated and untreated grape berries analysed for peroxidase activity, lipid peroxidation, and total phenols.

Extraction and assay of peroxidase

Grape berries (0.5 g fresh weight) collected at 4, 12, 24 and 36 h intervals of the fungal challenge extracted in 50 mM sodium phosphate buffer (pH 7.0) and assayed as described by Kato and Schimizu [44]. The reaction mixture (3 ml) consisted of 7.2 mM guaiacol, 11.8 mM hydrogen peroxide. Add about 0.1 ml of crude enzyme extract to start the reaction, which measured spectrophotometric ally (Genesys 10 views) at 470 nm. Enzyme activity expressed in terms of the change in absorbance per minute at 470 nm in the linear phase of the slope (Δ 470/min/g fresh weight) immediately after adding the substrate.

Extraction and determination of lipid peroxidation

Lipid peroxidation measured by the amount of malondialdehyde (MDA) as a product of peroxidation of unsaturated fatty acid (linolenic acid, 18:3). One gram of grape berries homogenized in three ml of 1.15M KCl. The homogenate filtered. MDA concentration measured by the method of Mesbah et al. [45]. Briefly, 0.5 ml of enzyme extract, 0.5 ml of TCA (20%) and 1 ml of TBA (0.67%) mixed together in centrifugation tubes and heated to 100°C for 15 min. After cooling, 4 ml butanol was added and the content was mixed by vortexing the tubes and centrifuged at 1500 rpm for 15 min. The absorbance of the supernatant measured at 530 nm in a spectrophotometer. The MDA activity expressed in nm/g fresh weight.

Extraction and estimation of total phenols

0.1 grams of dry grape berries powder kept in 10 ml ethyl alcohol (95%) in the dark for 24 hours and filtered through Whitman No 1 filter paper. Total phenols estimated by the method of Jindal and Singh [46]. Briefly, 1 ml of alcohol extract was pipetted in a graduated tube. Then 0.1 ml of Folin-ciacalteau reagent added followed by 1 ml of 20 present Na_2CO_3 solutions. The tube was shaken and heated in boiling water bath for 1min. The tube cooled under running tap water. The blue solution was diluted to 25 ml with distilled water and absorbance measured at 650 nm in a spectrophotometer. A control containing all reagents except fruit extract used to adjust the absorbance at zero. All treatments conducted at least three times per treatment for each time interval. The amount of phenolic content was expressed as phenol equivalents in mg/g dry tissue.

Statistical analyses

All results subjected to one-way ANOVA and the means compared according to the Student–Newman–Keuls (SNK) multiple range tests ($p \leq 0.05$).

Results

Detection and determination of ochratoxin A and aflatoxin B1

The results obtained from the analysis of ochratoxin A (OTA) and Aflatoxin B1 (AFB1) in *Aspergillus* species summarized in Table 1. 60% of the isolates belonged to *A. niger* presented detectable mean levels of 150 µg/g OTA. For AFB1, it occurred in 46 and 53% of *A. parasiticus* and *A. tubingensis* isolates, respectively. The mean values of positive samples were lower than OTA, ranging between 40 and 53 µg/g.

Impact of the antagonists on *Aspergillus* establishment, transmission, and rot development

Suspension of intact Taify table grape berries in live cells and crude cell-free extracts of the antagonistic epiphytic bacteria *P. aeruginosa*, *B. vallismortis* and *B. amyloliquefaciens* reduced *Aspergillus* infection to hundred percentages of those in the inoculated controls (data not presented). Application of living cells of the antagonist's reduced transmission thus induced rot resistance on grape berries, during the stages of 28 and 50-day incubation period at 20°C and 5°C, respectively (Figure 1). The results showed the relationship of average lesion diameter inhibition percentage of injuries treated with living cells of different bacteria and the incubation time after pathogen inoculation. The efficacy of bacterial treatments in inhibiting the lesion size was closely correlated with the type of bacterial cells used and incubation time. After sixteen days of inoculation, the lesion inhibition percentages reached a hundred percentages compared to the inoculated controls. This was clear in injuries treated with living cells of *P. aeruginosa*, *B. vallismortis* and *B. amyloliquefaciens* and infected with *A. niger* at 20°C (Figure $1A_1$), injuries treated with living cells of *B. amyloliquefaciens* and infected with *A. parasiticus* at 20°C (Figure $1B_1$), injuries treated with living cells of *P. aeruginosa* and *B. vallismortis* and infected with *A. tubingensis*at 20°C (Figure $1C_1$). Also, complete inhibition of injuries infected with *A. tubingensis* at 5°C was detected by *B. vallismortis*, *B.* amyloliquefaciens and *P. aeruginosa* after 10, 18 and 22 days, respectively (Figure $1C_2$). While, a relative low inhibition (less 50%) was observed by the tested bacteria against both *A. niger* and *A. parasiticus* (Figures $1A_2$ and $1B_2$, respectively). In case of *A. parasiticus* infection at 5°C, it noted that although living cells were effective in inhibiting the early stages of infection, it decreased as incubation time after pathogen inoculation bypassing (Figure $1B_2$).

Peroxidase, lipid peroxidation, and total phenols

The changes in peroxidase, lipid peroxidation, and total phenols recorded in pathogens inoculated, bacteria-treated, grape berries along with their respective non-inoculated controls. The samples collected at different time intervals from 4 to 36 h after the challenge. The activity of peroxidase enzyme and total phenols were higher in infected and bacteria-treated, grape berries than in uninfected or no bacteria treated ones. The vies was in the case of lipid peroxidation.

Fungus	No isolates				% positive isolates		Average mycotoxins (µg/g)	
	Assayed		Positive					
	AFB	OTA	AFB	OTA	AFB	OTA	AFB	OTA
A. niger BAVSH1	10	10	ND	6	ND	60	ND	150
A. parasiticus BAVSH4	15	15	7	ND	46	ND	42	ND
A. tubingensis BAVSH5	15	15	8	ND	53	ND	40	ND
ND: Not detected under the experimental conditions								

Table 1: The capacity of *Aspergillus* sp. isolated from Taify table grape to produce aflatoxin B1 (AFB) and ochratoxin A (OTA).

Peroxidase activity

The data in Table 2 showed that the peroxidase (PO) activity of the grape berries, treated with different antagonistic bacteria, was higher than that of the untreated berries, after times from application. Fungal challenged grapes caused higher PO activity that changed according to the fungus than the non-inoculated fruits, whether treated or untreated, particularly after 4 h of treatment. Pre-immersion of intact berries, either in living cells or crude cell-free extracts recorded higher enzyme activity than in wounded fruits. For all fungi, the induction of PO by all tested bacteria varied significantly compared to its control (Table 2). The highest levels of PO determined 4 h after treatment, then decreased and re increased with time in all cases. Control samples recorded the lowest activity of 9.33 U at 12 h after challenge with *A. niger*, with the maximal activity of 80 U at 4h. While the highest activity of PO for the infected control was at 4h of *A. parasiticus* infection. In berries suspended in living bacterial cells of *P. aeruginosa* and challenged with *A. parasiticus*, PO activity reached the peak at 4 h after fungal challenge with 193.31U. It was about 250% higher than the infested control. Grapevine treatment by living cells of *B. vallismortis* and *B. amyloliquefaciens* was the best inducer for PO activity against *A. tubingensis* at 4 h. They were 166.70 and 166.70U, respectively. It was about 28% higher than the infested control (Table 2). In case *A. niger* infection, PO activity induced by all tested bacteria was higher than the infested control and varied insignificantly. Moreover, more 100% of PO activity was induced using the crude cell-free extract of *B. vallismortis*.

Lipid peroxidation activity

The results indicated that the levels of MDA activity gradually decreased in all treatments with increasing the time after infection and then increased after 36h. MDA levels induced by all tested bacteria for infected berries that varied significantly compared to its control (Table 3). Fungal-infected and bacteria-free treated samples showed low MDA activity of 23.91 at 24 h, after the challenge with *A. tubingensis*. The highest value (103.39) was obtained at 4h for non-infected control. The activity was maximal at 36 h by the living cells of *P. aeruginosa* with *A. parasiticus* and *A. tubingensis*. It was 156.71 and 171.79, respectively. Also, it was detected by the living cells of *B. vallismortis* at 36 h against *A. niger* infection. In berries treated with bacteria, MDA levels were markedly decreased, as compared with non-infected and infected controls. Generally, bacteria cell-free extracts recorded lower MDA than living cells-treated wounds fruits, except those of *B. amyloliquefaciens* against *A. niger* infection at 24 h of infection. Crude cell-free extract of *B. amyloliquefaciens* increased MDA by 1.8 times, over the infested control.

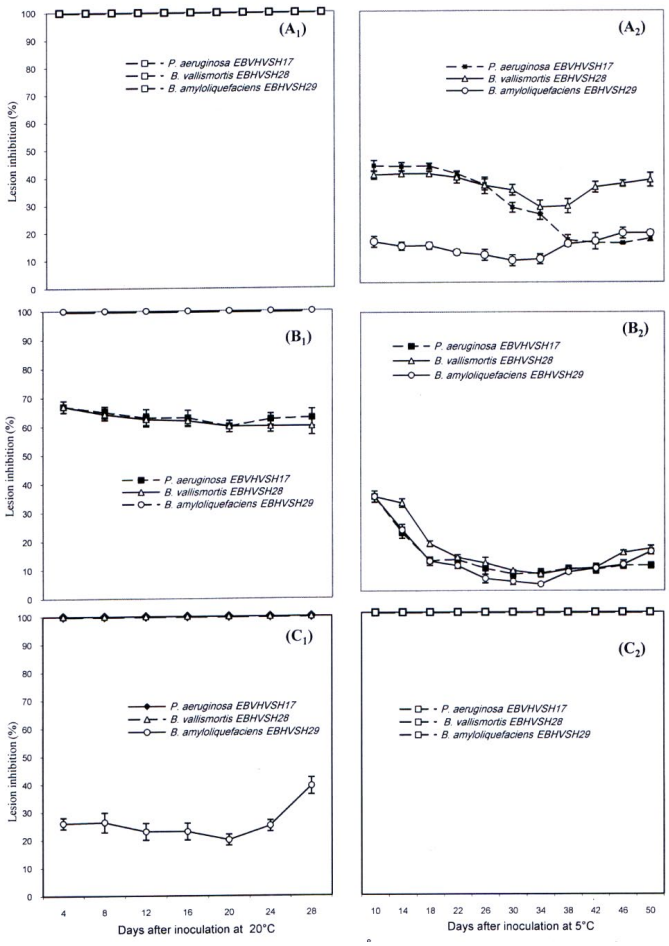

Figure 1: The effect of inoculating injured Taify table grape berries with living bacterial cells on *Aspergilli* rot disease development (lesion inhibition%), over time intervals at 20°C and 5°C. Grapefruits were injured and inoculated with bacterial cells at 1 × 10^8 cells/ml, 72 h prior to challenge with *Aspergillus niger* BAVSH1 (A_1, A_2), *A. parasiticus* BAVSH4 (B_1, B_2) and *A. tubingensis* BAVSH5 (C_1, C_2) at 1 × 10^4 spores/ml.

Total phenol content

The total phenols measured in the fungal inoculated and non-inoculated grape berries treated and untreated with the kinds of antagonistic bacteria (Table 4). Total phenol content was considerably increased simultaneously in infected berries, which increased with the increase in the period of infection, then diminished, compared to the dominance. It was obvious that fungi non-inoculated intact fruits and immersed in the crude cell-free extract and the living cells have considerable amounts of phenol compounds. Bacteria pre-treated, grape berries and challenged with the pathogens showed more levels of total phenols. The accumulation of phenols in the bacteria treated berries was relatively increased than in the non-inoculated fruits, during all periods of work. In the case of *A. niger* infection, total phenol content was maximal (37.33) at 4 h from the application by the cell-free extract of *P. aeruginosa*. Treatment by living cells of *P. aeruginosa* showed a high total phenols production for *A. tubingensis* infection at 24 h. While, crude cell-free extract induced a high total phenols production in case of *A. parasiticus* and *A. niger* at 24 and 4 h, respectively. After 12 h of infection a relatively high total phenols production recorded in grapes fruits, using crude cell free extract of *B. amyloliquefaciens* as a bio control agent to *A. tubingensis* and *A. niger* (Table 4). Generally, bacteria pre-treated, grape berries and challenged with the pathogens showed more levels of total phenols. The accumulation of phenols in the bacteria treated berries was highly increased than in the non-inoculated fruits, during all periods of work. Total phenol content was exhibited at the 4th h from the application, in the bacteria-treated and fungi-inoculated fruits, and then decreased progressively.

Ochratoxin A and aflatoxin B1 in grape berries

The grape samples before treated with the antagonistic bacteria and kept at 20°C and 5°C, that still showing fungal contamination, after 28 and the 50-day challenge, were subjected for determination of OTA and AFB1. Suspension of in place grapes in living cells and crude cell extracts of the epiphytic antagonistic bacteria *P. aeruginosa*, *B. vallismortis* and *B. Amyloliquefaciens* do contain neither AFB1 nor OTA that inhibited to 100 percentages of the inoculated controls (data not presented). Application of living cells of the bacteria reduced accumulation of OTA and AFB1 (Table 5). All the same, the inhibition percentages of

Treatment											
Challenge	Time	Control	*P. aeruginosa* EBVHVSH17			*B. vallismortis* EBHVSH28			*B. amyloliquefaciens* EBHVSH29		
			Living cells^{+}	Living cells^{++}	Crude cell-free extract^{+++}	Living cells^{+}	Living cells^{++}	Crude cell-free extract^{+++}	Living cells^{+}	Living cells^{++}	Crude cell-free extract^{+++}
Non-infected control	4 h	93.33^{a}	24.02^{c}	80.11^{a}	43.32^{b}	80.33^{a}	17.44^{e}	20.33^{d}	80.46^{a}	76.79 ^{a}	33.34^{c}
	12 h	30.33^{b}	14.02^{cd}	5.73^{d}	36.71^{b}	9.32^{cd}	16.73^{cd}	49.79^{a}	6.33^{d}	20.34^{c}	40.32^{b}
	24 h	13.32^{c}	17.32^{c}	11.73^{c}	75.37^{a}	16.71 ^{c}	18.32^{c}	42.33^{b}	13.33^{c}	43.33^{b}	74.37^{a}
	36 h	15.84^{e}	19.71^{de}	38.04^{bc}	72.39^{a}	22.73^{c}	44.35^{b}	74.78^{a}	70.77^{a}	34.36^{bcd}	80.63^{a}
	4 h	54.00^{f}	93.31^{e}	193.31^{a}	130.13^{c}	13.42^{h}	32.32^{g}	146.70^{b}	127.30^{c}	103.30^{d}	151.72^{b}
A. parasiticus BAVSH4	12 h	16.70^{d}	33.33^{c}	10.18^{d}	94.71^{a}	10.29^{d}	25.71^{c}	10.36^{d}	10.38^{d}	6.23^{e}	60.44^{b}
	24 h	24.00^{d}	124.03^{b}	121.32^{b}	17.32^{cd}	81.32^{c}	122.71^{b}	23.32^{d}	12.33^{d}	13.31^{d}	151.39^{a}
	36 h	22.01^{cd}	11.12^{d}	20.12^{cd}	11.39^{d}	77.11^{b}	81.31^{b}	19.72^{cd}	28.42^{c}	147.65^{a}	151.35^{a}
	4 h	130.00^{bc}	126.72^{c}	100.22^{b}	130.23^{b}	100.21^{d}	166.70^{a}	150.11^{a}	133.31^{b}	166.72^{a}	140.20^{b}
A. tubingensis BAVSH5	12 h	73.31^{cd}	73.31^{cd}	63.32^{d}	10.10^{f}	135.33^{b}	10.32^{f}	40.02^{e}	12.72^{f}	149.79^{a}	90.33^{c}
	24 h	12.33 ^{c}	13.00^{c}	17.72^{c}	18.72^{c}	170.44^{a}	13.11^{c}	14.31^{c}	167.31^{a}	134.39^{a}	57.35^{b}
	36 h	26.32^{b}	22.30^{b}	15.33^{b}	5.11^{c}	134.37^{a}	144.17^{a}	140.39^{a}	133.77^{a}	136.72^{a}	130.77^{a}
	4 h	80.00^{b}	146.70^{a}	150.08^{a}	146.77^{a}	140.28^{a}	140.37^{a}	160.44^{a}	133.35^{a}	123.37^{a}	136.76^{a}
	12 h	9.33^{e}	76.72^{bc}	61.71^{cd}	80.38^{b}	76.75^{bc}	9.34 ^{e}	13.33^{e}	18.12^{e}	147.78^{a}	57.04^{d}
A. niger BAVSH1	24 h	19.71^{d}	18.73^{d}	11.35^{d}	29.31^{c}	58.72^{b}	40.72^{bc}	74.36^{a}	71.38^{a}	77.23^{a}	70.33 ^{a}
	36 h	39.00^{d}	145.70^{a}	145.77^{a}	23.32^{d}	66.31^{c}	68.73^{c}	63.79^{c}	70.72^{c}	108.79^{b}	111.33^{b}

Means with different letters within the same row differ significantly at $p \leq 0.05$,$^{+}$Injuried berries inoculated with the living bacterial cells at 1 × 10^{8} cells/ml followed by challenge with different *Aspergillus* sp. at 1 × 10^{4} spores/ml after 72 h and kept at 20°C, ++ Intact berries suspended in the living bacterial cells at 1 × 10^{8} cells/ml, injurieed and challenged with different *Aspergillus* sp. at 1 × 10^{4} spores/ml after 72 h and kept at 20°C, +++ In place berries suspended in the crude cell-free extract from freeze-dried and thawed bacterial cell suspensions at 1 × 10^{8} cells/ml, injuried and challenged with different *Aspergillus* sp. at 1 × 10^{4} spores/ml after 72 h and kept at 20°C.

Table 2: Peroxidase activity (enzyme unit/g fresh weight/min) of the Taify table grape berries treated or untreated with the antagonistic epiphytic bacteria over different time intervals of the challenge with different *Aspergillus* sp.

Challenge	Time	Control	Treatment								
			P. aeruginosa EBVHVSH17			*B. vallismortis* EBHVSH28			*B. amyloliquefaciens* EBHVSH29		
			Living cells^{+}	Living cells^{++}	Crude cell-free extract^{+++}	Living cells^{+}	Living cells^{++}	Crude cell-free extract^{+++}	Living cells^{+}	Living cells^{++}	Crude cell-free extract^{+++}
Non-infected control	4 h	103.39^{c}	109.37^{c}	116.33^{c}	88.33^{c}	116.42^{c}	89.86^{c}	80.75^{c}	141.51^{b}	167.78^{a}	72.91^{c}
	12 h	40.67^{f}	88.40^{a}	58.90^{c}	30.19 ^{g}	56.54^{d}	37.95^{fg}	34.40^{fg}	52.74^{e}	68.89^{b}	39.10^{fg}
	24 h	52.33^{b}	27.14^{cd}	21.85^{d}	33.71^{c}	36.07^{c}	20.48^{d}	28.03^{cd}	35.67^{c}	73.11^{a}	20.58^{d}
	36 h	94.37^{c}	198.76^{a}	127.69^{bc}	117.21^{c}	88.00 ^{c}	98.98^{c}	123.86^{bc}	96.14^{c}	124.26^{bc}	134.16^{b}
A. parasiticus BAVSH4	4 h	80.06^{b}	104.86^{b}	106.79^{b}	67.91^{b}	79.58^{b}	108.09^{b}	101.92^{b}	116.22^{a}	91.63^{b}	110.71^{b}
	12 h	34.79^{d}	64.03^{b}	109.27^{a}	43.12^{c}	57.72^{b}	48.31^{b}	59.29^{b}	8.92^{f}	49.29^{b}	20.39^{e}
	24 h	43.71^{ab}	42.53^{abc}	39.30^{abc}	50.18^{a}	40.86^{abc}	20.78 ^{d}	36.36^{abcd}	40.28^{abc}	22.93^{cd}	26.17^{bcd}
	36 h	58.21^{e}	108.19^{bc}	156.71^{a}	77.52^{d}	117.62^{b}	101.14^{bc}	101.72^{bc}	89.95^{cd}	116.01^{b}	120.15^{b}
	4 h	73.01^{cd}	122.21^{a}	101.14^{bc}	108.29^{b}	50.18^{d}	90.36^{c}	75.27^{cd}	101.92^{bc}	101.33^{bc}	114.76^{a}
A. tubingensis BAVSH5	12 h	35.28 ^{c}	25.49 ^{d}	47.13^{b}	22.74^{d}	54.68^{a}	35.97^{c}	33.31^{c}	31.93 ^{c}	20.68^{d}	49.88^{b}
	24 h	23.91^{e}	70.26^{b}	47.33^{bc}	48.70^{bc}	48.80^{bc}	46.85^{bc}	26.98^{d}	83.04^{a}	52.82^{b}	29.60^{c}
	36 h	93.52^{d}	116.23^{cd}	171.79^{a}	49.98^{e}	102.93^{d}	135.53^{b}	51.25^{e}	83.59^{d}	165.03^{a}	117.66^{c}
	4 h	98.33^{c}	78.20^{cd}	94.96^{c}	77.13^{cd}	124.17^{b}	143.47^{a}	75.85^{cd}	78.50^{cd}	60.37 ^{d}	58.93^{d}
A. niger BAVSH1	12 h	53.51^{b}	17.25^{d}	54.73^{b}	28.52^{c}	80.07^{a}	24.01^{cd}	23.92^{cd}	29.01^{c}	54.40^{b}	24.72^{cd}
	24 h	24.52^{d}	46.55^{b}	40.57^{c}	30.38^{cd}	21.98^{d}	26.75^{d}	20.39^{d}	10.58^{e}	30.18^{cd}	68.20^{a}
	36 h	84.09^{c}	76.83^{c}	24.94^{e}	38.92^{d}	147.11^{a}	151.11^{a}	120.82^{b}	84.67^{c}	42.53^{d}	105.35^{bc}

Means with different letters within the same row differ significantly at $p \leq 0.05$,$^{+}$Injuried berries inoculated with the living bacterial cells at 1 × 10^{8} cells/ml followed by challenge with different *Aspergillus* sp. at 1 × 10^{4} spores/ml after 72 h and kept at 20°C, ++ Intact berries suspended in the suspension of living bacterial cells at 1 × 10^{8} cells/ml injuried and challenged with different *Aspergillus* sp. at 1 × 10^{4} spores/ml after 72 h and kept at 20°C, +++ Intact berries suspended in the crude cell extract from freeze-dried and thawed bacterial cell suspensions at 1 × 10^{8} cells/ml injuriedand challenged with different *Aspergillus* sp. at 1 × 10^{4} spores/ml after 72 h and kept at 20°C.

Table 3: Lipid peroxidation activity (MDA in nmol/g fresh weight) formed in Taify table grape berries treated or untreated with antagonistic epiphytic bacteria over different time intervals of the challenge with different *Aspergillus* sp.

Challenge	Time	Control	Treatment								
			P. aeruginosa EBVHVSH17			*B. vallismortis* EBHVSH28			*B. amyloliquefaciens* EBHVSH29		
			Living cells^{+}	Living cells^{++}	Crude cell-free extract^{+++}	Living cells^{+}	Living cells^{++}	Crude cell-free extract^{+++}	Living cells^{+}	Living cells^{++}	Crude cell- free extract^{+++}
Non-infected control	4 h	3.43^{c}	6.91^{b}	3.48^{c}	16.63^{a}	6.85^{b}	11.11^{ab}	8.91^{b}	7.39^{b}	11.46^{ab}	9.23^{b}
	12 h	4.43^{c}	16.93^{a}	7.03^{c}	9.27^{bc}	9.43^{bc}	5.13^{c}	8.67^{c}	9.17^{bc}	7.84^{c}	11.22^{b}
	24 h	1.07^{b}	13.33^{a}	1.41^{b}	4.17^{b}	3.53^{b}	0.31^{c}	2.93^{b}	2.83^{b}	1.03^{b}	4.53^{b}
A. parasiticus BAVSH4	36 h	18.57^{ab}	18.9^{ab}	14.03^{bc}	21.63^{a}	14.53^{bc}	11.57^{c}	18.77^{ab}	20.07^{a}	10.93^{c}	11.93^{c}
	4 h	12.17^{bc}	12.87^{b}	9.07^{c}	14.53^{b}	15.93^{ab}	14.12^{b}	10.77^{bc}	11.43^{bc}	10.67^{bc}	10.63^{bc}
	12 h	13.77^{a}	12.86^{a}	7.23^{c}	17.4^{a}	6.93^{c}	14.13^{a}	8.73^{b}	7.53^{c}	10.63^{a}	10.27^{a}
	24 h	4.53^{bc}	4.87^{bc}	10.11^{ab}	12.17^{a}	9.87^{ab}	6.22^{b}	2.27^{c}	2.27^{c}	2.47^{c}	2.43^{c}
A. tubingensis BAVSH5	36 h	16.43^{b}	12.07^{b}	11.71^{c}	10.92^{c}	20.47^{a}	15.43^{bc}	13.62^{bc}	11.43^{c}	12.6^{bc}	0.67^{d}
	4 h	23.83^{a}	14.63^{c}	12.03^{c}	10.73^{c}	13.93^{c}	12.91^{c}	7.73^{d}	17.42^{b}	9.44^{c}	13.03^{c}
	12 h	10.63^{a}	11.23^{a}	8.87^{a}	11.27^{a}	11.37^{a}	11.27^{a}	8.97^{a}	11.27^{a}	8.87^{a}	12.48^{a}
A. niger BAVSH1	24 h	2.31^{c}	24.87^{a}	2.43^{c}	2.33^{c}	2.12^{c}	1.33^{c}	2.73^{c}	2.33 ^{c}	2.23^{c}	18.57^{b}
	36 h	20.37^{a}	7.73^{b}	7.07^{b}	10.22^{b}	5.97^{b}	5.77^{b}	6.93^{b}	6.43^{b}	10.33^{b}	10.93^{b}
	4 h	13.17^{b}	8.27^{bc}	7.53^{c}	37.33^{a}	8.13^{bc}	11.63^{bc}	9.57^{bc}	8.13^{bc}	10.77^{bc}	0.74^{d}
	12 h	7.33^{bc}	7.27^{bc}	7.23^{bc}	9.37^{a}	6.13^{c}	7.43^{c}	5.13^{c}	14.13^{a}	7.83^{b}	11.33^{a}
	24 h	1.07^{d}	0.51^{e}	2.62^{b}	2.13^{e}	1.67^{c}	2.13^{c}	1.33^{c}	0.73^{e}	6.97^{a}	1.95^{c}
	36 h	8.77^{abc}	15.47^{a}	6.83^{bc}	11.87^{ab}	12.43^{ab}	12.55^{ab}	3.63^{c}	8.97^{abc}	4.53^{c}	6.53^{bc}

Means with different letters within the same row differ significantly at $p \leq 0.05$;$^{+}$Injuried fruits inoculated with the living bacterial cells at 1 × 10^{8} cells/ml followed by challenge with different *Aspergillus* sp. at 1 × 10^{4} spores/ml after 72 h and kept at 20°C, ++ The intact berries suspended in living bacterial cells at 1 × 10^{8} cells/ml injuried and challenged with different *Aspergillus* sp. at 1 × 10^{4} spores/ml after 72 h and kept at 20°C, +++ The intact berries suspended in the crude cell-free extract from freeze-dried and thawed bacterial cell suspensions at 1 × 10^{8} cells/ml injuried and challenged with different *Aspergillus* sp. at 1 × 10^{4} spores/ml after 72 h and kept at 20°C.

Table 4: Total phenols (mg/g dry weight) formed in Taify table grape berries treated or untreated with the antagonistic epiphytic bacteria over different time intervals of the challenge with different *Aspergillus* sp.

the mycotoxins were more pronounced at 20°C torn in 5°C. Levels of OTA and AFB1 were nil and the toxin inhibition percentages were a hundred percentages of the inoculated controls. This phenomenon was clear in the following treatments: wounds treated with living cells of *P. aeruginosa*, *B. vallismortis,* and *B. amyloliquefaciens*, infected with *A. niger* and incubated at 20°C; injuries treated with living cells of *P. aeruginosa* at 5°C and 20°C and *B. vallismortis* at 20°C, infected with *A. parasiticus*; injuries treated with living cells of *P. aeruginosa* at 5°C and *B. amyloliquefaciens* at 5°C and 20°C and infected with *A. tubingensis*. Other treatments showed detectable amounts of mycotoxins and thus the percentage of inhibition varied according to the treatment and the storage temperature. Referable to the treatments with the antagonistic bacteria, the present of inhibition of OTA ranged between 11-100 percentages and the inhibition percentage of AFB1 ranged between 38% to 100%.

Toxins of tested fungi	Treatment								
	Control			*P. aeruginosa* EBVHVSH17		*B. vallismortis* EBHVSH28	*B. amyloliquefaciens* EBHVSH29		
	5°C	20°C	5°C	20°C	5°C	20°C	5°C	20°C	Range of inhibition %
A. niger BAVSH1 (OTA)	3.13	4.36	2.81	0	0.79	0	0.38	0	(11-100)
A. parasiticus BAVSH4 (AFB1)	4.52	7.62	0	0	3.25	0	2.81	2.86	(38-100)
A. tubingensis BAVSH5 (AFB1)	3.78	37.61	0	17.48	1.63	2.81	0	0	(55-100)

Table 5: Levels of aflatoxin B1 (AFB1) and ochratoxin A (OTA) (ug/Kg dry weight), and inhibition percentage (%) in Taify table grape berries showed *Aspergilli* rot symptoms after injury treatment with living cells of the antagonistic epiphytic bacteria and challenge with different *Aspergillus* sp.

Discussion

The transmission of pathogenic microorganisms to table grapes during storage could be reduced by several treatments. Among these treatments, less of storage time and discarding visibly rotted bunches. The incidence of in post-harvest fruits could be reduced with sulfur dioxide in cold storage at 0°C [47]. Attempts to cut fungal colonization and mycotoxin content of grapes by agronomic practices, chemical, and biological treatments are sometimes controversial and have varying degrees of success [11]. Biocontrol agents (BCAs) as a tool to cut grape fruit berries diseases are reported, yeasts followed by bacteria and then fungi are the most potential BCAs [26,48,49]. Recently, *in vitro* studies conducted by El-Shanshoury and his coworkers [8] showed inhibition of *Aspergilli* sp., isolated from Taify table grape, with three epiphytic bacteria. In this study, preventive treatment of Taify table grape berries with *P. aeruginosa*, *B. vallismortis* and *B. amyloliquefaciens*, resulted in a dramatic reduction of *Aspergillus niger*, *A. parasiticus* and *A. tubingensis* infection and the rate of berries. The data show that the antagonistic bacteria interfered with berries to prevent the establishment and growth of *Aspergillus sp*., thus preventing transmission and progress of rot disease. Exist of any one of the three biocontrol agents, in the three, applied forms, resulted in lower or zero disease incidences, compared to the control. This was clear when these bacteria introduced; 3 days before the pathogens and the suppression continued until 28 days at 20°C or 50 days at 5°C. These points and storage degrees desirable for the bacteria to synthesize significant amounts of antimicrobial compounds to prevent the disease progression. Some researchers argued that carbohydrates and proteins implicated in the adhesion reaction of the bacteria [50,51]. Similarly, in this study, the inhibition of *Aspergillus* rot by *P. aeruginosa* could be due to the association of the bacterium and its products to the challenge fungi and the plant cells at the wound site containing the sugars. This concurs with the facts that *Pseudomonas* species promote biofilms [52] and adhere to plant cells [53,54] and fungi [55]. The inhibitory compounds produced by *B. vallismortis* and *B. amyloliquefaciens* seem bactericidal rather than bacteriostatic, by the outcomes received by Ranjbariyan et al. [56]. Populations of many BCAs that introduced into artificial environments decline with time [57]. Thus, the necessary quantities of antimicrobial agents may be lost with the resulting cessation in the synthesis of inhibitory compounds as observed in our study. Agree with studies conducted by Bruce and West [58], Sticher et al. [59] and Bakker et al. [34], the growth in the peroxidase activity in reaction to infection by the pathogens and bacterial treatments accounted in this work. The increased levels of total phenols in grape berries treated with different varieties of bacteria may offer an adequate substrate for oxidative reactions consuming oxygen [60] and producing fungi toxic for compounds, make the medium unfavourable to the fungal establishment, further growth of pathogens and disappearance of *Aspergillums* rot disease. Peroxidase production is stimulated by biotic and non-pathogenic bacteria, as documented in this study, and as one of the major mediators for oxidation of phenols to highly toxic compounds with antifungal potential [34]. This phenomenon supports our finding concerning total phenols and peroxidase in grape berries. Likewise, in a survey led by Mohamed et al. [61], the enzyme peroxidase is significantly induced by both biotic and abiotic factors that helped in controlling downy mildew of cucumber. Important metabolites from grapevine reviewed with their role against certain stress factors in grapevine physiology [1]. In this connection, several abiotic and biotic agents induce and enhance plant resistance to pathogens, mediated by stimulating the biosynthesis of phenol and phytoalexin inhibitory substances, PR-proteins and defines-related oxidative enzymes [29,30,36,60-62]. Bacterial determinants such as lipopolysaccharides [63] and lipopeptides [64], pathogen-resistant genes or pathways as well [65] or an enhancement of the bioactive secondary metabolites such as salicylic acid [66], siderophores [67], apigenin-7-O-glucoside [68]. In Taify table grape berries, significant amounts of the total phenols and the peroxidase enzyme were detected, that increased by epiphytic bacterial treatments. Lipid peroxidation was reduced, time after infection by fungi and with bacterial treatments, compared to control. Lipid peroxidation and lipid damage may be partly responsible for some of the cell changes and probably affect membrane function. When plants are subjected to pathogen attack, the equilibrium between productions and scavenging of ROS is broken, resulting in oxidative damage o for proteins, DNA and lipids. Malondialdehyde (MDA) content (a characteristic of lipid peroxidation) released from cellular membranes of tissues and is made by the reaction of ROS (HO or/and O•-) with lipid molecules [60,69]. Nevertheless, the reduction in lipid peroxidation in infected plants might be connected to the high activity of antioxidant enzymes, preventing accumulation of free radicals, and consequently membrane damage, in conformity with a study conducted by El- Khallal [70]. The outcomes described in this survey indicate that the increased total phenols and the peroxidase enzyme may have more antibacterial activity. The decrease in lipid peroxidation in infected and treated grapes might be linked to preventing membrane damage. The antimicrobial effect of the antagonists supported by the antibacterial activities of total phenols and the peroxidase enzyme. In increase, preventing membrane damage, all helped grape berries to prevent the formation of the mycotoxigenic fungi, further development, berry infection and rot disease development. Bearing out this idea, the role of defense-associated enzymes and total phenols in the resistance mechanisms of plants against fungal pathogens and phytophagous insects has been reviewed by Lattanzio et al. [71]. The subsequent products have been demonstrated to possess antibacterial properties as documented by Croft et al. [72] and signaling function as recorded by Melan et al. [73]. Preformed anti-fungal phenolic compounds are present in healthy plants at levels that are expected to be antimicrobial. Their grades may increase further in response to the challenge by pathogens. In accordance with the conclusion of Khan et al. [74], high phenol production in Taify table grape may have loss of virulence in the pathogens and induced resistance by bacterial cell determinants or cell-free extract may cause higher phenolic contents in the grapes and subsequently have deleterious effects on the pathogens. In accord with the last reported by Ciconova and his coworkers [27] and Somma et al.

[75], the answer recorded in the present study indicated exist of OTA and AFB1 in *Aspergillus* sp.- infected Taify table grapes. Some treatments of grape berries lowered the levels of OTA and AFB1 to nil. Suspension of Taify table grapes in living cells or crude cell extracts of the isolated antagonists was superior in this respect. Other treatments showed detectable amounts of the mycotoxins. The percentage of inhibition varied according to the treatment and the storage temperature. The stop of aggregation of OTA and AFB1 in the treated Taify table grape berries ascribable to the result of the bacterial antagonists, supported by the raised peroxidase enzyme and total phenols, in gain to lower lipid peroxidation in bacteria-treated groups. All shared grapes for destruction the establishment and developing of *Aspergillus spp,* thus preventing fungal transmission and gathering of OTA and AFB1. Other schemes prepared to minimize the levels of pre-or postharvest mycotoxins in different plants [23,76-80].

Conclusion

In this discipline, the antagonistic bacteria *Pseudomonas aeruginosa, Bacillus vallismortis* and *B. amyloliquefaciens*, their cell-free extracts, the incited peroxidase enzyme, total phenol and the brought down lipid peroxidation, made the medium ominous to the fungal establishment and further developing, and after that developing aggregate AFB1 and OTA ceased. Hence, the assemblage of aflatoxin B1 (AFB1) and ochratoxin A (OTA) in post-harvest Taify table grape was ceased. As pre-immersion treatment is potentially the most effective, this highlights its grandness to prevent the infection by the mycotoxigenic *Aspergillus* species and AFB1 and OTA assemblage. It suggests that *P. aeruginosa*, *B. vallismortis* and *B. amyloliquefaciens* could be held up as a surface treatment during the storage periods of grapes. It could be held up as the potential eco-friendly biocontrol agent for works protective cover strategy and recommended over other antagonistic organisms for field level management. This is because they well organized in big quantities in simple media and in very little time. The antagonistic bacteria, used in this study, may have certain determinants or synthesize certain compounds with antimicrobial activities. Therefore, further work needed to find the active agents and possible identification.

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