Genetic Characterization of Certain *Ascochyta* species Using Random Amplified Polymorphic DNA (RAPD)

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Abstract: The aim of this study was to use the random amplified polymorphic DNA (RAPD) technique as a tool for setting up a convenient and standard protocol to study the intergenetic variation between the three species of *Ascochyta* (*A.pinodella*, *A.pinodes* and *A.pisi*) and at the same time between the intragenetic variations within each species.

[Hanan A. Nor-El-Din and Ghada A. Kolaly. **Genetic Characterization of Certain *Ascochyta* species Using Random Amplified Polymorphic DNA (RAPD).** *Nat Sci* 2018;16(9):51-61]. ISSN 1545-0740 (print); ISSN 2375-7167 (online). http://www.sciencepub.net/nature. 8. doi:10.7537/marsnsj160918.08.

Keywords: Genetic Characterization; *Ascochyta;* species; Random Amplified Polymorphic DNA (RAPD)

1. Introduction

Pea (*Pisum sativum* L.) is a member of the diverse group of cultivated leguminous crops. The importance of peas comes next of cereals as a source of protein, carbohydrates, riboflavin, thiamine, and some minerals such as calcium, phosphorus, iron and magnesium (Hassan, 1989). The cultivated area of peas in Egypt was estimated by FAO as 2,400 feddans in 2014.

Peas are subjected to different fungal diseases. *Ascochyta* leaf, pod spotting and foot rot are considered the most serious diseases that affect pea's production in Egypt (Metwally, 1992).

Ascochyta spp. are differentiated from each other microscopically and also throughout the morphological symptoms on leaves, stems, roots, and pods. However, such methods have not the ability to differentiate between *Ascochyta* spp. perfectly.

Accordingly, a proper technique will be needed for differentiation between the three species of *Ascochyta* easily and perfectly. Therefore, this work was directed to use RAPD analysis (Random Amplified Polymorphic DNA) (Welsh and McClelland, 1990 and Williams *et al.*, 1990) which is considered a sensitive and quick technique to clear up the differences between such *Ascochyta* spp.

2. Material and Methods

2.1- Isolation from Infected Host:

Isolation trial was carried out from pea plants showing *Ascochyta* blight symptoms. The diseased pea plants were obtained from the major pea growing areas in Egypt. Infected pea tissues were washed thoroughly under running tap water, cut into small pieces then surface sterilized in 10% sodium hypochlorite solution (NaOCl). After that the disinfested pieces were rinsed in sterile distilled water, then dried between two layers of sterilized filter papers, plated on pea extract medium agar (PEA) [frozen pea grains (150 g) were boiled for 2 min in 500ml H_2O, filtered through cheesecloth, supplemented with glucose (5g) and agar (20 g), made up to 1 liter and autoclaved (Nasir and Hoppe 1991)] and incubated at 20^{o}C in 12 hours cycle alternating near ultraviolet light (NUV), (3800 ^{o}A), and darkness. Isolation was also made from infected seeds with the same previous manner. The isolated fungi were purified using single-spore technique and maintained on PEA slants and kept at 5^{o}C. Identification of the purified isolates was done according to the morphological features of the pycnida and the shapes and sizes of the pycnidiospores (Punithalingam and Holliday, 1972 a & b).

2.2- Pathogenicity test:

2.2.1- Spores induction:

The purified *Ascochyta* spp. were grown on PEA and incubated at 20 ^{o}C in 12 hrs cycle alternating NUV light and darkness for 14 days to encourage sporulation. Using a spatula or a single edge razor blade, the pycnidia were scraped or dislodged from the agar and rinsed with distilled water into a blinder canister, the suspension was filtered through two layers of cheesecloth to remove agar particles and mycelial fragments, then spore number was adjusted with the aid of a haemochtometer slide to $1x10^4$ spores/ml. A volume of 0.04% v/v of Tween 20 was added to break the surface tension of pea leaf inoculation. For soil inoculation, the concentration of $1x10^6$ spores/ml/g soil was used.

2.2.2- Host Cultivar: Pea seeds (cv. Little Marvel) were soaked in NaOCl solution (10%) for 10 min, washed in six changes of sterile distilled water, then soaked overnight in sterile distilled water to promote uniform germination.

2.2.3-Inoculation Techniques:

A- Foliage Inoculation: Seed of pea cv. Little Marvel were planted in a medium containing soil and sand (1:1 w/w) [10 seeds/pot, 4 replicates / treatment] in a greenhouse (temperature: 18-20^{o}C and 70-80% relative humidity). Pots were arranged in a complete randomized design (CRD) (Steel and Torrie 1980). When the leaflets of the third and fourth nodes were fully expanded (14 days old), 10 seedlings in each pot were sprayed with approximately 10 ml of spore suspension (1x10^{4} conidia/ml). After inoculation, plants were covered with clear plastic bags and left for 4 days over the plants to maintain high relative humidity. Re-isolation was carried out from the diseased plants.

Disease Assessment:

Symptoms on leaves were scored 7 days after inoculation using the following scale: 0= no visible reaction on leaves, 1=spots only, 2=deformation in 2-4 leaflets, 3=deformation in 4-6 leaflets, 4=the whole plant was completely infected. The percentage of infection was calculated using Wenzel equation (Wenzel, 1948).

B- Soil Infestation: For soil infestation, a mixture of autoclaved sand and soil (1: w/w), approximately 250 g/pot was irrigated before inoculation, then spore suspension was added at the rate of 1x10^{6} spores/ml/g soil, mixed thoroughly and left for one week to ensure establishment of the tested fungi. Seeds of pea cv. Little Marvel, pretreated with 10% NaOCl for 5 min, were sown at the rate of 10 seeds / pot. Each treatment consisted of 4 pots. The pots were arranged in a complete randomized design (CRD) (Steel and Torrie, 1980). The final count of damped-off seedlings and foot rot symptoms was taken after 21 days from sowing, and re-isolations were carried out from the diseased seedlings. Results were statistically analyzed by one-factor experiment after transforming the percentage of survivors to arcsine numbers (Steel and Torrie, 1980).

2.3- Molecular Techniques:

2.3.1-DNA Extraction:

The extraction of DNA from 47 isolates of *Ascochyta* spp plus one isolate of *Mycospaerella melonis* (a non-host fungus) was performed according to the method given by (Lee and Taylor 1990) with some modifications.

Ten ml of pea extract broth medium in a 50 ml Ehrlenmeyer flask were inoculated with *Ascochyta* isolates (10 flask/isolate) and incubated for 4 days at 18-20^{o}C under NUV lamps. The mycelium of each isolate was harvested and filtered through cheesecloth. Lyophilized mycelium (60 mg) was ground in the presence of liquid nitrogen and mixed with 400 ul lysis buffer (50 mM Tris HCl, pH 7.2, 50 mM Na-EDTA, pH 8.0, 3% SDS and1% 2-mercaptoethanol), at the rate of 1g/20 ml and vortexed gently. The suspension was then incubated at 65^{o}C for 1 hr in a water bath followed by adding 400 ul of phenol/saturated with Tris – HCl buffer (pH 8.0) and centrifuged in a microfuge (Eppendorf, USA) at 10,000 rpm for 15 min at 4^{o}C. The aqueous phase containing the DNA was transferred into a fresh tube, and equal volume of phenol: chloroform (1:1 v/v) was added. Then, the tube was inverted gently for 2-3 times, and centrifuged at 10,000 rpm for 15 min at 4^{o}C. This step was repeated 3 times, and the aqueous phase was extracted with a volume of chloroform by centrifugation at 10,000 rpm for 5 min. To remove of RNA contamination, 50 µl / ml from RNase (Sigma Co., USA) at the rate of 10 mg / ml were added to the upper clear phase and incubated for 1 hr at 37 ^{O}C in a water bath. The extracted DNA was deproteinized by adding 200 µl/ml proteinase K (10 mg/ml) (Sigma) at 35 ^{o}C in a water bath overnight (Gurr and Mcpherson, 1992). Phenol: chloroform (1:1 v/v) extraction was repeated until the interface between the aqueous and phenol: chloroform phase became clear. The aqueous phase was extracted with an equal volume of chloroform by centrifugation at 10,000 rpm for 5 min. The DNA was then precipitated with 0.1 volume of 3M sodium acetate (pH 6.5) plus 1 volume of isopropanol and incubated at -20^{o} C for 2 hrs or overnight followed by centrifugation at 10,000 rpm for 10 min at 4^{o}C. The pellet was washed with 70% ethanol, dried under vacuum and resuspended in 500-1000 µl of TE buffer pH 6.5 (10 mM Tris- HCl and 0.1 mM Na-EDTA pH 8.0) based on the size of pellet. DNA concentration was determined spectro-photometerically and adjusted to 10 ng / µl.

2.3.2- RAPD-PCR:

RAPD -PCR was carried out according to the procedure given by (Williams *et al*. 1990) with minor modification.

Amplification reaction was carried out in a volume of 50 µl. Each reaction mixture contained 50 ng genomic DNA (as a template), 0.5 µM decamer oligonucleotide primer from OPERON Technologies, Alameda, CA. (Kit A and B, 20 primers each), 2 units of *Taq* DNA polymerase (Promega Crop., Madison, WI, USA), 5 µ of 10X buffer [500 mM KCl, 100 mM Tris-HCl (pH9.0) and 1% Triton X-100], 3mM $MgCl_2$, 0.2mM, 0.2mMdNTPs (dATP, dCTP, dTTP, dGTP) and deionized dd H_2O. The reaction was overlaid with a drop of mineral oil. PCR amplification was performed in a Perkin – Elmer\DNA Thermal cycler 480 (Norwalk, CT) for 40 cycles after initial denaturation for 3 min at 94^{o}C. Each cycle consisted of denaturation at 94^{o}C for 1 min. annealing temperature at 36^{o}C for 1 min, extension at 72^{o}C for 2 min then the final primer extension segment was extended to 5 min, at 72^{o}C. The amplification products were resolved by electrophoresis in a 1.5% agarose at

60 volts for three and half hrs with 1X TAB buffer (10g Tris-base, 5.5g boric acid and 4 ml 0.5 M Na-EDTA pH 8.0 in 1 liter). PCR products were visualized by staining gel in ethidium bromide (0.5µg/ml) and photographed under UV light using a Polaroid camera. Amplified products were visually examined and the presence or absence of each size class was scored as 0 and 1, respectively.

2.3.3- RAPD Analysis: The banding patterns generated by RAPD-PCR analysis were compared to determine the genetic relatedness of *Ascochyta* isolates. The amplified fragments were scored either as present (1) or absent (0). Bands of the same mobility were scored as identical. The similarity coefficient (F) between two isolates was defined by the formula of (Nei and Li
1979), F= 2NxY/Nx+ NY, were NxY is the number of common bands between the isolates and Nx and NY are the number of bands in isolates X and Y respectively. A dendrogram was derived from the distance by the unweighted paired group method, arithmetic mean (UPGMA) algorithm contained in the computer program package NTSYS1.5 (Rohlf, 1990).

2.3.4- Southern Blotting and Hybridization:

2.3.4.1-Southern Blotting of DNA: Blotting of PCR products of *Ascochyta* isolates amplified using OPM-08 and OPB-15 onto positively charged nylon membrane (Boehringer Mannheim) was carried out according to (Sambrook *et al*. 1989). The PCR products were electrophoresed in 1.5% agarose gel and stained with ethidium bromide. Southern blotting was performed as described by (Southern 1975). The resulting membrane (mounting with the DNA bands) was then dried at room temperature (RT) on a sheet of 3MM Whatman paper and was cross-linked by using GS Gene Linker, UV chamber, Bio-Red, for fixing DNA on the nylon membrane. Finally the membrane was stored between 2 pieces of 3MM Whatman paper at RT until use for hybridization with the suitable probe.

2.3.4.2- Extraction of DNA from Agrarose Gel: The discriminating bands of *A.pisi* and *A.pinodes* derived from RAPD-PCR products with OPB-08 and *A.pinodella* derived from the RAPD-PCR products with OPB-15 were extracted from agarose gel using Gene clean Kit (Bio.101, Inc., USA. The eluted DNA was carefully transferred into a fresh tube. The concentration of the resulted DNA was spectrophotometrically determined.

2.3.4.3- Labeling of RAPD Derived DNA Probes: A prime-It® II Random Primer Labeling Kit (Stratagene, USA) was used to prepare 32 P- Labeled hybridization probes obtained from the discriminating bands of RAPD-PCR products of *Ascochyta* spp. fifty ng of DNA (31.5µl) was denatured by boiling for 5 min and was immediately cooled in an ice bath. To each of the denatured probes, 10 µl 5X labeling of buffer, 2 µl dNTPs (20 µM each), 0.5 µl 100X BSA, 5 µl α-^{32} P dCTP (10 µci/ µl) and 1 µl Klenow fragment (5 units/ µl) were added. The mixture was spun for few sec in a microcentrifuge at 40^{o}C for 10 min, and finally stored at - 20^{o}C until use.

2.3.4.4- Hybridization of Transferred PCR Products with RAPD Derived DNA Probes: The membranes were hybridized with (α-^{32} P) – DNA labeled probes according to the method given by (Sambrook *et al*. 1989). The membranes were rotated in hybridization tubes containing 50 ml of prehybridization solution (6X SSC, 0.5% SDS, 5X Denhardt's solution and 100 µg/ml denatured salmon sperm DNA and incubated overnight 42^{o}C in a hybridization oven (Techne hybridizer HB-1D, Techne Inc., Princeton N.J. USA). The α-^{32} P labeled probe was denatured by boiling for 5 min and immediately cooled in an ice bath. The prehybridization solution was replaced with 50 ml of hybridization solution (prehybridization solution containing 100 ng of denatured α-^{32} P probe) and incubated for 14 hrs. at 42^{o}C in the hybridization oven. Hybridization solution was drained from the tube and membranes were subsequently washed for 5 min at RT in 2X SSC containing 0.5% SSC then for 15 min at RT in 2X SSC containing 0.1% SDS and finally for 2 hrs. in 0.1X SSC containing 0.5% SDS at 42^{o}C.

Each membrane was left to dry at RT on a sheet of 3MM Whatman paper then wrapped in saran wrap.

2.3.4.5- Autoradiograph: For detection of (α-^{32}P) signals, membranes were wrapped in saran wrap and exposed to X- ray (Kodak, X-Omat K-Film 100) in a light proof cassette and stored at -80^{o}C for about 72 hrs following by developing machine (Kodak M35 X-Omat).

3. Results

Isolation and identification of *Ascochyta* spp.:

Forty seven isolates of *Ascochyta* were isolated from leaves, stems, pods, roots, and seeds of different pea plants showed *Ascochyta* disease complex symptoms. On the basis of size and shape of pycnidiospores, these isolates were identified and classified into three species as the following: *A.pinodella* (2 isolates), *A.pinodes* (43 isolates) and *A.pisi* (2 isolates).

Pathogenicity Test:

The pathogenic potentiality of each of the 47 isolates of *Ascochyta* was determined by both soil infestation and foliage spraying with pycnidiospores suspension.

Soil Infestation:

Results of soil infestation were presented in Tablel 1. Different levels of infection among *Ascochyta* isolates was observed. The three species of

Ascochyta caused pre- and post-emergence damping-off with noticeable difference between the 47 isolates. Clear differences were observed among the 43 isolates of *A.pinodes* in causing pre- and post-emergence damping-off of pea seedling Furthermore, most isolates of *A.pinodes* showed the highest percentage of pre- and post-emergence damping-off. A significant difference was observed between the two isolates of *A.pinodella* (Isolate 46 and 47). Isolate 46 showed higher percentage of damping-off on pea seedlings than isolate 47. No significant difference was observed between the two isolates of *A.pisi* (isolates 48 and 49) in causing damping-off symptoms on pea seedlings, however, they were the least in this respect.

Table (1): Percentage of pre-and post- emergence damping-off incited by 47 isolates of *Ascochyta* spp. on pea, cv. Little Marvel.

Ascochyta spp.	Isolate No.	Percentage of Infection		
		Pre-emergence damping- off.	Post-mergence damping- off.	Survivors
Control	c	0 (0)a	0 (0)c	100 (90)a
A.pinodes	3	42.5 (39.75)b	42.5 (39.75)b	15 (16.6)d
	4	90 (59.15)ab	7.5 (11.22)c	2.5 (4.6)d
	5	70 (42.95)b	7.5 (11.22)c	0 (0)e
	6	65 (57.65)ab	32.5 (30.75)bc	2.5 (4.6)d
	7	20 (26.15)b	80 (62.12)a	0 (0)e
	8	85 (67.45)ab	15 (22.45)bc	0 (0)e
	9	45 (38.1)b	55 (51.85)ab	0 (0)e
	10	52.5 (46.47)b	47.5 (43.47)ab	0 (0)e
	11	85 (67.45)ab	10 (18.4)bc	5 (9.2)d
	12	85 (70.4)ab	7.5 (11.22)c	7.5 (13.8)d
	13	32.5 (34.52)b	67.5 (55.4)ab	0 (0)e
	14	45 (42.02)b	55 (47.95)ab	0 (0)e
	15	32.5 (34.52)b	67.55 (55.45)ab	0 (0)e
	16	35 (35.97)b	65 (53.97)ab	0 (0)e
	17	47.5 (43.6)b	42.5 (40.6)ab	10 (15.82)d
	18	30 (33.02)b	70 (56.95)ab	0 (0)e
	19	87.5 (75.02)ab	12.5 (14.92)c	0 (0)e
	20	77.5 (62.25)ab	22.5 (17.85)c	0 (0)e
	21	52.5 (46.4)b	40 (39.2)bc	7.5 (11.22)d
	22	85 (67.45)ab	15 (22.45)bc	0 (0)e
	23	80 (71.17)ab	17.5 (17.27)c	2.5 (4.6)d
	24	82.5 (65.77)ab	7.5 (13.8)c	10 (15.82)d
	25	90 (40.9)b	10 (13.25)c	0 (0)e
	26	75 (62.85)ab	17.5 (20.45)bc	7.5 (13.8)d
	27	75 (63.7)ab	2.5 (4.6)c	22.5 (24.72)cd
	28	87.5 (75.55)ab	12.5 (14.4)c	0 (0)e

Table (1): (Continued)

Ascochyta spp.	Isolate No.	Percentage of Infection		
		Pre-emergence damping- off.	Post-mergence damping- off.	Survivors
A.pinodes (Continued)	29	25 (28.75)b	70 (57.72)ab	5 (9.2)d
	31	47.5 (43.52)b	52.5 (46.4)ab	0 (0)e
	32	42.5 (40.35)b	57.5 (49.57)ab	0 (0)e
	33	80 (64.87)ab	27.5 (30.77)bc	2.5 (4.6)d
	34	87.5 (75.55)ab	17.5 (23.6)bc	0 (0)e
	35	97.5 (85.37)a	2.5 (4.6)c	0 (0)e
	36	42.5 (27.9)b	55 (47.82)ab	2.5 (4.6)d
	37	87.5 (72.07)ab	12.5 (17.85)c	0 (0)e
	38	55 (47.12)b	42.5 (42.5)ab	2.5 (4.6)d
	39	95 (83.35)a	5 (6.62)c	0 (0)e
	40	52.5 (46.62)b	47.5 (41.25)ab	0 (0)e
	41	60 (51.6)b	40 (38.32)bc	0 (0)e
	42	25 (29.67)b	75 (60.25)ab	0 (0)e
	43	30 (27.6)b	70 (60.7)ab	0 (0)e
	44	35 (36.2)b	60 (50.85)ab	5 (9.2)d
	45	47.5 (43.5)b	50 (45.05)ab	2.5 (4.6)d
A.Pinodella	46	95 (80.75)ab	205 (4.6)c	2.5 (4.6)d
	47	35 (32.3)b	30 (32.27)bc	35 (32.8)c
A.pisi	48	27.5 (27.67)b	0 (0)c	72.5 (62.27)b
	49	27.5 (30.82)b	0 (0)c	72.5 (59.07)b

Values followed with the same letter (s) in each column are not significantly different at P=0.05. Values between bracts are the transformed percentage to arcsin numbers.

Foliage Inoculation:

Seedlings of pea were sprayed with spore suspension of the 47 isolates of *Ascochyta* spp. Moderate to severe symptoms (leaf symptoms and stem lesions), were appeared on leaves and stems after 7 days from spraying date with the pycnidiospores of the different isolates of *Ascochyta* spp**.**

Results in Table 2 indicated that isolates No.3 of *A.pinodes* was found to be more virulent on leaves and stems than the other isolates, however, isolate No. 15 was the least one in this respect. Also, *A.pinodella*, isolate No.47, was more virulent than isolate No. 46. It showed severe symptoms on pea foliage (leaves and stems). *A.pisi* isolates (48 and 49) showed moderate symptoms on leaves and stems of pea.

RAPD-PCR:

The RAPD technique was used as a tool for setting up a convenient and standard protocol to study the intergenetic variation between the three species of *Ascochyta* (*A.pinodella*, *A.pinodes* and *A.pisi*), and at the same time between the interagenetic variation within each species. All reactions were repeated at least twice and always included negative control (No template DNA). No amplified bands were observed in any of the control reaction.

Values followed with the same letter (s) in each column are not significantly different at P=0.05. Values between bracts are the transformed percentage to arcsin numbers.

Four (OPA-10, 12 and OPB-08, 15) out of forty

Primers (OPA and OPB) gave clearly differences among the three species of *Ascochyta* isolates on the basis of the amplified product patterns as shown in Figures 1,2,3and 4. The comparison between the three species of *Ascochyta* isolates showed difference in the size and numbers of the amplified fragments per primer for each species, indicating a high degree of variability between the three species with a final linkage between each other. Some bands were found to be common between the 47 isolates, but most of the amplificates were specific for each species.

Table (2): Percentage of infection of isolates of *Ascochyta* spp. on pea cv. Little Marvel.

***Ascochyta* spp.**	**Isolates No.**	**% of leaf infection**	***Ascochyta* spp.**	**Isolates No.**	**% of leaf infection**
Control	c	0 (0)^{e}		*26*	26.8 (30.52)^{cd}
	3	60.9 (55.35)^{a}		*27*	27.8 (31.75)^{cd}
	4	20.3 (26.55)^{cd}		*28*	42.7 (40.83)^{bc}
	5	29.5 (32.83)^{cd}		*29*	20.4 (26.85)^{cd}
	6	47.6 (43.78)^{b}		*30*	37.8 (37.78)^{bc}
	7	21.8 (27.88)^{cd}		*31*	25.3 (29.95)^{cd}
	8	25.2 (30.15)^{cd}		32	20.5 (26.9)^{cd}
	9	26.7 (31.03)^{cd}		33	24.3 (29.6)^{cd}
	10	15.2 (22.95)^{d}		34	20.5 (26.83)cd
	11	13.4 (21.43)^{d}		35	19.8 (26.3)^{cd}
	12	21.1 (27.33)^{cd}	***A.pinodes* (Continued)**	36	27.6 (31.67)^{cd}
A.pinodes	13	18 (25.05)^{cd}		37	35.3 (36.4)^{bc}
	14	17.5 (24.65)^{d}		38	30.4 (33.48)^{c}
	15	13.8 (21.82)^{d}		39	33.9 (35.37)^{bc}
	16	16.9 (24.15)^{d}		40	23.5 (28.85)^{cd}
	17	21.9 (27.7)^{cd}		41	46.4 (43.05)^{b}
	18	14.5 (22.22)^{d}		42	19.8 (26.45)^{cd}
	19	46.8 (43.2)^{b}		43	18.2 (25.23)^{cd}
	20	26.1 (30.68)^{cd}		44	16 (23.5)^{d}
	21	14.75 (22.6)^{d}		45	14.5 (22.3)^{d}
	22	28.6 (32.18)^{cd}	***A.pinodella***	46	14.3 (22.2)^{d}
	23	36.6 (37.33)^{bc}		47	56.3 (49.13)^{d}
	24	25.7 (30.3)^{cd}	***A.pisi***	48	12.4 (20.5)^{ab}
	25	34.5 35.88)^{c}		49	14.7 (22.4)^{d}

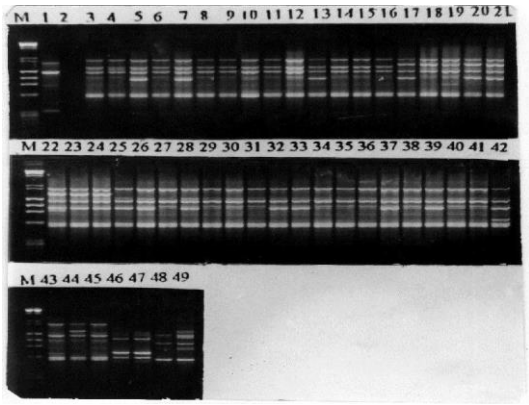

Figure 1: RAPD fragments from genomic DNA of different *Ascochyta* spp. Isolates and an isolate of *M. melonis* generated by primer (OPA-12). Lane M represents DNA marker (ØX174/*Hae III* and λ / *Hind III*), lane 1 represents the control isolate (*M. melonis*), lanes 3-45 represent isolates of *A.pinodes*, lanes 46-47 represent isolates of *A.pinodella* and lanes 48-49 represent isolates of *A.pisi.*

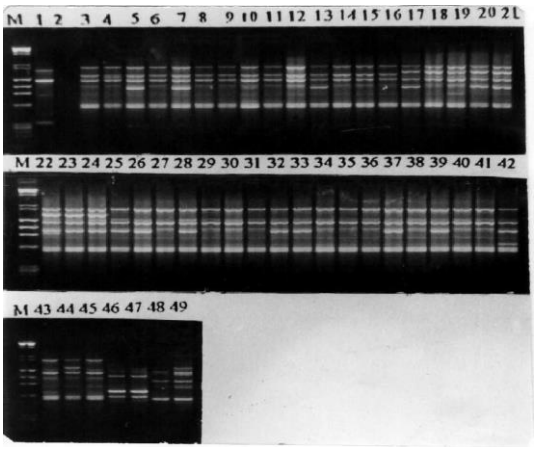

Figure 2: RAPD fragments from genomic DNA of different *Ascochyta* spp. Isolates and an isolate of *M. melonis* generated by primer (OPA-10). Lane M represents DNA marker (ØX174/*Hae* III and λ / *Hind* III), lane 1 represents the control isolate (*M. melonis*), lanes 3-45 represent isolates of *A.pinodes*, lanes 46-47 represent isolates of *A.pinodella* and lanes 48-49 represent isolates of *A.pisi.*

Results in Table 3 showed that a total of 19 DNA fragments were produced with OPA-10 primer among 47 isolates of *Ascochyta* spp. 7, 10, and 8 fragments were produced with OPA-10 primer among 47 isolates of *Ascochyta* spp. Seven, 10, and 8 fragments were present in isolates of *A.pinodella, A.pinodes* and *A.pisi* respectively. Furthermore, three different discriminating fragments were found to distinguish each isolate of the tested *Ascochyta* spp**.** Seventeen amplified DNA band were generated from isolates of the three species of *Ascochyta* employing primer OPB-

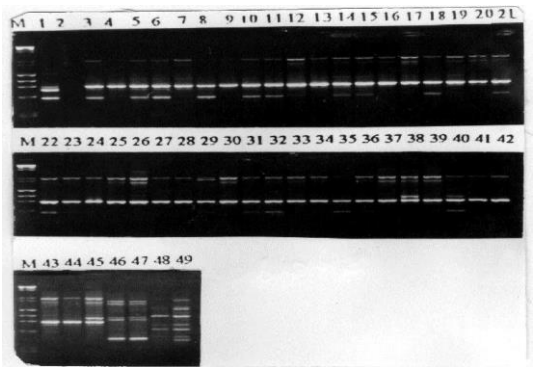

Figure 3: RAPD fragments from genomic DNA of different *Ascochyta* spp. Isolates and an isolate of *M. melonis* generated by primer (OPB-08). Lane M represents DNA marker (ØX174/*Hae* III and λ / *Hind* III), lane 1 represents the control isolate (*M. melonis*), lanes 3-45 represent isolates of *A.pinodes*, lanes 46-47 represent isolates of *A.pinodella* and lanes 48-49 represent isolates of *A.pisi.*

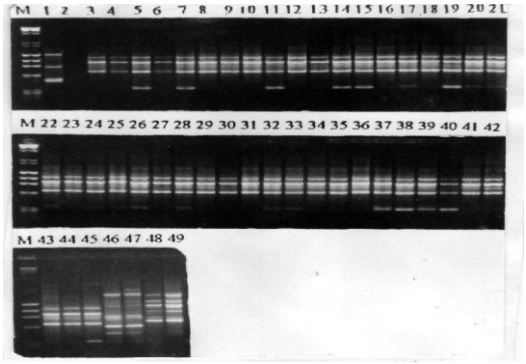

Figure 4: RAPD fragments from genomic DNA of different *Ascochyta* spp. Isolates and an isolate of *M. melonis* generated by primer (OPB-15). Lane M represents DNA marker (ØX174/*Hae* III and λ / *Hind* III), lane 1 represents the control isolate (*M. melonis*), lanes 3-45 represent isolates of *A.pinodes*, lanes 46-47 represent isolates of *A.pinodella* and lanes 48-49 represent isolates of *A.pisi.*

08. In general, 5, 6 and 7 DNA fragments out of 17 were found in *A.pinodella*, *A.pinodes* and *A.pisi* respectively. In addition, 4 of 5, 2 of 6 and 5 of 7 discriminating bands could be considered specific bands to differentiate *A.pinodella, A.pinodes* and *A.pisi* respectively. Primer OPB-15 produced three different discriminating bands for each species of the tested *Ascochyta* isolates. OPA-12 was able to discriminate both *A.pisi* and *A.pinodella* from *A.pinodes.* Although this primer could not produce (a) specific band (s) to discriminate *A.pinodes* it could classify individuals of *A.pinodes* to 4 different groups. These four groups could be assigned as pathotypes

.

Table 3: Oligonucleotide primers used for generating random amplified polymorphic DNA patterns from different isolates of *Ascochyta* spp.

Primer	Nucleotide sequence (5` to 3`)	Total number of bands among 47 isolates	*Ascochyta* spp.					
			A.pinodella		*A.pinodes*		*A.pisi*	
			NAB[1]	NDB[2]	NAB[1]	NDB[2]	NAB[1]	NDB[2]
OPA-10	GTGATCGCAG	19	7	3	10	3	8	3
OPA-12	TCGGCGATAG	23	8	4	15	0	9	3
OPA-08	GTCCACACGG	17	5	4	6	2	7	5
OPA-15	GGAGGGTGTT	14	7	3	6	3	6	3

NAB[1]: Total Number of Amplified Bands.
NDB[2]: Total Number of Discriminating Bands.

RAPD Analysis:

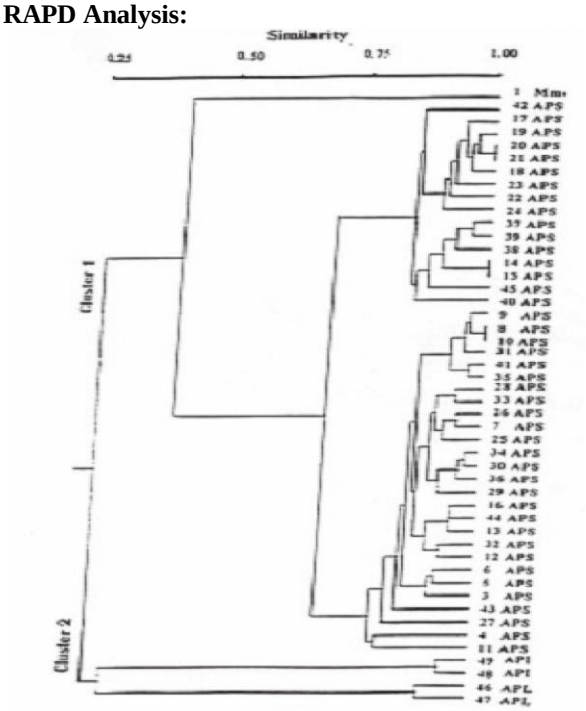

Figure 5: Dendrogram showing relationships among 47 isolates of *Ascochyta* spp. and one isolate of *M. melonis*, based on RAPD polymorphisms. Mm= *M. melonis*, APL= *A.pinodella*, APS= *A.pinodes*, API= *A.pisi*.

The combined data from all isolates were analyzed using SAHN program to produce a dendrogram (Figure 5). Variation in intensity was observed with some bands in the developed RAPD patterns, but this had no bearing upon the computer analysis. The only factor considered was presence or absence of any particular bands. *M. melonis*, was included as an out group fungus to create a rooted tree in the cluster analysis. The dendrogram divided into two main clusters (cluster I and II). The first cluster divided into two main groups, group 1 represented isolate of *M. melonis*, whereas group 2 contained the 43 isolates of *A. pinodes*. Consequently, group 2 divided into two subgroups that were not related to the pathogenicity. The cluster II divided into two groups, group1 contained two isolates of *A.pisi* and group 2 contained two isolates of *A.pinodella*.

Hybridization of Transferred PCR Products with RAPD Derived DNA Probes:

The discriminating bands of *Ascochyta* species were clearly identified in Figure 6. Primer OPB-08 produced two different size bands: 1078 bp and 872 bp when used to amplify DNA of *A.pisi* and *A.pinodes* respectively. Also, OPB-15 generated a band of 603 bp in *A.pinodella*. These PCR products were selected, labeled and used as DNA probes for Southern hybridization.

Results presented in Figures 7, 8 and 9 showed each probe specifically hybridized with its corresponding band in the same locus. No hybridization was detected with the rest of bands.

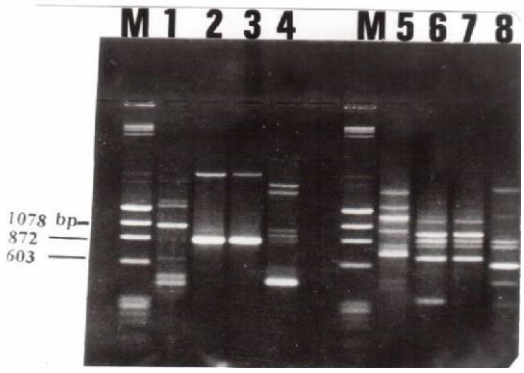

Figure 6: The molecular weight of the discriminating band of each *Ascochyta* spp. Lanes 1-4 represent RAPD patterns using OPB-08. Lanes 5-8 represent RAPD patterns using OPB-15. Lanes 1 and 5 each represents DNA profile of *A. pisi*. Lanes 2, 3, 6 and 7 each represents DNA profile of *A.pinodes*. Lanes 4 and 8 each represents DNA profile of *A.pinodella*. Lane M represents DNA marker (ØX174/*Hae* III and λ / *Hind* III).

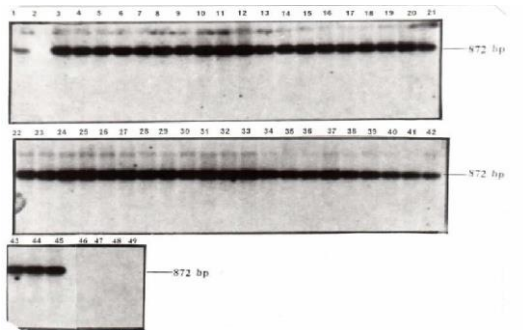

Figure 7: Southern hybridization of PCR products, (using OPB-08) of 47 isolates of *Ascochyta* spp. and one isolate of *M. melonis* hybridized with *A.pinodes*-specific probe. Lane 1 represents the isolate of *M. melonis*, lane 2 represents the negative control, lanes 3-45 represent isolates of *A.pinodes*, lanes 46-47 represent isolates of *A.pinodella* and lanes 48-49 represent isolates of *A.pis*

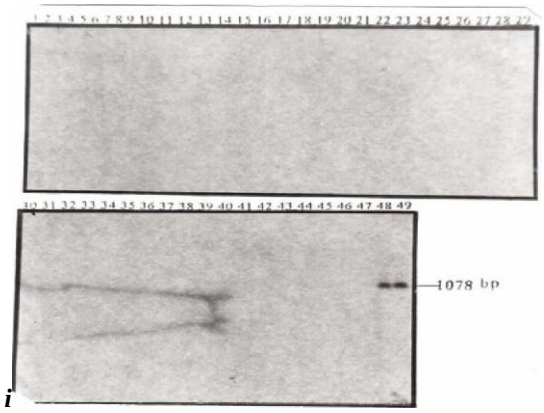

Figure 8: Southern hybridization of PCR products, (using OPB-15) of 47 isolates of *Ascochyta* spp. and one isolate of *M. melonis* hybridized with *A.pisi*-specific probe. Lane 1 represents the isolate of *M. melonis*, lane 2 represents the negative control, lanes 3-45 represent isolates of *A.pinodes*, lanes 46-47 represent isolates of *A.pinodella* and lanes 48-49 represent isolates of *A.pisi*.

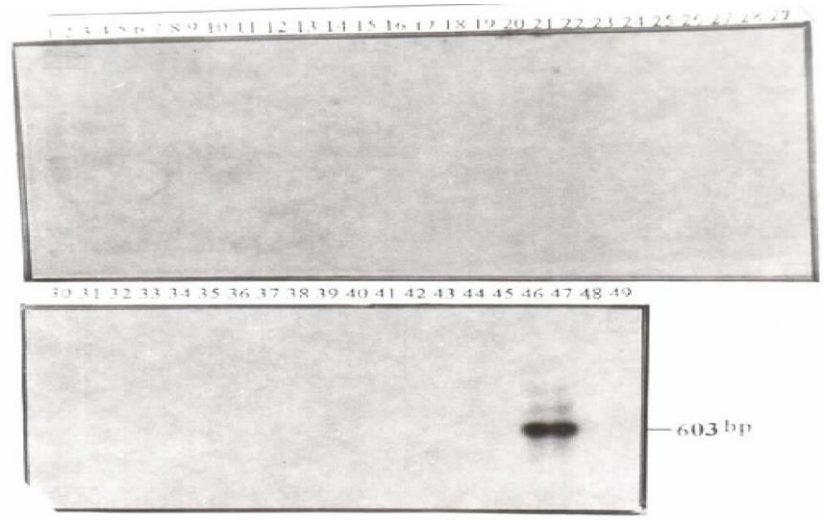

Figure 9: Southern hybridization of PCR products, (using OPB-08) of 47 isolates of *Ascochyta* spp. and one isolate of *M. melonis* hybridized with *A.pinodell*-specific probe. Lane 1 represents the isolate of *M. melonis*, lane 2 represents the negative control, lanes 3-45 represent isolates of *A.pinodes*, lanes 46-47 represent isolates of *A.pinodella* and lanes 48-49 represent isolates of *A.pisi*.

4. Discussion

The aim of this study was to find out (a) molecular marker (s) to discriminate between the closely related pea pathogens. *Ascochyta pinodella* (syn. *Phoma medicaginis* var. *pinodella*), *A.pinodes* (telemorph *Mycosphaerella pinodes*) and *A.pisi*. Their identification is currently based on diseases symptoms and morphological features particularly, pycnidial size conidial shape and size, and presence or absence of chlamydospores. Identification based on such criteria remains difficult and uncertain where minor differences in symptoms are often misleading, particularly during early phases of disease development and the morphological criteria are highly variable overlapping and dependent upon cultural conditions (Lawyer, 1989). Moreover certain isolates possess an intermediate colony morphology which makes it difficult to assign them to one species or another (Faris - Mokaiesh *et al*., 1996).

According to the available literature few trials have been done on using the biochemical and molecular markers to differentiate between those

worldwide-distributed *Ascochyta* spp. Limited success has been achieved in the serological differentiation of those closely related fungi (Bowen *et al.*, 1996, Faris - Mokaiesh *et al.*, 1996 and Lawyer. 1989). Isozyme analysis showed that the electrophoretic patterns of *A.pisi* were clearly different from those of *A.pinodes* and *A.pinodella.* Also, within each species some variation was noted in electrophoretic patterns among isolates (Faris – Mokaiesh *et al*., 1996) RFLP of the internal transcribed spacers (ITSs) of the three fungi showed no intraspecific and very little interspecific variation. However, RFLP of the intragenic spacer (IGS) revealed uniformiting in *A.pisi* patterns which consistently differed from those of *A.pinodes* and *A.pinodella*. No clear distinction could be made between the latter two fungi which both showed intraspecific variability (Faris – Mokaiesh *et al*., 1996).

The study presented here focused on using RAPD-PCR to differentiate the three closely related *Ascochyta* spp. Forty seven isolates of *Ascochyta* spp. were isolated from the pea growing areas throughout Egypt. These isolates were tentatively identified using the microscopic features of pycnidia and pycndiospores according to the key given by (Punthalingam and Holliday, 1972a and b) and (Lawyer 1989). The forty seven isolates contained *A.pinodella* and *A.pisi* two isolates each and forty three isolates of *A.pinodes*. These isolates caused *Ascochyta* blight disease on peas. Isolates of *A.pinodes* were more virulent than isolates of both *A.pinodella* and *A.pisi*. Similar results were reported by (Lawyer 1989), (Lepikhova, 1974), (Martens *et al.* 1984), (Rudakov and Lepikhova 1974) and (Wallen and Jeun 1968).

Random Amplified Polymorphic DNA (RAPD) analysis was used to differentiate those species of *Ascochyta*. Four decameric random oligonucleotide primers (OPA-10, 12 and OPB-08, 15), out of the 40 primers tested, were easily distinguished the three species of *Ascochyta* and each species had a unique pattern with each primer. Several reports have been demonstrated that RAPD markers have potential as a means of identifying different species belonging to one genus (Keinath *et al*. 1995 and Schilling *et al*. 1996). RAPD could distinguish between the closely related species of *F. culmorum* (FC) AND *F. graminearum* (FG). Amplified fragments clearly distinguished between FC and FG, among isolates and intraspecific genotyping of isolates. Also, species-specific RAPD patterns were extremely useful to identify false classified isolates of both species (Schilling *et al.* 1996).

Isolates of *A.pinodes* were distinguished into three subgroups using either OPA-10 or OPB-08 primers. Also, OPB-15 primer distinguished *A.pinodes* into two subgroups. Primer OPA-12 could classify isolates of *A.pinodes* into four distinguished subgroups; such a group could be assigned as a pathotype. The presence of the sexual cycle of *A.pinodes* could be responsible for this relative variability. Guthrie *et al*. (1992) used RAPD technique to detect the variability between isolates of the highly variable fungal pathogen, *Colletotrichum graminicola*, the causal agent of anthracnose of sorghum. Their results indicated that each isolate had a visual "signature" that allowed ready comparison among isolates. Also, some isolates had characteristic signature based on geographical origin. Since most the *Ascochyta* on peas are seed borne pathogens, and most pea seeds in Egypt are imported from outside country, this technology may, therefore have potential applications in quarantine and related fields.

The exclusive hybridization of amplified DNA probes derived from DNA products of certain isolates of *A.pinodella*, *A.pinodes* and *A.pisi* with DNA of the other isolates of each species could be considered DNA-specific probes for partial diagnosis of *Ascochyta* spp. with dot blot assay.

The aforementioned discussion clarifies the suitability of RAPD analysis to distinguish between the three species of *Ascochyta*-pea blight disease. The three pathogens possess different life cycle, and a rapid and accurate means of identification would lead to better planning for control measures: *i.e* preventive chemical seed treatments and foliar applications (Hwang *et al.* 1991). Accurate diagnosis is also essential in breeding programs and for epidemiological studies.

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7/15/2018