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GUAVA DIE-BACK IN EGYPT: THE CAUSAL AGENT AND PHYLOGENETIC ANALYSIS OF *BOTRYODIPLODIA THEOBROMAE* PAT

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ABSTRACT

Guava die-back disease is a destructive disease. It causes serious yield losses and reduced guava fruits. Inspection of guava plant for die-back revealed that Beheira governorate had the highest frequencies of all isolated fungi from natural infected guava trees, followed by Alexandria, while Kafr El-Sheikh obtained the lowest frequency. *Botryodiplodia theobromae* gave the highest frequency of isolated fungi from natural infected guava trees during seasons 2015 and 2016, followed by *Alternaria alternata*, *Alternaria* sp., whereas *Colletotrichum gloeosperiodes*, *Phomopsis* sp., and *Nigrospora* sp. obtained minor frequency. The highest frequency of *Botryodiplodia theobromae* was observed at Beheira, followed by Alexandria governorate. Meanwhile, the lowest frequency was found at Kafr El-Sheikh. Pathogenicity test indicated that *Botryodiplodia theobromae* was the most pathogenic fungus. RAPD analysis of DNA by using three random primers exhibited five groups of DNA bands with first primer, six groups with second primer and five groups with the third primer and the overall similarities between isolates were arranged from 69 to 100% with P1, from 62 to 100% with P2 and from 33 to 100% with P3. The present study aimed to finding correlation between these isolates groups which resulted of RAPD, its pathogenesis and its geographical isolated locations. Ten isolates of *B. theobromae* represented ten locations of five governorates in Egypt were tested to evaluate the ability of pathogenesis in guava transplants cultivar Banaty at pots under experiments greenhouse conditions for artificial inoculation. The obtained results were exhibited that all tested isolates were clearly pathogenic to guava transplants and produced typical symptoms of guava die-back disease. Such isolates were differences of differed in their pathogenicity. The ten isolates were coded according to their locations by three random primers (B.W.1, A.B.2, D.S.3, Q.K.4, A.A.5, B.N.6, K.Q.7, Q.S.8, D.B.9 and K.D.10). Isolate code No. Q.K.4 was the most virulent, followed by D.S.3 and Q.S.8, respectively which isolated of Qalyoubia and Damietta governorates. Meanwhile isolates K.D.10, A.A.5, K.Q.7 were moderately pathogenic, they were isolated from Kafr El-Sheikh and Alexandria, while A.B.2 and B.N.6 were the lowest pathogenic isolates isolated from Alexandria and Beheira governorates.

Key words: Guava, *Psidium guajava* L., *Botryodiplodia theobromae* Pat. (*Lasiodiplodia theobromae* Pat.), survey, associated fungi, guava die-back, Random Amplified Polymorphic DNA (RAPD), Molecular markers, DNA markers**.**

INTRODUCTION

Guava (*Pisidium guajava* L.,) which belongs to family *Myrtaceae*. Guava plantation is concentrated in Beheira, Alexandria, Qalyoubia, Damietta and Kafr El-Sheikh governorates in Egypt.

Guava trees suffer from several serious and destructive diseases at all stages of its life. Several fungi are attack guava causing root-rot, seed rot, seedling root rots, anthracnose, black spot, stem canker, stem-end rot, die-back and wilt diseases **(Nath, 1976, Ansari et al 2000, Imran-ul-Haq et al 2013, Asma Safdar et al 2015, Dwivedi, Neetu and Dwivedi, 2016).** Guava die-back disease is an important and economic serious problem causing severe guava losses in both nurseries and orchards in Egypt **(Abdel-Gawad, 2000, Baiuomy, et al 2003, Kamhawy, 2011, Rashed et al 2014).**

Guava die-back is a complex disease caused by *Botryodiplodia theobromae* Pat. (*Lasiodiplodia theobromae* Pat.) on the shoot causing fruit rot

(Baiuomy, et al 2003), fruit *Botryodiplodia* rot **(Junqueira, 2001)**, dry rot **(Rana, 1981, Sitansu, Pan and Mishra, 2010)**, wood stain, die back, stem rot **(Cardoso et al 2002)**, stem canker **(Rana, 1981 and Kamhawy, 2011)**, gummosis, wilt, seedling root rot, twig blight, tip die back and brown rot of panicle.

Several investigators have been recorded that *Botryodiplodia theobromae* Pat. (*Lasiodiplodia theobromae* Pat.) is a soil borne phytopathogen fungus and attack guava trees (*Pisidium guajava* L.,) and wide spread in the world **(Rana, 1981, Sitansu, Pan and Mishra, 2010, Kamhawy, 2011, Asma Safdar, et al 2015).**

The strategies of the present study were designed to survey guava die-back disease at ten different locations of five different governorates in Egypt, symptomatology, disease assessment, isolate the associated fungi with guava die-back disease and it's frequency, pathogenicity test of isolated fungi were also investigated. Moreover, DNA phylogenetic analysis in relation to their pathogenicity was taken into consideration**.**

MATERIALS AND METHODS

1. Quantitative Survey studies

During two successive 2015 and 2016 seasons field extensive survey of guava die-back disease was conducted on commercial cultivars of guava trees under natural infection in different orchards of ten locations of five governorates in Egypt i.e., Alexandria (A) (Burg El-Arab "A.B." and El-Ameria "A.A."); Beheira (B) (West El-Noubaria "B.W." and Wady El-Natroun "B.N."); Damietta (D) (Kafr El-Batteikh "D.B." and Kafr Saad "D.S."); Kafr El-Sheikh (K) (Dosook "K.D." and Qelleen "K.Q.") and Qalyoubia (Q) (El-Kanater El-Khayria "Q.K." and Kafr Shokr "Q.S.").

1.1. Sample collection

Samples were collected from infected tissues to isolate the associated fungi. The samples consisting of twig, branches and roots were collected from natural infected guava trees which exhibited typical symptoms of guava die-back disease.

The total number of 160 guava trees randomly chosen was examined in each location for development of guava die-back disease throughout the growing seasons 2015 and 2016. The total number of trees which showed symptoms of die-back disease was counted for each location and the percentage of diseased trees was calculated. Samples of twigs, branches and roots of diseased trees were collected and transferred to laboratory for disease severity (%) assessment. Samples were examined visually and different symptoms of guava die-back were defined. The frequency of isolated fungi was calculated for the different collected samples.

1.2. Disease assessment

A method of visual estimation of the disease was assessed on samples consisted 160 guava trees from each inspected locality where used for assign disease. The disease incidence (D.I.) and the disease severity (D.S.) percentages were calculating for each season through the following formula **(Cooke et al 2006).**

$$\% \text{ Disease incidence (D.I.)} = \frac{\Sigma x}{N} \times 100$$

Where:

Σ x= sum number of diseased trees

N= total number of inspected trees

$$\% \text{ Disease severity (D.S.)} = \frac{\Sigma (nxv)}{Nx} \times 100$$

Where:

n= number of examined trees; v= numerical rating of the scale (0-4); N= total number of trees; x= maximum value (5) of evaluation scale.

Disease reading was determined for each tree according to the disease severity rating. The following numerical rates were suggested for disease severity:

0= healthy trees, no symptoms; 1= 1-25% infected part of the tree; 2= 26-50% infected part of the tree; 3= 51-75% infected part of the tree; 4= 76-100% infected part of the tree.

2. Isolation, purification and identification of associated fungi with guava die-back disease

Isolation trials were made during the survey carried out in 2015 and 2016 seasons. Samples were washed carefully and cut into small pieces then surface disinfected with 2% sodium hypochlorite solution for two minutes and pieces rinsed several times in sterilized distillated water (SDW) and then dried between two folds of sterilized filter papers, pieces were placed on ready acidified potato dextrose agar (PDA) on Petri plates 9cm. in diameter under aseptic conditions and incubated at room temperature at 25±2°C for 7-10 days in the dark with daily observation. All the isolated fungi were purified using either single spore and/or hyphal tip techniques as mentioned by **(Dhingra and**

Sinclair, 1985) then sub-cultured on PDA medium. The isolated fungi were identified based on their morphological and cultural characters by using temporary slides were prepared from pure culture and examined under light microscope according to description of **(Sutton, 1980; Barnett and Hunter, 1987).** The isolated fungi were kindly identified in the Survey and Taxonomy of Fungi Department, Plant Pathology Research Institute, Agriculture Research Center, Giza, Egypt. Pure cultures stocks of isolated fungi were kept for further studies under 5°C.

$$Frequency\% = \frac{\text{Number of fungal colonies for each fungus}}{\text{Total number of fungal colonies for all isolated fungi}} \times 100$$

3. Pathogenicity test

All fungal isolates associated with guava die-back symptoms were used for testing pathogenicity on guava transplants cultivar Banaty one year old were kindly obtained from Production Unit of Fruit Section, Horticulture Research Institute, Agriculture Research Center (ARC) for pathogenicity screening against the isolated fungi under greenhouse conditions (10-14h. light-and- dark cycles) at 25°C±2 and 70-80% relative humidity (RH). Five replicates for each treatment, each treatment contained six homogeneous guava transplants and the same number of guava transplants was used as control treatment and pots were arranged in a randomized design.

3.1. Inocula preparation

Discs 5mm. of the mycelial growth of different isolated fungi were taken from 7 days old cultures on PDA medium. Each disc was set in the surface of sterilized corn meal medium in each 500ml. glass bottle. Corn meal medium was prepared by adding 200g. of corn meal to 100g. sand and 200ml. water in each glasses bottles (500ml.). All bottles were autoclaved at 120°C for one hour at 1.5 IB square inches. A set of 5 bottles were used as replicates of each fungus. A set of 5 bottles of sterilized corn meal sand medium were used without fungal inoculation as control treatment. All inoculated as well as uninoculated bottles were incubated at 25±2°C for two weeks to obtain sufficient mycelial growth of different fungi.

3.2. Soil infestation

Soil infestation was carried out using corn meal inoculated with a 5mm. disc from each of isolated fungi. Plastic pots 25cm. in diameter were sterilized by immersing in 5% formalin solution for 15 minutes and left to dry for two weeks for complete evaporation of formalin, and then were filled with autoclaved at 120°C for one hour at 1.5 IB square inches; sand and clay soil mixture (1: 1 w/w) was prepared for soil infestation. Both sterilized pots and soil mixture were left for few days under greenhouse before infestation. The prepared inocula of each isolated fungus on corn meal medium were mixed individually with the autoclaved soil sand mixture at the rate 5% (w/w). The inoculum was thoroughly mixed with the upper surface of soil and irrigated regularly 7 days before planting homogenous guava transplants to ensure the establishment of tested fungi. Thirty infested plastic pots (each pot contained 5kg. of soil sand mixture) were used for each isolated fungus. The soil was infested with each fungus alone at the rate of 5% of soil weight. Control treatment was applied using fungus free corn meal medium. One guava transplant cultivar Banaty (one year old) was cultivated in each pot. Five replicates were used for each treatment; each treatment contained six guava transplants. Plants were examined for guava die-back disease 15, 30, 45 and 60 days after inoculation. Pots were arranged in complete randomized design. Re-isolation from inoculated guava transplants was made as mentioned before.

4. Random Amplified Polymorphic DNA (RAPD) of *Botryodiplodia theobromae* Pat. (*Lasiodiplodia theobromae* Pat.) isolates

Molecular diversity of *Botryodiplodia* genus

Molecular diversity of *Botryodiplodia* genus was done at Biotechnology Central Laboratory, Finger Printing Lab. Plant Pathology Research Institute. Genetic markers after their DNA PCR Amplification with DNA (RAPD), three random primers i.e., P1, P2 and P3 to detect the genetic variations among different ten isolates of fungus *Botryodiplodia theobromae* Pat., isolated from natural infected guava trees which exhibited symptoms of guava die-back disease in ten locations of five governorates in Egypt during two successive 2015 and 2016 seasons.

4.1. Fungal growth on duplex media

Disposable polystyrene Petri dishes (4 cm.) were filled with 1800 µL solid medium (potato dextrose agar), on which a layer of liquid medium

(1400 µL peptone yeast glucose) was added. The fungal species isolates were cultured by inoculating a small loop from stock onto the prepared Petri dishes that were subsequently incubated for 2-3 days at 28°C. Mycelium was lifted from the medium using sterilized inoculating loops and transferred into sterile 1.5-mL microfuge tubes. For some fungal isolates, the cells were pelleted by centrifugation for 15 min at 4000 rpm a deepwell swing-bucket rotor (microcenrifuge 5804 R; Eppendorf). The mycelium pellet was washed with 600 mL TE buffer and centrifuged again for 5 min at 4000 rpm. Finally, the TE buffer was decanted.

4.2. DNA extraction

A modification of the traditional sodium dodecyl sulfate (SDS) extraction procedure was adopted. Fresh fungal pelt were homogenized in 400 µL sterile salt homogenizing buffer (200 mM Tris-HCl, pH 8.5, 250 mM NaCl, 25 mM EDTA, 0.5% SDS). Next, 6 µL 20 mg/mL RNase A was added and mixed well. The samples were incubated at 65°C for 10 min, after which 130 µL 3 M sodium acetate, pH 5.2, was added to each sample. Samples were vortexed for 30 s at maximum speed, and incubated at -20°C for 10 min. The lysate was centrifuged at 13,000 rpm at 4°C for 15 min, and the supernatant was transferred to fresh tubes. An equal volume of isopropanol was added to each sample, and after mixing well, samples were incubated at -20°C for 10 min. Samples were then centrifuged for 20 min at 4°C, at 6000 rpm. The DNA pellets were washed twice using 700 µL washing solution (100 and 70% ethanol, respectively). The DNA pellets were subsequently air dried in an oven at 40°C for at least 10 min. The resultant DNA pellet was then re-suspended in 100 µL 1X TE (10 mM Tris-HCl, 1 mM EDTA) buffer, pH 8.0 **(Dellaporta et al 1983, Guo et al 2005 and Abd-Elsalam et al 2007).**

4.3. DNA quantification and gel documentation

Seven microliters of the isolated DNA and 3 µL of 10X loading dye were loaded in a lane of 1.5% (w/v) agarose gel containing 0.05 µg/mL ethidium bromide, to check the quality of the DNA. For quantitative measurements, a charge-coupled device camera imaging system and UVI soft analysis (Gel Documentation and Analysis Systems, Uvitec, Cambridge, UK) were used to capture the image and to calculate the band intensities**.** The data for all three random primers were used to estimate the similarity based on the number of shared amplification products **(Nei and Li, 1979).** A dendrogram based on similarity coefficients was generated by using the un-weighted pair group method of arithmetic means (UPGMA) according to **Sneath and Sokai, 1993.**

4.4. RAPD-PCR analysis

RAPD-PCR analysis was undertaken using 10-mer primers (MWG, Germany; **Table 1**). RAPD analysis was performed in 25-µl reaction volumes containing PCR buffer (Promega, Mannheim, Germany), 0.2 mmol/l dNTPs, 0.5 mmol/l primer, 4.0 mmol/l MgCl2 , 1.25 units of Taq Polymerase (Promega, Mannheim, Germany) and 10–20 ng genomic DNA. PCR reactions were carried out in a T-Gradient thermal cycler (Biometra, Germany) using the following profile: 94 °C for 1 min, 36 °C for 1 min and 72 °C for 1 min for 30 cycles, and a final extension at 72 °C for 5 min. Following amplification, the samples were separated by electrophoresis in 1.4 % agarose gel, stained with 0.5 µg/ml of ethidium bromide and viewed under ultraviolet light. A 300- to 1500-bp ladder (Promega, Mannheim, Germany) was used as a molecular mass marker.

4.5. RAPD data collection, scoring and analysis

Image data were automatically collected and simultaneously recorded during electrophoresis. RAPD fragments were scored, and converted into numerical data using Gel documentation system, the fingerprint patterns were analyzed by using the software **AAB Program (Advanced American Biotechnology** and **Imaging, Fullerton CA 92831, USA)**. The fragments were scored for presence or absence of bands showing the same mobility in the gel, regardless of their optical density. RAPD fragments were treated as biallelic marker loci with two alleles encoding presence or absence of a band. The fragment data were coded as a binary matrix where "**1**" designated presence, and "**0**" absence of a particular band. Genetic similarities (GS) among all the isolates were calculated according to the definition of **Nel** and **Li (1979)**. Mathematically, Sij = 2a l (2a + b + c), where S*ij* is the similarity between the two individuals ***i*** and ***j*****;** ***b*** is the number present in ***i*** and absent in ***j*****;** and ***c*** is the number of bands present in ***j*** and absent in ***i*****.** The matrix of similarity was analyzed by the unweight pair group method using the arithmetic av-

erage **(UPGMA)**, as suggested by **Sneath** and **Sokal (1973)**.

Table 1. RAPD polymorphic decamer primers.

Primer	5'-Sequence-3'	G + C (%)
1	GGTCCCTGAC	70
2	TGCCGAGCTG	70
3	GGGTAACGCC	70

5. Statistical Analysis

All experiments were laid out in a completely randomized design (C.R.D.) there were five replicates for each treatment; each treatment contained six homogenous guava transplants, one transplant planted per/plastic pot (25cm. in diameter) experiment under greenhouse conditions.

Statistical analysis were carried out in Agricultural Informatics and Arithmetic Unit, Faculty of Agriculture, Ain Shams Univ. Data were subjected to ANOVA by using SAS statistical software **(SAS Institute, 2009)** and significant difference among the treatments was portioned by least significant difference test (LSD) at probability levels of P= 0.05 **(Steel et al 1997)**.

RESULTS

1. Quantitative survey

During two successive seasons 2015 and 2016, ten locations at five governorates in Egypt i.e., Alexandria (Burg El-Arab and El-Ameria); Beheira (West El-Noubaria and Wady El-Natroun); Damietta (Kafr El-Batteikh and Kafr Saad); Kafr El-Sheikh (Dosook and Qelleen) and Qalyoubia (El-Kanater El-Khayria and Kafr Shokr) were inspected for survey of guava die-back disease. Data presented in **Table (2)** indicate that Beheira governorate has the highest mean values of percentage of disease incidence (D.I.) and disease severity (D.S.) in both 2015 and 2016 seasons (59.53 and 40.16%), followed by Alexandria (43.74 and 30.00%), Qalyoubia (28.28 and 20.33%), Damietta (18.59 and 13.33%), Kafr El-Sheikh (12.65 and 9.50%). Meanwhile, West El-Noubaria location recorded the highest mean values of percentage of disease incidence and disease severity in both 2015 and 2016 seasons (65.31 and 42.32%), followed by, Wady El-Natroun (53.75 and 38.00%), Burg El-Arab (49.37 and 35.00%), El-Ameria (38.12 and 25.00%), El-Kanater El-Khayria (32.81 and 24.33%), Kafr Shokr (23.75 and 16.33%), Kafr El-Batteikh (20.00 and 14.33%), Kafr Saad (17.18 and 12.33%), Dosook (13.75 and 10.00%) and Qelleen (11.56 and 9.00%), respectively.

Table 2. Percentage of disease incidence (%) and disease severity (%) of natural infection of guava (*Psidium guajava* L.,) cultivar Banaty which exhibited typical symptoms of die-back disease tested at different ten locations of five governorates in Egypt during quantitative survey of two successive 2015 and 2016 year on number 160 trees per/Feddan of each location was surveyed.

Governorates	Locations	Disease Incidence (%)		Mean	Disease Severity (%)		Mean
		2015	2016		2015	2016	
Alexandria	Burg El-Arab	44.37	54.37	49.37	33.33	36.67	35.00
	El-Ameria	33.12	43.12	38.12	21.33	28.67	25.00
	Mean	38.74	48.74	43.74	27.33	32.67	30.00
Beheira	West El-Noubaria	52.50	78.12	65.31	31.33	53.33	42.33
	Wady El-Natroun	49.37	58.12	53.75	34.67	41.33	38.00
	Mean	50.93	68.12	59.53	33.00	47.33	40.16
Damietta	Kafr El-Batteikh	16.25	23.75	20.00	11.33	17.33	14.33
	Kafr Saad	13.75	20.62	17.18	9.33	15.33	12.33
	Mean	15.00	22.18	18.59	10.33	16.33	13.33
Kafr El-Sheikh	Dosook	10.00	17.50	13.75	7.33	12.67	10.00
	Qelleen	8.12	15.00	11.56	6.67	11.33	9.00
	Mean	9.06	16.25	12.65	7.00	12.00	9.50
Qalyoubia	El-Kanater El-Khayria	30.00	35.62	32.81	21.33	27.33	24.33
	Kafr Shokr	20.62	26.87	23.75	13.33	19.33	16.33
	Mean	25.31	31.25	28.28	17.33	23.33	20.33
	Mean Governorates	55.62	74.62	65.12	18.99	26.33	22.66
	Mean Locations	27.81	37.31	32.56	9.49	13.16	11.33

2. Isolation, purification and identification of fngi associated with guava die-back disease

Isolation trials from diseased guava exhibit typical guava die-back symptoms were done. Data revealed the occurrence of several fungi i.e., *Alternaria alternata, Alternaria* sp., *Aspergillus niger*, *Aspergillus flavus*, *Botryodiplodia theobromae*, *Fusarium semeitectum*, *Fusarium solani*, *Fusarium oxysporum*, *Pestalotia psidii*, *Colletotrichum gloeosperiodes, Phomopsis* sp. and *Nigrospora* sp. as shown in **Tables (3 and 4).**

Data presented **in Table (5)** clarified that total yield number of *Botryodiplodia theobromae* Pat. (*Lasiodiplodia theobromae* Pat.) isolates during 2015 season were 241 isolates, during 2016 season were 299 isolates.

These isolates were obtained from ten locations belong to five governorates in Egypt in both nurseries and orchards of guava die-back trees, coded numbers B.W.1, A.B.2, D.S.3, Q.K. 4, A.A.5, B.N.6, K.Q.7, Q.S.8, D.B.9 and K.D.10. These isolates were classified according to their molecular diversity to ten different isolates**.** Data also, cleared that Beheira governorate recorded the highest number and frequency of *Botryodiplodia theobromae* Pat. isolates during 2015 season reached 79 isolates and 2016 were 77 isolates. Meanwhile, values of frequency (%) during 2015 season were 32.78% and in 2016 season were 25.75%, followed by, Alexandria during 2015 season were 49 isolates and 2016 100 isolates. Meanwhile, values of frequency (%) during 2015 season were 20.33% and in 2016 season were 33.45%, Qalyoubia during 2015 season gave 55 isolates and 2016 gave 57 isolates. Meanwhile, values frequency (%) during 2015 season were 22.82% and in 2016 season were 19.06%, Damietta during 2015 season were 32 isolates and 2016 were 37 isolates. Meanwhile, values frequency (%) during 2015 season were 13.28% and in 2016 season were 12.37% and Kafr El-Sheikh during 2015 season were 26 isolates and 2016 were 28 isolates. Meanwhile, values frequency (%) during 2015 season were 10.78% and in 2016 season were 9.36%.

On the other hand, Burg El-Arab location recorded the highest total number and frequency (%) of *Botryodiplodia theobromae* Pat. isolate code No.A.B.2 during 2015 season were 27 isolates and 2016 were 56 isolates. Meanwhile, values frequency (%) during 2015 season were 11.20% and in 2016 season were 18.73%, followed by West El-Noubaria location (B.W.1 isolate) during 2015 season were 40 isolates and 2016 were 42 isolates.

Table 3. Number and frequency (%) of associated fungi isolated from natural infected guava trees which showing typical symptoms of die-back disease during two successive years 2015 and 2016.

Associated Fungi	Number of Isolated Fungi		Frequency (%) of Isolated Fungi	
	2015	2016	2015	2016
Alternaria alternata	9.00	14.00	3.082	3.753
Alternaria sp.	9.00	11.00	3.082	2.949
Aspergillus niger	4.00	7.00	1.369	1.876
Aspergillus flavus	3.00	4.00	1.027	1.072
Botryodiplodia theobromae	241.00	299.00	82.534	80.160
Colletotrichum gloeosperiodes	2.00	4.00	0.684	1.072
Fusarium oxysporum	7.00	11.00	2.397	2.949
Fusarium semeitectum	6.00	5.00	2.054	1.340
Fusarium solani	5.00	10.00	1.712	2.680
Nigrospora sp.	1.00	1.00	0.342	0.268
Pestalotia psidii	3.00	4.00	1.027	1.072
Phomopsis sp.	2.00	3.00	0.684	0.804
Total	292.00	373.00	----------	----------
Mean	24.33	31.08	----------	----------

Statistical analysis system (SAS), Duncan's studentized range (HSD) test at alpha= 0.05
Least Significant Difference (LSD) for associated fungi as frequency percentages = 1.2803

Table 4. Frequency (%) of isolated fungi caused guava die-back disease isolated from different ten locations of five governorates in Egypt during two successive years 2015 and 2016.

Associated Fungi	Frequency (%) of associated fungi																											
	Examined Governorates																											
	Alexandria					Beheira					Damietta					Kafr El-Sheikh					Qalyoubia							
	A.A.		A.B.		2015 and 2016	B.W.		B.N.		2015 and 2016	D.B.		D.S.		2015 and 2016	K.D.		K.Q.		2015 and 2016	Q.K.		Q.S.		2015 and 2016	2015	2016	2015 and 2016
	2015	2016	2015	2016		2015	2016	2015	2016		2015	2016	2015	2016		2015	2016	2015	2016		2015	2016	2015	2016				
Alternaria alternata	0.41	0.67	0.41	1.00	1.05	1.24	1.34	1.24	1.34	2.10	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.41	0.00	0.00	0.33	0.30	3.082	3.753	3.458
Alternaria sp.	0.41	0.67	0.83	1.00	1.20	1.66	1.34	0.83	0.67	1.80	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	3.082	2.949	3.007
Aspergillus niger	0.41	0.33	0.41	0.33	0.60	0.41	0.67	0.00	0.67	0.72	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.33	0.00	0.33	0.30	1.369	1.876	1.654
Aspergillus flavus	0.00	0.33	0.41	0.33	0.45	0.41	0.33	0.41	0.33	0.60	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	1.027	1.072	1.052
Botryodiplodia theobromae	9.13	14.71	11.20	18.73	22.40	16.60	14.05	16.18	11.70	23.46	7.47	6.69	5.81	5.68	10.37	6.22	5.35	4.56	4.01	8.12	12.45	8.69	10.37	10.37	16.84	82.534	80.160	81.203
Colletotrichum gloeosperiodes	0.00	0.33	0.00	0.33	0.30	0.41	0.33	0.41	0.33	0.60	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.684	1.072	0.902
Fusarium oxysporum	0.41	0.33	0.41	0.67	0.72	0.83	1.00	0.83	1.00	1.50	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.41	0.33	0.00	0.33	0.45	2.397	2.949	2.706
Fusarium semeitectum	0.41	0.00	0.41	0.33	0.45	0.41	0.67	0.41	0.33	0.72	0.41	0.00	0.00	0.00	0.15	0.00	0.00	0.00	0.00	0.00	0.41	0.00	0.00	0.33	0.30	2.054	1.340	1.654
Fusarium solani	0.41	0.33	0.00	0.67	0.60	0.83	1.00	0.41	0.67	1.20	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.41	0.33	0.00	0.33	0.45	1.712	2.680	2.255
Nigrospora sp.	0.00	0.00	0.00	0.00	0.00	0.41	0.33	0.00	0.00	0.30	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.342	0.268	0.300
Pestalotia psidii	0.00	0.33	0.41	0.33	0.45	0.41	0.33	0.41	0.33	0.60	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	1.027	1.072	1.052
Phomopsis sp.	0.00	0.00	0.00	0.33	0.15	0.41	0.33	0.41	0.33	0.60	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.684	0.804	0.751
All Fungi	11.62	18.06	14.52	24.08	28.42	24.07	21.74	21.58	17.72	34.28	7.88	6.69	5.81	5.68	10.53	6.22	5.35	4.56	4.01	8.12	14.11	9.69	10.37	12.04	18.65			
Mean	0.97	1.50	1.21	2.00	2.36	2.00	1.81	1.80	1.48	2.85	0.66	0.56	0.48	0.47	0.88	0.52	0.45	0.38	0.33	0.68	1.17	0.81	0.86	1.00	1.55			

Whereas, A.A.= Alexandria, Ameria; A.B.= Alexandria, Burg El-Arab; B.W.= Beheira, West El-Noubaria; B.N.= Beheira, Wady El- Natroun; D.B.= Damietta, Kafr El-Batteikh; D.S.=Damietta, Kafr Saad; K.D.= Kafr El-Sheikh, Dosook; K.Q.= Kafr El-Sheikh, Qelleen; Q.K.= Qalyoubia, El-Kanater El-Khayria; Q.S.= Qalyoubia, Kafr Shokr.

Statistical analysis system (SAS), Duncan's studentized range (HSD) test at alpha= 0.05

Least Significant Difference (LSD) for frequency percentages during seasons 2015 and 2016= 0.1119

Least Significant Difference (LSD) for associated fungi as frequency percentages = 0.2741

Least Significant Difference (LSD) for governorates as frequency percentages = 0.177

Least Significant Difference (LSD) for interaction between seasons, associated fungi and governorates= 0.8669

Table 5. Number and frequency (%) of *Botryodiplodia theobromae* Patouilard isolates isolated from natural infected guava trees which exhibited typical symptoms of guava die-back disease of different ten locations of five governorates in Egypt during two successive years 2015 and 2016.

Governorates	Locations	Code No. of *B. theo-bromae* isolates	Number of Natural Infected Trees (160 trees/Feddan) for each location		Frequency (%) of Natural Infected Trees (160 trees/Feddan) for each location	
			2015	2016	2015	2016
Alexandria	Burg El-Arab	A.B.2	27.00	56.00	11.20	18.73
	El-Ameria	A.A.5	22.00	44.00	9.13	14.71
	Total		49.00	100.00	20.33	33.45
	Mean		24.50	50.00	10.16	16.72
Beheira	West El-Noubaria	B.W.1	40.00	42.00	16.59	14.05
	Wady El-Natroun	B.N.6	39.00	35.00	16.18	11.70
	Total		79.00	77.00	32.78	25.75
	Mean		39.50	38.50	16.39	12.87
Damietta	Kafr El-Batteikh	D.B.9	18.00	20.00	7.47	6.69
	Kafr Saad	D.S.3	14.00	17.00	5.81	5.68
	Total		32.00	37.00	13.28	12.37
	Mean		16.00	18.50	6.64	6.19
Kafr El-Sheikh	Dosook	K.D.10	15.00	16.00	6.22	5.35
	Qelleen	K.Q.7	11.00	12.00	4.56	4.01
	Total		26.00	28.00	10.79	9.36
	Mean		13.00	14.00	5.39	4.68
Qalyoubia	El-Kanater El-Khayria	Q.K.4	30.00	26.00	12.45	8.69
	Kafr Shokr	Q.S.8	25.00	31.00	10.37	10.37
	Total		55.00	57.00	22.82	19.06
	Mean		27.50	28.50	11.41	9.53
	Total		241.00	299.00	----------	----------
	Mean Governorates		48.20	59.80	----------	----------
	Mean Locations		24.10	29.90	----------	----------

Statistical analysis system (SAS), Duncan's studentized range (HSD) test at alpha= 0.05

Least Significant Difference (LSD) for isolates of *Botryodiplodia theobromae* as frequency percentages = 5.6846

Meanwhile, values frequency (%) during 2015 season were 16.59% and in 2016 season were 14.05%, Wady El-Natroun (B.N.6 isolate) during 2015 season were 39 isolates and 2016 were 35 isolates. Meanwhile, values frequency (%) during 2015 season were 16.18% and in 2016 season were 11.70%, El-Ameria (A.A.5 isolate) during 2015 season were 22 isolates and 2016 were 44 isolates. Meanwhile, values frequency (%) during 2015 season were 9.13% and in 2016 season were 14.71%, El-Kanater El-Khayria (Q.K.4 isolate) and Kafr Shokr (Q.S.8 isolate) were occupied the same order during the two seasons 2015 and 2016, El-Kanater El-Khayria (Q.K.4 isolate) during 2015 was 30 isolates and 2016 were 26 isolates. Meanwhile, values frequency (%) during 2015 season were 12.45% and 2016 was 8.69%, Kafr Shokr (Q.S.8 isolate) during 2015 was 25 isolates and 2016 were 31 isolates. Meanwhile, values frequency (%) during 2015 season were 10.37% and 2016 was 10.37%, Kafr El-Batteikh (D.B.9 isolate) during 2015 season were 18 isolates and 2016 were 20 isolates. Meanwhile, values frequency (%) during 2015 season were 7.47% and in 2016 season were 6.69%, wherever, Kafr Saad (D.S.3 isolate) and Dosook (K.D.10 isolate) were occupied the same order during the two seasons 2015 and 2016, Kafr Saad (D.S.3 isolate) during 2015 was 14 isolates and 2016 were 17 isolates. Meanwhile, values frequency (%) during 2015 season were 5.81% and 2016 was 5.68%, Dosook (K.D.10 isolate) during 2015 was 15 isolates and 2016 were 16 isolates, Meanwhile, values frequency (%) during 2015 season were 6.22% and 2016 was 5.35% and Qelleen (K.Q.7 isolate) during 2015 was 11 isolates and 2016 were 12 isolates. Meanwhile, values frequency (%) during 2015 season were 4.56% and 2016 was 4.01%, respectively**.**

3. Pathogenicity test of isolated fungi

Pathogenicity test of twelve isolated fungi associate with guava die-back trees from ten different geographic locations of five governorates in Egypt were tested for their pathogenic capabilities on

guava transplants cultivar Banaty one year old under greenhouse conditions. **Data in Table (6)** revealed that the pathogenesis capabilities of tested fungi on guava transplants were differed from fungus to another. *Botryodiplodia theobromae* Pat. gave the higher mean values percentage of disease incidence (78.33%) and disease severity (74.79%). On the other hand, *Alternaria alternata, Alternaria* sp., *Fusarium solani, Fusarium oxysporum* and *Fusarium semeitectum* recorded very low percentage of disease incidence and disease severity in comparison to *B. theobromae*. Meanwhile, the fungi *Aspergillus niger*, *Aspergillus flavus*, *Colletotrichum gloeosperiodes*, *Nigrospora* sp., *Pestalotia psidii* and *Phomopsis* sp. were recorded 0.00% in disease incidence and disease severity (didn't have any pathogenic capability) and they were non-pathogenic fungi. Also, the disease incidence and disease severity of the tested fungi were increased by increasing periods after inoculation (15, 30, 45 and 60 days)**.**

Table 6. Pathogenicity test of different twelve fungi isolated from guava die-back disease on guava transplants, cultivar Banaty, one year old experiments were estimated 15, 30, 45 and 60 days after inoculation under greenhouse conditions during 2015 year.

Tested Isolated Fungi	No. of Tested Guava Transplants for Each Treatment	Days after Inoculation								Mean	
		15		30		45		60			
		*D.I.	**D.S.	*D.I.	**D.S.	*D.I.	**D.S.	*D.I.	**D.S.	*D.I.	**D.S.
Alternaria alternata	30.00	3.33	1.33	6.67	2.67	10.00	4.67	13.33	5.33	8.33	3.49
Alternaria sp.	30.00	0.00	0.00	3.33	1.33	6.67	2.67	10.00	4.67	5.00	2.16
Aspergillus niger	30.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Aspergillus flavus	30.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Botryodiplodia theobromae	30.00	56.67	49.33	76.67	72.67	86.67	85.33	93.33	90.00	78.33	74.33
Colletotrichum gloeosperiodes	30.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Fusarium semeitectum	30.00	0.00	0.00	0.00	0.00	0.00	0.00	6.67	2.67	1.66	0.66
Fusarium solani	30.00	0.00	0.00	0.00	0.00	6.67	2.67	10.00	4.67	2.33	1.83
Fusarium oxysporum	30.00	0.00	0.00	0.00	0.00	0.00	0.00	3.33	1.33	0.83	0.33
Nigrospora sp.	30.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Pestalotia psidii	30.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Phomopsis sp.	30.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Control (un-inoculated)	30.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Total	390.00	----------	----------	----------	----------	----------	----------	----------	----------	----------	----------
Mean	30.00	4.61	3.89	6.66	5.89	8.46	7.33	10.51	8.36	7.42	6.37

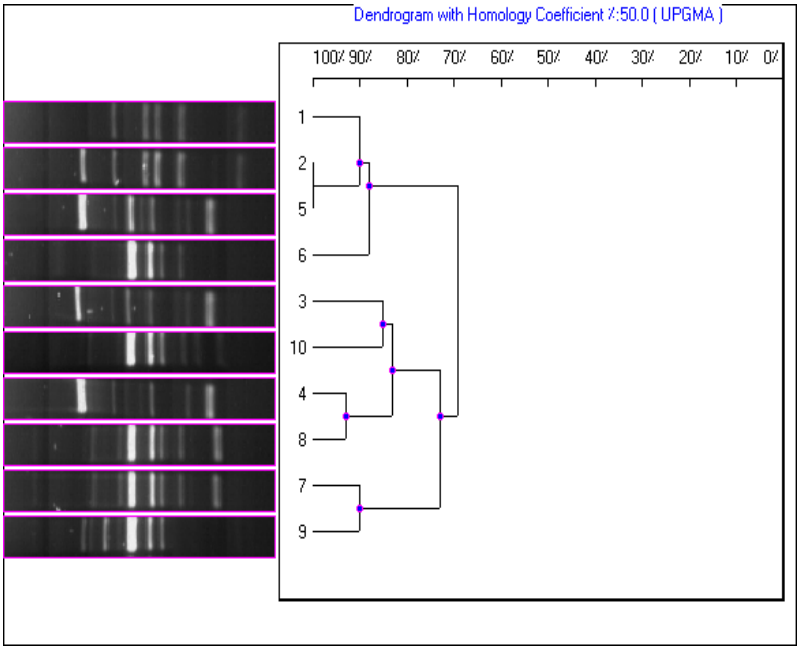
Whereas, *D.I.= Disease Incidence; **D.S.= Disease Severity
Statistical analysis system (SAS), Duncan's studentized range (HSD) test at alpha= 0.05
Least Significant Difference (LSD) for tested isolated fungi as percentages for disease incidence = 0.7502
Least Significant Difference (LSD) for days after inoculation (15, 30, 45 and 60) as percentages for disease incidence= 0.2393
Least Significant Difference (LSD) for days after inoculation as percentages for disease incidence after 15 days of inoculation= 0.6148
Least Significant Difference (LSD) for days after inoculation as percentages for disease incidence after 30 days of inoculation= 0.8249
Least Significant Difference (LSD) for days after inoculation as percentages for disease incidence after 45 days of inoculation= 1.3053
Least Significant Difference (LSD) for days after inoculation as percentages for disease incidence after 60 days of inoculation= 1.3336
Least Significant Difference (LSD) for tested isolated fungi as percentages for disease severity= 0.2181
Least Significant Difference (LSD) for days after inoculation (15, 30, 45 and 60) as percentages for disease severity= 0.0494
Least Significant Difference (LSD) for days after inoculation as percentages for disease severity after 15 days of inoculation= 0.1856
Least Significant Difference (LSD) for days after inoculation as percentages for disease severity after 30 days of inoculation= 0.1971
Least Significant Difference (LSD) for days after inoculation as percentages for disease severity after 45 days of inoculation= 0.2894
Least Significant Difference (LSD) for days after inoculation as percentages for disease severity after 60 days of inoculation= 0.3616

4. Differentiation of different obtained *Botryodiplodia theobromae* Pat. (*Lasiodiplodia theobromae* Pat.) isolates using Random Amplified Polymorphic DNA (RAPD technique)

Molecular finger printing of 10 isolates of *Botryodiplodia theobromae* Pat. (*Lasiodiplodia theobromae* Pat.), by using Random Amplified Polymorphic DNA (RAPD) analysis technique was used to characterize the different 10 coded isolates which tested in electrophoresis study. Such study was carried out to mark the genetic diversity in these isolates. **In Table (1)**, three random primers (1, 2 and 3) were used for screen for DNA clusters in the same sequence of *Botryodiplodia theobromae* Pat.

Data in **Fig. (1, 2 and 3)** indicate that each primer have different dendrogram figure of DNA clusters. As **primer No. 1 (Fig. 1)**, show a dendrogram derived by UVI software analysis of 10 isolates tested, also dendrogram showed clear distinction in two major clusters, 3 minor cluster and 5 groups at the genetic similarity of 69.00% of all isolates. All similarity levels of all 10 isolates were arranged from 69.00% to 100.00%. The first minor cluster contained two groups, the first group contained isolates code No. (B.W.1, A.B.2 and A.A.5 at similarity level 90.00%) of the *Botryodiplodia theobromae* Pat. isolates. Meanwhile, the two isolates code No. A.B.2 and A.A.5 exhibited very high genetic similarity level 100.00% which were isolated from Alexandria governorate, while the second group contained isolate code No. B.N.6 at 88.00% similarity level. The second minor cluster contained three groups, the first group contained isolates code No. (D.S.3 and K.D.10 at similarity level 85.00%), the second group contained isolates code No. (Q.K.4 and Q.S.8 at similarity level 94.00%). Isolates isolated from Qalyoubia governorate, the third group contained isolates code No. (K.Q.7 and D.B.9 at similarity level 90.00%). Where, isolates code No. D.S.3, K.D.10, Q.K.4 and Q.S.8 at similarity level 84.00%. Meanwhile, isolates code No. Q.K.4, Q.S.8, K.Q.7 and D.B.9 at similarity level 72.00%. Finally, the first minor cluster contained isolates code No. B.W.1, A.B.2, A.A.5 and B.N.6 at similarity level 90.00%. The second minor cluster contained isolates code No. D.S.3, K.D.10, Q.K.4, Q.S.8, K.Q.7 and D.B.9 were at least similarity level 69.00% compared with all 10 isolates.

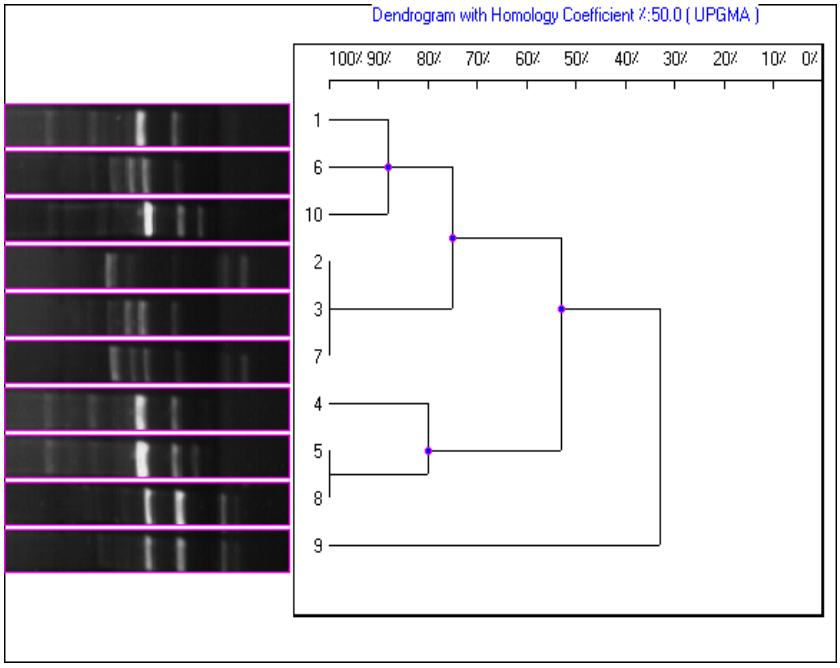
RAPD Results for Botryodiplodia isolates



Botryodiplodia-P1

Fig. 1. Banding and digitized patterns of random amplified polymorphic DNA (RAPD) profiles and similarity dendrogram of 10 *Botryodiplodia theobromae* Pat. isolates of die-back guava trees, obtained by using primer 1.

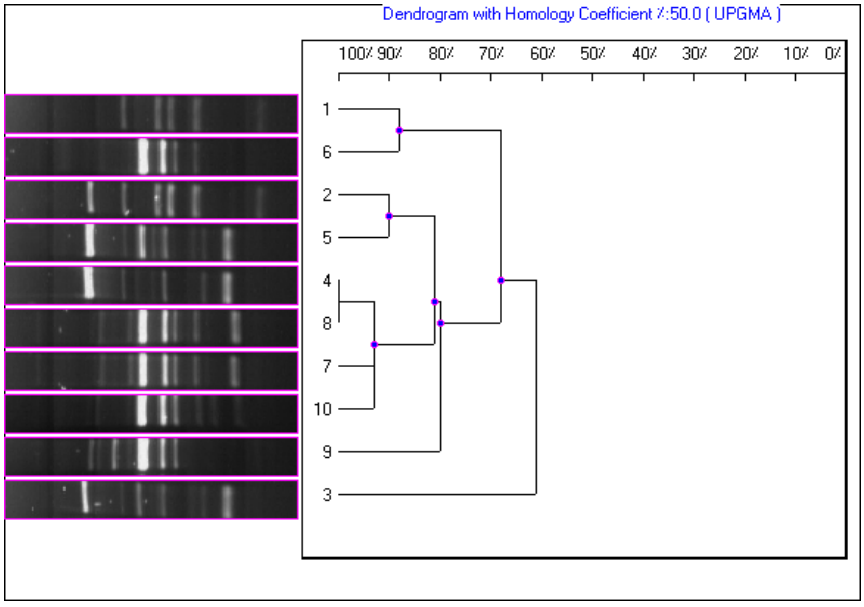
RAPD Results for Botryodiplodia isolates



Botryodiplodia-P2

Fig. 2. Banding and digitized patterns of random amplified polymorphic DNA (RAPD) profiles and similarity dendrogram of 10 *Botryodiplodia theobromae* Pat. isolates of die-back guava trees, obtained by using primer 2.

RAPD Results for Botryodiplodia isolates



Botryodiplodia-P3

Fig. 3. Banding and digitized patterns of random amplified polymorphic DNA (RAPD) profiles and similarity dendrogram of 10 *Botryodiplodia theobromae* Pat. isolates of die-back guava trees, obtained by using primer 3.

Primer No. 2. The UPGMA cluster analysis provided a better resolution of the relationships among the *Botryodiplodia theobromae* Pat. isolates, which were broadly clustered into two major clusters, 4 minor-clusters and 6 groups at the genetic similarity of 62.00% of all isolates. Similarity levels of all 10 isolates were arranged from 62.00% to 100.00%. The first major cluster contained one group consisted of isolates code No. B.W.1 and B.N.6 at similarity level 88.00%. The second major cluster contained 4 minor-clusters and 5 groups. The first group contained isolates code No. (A.B.2 and A.A.5 at similarity level 90.00%), the second group consisted of isolates code No.Q.K.4 and Q.S.8 showed very high genetic similarity level 100.00% were isolated from Qalyoubia governorate. The third group contained isolates code No. K.Q.7 and K.D.10 at similarity level 94.00%. Meanwhile, isolates code No. Q.K.4, Q.S.8, K.Q.7 and K.D.10 at similarity level 94.00%. The fourth group contained isolates code No. D.B.9 at similarity level 80.00% compared with the previous isolates. The fifth group contained isolate code No. D.S.3 at least level similarity 62.00% compared with all 10 isolates.

Primer No. 3. The UPGMA cluster analysis provided a better resolution of the relationships among the *Botryodiplodia theobromae* Pat. isolates, which were broadly clustered into two main groups at the genetic similarity of 33.00% of all isolates. **Primer No. 3** divided all *Botryodiplodia theobromae* Pat. isolates to 2 major -clusters and 3 minor -clusters and 5 groups. All similarity levels of all 10 isolates were arranged from 33.00% to 100.00%. The first minor -cluster consisted of two groups. The first group contained isolates code No. B.W.1, B.N.6 and K.D.10 at similarity level 88.00%. The second group contained isolates code No. A.B.2, D.S.3 and K.Q.7 cleared very high genetic similarity level 100.00% isolated from Alexandria, Damietta and Kafr El-Shiekh. Meanwhile, isolates code No. B.W.1, B.N.6 and K.D.10 (first group) and isolates code No. A.B.2, D.S.3 and K.Q.7 (second group) at similarity level 75.00%. The third group contained isolates code No. Q.K.4 at similarity level 80.00% compared with previously isolates. The fourth group contained isolates code No. A.A.5 and Q.S.8 exhibited very high genetic similarity level 100.00% isolated from Alexandria and Qalyoubia governorates. The finally fifth group contained isolates code No. D.B.9 at least level similarity 33.00% compared with all 10 isolates**.**

5. Pathogenicity test of *Botryodiplodia theobromae* Pat. isolates

The most frequent isolated fungus was *Botryodiplodia theobromae* and the highest pathogenic fungi, causing guava die-back disease. Ten different isolates of *Botryodiplodia theobromae* coded No. **B.W.1, A.B.2, D.S.3, Q.K. 4, A.A.5, B.N.6, K.Q.7, Q.S.8, D.B.9 and K.D.10.**, obtained from ten different geographic locations of five governorates in Egypt were tested for their pathogenic capabilities on guava homogenous transplants cultivar Banaty one year old under greenhouse conditions.

Data in **Table (7)** revealed that the pathogenesis capabilities of tested isolates on guava transplants were differed from isolate to another. Isolate code No. Q.K.4 was the most pathogenic isolate and recorded the highest mean values percentage of disease incidence (21.67%) and disease severity (22.83%), followed by D.S.3 isolate (20.83 and 22.16%), Q.S.8 isolate (19.17 and 20.83%), K.D.10 isolate (17.50 and 19.66%), A.A.5 isolate (16.67 and 18.66%), K.Q.7 isolate (15.83 and 17.33%), B.W.1 isolate (15.00 and 16.16%), D.B.9 isolate (14.17 and 14.83%) and A.B.2 isolate (9.17 and 11.83%), while B.N.6 isolate (5.83 and 9.16%) was the lowest pathogenic isolate. Data also, recorded that all the tested *Botryodiplodia theobromae* Pat., isolates were pathogenic and differed from isolate to another. The previously data also showed significant differences between all the tested isolates and control treatment. Also, the disease incidence and disease severity of the tested isolates were increased by increasing periods after inoculation (15, 30, 45 and 60 days).

Data in Table (8) indicate that the correlation between molecular diversity of isolates and their capabilities to infect guava transplants (pathogenicity groups of isolates) gave three groups, the first group (I) of *Botryodiplodia theobromae* Pat. isolates Q.K.4, D.S.3 and Q.S.8 were recorded the highest percentage of disease incidence (from 19.17 to 21.67%) and disease severity (from 20.83 to 22.83%), respectively. While, the second group (II) K.D.10, A.A.5, K.Q.7 and B.W.1 isolates were recorded moderate of percentage disease incidence (from 15.00 to 17.50%) and disease severity (from 16.16 to 19.66%), respectively. Meanwhile, the third group (III) D.B.9, A.B.2 and B.N.6 isolates were recorded the lowest group of percentage disease incidence (from 5.83 to 14.17%) and disease severity (from 9.16 to 14.83%), respectively**.**

Table 7. Pathogenicity test of ten isolates of *Botryodiplodia theobromae* Pat. *(Lasiodiplodia theobromae,* Pat.*)* obtained from guava die-back disease on guava transplants, cultivar Banaty, one year old experiments were estimated 15, 30, 45 and 60 days after inoculation under greenhouse conditions during 2015 year.

Tested *B. theobromae* Isolates	No. of Tesed Guava Transplants for Each Treatment	Days after Inoculation							
		15		30		45		60	
		*D.I.	**D.S.	*D.I.	**D.S.	*D.I.	**D.S.	*D.I.	**D.S.
B.W.1	30.00	0.00	0.00	16.67	18.67	20.00	22.67	23.33	23.33
A.B.2	30.00	0.00	0.00	0.00	0.00	16.67	16.67	20.00	30.67
D.S.3	30.00	13.33	14.00	20.00	17.33	23.33	26.67	26.67	30.67
Q.K.4	30.00	13.33	16.00	16.67	18.67	26.67	23.33	30.00	33.33
A.A.5	30.00	6.67	8.67	16.67	11.33	16.67	17.33	26.67	37.33
B.N.6	30.00	0.00	0.00	0.00	0.00	10.00	10.00	13.33	26.67
K.Q.7	30.00	3.33	4.67	6.67	9.33	23.33	25.33	30.00	30.00
Q.S.8	30.00	16.67	10.67	16.67	18.00	20.00	22.67	23.33	32.00
D.B.9	30.00	0.00	0.00	0.00	0.00	26.67	18.67	30.00	40.67
K.D.10	30.00	10.00	5.33	13.33	15.33	20.00	27.33	26.67	30.67
Control (un-inoculated)	30.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Total	330.00	----------	----------	----------	----------	----------	----------	----------	--------

Whereas, *D.I.= Disease Incidence; **D.S.= Disease Severity

Statistical analysis system (SAS), Duncan's studentized range (HSD) test at alpha= 0.05

Least Significant Difference (LSD) for tested *B. theobromae* isolates as percentages for disease incidence= 1.6511

Least Significant Difference (LSD) for days after inoculation (15, 30, 45 and 60) as percentages for disease incidence= 0.436

Least Significant Difference (LSD) for days after inoculation as percentages for disease incidence after 15 days of inoculation= 1.5878

Least Significant Difference (LSD) for days after inoculation as percentages for disease incidence after 30 days of inoculation= 1.9802

Least Significant Difference (LSD) for days after inoculation as percentages for disease incidence after 45 days of inoculation= 2.4497

Least Significant Difference (LSD) for days after inoculation as percentages for disease incidence after 60 days of inoculation= 2.2187

Least Significant Difference (LSD) for tested *B. theobromae* isolates as percentages for disease severity= 0.3457

Least Significant Difference (LSD) for days after inoculation (15, 30, 45 and 60) as percentages for disease severity= 0.1673

Least Significant Difference (LSD) for days after inoculation as percentages for disease severity after 15 days of inoculation= 0.1619

Least Significant Difference (LSD) for days after inoculation as percentages for disease severity after 30 days of inoculation= 0.1501

Least Significant Difference (LSD) for days after inoculation as percentages for disease severity after 45 days of inoculation= 0.2899

Least Significant Difference (LSD) for days after inoculation as percentages for disease severity after 60 days of inoculation= 1.1388

Table 8. Infection rates of pathogenicity test of ten *Botryodiplodia theobromae* Pat. isolates obtained from guava die-back trees from ten locations of five governorates on guava cultivar Banaty one year old as means of percentage of disease incidence and disease severity 15, 30, 45 and 60 days after inoculation in pot experiments under greenhouse conditions.

Tested *B. theobromae* Isolates	Governorates	Locations	Means after 15, 30, 45 and 60 days after Inoculation		Groups*
			Banaty Cultivar		
			% Disease Incidence	% Disease Severity	
Q.K.4	Qalyoubia	El-Kanater El-Khayria	21.67	22.83	I
D.S.3	Damietta	Kafr Saad	20.83	22.16	
Q.S.8	Qalyoubia	Kafr Shokr	19.17	20.83	
K.D.10	Kafr El-Sheikh	Dosook	17.50	19.66	II
A.A.5	Alexandria	El-Ameria	16.67	18.66	
K.Q.7	Kafr El-Sheikh	Qelleen	15.83	17.33	
B.W.1	Beheira	West El-Noubaria	15.00	16.16	
D.B.9	Damietta	Kafr El-Batteikh	14.17	14.83	III
A.B.2	Alexandria	Borg El-Arab	9.17	11.83	
B.N.6	Beheira	Wady El-Natroun	5.83	9.16	

Groups*: Infection rates of means percentage of disease incidence (%) and disease severity (%).
Highest group (I) of disease incidence (from 19.17 to 21.67%) and disease severity (from 20.83 to 22.83%).
Moderate group (II) of disease incidence (from 15.00 to 17.50%) and disease severity (from 16.16 to 19.66%).
Lowest group (II) of disease incidence (from 5.83 to 14.17%) and disease severity (from 9.16 to 14.83%)

DISCUSSION

Guava (*Pisidium guajava* L.,) is considered one of the most important popular fruit crops to the Egyptian people. Guava die-back disease is an important and economic serious problem causing severe guava losses in both nurseries and orchards in Egypt **(Baiuomy et al 2003 and Kamhawy, 2011)** caused by *Botryodiplodia theobromae* Pat. (*Lasiodiplodia theobromae* Pat.). Several investigators have been reported that *Botryodiplodia theobromae* Pat. is a soil-borne phytopathogen fungus and attack guava trees and wide spread in the world **(Rana, 1981, Adisa, 1985, Junqueira et al 2001, Cardoso et al 2002, Pandit and Samajpati, 2002, Baiuomy et al 2003, Pandit and Samajpati, 2005, Mishra & Sitansu, Pan, 2007, Nunes et al 2008, Mishra et al 2009, Sitansu, Pan & Mishra, 2010, Kamhawy, 2011 and Asma Safdar et al 2015).**

Disease survey revealed that disease is widespread and causes serious losses in surveyed fields. Confirming with these statements, the survey study of the disease in the present work cleared that guava die-back disease has expanded to cover ten locations in five governorates of Egypt i.e., Alexandria (Burg El-Arab and El-Ameria); Beheira (West El-Noubaria and Wady El-Natroun); Damietta (Kafr El-Batteikh and Kafr Saad); Kafr El-Sheikh (Dosook and Qelleen) and Qalyoubia (El-Kanater El-Khayria and Kafr Shokr), this survey was carried out during the two successive seasons 2015 and 2016. Data obtained during the survey showed that guava die-back disease was presented in all examined locations and its occurrence varied from location to another. This may be attributed to the prevailing meteorological conditions, soil conditions i.e., temperature, relative humidity, mechanical and chemical components and behaviour of pathogens. The highest total number of natural infected trees, percentage of disease incidence and percentage of disease severity were obtained at Beheira governorate while the lowest was found at Kafr El-Sheikh governorate. The former finding was confirmed by **Kamhawy, 2011** in Egypt, such results are also in harmony with those obtained by several foreign authors **(Gomes et al 2012, Imran-ul-Haq et al 2013, Safdar, Asma et al 2015).**

Isolation and identification of the associated fungi showed that obtained fungi were *Alternaria alternata, Alternaria* sp., *Aspergillus niger*, *Aspergillus flavus*, *Botryodiplodia theobromae*, *Fusarium semeitectum*, *Fusarium solani*, *Fusarium oxysporum*, *Pestalotia psidii*, *Colletotrichum gloeosperiodes, Phomopsis* sp. and *Nigrospora* sp. These results are in agreement with several authors in Egypt **(Abdel-Gawad, 2000, Baiuomy et al 2003, Kamhawy, 2011, Rashed et al 2014)** and in different countries in the world **(Rao et al 1976, Acosta et al 2002, Asma Safdar, et al 2015 and Mishra, Maneesh, 2016).** They were differed in their frequencies. The highest frequency was belonged to *Botryodiplodia theobromae* during seasons 2015 and 2016 (81.203%). Also, frequencies of obtained fungi varied from location to another. Beheira governorate (34.28%) revealed the highest frequencies of all isolated fungi, followed by Alexandria (28.42%). The lowest frequency was obtained at Kafr El-Sheikh (8.12%). While, Qalyoubia (18.65%) and Damietta (10.53%) were occupied moderate position of their frequencies. The differences between fungal frequencies through the various governorates may be attributed to soil variation, cultivar susceptibility (root exudate) and the prevailing environmental their circumstances. These factors affected on fungal survival and distribution. Although even the presence of each fungus was differed from governorate to another i.e., the highest frequency of *Botryodiplodia theobromae* was observed at Beheira (23.46%), followed by Alexandria governorate (22.40%). The lowest frequency of *Botryodiplodia theobromae* was found at Kafr El-Sheikh (8.12%) **(Baiuomy, *et al.,* 2003; Tohamy *et al.,* 2005 and Kamhawy, 2011).** These results are in agreement with those obtained by several foreign investigators **(Adisa, 1985, Misra, 2007, Gupta & Misra, 2012 and Asma Safdar et al 2015).**

Testing the pathogenesis capability of each isolated fungus associated with guava die-back disease showed significant differences between all the tested fungi and control treatment. Although, they were varied in their capabilities to cause guava die-back disease. *Botryodiplodia theobromae* was the most pathogenic fungus (mean values percentage of disease incidence was 78.33% and disease severity was 74.79%), followed by *Alternaria alternata* (8.33 and 3.49%), *Alternaria* sp. (5.00 and 2.16%), *Fusarium solani* (2.33 and 1.83%), *Fusarium semeitectum* (1.66 and 0.66%), while *Fusarium oxysporum* (0.83 and 0.33%) was the lowest pathogenic fungus. Meanwhile, the fungi *Aspergillus niger*, *Aspergillus flavus*, *Colletotrichum gloeosperiodes*, *Nigrospora* sp., *Pestalotia psidii* and *Phomopsis* sp. were recorded 0.00% in disease incidence and severity (didn't have any pathogenic capability) and they were non-pathogenic fungi. The former data was relatively in harmony with **Baiuomy et al 2003, Tohamy et al 2005 and Srivastava et al 2008**.

The results of the present study revealed an influence of geographic origin on genetic variability among the population of *Botryodiplodia theobromae* isolates of guava die-back disease in Egypt. Simultaneously, the dendrogram figures of RAPD reactions using three different primers have arranged the 10 isolates of *Botryodiplodia theobromae* Pat. according to DNA clusters analysis. Obtained data cleared that similarities and differences in banding patterns obtained by RAPD, could be useful molecular tool for identification and phylogenetic studies of the pathogenic groups. As **primer No. 1** showed a dendrogram derived by UVI software analysis of 10 tested isolates of *Botryodiplodia theobromae* Pat., the UPGMA cluster analysis cleared distinction in two major clusters, 3 minor cluster and 5 groups at genetic similarity level arranged from 69.00% to 100.00% of all isolates. First minor cluster contained two groups, first group contained isolates code No. (B.W.1, A.B.2 and A.A.5 at similarity level 90.00%). Meanwhile, the two isolates code No. A.B.2 and A.A.5 exhibited very high genetic similarity level 100.00% were isolated from Alexandria governorate, while the second group contained isolate code No. B.N.6 at 88.00% similarity level compared with previous isolates. Second minor cluster contained three groups, first group contained isolates code No. (D.S.3 and K.D.10 at similarity level 85.00%), second group contained isolates code No. (Q.K.4 and Q.S.8 at similarity level 94.00%) isolated from Qalyoubia governorate, third group contained isolates code No. (K.Q.7 and D.B.9 at similarity level 90.00%). Where, isolates code No. D.S.3, K.D.10, Q.K.4 and Q.S.8 at similarity level 84.00%. Meanwhile, isolates code No. Q.K.4, Q.S.8, K.Q.7 and D.B.9 at similarity level 72.00%. Finally, first minor cluster contained isolates code No. B.W.1, A.B.2, A.A.5 and B.N.6 at similarity level 90.00%. Second minor cluster contained isolates code No. D.S.3, K.D.10, Q.K.4, Q.S.8, K.Q.7 and D.B.9 were at least similarity level 69.00% compared with all 10 isolates.

Primer No. 2 showed that UPGMA cluster analysis provided a better resolution of the relationships among the isolates, which were broadly

clustered into two major clusters, 4 minor-clusters and 6 groups at genetic similarity level were arranged from 62.00% to 100.00% of all 10 isolates. First major cluster contained one group included isolates code No. B.W.1 and B.N.6 at similarity level 88.00%. Second major cluster contained 4 minor -clusters and 5 groups. First group contained isolates code No. (A.B.2 and A.A.5 at similarity level 90.00%), second group included isolates code No.Q.K.4 and Q.S.8 showed very high genetic similarity level 100.00% were isolated from Qalyoubia governorate. Third group contained isolates code No. K.Q.7 and K.D.10 at similarity level 94.00%. Meanwhile, isolates code No. Q.K.4, Q.S.8, K.Q.7 and K.D.10 at similarity level 94.00%. Fourth group contained isolates code No. D.B.9 at similarity level 80.00% compared with the previous isolates. Fifth group contained isolate code No. D.S.3 at least level similarity 62.00% compared with all 10 isolates.

Primer No. 3 showed that UPGMA cluster analysis provided a better resolution of the relationships among the isolates, which were broadly clustered into two major clusters at the genetic similarity level were arranged from 33.00% to 100.00% of all ten isolates. **Primer No. 3** divided all isolates to 2 major-clusters and 3 minor-clusters and 5 groups. First minor-cluster contained two groups, first group contained isolates code No. B.W.1, B.N.6 and K.D.10 at similarity level 88.00%. Second group contained isolates code No. A.B.2, D.S.3 and K.Q.7 cleared very high genetic similarity level 100.00% isolated from Alexandria, Damietta and Kafr El-Shiekh. Meanwhile, isolates code No. B.W.1, B.N.6 and K.D.10 (first group) and isolates code No. A.B.2, D.S.3 and K.Q.7 (second group) at similarity level 75.00%. Third group contained isolates code No. Q.K.4 at similarity level 80.00% compared with previously isolates. Fourth group contained isolates code No. A.A.5 and Q.S.8 exhibited very high genetic similarity level 100.00% isolated from Alexandria and Qalyoubia governorates. The finally fifth group contained isolates code No. D.B.9 at least level similarity 33.00% compared with all 10 isolates.

Generally, analysis of Egyptian *Botryodiplodia theobromae* Pat. isolates by RAPD technique has provided evidence for the conclusion that *B. theobromae* isolates, a causal agent of the guava dieback disease, present a high genetic variability. This variability should be taken into consideration in clonal selection programs against the disease. This variability is depended, generally, on geographical locations. Isolate code No.D.S.3 and D.B.9 were particularly different from other *B. theobromae* isolates. The former data was relatively in harmony with **Tohamy et al 2005 and Shah et al 2010.**

Data obtained revealed that the pathogenesis capabilities of tested isolates on guava transplants were differed from isolate to another. *Botryodiplodia theobromae* Pat. isolate code No. Q.K.4 was the highest of mean values percentage of disease incidence (21.67%) and disease severity (22.83%), followed by D.S.3 isolate (20.83 and 22.16%), Q.S.8 isolate (19.17 and 20.83%), K.D.10 isolate (17.50 and 19.66%), A.A.5 isolate (16.67 and 18.66%), K.Q.7 isolate (15.83 and 17.33%), B.W.1 isolate (15.00 and 16.16%), D.B.9 isolate (14.17 and 14.83%) and A.B.2 isolate (9.17 and 11.83%), while B.N.6 isolate (5.83 and 9.16%) was the lowest pathogenic isolate. Data also, recorded that all the tested *Botryodiplodia theobromae* Pat., isolates were pathogenic and differed from isolate to another. The previously data also showed significant differences between all the tested isolates and control treatment. Also, the disease incidence and disease severity of the tested isolates were increased by increasing periods after inoculation (15, 30, 45 and 60 days)**.**

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الموت الرجعى للجوافه في مصر: العامل المسبب والتحليل الفيلوجيني للفطر *Botryodiplodia theobromae* Pat.

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الموجـــــز

يعتبر مرض الموت الرجعي على الجوافة واحداً من الأمراض الهامة والإقتصادية على الجوافة. يتسبب عنه خسائر كبيرة فى المحصول كما ونوعا. إزداد إنتشار المرض فى السنين القليلة الماضية على محصول الجوافه في مصر نظرا للتوسع في زراعة أشجار الجوافه فى أراضى الإستصلاح الجديدة. تم عزل الفطريات المصاحبة لمرض الموت الرجعى على الجوافه حيث وجد أن فطر *Botryodiplodia theobromae* هو أعلى الفطريات المعزولة تكرارا من أشجار الجوافه المصابة طبيعيا أثناء موسم 2015 و2016 ، يتبعه الفطر *Alternaria alternata*، *Alternaria* sp.، بينما كانت الفطريات *Colletotrichum gloeosperiodes*، *Phomopsis* sp. ، *Nigrospora* sp. أقل الفطريات تكرارا. وجد أن أعلى نسبة تكرارية للفطر *Botryodiplodia theobromae* كانت فى محافظة البحيرة ، تليها الإسكندرية ، بينما كانت أقل نسبة تكرارية في محافظة كفر الشيخ. فى المقابل حققت الفطريات *Colletotrichum gloeosperiodes* ، *Phomopsis* sp. ، *Nigrospora* sp. أقل نسبة تكرارية في جميع المحافظات. وجد أن الفطر *Botryodiplodia theobromae* هو أعلى الفطريات قدرة مرضية على إحداث المرض ، يليه الفطر *Alternaria alternata* ، *Alternaria* sp. ، بينما كان الفطر *Fusarium oxysporum* هو أقل الفطريات قدرة مرضية على إحداث المرض. فى حين ، كانت الفطريات *Aspergillus niger* ، *Aspergillus flavus* ، *Colletotrichum gloeosperiodes* ، *Nigrospora* sp. ، *Pestalotia psidii* ، *Phomopsis* sp. غير ممرضة تماما. تم إجراء تقييم للإختلافات الوراثية المتواجدة فى عزلات فطر *Botryodiplodia theobromae* بإستخدام تقنية الإكثار العشوائى للحمض النووي (RAPD) من خلال عدد 3 بادئات عشوائية بغرض عمل تصنيف لهذه العزلات إلى مجموعات متشابهة أو مختلفة من حيث عدد الحزم من الحمض النووي DNA الناتجة مع كل بادىء على حده. وأثبت تحليل الإكثار العشوائى للحمض النووي (RAPD) بإستخدام ثلاثة بادئات عشوائية ظهور 5 مجموعات من حزم الحمض النووي DNA مع البادىء الأول (رقم1) و6 مجموعات مع البادىء الثاني (رقم2) و5 مجموعات مع البادىء الثالث (رقم3). وأظهر تصنيف هذه المجاميع درجات تقارب مختلفة بين العزلات تراوحت من 69 إلى 100% للبادىء الأول، ومن 62 إلى 100% للبادىء الثانى، ومن 33 إلى 100% للبادىء الثالث حسب مجاميع العزلات والبادىء العشوائى المستخدم. ولقد إستهدفت الدراسة محاولة إيجاد إرتباط بين هذه المجاميع التصنيفية من نتائج RAPD وقدرتها المرضية ومناطق عزلها

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الجغرافية. تم إختبار عدد 10 عزلات من فطر *B. theobromae* تمثل 10 مراكز في خمس محافظات في مصر لتقييم قدرتها على إحداث المرض على شتلات الجوافه فى تجربة الأصص على صنف البناتى تحت ظروف الصوبة نتيجة العدوى الصناعية. وقد أظهرت نتائج العدوى الصناعية أن كل العزلات المختبرة لها قدرة مرضية (ممرضة) وإعادة إنتاج الأعراض النموذجية لمرض الموت الرجعى على أشجار الجوافه وأن هناك تباين واضح فى القدرة المرضية لهذه العزلات. وتم تصنيف العزلات إلى عدد 10 عزلات أكواد B.W.1, A.B.2, D.S.3, Q.K.4, A.A.5, B.N.6, K.Q.7, Q.S.8, D.B.9, K.D.10 والتي تم تسميتها طبقاً للمراكز المعزولة منها بواسطة الثلاثة بادئات العشوائية. كانت العزلة Q.K.4 هي الأكثر قدرة على العدوى وإحداث المرض، تليها العزلة D.S.3 ثم العزلة Q.S.8 على التوالي وهذه العزلات تم عزلها من محافظتى القليوبية ودمياط ، بينما أظهرت العزلات K.D.10، A.A.5 ، K.Q.7 قدرة مرضية متوسطة والتى تم عزلها من محافظتى كفر الشيخ والإسكندرية على التوالى، فى حين كانت العزلات A.B.2 و B.N.6 أقل العزلات قدرة مرضية على إحداث المرض.

الكلمات الدالة: الجوافة *Psidium guajava* L.، *Botryodiplodia theobromae* Pat.، المسح المرضي، الفطريات المصاحبة، مرض الموت الرجعي في الجوافة، الإكثار العشوائي للحمض النووي DNA (RAPD)، المعلمات الجزيئية، معلمات الـ DNA