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DIVERSITY AMONG *Botryodiplodia theobromae* PAT., THE CAUSAL OF MANGO DIE- BACK DISEASE, IN EGYPT

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ABSTRACT: Mango branches and twigs of seedlings and/ or trees showing typical die-back symptoms were collected from mango orchards in three Egyptian governorates. *Botryodiplodia theobromae* Pat., Was the most frequently isolated fungus *B. theobromae*, caused typical die-back symptoms on mango seedlings, branches and twigs.

The growth characteristics and the pigment production *in vitro* tested at 36°C did not distinguish the differences between the three *B. theobromae* isolates.

While, there were obvious differences between the three isolates of *B. theobromae*, revealed by both SEM (scanning electron microscope) and RAPD-PCR investigations. The dendogram of RAPD-PCR classified two of the obtained isolates (№ 1&2) in one group, and the isolate (№ 3) in another group.

The present study suggest that, *B. theobromae* classification still need more advanced studies on the molecular levels to elucidate the diversity of this specie.

Key words: *Botryodiplodia theobromae*, pigment production, growing on 36°C, RAPD-PCR, SEM, mango trees and seedlings.

INTRODUCTION

Various diseases attacked mango trees (Khurana, 1998). Mango die-back disease caused by *Botryodiplodia theobromae* Pat., one of the serious diseases that significantly reduced the fruit yield (Ahmed *et al.*, 1996 and Al Adawi *et al.*, 2003). The typical symptoms of the disease were described by various workers (Ramos *et al.*, 1997 and Al Adawi *et al.*, 2003).

Several powerful biochemical techniques that has long been used by geneticists to study the

population genetics of plant pathology. These techniques such as Random amplified polymorphic DNA (RAPD-PCR) are now being used to settle analyze genetic variability between isolates of pathogenic fungi (Singh and Singh 1994).

Traditional technique might be not accurate to differentiate between isolates either moleculary or morphologically. It had been reported that RAPD-PCR is accurate method to differentiate genetic structure between the isolates of the pathogenic fungi (Almeida *et al.*, 2003; Jana *et al.*, 2003; Fernandez *et al.*, 2006; Purkayastha *et al.*, 2006 and Aboshosha *et al.*, 2007). Morphologically, it has been stated that spore surface of fungi might be different from one isolate to another (Hassan, 1980).

The present work was aimed to study the following points:

1. Isolate, identify and pathogenic capability of various *Botryodiplodia theobromae* isolated from mango die-back branches and twigs.
2. Evaluate and differentiate between the surface of pycnidium and conidio spores of three *Botryodiplodia theobromae* isolates, using scanning electron microscope (SEM)
3. Find out the degree of genetic homology of *Botryodiplodia theobromae* isolates through RAPD-PCR investigation.

MATERIALS AND METHODS

Isolation, Purification and Identification of *Botryodiplodia theobromae* Isolates from Diseased Mango Die-back Branches and Twigs

Samples of mango branches and twigs of seedling and/or trees exhibited die-back typical symptoms as shown in Fig. 1, were collected from three mango production locations at El-Ismaelia (El-Kantara shark), Cairo (Ahmed Orabby) and Giza (Nekla). Isolation was conducted as described by Khurana (1998) and Al-Adawi *et al.* (2003), purification was done using single spore isolate as mentioned by Kiet (1915). Identification was carried out according to Barnett and Hunter (1998) as well as Khurana (1998).

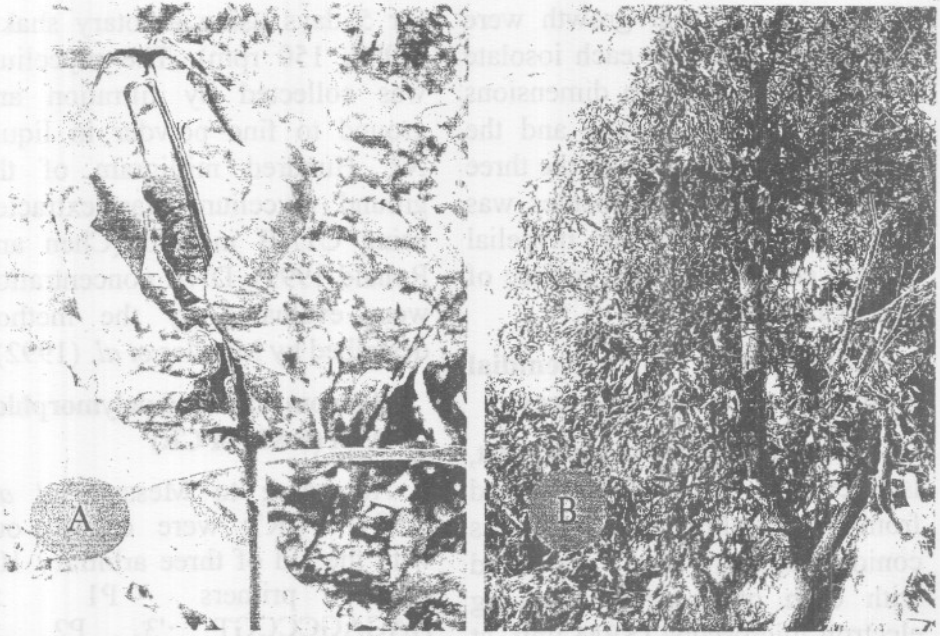

Fig. 1. (A) Typical symptoms of die-back on mango seedlings grown in nurseries: notice, drying of twigs from tip downward (B) symptoms on mango tree in open field: notice, different stages of infection on individual tree

Pathogenicity Tests

The pathogenic capabilities of the *Botryodiplodia theobromae* isolates was carried out according to (Khanzada *et al.* 2004).

Variations between *Botryodiplodia theobromae* Pat. Isolates

To determine the differences between 3 representative isolates of *Botryodiplodia theobromae* Pat. Collected from three locations in Egypt, morphological characteristics, pigment production and the ability to grow on 36ºC tests were conducted according to Krupinsky (1983) and Leavitt and Munnecke (1987).

Pigment production and the ability to growth at 36ºC

The ability of the isolated *B. theobromae* Pat, isolates № 1, 2 and № 3 to produce red pigments as well as growth rate at 36ºC were studied. The inoculated plates with the tested isolates were examined for the presence of visible red

pigment. The linear growth were determined daily for each iosolate by measuring the two dimensions of growth in each plate and the mean was estimated from the three replicates. This experimentes was terminated whenever the mycelial growth coverd the plate surface of any treatment.

Morphology of pycenidial surface and conidio spores

Spores of *B. theobromae* Pat, isolates № 1, 2 and № 3 obtained from individual pycnidia as well as conidio spores were compared with each other using scanning electron microscope (SEM unit, at Agric. Fac., Ain Shams Univ., Egypt). Differences in length and width between spores and pycnidia, also the presence of stromata and ostioles of pycnidia were also recorded.

Molecular characterization of different isolates

The DNA extraction and the PCR reactions were preformed in the central lab., Plant Pathology research institute, ARC, Egypt.

DNA extraction

The selected *Botryodiplodia theobromae* isolates were cultured in 100ml Erlenmeyer-flasks containing 20 ml Czapek's medium for 5 days using a rotary shaker (30°C, 150 rpm). The mycelium was collected by filtration and ground to fine powder in liquid N2. Hundred milligram of the ground mycelium was extracted using CTAB method (Chen and Ronald 1999). DNA concentration were evaluated by the method described by Moeller *et al.* (1992).

Random amplified polymorphic DNA (RAPD-PCR)

According to Messner *et al.* (1994) PCR's were carried out with the aid of three arbitrary 10-base primers P1 5' AAGAGCCCGT '3, P2 5' AACGCGCAAC '3 and P3 5'GTAGACCCGT '3. PCR amplifications were performed in 50 µL volumes containing 1unit Taq DNA polymerase (sigma) dNTP mix (0.2 mM each of RAPD analysis- PCR conditions and separation of RAPD-PCR fragments were done dCTP, dGPT, dATP and dTTP); 20 mM Tris-HCl (pH 8.4); 50 mM MgCl ; 0.5 mM primer and 15-20 ng of genomic DNA. Amplification was performed in a thermalcycler (MJ research PTC200) PCR was programmed with a first cycle at 92°C for 4 min, followed by 35 cycles at 92°C for 2 min, 36°C for 1.5 min and 72°C for 2 min with a final extension at 72°C for 3 min.

The PCR product were resolved by electrophoresis on 1.5% agarose gel in 0.5 X Tris- Borate-EDTA (TBE) buffer, at 100 V for 3 h. Gels were stained with ethidium bromide and photographed under UV light using Herolab gel documentation system model Mididoc (Herolab, Germany).

RESULTS AND DISCUSSION

Isolation, Purification and Identification of *Botryodiplodia theobromae* Isolates from Diseased Mango Die-back Branches and Twigs

Isolation trails from diseased branches and twigs of mango at three growing locations in Egypt yielded *Botryodiplodia theobromae.* isolates Data showed that, *Botryodiplodia theobromae* was the most frequent isolated fungus not only from infected seedling but also from infected trees, (4.63 and 12.36). Data also showed that samples from the trees of El-Ismaelia governorate yielded the highest percentage of *Botryodiplodia theobromae* (15.3%) followed by El-Giza (12.6%) and lastly Cairo (9.2%). Similar observations have been previously reported by (Ahmed *et al.,* 1996; Ploetz *et al.,* 1996; Ramos *et al.,* 1997 and Al Adawi *et al.* 2003).

Pathogenicity Tests

Result of pathogenicity testes of the isolated *Botryodiplodia theobromae* isolates (№ 1, 2 and 3) proved that, *Botryodiplodia theobromae* isolates (№ 1, 2 and 3) were pathogenic to mango seedlings. Data in Table 1 indicate that, *Botryodiplodia theobromae* isolates exhibited typical symptons of die-back disease **(Savant and Raut, 2000).** It is also clear from data in Table 1 that, *Botryodiplodia theobromae* isolate №3 was the most pathogenic fungus followed by № 2 and №1 (73, 70 and 66mm average length of necrotic part, respectively).

Variations between *Botryodiplodia theobromae* Isolates

No differences were obtained between the tested *Botryodiplodia theobromae* isolates neither in its ability to produce red pigments in the agar culture media nor its growth rate at 36°C. On the other hand, data in Table 2 and Fig. 2 reveal that, there are structural differences in the formation of stromata, and the presence of the ostiole. The stromata was observed only in isolate №. 3 while the ostiole, observed only in

Table 1. Pathogenicity of different fungal isolates to mango seedling, 28 days after inoculation

Tested isolates	No. tested plants	No.tested plants with die-back	average length of necrotic part in mm.
Botryodiplodia theobromae **isolate №1**	6	4.0	66.0
Botryodiplodia theobromae **isolate №2**	6	4.0	70.0
Botryodiplodia theobromae **isolate №3**	6	5.0	73.0

Isolate № 1 (Cairo), isolate № 2 (El-Giza), isolate № 3 (El-Ismaelia)

Table 2. Differences in measurements of pycnidial and conidia length as well as width of *B.theobroma* isolates

B. theobromae Isolates	Pycnidia		Conidia	
	Width(µm)	Length(µm)	Width(µm)	Length(µm)
Isolate № 1	103.57- 117.86 (113.96)	159.09-179.54 (168.386)	9.54-10.23 (9.66)	17..05-17..27 (17.18)
Isolate № 2	103.57- 117.86 (113.96)	117.17- 127.85 (123.96)	9.37-10.94 (9.94)	15.62-17.19 (16.10)
Isolate № 3	128- 152 (140)	160-192 (191.72)	7.81-12.50 (10.155)	14.06-18.9 (16.66)
L.S.D mean at 5% level	7.57	6.92	0.533	0.529

isolates №. 2 and 3. Such results was in harmony with those obtained by Hassan (1980) who found that *Botryodiplodia theobromae* isolates obtained from different hosts varied in the formation of pycnidia, pycnidiospores and stroma. Aly *et al.* (2002) obtained similar results with *B. theobromae* on grapevine as well as Tohamy *et al.* (2005) and Alves *et al.* (2008) on mango.

Genetic Variations among *B. theobromae* Isolates

The genetic variations among 3 isolates of *B. theobromae* were analyzed using three different primers (P1 5' AAGAGCCCGT '3, P2 5' AACGCGCAAC '3 and P3 5' GTA GAC CCG T '3). The used RAPD fragment pattern (Fig. 3) revealed high degree of similarity between *B. theobromae* isolates. It is also clear from Fig. 4 which represented phylogenetic tree contracted from data obtained from the three tested primers which divided the three isolates into two groups, the first one contained *B. theobromae* the isolates № 1and 2 with a 88% homology between them, while the second group contained *B. theobromae* isolate № 3 with a 75% homology to the first group. This study also showed that, there is considerable genotypic variability among the Egyptian isolates of *Botryodiplodia theobromae* obtained from different isolation localities. The obtained data is in harmony with those recently found by Alves *et al.* (2008).

Conclusion

It might be concluded that both isolates (№s 1 and 2) are progeny of the same isolate since they had 88% genetic relation. While isolate № 3 differ from the above mentioned isolates but, share them with similarity of 75%.

In brief, although our work indicated that, the three examined *Botryodiplodia theobromae* isolates are similar by morphological examinations and pigment production, however, using more accurate methods detecting that, the three tested isolates were differed using RAPD-PCR and SEM .It was easy to indicate some differences in pycnidial and conidial surface.

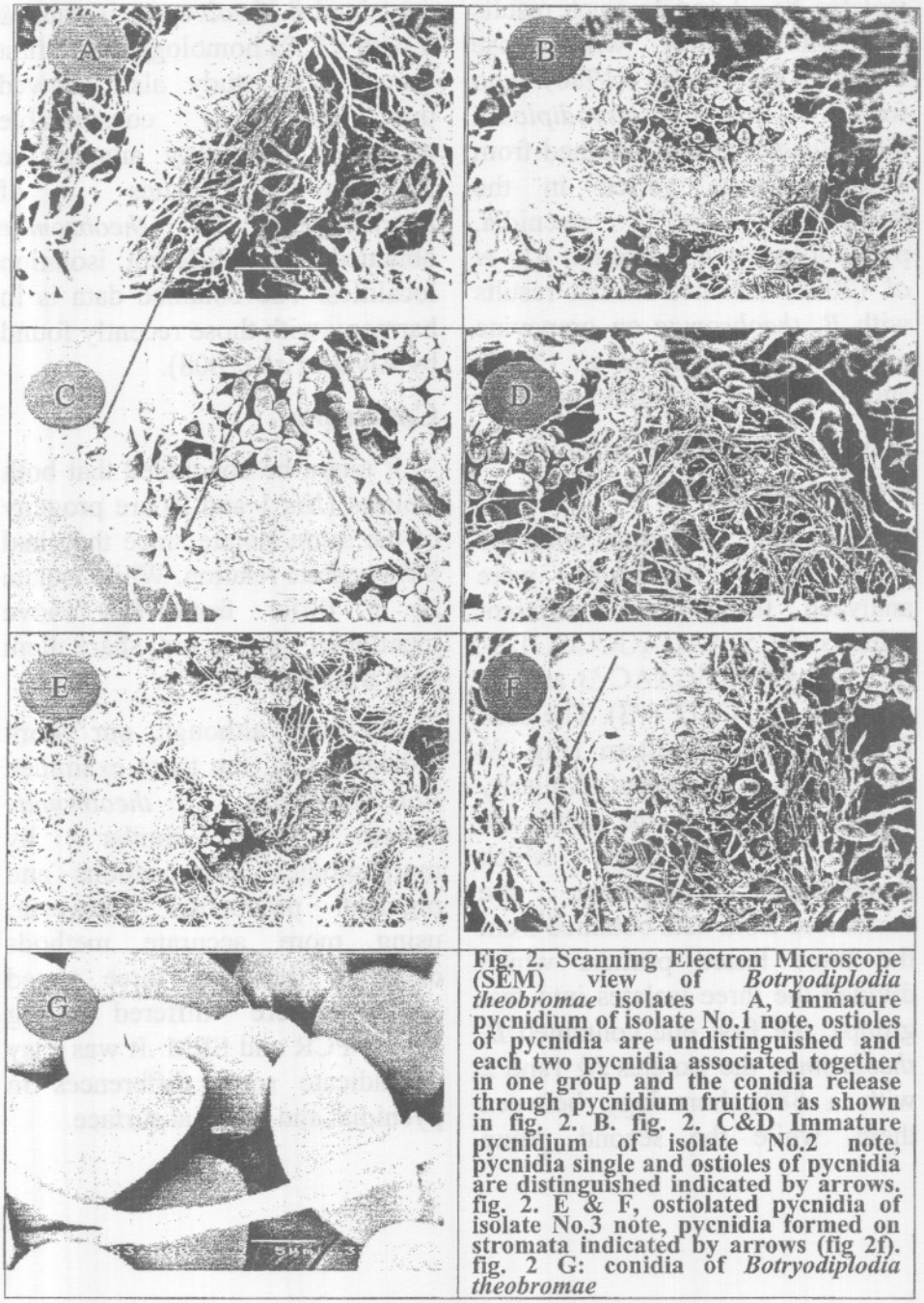

Fig. 2. Scanning Electron Microscope (SEM) view of *Botryodiplodia theobromae* isolates A, Immature pycnidium of isolate No.1 note, ostioles of pycnidia are undistinguished and each two pycnidia associated together in one group and the conidia release through pycnidium fruition as shown in fig. 2. B. fig. 2. C&D Immature pycnidium of isolate No.2 note, pycnidia single and ostioles of pycnidia are distinguished indicated by arrows. fig. 2. E & F, ostiolated pycnidia of isolate No.3 note, pycnidia formed on stromata indicated by arrows (fig 2f). fig. 2 G: conidia of *Botryodiplodia theobromae*

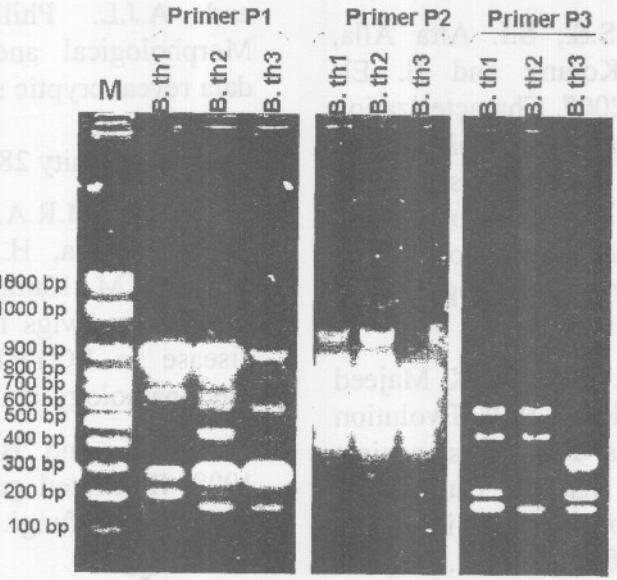

Fig. 3. DNA banding patterns from random amplified polymorphic DNA analysis of *Botryodiplodia theobromae* isolates (*B. th* 1, *B. th* 2 and *B. th* 3) primed by three arbitrary 10-base primers (P1, P2 and P3). Lane M is a 150 Kb DNA

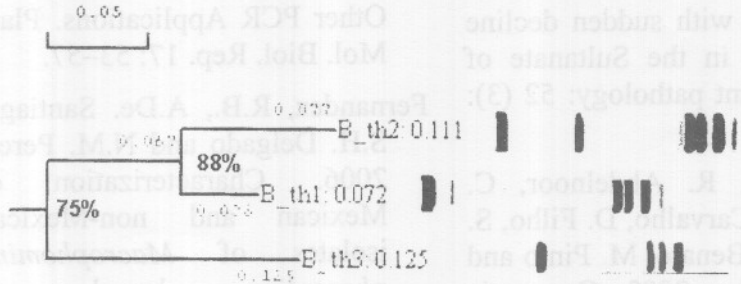

Fig. 4. The genetic variations among 3 isolates of *B. theobromae* isolated from different regions in Egypt. The Phylogenetic tree constructed from the combined RAPD-PCR data generated from the primers P1 5' AAGAGCCCGT '3, P2 5' AACGCGCAAC '3 and P3 5' GTA GAC CCG T '3

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التباين في عزلات فطر *بوتريوديبلوديا ثيوبروم* المسبب لمرض الموت الرجعي للمانجو في مصر

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تهاجم أشجار المانجو بالعديد من الامراض التي تؤدي الي خساره شديده في محصول الثمار، ويعتبر مرض الموت الرجعي واحدا من هذه الامراض. من اجل ذلك تم اجراء هـذا البحث لعزل وتنقية وتعريف العزلات المحتلفه من فطر *بوتريوديبلوديا ثيوبروم* مـن افـرع واغصان المانجو المصابه بهذا المرض حيث تم جمع عينات ممثله للأفرع والأغصان مـن أشجار وشتلات المانجو الظاهر عليها أعراض واضحة لمرض الموت الرجعي من بسـاتين المانجو في ثلاث محافظات مصرية هي القاهره (احمد عرابي)، الجيزة (نكلة)والاسـماعيليه (القنطره شرق).

أثبتت نتائج العزل ان فطر *بوتريوديبلوديا ثيوبروم* كان اكثر الفطريات تكرارا للعزل من المناطق المختلفة.

أظهرت اختبارات العدوى أن العزلات المختلفه لفطر *بوتريوديبلوديا ثيوبروم* كانت لهـا قدره كبيره على إعطاء الأعراض الظاهرية لمرض الموت الرجعي علي نباتـات المـانجو (أفرع وأغصان).

أجريت عدة تجارب للتفرقه فيما بين العزلات المختلفه للفطر *بوتريوديبلوديا ثيـوبروم* سواء عن طريق القدره علي انتاج الصبغه الحمراء علي البيئه او النمو علي درجـه ٣٦°م او الشكل الظاهري للاوعيه البكنيديه او الجـراثيم الكونيديـه باسـتخدام الميكروسـكوب الالكتروني الماسح وايض باستخدام تفاعل البلمره المتسلسل للقطع العشـوائيه المضـاعفه للحمض النووي.

أثبتت النتائج أن اختبار إنتاج الصبغات أو النمو علي درجه ٣٦°م لم يعطي اي نتـائج تمكننا من التفرقه بين العزلات المختله المتحصل عليها للفطر *بوتريوديبلوديا ثيوبروم* .بينما أدى الفحص باستخدام الميكروسكوب الالكتروني الماسح الـي وجـود بعـض الاختلافـات الظاهريه للاوعيه البكنيديه والجراثيم الكونيديه التي تنتجهـا العـزلات الثلاثـه. كمـا ادي استخدام اختبار تفاعل البلمره المتسلسل للقطع العشـوائيه المضـاعفه للحمـض النـووي والتقسيم الشجيري الناتج عنه الي تقسيم العزلات المختلفه المتحصل عليها الي مجموعتين بحيث تقع العزله رقم ١ ،٢ في مجموعه واحده بينما تقع العزلـه الثالثـه فـي مجموعـه اخري.

وباختصار يمكن أن نوضح أن تقسيم الفطر بوتريوديبلوديا ثيوبروم مازال في حاجـه كبيرة إلي اجراء دراسات جاده في مجال البيولوجيا الجزيئية لتوضيح التنوع الوراثي لتحت أنواع هذا الفطر بمصر.