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DEVELOPMENT OF BIOAGENTS AS FUNGICIDES FOR PLANT DISEASE CONTROL

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ABSTRACT - **The commercial development of biofungicides received a significant boost in recent years, primarily because of impressive progress in the isolation and characterization of novel strains of microorganisms that can fulfill the mam characteristics of a biofungicide, which are the consistent suppression of pathogens under field conditions, and easy mass production in standard fermentation facilities. To develop biocontrol agents (BCAs) as commercial biofungicides, an extensive identification is essential. Therefore, biological, biochemical and molecular technique were conducted with the aim of identification of promising isolates for development as biological control agents as biofungicides. In recent years, biocontrol agents Pseudomonas fluorescence , P. putida, Paenibacillus polymyxa, Bacillus brevis, Streptomyces sp., Tilletiopsis pallescens, Rhodotorula glutinis, Verticillium lecanii and Penicillium oxalicum have been isolated from various parts of plants and used successfully as biological control agents against numerous foliar plant pathogens. Isolates were characterized by production of secondary metabolites, include enzymes, peptides and siderophore. We found that metabolites secreted by BCAs isolates had an inhibitory growth effect on target pathogens, suggesting that these metabolites play a role in suppression of plant pathogens. Molecular genetic markers have been developed to discriminate these biocontrol agents from others and to identify specific applied isolates by the use of different PCR-based methods. SDS electropherograms obtained in the present study reveled substantial differences between the investigated strains, proving that SDS-PAGE of total protein is a reliable technique applicable for discriminating different genotypes . The results of RAPD analysis obtained in the present study showed a considerable amount of genetic variation present in the nine strains investigated, although individual primers differed in the amount of variation they detected. More importantly, the level of polymorphism detected by using RAPD'S will provide microbiologists with environment independent DNA marker, which should be regarded as essential tools for selection.**

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INTRODUCTION

Control of fungal pathogens is based on the use of agronomic practices and pesticides, but widespread application of chemicals inundates the agroecosystems with toxic compounds that affect the balance of the natural food chain. Most sustainable and environmentally acceptable control may be achieved using BCAs (Haggag, Wafaa 2002 and 2003). Antagonistic microorganisms are being studied in depth and considered as an attractive option for the development of microbial-based biofungicides and mixed formulations containing biocontrol agent(s) and low doses of chemical fungicides. At the same time. identifying, and utilizing microorganisms to control plant diseases and to enhance crop production are integral parts of sustainable agriculture. When selecting microorganisms for a specific purpose or function it is essential to correctly classify and identify isolates for the selection of appropriate candidates in given situations. This precise identification is also necessary to ensure consistency in experimental, industrial, and commercial processes. Classical approaches based on the use of morphological criteria are difficult to apply to biocontrol isolates, due to the plasticity of characters. Use of molecular techniques has facilitated identification of different micro-organisms quickly, efficiently and reliably (Tyler *et al.,* 2001). Also, the identification and molecular characterization of microorganisms, useful as producers of bioactive compounds, are of great interest for the modern and echo-compatible agriculture (Haggag, *et al*., 2007 and Haggag, Wafaa 2013). A combination of physiological, biochemical (enzyme production), and molecular criteria were used to establish intraspecific groups, which could be related to different levels and spectrum of plant pathogens control. Numerous molecular techniques have been employed in the detection, identification and phylogenetic analysis of biocontrol agents populations (Oliveira *et al.,* 2004 and Castelli, *et al*., 2008). PCR amplification serves as the basis for the above mentioned molecular techniques and a large variety of primers currently exist for the amplification of different regions of fungal DNA.

Thus, analysis of the genetic structure of microbial isolates has practical importance; the results can be used to assess the fate of released strains and their impact on resident microbial communities and plant pathogens (Yang *et al.,* 2008). Some bioagents, previously isolated

from the phyllosphere, which showed strong *in vitro* and *in vivo* antifungal activity against some plant pathogens (Haggag, Wafaa et al., 2013a, b and 2014). We intend to stimulate the development of a biotechnological identification and development of bioproducts as bio-fungicides . Thus, the aim of this study is to molecular characterization of the beneficial promising local biocontrol microorganisms include *Tilletiopsis* spp., *Bacillus brevis*., *Verticillium lecanii*, *Rhodatorula glutinis* , *Bacillus brevis*, *Paeniobacillus polymyxa*, *Pseudomonas* spp. *Streptomyces* spp. and *Penicillium oxcillicum* . In addition, the present study was undertaken with the primary objective of further evaluating genetic variability among biocontrol isolates through the application of an extended range of molecular techniques.

MATERIALS AND METHODS

Microorganisms

Pathogens: Pathogenic fungi and their host plants used for this work are listed in Table (1). These isolates were maintained at –20°C as spore suspensions in 20% glycerol and were activated in potato dextrose agar for 5 to 7 days. Spores were washed from the agar with tap water containing 0.01% tween-80 to prepare inoculum suspensions. Inoculum concentrations were 10^4 spores /ml. Grape and mango leaves naturally infected with powdery mildew fungus (*Uncinula necator* and *Oidium mangiferae* , respectively) , were cut into leaf disks (40 mm2). Spores were harvested from mildew-infected leaves by washing with distilled water containing 0.01% Tween 20. The spore concentration was adjusted to 15,000 spores/ml and lightly atomized onto grape plants.

Bioocontrol agents (BCAs)

Biocontrol agents used for this work are listed in Table (1) These isolates were identified in Plant Pathology Department, National Research Centre based on a great variety of morphological, cultural, physiological and biochemical characteristics. These isolates were maintained at –20°C as spore suspensions in 20% glycerol and were activated in potato dextrose agar or nutrient yeast salt medium (NYSM) for 3 to 5 days. The fungi, *V. lecanii and P. oxalicum* were grown on Potato Dexstrose agar medium for 10 days at 25°C. The conidial suspensions were obtained by flooding the colonies with sterile water containing 0.01% Tween 80, then weltered and adjusted to $5X10^5$ colony forming units (CFUs/ml).

Bacteria , *B. brevis* , *P. polymyxa* , *P. fluorescens* and *P. putida* were grown in nutrient broth at 26^0C for 24 h. Bacteria were spread onto nutrient agar plates from stocks. These plates were incubated overnight at 25^0C and used to inoculate 100 ml nutrient broth (Oxoid) in 250 ml conical flasks

For *Streptomyces,* starch-nitrate medium was used. Cells were harvested after four days, centrifuged at 3000 g for 10 min. Pellet were resuspended in 15 ml distilled water containing 0.01% tween 80 and phosphate-buffered saline (PBS) to a density of 10^5 bacteria ml^{-1}.

Yeast ,*T. pallescens* and *R. glutinis* were maintained on YMPG and incubated at 4 °C for long-term storage. The spores were diluted to 2 x 10^6 blastospores/ml with sterile water and then resuspended in 0.01 M potassium phosphate buffer (pH 7.0) to 2 x 10^5 / cfu /ml^{-1} plus 0.01% Tween-80 as surfactant.

Biological evaluation

Antagonistic activity of metabolites secreted by BCAs was measured as growth reductions of target pathogens, where volume of each BCAs culture filtrate was added to pathogen medium to provide a final concentration of 50% . Isolates growth were determined by measuring the optical density at 610 nm. *In vitro* bioassay, antagonistic activity of the biocontrol agent against *U. necator* or *Oidium mangiferae* on leaf disks of grapevines cultivar King Ruby (20-mm diam.) was determined under controlling conditions. Leaf disks were sprayed with culture filtrate (10 %). Sterile water was used as control treatment. Leaves were incubated at 25°C for 16 h under light and 90% relative humidity. The experimental design was a randomized block with four replicates. Reduction of spore germination of pathogen was determined 72 h after their application.

Biochemical Identification.

Hydrolysis enzymes activities assay

Activities of hydrolysis enzymes were assessed in culture filtrates of the tested yeast isolates over 3 - 21 days using a Labsystems Uniskan II microtiter plate spectrophotometer. Protease activity was measured in dimethylcasein (5 mg/ ml in 20 mM phosphate buffer pH 7.0) as substrate using autoanalyser. The release of alanine was measured and used as a basis for the expression of protease activity (I U = 1 µmol alanine/ min/ g) (Tsuchida *et al.,* 1986). Exo-glucanase activity was detonated using glucose oxidase-O-dianisidine reaction (Sigma Chemicals, glucose determination kit 510-A) which specifically measures glucose produced from laminarin hydrolysis. ß-1,3-Glucanase activity was assayed by incubating 0.2 ml of culture filtrate in 50 mM potassium acetate buffer (pH 5.5) with 50 µl of enzyme solution appropriately diluted in the same buffer. Reaction mixtures were incubated at 37°C for 30 min and were stopped by boiling for 5 min. One unit of ß-1,3-glucanase activity was defined as the amount of enzyme that releases 1 µmol of reducing sugar equivalents (expressed as glucose) per min under the standard assay conditions (Nelsonm, 1955). The activity of exo-chitinase, was measured as the release of N-acetylglucoseamine from chitin and one unit (U) of enzyme activity was defined as the amount of enzyme that release 1 µmol of reducing groups/ min/ ml of the filtrate (Bradford, 1976).

Extraction and purification of polypeptides antibiotics :

Antibiotics and antibiotic peptides produce by biocontrol agents i.e. *P. polymyxa , B. brevis , P.fluorescence, P.Putida and Streptomyces sp.* were

determine by HPLC and compared with Standard (Sigma Chemicals).

Siderophores assay. The amount of siderophores excreted into the culture medium was determined by spectrophotometer. Concentration was calculated using absorption maximum and the molar absorption coefficient (max; 400 nm) according to the method of (Meyer and Abdallah, 1978).

Measurements of BCAs growth and protein production.

Protein production by BCAs were measured in culture filtrates after four and seven days of incubation. Isolates growth were monitored by measuring the difference in protein contents between culture broth and culture supernatant. Isolates growth were determined by measuring the optical density at 610 nm. Protein content in the supernatant was determined at 595 nm by the method of Bradford (1976) using bovine serum albumin as a standard.

Molecular Identification

Protein analysis

Protein extraction was carried out according to Bollag & Edelstein (1990) . and separated by polyacrylamide gel electrophoresis (SDS-PAGE) which carried out according to a protocol proposed by Laemmli (1970). Electrophoretic separation was carried out using mini gel unit at 60 volt for 4 hours. The gel was stained using silver stain and distained with methanol solution. Molecular weights of polypeptide bands (KD) was calculated from a calibration curve of low molecular weight marker kit of *Pharmacia* .

a. Gel preparation

The polyacrylamide separating gel (main gel) (12%) and stacking gel (5%) were prepared according to Laemmli (1970) from the stock solutions as showen in Table 2. 30 µl of the protein extract were mixed with 5 µl of sample buffer, then heated in a boiling water bath for 5 minutes for protein denaturation.

c. Electrophoretic separation of denatured proteins

Electrophoresis was carried out using Hoefer Vertical Slab gel unit, Model SE-400. The prepared samples were loaded on the gel at constant Voltage of 150V and 15 mA, until the bromophenol blue dye reached the bottom of the gel. The duration of the separation was about 5-6 hours. The gel was directly placed in the Coomassie brilliant blue staining solution overnight. The gels were destained several times for twelve hours in the destaining solution. After destaining, the gel was photographed, dried and kept for comparison.

d. Subunit molecular weight estimation by SDS-PAGE

The method of Weber and Osborne (1969) was used to determine the apparent (subunit) molecular weight of proteins dissolved or extracted in the presence of SDS. Electrophoretic mobilities were calculated relative to the mobility of the bromophenol blue marker band in 12 % and 5 % polyacrylamide slab gel. The following proteins were used as molecular weight standards for tissue culture derived plants: β-galactosidase (116.0 KDa), bovine serum albumin (66.2 KDa), ovalbumime (45.0 KDa), lactate dehydroginase (35.0KDa), REase β-sp- 981 (25.0 KDa), β- Lactoglobuline (18.4 KDa) and Lysozyme (14.4 KDa).

RAPD-PCR

A-DNA extraction

1. Bacterial DNA isolation

Total DNAs were isolated by a versatile quick-prep method for genomic DNA isolation. Cells grown in broth shake culture were centrifuged, rinsed with TE and resuspended in 4 ml SET buffer (75 mM NaCl, 25 mM EDTA, 20 mM Tris, pH 7.5). Lysozyme was added at a concentration of 1 mg /ml and incubated at 37 ^{o}C for 0.5–1 h. One-third volume of 10% SDS and 0.5 mg Proteinase K mg/ml were added and incubated at 55 ^{o}C with occasional inversion for 2 h. One-third volume 5 M NaCl and 1volume of Chloroform was added and incubated at room temperature for 0.5 h with frequent inversion. The mixture was centrifuged at 4500 xg for 15 min and the aqueous phase was transferred to a new tube using a blunt-ended pipette tip. Chromosomal DNA was precipitated by the addition of 1 volume 2-propanol with gentle inversion. The DNA was transferred to a new tube, rinsed with 70% ethanol, dried under vacuum and dissolved in about 100 ml of distilled water. The dissolved DNA was treated with 20 mg RNase A mg/ ml at 37 ^{o}C for 1 h. Samples were extracted in the same volume of phenol/chloroform/isoamyl alcohol (25: 24: 1) and precipitated with0.1 volume of 3 M sodium acetate and 2.5 volume of cold ethanol. The pellets were washed with 70% ethanol, dried and dissolved in TE or distilled water.

2. Fungal DNA isolation

DNA isolation was performed using the CTAB method of Doyle and Doyle (1990). 0.5 g fresh sample was ground to powder in liquid nitrogen with a prechilled pestle and mortar, suspended in 1 ml preheated CTAB buffer, and incubated at 65^{0} C for 1 hour with occasional shaking then centrifuge for 10 min at 1000 rpm. Transfer the supernatant to a new tube by wide pore, add 0.5 ml of (chloroform: isomyl) 24:1 then centrifuge for 15 minutes at 14000 rpm and the aqueous layer was transferred to a new sterilized tube (avoid protein surface). The ice cold isopropanol was added to precipitate the nucleic acid (RNA, DNA) then Incubate at – 20 ^{0}C overnight and centrifugation was happened at 14000 rpm for 20 minutes. The supernatant was discard and the pellet was washed carefully twice with cold 70% ethanol, dried at room temperature and resuspend in 100 µl of sterile deionized distilled water. DNA concentration was determined by electrophoresis of 5 µl of DNA with 2µl of loading buffer and run at 100 volt for approximately 30 minutes.

B- RAPD analysis.

RAPD was performed as described by Williams *et al.* (1990**)** with minor modifications. Briefly, PCR amplification was performed in 25 µl reaction mix (Table. 3) containing 20.40 ng genomic DNA, 0.5 unit Taq polymerase (Sigma), 0.2 mM each of dATP, dCTP, dGTP, dTTP, 5 Pico mole random primer and appropriate amplification buffer. The reaction was assembled on ice, overlaid with a drop of mineral oil. Amplification was performed for 45 cycles (Table, 4 & 5) using Biometera Uno thermal cycler, as

follows: One cycle at 95^0c for 3 minutes and then 44 cycles at 92^0 C for 2 minutes, 37 ^{0}c for 1 minute and 72 ^{0} C for 2 minutes (for denaturation, annealing and extension, respectively). Reaction was finally incubated at 72 ^{0} C for 10 minutes and further incubated on 4 ^{0}C .Five primers were used for RAPD analysis based on their ability to amplify bacterial and fungal genome and producing reproducible amplification patterns (Table 6).

Table (1): Bio-control isolates used in this work and their target pathogens and hosts

Bio-control	*Pathogens*	*Host*	*References*
Bacillus brevis	*Botrytis cinerea pers.*	*Strawberry*	*Haggag, (2008), Haggag and Timmusk (2008).*
	Botryodiplodia theobromae (Lat.) (Lasiodiplodia theobromae)	*Grape*	
Paeniobacillus polymyxa	*Botrytis cinerea and*	*Strawberry*	
	Phytophthora sp.		
P. putida	*Phomopsis and Botryodiplodia*	*Mango*	*Haggag, and Nofal, (2006).*
Pseudomonas fluorescens	*Botrytis cinerea and Phytophthora*	*Strawberry*	
Streptomyces	*Fusarium subglutinis and Colletotrichum gleosporioides*	*Mango*	
Penicillium oxalicum	*Fusarium subglutinis*	*Mango*	
Verticillium lecanii	*Oidium mangiferae*	*Mango Grape*	*Haggag, et al., (2007)*
Tilletiopsis pallescens	*Uncinula necator*		*Nofal , and Haggag (2006)*
Rhodatorula glutinis	*Botrytis cinerea*	*Strawberry Grape*	*Haggag et al., (2004 and 2005)*

Table 2. The component of 10% acrylamide separating and 5% acrylamide stacking gel.

Stock solutions	**Main gel (12%)**	**Stacking gel (5%)**
Distilled H2O	3.3 ml	2.1 ml
30% Acrylamide mixture	4 ml	0.5 ml
1.5M Tris-buffer(8.8)	2.5 ml	—
0.5M Tris-buffer(6.8)	—	0.38 ml
10% SDS	0.1 ml	0.03 ml
10% APS	0.1 ml	0.03 ml
TEMED	0.004 ml	0.003 ml
Total volume	10 ml	3 ml

b. Sample preparation

Table 3. Components of RAPD-PCR mixture

Reagent	**Concentration**	**Volume**
d NTP_S	0.2 mM	2.5 µl
PCR buffer 10X	1X	2.5 µl
Ampli Taq polymerase (RTS Taq DNA polymerase).	2 Units	0.25 µl
$MgCl_2$		
Primer	2mM	1.5 µl
Distilled sterile water	10 p mole	3 µl
Total genomic DNA	-	9.75 µl
	20:40 ng	3 µl
Total volume	-	25 µl

Table 4. PCR program (temperature profile)

Order	Action	Temperature	Duration	No. of cycles
1	1stDenaturation	95°C	3 minutes	1 cycle
2	Denaturation	92 °C	2minutes	
3	Annealing	37 °C	1 minutes	44 cycles
4	Extension	72 °C	2 minutes	
5	Last extension	72 °C	10 minutes	1 cycle
6	Incubation	4 °C		

Table 5. Composition of stocks.

Stock	Composition
CTAB	1.4 M Nacl, 0.2 % β-mercaptoethanol, 100mM Tris-Cl and 20 mM EDTA
50X Tris-Acetate Buffer	242 g Tris- base, 57.1 ml Glacial acetic acid and 100 ml EDTA (0.5 M- pH 8.0
Loading buffer	0.25 g bromophenol blue and 100 ml Glycerol (30%)
Ethidium bromide	0.2 µg/ml ethidium

Table 6. Name and sequences of the selected random primers used in RAPD-PCR analysis

Primer code	Nucleotide sequences (5`- 3`)
OPA1	CAGGCCCTTC
OPA2	TGCCGAGCTG
OPA3	AGTCAGCCAC
OPA4	AATCGGGCTG
OPG1	CTACGGAGGA

C-Agarose electrophoresis

The amplification products were analyzed by electrophoresis in 2% agarose in TAE buffer stained with 0.2 µg/ml ethidium bromide and photographed under UV light. The buffer was added to the agarose then heated in a microwave tell melting, cooling to 60 ^{0}C then the ethidium bromide was added. Sample was prepared by using 10 µl PCR-product and 2 µl loading buffer. One marker was used, 100 bp DNA ladder (Axygen).

Statistical analysis. The percentages of disease severity were arcsin transformed before analysis of variance to improve homogeneity of variance. LSD values were computed for comparison between treatments.

RESULTS

Biological evaluation

Results in Table (7) showed that all filtrates from BCAs decreased the growth of target pathogens (%).In particular, *Streptomyces* spp and *Rhodotorula glutinis* showed significant activity against *Fusarium subglutinis* and *Botrytis cinerea*, respectively . Results indicated that *B. brevis* and *P. putida* showed the highly effect on reducing the mycelial growth of *Botrytis cinerea* and *Phomopsis, sp.* respectively.

Enzymes assays

The general ability of tested BCAs to produce secondary metabolites include hydrolysis enzymes were determined (Table 8). Exochitinase, exoglucanase, β-1,3- glucanase and protease, appeared to be common metabolites produced by the tested BCAs. Maximum production of chitinase by the tested *Streptomyces sp.* and *V. lecanii*. in shaken broth culture occurred after 15 days. *Rhodotorula glutinis* produced the highest amounts of all four hydrolysis enzymes. Results also showed production of high levels of protease by the tested *Tilletiopsis*. Meanwhile, cellulase was detected in the tested *P. oxalicum*. Furthermore, the isolate *Rhodotorula glutinis* was superior to the other isolates for enzyme production.

Production of antibiotics and Siderophore

The yield of antibiotics from *BCAs* was studied (data not showed). Gramicidin S and polymyxin B antibiotics were purified from culture filtrates of *B. brevis* and *P. polymyxa*, respectively using HPLC . A total of 75 and 80 µg /L of gramicidin S and polymyxin B, respectively was yielded. Antibiotics phenazine was purified from the culture filtrates (50 liters) of *P . fluorescens* and *P. putida* using HPLC . A total of 48.9 and 41.0 µg of the antibiotic was yielded from liter of culture extracts of *P . fluorescens* and *P. putida,* respectively.

Siderophore production

The clearest siderophores production by *Pseudomonas fluorescens* and *P. putida* was detected in succinate medium . Final siderophore concentration achieved was almost 75 and 60 µM. for *Pseudomonas fluorescens* and *P. putida,* respectively. Maximum siderophore production was obtained when glycerol was used as carbon source. (data not showed).

Growth and protein production by BCAs

Isolates growth, and protein concentrations of BCAs production in liquid medium, are recorded in Table 9. Data were recorded after four days for yeast, and bacteria and seven days for fungi. Maximum level of protein production was observed in *P. polymyxa* in culture broth.

Table 7. Effect of culture filtrates of BCAs on growth of pathogenic fungi after 7 days incubation at 25°C.

Bio-control	Pathogens	Growth reduction %
B. brevis	*Botrytis cinerea*	99.1ab
	Botryodiplodia	95.4d
B. polymxya	*Botrytis cinerea*	98.5b
	Phytophthora	97.4bc
P. putida	*Phomopsis*	99.2ab
	Botryodiplodia	98.5b
P. fluorescens	*Botrytis cinerea*	98.6b
	Phytophthor	96.7c
Streptomyces	*Fusarium subglutinis*	100.0a
	Colletotrichum gloeosporioides	92.6ef
Penicillium oxalicum	*Fusarium subglutini*	93.8e
Verticillium lecanii	*Uncinula necator*	93.2e
	Oidium mangiferae	91.6f
Tilletiopsis pallescens	*Uncinula necator*	97.8bc
	Oidium mangiferae	96.4c
Rhodotorula glutinis	*Botrytis cinerea*	100.0a

Values represent the mean percentage of six replicates. Values in each column followed by the same letter are not significantly different (P<0.05)

Table 8. Antifungal Enzymes produce by biocontrol agents

BCAs	**Antifungal products (Unit/ ml)**			
	Alkaline Protease	**Chitinase**	**β-1,3 glucanase**	**Cellulase**
Pseudomonas fluorescens	--	--	--	--
P. putida	--	--	--	--
Bacillus brevis	--	--	--	--
Paeniobacillus polymyxa	--	0.23d	--	2.6a
Tilletiopsis	3.5a	0.46cd	2.7b	--
Rhodotorula glutinis	1.8b	0.67c	3.8a	0.2c
Streptomyces	--	1.98a	2.9b	--
Verticillium lecanii	0.3c	1.23b	--	--
Penicillium oxalicum	--	0.28d	3.2ab	1.7b

Values represent the mean percentage of six replicates. Values in each column followed by the same letter are not significantly different (P<0.05)

Table 9 . Growth and total soluble protein produced by various BCA

Values represent the mean percentage of six replicates. Values in each column followed by the same letter are not significantly different (P<0.05)

Biocontrol agents	**Cell concentration (OD 610 nm)**	**Total soluble protein**		
		Ab	**Conc. (g/l)**	**Dil (1:10)**
Rhodotorula glutinis	1.715c	2.145a	10.412899a	104.129a
Verticillium lecanii	1.570d	1.035e	3.0325798e	30.3258e
***Penicillium* oxalicum**	2.895a	1.08e	3.3317819e	33.31782e
Bacillus brevis	0.976f	1.8b	8.119016b	81.19016b
Pseudomonas putida	1.238e	1.82b	8.2519947b	82.51995b
Tilletiopsis pallescens	1.860b	1.45c	5.7918883c	57.91888c
Paenibacillus polymyxa	0.660j	1.265d	4.5618351d	45.61835d
Streptomyces sp.	0.745h	1.135de	3.6974734e	36.97473e
Pseudomonas fluorescence	0.870g	1.23c	4.3291223d	43.29122d

Protein analysis

The electrophoretic banding patterns of the SDS-PAGE of *Streptomyces sp., Rhodotorula glutinis, Bacillus brevis, Paenibacillus polymyxa, P. Putida, Verticillium lecanii, Tilletiopsis pallescens, Penicillium oxalicum and Pseudomonas fluorescence* are shown in Figure (1). They manifested a maximum number of 25 bands, which were not necessarily being present in all strains. Table (10) represents the occurrence of the bands as (+) and their absence as (-). The nine strains investigated showed variability in their protein patterns. However, each strain was characterized by the presence of one or more specific band, i.e. three bands of MWs 44, 37 and 35 kDa in *Streptomyces sp.*, five bands of MWs 93, 84, 22, 17 and 16 *K*Da in *Paenibacillus polymyxa* and two characteristic bands of MWs 57 and 29 kDa in *P. putida*. Only additional band of MW 32 kDa was detected in *Bacillus brevis* and another one of MW 23 kDa in *Penicillium oxalicum*. On the other hand, band of MW 63 kDa was characteristic to *Bacillus brevis*, *Tilletiopsis pallescens, Penicillium oxalicum* and *Pseudomonas fluorescenc.* Also, band of MW 61 kDa was characterized to *Streptomyces sp.* and *Rhodotorula glutinis*.

Penicillium oxalicum and *Pseudomonas fluorescence* shared in band of MW 58 kDa and *Bacillus brevis* and *Pseudomonas fluorescence* shared in band of MW 55 kDa. Band of MW 47 kDa was characterized to *Streptomyces sp., Rhodotorula glutinis, P. Putida, Verticillium lecanii* and *Penicillium oxalicum*. Bands of MWs 43 and 34 kDa were detected only in *Paenibacillus polymyxa* and *P. Putida*. Band of MW 41 kDa was present only in *Paenibacillus polymyxa* and *Pseudomonas fluorescenc.* B and of MW 33 kDa was a characteristic to *Rhodotorula glutinis* and *Tilletiopsis pallescens*. Also, band of MW 31 kDa was found only in *Tilletiopsis pallescens* and *Pseudomonas fluorescenc*. On the other hand, *Verticillium lecanii* and *Tilletiopsis pallescens* had a characteristic band of MW 30 kDa that act as a genetic marker for these strains. Finally, band of MW 27 kDa was present in *P. putida* and *Verticillium lecanii*.

RABD analysis

All the primers examined reacted with the nine strains investigated producing a polymorphic band pattern for each strain. Amplifications using five primers produced RAPD fingerprints with varying numbers of bands ranging in size from 114 bp to 12047 bp. Depending on the primers, the number of bands varied between 19 and 26. The primers have amplified 119 bands (Figs. 2, 3, 4, 5, 6 and Tables 11 and 12), out of them 60 bands were polymorphic with percentage 50.42%. The size and number of amplified fragments also varied with different primers. The size of fragments ranged from 114 bp to 12047 bp approximately while the number of amplified fragments ranged from 19 and 26.

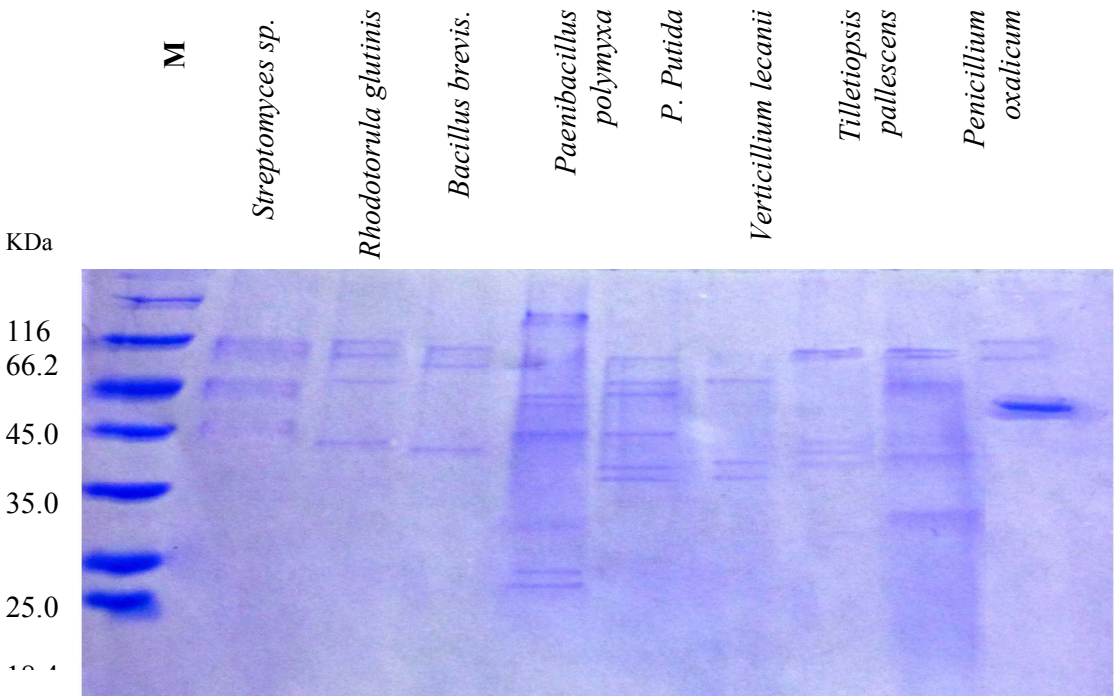

Fig. 1. SDS-PAGE pattern of *Streptomyces sp., Rhodotorula glutinis, Bacillus brevis, Paenibacillus polymyxa, P. putida, Verticilliu lecanii, Tilletiopsis pallescens, Penicillium oxalicum and Pseudomonas fluorescence*

Table (10): Variation in the electrophoretic protein banding patterns of *Streptomyces sp., Rhodotorula glutinis, Bacillus brevis, Paenibacillus polymyxa, P. putida, Verticillium lecanii Tilletiopsis pallescens, Penicillium oxalicum and Pseudomonas fluorescence* where (+) means presence and (-) means absence.

Band No.	Band molecular weight in KD	Streptomyces sp.	Rhodotorula glutinis	*Bacillus brevis.*	*Paenibacillus polymyxa*	*P. Putida*	*Verticillium lecanii*	*Tilletiopsis pallescens*	*Penicillium oxalicum*	*Pseudomonas fluorescence*
1	**93**	-	-	-	+	-	-	-	-	-
2	**84**	-	-	-	+	-	-	-	-	-
3	**66**	+	+	-	-	-	-	-	-	-
4	**63**	-	-	+	-	-	-	+	+	+
5	**61**	+	+	-	-	-	-	-	-	-
6	**58**	-	-	-	-	-	-	+	+	-
7	**57**	-	-	-	-	+	-	-	-	-
8	**55**	-	-	+	-	-	-	-	-	+
9	**47**	+	+	-	-	+	+	-	+	-
10	**44**	+	-	-	-	-	-	-	-	-
11	**43**	-	-	-	+	+	-	-	-	-
12	**41**	-	-	-	+	-	-	-	-	+
13	**37**	+	-	-	-	-	-	-	-	-
14	**35**	+	-	-	-	-	-	-	-	-
15	**34**	-	-	-	+	+	-	-	-	-
16	**33**	-	+	-	-	-	-	+	-	-
17	**32**	-	-	+	-	-	-	-	-	-
18	**31**	-	-	-	-	-	-	+	+	-
19	**30**	-	-	-	-	-	+	+	-	-
20	**29**	-	-	-	-	+	-	-	-	-
21	**27**	-	-	-	-	+	+	-	-	-
22	**23**	-	-	-	-	-	-	-	+	-
23	**22**	-	-	-	+	-	-	-	-	-
24	**17**	-	-	-	+	-	-	-	-	-
25	**16**	-	-	-	+	-	-	-	-	-

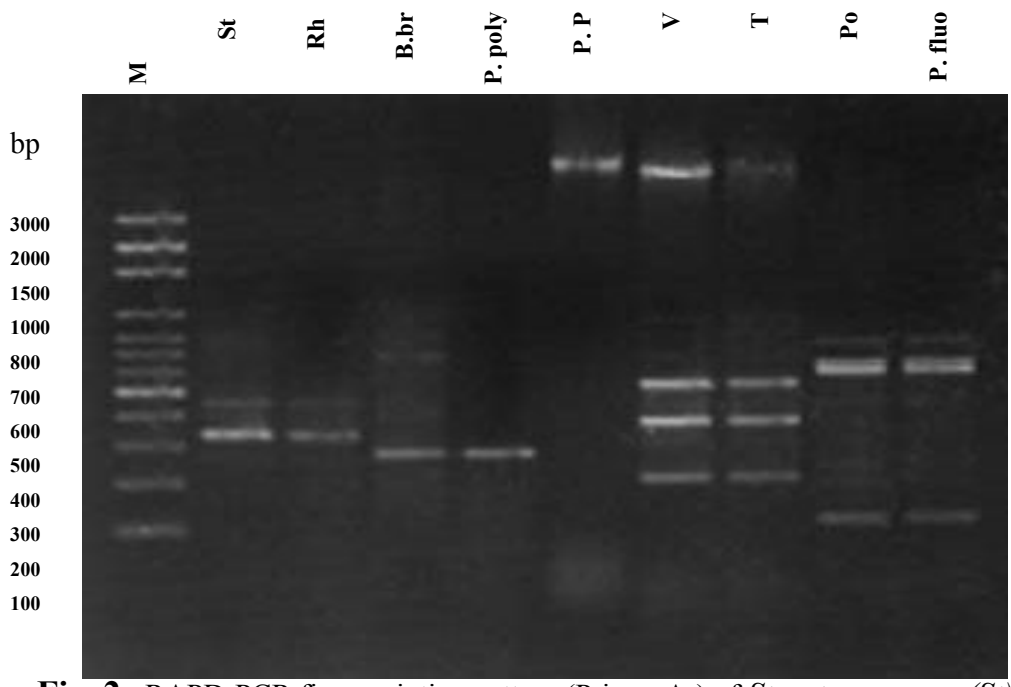

Fig. 2. RAPD-PCR fingerprinting pattern (Primer A_1) of *Streptomyces sp.(St), Rhodotorula glutinis (Rh), Bacillus brevis (P.br), Paenibacillus polymyxa(P.poly), P. putida*(P.P*), Verticillium lecanii (V), Tilletiopsis pallescens(T), Penicillium oxalicum* (Po) and *Pseudomonas fluorescence(P.fluo)*

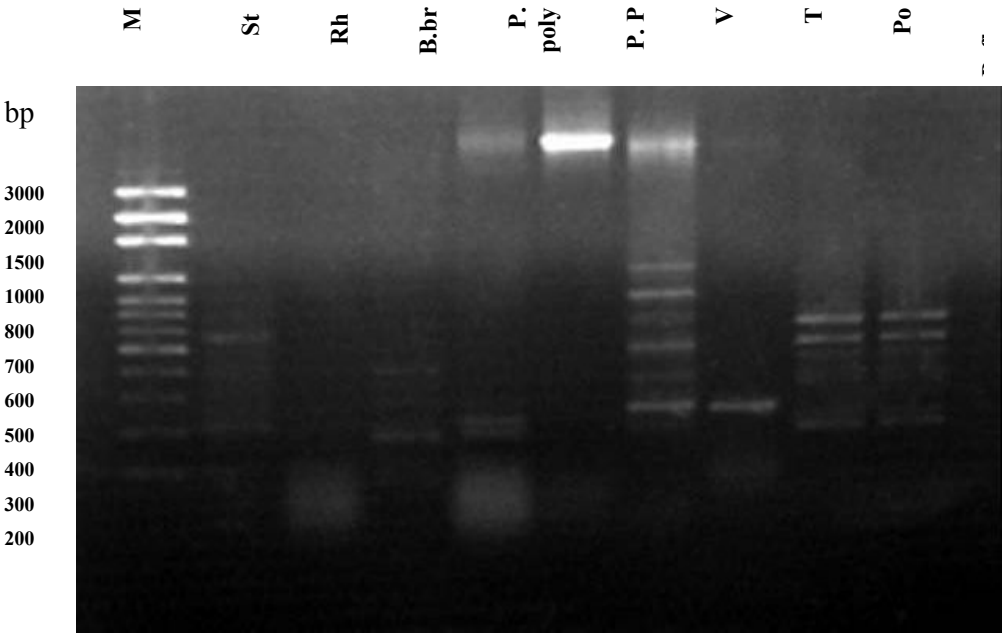

Fig. 3. RAPD-PCR fingerprinting pattern (Primer A_2) of *Streptomyces sp.(St), Rhodotorula glutinis (Rh), Bacillus brevis (P.br), Paenibacillus polymyxa(P.poly), P. putida*(P.P*), Verticillium lecanii (V), Tilletiopsis pallescens(T), Penicillium oxalicum* (Po) and *Pseudomonas fluorescence(P.fluo)*

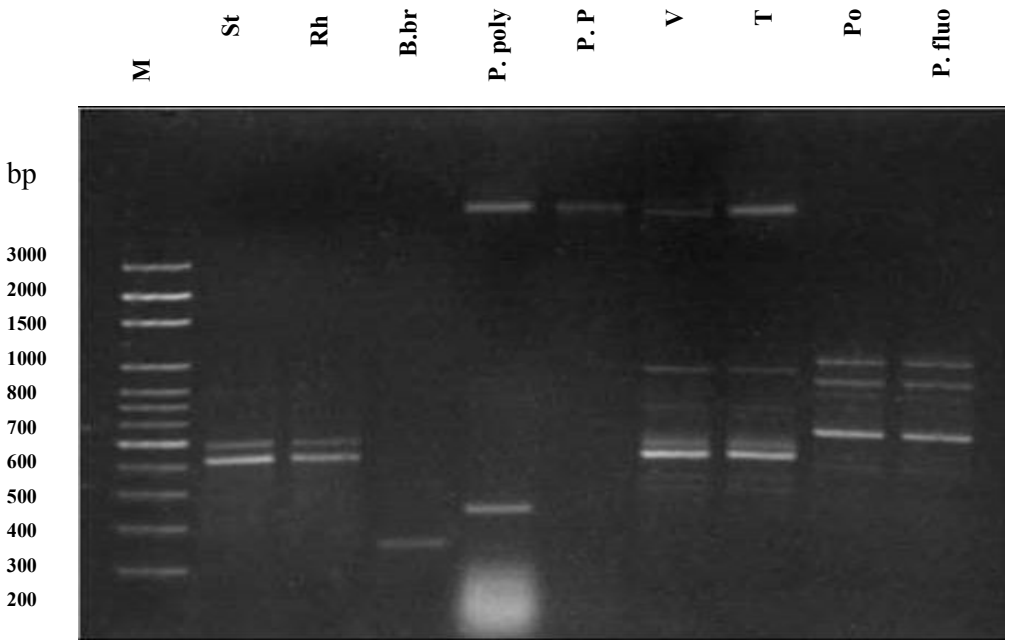

Fig. 4. RAPD-PCR fingerprinting pattern (Primer A_3) of *Streptomyces sp.(St), Rhodotorula glutinis (Rh), Bacillus brevis (P.br), Paenibacillus polymyxa(P.poly), P. putida*(P.P*), Verticillium lecanii (V), Tilletiopsis pallescens(T), Penicillium oxalicum* (Po) and *Pseudomonas fluorescence(P.fluo)*

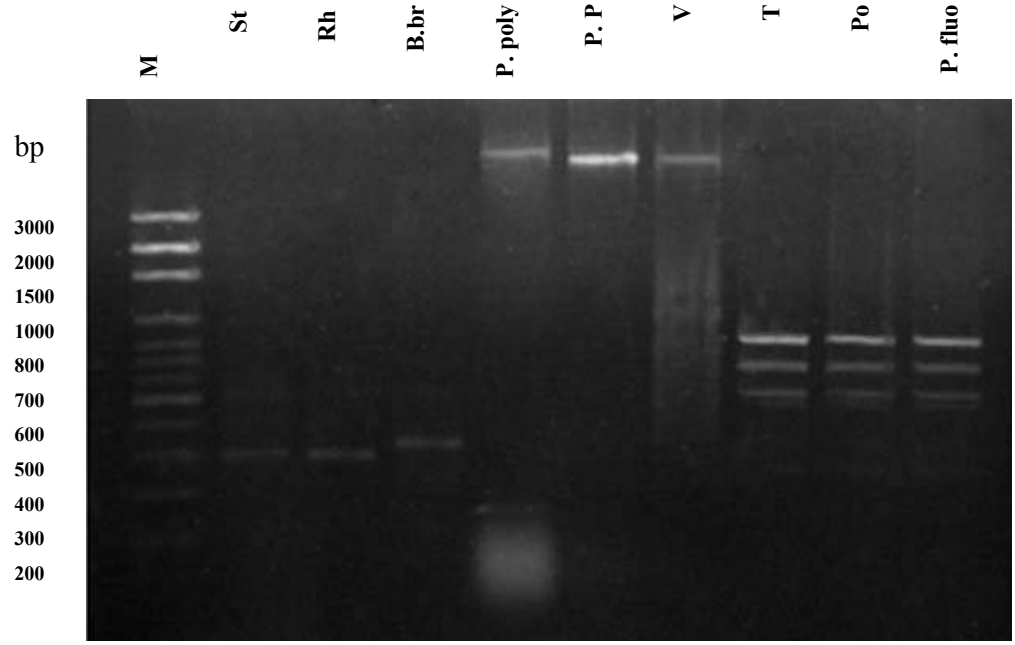

Fig. 5. RAPD-PCR fingerprinting pattern (Primer A_4) of *Streptomyces sp.(St), Rhodotorula glutinis (Rh), Bacillus brevis (P.br), Paenibacillus polymyxa(P.poly), P. putida*(P.P*), Verticillium lecanii (V), Tilletiopsis pallescens(T), Penicillium oxalicum* (Po) and *Pseudomonas fluorescence(P.fluo)*

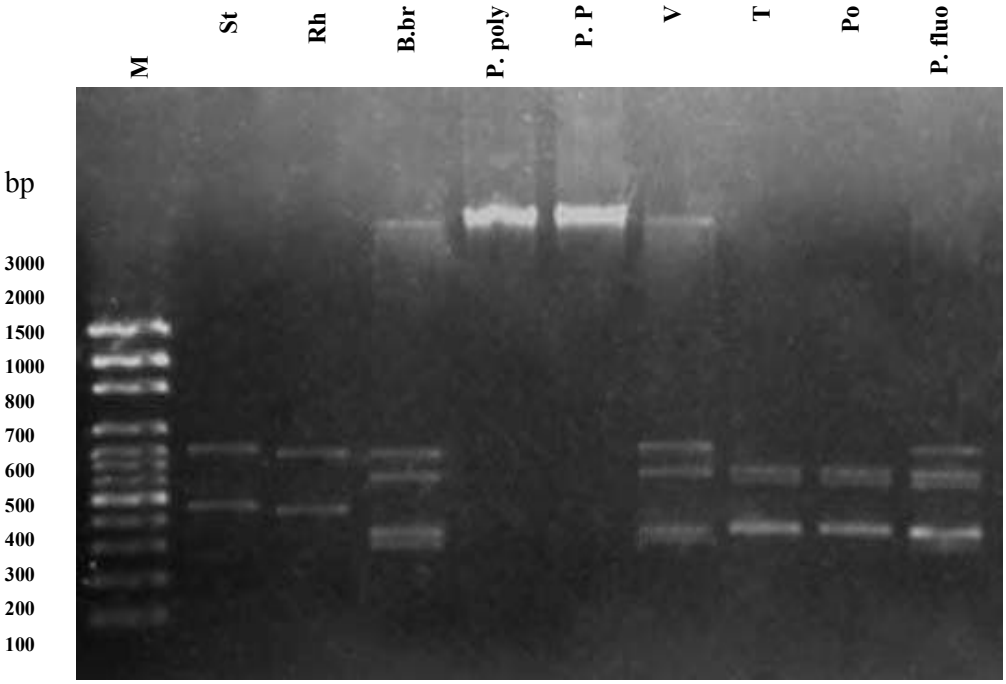

Fig. 6. RAPD-PCR fingerprinting pattern (Primer G_1) of *Streptomyces sp.(St), Rhodotorula glutinis (Rh), Bacillus brevis (P.br), Paenibacillus polymyxa(P.poly), P. putida*(P.P*), Verticillium lecanii (V), Tilletiopsis pallescens(T), Penicillium oxalicum* (Po) and *Pseudomonas fluorescence(P.fluo)*

Table 11. Survey of the RAPD markers in *Streptomyces sp., Rhodotorula glutinis, Bacillus brevis, Paenibacillus polymyxa, P. putida,Verticillium lecanii,Tilletiopsis pallescens, Penicillium oxalicum* and *Pseudomonas fluorescence*

where (+) means presence and (-) mean absence.

Table 12. The total number of amplification products per primer, number of polymorphic bands and

Band No.	RAPD Marker Base Pair	*Streptomyces sp.*	*Rhodotorula glutinis*	*Bacillus brevis.*	*Paenibacillus polymyxa*	*P. Putida*	*Verticillium lecanii*	*Tilletiopsis pallescens*	*Penicillium oxalicum*	*Pseudomonas fluorescence*
1	Primer A_1 6750	-	-	-	-	+	-	+	-	-
2	5958	-	-	-	-	-	+	-	-	-
3	788	-	-	-	-	-	-	-	+	+
4	710	-	-	+	-	-	-	-	-	-
5	665	-	-	-	-	-	-	-	+	+
6	600	-	-	-	-	-	-	-	+	+
7	538	-	-	-	-	-	+	+	-	-
8	461	+	+	-	-	-	-	-	-	-
9	383	-	-	-	-	-	+	+	-	-
10	335	+	+	-	-	-	-	-	-	-
11	279	-	-	+	+	-	-	-	-	-
12	220	-	-	-	-	-	+	+	-	-
13	114	-	-	-	-	-	-	-	+	+
1	Primer A_2 6416	-	-	-	+	+	+	-	-	-
2	1145	-	-	-	-	-	+	-	-	-
3	853	-	-	-	-	-	+	-	-	-
4	671	-	-	-	-	-	+	-	+	+
5	558	+	-	-	-	-	-	-	+	+
6	509	-	-	-	-	-	+	-	-	-
7	484	-	-	-	-	-	-	-	+	+
8	406	-	-	+	-	-	-	-	-	-
9	386	-	-	-	-	-	+	-	-	-
10	277	-	-	-	-	-	+	+	-	-
11	235	-	-	-	+	-	-	-	-	-
12	226	-	-	-	-	-	-	-	+	+
13	213	+	-	-	-	-	-	-	-	-
14	200	-	-	+	+	-	-	-	-	-
15	188	-	-	-	-	-	-	-	-	-
1	Primer A_3 6888	-	-	-	+	+	-	+	-	-
2	6224	-	-	-	-	-	+	-	-	-
3	1043	-	-	-	-	-	-	-	+	+
4	987	-	-	-	-	-	+	+	-	-
5	866	-	-	-	-	-	-	-	+	+
6	779	-	-	-	-	-	-	-	+	+
7	709	-	-	-	-	-	+	+	-	-
8	548	-	-	-	-	-	-	-	+	+
9	500	-	-	-	-	-	+	+	-	-
10	493	+	+	-	-	-	-	-	-	-
11	453	-	-	-	-	-	+	+	-	-
12	435	+	+	-	-	-	-	-	-	-
13	252	-	-	-	+	-	-	-	-	-
14	152	-	-	+	-	-	-	-	-	-
1	Primer A4 6913	-	-	-	+	-	-	-	-	-
2	6284	-	-	-	-	+	+	-	-	-
3	842	-	-	-	-	-	-	+	+	+
4	679	-	-	-	-	-	-	+	+	+
5	525	-	-	-	-	-	-	+	+	+
6	491	-	-	-	-	-	-	+	+	+
7	332	-	-	+	-	-	-	-	-	-
8	300	+	+	-	-	-	-	-	-	-
9	151	-	-	-	+	-	-	-	-	-
1	Primer G_1 12047	-	-	-	+	+	-	-	-	-
2	11044	-	-	+	-	-	+	-	-	-
3	800	+	+	-	-	-	-	-	-	-
4	785	-	-	+	-	-	+	-	-	+
5	641	-	-	+	-	-	+	+	+	+
6	600	-	-	-	-	-	-	+	+	+
7	464	+	+	-	-	-	-	-	-	-
8	357	-	-	+	-	-	+	+	+	+
9	300	-	-	+	-	-	+	-	-	-

percentage of polymorphism

Table 12. The total number of amplification products per primer, number of polymorphic bands and percentage of polymorphism

Primer	*Streptomyces sp.*	Rhodotorula glutinis	*Bacillus brevis.*	*Paenibacillus polymyxa*	*P. Putida*	*Verticillium lecanii*	*Tilletiopsis pallescens*	*Penicillium oxalicum*	*Pseudomonas fluorescence*	**Total number of amplified bands**	**Polymorphic bands**	**Polymorphism percentage(%)**
OPA_1	**2**	**2**	**2**	**1**	**1**	**4**	**4**	**4**	**4**	**24**	**13**	**54.16**
OPA_2	**2**	**-**	**2**	**3**	**1**	**7**	**1**	**4**	**4**	**24**	**15**	**63.5**
OPA_3	**2**	**2**	**1**	**2**	**1**	**5**	**5**	**4**	**4**	**26**	**14**	**53.84**
OPA_4	**1**	**1**	**1**	**2**	**1**	**1**	**4**	**4**	**4**	**19**	**9**	**47.36**
OPG_1	**2**	**2**	**5**	**1**	**1**	**5**	**3**	**3**	**4**	**26**	**9**	**34.61**
Total	**9**	**7**	**11**	**9**	**5**	**22**	**17**	**19**	**20**	**119**	**60**	**50.42**

DISCUSSION

Plant pathogens include fungi are the most visible threats to sustainable food production. The decreasing efficacy of the fungicides as well as risks associated with fungicide residues on the leaves and fruit, have highlighted the need for a more effective and safer alternative control measures. Most sustainable and environmentally acceptable control may be achieved using biocontrol agents due to the effort to reduce the use of agrochemicals and their residues in the environment and in food. Numerous studies have described the isolation of micro-organisms and demonstrated their potential to antagonize. Considering that environmental conditions are important, the right selection of BCAs, is equally important since the exact identification of strains to the species level is the first step in utilizing the full potential of BCA in specific applications (Lieckfeldt, *et al*., 1999). At the same time, molecular markers provide gigantic sources of data that can assist scientists in developing tools to monitor the genetic and environmental fate of these agents. Selection of appropriate molecular techniques should be based on the specific characteristics of the organism and on the desired type of information necessary to evaluate a particular step in the developmental process of a biofungicides. The identification and the biological and molecular characterization of microorganisms, useful as biocontrol agents or as producers of bioactive compounds, are of great relevance for the modern and echo-compatible agriculture (Spadaro and Gullino, 2005). Numerous molecular techniques have been employed in the detection, identification and phylogenetic analysis of biocontrol agents populations (Oliveira *et al.,* 2004)**.**

The protein-banding pattern of each organism represents a biochemical genetic fingerprint of that organism. (Hussein and Salam, 1985) stated that each band in the protein-banding pattern of an organism reflects a separate transcriptional event. Furthermore, electrophoretic analysis of the protein provides information concerning the structural genes and their regulatory systems that control the biosynthetic pathways of that protein. An important proportion of the protein-coding genes are polymorphic, i.e. they exist in the form of one or more alleles. Several different kinds of mutation may occur in the codon, in particular the replacement of certain nucleotides by others. Mutation events, therefore, have to be considered as the reasonable interpretation for the observed banding pattern

changes. The correlation between the alterations in the electrophoretic banding pattern and mutational events has been demonstrated by work of (Muller and Gottschalk, 1973 and Gamal El-Din *et al.,* 1988).

SDS electropherograms obtained in the present study reveled substantial differences between the investigated strains, proving that SDS-PAGE of total protein is a reliable technique applicable for discriminating different genotypes as indicated by (Mulvey and Vrijenhock 1981 and 1984; Red, 1985; Mulvey *et al.,* 1988; Paul and Venkatesan, 1995 and Chen *et al.,* 1999).

RAPD is widely used to study the variation at the DNA level among the variants (Rout *et al.,* 1998; Soniya *et al.,* 2001 and Bennici *et al.,* 2003).

The results of RAPD analysis obtained in the present study showed a considerable amount of genetic variation present in the nine strains investigated, although individual primers differed in the amount of variation they detected. In this study, RAPD analysis was useful since genetic variability was observed among such species using five random primers. The markers used in the present investigation proved to be quite powerful in distinguishing different strains.

The molecular analysis used in the present study provide a simple means for the verification of phylogenic relationships and strain identification without the need for additional sources of information that might be subjected to experimental error over time. More importantly, the level of polymorphism detected by using RAPD'S will provide microbiologists with environment independent DNA marker, which should be regarded as essential tools for selection.

In summary, our characterization of *BCAs* by different molecular methods revealed an elevated level of similarity between isolates, demonstrating a low or high level of genetic polymorphism within this isolates . The pathogenic potential of *BCAs* against pathogens has been known for some time, yet there is a dearth of literature concerning the biological control potential of this isolates.

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