Pathogenicity Test and Anastomosis Group of *Rhizoctonia solani* the Causal Organism of Stem Canker and Black Scurf Disease of Potato in Egypt

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Abstract: Stem canker and black scurf caused by *Rhizoctonia solani* Kuhn is considered the most destructive disease and reduce productivity of potato. Pathogenicity test of 12 isolates of *R. solani* obtained from two governorates, El-Sharkiya (New El–Salhia region) and Ismailia (El-Shabab region) revealed that all isolates were pathogenic, however isolate No.RS7 was the most virulent isolate in the development of the stem canker and black scurf disease. Molecular technique using PCR applied to determine anastomosis group of isolate No. RS7 showed that this isolate belong to AG3.

Keywords: Anastomosis group, Molecular technique, *Rhizoctonia solani,* Stem canker and black scurf, Pathogenicity test, PCR

INTRODUCTION

Potato (*Solanum tuberosum* L.) is one of the most important crops in Egypt as well as all over the world and produces a tuber very rich in starch that it ranks as the world's fourth most paramount food crop, after maize, wheat and rice (Cunnington, 2008). A recent special report by the Food and Agriculture Organization of the United Nations (FAO) highlighted the potato crop as "a vital part of the global food system, and will play an ever greater role in strengthening world food security and alleviating poverty" (Anonymous, 2009).

Stem canker and black scurf caused by *R. solani* Kuhn is considered the most destructive disease, particularly in warm – climate countries and warm sandy soils of temperate regions. *Rhizoctonia* isolates both multinucleate and binucleate species which are divided into anastomosis groups (AGs). Presently, the multinucleate species of *R. solani* Kuhn (teleomorph: *Thanatephorus cucumeris* (Frank) Donk.) are divided into 14 anastomosis groups (Tuncer and Eken, 2013): AG-1 to AG-10, AG-BI Sneh *et al.* (1991), AG-11 Carling *et al.* (1994), AG-12 Carling *et al.* (1999) and AG-13 Carling *et al.* (2002). Each AG has either host specific or with a wide host ranges (Carling *et al*., 2002; Ogoshi, 1987). For example AG-2 is associated with diverse host plants, but AG 8 is more specifically on cereals. AG-3 is divided into two genetically different subgroups, AG 3 PT associated with potatoes and AG-3 TB on tobacco (Woodhall *et al*., 2008). *Rhizoctonia solani* AG-3 PT reduces tuber quality by producing sclerotia (black scurf) on progeny potato tubers. The pathogen also can infect underground organs (stems, stolons and roots), which affects crop yield, tuber size and number (ElBakali and Martin, 2006). In addition to those typical symptoms *R. solani* is associated with several types of blemishes on potato tubers (Fiers *et al.*, 2010). This investigation aims to determine the pathogenicity test of *R. Solani* isolates obtained from two governorates, El-Sharkiya (New El–Salhia region) and Ismailia (El-Shabab region) in the events of stem canker and black scurf on potatoes and determine anastomosis group of aggressive isolate.

MATERIALS AND METHODS

Isolation & identification of the pathogen

For fungal isolation, collected samples of potato were washed carefully under running tap water to remove the adjacent soil particles followed by sterile water, and then was dried between two sterilized filter papers. Infected parts were cut using sterilized scalpel into small pieces (1cm), then surface sterilized by immersing in sodium hypochlorite (1%) for three minutes subsequently rinsed with sterile water several times. This was followed by soaking in 70% ethanol for one minute**.** After rinsing with sterile water three times, 1 cm infected (stem or tubers) dried segments were sterilized, transferred to plates of PDA or CYA (Czapek yeast extract agar) supplemented with rose bengal (1/15) and chloramphenicol (50 ppm) to suppress bacterial growth then incubated at 28±2ºC for 7 days. Plates were daily observed for mycelial growth. Hyphal tips of mycelium emerging from the infected pieces were transferred to fresh plates of PDA and CYA.

Pure cultures of *R. solani* isolates were preliminary identified by visual observation of macromorphological characters using naked eye, and the identification was confirmed microscopically on the basis described by Ogoshi (1987). The identified fungi were subcultures on PDA slants and kept at 5ºC for further studies.

Pathogenicity tests

These experiments were started with pathogenicity test with12 pure cultures of *R. solani* (7-days – old) toward potato plants. These cultures were obtained from two governorates (EL-shakia and Ismailia). Randomly selected for 6 isolates for every governorate; *R. solani* isolates were artificially inoculated on potato plants under greenhouse conditions.

Preparation of inoculum

The fungal inoculum was prepared by soaking 100 g of sorghum grain in 50 ml distilled water in a 500-ml glass bottles overnight, then autoclaved for 60 min at 121ºC (Mueller *et al.,* 2003). This was followed by inoculating sterilized sorghum with Rhizoctonia

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mycelial plugs (5 mm in diameter) obtained from one week old potato dextrose agar culture and incubated for 15 days at 27±2ºC. Plastic pots, 50 cm diameter, filled with 8 kg sterilized soil used for soil infested by the pathogen inoculum at rate 10g/kg. To ensure good colonization of the pathogen, inoculated pots watered and kept for 48 hours before sowing potato seeds.

Preparation of seed tubers

Potato seeds, spounta, cultivar (most susceptible and the common grown cultivar in Egypt) according to El-Naggar *et al.* (2013), were surface sterilized by soaking in sodium hypochlorite (1%) for three minutes then in ethyl alcohol (70%) for one minute. After that washing several times with sterilized water.

Experimental layout

Soil (clay and sand 1: 1) were sterilized by using 37% commercial formalin. One part of the formalin was diluted with nine parts of water. Soil was irrigated with the formalin solution and covered with a polyethylene sheet for 48 hours. Soil was exposed to air for 4 to 5 days until the formalin completely evaporated, according to El-Aziz *et al*. (2013). Pots (50 cm in diameter) were filled with sterilized soil, then inoculated with *R. solani* isolate at a rate of 10 g/kg soil and watered for 4 days before planting. The pots were randomly distributed in open field at Experimental Farm, Faculty of Agriculture, Suez Canal University during winter season at ambient temperature, and three pots were used as replicates for each treatment, each replicate contains three plants.

Disease Severity

Severity of stem canker disease was evaluated using a scale of (0-4) according to Carling and Leiner (1990) after 50 days from sowing. Black scurf disease severity was determined by using 0-5 disease severity grades based on the percent tuber surface showing disease symptoms, according to Ahmad *et al.* (1995)

Anastomosis group identification by molecular technique

This experiment was conducted in Biotechnology Research Center, Suez Canal University during February 2015.

Fungal isolates

Isolate of *R. solani* from potato tubers (RS No7) were collected from El-Sharkia Governorate (New EL–Salhia region) and was the most virulent isolate among 12 isolates on pathogenicity test trial, were chosen to identify Anastomosis group **(**AG) of this isolate.

The DNA of the fungal isolate was extracted from the freeze-dried powder of mycelium with the Genomic DNA preparation kit (Jena Bioscience).

DNA extraction

Discs (5-mm-diam), taken from a – 7-dayes-old *R. solani* culture grown on PDA medium, were transferred into 250 ml conical flask containing 100 ml of potato-dextrose broth with shaking at125rpm using an orbital shaker (Gallenkamp), at 23ºC for 4 days. Growing mycelia were harvested by filtration through sterile cloth, washed with sterile deionized water, frozen in liquid nitrogen freeze-dried and stored at – 20ºC.

DNA extracted from 50 mg of mycelial growth using Qiagen Kit was dissolved in 100 µl of elution buffer. The concentration and purity of the obtained DNA was determined by using Genomic DNA preparation kit (Jena Bioscience). For DNA sample, the concentration was adjusted to 6 ng/µl using TE buffer (pH.8.0); meanwhile the purity reached 90-97% with ratios between 1.7- 1.8 according to (Sambrook *et al*., 1989).

PCR amplification

The internal transcribed spacer (ITS) region of the isolate was amplified using the universal primers

ITS-1 (5’ TCC GTA GGTGAA CCT GCGG 3’)

ITS-4 (5’TCC TCC GCT TAT TGA TATGC3’).

The ITS region of the rDNA and part of DNA template were amplified by PCR reaction: for total volume of 25 µl:

12.5 µl Master Mix (Qiagen)
2 µl DNA template (20 ng/ µl)
2 µl Primer (20 pmole)
8.5 µl deionized H2O

The amplification protocol using PCR was carried out as follows:

Denaturation at 94ºC for 2min.(one cycle).
35 Cycles, each consists of the following steps:
Denaturation at 94ºC for 30s
Annealing at 52ºC for 30s
Extension at 72ºC for 3min
Final extension at 72ºC for 10min. (one cycle)

The sequences were analyzed with BLAST® (Altschul *et al.,* 1997) against the NCBI sequence database (National Center for Biotechnology Information, GenBank) (www.ncbi.nlm.nih.gov/genbank/) to detect similar sequences of known AGs

Gel analysis

The PCR product was analyzed via agarose gel electrophoresis in 0.5 X TBE to verify the reaction and to check for contamination. A part is taken of PCR products were checked by electrophoresis on a 1.5% agarose gel, revealed with ethydium bromide and visualized by UV transillumination according to (Sambrook *et al*., 1989)

RESULTS

Isolation and purification of the causal pathogen

Fungal cultures belonging to *R. solani* were isolated and identified according to morphological and microscopical characters.

Mycelia of all isolates were light brown during early growth and all produced large amounts of aerial hyphae throughout the growth cycle. As cultures aged, their color darkened and most was very dark brown after 21 days. Concentric rings formed on all cultures by day three, but rings tended to disappear as cultures matured and darkened. By day six, sclerotia formed near the edge of the Petri dishes. However, 21 days after growth, sclerotia were scattered randomly about the agar surface as well as in the agar. Individual sclerotia were tan when young, generally dark brown when mature, and up to 1.5 mm in diameter with clumps up to 5 mm in diameter

Microscopical examination shows a long mycelium with a dimension of 14.8µm. Hyphae produce

branches at right and acute angles and there is often a septum near the developing branch (Fig. 1).

Pathogenicity tests

Twelve Rhizoctonia isolates were selected from 140 *R. solani* isolates according to locations, seasons, and potato infected parts were screened for their pathogenicity toward potato cultivar Spounta in pots plants under greenhouse conditions were conducted in Experimental in the open field botanical garden, Faculty of Agriculture, Suez canal University during winter season at ambient temperature.

Rhizoctonia diseases on stems were presented as disease index (DI) (Fig. 2).

Potato black scurf disease index using 0-5 scales (Fig. 3).

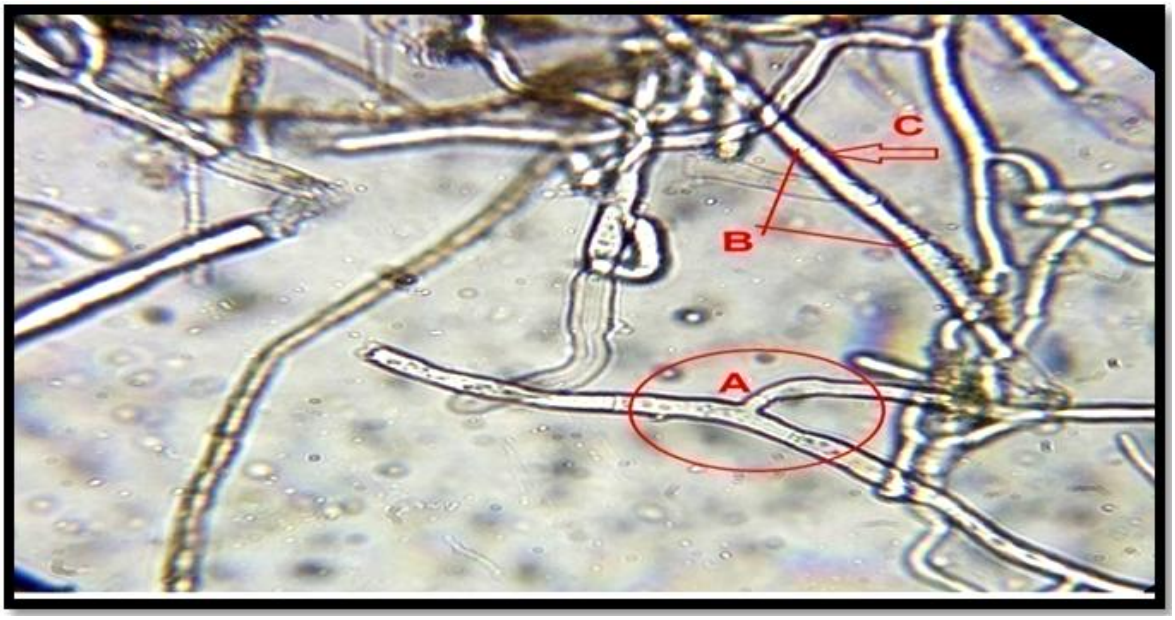


Fig. (1): Morphological characteristics to *R. solani* under light microscope
Branching hyphae (A) Septum (B) Main hyphae (C).

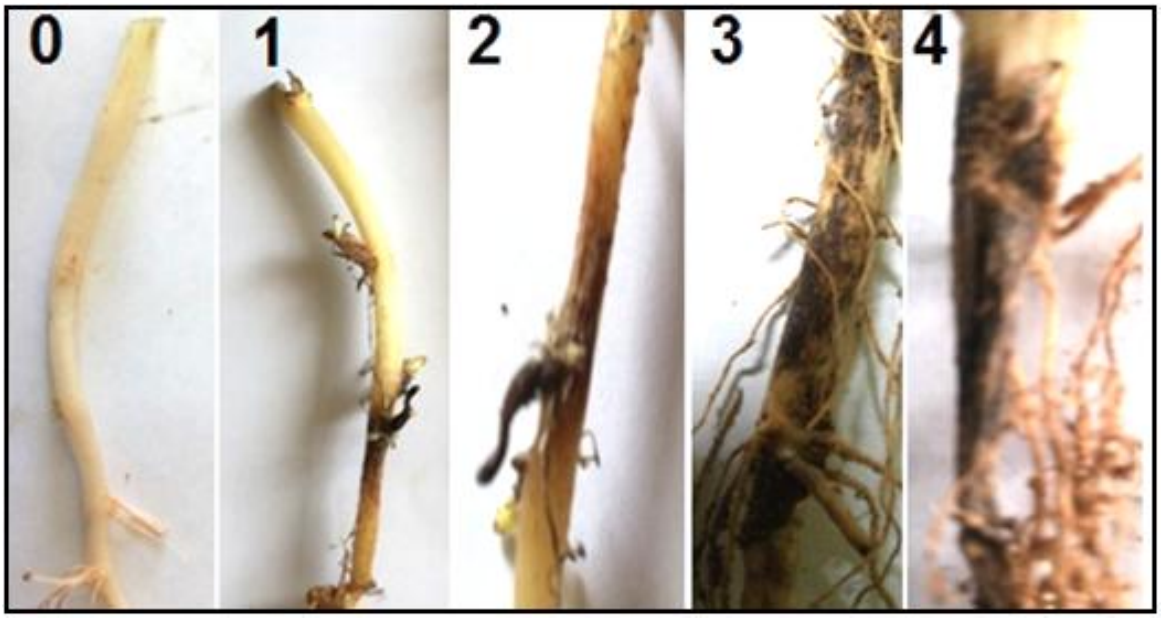


Fig. (2): Stem canker index in potato (0 -4 scales)

Fig. (3): Black scurf index in potato(0 -5 scales)

During testing the ability of *R. solani* isolates to induce potato Rhizoctonsis, representing data in Fig. (4) show significant differences of potato stem canker and black scurf disease severity. According to disease severity of stem canker and black scurf by *R. solani* isolates under test, isolates classified into three main pathogenic groups:

A- **Strong isolates,** this group represented by isolate RS7 isolated from ElSharkiya Governorate (New El–Salhia Region). This isolate was used throughout this investigation.

B- **Moderate isolates,** this group exemplified by isolates RS6, RS8 and RS10 obtained from Ismailia Governorate (El-Shabab Region).

C- **Weak isolates,** this group includes three isolates (RS5, RS11, and RS10), isolated from Ismailia and El-Sharkiya Governorates, respectively.

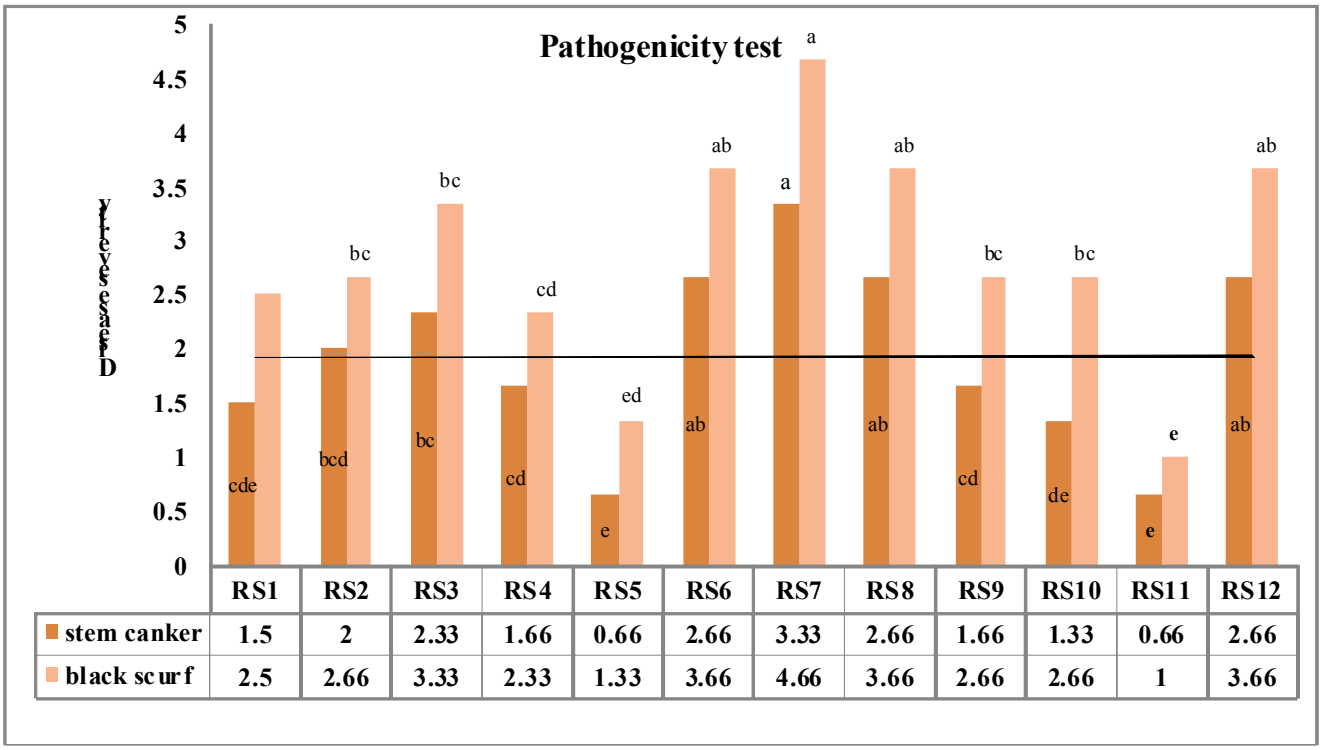


Fig. (4): Pathogenicity test with 12 *R. solani* isolates on potato plants.
Each value represents the mean of 3 replicates
Values followed by the same letter do not differ significantly according to (LSD) least significant difference ($P \leq 0.05$).

Anastomosis group identification by specific PCR and sequencing of ribosomal DNA

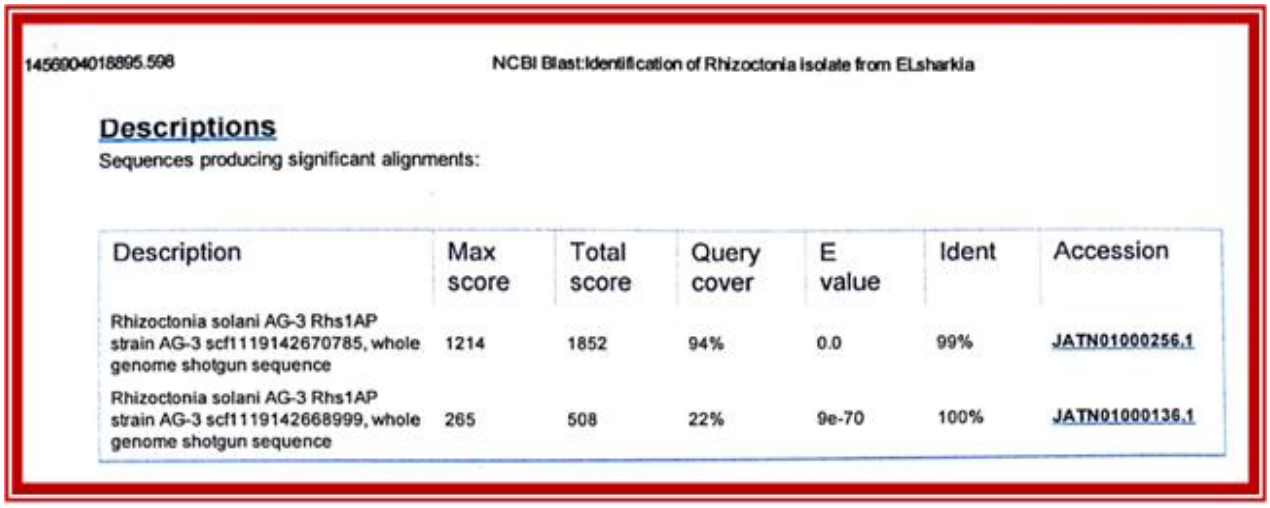
The resulting data of *R. solani* sequences isolated from potato tubers (RS No7), collected from El-Sharkiya (New El–Salhia Region), revealed that a base length of 1169 base.

Sequencing of rDNA of isolate RS7 showed that sizes of the ITS region varied from 680 to 700 base pairs and their sequences were identical (100%) in the ITS region.

The resulting data of sequences which analyzed with BLAST® Altschul *et al.* (1997) against the sequence database National Center for Biotechnology Information, GenBank (NCBI) to detect similar sequences of known AGs. showed that the tested isolate is completely related to AG-3 .

The comparison between sequences registered in GenBank and sequences of RS7 tested isolate showed that the isolate had sequence homology 99% and 100% with the referent isolates. ITS sequences of the tested isolate showed 100% complementarily with representative of anastomosis group 3.

Data illustrated in Figure (5) represent two sequences of *R. solani* AG-3 from GenBank (JATN01000256.1 and JATN01000136.1) were used as references. Nucleotide identity among sequences was determined for anastomosis group identified.


1456004018895.598 NCBI Blast:Identification of Rhizoctonia isolate from ELsharkia

Descriptions

Sequences producing significant alignments:

Description	Max score	Total score	Query cover	E value	Ident	Accession
Rhizoctonia solani AG-3 Rhs1AP strain AG-3 scf1119142670785, whole genome shotgun sequence	1214	1852	94%	0.0	99%	JATN01000256.1
Rhizoctonia solani AG-3 Rhs1AP strain AG-3 scf1119142668999, whole genome shotgun sequence	265	508	22%	9e-70	100%	JATN01000136.1

Fig. (5): Two sequences reference of *R. solani* AG-3

The ITS1 – ITS4 regions of the rDNA from the *R. solani* sequences showed high sequence similarity (99-100%) among them and with two AG-3PT sequences (JATN01000256.1 and JATN01000136.1), respectively from Gen Bank (Fig. 5).

Gel electrophoresis after PCR reaction, the presence of amplified products was tested by running an agarose gel in TAE buffer. DNA bands were approximately 687 base pairs long (Fig. 6).

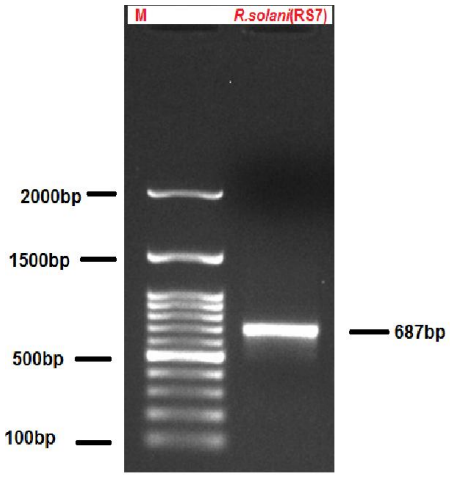


Fig. (6): Gel electrophoresis after PCR reaction to identify AGs of *R. solani* isolate RS7

DISCUSSION

Increasing importance of the potato crop year after year in Egypt because of its nutritional value, as well as economic value due to the increase of quantity exported from it, especially for the countries of the European Union and Russia. Egypt is the first country on the level of the continent of Africa in the amount of potatoes produced in 2013 (Anonymous, 2013).

Result of pathogenicty test with 12 isolates showed significant differences of potato stem canker and black scurf disease severity, isolates classified into three main groups: Strong isolates**,** represented by isolate RS7 isolated from El-Sharkiya Governorate (New El–Salhia Region). Moderate isolates, this group exemplified by isolates RS6, RS8 and RS10obtained from Ismailia Governorate (El-Shabab Region).Weak isolates, this group includes three isolates (RS5, RS11, and RS10), isolated from Ismailia and El-Sharkiya governorates, respectively.

Virulence of *R. solani* isolate on potato may be depending on the source of isolates from lesions or sclerotia, as reported by Carling and Leiner (1990). They found height virulence between isolates which isolated from lesions, while low virulence was observed from sclerotia isolates. Virulence of isolates in pathogenicity attributed to difference between (AGs), *R. solani* other than AG3 generally possess low virulence

against potato. These results agree with several researches (Balkana and Wenham, 1973; Yanar *et al*., 2005; Khandaker *et al.,* 2011). Some researchers has made it clear that the reason of differences in susceptibility of infection due to the nature of resistance in potato cultivars (Zhang *et al*., 2016), another researches attributed this to difference in crop hosts (Heremans *et al*., 2007). While some researchers attributed this to the difference in anastomosis groups (AGs) (Balali *et al*., 1995; Woodhall *et al*., 2008; Ferrucho *et al.,* 2012; Yang *et al*., 2015). As clarified by Truter and Wehner (2004) who found that AG-3 was the most virulent, with isolates from sclerotia on tubers and lesions, on stems more aggressive than those from symptomless tubers or soil. AG-4 and AG-5 caused significantly less disease than AG-3, but none of the AG-7 and AG-8 isolates showed any virulence to potato sprouts.

Rhizoctonia isolates differed among themselves in severity of the infection and the symptoms brought about it, although similar conditions, several studies have indicated that there are significant differences in the ability of *R. solani* isolates on potatoes infection. Even among optimum conditions, this is an indication the role of genetic factors in different virulence (Rubio et *al.,* 1996). Some studies have recorded a vital variation between isolates AG3, in the difference in the growth rate, and the severity of symptoms and seriousness (Carling and Leiner, 1990; Bains and Bisht 1995; Balali *et al.*, 1995).

Rhizoctonia encompass both multinucleate and binucleate species which are furthermore divided into anastomosis groups (AGs). Presently, the multinucleate species of *R. solani* Kuhn (teleomorph: *Thanatephorus cucumeris* (Frank) Donk.) are divided into 14 anastomosis groups (Tuncer and Eken, 2013): AG-1 to AG-10, AG-BI (Sneh *et al.,* 1991), AG-11 (Carling *et al.,* 1994), AG-12 (Carling *et al.,* 1999), and AG-13 (Carling *et al.,* 2002), this subgroup may differ for pathogenic, biochemical or genetic characteristics (Carling *et al.,* 2002).

Experiments conducted to determine the anastomosis group for aggressive isolate {isolate RS7 isolated from Sharkiya Governorate (New El–Salhia Region)} by use Molecular technique (sequence analyses of ribosomal DNA (rDNA) regions) made it clear that they belong to AG3, This result comes from previous approval of the results indicated that the group AG3 are prevail in potato fields (Ferrucho *et al.,* 2012). The results are consistent with what was obtained in Britain by Woodhall *et al.* (2007) where he focused most of the isolates (92.6%) to the group AG3 PT. Similar results were reported in France by Campion *et al*. (2003), the same results happened in Northern Ireland (Chand and Logan, 1983). In the State of Michigan America, three anastomosis groups were found; these are: AG2-2, AG3 and AG4, AG3 and considered the pathogenic isolates of the potato (Wharton *et al.,* 2007). In warm weather, at Venezuela, Cedeno *et al.* (2001) found that the percentages were similar for the AG3 and AG2-1. This indicates that agricultural operations are more important than environment in determining the existence of any of the anastomosis groups (Woodhall *et al.,* 2007). In Pakistan, most of the isolates of *R. solani* was the group of potato-growing areas belong to AG3 (81.89%) and 8.66% for the group AG5 and 5.5% for the group AG4 and 1.57% for the group AG2-1 and 0.79% for each of the AG1-1A and AG2-2 and AG9 groups (Rauf *et al*., 2007). On the other hand, some investigators stated that AG3 be more aggressive in cold conditions, such as some parts of North America such as Canada, and Northern and Central Europe (Otrysko *et al.*, 1985; Bandy *et al.,* 1988; Bains and Bisht 1995; Campion *et al.*, 2003). This is in contrary to what is found in Venezuela. Yang *et al*. (2015) found that AG-3 PT of *R. solani* was the predominant pathogen causing stem canker and black scurf on potatoes in China.

CONCLUSIONS

Pathogenicity test with 12 isolates of *R. solani* using susceptible cultivar of potato cv. Spounta, revealed that all isolates were pathogenic, with different degrees of pathogenicity (virulent strong, moderate and virulent weak isolates) on potato giving the typical stem canker and black scurf disease symptoms.

Isolate No.RS7 from El-Sharkia Governorate (New El–Salhia Region) was most the virulent isolate and caused the highest stem canker and black scurf disease severity.

Specific PCR-based methods or DNA sequencing of the ITS-rDNA were useful for detection and identification of AGs.

R. solani AG-3PT was associated with potato stem canker and black scurf diseases in Egypt. The adoption of a crop rotation system should consider on-hosts of AG-3PT.

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اختبار القدرة المرضية وتحديد مجموعة الالتحام للفطر ريزوكتونيا سولانى المسبب لمرض تقرح الساق والقشرة السوداء على البطاطس في مصر

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يعتبر مرض تقرح الساق والقشرة السوداء المتسبب عن الفطر *Rhizoctoni solani* من أخطر الأمراض الفطرية التي تؤدى إلى خسائر كبيرة في إنتاجيه وجودة الدرنات لمحصول البطاطس، تم اختبار القدرة المرضية لـ ١٢عزلة للفطر *R. solani* تم عزلها من عينات مأخوذة من مناطق زراعة البطاطس في الصالحية الجديدة بمحافظة الشرقية ومنطقة الشباب بمحافظة الإسماعيلية وأتضح أن جميع العزلات كان لها القدرة على إحداث الإصابة، وكانت العزلة RS7 هي أعلاها قدرة على إحداث الإصابة بتقرح الساق والقشرة السوداء وقد تم تحديد مجموعة الالتحام لهذه العزلة عن طريق تكنيك تفاعل البلمره المتسلسل (PCR) التي أوضحت أنها تنتمي إلى مجموعة الالتحام AG3.

كلمات مفتاحية: تقرح الساق – القشرة السوداء - القدرة المرضية - مجموعة الالتحام - تفاعل البلمره المتسلسل (PCR).