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Isolation and identification of *Sclerotinia sclerotiorum* the casual agents of white mold in eggplants by polymerase chain reaction (PCR) technique

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Key words: *Sclerotinia sclerotiorum,* PCR , HPLC, oxalic acid.

Abstract

This study was carried out at Plant Protection Dept. / College of Agriculture/ University of Baghdad, in 2016-2017. Due to phenotypic differences between four *Sclerotinia sclerotiorum* isolates, the polymerase chain reaction (PCR) technique using the universal fungal primer pairs of the Internal transcribed spacer 4 and 5 (ITS4/ITS5) was depended to identify. The four *S. sclerotiorum* isolates (SS1, SS2, SS3 and SS4) were isolated from infected eggplants cultivated in plastic houses in Karbala (one isolate) and Baghdad (three isolates). DNA of each isolate was extracted from the sclerotia. Results of sequences analysis revealed that the four isolates shared 99-100% identities with the equivalent sequences of the fungal isolates conserved international Gen Bank, using MEGA6 program variations in morphological characteristics (mycelium growth rate, sclerotia numbers and their distribution in the plate), were found between the isolates on PDA at 25±2°c. Isolates pathogenicity were evaluated on egg plants, 30 days old, in pots under plastic house conditions by inoculating the stem under meristem, without injury, with 5 mm disk of fungal mycelium growth from edge of isolate cultural, 5 days old, and measuring the spot length after 7 days of inoculation. Significant difference in the pathogenicity between the isolates was recorded. SS1 from Karbala was found the more active with lesion length attained to 4.25 cm. Variations in oxalic acid production were manifested between isolates in potato sucrose broth medium (PSB) as determined by high performance liquid chromatography (HPLC) which found to be 10.95, 8.70, 4.90, 8.60 mg/L. of PSB medium for SS1, SS2, SS3, SS4 respectively.

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Introduction

Sclerotinia sclerotiorum, Family: Sclerotiniaceae, Order: Helotiales, Class: Discomycotina, Phylum: Discomycetes, and Fungi kingdom (Bolton *et al*., 2006) .It is considered among the most economic important fungi infecting wide range of economic crops and representing a serious problems in crops production in cold and humid regions as well as in protected cultivations. The pathogen was reported to infect 408 plant species belongs to 278 genus and75 families (Bolland and Hall, 1994) .The fungus was recorded on different plant hosts to Solanaceae, Crucifaceae, Umbellifacerae, Compositae, Chenopodiaceae and Leguminoseae (Willets and Wong , 1980) .

The symptoms caused by the pathogen were found vary with the environmental conditions, host and infected host part (stems, leaves, fruits and petioles).The typical symptoms appeared as water soaked lesions, light to dark brown ridgeling the stem, associated with partial wilt. White cottony mycelium with an irregular dark sclerotia appeared in the pith or on the lesions of infected stem that considered as characteristic sign of pathogen infection (Dilantha Fernando *et al*., 2004).

In 1979, Al-Behadli and Al-Azawi have recorded this disease on eggplants at infection percentages 30-60% under plastic house conditions in Iraq and referred to as white mold disease (Jaber and Habeb,1986) .It has been reported that White mold disease was recorded on mustard cultivation at percentage up to 73.8% in Rajstan (Kang and Chanal, 2000). The disease caused heavy losses reached in sunflower production reached to 15 million dollars in USA (Sayler, 2003).It was indicated that *S. sclerotiorum* produce number of decomposing enzymes that induce decomposition of host cell wall components, including proteases, cellulase, hemicellulase, pectinases (Riou *et al.* , 1991) and acidic protease (Girard , 2004).

The pathogen produce oxalic acid (OA) in the infected tissue which considered as a limiting and essential factor in disease development, where at make acidic environment that activate decomposing enzymes , pulling calcium ions from cell wall rendering pectin part more easy to by decomposed by fungus enzymes as well as inhibited oxidative burst reactions , that represent an important defense response in plant (Marciano, 1982). It was reported that oxalic acid production affected by environmental temperature and pH. The highest production of on by *S. sclerotiourm* was found at 25 °C while the lowest was at30°C,the optimum pH for OA production was 5.5 (Mohammed and Al-Mothefar, 2013). The aim of this study is to isolate and identify *S. sclerotiourm* from different areas in Iraq by polymerase chain reaction (PCR) technique, determine the pathogenicity of the isolates on eggplants and evaluate their ability to produce oxalic acid (OA) under laboratory conditions.

Materials and methods

Sclerotinia sclerotiourm Isolation

The sclerotia were collected from infected eggplant stems showing symptoms suspected to be caused by *S. sclerotiourm* grown in plastic houses in Karbala and Baghdad (Al-Gadria, Abu-Graib/College of Agriculture, Al-Rathwania) areas. The sclerotia were surface sterilized by Alcohol 70% for 3 min, washed 3 times with distilled water and air dried on sterilized filter paper.

The sclerotia were cultivated on PDA amended with Amoxicillin, 250 mg/L 9 cm diameter (2 sclerotia/plate) and maintained at 25±2 C^o for 5 days .Small part of fungal mycelium growth, from plate edge, was transferred into new plate containing PDA for obtaining pure culture. Three plates for each isolate were used.

The average of the fungal redial growth, color and nature of mycelium growth, number of the sclerotia/isolate, were recorded .The pure isolates were transferred to slant PDA in test tubes, maintained at 25±2 C^o for 7 days and conserved in refrigerator for next experiments.

Pathogen identification by polymerase chain reaction technique (PCR)

DNA extraction

Single sclerotium from pure culture of each isolate were re-cultivated on new PDA in petri plate for 14-18 days, and sclerotia formed were collected and put in sterilized 2ml Ependorf tube, surface sterilized with 70% alcohol for 2 minutes, washed 3 times with sterile distilled water and let too dry on filter paper .The dried Sclerotia were ground in sterile mortar with liquid nitrogen, and the powder was transferred into sterile small tube with 180 microliter of universal digestion buffer and 20 microliter of proteinase k . The mixture was mixed thoroughly with vortex and maintained at 56 °C in water path for 30-60 min (Li and Rollins, 2009). DNA extraction from sclerotia powder was done by AL-Museab Bridge Company/ Baghdad using a kit supplied by Korean Bioneer Company.

Determination of S. sclerotiorum DNA concentration and purity

The ITS region of the four isolates was amplified with the primer of the pathogen (Table 1.) (White *et al*., 1990; Qin *et al.* , 2011 ; Kapatia *et al*., 2016).

DNA obtained was analyzed by electrophoresis on 1% agarose gel, 1gm agarose : 99 ml TAE buffer, (Tris hydroxyl methyl, Amino methane - acetate - ethylene di amine tetra acetic acid).

The mixture was maintained in a micro-wave for 3 min and 0.1 microliter of Ethidium bromide stain was added. The agarose gel was poured in agarose block containing a comb at one end and let to solidify at laboratory temperature.

The comb was lifted and the block fixed in the electrophoresis system gel basin. The basin was filled with TAE buffer , the well in the gel were charged with a mixture of 5 µl of DNA with 3µl of dye loading DNA and electrophoresis was operated at 70 volt for 50 minutes. The gel was elevated and exposed to ultra violet light for detecting DNA bands.

The contents were mixed and put in automated Thermal cycler (PCR) to Amplification *S. sclerotiorum* DNA. The device was checked on the program that was shown in Table 3 (Qin *et al*., 2011). The electrophoresis system method was used as it was mentioned lastly for test on the product of magnification operation with 1.5% agarose gel was used. DNA ladder 100 base pair.

DNA sequence analysis of Sclerotinia sclerotiorum isolates

The nucleotide sequences of amplified DNA by using primer set (ITS4/ITS5) for 4 isolates were determined and compared with the equivalent sequences of *S. sclerotiorum* isolates stored in the genes bank from USA (no. KF 545320), Tunis (no. KT 369008), Turkey (no. KX609406), Bangladesh (no. IC 318721 and IC 318722), Iran (no. KY 694474) and China (no. KX 781301, KJ 576850 and HQ 833450), by using MEGA6 sequences program. The same sequences were analyzed by using Sequence demarcation toll version (1.2 SDT0) to confirm fungus species identification.

The quantitative determination of oxalic acid produced by Sclerotinia sclerotiorum isolates by High Performance liquid Chromatography (HPLC)

Oxalic acid (OA) produced by fungus isolates was estimated by high performance liquid chromatography (HPLC) in potato sucrose broth (PSB).The medium was prepared, sterilized at 121°Cand1.5 Kg/cm² for 15 min, and distributed in 250 ml flasks, 100 ml/flask; the medium in the flask was inoculated with 5mm of *S. sclerotiorum* isolates (5 disks / flask) from fungus culture, 5 days old and maintained at 25 ± 2°Cfor 15 days. Cultures filtrate were obtained by paving the culture through muslin cloth. Fifty ml of the filtrate were mixed with 50 ml of methanol in flask and homogenized in a shaker for 15 min .The mixture was passed through filter paper and the filtrate conserved under freezing for on estimation (Nozal *et al*., 2003).The samples were analyzed by (HPLC) (Sykam/Germany) in environment and water department laboratories/Ministry of sciences and

technology/Baghdad. HPLC is formed of mobile phase, a mixture of acetonitrile and water (60:40) ml and immobile phase formed of silica particles in column, C18 (25cm* 4.6mm* 5um) at flow rate 1ml/min, using fluorescence: EX= 365 nm/Em explorer at 30 °C. Five microliters of standard oxalic acid at 5ppm followed by samples were injection in the column, the retention time of each sample was calculated and the concentration of OA of each sample was estimated by the following equation:

$$\frac{\text{Standard OA concentration}}{\text{Curve area of the standard OA}} \times \frac{\text{Curve area of sample}}{\text{original weight of the sample}} \times \text{dilution factor}$$

Pathogenicity test on eggplant under plastic house condition

The pathogenicity tests of the isolates were carried out on eggplant under plastic house conditions .A mixture loamy soil with peat moss at 1:2 was humidified and sterilized at 121°Cand 1.5kg/cm² for one hour, twice at two successive days. The sterilized soil was distribute in pots (3 kg/pot) and cultivated with eggplant seeds (Thoria, F1) from Enzazaden company/Holland, supplied by Dabana company .(2 seeds/pot). The eggplants 1 months old were sprayed with sterilized distilled water and inoculated with 5 mm diameter disks from (SS1, SS2, SS3 and SS4) isolates culture on PDA 5 days old, at the main stem directly under meristem according to modified method by Al-jarah, 2016. Sterilized piece of wetted cotton was fixed around the inoculum and the plants were covered with polyethylene bags for two days to maintain moisture. Four pots of each isolate were used and 4 pots were left without inoculation for control. The pots were distributed as complete randomized design in the plastic house. Spot length on the stem was recorded after 10 day of inoculation. Apiece of 5 mm near infection area was taken, sterilized with commercial Clorox (10%), washed 3 times with sterilized distilled water, let too dry on filter paper and cultivated on PAD to confirm isolate identification.

Results

Pathogen isolation

The cultivation sclerotia, collected from eggplants showing white mold symptoms, on the PDA has induced the formation of white mycelium growth that expanded to include all the plate after 4 days of cultivation. Aggregates of circular transparent, colorless to slightly yellowish seclerotia were formed on the mycelium after 7 days of mycelium growth. The sclerotia have turned to black gradually and differed in size and in distribution in the plate with white to yellow fluid on the outer surface (Fig. 1). The isolate were given the symbols SS1 (Karbala isolate) SS2, SS3, SS4 Baghdad (Al-Gadria, Abo- Graib and Al- Rathwania) respectively. The average diameters of radial growth were found to be 6.33 and 8.27 cm for SS4 and SS1 respectively.

Table 1. The primer used in polymerase chain reaction (PCR) for molecular characterization of ITS4/ITS5 region of *Sclerotinia sclerotiorum.*

Primer	sequence of the primer	Amplicon (bp)size
ITS4	`5-TCCTCCGCTTATTGATATGC-3`)-F	564pb
ITS5	`5-GGAAGTAAAAGTGGTAACAAGG-3`)-R	

Table 2. Mixture of polymerase chain (PCR) reaction.

volume(ul)	Materials
2	Primer forward
2	Primer reverse
5	DNA
11	Deionized water
20	Total volume

A-SS1 (Karbala), B-SS2 (Gadria), C-SS3(College of Agriculture, Abu-Ghraib),D-SS4 (Al-Rathwaneia).

SS1 isolate showed significant difference in average of the number of sclerotia formed /plat, 48.00 sclerotia /plat compared with 37.67, 29.33, 16.33 sclerotia /plat for SS2, SS3, and SS4 respectively.

The higher weight of sclerotia was found in SS1 0.54 gm/plate.

Table 3. Program of thermal cycler (PCR) for amplification the extracted DNA from *S. sclerotiorum* isolates.

Cyclic number	Time	Temp. ˚C	Steps
1	4 min.	95	Initial denaturation
30	30 sec.	94	Denaturation
	45 sec.	50	Annealing
	1 min.	72	Extension
1	5 min.	72	Final extension

The high number of sclerotia was found to affect negatively with sclerotia weight (0.11 gm), compared with 0.29 gm / plate in SS4 at 0.018 gm/ sclerotium .The higher growth rate was found in SS1 (2.07 cm/ day) while the lowest growth rate was in SS4 (1.58 cm/day) (table.4).

Table 4. Some morphological characters of *Sclerotinia sclerotiorum* isolates that grown on the PDA media at 25±2 C° in incubator condition.

Isolates	average diameters Length (cm)*	Average of sclerotia number /plate*	Average of sclerotia weight (gm./plate)**	Average of sclerotium weight (gm./plate)	Average of daily growth rate (cm/day)
SS1	8.27	48.5	0.54	0.011	2.07
SS2	7.40	37.67	0.48	0.013	1.85
SS3	7.17	29.33	0.42	0.014	1.79
SS4	6.33	16.33	0.29	0.018	1.58
L.S.D(%5)	0.13	5.16	0.26	0.0023	0.033

*After 4 days from inoculation on PDA media in an incubator. Every number represents average of four readings. A -SS1 isolation (Karbala), B-SS2 isolation (AL-Ghadria), C-SS3 isolation-College of Agriculture (Abo- Graib) and D- SS4 isolation (Al-Radwania).

**Average of scleroitum weight = the weight of all sclerotia in plate/sclerotia No.

Daily growth rate= Colony diameter /Number of growth days.

Sclerotinia sclerotiorum identification by polymerase chain reaction technique (PCR)

The analysis of PCR products by electrophoresis on 1.5% agarose gel showed one band at 564bp of four isolates (1,2,3,4,) (Fig.2). Which indicate that these isolates are belong to the same pathogen.

The result of DNA sequencing of the regions, ITS4 and ITS5 of Iraqi isolates showed similarity of 99.3-100% with the sequence of the genetic region located between 18S and 28S of ribosomal RNA of *S. sclerotiorum* isolates stored in Gene bank from USA (no. Kf 545320), Tunis (no. KT 369008), Turkey (no. KX 609406), Bangladesh (no. IC 318721 and IC 318722), Iran (no. KY 694474) and China (no. KX 781301, KJ 576850 and HQ 833450).

The same results were obtained when the nucleotide sequence of isolates compared with the nucleotide sequence of *S. Sclerotiorum* using SDTV1.2 program (Fig.3, 4) which confirms fungus identification.

Table 5. Oxalic acid produced from *Sclerotinia sclerotiorum* isolates grown on PSB media by HPLC analysis.

Treatments*	Oxalic acid concentration mg/L
Standard oxalic acid	0.6
SS1	10.95
SS2	8.7
SS3	4.9
SS4	8.6

*the acid was extracted after 15 days from grown 5 discs of *S. sclerotiorum* mycelium growth on 100ml of PSB media by using methanol (1:1).

Table 6. Lesion length (cm) on eggplant stem (Thoreya variety) that was inoculated by different isolates of *S. sclerotitorum* in pots conditions.

Isolates	Collection regions	Lesion length(cm)*
SS1	Karbala	4.28
SS2	Gadria	3.40
SS3	Abu-Graib	3.23
SS4	Radwania	2.00
L.S.D(**%5**)		0.67

The neighbor joining phylogenetic tree constructed from nucleotide sequences of the genetic region located between 18 S ribosomal RNA gen and 28 S ribosomal RNA gen of *S. sclerotiorum* indicated the genetic relationship between Iraqi isolates with those brought from Gene Bank database. These results with the morphological characteristics confirm the identity of the Iraqi 4 isolates with that belong to *S. sclerotiorum*.

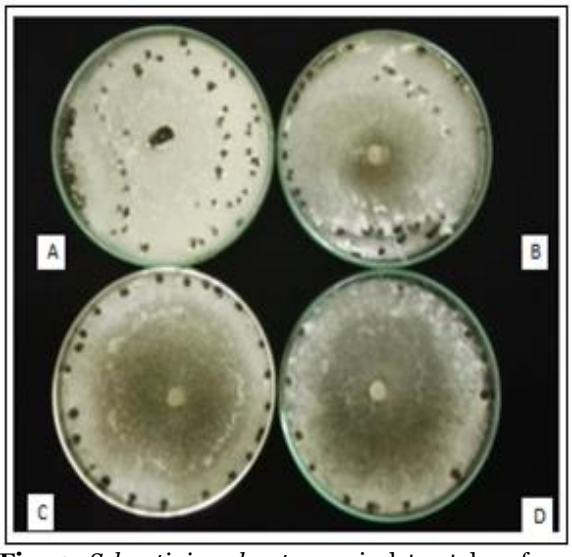

Fig. 1. *Sclerotinia sclerotorum* isolates taken from different regions in Iraq.

A. SS1 (Karbala), B-SS2 (Gadria) ,C-SS3(College of Agriculture, Abu-Ghraib),D-SS4 (Al-Rathwaneia).

The quantitative determination of oxalic acid produced by Sclerotinia sclerotiorum isolates by High Performance liquid Chromatography (HPLC)

The result in Table 5. shows concentration of oxalic acid produced by *S. sclerotiorum* isolates that were determined by HPLC analysis. The SS1 isolate showed highest acid concentration (10.95 mg/L), while the SS3 isolate shows less acid concentration (4.9mg/ L).

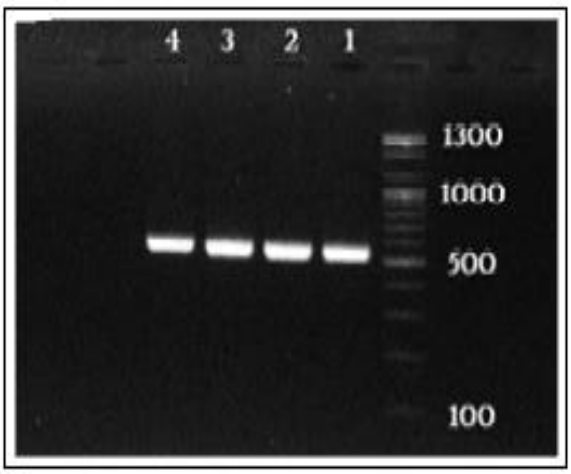

Fig. 2. PCR banding on 1.5% agarose gel of *S. sclerotiorum* isolates DNA using ITS4/ITS5 primer which its molecular weight is 564 base pair (the numbers on the right side of the figure represent the number of the marker base pair used. 1,2,3 and 4 symbols represent the *S. sclerotiorum* isolates).

Pathogenicity test on eggplant under plastic house condition

The Four isolates caused diseased infections on eggplant with variance of the spot length as it is shown in table 6. The symptoms were presented as watery, brown color lesion expanded from inoculation point to up and down of stem with girdles the stem.

The average length with isolates variances. The SS1 isolate was superior in causing highest lesion length on the main stem of eggplant in which was 4.28 cm length, the less lesion length average was 2 cm on the main stem of the SS4 isolation after 7 days of inoculation and regrown of the infected part of stem of the tested plants presence of *S. sclerotitorum* at 100% rate in all the planted parts and this emphasized Koch' postulates.

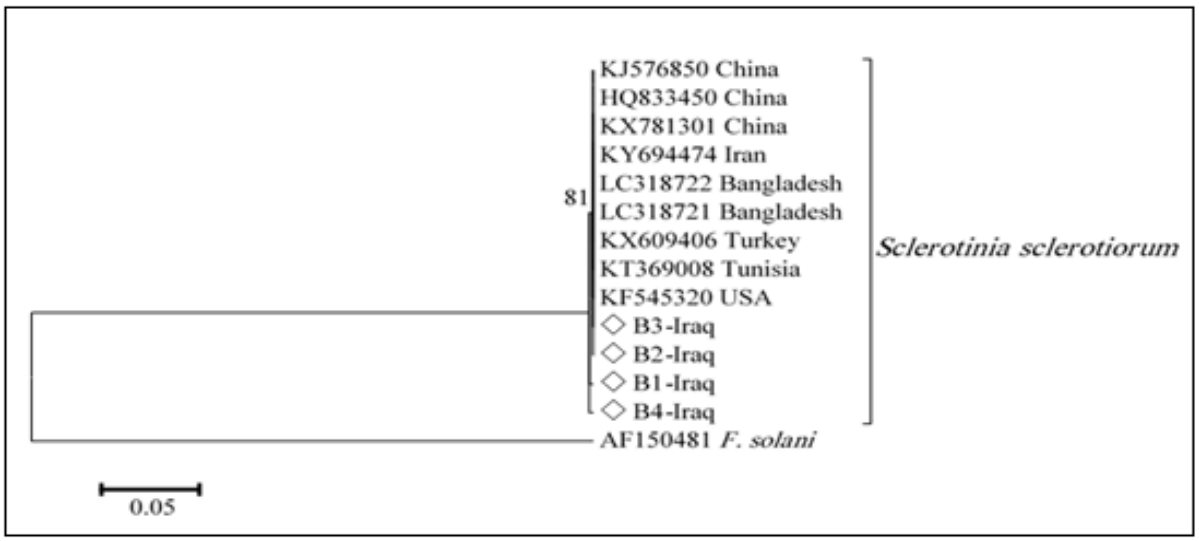

Fig. 3. Phylogenetic of *S. sclerotiorum* isolates.(B1=SS1 ,B2=SS2 ,B3=SS3 ,B4=SS4).

Discussion

The formation of white cottony growth on PDA from sclerotia that collected from infected eggplants, then the appearance of black aggregates of sclerotia with irregular shapes and different distribution in the plate (Fig. 1), protection of light brown-rosy fruiting bodies bearing asci, and each ascus containing eight hyaline oval ascospores indicated that the isolates under study is *S. sclerotiorum*: Similar symptoms concerning *S. sclerotiorum* on PDA were reported (cooks, 1977; Willets and Wong, 1980).

This conclusion was supported by PCR analysis and sequencing the region 18S/28S of the DNA amplified , that showed similarity at 100% with the equivalent isolates of *S. sclerotiorum* stored in Gen Bank from USA, Tunis , Turkey ,Bangladesh , Iran and China, using MEGA6 and neighbor joining phylogenetic tree (Fig.3,4). The morphological characteristics of the isolates on PDA (Table 4.) together with DNA sequencing confirm the identity of all isolates and that belong to *S. sclerotiorum* (Fig.2).

Variations in pathogenicity on eggplant (Table 6.) and in producing oxalic acid in liquid culture between isolates (Table 5.) were manifested. These variations may be attributed to genetic variation in other region of DNA nucleotides sequence leading to variation in isolated capacity to produce pathogenicity related enzymes especially those decomposing host cell wall constituents of cellulose and pectin (cellulose and pectinase).

It was reported that *S. sclerotiorum* produce several decomposing enzymes capable to decompose host cell wall constituents including, protease, cellulase, hemicellulose, pectinase (Riou *et al.*, 1991) and protease acidic (Girard, 2004).

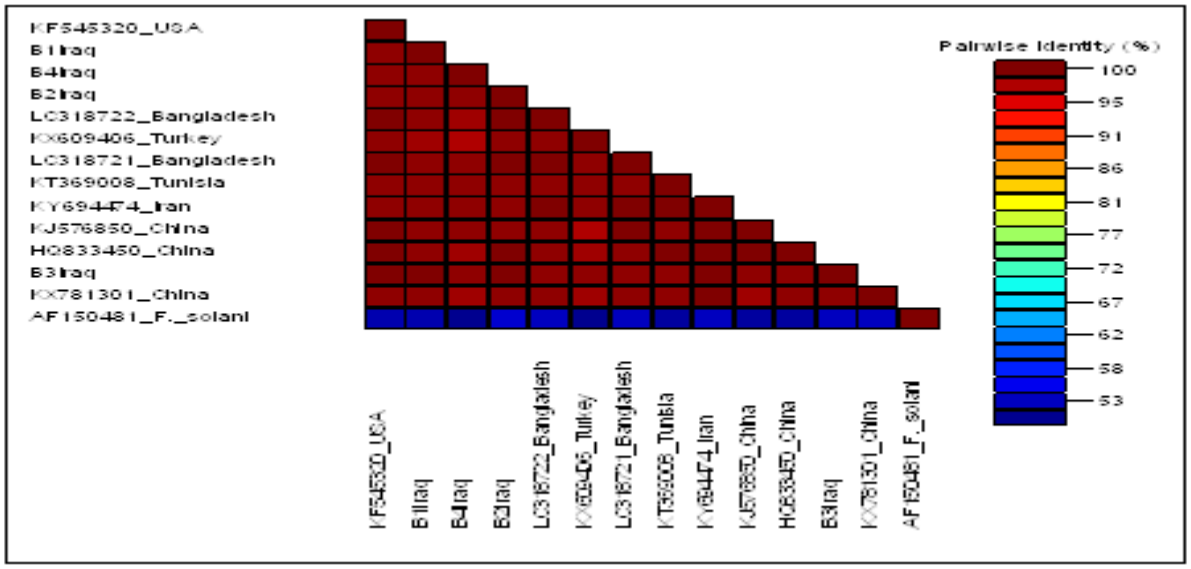

Fig. 4. Nucleotide matching ratio of the region located between 18S ribosomal RNA gene and28S ribosomal RNA that belongs to the Iraqi *S. sclerotiorum* isolates and their counterparts of genes bank which was estimated by using sequence demarcation tool version 1.2 (v1.2 SDT).

The activity of these enzymes was increased in acidic environment provided by oxalic acid produced by the fungus which consider the main factor of fungus pathogenicity and its ability to infect the host (Marciano, 1982).

It has been noted that isolates pathogenicity varied with collection region. SS1 from Karbala was found the more pathogenic than the others (Table 6.) ,which may indicate that the ecological factors play a role in DNA nucleotides sequence leading to variation in OA and pathogenicity related enzymes production. Mohamed and Al-Mothfar (2013) mentioned indicated that the higher production of OA by *S. sclerotiorum* was at 25°C, decreased at 30°C , the higher production of OA was found at pH 5.5, attained to 6.43 mg/L media decreased to 1.52 and 1.77 mg/L at pH 9.5 and 7.2 respect the variation in isolate pathogenicity may attributed to other microclimates related to plant affecting the capacity, rapidity, and type of enzymes produced and depend on the interaction between host and pathogen.

Conclusion

The nucleotide sequences of amplified DNA by using primer set (ITS4/ITS5) for *S. sclerotiorum* isolates gave good results to identifying the pathogen and when compared with the equivalent sequences of fungus stored in the gene bank.

The variation in pathogenicity on eggplant and in producing oxalic acid may be attributed to genetic variation in other region of DNA nucleotides sequence leading to variation in isolated capacity to produce pathogenicity related enzymes.

Recommendation

Further studies must be done to compare the genetic variation and virulence between isolates from different host in the same geographic area. The variation in the S. sclerotiorum virulence will be useful in disease management strategies, particularly in screening for host plant resistance to this pathogen, which is considered an important disease causal agent around the world.

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