Egypt. J. Phytopathol., Vol. **42**, No. 1, pp. 25-42 (2014)

Morphological, Pathological and Genetic Diversities among *Cephalosporium maydis* Isolates

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Morphological, pathological and possible genetic variations among forty two *Cephalosporium maydis* isolates, obtained from 13 Egyptian Governorates, were evaluated. Clear differences among *C. maydis* cultures grown on PDA+0.2% yeast extract (PDAY) were found with respect to their growth rate and sporulation. However, the majority of isolates grew at the normal growth rate (7 days after incubation at 27±2°C), showed whitish grey and white colour, had rhizoid margin and displayed intermediate growth density. In most isolates, the maximum number of spores was observed at the third day of incubation at 27±2°C, but no spores were produced after the sixth day of growth.

Under greenhouse conditions, the virulence of *C. maydis* isolates against maize cv. Boushy revealed that, in general, all the tested isolates were pathogenic and differed in their aggressiveness. Some isolates showed the onset late wilt symptoms, 65 days after sowing, whereas symptoms started to appear, 75 and/or 95 days after sowing. However, isolate No. 9 from Gharbiya caused the highest infection, 95 days after sowing (93.3%); meanwhile isolate No. 48 from Wadi El-Natroon recorded the least infection (13.3%). A highly negative correlation was observed between infection percentages and each of plant height or dry weight of plants. Also, no correlation was observed between morphological and pathological characters of the tested isolates.

Genetic variability among 14 *C. maydis* isolates was also carried out in the present study using RAPD-PCR technique. Different levels of genetic variability were recorded among the tested isolates and the reference ones, where they clustered into four phylogenetic lineages. Moreover, slight relationship between the lineage of isolates and any of their geographic origin and their virulence was observed. However, no correlation was noticed between the lineage and the morphological characters of the tested isolates.

Keywords: *Cephalosporium maydis*, late wilt, maize, phylogenetic and RAPD-PCR.

Cephalosporium maydis Samra, Sabet and Hingorani, the causal agent of maize late wilt disease, was recorded for the first time in Egypt in 1960, where it can cause yield losses up to 40% in susceptible cultivars (Samra *et al*., 1966).

C. maydis originally was described based on growth characters and the morphology of hyphae, conidia and conidiophores. Sabet *et al.* (1966) demonstrated that *C. maydis* colonies on PDAY were of low growth and felty with a characteristic rhizoid margin and grayish white to slate grey colour. Moreover, Ali (2000) found that clear differences among *C. maydis* isolates exhibited with respect to their radial growth, colony colour, margin and growth density on PDAY, where the colony colour of the isolates varied from whitish grey to dark grey and the colony margin differed clearly from rhizoid to entire or semi-rhizoid. Furthermore, Drori *et al.* (2013) reported that on PDA medium, the colonies of *C. maydis* reached 90-mm-diam. within 8 days at 28°C. The colonies were flat, white at first then turning from grey to black with age.

Ali (2000) stated that *C. maydis* isolates differed in their aggressiveness towards maize cultivars. Also, many researchers (Sabet *et al.*, 1966 and Degani and Cernica, 2014) found that the first symptoms of late wilt appeared 50 - 60 days after sowing and *C. maydis* isolates differed in their pathogenicity to maize varieties. In infested soil, maize plants showed severe wilting symptoms at 61 days after sowing, meanwhile at 75 days; all plants were dead (Drori *et al.*, 2013).

There are several studies on genetic variability of *C. maydis*. Zeller *et al*. (2000) characterized 48 isolates of *C. maydis* from Egypt with isozymes and amplified fragment length polymorphisms (AFLPs) using 69 primer-pair combinations and found that these isolates divided into 25 clones and four putative phylogenetic lineages. Furthermore, Saleh *et al.* (2003) characterized 866 isolates of *C. maydis* collected from 14 Governorates in Egypt with AFLP markers. They noticed that the four tested AFLP primer-pair combinations generated 68 bands, 25 of which were polymorphic, resulting in 52 clonal haplotypes that clustered the tested isolates into four phylogenetic lineages. Molinero-Ruiz *et al.* (2010) identified several *C. maydis* isolates using internal transcribed spacer (ITS) region of mycelial DNA and found that BLAST analysis showed 99% homology with *C. maydis* (Gen Bank Accession Nos. CM2A1, CM884, CM3B, and CM1A).

The present investigation was designed to study the morphological, pathological and genetic variations among some *C. maydis* isolates. The correlation among characters of this fungus was also considered.

Materials and Methods

1. Source of Cephalosporium maydis isolates:

Forty-two isolates of *C. maydis*, isolated from late wilt-infected maize plants collected from thirteen Egyptian Governorates during 2011 growing season, and identified according to Samra *et al.* (1963), were used in the present study.

2. Morphological diversities among C. maydis isolates:

Cephalosporium maydis isolates were evaluated for growth characters, *i.e.* radial growth, growth- rate and density, colony- colour and margin, as well as sporulation rate, on PDA+0.2% yeast extract (PDAY) medium. Disks (5-mm-diam.), taken from the margin of 7-day-old *C. maydis* cultures, were individually transferred into the centre of Petri dishes (9-cm-diam.) containing about 15-20 ml PDAY medium and

incubated at 27±2ºC for up to 10 days. Two days after incubation, colony diameter was daily measured, when the mean of radial growth (cm) was calculated. Five plates were kept as replicates for each isolate. Growth rate was calculated using the following equation of Meletiadis *et al.* (2001):

$$\text{Growth rate (cm/day)} = (R_2-R_1)/(T_2-T_1)$$

Whereas: R_1 and R_2 are the average of radial growth (cm) at the beginning and the end of the measurement period, respectively. T_1 and T_2 are the times (day) at the beginning and the end of the measurement period, respectively.

However, colony- colour and margin as well as growth density were visually observed and recorded at the end of incubation period as described by Samra *et al*. (1966). Also, number of spores/ml was daily counted using a haemocytometer slide.

3. Pathological diversities:

Pathogenicity tests of *C. maydis* isolates were carried out in potted soil under greenhouse conditions at the Maize and Sugar Crops Disease Dept., Plant Pathol. Res. Inst., ARC, Giza, Egypt, following the soil infestation technique.

3.1. Inoculum preparation and soil infestation:

Fungal inoculum was prepared by aseptically growing the *C. maydis* isolates on autoclaved sorghum grains in 500 ml glass bottles and incubation for 2 weeks at 27±2°C until sufficient growth of the fungus was obtained (El-Shafey *et al.*, 1979). The content of the bottles of each isolate was poured out and mixed to get homogenized inoculum, and then inoculum was used for soil infestation at the rate of 30 g/kg soil as described by Samra *et al*. (1966). Three pots (25-cm-diameter) were used as replicates for each isolate. Autoclaved sterilized sorghum grains, mixed thoroughly with soil at the same rate (30 g/kg soil) and kept as control (check) treatment. Eight maize grains (cv. Boushy) were sown in each pot and thinned 20 days after sowing into 5 plants.

3.2. Disease assessment:

Wilted plants were recorded 65, 75 and 95 days after sowing and disease incidence percentage was calculated according to Sabet *et al*. (1966). Also, seed germination percentage was recorded 10 days after sowing. Moreover, plant growth characters, *i.e.* plant height and dry weight, were calculated 95 days after sowing.

4. Molecular studies:

The possible genetic variations among *C. maydis* isolates were determined using Random Amplified Polymorphism DNA technique (RAPD) at Cairo Univ. Res. Park (CURP), Fac. Agric., Cairo Univ., Giza, Egypt.

4.1. Source of materials:

A number of 14 isolates (the highly virulent isolate from each Governorate) as well as four old isolates from the culture type collection of the Maize and Sugar Crops Dis. Dept., Plant Pathol. Res. Inst., A.R.C., Giza (previously identified by Amplified Fragment Length Polymorphism (AFLP) technique) were used as references. Amount of genetic variation was evaluated by PCR amplification with set of 11 random primers (Bio Basic Inc.) as shown in Table (1).

Table 1. Code and nucleotide sequence of primers used in the RAPD reactions

Primer	Sequence
1	5´-GAAACAGCGG-3´
2	5´-GGAGCCCAC-3´
3	5´-GCCGTCTACG-3´
4	5´-GGCATCGGCC-3´
5	5´-GTGAGCGTC-3´
6	5´-GGTGCGGGAA-3´
7	5´-GTTTCGCTCC-3´
8	5´-GTAGACCCGT-3´
9	5´-AAGAGCCCGT-3´
10	5´-AACGCGCAAC-3´
11	5´-CCCGTCAGCA-3´

4.2. DNA extraction:

Disks (5 mm-diam.), taken from a 7 day-old cultures, were transferred into 250 ml conical flasks containing 100 ml of potato dextrose broth + 0.2% yeast extract and incubated at 27±2ºC for one week. Growing mycelium was harvested by filtration through sterile cloth. Excess water was removed by blotting mycelia between clean paper towels, and dried mycelia stored frozen at -20ºC until ground. The mycelium was ground to a powder with liquid nitrogen in a mortar and pestle, placed into a 1.5 ml microfuge tube and stored at -70ºC until DNA was extracted. Fungal DNA was extracted with the CTAB method of Murray and Thomposon (1980).

4.3. RAPD-PCR technique:

For each tested *C. maydis* isolate, 30 ng of the extracted DNA were used for the amplification reaction. All solutions were gently vortexed and briefly centrifuged after thawing. A thin-walled PCR tube was placed on ice and the PCR mixture (2.5 µl of 10X Dream Taq Buffer, 2 µl of dNTP Mix, 2 mM each, 2 µl of primer, 3 µl of 25 mm $MgCl_2$, 0.25 µl of dream Taq DNA polymerase and 1 µl of tamplet DNA) was added. The total volume was adjusted to 25 µl by adding sterile distilled water. The samples were gently vortexed and spined down.

The reactions were placed in a thermal cycler. Perform PCR using the recommended thermal cycling conditions outlined in Table (2).

Table 2. Thermal cycling conditions of PCR reaction

Step	Temperature (°C)	Time	Number of cycles
Initial denaturation	94	5 min	1
Denaturation	94	30 sec	40
Annealing	35	1 min	
Ramp up to 72º C		5 min	
Extension	72	2 min	
Final Extension	72	7 min	1
Final hold	4	∞	-

4.4. DNA electrophoresis:

For all samples, the amplified DNA (7 µl) was electrophoresed using electrophoresis unit (WIDE mini-sub-cell GT Bio-RAD) on 2% Agarose containing ethedium bromide (0.5 µg/ml), at a constant 120 volt and 60 mA, and visualized with UV transilluminator.

4.5. Gel analysis:

DNA gel was scanned for band R_f using gel documentation system (AAB Advanced American Biotechnology 1166E. Valencia Dr. Unit 6C, Fullerton, CA 92631). The different molecular weights of bands were determined against a DNA standard (Gene Ruler^{TM} 100 bp Plus DNA Ladder) with molecular weights of 100, 500, 1000, 3000 and 10000 bp. The similarity level was determined by unweight pair-group method based on arithmetic mean (UPGMA).

Results

1. Morphological diversities of C. maydis isolates:

1.1. Radial growth and growth rates of C. maydis isolates:

Results presented in Table (3) show that *C. maydis* isolates showed clearly variations among them in their growth rates. However, all isolates began to grow with slight differences among them 2 days after incubation, where the radial growth ranged from 0.8 to 2.3 cm. Only, one isolate (isolate No. 28 from Assiut Governorate) was very fast grower, where it filled the whole plates (9 cm) 6 days after incubation with growth rate of 1.8 cm/day. Meanwhile, the majority of isolates (25 isolates) grew at the normal growth rate and filled the whole plates after 7 days and 11 isolates gave the complete growth, 8 days after incubation. The growth rate of these isolates was varied from 1.1 to1.6 cm/day. The slowest isolates were isolates No. 1 and No. 10, which isolated from Gharbiya and Menoufiya Governorates, respectively. They took 10 days to give the complete growth with growth rate 0.9 cm/day.

1.2. Colony colour:

Results shown in Table (4) reveal that *C. maydis* isolates exhibited four different colours, *i.e.* whitish grey, white, grey and dark grey. The majority of isolates seemed to have whitish grey (21 isolates) and white colour (18 isolates). However, only one isolate showed grey colour (isolate No. 19 from Giza). It was found, also, that two isolates, *i.e.* No. 1 from Gharbiya and No. 10 from Menoufiya exhibited the dark grey colour.

1.3. Colony margin:

The aggregates of entwined hyphae terminate into fine ropes, which are often oriented in a clockwise direction, are characterized as rhizoid or semi-rhizoid margin. Meanwhile, slimy colonies with no aerial hyphae are described with entire margins. Results presented in Table (4) reveal that the majority of cultures (31 isolates) showed to have the rhizoid margin. Meanwhile, 7 isolates (Nos. 6, 8, 9, 22, 25, 26 and 29) appeared to have semi-rhizoid margin and 4 isolates (Nos. 1, 10, 19 and 31) seemed to have entire margin.

Table 3. Radial growth (cm) and growth rate (cm/day) of some *C. maydis* isolates, 2-10 days after incubation at 27±2°C

Govern-orate	Isolate No.	Radial growth (cm after day)								Growth rate
		2	3	4	6	7	8	9	10	
Gharbiya	1	1.5	2.9	4.1	5.6	6.7	7.7	8.4	9.0	0.9
	2	1.4	4.5	6.1	8.2	9.0				1.5
	3	1.4	2.9	4.5	7.4	9.0				1.5
Kafr El-Sheikh	4	1.2	3.6	5.2	6.9	8.2	9.0			1.3
	5	1.4	4.7	6.5	8.2	9.0				1.5
	6	1.8	3.2	4.9	6.4	8.2	9.0			1.2
Beheira	7	2.0	4.2	5.7	8.3	9.0				1.4
	8	2.3	3.4	5.2	6.1	7.4	9.0			1.1
	9	1.7	2.8	4.5	7.3	8.2	9.0			1.2
Menoufiya	10	1.9	3.1	3.7	6.0	6.4	7.0	8.1	9.0	0.9
	11	1.9	3.7	5.5	8.0	9.0				1.4
	12	1.8	2.6	3.9	6.3	8.0	9.0			1.2
Dakahliya	13	1.4	4.1	5.8	7.7	9.0				1.5
	14	1.8	5.0	6.8	8.1	9.0				1.4
	15	1.5	4.6	6.3	8.0	9.0				1.5
Qaluobiya	16	2.1	3.8	5.4	8.5	9.0				1.4
	17	1.6	4.2	5.4	8.0	9.0				1.5
	18	1.3	4.8	6.5	8.2	9.0				1.5
Giza	19	2.3	3.4	4.3	6.2	7.3	8.2	9.0		1.0
	20	1.8	4.4	5.7	8.3	9.0				1.4
	21	0.8	4.2	6.0	7.3	9.0				1.6
Beni Suef	22	1.5	2.9	4.3	6.1	7.1	8.1	9.0		1.1
	23	1.5	3.9	5.3	7.5	9.0				1.5
	24	2.2	3.8	5.6	8.3	9.0				1.4
Menia	25	0.9	3.3	5.1	6.6	8.2	9.0			1.4
	26	1.6	4.0	5.7	7.8	9.0				1.5
	27	1	3.9	5.2	5.6	7.6	9.0			1.3
Assiut	28	2.0	4.3	5.9	9.0					1.8
	29	1.5	4.1	5.8	7.7	9.0				1.5
	30	1.2	3.9	5.5	7.9	8.5	9.0			1.3
Qena	31	1.4	2.6	3.7	5.8	6.9	8.4	9.0		1.1
	32	1.4	3.6	5.6	5.9	7.8	9.0			1.3
	33	2.3	4.5	6.1	7.3	9.0				1.3
Sohag	34	1.8	4.3	5.7	8.4	9.0				1.4
	35	1.6	4.1	5.3	8.0	9.0				1.5
	36	1.3	4.1	5.8	7.9	9.0				1.5
Sharkiya	37	1.5	3.9	5.5	7.9	9.0				1.5
	38	1.6	2.5	4.1	7.0	8.2	9.0			1.2
	39	1.2	3.5	4.6	6.8	9.0				1.6
Wadi El-Natroon	40	1.3	4.5	6.5	8.5	9.0				1.5
	41	1.4	4.4	6.1	6.9	8.5	9.0			1.3
	42	1.6	3.5	5.0	8.5	9.0				1.5

Table 4. Growth characters of *C. maydis* isolates: colony colour, colony margin and growth density, 7-10 days after incubation at 27±2°C

Govern-orate	Isolate No.	Character		
		Colony colour	Colony margin	Growth density
Gharbiya	1	Dark gray	Entire	Intermediate
	2	Whitish grey	Rhizoid	Intermediate
	3	Whitish grey	Rhizoid	Thin
Kafr El-Sheikh	4	Whitish grey	Rhizoid	Intermediate
	5	Whitish grey	Rhizoid	Intermediate
	6	Whitish grey	Semi-rhizoid	Intermediate
Beheira	7	White	Rhizoid	Intermediate
	8	White	Semi-rhizoid	Thin
	9	Whitish grey	Semi-rhizoid	Intermediate
Menoufiya	10	Dark grey	Entire	Intermediate
	11	White	Rhizoid	Intermediate
	12	White	Rhizoid	Thin
Dakahliya	13	Whitish grey	Rhizoid	Intermediate
	14	Whitish grey	Rhizoid	Intermediate
	15	White	Rhizoid	Thick
Qaluobiya	16	Whitish grey	Rhizoid	Thin
	17	Whitish grey	Rhizoid	Intermediate
	18	Whitish grey	Rhizoid	Intermediate
Giza	19	Grey	Entire	Intermediate
	20	White	Rhizoid	Intermediate
	21	White	Rhizoid	Intermediate
Beni Suef	22	White	Semi-rhizoid	Thin
	23	Whitish grey	Rhizoid	Intermediate
	24	Whitish grey	Rhizoid	Intermediate
Menia	25	Whitish grey	Semi-rhizoid	Intermediate
	26	White	Semi-rhizoid	Intermediate
	27	Whitish grey	Rhizoid	Intermediate
Assiut	28	Whitish grey	Rhizoid	Intermediate
	29	Whitish grey	Semi-rhizoid	Intermediate
	30	Whitish grey	Rhizoid	Intermediate
Qena	31	White	Entire	Thick
	32	White	Rhizoid	Intermediate
	33	Whitish grey	Rhizoid	Thin
Sohag	34	White	Rhizoid	Intermediate
	35	White	Rhizoid	Thin
	36	White	Rhizoid	Intermediate
Sharkiya	37	Whitish grey	Rhizoid	Intermediate
	38	White	Rhizoid	Intermediate
	39	White	Rhizoid	Thin
Wadi El-Natroon	40	White	Rhizoid	Intermediate
	41	Whitish grey	Rhizoid	Intermediate
	42	White	Rhizoid	Intermediate

1.4. Growth density:

Results presented in Table (4) show that *C. maydis* isolates exhibited three degrees of growth density, *i.e.* thick, intermediate and thin mycelial growth when evaluated at the end of incubation period. The majority of *C. maydis* isolates (32 isolates) displayed intermediate growth. Meanwhile, 8 isolates (isolate No. 3, 8, 12, 16, 22, 33, 35 and 39) exhibited thin growth and two isolates (isolate No. 15 and No. 31) showed thick growth.

1.5. Sporulation rate:

Results in Table (5) reveal that, on the average, *C. maydis* isolates showed apparent variations among them in the number of spores. However, it was found that all *C. maydis* isolates started spores production at the second day of incubation at 27±2°C. Although, in most isolates (36 isolates) the maximum number of spores was observed at the third day of incubation, the other isolates reached the maximum number at the 4^{th} day. Some isolates (18 isolates) stopped production of spores, 5 days after incubation. Meanwhile, the other ones produced a little numbers of spores at the 6^{th} day of incubation. At the seventh day of incubation, it was found that no spores were produced by any isolate. The isolates No. 42 (from Wadi El-Natroon), No. 9 (from Beheira) and No. 24 (from Beni Suef) produced the highest number of spores, being 11.0, 9.6 and 9.5×10^4 spore/ml, respectively. Meanwhile, isolate No.7 from Beheira produced the least number of spores (3.0×10^4 spore/ml).

2. Pathological diversities of C. maydis isolates:

Obtained results (Table 6) reveal that all tested isolates were pathogenic, to different degrees, on Boushy maize cultivar compared with check treatment. Even though, some isolates caused the onset wilt symptoms, 65 days after sowing, wilt symptoms of the other isolates started to appear, 75 and/or 95 days after sowing. However, most isolates, which caused the beginning of symptoms 65 days after sowing, were highly virulent after 95 days, with a few exceptions.

Generally, all the tested *C. maydis* isolates differed in their aggressiveness toward maize plants, where wilt percentages varied between 13.3 and 93.3%, 95 days after sowing. However, isolates No. 1, 35, 42, 26 and 28 (from Gharbiya, Sohag, Wadi El-Natroon, Menia and Assiut, respectively) caused the highest wilt percentages (93.3, 86.7, 86.7, 80.0 and 80.0%, respectively), 95 days after sowing. Meanwhile, isolates No. 15 (from Dakahliya), No. 31 (from Qena) and No. 40 (from Wadi El-Natroon) caused the lowest wilt percentages, being 26.7, 26.7 and 13.3%, respectively, (Table 6).

On the other hand, results presented in Table (6) show that slightly effects of infestation with *C. maydis* isolates were observed on seed germination, plant height and dry weight of the plants. Significant variations in the virulence among *C. maydis* isolates were recorded, whether 65 and 75 or 95 days after sowing. Insignificant variations were observed among isolates in their effect on seed germination. Meanwhile, significant differences were recorded in their effect on plant height and dry weight of plants.

Table 5. Number of spores of *C. maydis* isolates (spore/ml) after several days of incubation at 27±2°C

Govern-orate	Isolate No.	Number of spores per ml after day					
		2	3	4	5	6	Mean
Gharbiya	1	6.0×10^4	14.2×10^4	12.0×10^4	7.6×10^4	0.5×10^4	8.1×10^4
	2	6.4×10^4	21.4×10^4	10.4×10^4	5.8×10^4	1.8×10^4	9.1×10^4
	3	5.6×10^4	17.4×10^4	7.6×10^4	4.8×10^4	0.3×10^4	7.1×10^4
Kafr El-Sheikh	4	5.2×10^4	12.4×10^4	7.2×10^4	3.5×10^4	0.3×10^4	5.7×10^4
	5	1.4×10^4	9.0×10^4	7.4×10^4	0.8×10^4		4.6×10^4
	6	4.2×10^4	17.4×10^4	6.8×10^4	1.3×10^4		7.4×10^4
Beheira	7	1.4×10^4	7.0×10^4	3.3×10^4	0.3×10^4		3.0×10^4
	8	2.3×10^4	10.4×10^4	7.4×10^4	2.3×10^4	0.3×10^4	4.5×10^4
	9	6.6×10^4	19.6×10^4	12.8×10^4	7.5×10^4	1.5×10^4	9.6×10^4
Menoufiya	10	2.0×10^4	8.2×10^4	6.0×10^4	1.0×10^4		4.3×10^4
	11	5.5×10^4	20.6×10^4	9.0×10^4	4.8×10^4	0.3×10^4	8.0×10^4
	12	4.2×10^4	11.2×10^4	10.3×10^4	6.5×10^4	0.5×10^4	6.5×10^4
Dakahliya	13	2.9×10^4	6.4×10^4	8.6×10^4	3.5×10^4	0.3×10^4	4.3×10^4
	14	1.8×10^4	8.4×10^4	7.8×10^4	1.3×10^4		4.8×10^4
	15	6.2×10^4	11.4×10^4	9.2×10^4	2.5×10^4	0.2×10^4	5.9×10^4
Qaluobiya	16	7.4×10^4	16.6×10^4	8.4×10^4	2.9×10^4	0.2×10^4	7.1×10^4
	17	1.6×10^4	9.0×10^4	4.0×10^4	1.6×10^4		4.1×10^4
	18	1.5×10^4	9.4×10^4	5.0×10^4	1.0×10^4		4.2×10^4
Giza	19	3.6×10^4	13.8×10^4	9.2×10^4	3.4×10^4	0.2×10^4	6.0×10^4
	20	2.8×10^4	8.5×10^4	6.8×10^4	1.5×10^4		4.9×10^4
	21	5.6×10^4	18.4×10^4	8.6×10^4	2.5×10^4	0.2×10^4	7.1×10^4
Beni Suef	22	4.8×10^4	11.2×10^4	4.0×10^4	0.8×10^4		5.2×10^4
	23	2.0×10^4	8.0×10^4	9.0×10^4	2.5×10^4	0.2×10^4	4.3×10^4
	24	8.5×10^4	21.0×10^4	11.5×10^4	5.3×10^4	1.3×10^4	9.5×10^4
Menia	25	3.2×10^4	7.2×10^4	3.5×10^4	0.5×10^4		3.6×10^4
	26	7.5×10^4	13.2×10^4	1.6×10^4	7.0×10^4	0.8×10^4	6.0×10^4
	27	8.3×10^4	13.8×10^4	15.6×10^4	5.5×10^4	0.3×10^4	8.7×10^4
Assiut	28	2.5×10^4	7.0×10^4	8.2×10^4	1.5×10^4		4.8×10^4
	29	4.6×10^4	9.4×10^4	15.0×10^4	7.3×10^4	0.8×10^4	7.4×10^4
	30	4.2×10^4	16.0×10^4	7.2×10^4	2.8×10^4	0.5×10^4	6.1×10^4
Qena	31	1.4×10^4	9.8×10^4	3.4×10^4	0.2×10^4		3.7×10^4
	32	2.8×10^4	17.2×10^4	9.2×10^4	5.3×10^4	1.0×10^4	7.1×10^4
	33	1.8×10^4	12.3×10^4	5.6×10^4	1.2×10^4		4.2×10^4
Sohag	34	2.6×10^4	13.0×10^4	4.6×10^4	0.8×10^4		5.2×10^4
	35	1.6×10^4	9.4×10^4	6.4×10^4	1.0×10^4		4.6×10^4
	36	5.6×10^4	14.0×10^4	10.0×10^4	4.3×10^4	0.2×10^4	6.8×10^4
Sharkiya	37	6.4×10^4	14.0×10^4	10.4×10^4	5.5×10^4	0.8×10^4	7.4×10^4
	38	6.0×10^4	8.6×10^4	5.8×10^4	0.6×10^4		5.2×10^4
	39	2.2×10^4	13.2×10^4	5.2×10^4	1.5×10^4		5.5×10^4
Wadi El-Natroon	40	1.8×10^4	5.0×10^4	8.6×10^4	2.5×10^4	0.2×10^4	3.6×10^4
	41	1.2×10^4	8.5×10^4	3.2×10^4	0.3×10^4		3.3×10^4
	42	8.8×10^4	26.4×10^4	10.8×10^4	7.5×10^4	1.3×10^4	11.0×10^4

Table 6. Virulence of *C. maydis* isolates on maize (cv. Boushy) under greenhouse conditions during 2012 growing season

Governorate	Isolate No.	Wilt (%) after days			Plant growth vigour's		
		65	75	95	G (%)*	P.H. (cm)	D.W. (g)
Gharbiya	1	20.0	73.3	93.3	85.7	68.3	14.7
	2	0.0	13.3	40.0	85.7	73.5	27.3
	3	0.0	33.3	60.0	81.0	71.8	24.7
Kafr El-Sheikh	4	6.7	60.0	66.7	81.0	72.5	25.7
	5	0.0	26.7	46.7	90.5	73.8	27.1
	6	0.0	13.3	46.7	81.0	74.1	26.5
Beheira	7	6.7	33.3	53.3	90.5	73.3	25.6
	8	0.0	0.0	40.0	90.5	73.8	26.0
	9	6.7	73.3	73.3	81.0	70.3	19.6
Menoufiya	10	6.7	40.0	73.3	90.5	69.6	20.1
	11	6.7	46.7	66.7	85.7	72.7	24.8
	12	6.7	33.3	46.7	90.5	73.9	26.8
Dakahliya	13	6.7	20.0	53.3	90.5	73.6	24.2
	14	6.7	6.7	60.0	85.7	71.6	24.2
	15	0.0	20.0	26.7	85.7	75.5	28.2
Qaluobiya	16	6.7	33.3	46.7	85.7	73.9	25.2
	17	0.0	20.0	53.3	81.0	73.1	25.0
	18	6.7	40.0	60.0	76.2	72.2	24.6
Giza	19	13.3	53.3	66.7	81.0	72.0	24.5
	20	0.0	26.7	46.7	85.7	74.1	26.5
	21	0.0	33.3	66.7	85.7	71.4	24.6
Beni Suef	22	0.0	6.7	66.7	81.0	72.1	24.5
	23	6.7	20.0	46.7	90.5	73.3	25.8
	24	20.0	60.0	73.3	85.7	71.5	19.4
Menia	25	6.7	53.3	66.7	85.7	71.5	24.7
	26	13.3	60.0	80.0	81.0	69.7	18.0
	27	0.0	0.0	40.0	90.5	74.2	27.5
Assiut	28	6.7	40.0	80.0	85.7	69.4	17.3
	29	0.0	20.0	53.3	85.7	73.2	24.8
	30	0.0	46.7	73.3	90.5	71.4	18.2
Qena	31	6.7	13.3	26.7	85.7	75.2	26.5
	32	0.0	33.3	60.0	76.2	72.8	25.3
	33	0.0	13.3	40.0	81.0	73.4	27.3
Sohag	34	6.7	53.3	73.3	81.0	71.4	17.9
	35	13.3	60.0	86.7	85.7	70.1	17.2
	36	0.0	6.7	46.7	90.5	73.4	25.8
Sharkiya	37	13.3	66.7	66.7	90.5	72.5	24.5
	38	0.0	13.3	60.0	85.7	72.8	23.9
	39	0.0	6.7	60.0	76.2	73.0	23.6
Wadi El-Natroon	40	0.0	0.0	13.3	85.7	76.6	29.0
	41	0.0	0.0	66.7	90.5	72.7	24.2
	42	20.0	66.7	86.7	85.7	68.9	16.1
Control		0.0	0.0	0.0	90.5	76.7	29.0
LSD at 0.05		0.6	0.5	0.5	-	1.9	2.3

* Seed germination 10 days after sowing. ** Wilt data were transformed into arc sine angles while seed germination data were square root transformed before carrying out the analysis of variance.

Results in Table (7) representing the correlations among variables which used for evaluating virulence of the tested *C. maydis* isolates. Highly significant positive correlation was observed between wilt percentage after 65 days and each of wilt (%) after 75 and/or 95days. In addition, wilt percentage after 95 days was positively correlated with wilt (%) after 75 days. On the other hand, a highly negative correlation was observed among wilt percentages after 65, 75 and 95 days and each of plant height and dry weight of plants. Moreover, insignificant correlation was recorded among wilt percentages after 65, 75 and 95 days and seed germination percentage.

Table 7. Correlation coefficients among variables used for evaluating pathogenicity of *C. maydis* isolates tested on maize cv. Boushy

Tested variable	Infection (%) after 65 days	Infection (%) after 75 days	Infection (%) after 95 days	Germination (%)	Plant height	Dry weight
Infection (%) after 65 days	1.000	0.761**	0.607**	0.031	- 0.610**	- 0.654**
Infection (%) after 75 days	----	1.000	0.732**	- 0.153	- 0.698**	- 0.683**
Infection (%) after 95 days	----	----	1.000	- 0.158	- 0.942**	- 0.870**
Germination (%)	----	----	----	1.000	0.137	0.076
Plant height	----	----	----	----	1.000	0.894**
Dry weight	----	----	----	----	----	1.000

** Person's correlation is significant at the 0.01 level.

3. Correlation between morphological and pathological characters of C. maydis isolates:

The Person's correlation coefficients between *C. maydis* isolates characters were calculated by analysing the essential data using SPSS program. Results presented in Table (8) reveal the correlations among variables used for evaluating morphological and pathological variations of the tested *C. maydis* isolates. It was found that no correlation was observed between any of the studied characters, except the negative correlation between colony margin and growth rate.

Table 8. Correlation coefficients among variables used for evaluating morphological and pathological characters of *C. maydis* isolates

Tested variable	Colony colour	Colony margin	Growth density	Growth rate	No. of spores/ ml	Virulence (%)
Colony colour	1.00	----	----	----	----	----
Colony margin	- 0.04	1.00	----	----	----	----
Growth density	- 0.21	- 0.05	1.00	----	----	----
Growth rate	0.08	- 0.45**	0.10	1.00	----	----
No. of spores/ ml	- 0.11	0.10	0.16	0.02	1.00	----
Virulence (%)	- 0.18	0.12	0.19	- 0.07	0.29	1.00

** Person's correlation is significant at the 0.01 level.

4. Genetic variability in C. maydis isolates:

Results presented in Table (9) and Figs. (1 and 2) suggest that only two isolates (No. 10 and 14) belonged to lineage I with 63 and 74% genetic similarity (GS), respectively. Meanwhile, lineage II included isolates No. 24, 19, 28 and 26 with 80, 73, 67 and 76% GS, respectively. Furthermore, Lineage III incorporated isolates No. 35, 9, 37, 32, 1, 18 and 50 with 80, 77, 69, 76, 74, 76 and 74% GS, respectively. Meanwhile, only isolate No. 4 belonged to lineage IV with 69% GS.

Generally, two major groups were observed in the dendrogram based on the banding patterns of the tested *C. maydis* isolates with 61% GS between them. The first group divided into two overlapping clusters with 74% GS, which included lineages I and II as well as its belonged isolates. The second major group, also, divided into two sub-groups with 74% GS, the first one incorporated the lineage IV and isolate No. 4 that belonged it. Meanwhile the second sub-group separated into three overlapping clusters, the first one included the Lineage III while the other clusters incorporated the isolates that belonged this lineage with 76 and 80% GS among the three clusters (Figs. 1 and 2).

Obtained results, also, reveal that most of Lower Egypt Governorates isolates belonged to lineages I, III and IV. Meanwhile, isolates of Middle Egypt Governorates belonged to lineage II. Moreover, isolates of Upper Egypt Governorates belonged to lineages II and III.

With reference to morphological and pathological characters of the tested isolates, no relationship between RAPD patterns and the morphological characters was observed. Meanwhile, it was noticed that, on average, isolates of lineages III caused the highest infection percentage (75.2 %) followed by isolates of lineage II (75%). Meanwhile, isolates of lineages I and IV caused the lowest infection percentages, being 66.7%.

Table 9. Genetic similarity (%) among 14 isolates of *C. maydis* and the reference ones represented the four lineages

Isolate No.	Governorate	Lineage I	Lineage II	Lineage III	Lineage IV
1	Gharbiya	67	59	74	52
4	Kafr El-Sheikh	67	50	67	69
9	Beheira	64	48	77	59
10	Menoufiya	63	61	45	44
14	Dakahliya	74	53	56	48
18	Qaluobiya	67	59	76	52
19	Giza	53	73	48	59
24	Beni Suef	71	80	40	63
26	Menia	63	67	45	67
28	Assiut	50	67	55	44
32	Qena	53	53	76	56
35	Sohag	58	50	80	62
37	Sharkiya	61	53	69	64
42	Wadi El-Natroon	67	59	74	61

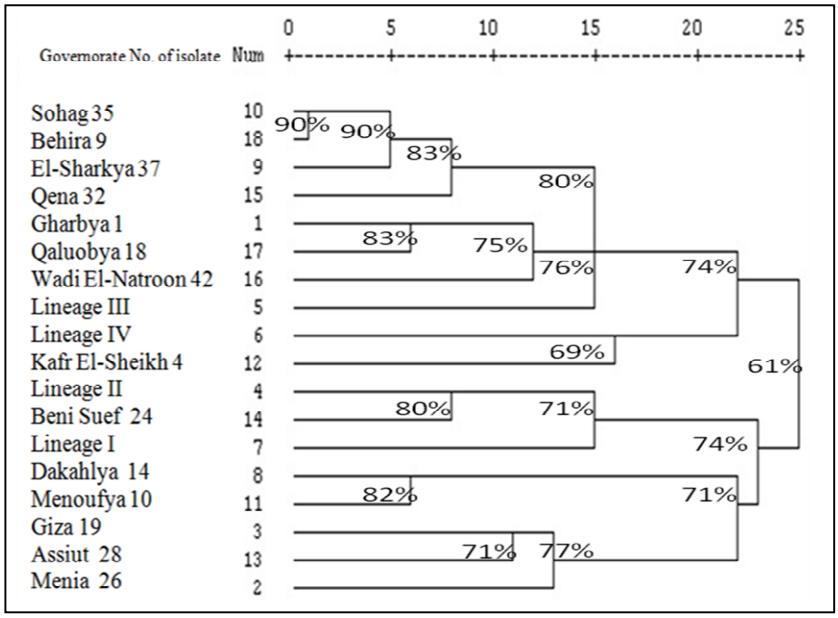

Fig. 1. Dendrogram derived from RAPD analysis of 18 isolates of *C. maydis*.

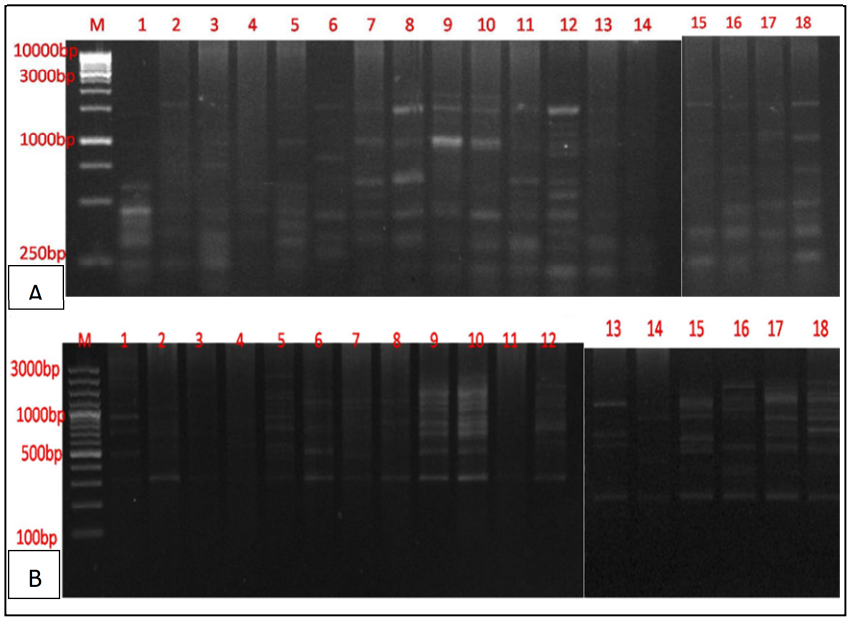

Fig. 2. Banding pattern of 18 isolates of *C. maydis* determined by primers 4 and 8 (A and B, respectively).

Discussion

In current study, the morphological, pathological and possible genetic variations among *C. maydis* isolates, obtained from 13 Egyptian Governorates, were studied. Also, the correlation between the fungus characters was considered.

Obtained results revealed that clear differences among isolates in their growth rates were recorded. The radial growth of most isolates under study appeared to be moderately rapid giving 9-cm-diam. on PDAY after 7 days (60% of isolates) and 8 days (26% of isolates) of incubation at 27±2°C as recorded by Samra *et al.* (1963). Some isolates were slow growers and others were fast growers comparable to those of normal radial growth. The fast growing isolates (2% of isolates) filled the whole plates 6 days after incubation. Meanwhile, the slow growing ones (12% of isolates) needed 9-10 days of incubation to fill the plates. These results are in accordance with those obtained by many investigators (Samra *et al.*, 1966, Ali, 2000 and Drori *et al.*, 2013). Also, Refaat (1979) found wide differences among *C. maydis* isolates in their radial growth.

In this study, the majority of *C. maydis* isolates appeared whitish grey (50% of isolates) and white (43% of isolates) colour, while other isolates showed grey (2% of isolates) and dark grey (5% of isolates) colour. These results are in agreement with those obtained by Samra *et al.* (1963) in their original description of the fungus. They noted that the culture of the fungus vary in colour from greyish white to slate grey. Also, Ali (2000) found that the majority of *C. maydis* isolates had greyish colour.

On the other hand, the colony margin of the tested *C. maydis* cultures were clearly differed, which had rhizoid, semi-rhizoid or entire margin. The majority of cultures (74% of isolates) showed the rhizoid margin, while 17% of isolates had semi-rhizoid and 9% of them had entire margin. These findings are in agreement with those stated by Samra *et al.* (1963) and Ali (2000) who reported that the majority of *C. maydis* isolates had rhizoid or semi-rhizoid margins. Also, Drori *et al.* (2013) mentioned that the margins of the older colonies of *C. maydis* (10 to 21 days) had a rhizoidal or hyphal rope appearance.

Concerning to growth density of *C. maydis* cultures, results showed that 76% of the tested isolates exhibited intermediate growth comparable to other cultures, which showed the thin (19% of isolates) or thick growth (5% of isolates). Similar results were obtained by many researchers (Sabet *et al.*, 1966 and Ali, 2000).

Also, obtained results revealed that, on the average, apparently differences in the number of spores were recorded among the tested isolates. In all isolates, spore production was initiated at the second day of incubation at 27±2°C, and stopped after the 6th day. The maximum number of spores was recorded at the third and fourth day of incubation. These findings are in harmony with that reported by Sabet *et al.* (1966) who stated that conidia of *C. maydis* disappear in agar cultures more than 6-day-old. It is well known that there is variability in the sporulation capability within the same fungal species isolates (Kanatani and Takeda, 1991).

Regarding differences in virulence, the results revealed that all the tested *C. maydis* isolates were pathogenic and differed in their aggressiveness toward maize (cv. Boushy) plants. Last recorded wilt data (95 days after sowing) were very high scoring, with a few exceptions, in the majority of the isolates. Meanwhile, readings made 65 and 75 days after sowing were variable. These findings are in agreement with those obtained by several researchers (Sabet *et al.,* 1966; Drori *et al.,* 2013 and Degani and Cernica, 2014). Also, García-Carneros *et al.* (2012) found that the initial incidence of late wilt symptoms in maize plants was significantly dependent on the isolate of *C. maydis* and on the maize variety.

It has been found that, a highly negative correlation was observed between infection percentages and each of plant height or dry weight of plants, while insignificant correlation was recorded between disease incidence and seed germination percentage. These results are in harmony with the findings of Molinero-Ruiz *et al.* (2010) and García-Carneros *et al.* (2012) who reported that the infestation with *C. maydis* isolates significantly reduced the root weight of maize and led to significant low weight of aboveground parts.

The obtained results revealed that no correlation was observed between any of studied characters, *i.e.* growth rate, colony colour, colony margin, growth density and number of spores and the virulence of the tested isolates. Also, no correlation between growth rates and number of spores was noticed. These findings are in consistence with those reported by Ali (2000). But it was in contrast with the results of Samra *et al.* (1966) who correlated between morphological characters and virulence of *C. maydis* and stated that the black variants of the fungus were avirulent to maize plants compared to the white variants, while the grey variants were the most virulent to the host.

The genetic variability among 14 isolates of *C. maydis* and four reference ones (representing the four lineages) was investigated using RAPD-PCR technique. The banding patterns generated from tested isolates showed different levels of genetic variability among the isolates and/or the four lineages of *C. maydis*. Moreover, no correlation between RAPD patterns and the morphological characters of tested isolates was observed. On the other hand, the four lineages of isolates correlated somewhat to their geographic origin where lineage II and III were found in both the Nile River Delta and southern Egypt; while lineage I and IV was recovered only from Governorates in the Nile River Delta. These results are in harmony with those reported by Saleh *et al.* (2003) who found that the Egyptian *C. maydis* isolates clustered into four phylogenetic lineages; three of them were found in both the Nile River Delta and southern Egypt while lineage IV was recovered only from the Nile River Delta Governorates. Moreover, Zeller *et al.* (2002) reported that the four clonal lineages of Egyptian *C. maydis* isolates show diversity in AFLP, and differ in colonization ability and virulence on maize.

Also, obtained results revealed that the majority of isolates (50% of isolates) belonged to lineage III and 28.6% of isolates belonged to lineage II. Meanwhile, 14.2 and 7.1% of isolates belonged to lineages I and IV, respectively. By reference to pathogenicity test, isolates of lineages III and II caused the highest infection percentages (on average, 75.2 and 75%, respectively). Several investigators (Zeller

et al., 2002; Molinero-Ruiz *et al.*, 2010 and Drori *et al.,* 2013) studied the genetic diversity among *C. maydis* isolates by using AFLP and ITS techniques. However, Zeller *et al.* (2000) identified four AFLP primer pairs that could be used as markers to determine the distribution of *C. maydis* lineages and to identify new lineages in field populations.

It could be concluded that *C. maydis* isolates were differed in their morphological and pathological characters. No correlation was found between growth characters and the virulence of the tested *C. maydis* isolates. The tested isolates represented the four phylogenetic lineages of fungus that differed in their geographic distribution and in their virulence toward maize plants. The lineages II and III incorporated the highly virulent isolates and distributed in most Egyptian Governorates.

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(Received 20/01/2014;
in revised form 23/02/2014)

التباين بين عزلات الفطر *Cephalosporium maydis* من الناحية المورفولوجية والمرضية والوراثية

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تم دراسة التباين بين ٤٢ عزلة من الفطر *Cephalosporium maydis* المتحصل عليها من ١٣ محافظة واقعة بشمال ووسط وجنوب مصر وذلك من الناحية المورفولوجية والمرضية والوراثية. وقد أظهرت العزلات اختلافات واضحة فيما بينها من حيث نموها الشعاعي ومعدل النمو وتكوين الجراثيم على البيئة الصناعية. عموماً أغلب العزلات نمت بالمعدل الطبيعي (٧ أيام من التحضين) وأظهرت مستعمراتها اللون الأبيض الرمادي أو الأبيض كما كانت حافة المستعمرة جذرية وأظهرت نمو متوسط الكثافة. أيضاً معظم العزلات أنتجت العدد الأكبر من الجراثيم في اليوم الثالث من التحضين بينما توقفت العزلات عن إنتاج الجراثيم بعد اليوم السادس من التحضين.

وكانت جميع عزلات الفطر *C. maydis* المدروسة قادرة على إصابة نباتات الذرة الشامية (صنف بوشي) تحت ظروف الصوبة ولكن اختلفت في شدتها المرضية. ظهرت بداية أعراض الإصابة بالذبول المتأخر ببعض العزلات بعد ٦٥ يوم من الزراعة في حين لوحظت أعراض الإصابة بالذبول بالعزلات الأخرى بعد ٧٥ و ٩٥ يوم. وكانت العزلة رقم ٩ من محافظة الغربية الأكثر شدة مرضية في حين كانت العزلة رقم ٤٨ من وادي النطرون الأقل شدة مرضية. ووجد ارتباط سلبي عالي بين نسبة الإصابة وكل من ارتفاع النبات والوزن الجاف للنبات. في حين لم يلاحظ وجود أي ارتباط بين الخواص المورفولوجية والشدة المرضية للعزلات المدروسة.

كما تمت دراسة التباين الوراثي بين ١٤ عزلة من الفطر *C. maydis* باستخدام تقنية RAPD-PCR ولوحظ وجود نسب مختلفة من التباينات الوراثية بين العزلات المدروسة والعزلات المرجعية حيث توزعت العزلات المدروسة الى أربع مجموعات وراثية. لم يلاحظ وجود أي ارتباط بين أنماط RAPD والخصائص المورفولوجية للعزلات المدروسة ، في حين لوحظ أن المجموعات الأربع للعزلات ارتبطت نوعاً ما بكل من المنطقة الجغرافية للعزلة والشدة المرضية.