

PCR-Based Assay for Detection of *Harpophora maydis* the Causal Agent of Late Wilt Disease in Maize Plant Parts

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Importance of maize late wilt caused by *Harpophora maydis* (*Cephalosporium maydis* Smra, Sabet & Hingorani) comes from the sudden and relatively rapid wilt of maize plants at the time of flowering and until shortly before maturity. So, the detection of the causal agent early before symptoms appearance may be useful for managing the disease. For this purpose, two maize plants were chosen randomly at 7, 21, 35, 49, 63, 77, 91 and 105 days after seeding in greenhouse and field infested soils, during 2016 and 2017 seasons. Fungal DNA was detected in emerged plant parts using the specific primer of *H. maydis*. The first detection of the fungal DNA was at 7d after seeding in roots and leaves of greenhouse and field seedlings. At the age of 21, 35, 49, 63 days the fungus was detected in all tested plant parts. With pathogen progression, from 77d to 105 d after sowing, clear amplified fungal DNA was found in samples taken from stalk-internodes, tassels, ear-shanks, cobs, kernels and leaves with the consistent of disease symptoms. But the unique band of *H. maydis* did not appear in the amplified DNA taken from mature kernels at the age of 105 days. As a conclusion of the obtained results, it is clearly pronounced that *H. maydis* colonized all maize plant parts emerged during the growing season. Also, polymerase chain reaction (PCR)-based assay was successfully employed to identify *H. maydis* directly from the mycelia that embedded in maize plant tissues, with no need to obtain pure fungal cultures for detection compared with traditional method. Thus, this protocol can be used for diagnosis of infected plants by late wilt causal agent.

Keywords: PCR-based assay, *Harpophora maydis*, *Cephalosporium maydis*, detection, specific primer, Maize, Late wilt.

Harpophora maydis Gams (Synonym *Cephalosporium maydis* Samra, Sabet & Hingorani), is one of the most important soilborne and seedborne fungi (El-shafey *et al.*, 1991 and Michail *et al.*, 1999), attacks maize in Egypt causing late wilt. The disease acts as a principle limiting factor in maize production. It was first reported in Egypt in 1960 and the causal organism was identified as *Cephalosporium maydis* (Samra *et al.*, 1962 and 1963). *C. maydis* renamed by Gams (2000) as *H. maydis*, hence *Harpophora* spp are characterized by fast-growing, thin colonies with sickle-shaped conidia. As well as older hyphae are heavily pigmented, younger hyphae are

nearly hyaline, and phialides are intermediate in pigmentation relative to the younger hyphae. *H. maydis* reproduces asexually, and no perfect state has been identified (Saleh and Leslie, 2004). Infection of maize occurs through the roots or mesocotyl (Sabet *et al.*, 1970a). The fungus grows superficially on the roots, producing hyphae with short, thick-walled, swollen cells. After penetration, the fungus colonizes xylem tissue at 21 days after sowing and rapidly translocated to the upper parts of the plant. When infections are severe, the fungus colonizes the kernels, resulting in seed-borne dissemination and also causes seed rot and damping off (El-Shafey and Claflin, 1999; Michail *et al.*, 1999 and Payak *et al.*, 1970). The most common character distinguished late wilt disease is relatively rapid wilting of maize plants appeared at flowering stage and until shortly before maturity. The wilting progresses from the lower to the upper portions of the plant. Leaves become dull green, eventually lose color and become dry. Vascular bundles in the stalk become reddish brown, lower internodes shrunken and dry (Samra *et al.*, 1963; El-Shafey and Claflin, 1999). The geographic distribution of the disease is expanded; India (Payak *et al.*, 1970), Portugal and Spain (Molinero-Ruiz *et al.*, 2011), Italy and Hungary (Pecsi and Nemeth, 1998) and Israel (Drori *et al.*, 2013). In addition, latent infection of the causal organism is existed (El-Naggar and Sabry, 2011 and Drori *et al.*, 2013). Seed quantity is negatively correlated to disease severity (El-naggar *et al.*, 2015). Yield losses of up to 52 % have been reported (El-Naggar *et al.*, 2015). The degree of losses depends on the reaction of cultivated varieties and the time of symptoms appearance (El-Shehawy *et al.*, 2014; El-Naggar *et al.*, 2015 and Johal *et al.*, 2004). During the last decades, the use of molecular markers based on the polymerase chain reaction (PCR) for species identification and as a diagnostic tool became very popular (Nicholson *et al.*, 1996; Murillo *et al.*, 1998; Moller *et al.*, 1999; Drori *et al.*, 2013 and Degani and Cernica, 2014). *H. maydis* specific oligonucleotides were designed and used as primers for *in vitro* DNA amplification by the polymerase chain reaction (Saleh and Leslie, 2004). Recently, Drori *et al.* (2013) modified the primer and produced a new sequence (200 bP) which is a major part of a larger AFLP fragment that was previously proved to be species-specific (Saleh and Leslie, 2004). Drori *et al.* (2013) seeded two sweet corn cultivars (susceptible and partial resistant to late wilt) in *H. maydis* infested field for assessing resistance of maize varieties and fungus molecular diagnosis. They arbitrary selected plants at the age of 22-78 days with approximately 7 days intervals for DNA isolation from roots, stems, leaves and kernels. They found that the first detection of the fungus was in the age of 43 days in roots and stems and at 78 d in kernels at eating stage. The authors used a narrow period (78 days) out of long maize growing season (approximately 120 days) and also selected portions of some not all plant parts in their studies. So, we need more details about the movement or development of *H. maydis* in maize plant parts during the growing period. The aim of this study is to answer the question of; in which maize plant parts *H. maydis* colonizes during greenhouse and field growing season using polymerase chain reaction (PCR)-based

assay with its unique primer?. This may help to apply any possible treatment to reduce disease appearance especially at early stages of plant age.

Materials and Methods

Harpophora maydis isolates:

Five single spore cultures of *H. maydis* were used in this study. The isolates were recovered from wilting plants obtained from Gharbia (Hm-1), Kafr El Sheikh (Hm-2), Beheira (Hm-3), Beni Sweif (Hm-4) and Minia (Hm-5) governorates maize fields. All isolates were previously identified as *H. maydis* depending on cultural and microscopic characteristics (Samra *et al.*, 1963). Herein, we used the modified unique oligonucleotides primer for *H. maydis* diagnosis (Drori *et al.*, 2013) as the molecular diagnostic tool to satisfy the previous identification.

Inoculum preparation:

A clean grain sorghum seeds (100g) were soaked in water overnight in 500 ml glucose glass bottle, and then the excess water was decanted and autoclaved for 1 hour. One piece, 2 cm², from 7-d-old *H. maydis* culture (grown on PDA + 0.2% yeast extract at 27°C) was transferred to each bottle and incubated at room temperature (27°C±2) for 15 days and afterward the bottles were used for soil infestation of greenhouse experiments (Sabet *et al.*, 1966; El-Shabrawy and Shehata, 2018; El-Gazzar *et al.*, 2018).

Maize hybrids screening for susceptibility to late wilt:

Thirteen maize hybrids (Table 1) were cultivated for testing their reaction against *H. maydis* to select the susceptible one for further studies. The tested hybrids were obtained from Agricultural research Center, Maize Research Department (ARC-MRD); HYTECH and PIONEER companies. For soil infestation, *H. maydis* – colonized sorghum seeds of each of the five selected isolates was mixed with equal quantity. Eventually, 200 g of the mixture were added to the loam soil of 10 Kg capacity pots (35 cm in diam.) and mixed (4 pots/ hybrid). Seeds were planted on May 5, 2015 at 2 cm depth, eight seeds per pot. Five maize plants were left in each pot after 15 days of seedling emergence. All cultural practices were done as recommended. The experiment was carried out in a completely randomized design. Disease reaction was recorded as a percent of infection after 90 days from seeding date. Data were analyzed by ANOVA using Duncan's multiple range test with significant level 5% after transformed to Arcsine. ANOVA was performed using COSTAT version 3.03 software (Steel and Torrie, 1980).

Molecular detection of H. maydis:

According to the results of the previous experiment, Maize hybrids screening for susceptibility to late wilt, the susceptible hybrid Pioneer SC 3062 was chosen for greenhouse and field assay.

Greenhouse experiments:

Five seeds of the susceptible hybrid SC3062 were seeded (May 24, 2016) in infested soil of pots (35 cm in diam.), as mentioned before for soil infestation. Two plants were randomly sampled from the age of 7 to 91 days with 15d intervals. Plants were divided according to their emerged principal parts, i.e. roots, stalk-internodes, leaves, ear-shank, cob, kernels and tassel. The stalk internodes of each plant were separated individually and numbered, i.e. stalk-internode number 1, 2 ... etc. Portion (2-3 cm long) of each plant part was wrapped in aluminum foil stripes, labeled and immediately stored at -20°C until further use for DNA extraction. The experiment was repeated as it is on May 29, 2017. All cultural practices were done as recommended.

Field experiment:

Seeds of the susceptible hybrid Pioneer SC 3062 were seeded on May 14, 2016 in late wilt nursery field (Giza Agricultural Research Station, ARC, Egypt). The experiment was carried out in a randomized complete block design with three replicates. Each replicate comprised of 6 rows, 4m long and 70cm apart with 20 cm plant distance. Rows were infested by adding 600 g of *H. maydis* colonized sorghum seeds (prepared as described before) into a groove, 3cm depth and 5cm width, made in each row. Afterward two seeds were planted and covered with soil and irrigated. All cultural practices were done as recommended. Two plants were randomly sampled at the age of 7, 21, 35, 49, 63, 77, 91 and 105 days after planting. Then, each plant was divided separately according to their emerged principal parts as mentioned before in the greenhouse. Samples of 5-10 kernels from the selected plants were used. All samples were wrapped in aluminum foil stripes, labeled and immediately stored at -20°C until further DNA extraction.

Fungal culture:

Fungal culture (Hm-4 isolate) was grown in potato dextrose broth (PDB) (Murillo *et al.*, 1998) supplemented with 0.2 % yeast extract (Sabet *et al.*, 1966) and incubated at 27°C for 7 days. After that, the mycelium was harvested, rinsed thoroughly with sterile distilled water, dried using filter paper (No. 102) and stored at -20°C until used.

DNA extraction from fungal culture and maize plant parts:

To extract DNA from *H. maydis* culture and maize plant parts possibly infected by the fungus, frozen samples were ground to a fine powder by liquid nitrogen using mortar and pestle. Afterward, 0.5 g of each ground sample immediately was stored at -20°C in separate micro-centrifuge tubes (1.5 mL) until used. A modified CTAB method of Allen *et al.* (2006) for DNA extraction was used with a slight modification. Briefly, 1 ml of preheated (65°C for 1-2 hr in water bath, KOTTERMANN - Germany) extraction buffer (100mM Tris-HCL, pH 8; 20 mM EDTA, pH 8; 1.4 M NaCL; 2% CTAB, (W/V) and 1% beta-mercaptoethanol) was added to each tube, vortexed thoroughly for 5-10 s and incubated at 65°C for 30 min

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with inverting the tubes every 5-10 min. After centrifugation (Sigma 1-14) at 12,000 rpm for 5-10 min, the supernatant was transferred into new 2ml microfuge tube then 800 µl of chloroform: isoamyl alcohol (24:1 v/v) were added with mixing 2-3 min. The mixture was centrifuged again (12,000 rpm for 5 min). Afterward, the upper phase was transferred to a fresh, sterile microfuge tube and mixed gently through inversion with 800 µl of phenol: chloroform: isoamyl alcohol (25: 24: 1) at room temperature (RT) for 20 min. Centrifugation was at 12,000 rpm for 5min at RT thereafter the upper layer was carefully transferred to new 2-ml microfuge tube. A pre-cooled isopropanol (stored at -20°C), 800 µl, were added then mixed by inverting the tubes and incubated at RT for 20 min to precipitate the DNA. The supernatant was removed after centrifugation at 10,000 rpm for 5 min at RT and then the pellet was re-suspended in 500 µl TE buffer (10mM Tris-HCL, PH 8.0, 1mM EDTA). A volume of 1 µl of RNase A (10 mg/ml) was added into the sample mixture and then incubated at 37°C for 30 min. Next, 25ul of 3M Sodium acetate (NaAc) were added and mixed, afterward 600 µl of pre-cooled absolute ethanol (-20°C) were added, mixed, then the tubes were incubated at -20°C for 20 min. The mixture was centrifuged at 10,000 rpm for 5 min and the supernatant was carefully decanted. DNA pellet was washed by adding 500 µl of cold (-20°C) 70% (v/v) ethanol, dislodged by quick 1-2 s vortexing and the ethanol was removed after centrifugation at 10,000 rpm for 5 min at RT. DNA pellet was dried at RT under laminar flow to remove the residual ethanol then DNA was dissolved in 50 µl of TE buffer at 4°C. After the DNA was completely dissolved the microfuge tubes were stored at -20°C until used.

Polymerase chain reaction (PCR):

PCR reaction was carried out in a thermal cycler (AB Applied Biosystems, 2720 - Singapore) in a volume of 25 µl: 2 µl primers (20 µM of each primer; A200a, Forward primer: 5'-CCGACGCCTAAAATACAGGA-3'; A200b, Reverse primer: 5'-GGGCTTTTTAGGGCCTTTTT-3'), 12.5 µl of ready-to-PCR reaction mixture (OnePCRTM , GeneDirex), 1 or 2.5 µl DNA sample and 9.5 or 8 µl sterile distilled water (SDW) for fungal and plant samples respectively. A program of one cycle (94°C for 2 min) followed by 35 cycles (94°C for 30 s, 55°C for 30 s, 72°C for 1 min) and a final step of 72°C for 5 min was performed (Drori *et al.*, 2013). Aliquots of 50 % of the PCR product mixture were analyzed on 1.4 % agarose mediumgels (SCIE-PLAS) in TE buffer (10mM Tris-HCL, PH 8.0, 1mM EDTA), containing 0.5 µg/ml ethidium bromide. After electrophoresis (90 V for 1.5 h), a 200-bp amplified DNA band (Drori *et al.*, 2013) was identified under UV transilluminator (Herolab-UVT-40 S/L) in comparison with 100bp DNA ladder (Thermo Scientific, GeneRuler 100bp plus DNA Ladder or 100 bp DNA Ladder, Solis BioDyne) and was photographed by digital still Camera (Sony, Cyber-Shot DSC-W510).

Recovery of H. maydis on PDA:

The same plant parts used for molecular detection of *H. maydis* were used for selecting segments to recovery the fungus on potato dextrose agar (PDA) medium
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(Table 2). Selected segments were sterilized in 70% ethyl alcohol, flamed and split or cut in cross sections with sterile scalpel; small pieces of tissues (5 pieces / plate) were placed on PDA supplemented by 0.2 % yeast extract. *H. mydis* appearance was recorded after 7 days of incubation at 27°C, and then was compared visually and microscopically with the previously identified isolates.

Results

Isolates identification using H. maydis-specific primer:

The 200bp unique band of *H. maydis* was identified with the amplified DNA of the selected five isolates, Hm-1, Hm-2, Hm-3, Hm-4 and Hm-5, used in this study (Fig. 1).

Reaction of thirteen maize hybrids against late wilt:

Data in Table (1) reveal that the tested hybrids showed different reactions to late wilt since the infection ranged from 14.9 to 81.1 %. The hybrid HYTIC SC 2066 exhibited low degree of infection (14.9 %) followed by the hybrids HYTIC SC2055, PIONEER SC30N11 and PIONEER SC30K08 (21.4, 21.4 and 24.1 %, respectively). On the other hand, the hybrid PIONEER SC3062 showed high degree of susceptibility (81.1 %). so, it was selected for further greenhouse and field investigations. Meanwhile, other hybrids were also susceptible (the infection ranged from 26.6 to 60 %).

Table (1): Disease infection (%) of 13 tested maize hybrids against late wilt under greenhouse conditions, 2015.

Tested hybrids	% infection
ARC-MRD-SC 162	60 b*
ARC-MRD-SC173	33.2 de
ARC-MRD-SC168	63.4 b
ARC-MRD-SC130	50.8 c
ARC-MRD-SC131	35 d
ARC-MRD-SC122	43.1 c
HYTECH-SC2066	14.9 g
HYTECH -SC2031	26.6 ef
PIONEER-SC3062	81.1 a
PIONEER-SC30K09	50.8 c
HYTECH -SC2055	21.4 fg
PIONEER-SC30K08	24.1 f
PIONEER-SC30N11	21.4 fg

*= Means followed by the same letter are not significantly different at 5 % level.

Detection of H. maydis DNA in emerged maize plant parts:

To detect *H. maydis* in emerged maize plant parts; roots, stalk-internodes, tassels, cobs, ear-shanks, kernels and leaves during plant development, two greenhouse and one field experiments were conducted in Giza Agricultural Research Station, ARC, Egypt during 2016 and 2017. The sampling program included the susceptible hybrid, PIONEER SC3062, samples were taken from the age of 7d to 91d in the greenhouse and from 7d to 105 d in the field with 15 days intervals. In 2016-greenhouse trail, a unique band (200-bp) of *H. maydis* DNA was detected in roots and leaves of 7d old seedlings (Figs. 2 and 6A). After 21 d and 35 d of seeding, the pathogen DNA was detected also in roots, stalk-internode no.1 and in leaves (Figs. 2 and 6A). In addition, a clear 200-bp band was visualized in all tested DNA samples of roots, leaves and stalk-internodes until number 5, 7 and 10 at the age of 49, 63 and 77 days, respectively (Figs. 2 and 6A). Eventually, after 91 d after sowing the causal agent colonized all tested samples of stalk-internodes, leaves, ears (cobs with initial kernels) and tassels since the 200-bp DNA band was appeared (Figs. 2 and 6).

On the other hand, in 2017-greenhouse experiment, the first detection of *H. maydis* distinguishing band (200-bp) was in roots and leaves samples of 7d old seedlings (Figs.3 and 6B). At the age of 21, 35, 49 and 63 days, the fungus progressed consistently with the emerged plant parts since the pathogen DNA was detected in roots, stalk-internodes and leaves. Also, at the age of 77 days fungal DNA was detected in the tested samples with the exception of roots and tassels. Finally, except of kernel's sample, a clear 200bp band was detected in roots, stalk-internodes, cob and tassel samples taken after 91 days from sowing.

In respect of *H. maydis* detection in 2016-field trail, a 200-bp amplified DNA band was identified in 7d old seedling roots and leaves (Figs. 4 & 6C). Also, *H. maydis* unique band was detected in DNA of roots, stalk-internodes and leaves taken from 21, 35 and 49d old plants (Figs. 4 & 6C). In addition, at the age of 63 days, a clear 200-bp band was appeared in stalk-internodes until no. 9, tassels, leaves, ear-shanks and ears (cobs with initial kernels) (Figs. 4 & 6C). Moreover, at 77 and 91 d after sowing the causal fungus was distributed in all emerged plant parts and colonized roots, leaves, stalk-internodes, tassels, cobs and kernels which reflected by a clear 200-bp DNA band appearance (Figs. 4, 5 & 6C). Later, in samples taken from 105 d old plants, the unique *H. maydis* DNA band was found in stalk-internodes until no. 11, leaves, tassels and cobs but was not detected in kernel's sample (Figs. 5 & 6C).

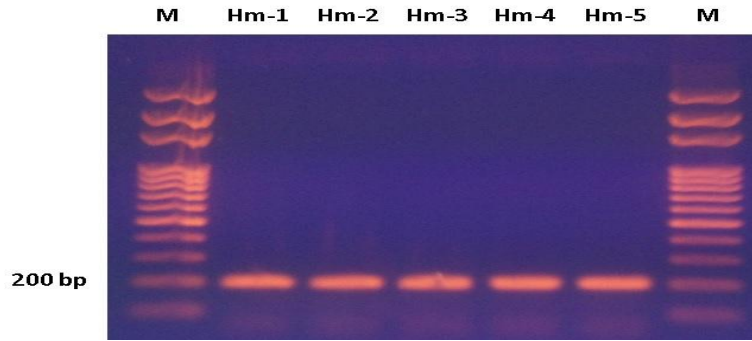


Fig. (1): PCR amplification products obtained with the specific primer of *Harpophora maydis* to identify the fungal isolates. Lanes: M, 100 bp DNA Ladder (Solis BioDyne); Hm-1, genomic DNA of *H. maydis* from Gharbia; Hm-2, genomic DNA of *H. maydis* from Kafr El Sheikh; Hm-3, genomic DNA of *H. maydis* from Beheira; Hm-4, genomic DNA of *H. maydis* from Beni Sweif; Hm-5, genomic DNA of *H. maydis* from Minia governorate maize fields.

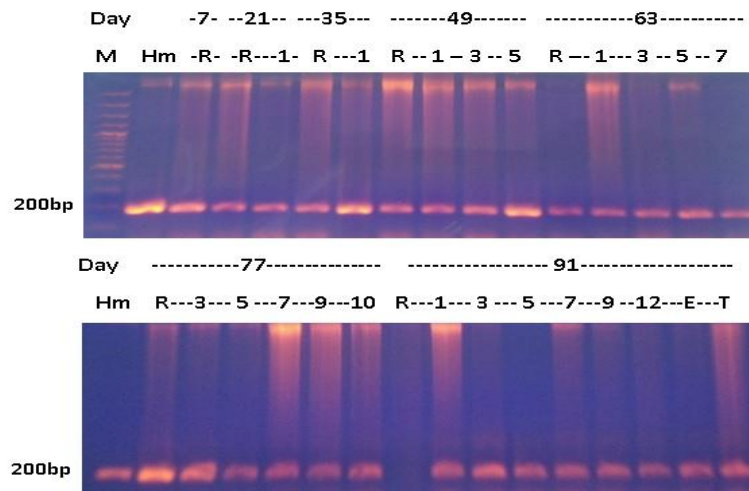


Fig. (2): PCR amplification products obtained with unique *Harpophora maydis* oligonucleotide to detect the fungus in greenhouse maize plant parts (PIONEER SC3062, 2016) from 7 d post sowing until 91 days. Lanes: M, GeneRuler 100 bp Plus DNA Ladder (Thermo Scientific); Hm, genomic DNA of *H. maydis* isolate Hm-4 (Positive control); R, genomic DNA of roots; 1-12: genomic DNA of stalk-internodes from number 1 to 12; E, genomic DNA of cob with initiated seeds; T, genomic DNA of tassel.

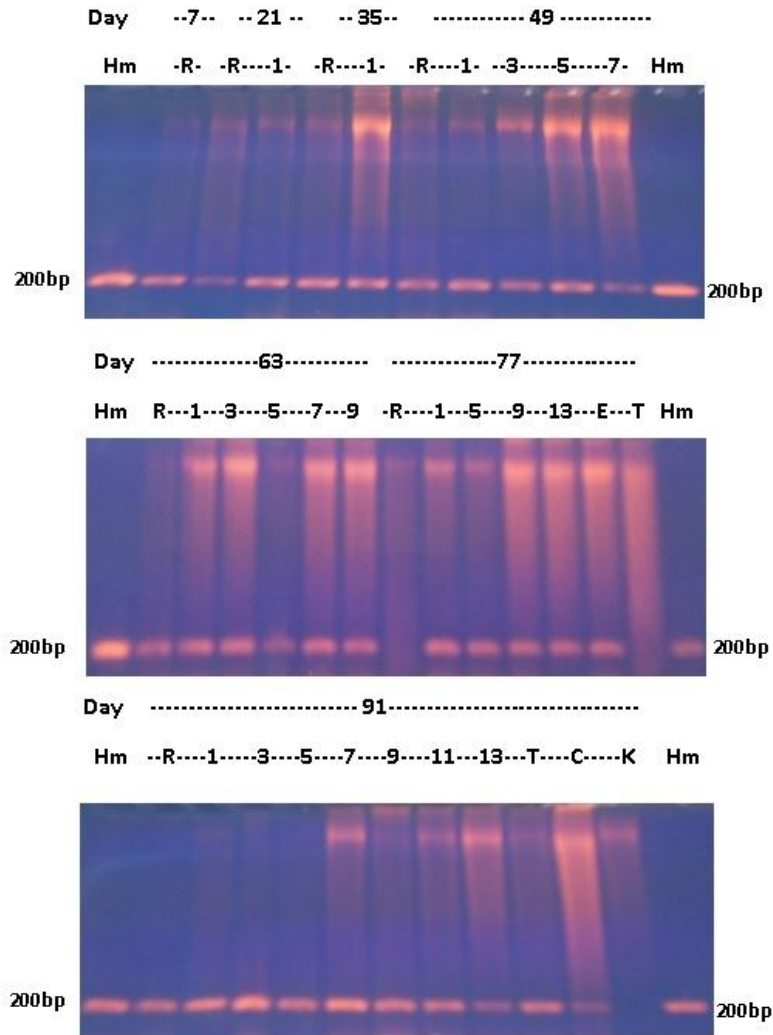


Fig. (3): PCR amplification products obtained with the unique *Harpophora maydis* oligonucleotide to detect the fungus in greenhouse maize plant parts (PIONEER SC3062, 2017) from 7 d post sowing until 91 days. Lanes: Hm, genomic DNA of *H. maydis* isolate Hm-4 (Positive control); R, genomic DNA of roots; 1-13: genomic DNA of stalk-internodes from number 1 to 13; E, genomic DNA of cob with initial seeds; T, genomic DNA of tassel; C, genomic DNA of cob and K, genomic DNA of kernels.

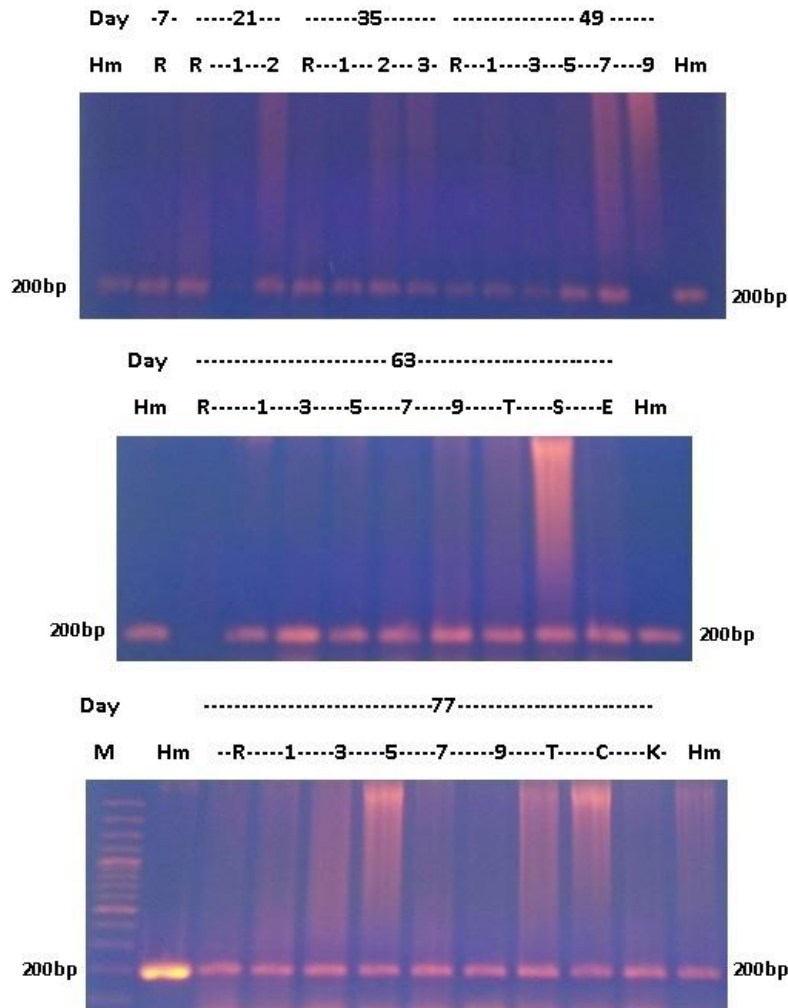


Fig. (4): PCR amplification products obtained with the unique *Harpophora maydis* oligonucleotide to detect the fungus in field maize plant parts (PIONEER SC3062, 2016) from 7 d post sowing until 77 days. Lanes: M, GeneRuler 100 bp Plus DNA Ladder (Thermo Scientific); Hm, genomic DNA of *H. maydis* isolate Hm-4 (Positive control); R, genomic DNA of roots; 1-9: genomic DNA of stalk-internodes from number 1 to 9; T, genomic DNA of tassel; S, genomic DNA of ear-shank; C, genomic DNA of cob; E, genomic DNA of cob with initial seeds; K, genomic DNA of kernels.

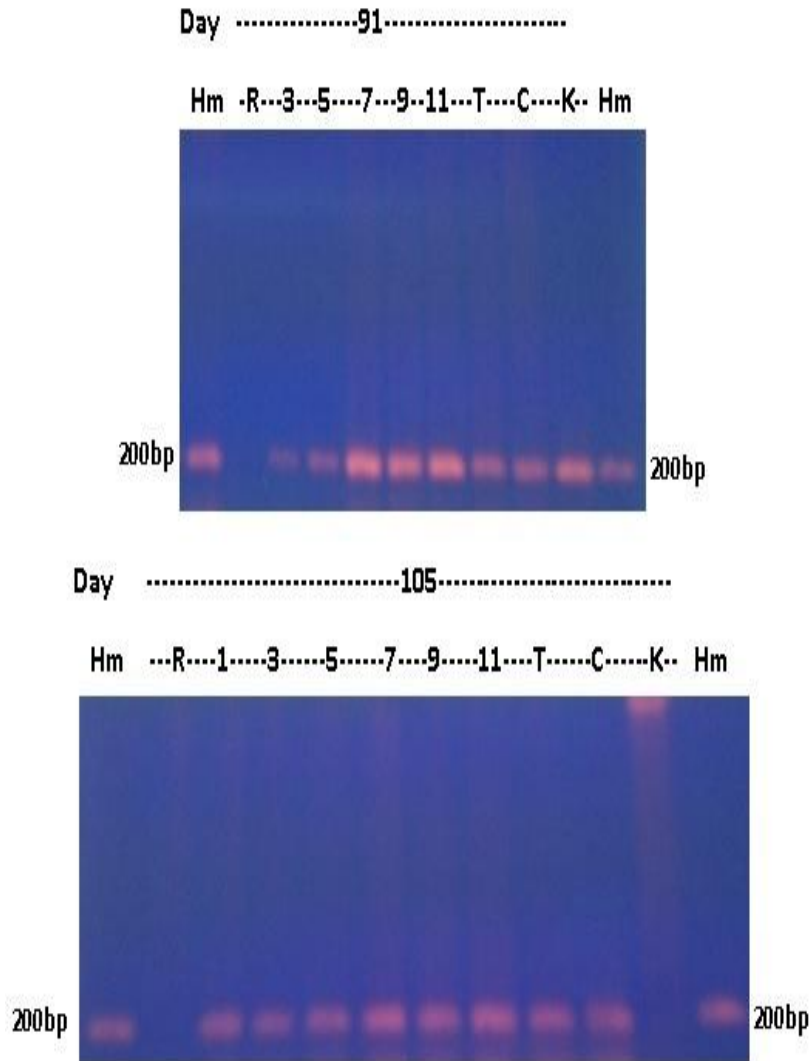


Fig. (5): PCR amplification products obtained with the unique *Harpophora maydis* oligonucleotide to detect the fungus in field maize plant parts (PIONEER SC3062, 2016) at the age of 91 and 105 days. Lanes: Hm, genomic DNA of *H. maydis* isolate Hm-4 (Positive control); R, genomic DNA of roots; 1-11: genomic DNA of stalk-internodes from number 1 to 11; T, C and K, genomic DNA of tassels, cobs and kernels, respectively.

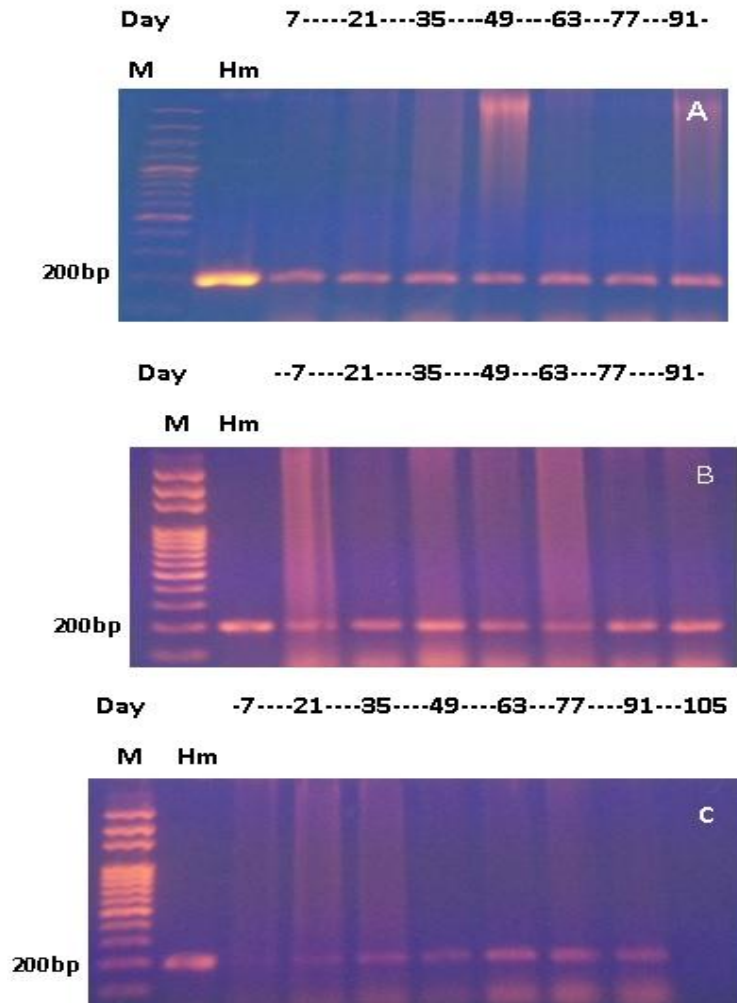


Fig. (6): PCR amplification products obtained with the unique *Harpophora maydis* oligonucleotide to detect the fungus in maize plant leaves (PIONEER SC3062, 2016) from 7 d post sowing until 91 d in the greenhouse (A & B panels; 2016 and 2017, respectively) and until 105 d in the field (C panel; 2016). Lanes: M in panel A, GeneRuler 100 bp Plus DNA Ladder (Thermo Scientific); M in panels B & C, 100 bp DNA Ladder (Solis BioDyne); Hm, genomic DNA of *H. maydis* isolate Hm-4 (Positive control).

Recovery of H. maydis on PDA

The obtained results (Table 2) show that the first recovery of *H. maydis* on PDA was found only in stalk-internode samples taken from greenhouse plants (2016 and 2017) with the age of 63 days. While, at the age of 77 days, the fungus was recovered from root and stalk-internode samples. On the other hand, the fungus was recovered from root, stalk-internode and tassel samples of 91 days old plants. In 2016-field trail, the first recovery of the causal agent of late wilt was appeared in both root and stalk-internode samples taken from plants with the age of 63 and 77 days. Also, the fungus was recovered from root, stalk-internode and tassel samples of both 91 and 105 days old plants. However, the fungus was not recovered from either leaf or kernel samples taken from any plant age.

Table (2): *Harpophora maydis*-recovery from different maize plant parts with or without symptoms at different plant ages ranged from 7 to 105 days after sowing under greenhouse and field conditions, 2016 and 2017 growing seasons.

Exp. name	Plant age /day	Symptoms	<i>H. maydis</i> -recovery from					
			Roots	Stalk internode	Leaves	Tassel	Cob	Kernel
Greenhouse 2016	7	No	-1	-	-			
	21	No	-	-	-			
	35	No	-	-	-			
	49	No	-	-	-			
	63	Two dried leaves	-	+	-	-		
	77	Four dried leaves	+2	+	-	-		
	91	Five dry leaves with streaking in stem	+	+	-	+	-	
Greenhouse 2017	7	No	-	-	-			
	21	No	-	-	-			
	35	No	-	-	-			
	49	No	-	-	-			
	63	Two dried leaves	-	+	-	-		
	77	Three dried leaves	+	+	-	-		
	91	All leaves were dried with streaking in stem	+	+	-	+	-	-
Field 2016	7	No	-	-	-			
	21	No	-	-	-			
	35	No	-	-	-			
	49	No	-	-	-			
	63	No	+	+	-	-		
	77	five dried leaves	+	+	-	-	-	-
	91	All leaves were dried with streaking in stem	+	+	-	+	-	-
	105	All leaves were dried with streaking in stem	+	+	-	+	-	-

1: *H. maydis* was not recovered; 2: *H. maydis* was recovered

Discussion

The importance of the late wilt disease in Egypt and in other countries where it has been reported came from the sudden and rapid wilt of maize plants at the time of flowering until shortly before maturity (Sabet *et al.*, 1966 and Payak *et al.*, 1970) and its impact on yield depends on the time of symptoms appearance (El-naggar *et al.*, 2015). Moreover, in the past late wilt of maize caused by *H. maydis* has existed in Egypt only (Samra *et al.*, 1962 and 1963) and was remained mostly restricted in this country for eight years. But today, the disease is distributed at other countries such as India, Portugal, Spain, Italy, Hungary and in Israel (Payak *et al.*, 1970; Molinero-Ruiz and Melero-Vara *et al.*, 2011; Pecsí and Nemeth, 1998; Drori *et al.*, 2013 and Ortiz-Bustos *et al.*, 2016). So, the detection of the causal agent before the appearance of plant wilting (especially in early stages of plant age) is useful for managing the disease.

Results of the present investigation revealed that the first detection of the fungal DNA was at 7d after seeding in roots and leaves of greenhouse and field seedlings. This means that when the fungus touches with maize roots, its penetration and distribution through the roots and primary leaves tissues required 7days. These results are inconsistent with those obtained by Sabet *et al.* (1970a); Drori *et al.* (2013) as well as Degani and Cernica (2014). Sabet *et al.* (1970a) reported that after the fungus penetrate the plant roots, reached to the root endodermis and the xylem after 15 and 21days of sowing date, respectively. Also, in 2013 Drori *et al.*, used the same specific primer to diagnose the fungus in field sweet corn roots and leaves of 22-78d old plants and they reported that the first detection of the fungus was at 43 days after sowing. Recently, Degani and Cernica (2014) demonstrated that *H. maydis* DNA was clearly identified in roots, stems and leaves of 22d old maize seedlings grown in infested soils. Meanwhile, the obtained results revealed that the DNA of the fungus was existed in roots and primary leaves of 7 days old seedlings.

In this study, at the age of 15, 35, 49, 63 days, the fungus was detected in all emerged plant parts. This result was some consistent with that obtained by Drori *et al.* (2013), since they could diagnose *H. maydis* in sweet corn roots and stems of plants with the age of 43 days. Also, these results are in agreement with those obtained by Sabet *et al.* (1970a) who reported that after 21 days of sowing, the hyphae of the pathogen were almost confined to upwards growth in the vessels, and finally reached vessels of the fourth internode above ground at the age of 7 weeks. Furthermore, with pathogen progression, from 77d to 91 d. after sowing, the obtained results showed that the fungal DNA was clearly detected in all tested stalk-internodes, tassels, ear-shanks, cobs, kernels and leaves consistently with symptoms appearance. These results are consistent with the fact that the causal agent of late wilt is vascular pathogen as well as seed transmitted (Samra *et al.*, 1966; El-Shafey *et al.*, 1991; Michail *et al.*, 1999 and Drori *et al.*, 2013).

In this research, the author could not detect the pathogen in mature kernels (at 105 d after planting) sampled from a plant existed positive detection of pathogen DNA in other tested parts even the cob. Indeed, this may be due to: the fungus did not reach to the kernels, or the fungus entered in dormancy stage with very little amount of DNA unable to detect, or the high levels of polysaccharides frequently present in kernels which significantly affect the efficiency of DNA extraction, and/or due to the presence of PCR inhibitors that can negatively impact on successful amplification of the recovered DNA (De Boer *et al.*, 1995; Hu *et al.*, 1999 and Murillo *et al.*, 1998). So, further attempts are needed to adapt method for *H. maydis* detection or diagnosis in mature or commercial maize seeds.

Furthermore, the obtained results showed that the PCR based method is more efficient for the pathogen detection than that of traditional method. These results have matched with the nature of weak competitive saprophytic ability of *H. maydis* with the other stalk rot pathogens attack maize plants (Sabet *et al.*, 1970b).

In this study, the PCR based assay using *H. maydis* specific primer, (Saleh and Leslie, 2004) which developed by Drori *et al.* (2013), was successfully suitable to detect the causal agent of late wilt in different maize plant parts in relation to the plant age; providing more information about the movement of the fungus in maize from sowing to maturity with clear vision of the causal organism disease cycle. On the other hand, early detection of the fungus in leaves, from 7 to 50 d after sowing, may help researchers and maize breeders to test the plants for *H. maydis* colonization without cutting any plant, hence restrict disease spread (Samra *et al.*, 1966; El-shafey *et al.*, 1991 and Michail *et al.*, 1999). Moreover, early detection of the causal agent may help to apply, agro-mechanic techniques such as intensive watering (Samra *et al.*, 1966), and possibly other treatments to reduce late wilt such as phosphorus, organic amendments, micronutrients (Cu, Fe, Mn, and Zn) and chemical control (Singh and Siradhana, 1990 and Degani *et al.*, 2018). As well as, the detection of the pathogen in leaves at any plant age may help to test the effect of available control methods on *H. maydis* movement inside maize plants (Degani and Cernica, 2014). Eventually, this PCR analysis was successfully employed to identify *H. maydis* directly from the mycelia that embedded in maize plant parts, with no need to obtain pure fungal cultures for diagnosis. Thus, this protocol can be used for diagnosis of infected plants (Mbofung and Pryor 2010 and Lee *et al.*, 2001) by late wilt causal agent.

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الكشف عن الفطر *Harpophora maydis* المسبب
لمرض الذبول المتأخر في أجزاء نباتات الذرة الشامية
باستخدام تحليل يعتمد على ال PCR

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قسم بحوث أمراض الذرة والمحاصيل السكرية ، معهد بحوث أمراض
النباتات ، مركز البحوث الزراعية ، الجيزة ، مصر

تأتي أهمية مرض الذبول المتأخر في الذرة الشامية المتسبب عن الفطر
Harpophora maydis (*Cephalosporium maydis* Smra,
Sabet & Hingorani) من الذبول الفجائي والسريع نسبيا لنباتات الذرة
وقت التزهير والذي قد يمتد حتى قبل النضج بقليل ، لذا فإن الكشف عن
المسبب المرضي في مراحل مبكرة من عمر النبات قبل ظهور الأعراض
ربما يفيد في التحكم بالمرض. ومن أجل هذا الغرض أختبر عشوائيا نباتان
من نباتات الذرة الشامية المنزرعة في تربة معدة بالفطر تحت ظروف
الصوبة والحقل موسمي ٢٠١٦ و ٢٠١٧ وذلك عند عمر ٧ ، ٢١ ، ٣٥ ،
٤٩ ، ٦٣ ، ٧٧ ، ٩١ و ١٠٥ يوم بعد الزراعة. تم الكشف عن المادة
الوراثية للفطر في كل أجزاء النبات المتكشفة باستخدام الريمير (باديء)
المتخصص للفطر *Harpophora maydis*. كان أول إكتشاف ل DNA
(المادة الوراثية) للفطر عند عمر 7 ايام في كل من الجذور والأوراق
للبادرات المنزرعة في كل من الصوبة والحقل. كذلك عثر على الفطر في
كل أجزاء نبات الذرة المختبرة عند عمر ٢١ ، ٣٥ ، ٤٩ و ٦٣ يوم بعد
الزراعة. ومع تقدم الكائن الممرض (من عمر ٧٧ إلى ١٠٥ يوم) إكتشفت
بوضوح المادة الوراثية للفطر (DNA) في كل عقل الساق المختبرة حتى
الأخيرة ، وكذلك في كل من السنابل ، حوامل الكيزان ، القوالج ، الحبوب
، والأوراق وكان ذلك متلازما مع ظهور الأعراض. لم تظهر الباند المحددة
للفطر في ال DNA المستخلص من الحبوب عند عمر ١٠٥ يوم. أوضحت
النتائج المتحصل عليها أن الفطر *Harpophora maydis* يستعمر كل
أجزاء نبات الذرة المتكشفة طوال موسم النمو. وأيضا فإن هذا الإختبار
المعتمد على ال PCR قد نجح في تعريف الفطر *Harpophora*
maydis مباشرة من هيفاته المغمورة داخل أنسجة نباتات الذرة الشامية
دون الحاجة إلى الحصول على المزارع النقية منه من أجل التشخيص
مقارنة بالطرق التقليدية. لذا فيمكن إستخدام هذا الإختبار في تشخيص
النباتات المصابة بالفطر المسبب لمرض الذبول المتأخر.