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PHENOTYPE AND GENOTYPE CHANGES EFFECTS OF THE FUNGICIDE BENLATE ON BACTERIAL ANTAGONISTIC AGAINST PATHOGENIC FUNGI CAUSING DERMATOLOGICAL DISEASES

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ABSTRACT

Various fungicides are used to protect different crops from pathogenic fungi attacks, but very little is known about the damaging effects of fungicides' application on non-target, beneficial soil microflora. Laboratory experiments were conducted to study the effects of Benlate fungicides on the antimicrobial activities of eight antimicrobial strains of Bacillus and E. coli isolated from the Kingdom of Saudi Arabia. The Bacillus and E. coli strains were exposed to 0.5% Benlate, then tested for their inhibition effects of the pathogenic fungi that cause wound inflammations and ulcers, i.e., Acremonium sp., Microsporium gypseum, Microsporium gallinae, Exserohilum sp. and A. niger. Their DNAs were also analyzed for any changes using RAPD-PCR. Results showed different effects of Benlate on bacterial antagonistic activities including more and less antagonistic efficiencies against target pathogenic fungi. Random amplified polymorphic DNA (RAPD) technique was used to check for any changes in DNA after their treatment with Benlate. Different changes in the genome was indicated including variation in band intensity; loss and appearance of new DNA bands. In conclusion, results revealed that treatment with Benlate induced great changes of genomic DNA of non-target bacteria and in their physiological activations. In addition, the results demonstrated that RAPD technique is useful for detection of alterations in the DNA. We observed the inhibitory effect of Bacillus strains and E. coli M2 on pathogenic fungi; however, what molecule was endowed with antifungal activity is not yet known. Further studies are needed to identify and characterize the antifungal protein molecule (s) of Bacillus and E. coli having potential against fungi. The work on identification of the molecule endowed with antimycotic properties is in progress.

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Introduction

Fungicide seed treatment is used to improve early plant emergence and to control the early attack by the pests. This strategy is useful in reducing fatalities from seed borne pathogens and seedling damping off agents [1]. These fungicides may harmfully affect the non-target soil microflora [2], especially when these fungicides are used in conjunction with microbial inoculants, they cause damage to the inoculants by affecting the bacterial infection to the root hair, nodule formation and bacterial growth hormone production. This important issue attracted a negligible amount of research in the past and not much more in recent

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years. The majority of work has been done under *in vitro* conditions [3] for leguminous crops on the basis of biochemical [4], as well as molecular technologies [5, 6]. Benomyl (methyl 1-(butylcarbamoyl) benzimidazol- 2-ylcarbamate) and carbendazim (methyl benzimidazol-2-ylcarbamate) are two very popular fungicides. The methyl benzimidazole carbamate (MBC) fungicides are widely used in crop production, inhibit mitosis in fungi. They can also influence the beneficial arbuscular mycorrhiza fungi (AMF) [7, 8 &9] and mammalian cells [10, 11]. [12] reported that the fungicide benomyl (formulated as Benlate 50 DF) has been implicated in damage to several crops grown under warm, moist conditions. Application of benomyl may have harmful non-target effects, leading to problems associated with its use. The study of pesticide effects on non-target populations is an accepted strategy to evaluate their associated potential environmental risks. The impact inflicted on soil microbial populations caused by a specific pesticide is a potential indicator of the toxicity level of this product, and may represent a component of a broad study aiming to evaluate its potential impact on the environment [13]. Microbial diversity has been studied through molecular methods, by the study of microbial community profiles obtained using molecular tools such as Random Amplified Polymorphic RAPD [14, 15], as they are quick, simple and inexpensive. RAPD analysis has become one of the most popular DNA based methods for assessing genetic diversity in plants [16] and has been used in DNA analysis of soil microbial community [17]. RAPD fragments are detected after agarose gel electrophoresis and ethidium bromide (EB) staining by visualizing band shifts, missing bands or the appearance of new bands in a DNA gel elctrophoresis. Detection of genotoxic effect using RAPD involves the comparison of profiles generated from control and treated DNA. Indeed, the gain/loss or intensity differences of RAPD bands may be related to DNA damage, mutations or structural rearrangements induced by genotoxins affecting the primer sites and/or interpriming distances [18]. Therefore, the main aim of this study is to investigate the effects of fungicide Benlate on antimicrobial activities of two different types of bacterial strains, Bacillus and *Escherichia coli* against pathogenic fungi which cause dermatological diseases; *Acremonium* sp., *Microsporium gypseum, Microsporium gallinae, Exserohilum* sp., *Trichphyton* sp. and *Aspergillus niger*. The other aim is to evaluate genetic stability of the bacterial strains after treatment with the fungicide Benlate using RAPD-PCR method for detection of possible DNA changes which are going to be induced.

Materials and methods

Bacterial Strains

All Bacterial strains used in these experiments were isolated from the Kingdom of Saudi Arabia soil, from El-Madina El-Monawara, El–Taif, El-Jouf and El- Dammam. They are *Bacillus subtilis* M10, T2, T3, J1, J3, D2, D4 strains, *B. megaterium* M3 strain and *E. coli* M2 [19]. All strains demonstrated antagonistic activities under *in vitro* conditions against pathogenic fungi which cause dermatological diseases; *Acremonium* sp., *Microsporium gypseum, Microsporium gallinae, Exserohilum* sp., *Trichphyton*s sp. and *Aspergillus niger*.

Effect of Benlate fungicide on antagonistic strains

The antimicrobial activities of studied bacteria without any Benlate were compared with those activities at 0.5% concentration. The fungicide (Benlate) with 0.5% concentration was used to test its influence on Bacillus and *E. coli* strains. The antagonistic effects were tested according to the method developed by [20]. The Petri plates were incubated at 27 ± 2°C and all the treatments were replicated in three.

Molecular characteristics of Bacillus and E. coli fungicide treated strains Isolation of total DNA from bacteria

Total DNA was isolated from Benlate-treated bacterial strains according to [21]. The quantity and purity of the obtained DNA were determined according to the ultraviolet (UV)-absorbance at 260 and 280 nm using spectrophotometer (Shimadzu UV-VIS model UV-240).

DNA amplified banding patterns based on RAPD:

The DNAs of the Bacillus and *E. coli* were analyzed by universal primer PCR marker, a multi-site amplification technique. Polymerase Chain Reaction (PCR)- GOLD Master-Mix Beads (BIORON, Germany) were used for PCR technique. Each bead contains all of the necessary reagents, except primer and DNA template, for performing 25 µl PCR amplification reactions. Two different primers were used in the present study. The first primer (RAP3) sequence 5′-GTG TTG TGG TCC ACT-3'. The second primer (RAP5) sequence was 5′-TGA GTG GTC TAC GTG-3'. All primers were supplied by Operon Technologies Company, Netherlands. To each ready-to-go PCR bead, 12 ng of the used random primer and 40 ng of the purified DNA sample were added. The total volume of the amplification reaction was completed to 25 µl using sterile distilled water. The amplification protocol was carried out as follows: Denaturation at 95°C for 5 min., 35 cycles each consists of the following steps: denaturation at 95°C for 1 min; primer annealing at 32°C for 2 min according to guanine cytosine (GC) ratio of each primer and incubation at 72°C for 2 min, for DNA polymerization. At the end, the PCR was held at 4°C till analysis. The

amplified DNA products from RAPD analysis were electrophorated in 1% agarose gel and 1 X TBE buffer at consistent100 volt for about 2 h. The different band sizes were determined against 100 bp ladder and the separated bands were stained with 0.5 µg/ml ethidium bromide and photographed using both Polaroid Instant Camera and UV Transilluminator.

Results and Discussion

Effect of Benlate on bacterial antagonism.

As pesticides are not species specific, their biological activity gets extended to a wide variety of non-target organisms. Nine bacterial strains (eight Bacillus isolates and one *E. coli*) were grown with 0.5% Benlate supplemented to LB agar medium. Antifungal strains were recovered from agar plates and evaluated for their antimicrobial activities against the pathogenic fungi which cause dermatological diseases; *Acremonium* sp., *M. gypseum, M. gallinae, Exserohilum* sp. and *A. niger*. The antagonism test was measured as the inhibition zone. Bacterial strains had different levels of antagonistic activities against the pathogenic fungi (Table 1). It was observed that *B. subtilis*T3 strain had kept its non-antagonism against all the pathogenic fungi similar to the control, but lost inhibition zone after treatment against *A. niger* (Table 1). Comparison of the inhibition zones' mean values before and after treatment displayed by fungicide treated strains indicated that some strains had inhibition zones higher than the non-treated control strain. Results showed that *B. megaterium* M3 had lower antifungal activity after fungicide treatment against all the tested fungi, the inhibition zones decreased from 69 to 25, 60 to 18, 75 to 10, 85 to 30 and 25 to 12 mm, respectively. Both *E. coli* M2 and *B. subtilis* M10 showed lower antifungal activity after fungicide treatment against four tested fungi, *M. gallinae, M. gypseum, Exserohilum* sp. and *Acremonium* sp., with different inhibition zones. Antifungal activity increased against *A. niger* from 15 to 24, 24 to 40 and 0 to 17 for *E. coli* M2, *B. subtilis* M10 and *B. subtilis* D4, respectively. *B. subtilis* T3 lost antifungal activity against *A. niger*. *B. subtilis*T2 showed increased inhibition zones against *M. gallinae M. gypseum* and *Acremonium* sp., lower antifungal activity after fungicide treatment against *Exerohilum* and the same antifungal activity against *A. niger*. *B. subtilis* J3, its inhibition zone increased against *M. gallinae*, lost inhibition against *A. niger* and decreased for *M. gypseum*, *Exerohilum* sp. and *Acremonium* sp. *B. subtilis* J1, its inhibition zone increased against *Acremonium* sp. , lost inhibition against *A. niger* and decreased for *M. gallinae, M. gypseum* and *Exerohilum* sp., respectively. *B. subtilis* D2 inhibition zones decreased against *M. gallinae* and *Exerohilum* sp. from 50 to 35, 50 to 20, respectively. *B. subtilis* D4 inhibition zone increased against *A. niger* and showed lower antifungal activity after fungicide treatment against three tested fungi for *M. gallinae, M. gypseum* and *Exerohilum* and the same with *Acremonium* sp. The use of fungicides, besides being expensive and involving risks to the environment associated with the application of chemicals, is not totally effective and may lead to the appearance of new, resistant strains of pathogens [22]. Bacillus strains used in this study were isolated from different geographical areas and from different sources. In this study, results showed different behavior of antagonistic activity against different pathogenic fungi after treatment with Benlate fungicide on agar medium for Bacillus strains. It was reported that high metabolic activities of *Bacillus* strains often correspond to their specific genotypes [23]. Fungicidal seed dressings used to improve the early plant emergence are often damaging Rhizobium applied as inoculants to legume seed. Some reports claim little damage, which may reflect the considerable variation within and in between different groups of Rhizobium in their sensitivity to fungicides [24]. That the secreted inhibitory factors might be affected by the treatment.

In this study, in most cases, the antagonism capacity of the Bacillus strains was affected by the exposure to the chemical fungicide. The antagonism's capacity under *in vitro* condition was negatively affected in all of the treated strains with some pathogenic strains, the antagonistic capacity lost for *A. niger* (*B. subtilis* J3, J1 and T3). A similar phenomenon was found in Trichoderma on Captan and Thiabendazol resistance, and sensitive strains on which were only negatively affected in one out of the 10 tolerant lines obtained [25]. In many cases, the antagonistic capacity was increased (Antifungal activity increased against *A. niger* for *E. coli* M2, *B. subtilis* M10, *B. subtilis* D4 and *B. megaterium* M3). Earlier studies showed that fungicides Carbendazim and Mancozeb were exerting an adverse effect on various soil bacteria including Bradyrhizobium, Rhizobium, Sinorhizobium and *Psudomons* sp. [26, 27, 28 & 29]. A correlation between fungicide resistance and antagonistic activity is suggested [30]. [31] stated that mechanisms of the antagonism of many microorganisms like fungi and bacteria against different pathogens may be due to mycoparasitism, competition and antibiosis. Antifungals-Mycobacillin, subtilin, lipopeptides, bacillomycin, bacilysin, fengimysin, levansucrase and 2, 3-dihydroxy benzoyl glycine were reported to be produced by *Bacillus* sp. [32]. Chitinase and β-1,3-glucanase produced by biological control agents (BCAs) have been reported as key enzymes responsible for fungal cell and sclerotial wall lysis and degradation. [33] reported that Carbendazim Benomyl had no significant fungicide inhibition on chitinase activity of *T. harzianum* in range of 0 – 1500 mg/ml.

[34] investigated the effect of benomyl on the growth and the production of cellulase by *Trichoderma spp.* Although *T. reesei* produced the highest, and *T. harzianum* the lowest cellulase amounts, the growth of both strains was equally inhibited by benomyl concentration of 2µg/ml. However, sublethal doses of benomyl (0.2–0.5 g/mL) promoted growth and stimulated cellulase production. As pesticides are not species specific, their biological activity gets extended to a wide variety of non-target organisms. Decrease in PSB population with fungicides was also reported by [35] who concluded that, the PSB showed decline in their viable population on prolonged contact with fungicides. In 2007 and 2010, [36, 37] reported that cytosolic

proteins of *E. coli* are responsible for antifungal potential against pathogenic strains of *Aspergillus fumigatus*, *Aspergillus flavus*, *A. niger*and *Candida albicans*. Also, [38] reported that *E. coli* showed almost complete inhibition against pathogenic fungi such as *A. niger* and *A. flavus.* [39] reported that Benlate is less toxic at full-recommended dose. Bacillus strain F14 is sensitive to all test fungicides. Bacillus strains F3, is sensitive to Darosal, Captan, and resistant to Benlate and Vitavax while F4 is sensitive to Captan, Vitavax and resistant to Darosal and Benlate. Azospirillum strains BtJ-16 is most resistant and better survived in all fungicide supplemented medium, even its growth is stimulated in the presence of fungicides. Comet assay was used for the first time, to assess the genotoxicity of fungicides for PGPR, indicate that fungicides viz., Alert Plus, Darosal and Mancozeb do have the inhibitory effect, while Benlate have stimulatory effect on growth, though the effect varies from strain to strain. No tested fungicides cause the genotoxicity. The use of Comet assay technique for the visual counting of DNA double strand breaks in single electrostreched PGPR DNA molecule. Double- strand breaks may lead to chromosomal breaks lethal to bacterial cell [40]. It was reported that fungicides; [41], [42, 43],[44],[45],[41, 43] are responsible for cytogenetic effect in human population after a prolonged exposure. In case of prokaryote genotoxicity of Captan, it was reported [46] for Salmonella using *in vitro* Ames test and SOS chromo test. [39] regarding genotoxicity due to fungicides through comet assay no significant difference was observed under fluorescence microscope in slides with fungicide treatment as compared to the control. No distinct comet like structure was observed in any slide reveals that there is no DNA double strand break in any PGPR strain by any of the fungicide treatment. Though these results are not depicting the toxic effect of fungicide as compared to the effect on viable count and resistant pattern experiments but it is clear that growth inhibition of PGPR strains is not due to the damage of bacterial DNA. Results of comet assay deduced that the test fungicides do not cause bacterial DNA damage. [37] reported that *A. fumigatus* and *A. flavus* were more susceptible than other species of fungi to the lethal effects of PPEBL21 they indicated that the surface molecules in different species of pathogens may determine the susceptibility to treatment with PPEBL21 from *E. coli*. The difference in sensitivity of different Aspergillus species to treatment with proteins isolated from Moringaoleifera and Indigoferaoblongifolia was also observed by [47, 48].

Table 1: The antagonistic effect of bacterial suspensions of species treated by fungicide against pathogenic fungi expressed by inhibition zones (mm).

Bacterial Isolate	*M. gallinae*		*M. gypseum*		*Exerohilum* sp.		*Acremoniu* sp.		*A. niger*	
	Before	After	Before	After	Before	After	Before	After	Before	After
E. coli M2	54	44	51	51	51	51	51	42	51	42
*B. megaterium*M	06	41	02	55	51	52	51	52	41	54
*B. subtilis*M10	51	22	05	51	01	52	02	55	42	22
B. subtilis T2	2.2	45	2.2	55	21	51	2.2	51	2.2	2.2
B. subtilis T3	2.2	2.2	2.2	2.2	2.2	2.2	2.2	2.2	54	2.2
B. subtilis J1	51	45	22	41	51	51	54	45	42	2.2
B. subtilis J3	44	52	22	52	51	55	52	52	42	2.2
B. subtilis D2	12	51	2,2	51	12	42	54	52	51	45
B. subtilis D4	15	52	21	45	12	52	52	52	2,2	55

Table 2. Number of DNA polymorphic bands in eight bacterial strains amplified with two oligonucleotide primers after Benlate treatment.

Primer 1 Bacterial strains **Number of fragments scored of polymorphic loci** after Benlate treatment			**Primer 2** **Number of fragments scored of polymorphic loci** after Benlate treatment
E. coli M2	(lane6)	differences in bands of DNA with sizes of (600,700,900, 1400 and 1500 bp) compared with the control (700, 900, 1200 and 1800) bp	disappearance of DNA bands with size of 1000 and 1500 bp
*B. megaterium*M	(lane2)	appearance of DNA bands with size of 900, 1000 and 1500 bp	appearance of DNA band with size of 1000 bp

*B. subtilis*M10	(lane1)	appearance of DNA bands with size of 900 and 1000 bp	
B. subtilis T2	(lane3)	disappearance of DNA band with size of 1800 bp	disappearance of DNA band with size of 1800 bp
B. subtilis T3	(lane7)	No change	No change
B. subtilis J3	(lane8)	appearance of DNA bands with size of 600 and 1500 bp	Appearance of DNA bands with size of 500, 800 and 1500 bp
B. subtilis D2	(lane4)	No change	No change
B. subtilis D4	(lane5)	disappearance of DNA band with size of 600 bp	disappearance of DNA band with size of 600 bp

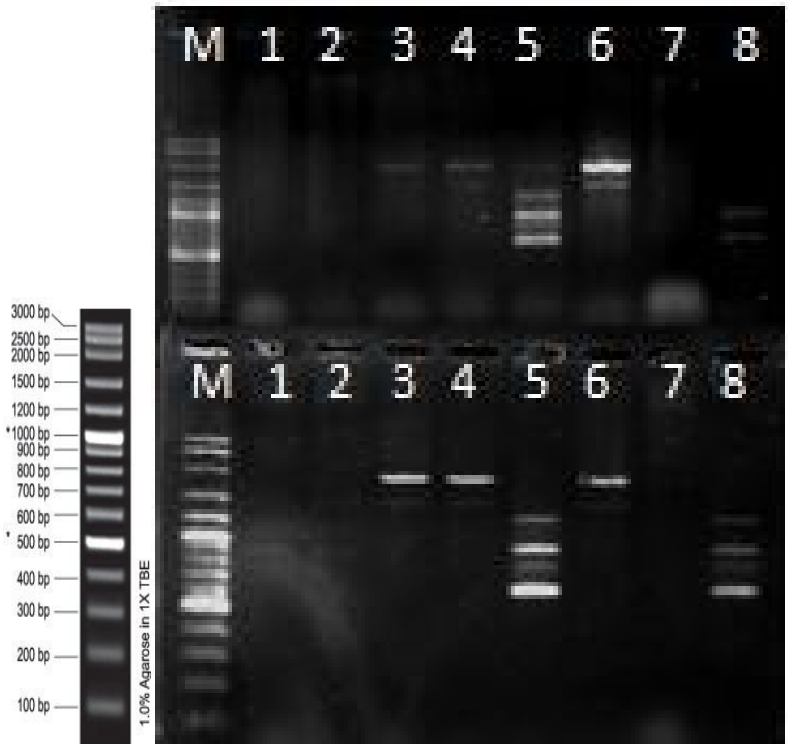

Figure 1: DNA amplified banding patterns based on RAPD for eight bacterial strains using RAP3 primer. VC100 bp Plus DNA ladder, Vivantis NL 1407(Lane M). Bacterial strains without treatment (Upper) and after treatment with fungicide (Lower). Lanes 1 to 8 are bacterial strains, M_{10}, M_3, T_2, D_2, D_4, M_2, T_3 and J_3, respectively.

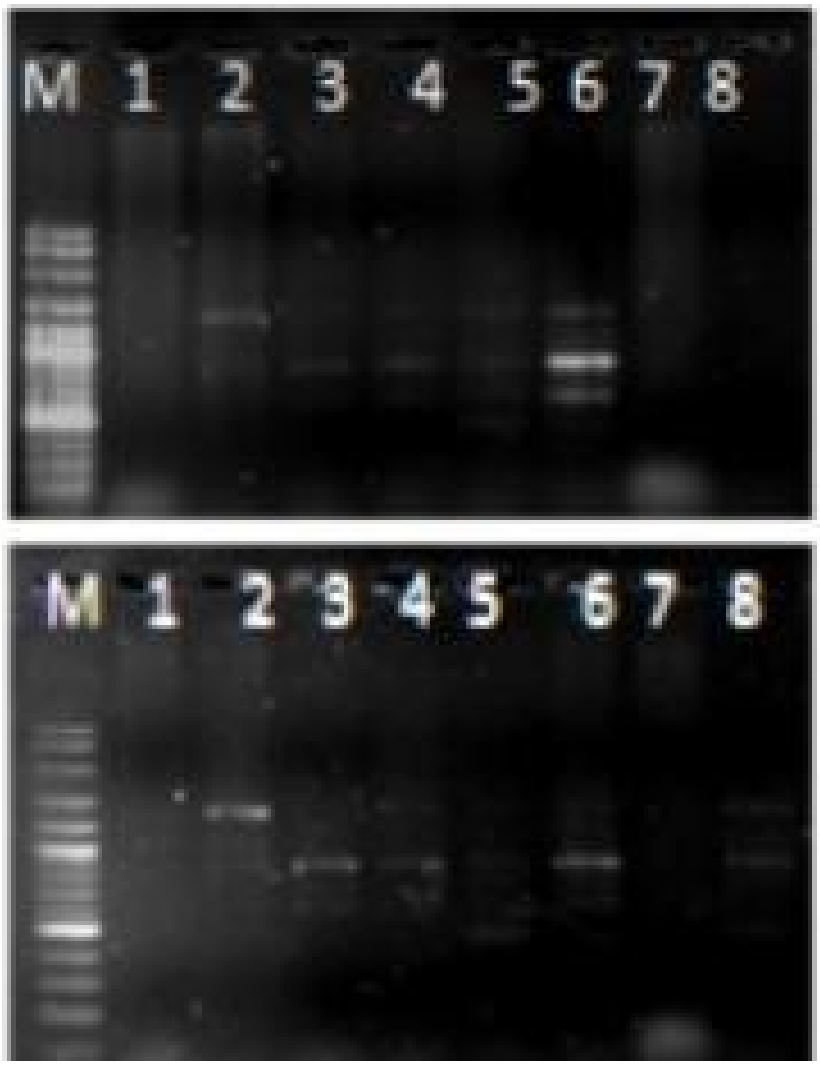

Figure 2: DNA amplified banding patterns based on RAPD for eight bacterial strains using RAP5primer. VC100bp Plus DNA ladder, Vivantis N 1407(Lane M). Bacterial strains without treatment (Upper) and after treatment with fungicide (Lower). Lanes 1 to 8 are bacterial strains, M_{10}, M_3, T_2, D_2, D_4, M_2, T_3 and J_3, respectively.

RAPD profiles and genetic similarity

A random amplified polymorphic DNA (RAPD) technique was applied to detect the genetic effects at the DNA level in two different types of antimicrobial bacterial, *E. coli* and *Bacillus* strains after treatment with Benlate. This study compared the effects occurring at molecular level in bacterial cells treated with fungicide Benlate with untreated bacterial cells as a control. Two 10- meroligonucleotide primers were used for screening genomic DNA of bacterial cells treated and untreated with Benlate that produced visible and reproducible bands. The presence of changes in the RAPD profiles obtained from treated bacterial samples depended on the primer used. The total number of amplified bands were 12 and 55 bands for *E. coli* and *Bacillus* bacterial strains, respectively. Different size bands obtained in RAPD profile was from 4800-1800 bp. The results of RAPD profiles generated by the two primers revealed differences between treated and non treated bacterial samples, with visible changes in the number and size of amplified DNA fragments and loss of original bands and appearance of new ones. Table (2), Figures 1 and 2 present a summary of all RAPD profiles alterations and RAPD products of selected primers. Primer 1 (Fig.1) produced polymorphic bands with genomic DNA of *B. subtilis* M10 (lane1) after treatment, caused appearance of DNA bands with size of 900 and 1000 bp compared with the control. Produced polymorphic bands with genomic DNA of *B. megaterium* M3 (lane 2) after treatment, caused appearance of DNA bands with size of 900, 1000 and 1500 bp compared with the control. RAPD profiles were generated similar bands using primer1 for controls and treatment groups in the genomic DNA of *B. subtilis*T2 (lane3) and *B. subtilis* D2 (lane 4), rendering it uninformative in revealing alterations. Primer1 produced polymorphic bands with genomic DNA of *B. subtilis* D4 (lane 5) after treatment, caused disappearance of DNA band with size of 600 bp compared with the control. Primer1 produced polymorphic bands with genomic DNA of *E. coli* M2 (lane 6) after treatment, caused differences in bands of DNA with sizes of (600,700, 900, 1400 and 1500 bp) compared with the control (700, 900, 1200 and 1800). Primer 1 produced polymorphic bands with genomic DNA of *B. subtilis*J3 (lane 8) after treatment,

caused appearance of DNA bands with size of 600 and 1500 bp. For example primer 2 (figure 2) produced polymorphic bands with genomic DNA of *B. megaterium*M3 (lane 2) after treatment, caused appearance of DNA band with size of 1000 bp compared with the control. For example, primer 2 figures 2 produced polymorphic bands with genomic DNA of *B. subtilis*T2 (lane 3) after treatment, caused disappearance of DNA band with size of 1800bp. RAPD profiles generated similar bands using primer2 for controls and treatment groups in the genomic DNA of *B. subtilis*D2 (lane 4), rendering it uninformative in revealing alterations. Primer 2 produced polymorphic bands with genomic DNA of *B. subtilis*D4 (lane 5) after treatment, caused disappearance of DNA band with size of 600 bp compared with the control. Primer2 produced polymorphic bands with genomic DNA of *E. coli* M2 (lane 6) after treatment, caused disappearance of DNA bands with size of 1000 and 1500 bp, primer 2 produced polymorphic bands with genomic DNA of *B. subtilis*J3 (lane 8) after treatment, caused appearance of DNA bands with size of 500, 800 and 1500 bp. The RAPD profiles of the eight strains were compared before and after treatment with fungicides, and variation in the band profiles was observed for each primer; each strain has specific bands which were absent in others. In order to score fingerprints, one band has assumed to be corresponded to one locus. Analysis of the eight isolates by using two RAPD primers (Table 2) revealed that *B. megaterium* M3 (lane2) after treatment, caused appearance of DNA bands with size of 900, 1000 and 1500 bp and appearance of DNA band with size of 1000 bp with primer 1 and 2, respectively. *B. megaterium* M3 had lower antifungal activity after fungicide treatment against all the tested fungi, the inhibition zones decreased from 69 to 25, 60 to 18, 75 to 10, 85 to 30 and 25 to 12 mm, respectively. *B. subtilis*T2 (lane3) caused disappearance of DNA band with size of 1800 bp using primer 1 and 2, respectively. *B. subtilis*T2 showed increased inhibition zones against *M. gallinae, M. gypseum* and *Acremonium* sp., lower antifungal activity after fungicide treatment against *Exerohilum* and the same antifungal activity against *A. niger***.** *B. subtilis*D2 (lane 4), rendering it uninformative in revealing alterations with primer 1 and 2, respectively. *B. subtilis* D2 inhibition zones increased against *M. gypseum*, *Acremonium* and *A.niger* from 0 to 35, 12 to30 and 15 to 28, respectively, decreased against *M. gallinae*, *Exerohilum* sp. from 50 to 35, 50 to 20 respectively. *B. subtilis* D4 (lane 5) after treatment, caused disappearance of DNA band with size of 600 bp, with primer 1 and 2, respectively. *B. subtilis* D4 inhibition zone increased against *A. niger* and showed lower antifungal activity after fungicide treatment against three tested fungi for *M. gallinae, M. gypseum*, *Exerohilum* and the same with *Acremonium* sp. *E .coli* M2 (lane6) after treatment, caused differences in bands of DNA with sizes of (600,700,900, 1400 and 1500 bp) compared with the control (700, 900, 1200 and 1800), disappearance of DNA bands with size of 1000 and 1500 bp. *E. coli* M2 and *B. subtilis*M10 showed lower antifungal activity after fungicide treatment against four tested fungi, *M. gallinae, M. gypseum, Exserohilum* sp., *Acremonium* sp., with different inhibition zones. Antifungal activity increased against *A. niger* from 15 to 24, 24 to 40 and 0 to 17 for *E. coli* M2, *B. subtilis*M10 and *B. subtilis*D4. *B. subtilis*T3 lost antifungal activity against *A. niger*. *B. subtilis* M10 (lane1) after treatment, caused appearance of DNA bands with size of 900 and 1000 bp compared with the control. *B. subtilis*J3 (lane8) after treatment, caused appearance of DNA bands with size of 600 and 1500 bp compared with the control. Appearance of DNA bands with size of 500, 800 and 1500 bp compared with the control for primer 1 and 2, respectively. Its inhibition zone increased against *M.gallinae*, lost inhibition against *A. niger* and decreased for *M. gypseum*, *Exerohilum* sp. and *Acremonium* sp. Combined pollution or elevated toxicant level would increase disappearing RAPD bands compared to control. In all cases, polymorphisms were due to the loss and gain of amplified bands in the treated samples compared with the control. DNA changes were observed in 6 Bacillus strains and *E. coli* M2 treated with Benlate with primer 1 and 2 (Fig.1 and 2). Methyl benzimidazole-2-yl carbamate (MBC) is the active component of the widely used fungicide Benomyl. Contrasting reports that benomyl or MBC is mutagenic in some organisms have been attributed to the ability of these compounds to act as purine analogs that are incorporated into DNA [49, 50, 51 &52]. This low level of mutagenicity was dependent on the efficiency of DNA repair synthesis: excision repair- deficient strains of *E. coli* or *S. typhimurium* had a higher frequency of mutation than excision-proficient strains when treated with Benomyl [50]. Differences in the number of genetic changes observed in the *Trichoderma* strains treated with chemical fungicides could be due to their mode of action or to the approach used for the tolerance development. It has been described that protectant fungicides such as Captan, induce mutations in several genes, contrary to systemic fungicides in which target is a particular gene or gene product [53, 54 &55]. This coincides with the results, since high genetic changes observed in the Captan tolerant *Trichoderma* lines as compared to the wild type strains. Benomyl (methyl-l-(butylcarbomoyl)- 2-benzimidazole carbamate) is widely used as a systemic fungicide. It has been found to induce base-pair substitution mutations in *S. typhimurium* spot tests in Ames' tester strains [50, 51]. However, some investigators did not find any mutagenic effect of benomyl in Ames' tester strains [56, 57].The results obtained in this investigation are found to be similar with studies of [58, 59] in which, the species of the genus Bacillus Particularly *B. subtilis* have been shown to produce a range of antimicrobial dipeptides and cyclic lipopeptedes. Some of the metabolites are strain- specific and may be associated with certain species or subspecies of the *B. subtilis*.

Conclusion

In conclusion, results revealed a correlation between Benlate fungicide treatment and antagonistic activity of Bacillus and *E. coli*. The antagonistic capacity under *in vitro* condition was negatively affected in all of the treated strains with some pathogenic strains, in many cases the antagonistic capacity was increased. Results also revealed that treatment with Benlate induced great changes of genomic DNA of non target bacteria and in their physiological activates. In addition, the results demonstrated that

RAPD technique is useful for detection of alterations in the DNA. We observed the inhibitory effect of Bacillus strains and *E. coli* M2 on pathogenic fungi. However, what molecule was endowed with antifungal activity is not yet known. Further studies are needed to identify and characterize the antifungal protein molecule (s) of Bacillus and *E. coli* having potential against fungi. The work on identification of molecule endowed with antimycotic properties is in progress.

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