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Molecular, physiological and infection behaviour studies of *Rhizoctonia solani* causing the Rice Sheath Blight disease

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ABSTRACT

Rhizoctonia solani is the causal agent of rice sheath blight, which is one of the most serious diseases of rice worldwide. In this study, seventeen Egyptian isolates of sheath blight pathogen, *R. solani* AG1 IA, were identified using morphological and molecular characterization by the internal transcribed spacer (ITS) sequences. Extracellular enzymes were assessed in in vitro and infected rice samples. Also, pathogenicity tests for all isolates were done on two rice cultivars under greenhouse conditions. Growth behavior by RS13 isolate was studied using two rice cultivars Sakha 101 (Japonica type) and Egyptian Yasmine (Indica type). The morphological and pathogenicity properties of isolates showed a wide range of diversity in the results. Two isolates RS10 and RS13 (AB5) were sequenced and found aligned with Egyptian and Chinese isolates. RS13 and RS10 isolates exhibit the highest production of cellulase, pectinase and amylase enzymes in plate culture (in vitro). While on the plant sample tissue cellulase and pectin methylesterase (PME) activity enzymes are relating to development of rice sheath blight disease. Under the artificial condition, SK 101 rice proved the most susceptible to infection with sheath blight pathogen compare with E. Yasmine. The hyphae growth on SK101 cultivar was more than in the E. Yasmine cultivar. Furthermore, we examined the morphological developments of hyphae and sclerotial on both cultivars using light and scanning electron microscopy (SEM). It was observed that the fungal pathogen could intercept host surface structures for improving cell penetration and anchorage.

Keywords: Sheath blight, Rice, *Rhizoctonia solani*, internal transcribed spacer, extracellular enzymes

INTRODUCTION

Rice is the most important grain crop for more than half of the world's population. In Egypt, in 2018 the area cultivated with rice reached 724 000 feddans (304000 hectares) produced 6.1 million tonnes (FAO, 2018). Ou (1985) has listed 40 fungal diseases of rice, representing approximately 50% of the recorded rice diseases. *R. solani* is a soilborne fungal pathogen causing rice sheath blight, which is one of the most serious diseases of rice worldwide (Wamishe *et al*., 2007). *R. solani* Kühn teleomorph (*Thanatephorus cucumeris*) anastomosis group (AG) AG-1 IA was first described in Japan at the beginning of the 20th century as the causal fungus of rice sheath blight disease (Lee, 1983), then it has been recorded in major rice-growing areas of the world (Ou, 1985). In Egypt, the rice sheath blight was noted in 2013 and reported in 2019 by (El-Shafey *et al*., 2019). By multinucleate cells in young vegetative hyphae species of Rhizoctonia can be differentiated and prominent septa pore apparatus (dolipore septum) branching towards the distal septum of cells, formation of a septum in the branch near the point of origin, and branch constriction and some shade of brown and *T. cucumeris* is the perfect stage no conidia, no clamp connections, no sclerotia differentiated into medulla and a rind, no rhizomorphs. No colour another pigment except brown (Ogoshi, 1975). ITS regions of the ribosomal DNA (rDNA) were used for the identification of *R. solani* as specific primers (Lehtonen *et al*., 2008). In rice germplasm, few sources of complete resistance have been identified for sheath blight disease.

Also, the finding for resistance sources of rice genotypes is low. Partial resistance may provide a road to reduce epidemics to given the low level of it's especially of the sheath blight fungus (Srinivasachary *et al*., 2011). Before and during infection, the sheath blight pathogen *R. solani* was examined differently in tolerant and susceptible rice genotypes (Basu *et al*., 2016). The rice plant interaction between *R. solani* fungus and the host is suggested by (Chowdhury *et al*., 2014); During the early stages of infection, the fungus may recognise and select a resistant or vulnerable host as a favoured candidate for further spread. In addition, the pathogen's behaviour may alter depending on whether the host is resistant or vulnerable. When *R. solani* infects cauliflower plants, single hyphae branch into clusters of bulbous ends, resulting in the formation of

complex appressoria, also known as infection cushions (Pannecoucque and Höfte, 2009). *R. solani* with anastomosis group AG1 IA was identified as the cause of rice sheath blight. Several secondary metabolites, carbohydrate-active enzymes, and secreted protein effectors have been discovered to be key pathogenicity factors in *R. solani* during its development contact with host plants (Li *et al*., 2021). In rice plants, during the period of infection by *R. solani* some metabolic changes exhibit between plant host and pathogen the majority of which were focused on carbohydrate metabolism (Molla *et al*., 2020). The pathogen exploited the original source of nutrients in the host-pathogen relationship, triggering chlorophyll loss and cell death, which resulted in necrotic lesions on the host.The lesions begin as little water-soaked pale green spots and then rapidly increase, causing the plant to wilt and die (Taheri and Tarighi, 2011).

In the current study, Egyptian isolates of sheath blight pathogen, *R. solani* AG1 IA, were identified based on their morphological and molecular characterization. Also, the pathological and physiological of the pathogen were determined. The growth behaviour of *R. solani* on two different types of rice cultivars during infection was studied.

MATERIALS AND METHODS

Laboratory and greenhouse studies: Experiments were conducted at the laboratory and greenhouse of the Rice Pathology Department, Sakha, Egypt.

1- Samples collection:

Seventeen samples of rice leaves and sheath of different cultivars showed sheath blight symptoms were collected from four governorates, i.e. Kafrelsheikh, Dakahlia, Gharbia and Beheira in Egypt during 2017 and 2018 in Table 1.

Isolation and identification of the causal organism:

Infected rice sheaths and leaves were cut as small pieces, nd surface sterilised by soaking them in a 0.5 percent sodium hypochlorite solution for 2 minutes before being washed with sterilised distilled water. Drying the sterilised pieces between sterile filter papers before transferring them to Petri dishes with water agar (WA). At 25°C, the plates were incubated. The isolates were purified using the hyphal tip technique (Burgess *et al*., 2008) on a water agar medium and identified using Barnett and Hunter parameters (1998).

Table 1: Sources of different isolates of *Rhizoctonia solani*.

Isolate no.	Governorate	District	Rice cultivar	Year
RS1	Kafrelsheikh	Sakha	Giza 178	2017
RS2	Kafrelsheikh	Desouq	Sakha 101	2017
RS3	Kafrelsheikh	Metbol	Giza 178	2018
RS4	Kafrelsheikh	Foaa	Sakha 101	2018
RS5	Kafrelsheikh	Qallin	Sakha 104	2018
RS6	Gharbia	Gemmiza	Sakha 101	2018
RS7	Gharbia	Basuooen	Sakha 104	2018
RS8	Kafrelsheikh	Misar	Sakha 108	2018
RS9	Kafrelsheikh	Sakha	Sakha 104	2017
RS10	Dakahlia	Mansoura	Sakha 101	2017
RS11	Dakahlia	Met Sweed	Sakha 101	2018
RS12	Gharbia	Qotour	Giza 178	2018
RS13	Gharbia	Basuooen	Sakha 101	2018
RS14	Beheira	Mahmoudia	Sakha 101	2018
RS15	Beheira	Itai-El-Barood	Sakha 104	2018
RS16	Dakahlia	Meit Azon	Giza 177	2017
RS17	Dakahlia	Kafr Tanah	Hybrid 1	2018

2- Morphological characterization:

The fungal isolates of *R. solani* were cultured on Petri dishes (9 cm) containing potato dextrose ager (PDA) medium. Discs of 5mm diameter of the margins of 2 - day old cultures were used to transfer to the center of Petri plates containing PDA medium and incubated at 25 ± 2°C for 12 days. Cultural characteristics of all tested isolates and hyphal anastomosis characteristics of *R. solani* RS 13 and RS10 isolates were studied according to (Moni *et al.,* 2016).

3- Molecular identification:

Fungal DNA extraction:

Two isolates (R10 and R13) represent two governorates Dakahlia and Gharbeia and showed the highest aggressively were subjected to molecular identification. DNA extraction was performed by the method used by Kuramae-Izioka (1997). The

primer combination ITS1 (TCC GTAGGTGAACCTGCGG) and ITS4 (CCTCCGCTTATTGATATGC) were used to amplify the ITS region using an automated thermal cycler (C1000 TM Thermal Cycler, Bio-RAD, Hercules, CA) (White *et al*., 1990). Sigma Company in Germany sequenced purified PCR products.

DNA sequencing and evolutionary relationships of taxa data analysis:

Through Sigma Company, Germany DNA sequencing was performed. Chromatograms of the raw sequence were assembled and edited using GAP4 (Bonfield *et al*., 1995). Using the BLAST algorithms the known fungal sequences were detected according to (Altschul *et al*., 1997) with a database of GenBank at http://www.ncbi.nlm.nih.gov/blast. The evolutionary history was inferred using the Neighbor-Joining approach (Saitou and Nei, 1987). The optimal tree is shown, with the sum of branch lengths. The branch lengths are in the same units as the evolutionary distances used to estimate the phylogenetic tree, and the tree is drawn to scale. The evolutionary distances were calculated using the maximum Composite Likelihood technique (Tamura *et al*., 2004), and are in base substitutions per site units. A total of 18 nucleotide sequences were examined. 1st+2nd+3rd+Noncoding codon locations were included. For each sequence pair, all unclear locations were deleted. MEGA X was used to undertake evolutionary analysis (Kumar, 2018).

3- Screening of *R. solani* isolates for hydrolytic cellulolytic, amylolytic and pectolytic enzymes production in vitro:

The screening of cellulase activity enzymes was done by inoculates the *R. solani* isolates on the medium containing carboxymethyl cellulose (CMC) as described by Leopold and Samsˇinˇáková (1970). For pectinase activity isolates of *R. solani* were growing on the medium containing pectin as described by Hankin & Anagnostakis (1975). A 5 mm mycelial disc from each *R. solani* isolate's 7-day-old culture was placed in the center of the plates for CMC and pectin media, and then incubated at 25±2°C. 1 percent aqueous hexadecyl-trimethyl-ammonium-bromide was pumped into culture plates (HTAB) after three days of incubation, a clear zone formed surrounding the culture growth, indicating cellulose and pectinase enzymes activity. The clean zone's average diameter was measured and recorded (Gawade *et al*., 2017). Amylase activity was inoculated on starch agar medium and incubated for 3 days at 25±2°C. The growth plates were soaked in iodine solution for a minute before being drained out. Plates were visually inspected for the presence of. Plates were screened visually for the appearance of clear zones around the colonies (Jalgaonwala and Mahajan, 2011).

4- Extraction of extracellular enzymes activity from infected tissue:

Rice sheath plants at tillering stage of SK101 and E. Yasmine were collected 14 days after inoculation with different isolates of *R. solani* and quickly frozen in liquid nitrogen and stored at -20°C.

A) Cellulase enzyme assay:

Calorimetric measurements of reducing sugars produced by hydrolysis of carboxymethyl cellulose (CMC) as a substrate were used to quantify enzymatic activity. A 50 mg sheath sample was homogenised for one minute in a blender with 100 mM Sodium acetate buffer (pH 6.0) containing 0.2 % sodium dithionite ($Na_2S_2O_4$) and 1 % PVP (MW 44000), centrifuged at 10,000 rpm for 20 minutes, and the supernatant was kept at -12°C. Centrifuged, filtered through Whatman No.1 filter paper, and dialyzed against water for 48 hours after adjusting the pH to 8.2 with NaOH and incubating overnight at 4°C with continuous stirring. For enzyme assay, 0.5 ml of 1% CMC and 0.5 ml 100 mM sodium acetate buffer (pH 5.0) were incubated at 50°C for 30 min. the reaction was started by adding 3.0 ml of dinitrosalicylic Acid (DNS) reagent and 0.5g crude enzymes and complete with 20 ml distilled water. After 5 minutes in a boiling water bath, the tubes were cooled to room temperature and the absorbance was measured at 540 nm. The same material without crude enzyme was used as blank. By DNS method using D-glucose as a standard, the reducing sugars were estimated (Ghose, 1987).

B) Assay of PME:

Spectrophotometric test was used to evaluate PME enzyme activity at 25°C. Using a pestle and mortar, a 200 mg rice sheath sample was homogenised with 15 ml cold 8.8% NaCl, centrifuged at 12000 rpm for 10 minutes, collected the supernatant, adjusted its pH to 7.5 with NaOH, and utilised for enzyme assay. The continuous spectrophotometric assay of the PME enzyme activity was determined at 25ºC. 0.15 ml of 0.01% (w/v) bromothymol blue solution was mixed with 2 ml of 0.5% pectin and 0.83 ml water and incubated at 25°C in a circulating water bath, in a cuvette. By adding 100 µl enzyme solution the reaction was started and measured the rate of decrease at 620 nm and calculated the activity of enzyme at intervals of 20, 40, 60 and 80 s. For PME enzyme against water blank the determined initial absorbance at 620 nm (A620) (Gawade *et al*., 2017).

5- Pathogenicity test of *R. solani* isolates under greenhouse conditions:

Two rice cultivars SK 101 and E. Yasmine were used in the pathogenicity test under greenhouse conditions. Before sowing, rice seeds were surface sterilised for 10 minutes with 1 percent sodium hypochlorite. Five sterilised seeds of each cultivar were sown in plastic pots (30 cm in diameter) with sterilised soil and stored at 25–30°C. For each isolate, three pots were utilised. Seedlings were thinned as one seedling per pot after 21 days following seeding. Urea 46.5 % N (1 g/pot) was used to fertilise the plants. Rice plants at a maximum tillering stage were inoculated with *R. solani* isolates by placing a mycelial disk around the leaf sheath. The inoculated sheaths are covered immediately with aluminium foil (Park *et al*., 2008). The plants

were daily observed, and when infection lesions started to appear, the aluminium foil was removed and plants were left in humidity between 80 and 100% at 28°C for further 14 days.

Disease evaluation:

According to the standard evaluation system (SES) (IRRI, 2013), the disease score was taken from the scale for sheath blight (ShB) Relative lesion height (RLH): disease progress relative to plant height. (0) No infection observed, (1) Lesions limited to lower 20% of the plant height, (3) 20-30%, (5) 31-45%, (7) 46-65%, (9) more than 65%. RLH% = (Lesion height/ Plant height) X100 according by Sharma *et al*. (1990). The percentage of infected plants was calculated from the total number of infected plants for each isolate/ total plant.

6- Behavior study of *R. solani* for susceptibility of rice seedlings:

Seeds of two rice cultivars i.e SK 101 (Japonica type) and E. Yasmine (Indica type) were germinated in incubator conditions. Seeds of each cultivar were first surface-sterilized with immersing in 1% sodium hypochlorite solution for 2 min, followed by washing with sterile distilled water three times. Culture bottles containing sterilized soil was used to sowing surface-sterilized seeds. Seeds were grown in growth chambers at a temperature of 25-27 °C with 16 h light and 8 h dark. Four replicates were used for each cultivar. Seedlings were inoculated at 14 days old using a single mycelium disc growing for 7 days old from RS13 isolate and placed at a distance of 1 cm from the middle of each leaf, then covered immediately with apices of cotton and rolled with aluminium foil. The development of fungal hyphal growth and sclerotia on the surface of leaves was observed under a light microscope and the inoculated leaves were studied 1, 2 and 3 days post-inoculation (dpi) until 7 dpi.

Preparation of rice leaves for compound microscopy

Infested leaves of 0.5 cm long were stained with 0.05% trypan blue solution for 10 min. then washing three times with lactophenol and put on glass slides. Three microscopic fields were observed for each leaf. The samples were mounted for observation under a compound microscope (Basu *et al*., 2016).

A scanning electron microscope (SEM): The surface scan was performed using an SEM (JEOL, JSM-6400, and JAPAN) for detailed observation of the hyphal behavior in rice leaves. Before the scanning process, all non-inoculated (control) and inoculated rice leaf samples were dried and coated with gold to enhance the electron conductivity (Chowdhury *et al*, 2014). Samples were observed at 1, 2 and 3 dpi for each cultivar of rice.

Data Analysis: Data were statistically analyzed using standard statistical analysis with MSTATC. in the table of main treatments, Duncan's Multiple Range, T. (1955) was used to compare the significantly different averages.

RESULTS

Identification of the causal organism: -

All isolates were varied on culture colour and divided into three colour groups; cream, dark brown and light brown. Eight isolates having dark brown colour followed by 5 light brown isolates and 4 isolates are cream (Table 2 and Fig. 1). The nature of mycelial growth was varied between areal/flat and flat growth patterns. Flat mycelial growth formed in 9 isolates and 8 isolates as areal/flat growth. Rapid growth rates were exhibited in 11 isolates followed by 6 isolates were moderately (Table 2), so in almost 2 days the surface of the entire plate was filled. The major runner hypha breadth of the mycelium hyphae masses ranged from 7.5 to 12.5 µm. The morphological variation of all isolates exhibited a wide range (size of sclerotia and abundance of sclerotia production). On PDA media the sclerotial colors were developed from white at first and became brown to black-brown after maturation in all isolates (Fig. 1). Different sclerotial distribution patterns were showed as peripheral, centered and scattered (Fig. 1). At the point of branching, typical right angles are usually seen at hyphal constrictions (Fig. 2) with the use of a light microscope the mycelium is made up of hyphae that are divided into individual cells by dolipore septa, which have a wide range of taxonomic characteristics. (Fig. 2E) hyphal anastomosis characteristics of *R. solani* isolate RS 13 (Fig. 2F and G) and RS10 (Fig. 2H).

Table 2: Morphological characteristics of *Rhizoctonia solani* isolates, the causal fungal of rice sheath blight disease

Isolate No.	Mycelial colony*				Sclerotial characteristics*			
	Growth nature of mycelium	Colour	Growth rate	Hyphal dimension (µm)	No. of days to produce sclerotia	Colour	Sclerotia size (mm)	Formation pattern
RS1	Flat/aerial	Cream	Rapid	7.5	5	Dark brown	1.2x1.3	Scattered
RS2	Flat/aerial	Cream	Rapid	11.2	5	Black brown	3.0x1.0	Peripheral
RS3	Flat	Dark brown	Rapid	7.5	5	Dark brown	1.0x1.0	Central + Peripheral
RS4	Flat	Dark brown	Moderate	11.25	9	Dark brown	0.1x0.2	Central
RS5	Flat	Dark brown	Rapid	7.5	5	Dark brown	0.2x0.39	Central
RS6	Flat	Dark brown	Rapid	7.8	5	Dark brown	0.2x0.25	Central
RS7	Flat	Light brown	Rapid	10.5	5	Dark brown	3.0x2.5	Central+ scattered
RS8	Flat/aerial	Cream	Rapid	10.0	7	Black brown	1.5x1.9	Central
RS9	Flat/aerial	Dark brown	Rapid	12.5	9	Dark brown	0.25x0.34	Scattered
RS10	Flat/aerial	Dark brown	Rapid	12.5	3	Dark brown	0.24x0.12	Central+ Peripheral
RS11	Flat/aerial	Cream	Rapid	7.5	7	Black brown	1.0x2.0	Peripheral
RS12	Flat/aerial	Light brown	Moderate	7.5	5	Dark brown	3.0x2.0	Scattered +Peripheral
RS13	Flat/aerial	Light brown	Rapid	7.5	5	Dark brown	2.0x3.0	Central + scattered
RS14	Flat	Light brown	Moderate	10.0	6	Dark brown	3.0x2.0	Central+ Peripheral
RS15	Flat	Dark brown	Moderate	7.5	7	Dark brown	0.9x0.7	Central+ Peripheral
RS16	Flat	Dark brown	Moderate	9.75	4	Dark brown	1.25x1.25	Central+ Peripheral
RS17	Flat	Light brown	Moderate	12.5	12	Dark brown	0.12x0.15	Central+ scattered

Observations recorded 12 days after incubation; Colum is average of 5 observations. Moderate = covering Petri plate (90mm diameter) in 60hrs; Rapid =covering Petri plate (90mm diameter) in 48 hrs

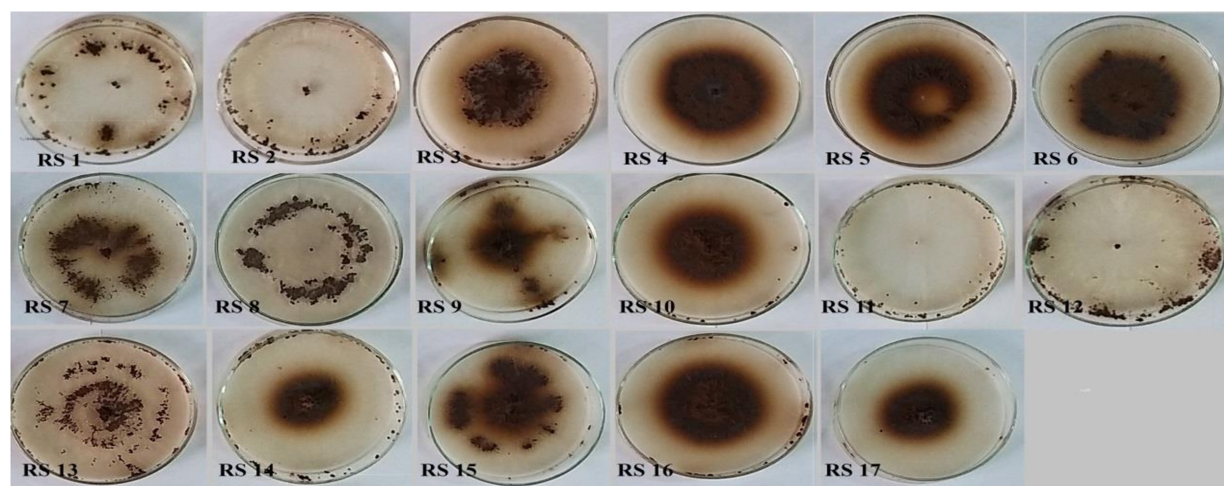

Fig. 1: Cultural variation of *R. solani* (RS1 to RS 17) showing sclerotial distribution

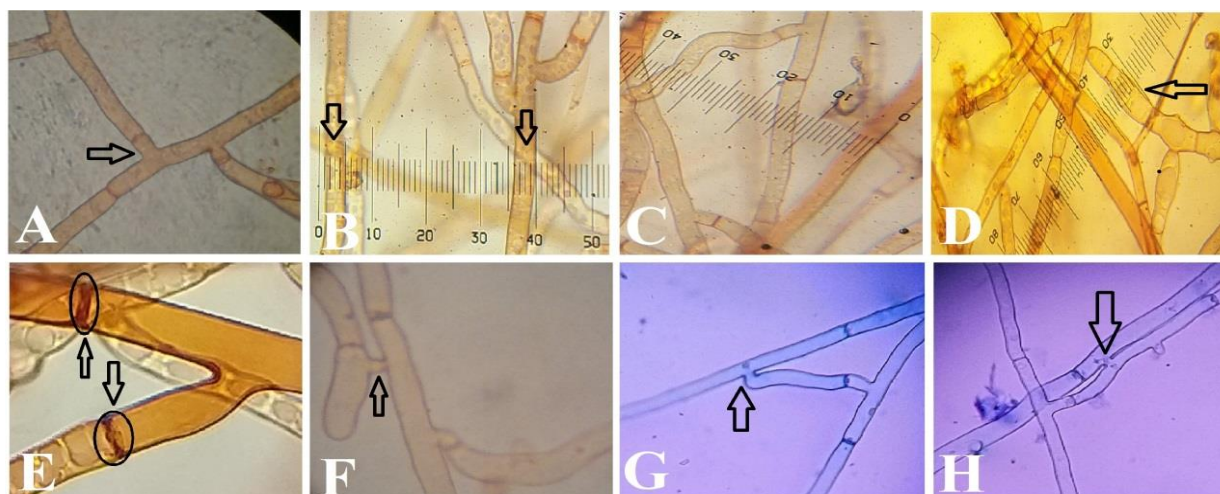

Fig. 2: Hyphal characteristics of *R. solani*, (A, B, C and D) right angle, Foot cell with restriction, multinucleated cells (B), and mycelia branching with a slight constriction at the point of branching and septum (E). Hyphal anastomosis of RS13 isolate (F and G) and RS10 isolate (H). Bar =10µm (A, B, C, D,F,G and H) and 5 µm (E)

Molecular identification

Two isolates; RS10 and RS13 were selected for molecular identification, A single PCR product of 661 bp was produced using ITS1/ITS4-specific primer pair. The ITS1/ITS4 sequences of the tested two Egyptian Rhizoctonia isolates were submitted and get accession numbers; MZ357689 for RS10 (Hassan, A.A) and MZ040497 (AB5) for RS13 (Abdelbary, A.A) in (NCBI) Genebank as follows. Phylogenetic trees (Fig. 3) revealed high similarity between isolates RS10 (MZ357689) and RS13 (AB5) (MZ040497) sequences and other isolates Blasted in the GenBank sequences and unknowns were excluded from the analyses as well. The most closely related sequence to our isolate's sequences was *R. solani* AG1-IA isolates from Egypt with accession number (KY570520) and China with accession number MG763678, MG397054, and MG770085).

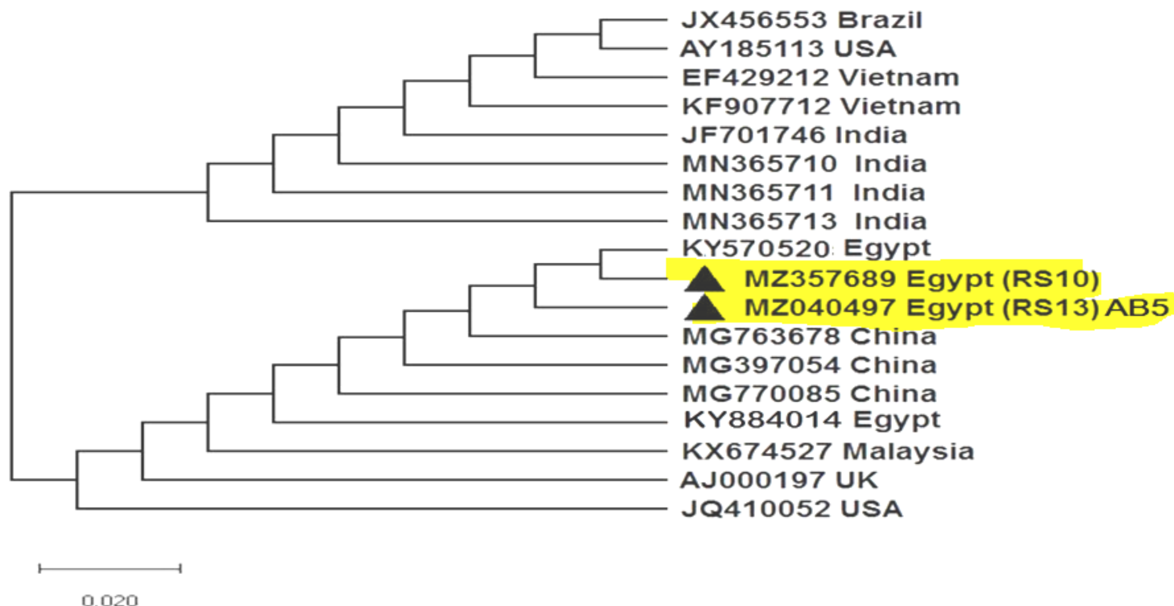

Fig. 3: Phylogenetic tree based on rDNA sequences of RS10 and RS13 isolates and related sequences retrieved from the GenBank using the Neighbor-Joining method. Bootstrap support on the nodes represents maximum-likelihood (ML) and Maximum parsimony (MP) ≥ 50%.

Production of extracellular enzymes (plate assay) by *R. solani* isolates:

The test isolates exhibited cellulase, pectinase and amylase enzymes production (Table 3), around the fungus growth by the clear zone produced (mm) to measurement the enzymes activity. Wide variability in cellulase enzyme was showed by *R. solani* isolates it was highest with the isolate RS13 (78.00 mm), followed by RS10 (65.0 mm), whereas, it was lowest with the isolate RS3 (10.33 mm). Pectinase enzyme production of the test isolates ranged from 29.00 to 60.00 mm of (Table 3). However, it was highest as evidenced by clear zone (mm) with the isolate RS10 (60.00 mm), followed by RS13 (59.00 mm), and whereas, it was moderate with the isolate RS9 (55.67 mm) and RS7 (54.33 mm) and in the control plate did not indicate any pectinase enzyme production. The amylase enzyme production ranged from 45.00 to 88.33 mm of the test isolates.

However, RS10 (88.33 mm) was highest amylase production followed by RS13 (86.67 mm), while it was lower with isolates RS3 (45.00 mm) and RS8 (51.67 mm).

Profile activity of Extracellular enzymes from infected rice samples (Cell-wall-degrading enzymes):

R. solani test isolates produced extracellular cellulase enzyme from infected rice plant specimens. The results in Table (3) showed wide variability in cellulase enzyme activity it was highest with the isolate RS13 on both SK101 and E. Yasmin (420.07 and 325.0 mg, respectively), followed by isolate RS10 (389.87 and 299.45 mg, respectively) and RS9 (379.8 and 275.67 mg, respectively). The cellulase activity in sheath leaves on SK 101 was a minimum of isolates RS5 and RS3 (208.78 and 204.28 mg, respectively), and the same isolates RS3 and RS5 (105 and 108 mg, respectively) proved minimum cellulase activity with sheath leaves on E. Yasmine. Also, PME activity results in (Table 3) indicate that the tested isolates exhibited varied PME enzyme activity in both cultivars, which ranged from 0.104 to 0.54 optical densities (OD/min/g) with isolate RS5 and RS13, respectively on SK101 rice cv. While, with E. Yasmine the PME enzyme of the tested isolates ranged from 0.102 to 0.359 OD/min/g with isolate RS5 and RS10, respectively. However, it was maximum (OD/min/g) with the isolate RS13 on SK 101 (0.54), followed by the isolate RS10 (0.428). While, on E. Yasmine maximum (OD/min/g) with the isolate RS10 (0.359), followed by the isolates RS13 and RS2 (0.272) and it was lowest in untreated control for SK101 and E. Yasmine (0.101 and 0.093 OD/min/g, respectively). Even though some of the isolates were highly or moderately pathogenic, there was reduced PME enzyme production. When comparison to SK101, E. Yasmine produced fewer PME enzymes.

Table 3: Extracellular enzymes production by *R. solani* isolates in vitro and infected plant tissue samples collocted from Sakha 101 and Egyptian Yasmnie

Isolate	Plate assay			Infected rice samples after 14 days			
				Cellulase activity (mg of D-glucose/hr/ml extract		PME (ΔA620min/1g/1 fresh weight)	
	Cellulase (mm)	Pectinase (mm)	Amylase (mm)	SK101	E. Yasmine	SK101	E. Yasmine
RS1	41.67^{de}	50.00^{de}	68.67^{cd}	269.56^{de}	170.0^{g}	0.25^{e}	0.204^{d}
RS2	43.67^{d}	50.00^{de}	71.67^{cd}	273.4^{de}	173.0^{g}	0.373^{e}	0.272^{b}
RS3	10.33^{g}	29.00^{j}	45.00^{g}	204.28^{i}	105.0^{l}	0.194^{i}	0.171^{efg}
RS4	37.00^{ef}	40.33^{hi}	65.00^{cde}	253.88^{fg}	162.0^{h}	0.198^{i}	0.158^{g}
RS5	34.67^{f}	38.67^{i}	61.67^{def}	208.78^{i}	108.0^{l}	0.104^{k}	0.102^{i}
RS6	38.67^{def}	46.67^{efg}	53.33^{fg}	249.11^{fgh}	145.75^{i}	0.187^{i}	0.167^{efg}
RS7	57.67^{c}	54.33^{bcd}	73.33^{bc}	283.54^{cd}	184.5^{e}	0.39^{d}	0.161^{fg}
RS8	33.33^{f}	39.00^{i}	51.67^{fg}	217.14^{i}	119.16^{k}	0.158^{j}	0.128^{h}
RS9	62.33^{bc}	55.67^{abc}	83.33^{ab}	379.8^{b}	275.67^{c}	0.41^{c}	0.247^{c}
RS10	65.00^{b}	60.00^{a}	88.33^{a}	389.87^{b}	299.45^{b}	0.428^{b}	0.359^{a}
RS11	38.00^{def}	42.67^{ghi}	61.67^{def}	277.89^{cd}	177.5^{f}	0.19^{cd}	0.186^{de}
RS12	61.67^{bc}	52.33^{cd}	74.00^{bc}	292.04^{c}	193.05^{d}	0.398^{cd}	0.183^{def}
RS13	78.00^{a}	59.00^{ab}	86.67^{a}	420.7^{a}	325.0 ^{a}	0.54^{a}	0.272^{b}
RS14	35.67^{ef}	49.67^{def}	65.00^{cde}	242.45^{gh}	145.0i	0.224^{h}	0.124^{h}
RS15	35.00^{f}	42.67^{ghi}	56.00^{ef}	235.24^{h}	135.0^{j}	0.198^{i}	0.169^{efg}
RS16	38.00^{def}	44.67^{fgh}	55.00^{efg}	245.04^{gh}	145.9^{i}	0.281^{f}	0.181^{efg}
RS17	41.33^{de}	46.33^{efg}	71.67^{cd}	260.96^{ef}	160.0^{h}	0.194^{i}	0.174^{efg}
Control	0.00^{h}	0.00^{k}	0.00^{h}	102.62^{j}	94.5^{m}	0.101^{k}	0.093^{i}

In a column, means followed by a common letter are not significantly different at the 5% level by DMRT.

Pathogenicity tests of *R. solani* isolates using Sakha 101 and Egyptian Yasmine rice cultivars under greenhouse conditions:

In a greenhouse, the artificially inoculated rice plants (SK101 and E. Yasmine) exhibited typical lesions of sheath blight (Fig. 4). Data presented in Table (4) indicated that all the tested isolates varied in their virulence on SK101 and E. Yasmine. The isolates were divided into three categories of relative lesion height (RLH) % i.e. 21–30%, 31–45 % and 46–65% on SK101 rice cv. The isolate RS13 recorded high disease score (5) in maximum tillering stages (Table 4). While, on E. Yasmine rice cv, all isolates were divided into three categories of RLH % i.e. 0-20%, 21–30% and 31–45 %. Isolates number RS 13, 10 and 9 collected from Gharbia, Dakahlia and Kafrelsheikh governorates, respectively, proved to be the most aggressive isolates for SK101 and E. Yasmine rice cvs. However, isolates RS5 and RS3 which were collected from Kafrelsheikh gov. was the least virulent among all the tested isolates. High disease score, infection % and a number of sclerotia were found on isolates RS13 followed by RS10, RS9, RS12, RS7, RS2 and RS1. E. Yasmine rice cv had fewer values in the disease infection % and a number

of sclerotia compared with SK 101 rice cv. Sakha 101 rice cv proved the most susceptible for infecting with sheath blight pathogen compare with E. Yasmine.

Table 4: Pathogenicity test on two rice cultivars against *Rhizoctonia solani* isolates under greenhouse condition at tillering stage

Isolate No.	District	Sakha 101				Egyptian Yasmine			
		Infection %	RLH%	DS (0–9)	No. of Scerlotia/ total	Infection %	RLH%	DS (0–9)	No. of Scerlotia / total
RS1	Kafrelsheikh	70.01^{cd}	30.59^{def}	5	9.00^{e}	30.08^{cd}	21.93^{d}	3	3.00^{cde}
RS2	Kafrelsheikh	70.74^{cd}	31.79^{cde}	5	9.16^{e}	35.00^{c}	27.9^{c}	3	3.67^{bcd}
RS3	Kafrelsheikh	47.94^{f}	24.59^{gh}	3	2.00^{ij}	11.65^{f}	12.35^{h}	3	0.00^{g}
RS4	Kafrelsheikh	66.42^{cde}	29.19^{ef}	3	7.00^{fg}	27.67^{d}	20.29^{def}	3	3.00^{cde}
RS5	Kafrelsheikh	44.44^{f}	21.24^{h}	3	1.50^{jk}	10.19^{f}	12.13^{h}	1	0.00^{g}
RS6	Gharbia	62.16^{de}	27.65^{efg}	3	4.00^{h}	17.84^{e}	17.31^{fg}	1	1.00^{fg}
RS7	Gharbia	73.25^{c}	33.43^{cd}	5	14.0^{d}	35.10^{c}	28.45^{c}	3	4.00^{bc}
RS8	Kafrelsheikh	60.1^{e}	27.26^{fg}	3	4.00^{h}	13.81^{ef}	16.26^{g}	1	0.00^{g}
RS9	Kafrelsheikh	85.45^{b}	35.89^{bc}	5	20.50^{c}	45.7^{ab}	31.30^{ab}	5	5.00^{b}
RS10	Dakahlia	87.6^{ab}	38.74^{b}	5	24.0^{b}	49.95^{a}	31.50^{ab}	5	7.34^{a}
RS11	Dakahlia	48.2^{f}	27.25^{fg}	3	3.50^{hi}	11.65^{f}	15.78^{g}	1	0.00^{g}
RS12	Gharbia	82.65^{b}	33.49^{cd}	5	20.0^{c}	41.67^{b}	28.69^{bc}	3	5.00^{b}
RS13	Gharbia	95.00^{a}	55.50^{a}	7	30.5^{a}	50.67^{a}	32.43^{a}	5	8.00^{a}
RS14	Beheira	62.79^{de}	27.85^{efg}	3	4.00^{h}	26.16^{d}	18.73^{efg}	1	2.00^{ef}
RS15	Beheira	62.73^{de}	27.67^{efg}	3	4.00^{h}	24.98^{d}	18.39^{efg}	1	1.00^{fg}
RS16	Dakahlia	64.33^{de}	28.26^{efg}	3	6.16^{g}	27.62^{d}	19.61^{def}	1	2.34^{def}
RS17	Dakahlia	66.45^{cde}	29.77^{def}	3	8.0^{ef}	29.50^{cd}	20.92^{de}	3	3.00^{cde}
Control	-	0.0^{g}	0.0^{i}	-	0.0^{k}	0.0^{g}	0.0^{i}	-	0.00^{g}

RLH; Relative lesion height DS; Disease score (0-9)

In a column, means followed by a common letter are not significantly different at the 5% level by DMRT.

Fig.4: Rice sheath blight symptoms related to infection by *Rhizoctiona solani* isolates (RS 13). Method of inoculation on SK101 (A) and development of infection on SK101 (A, B, C and D), and method of inoculation on E. Yasmine (E) disease development on E. Yasmine sheath leaves (E, F, G and H), brown sclerotia development on SK101 (C), whit sclerotia on E.Yasmine(G).

Behaviour of *R. solani* in both hyphal growth and development of sclerotia on the leaf surface of two rice cultivars:

At 2 days post-inoculation, the hyphae started to grow from the mycelial discs of isolate RS 13 placed on the surface of rice leaves. on SK101 rice susceptible leaves the hyphae growth of *R. solani* was visible (Fig. 5B1) as well as on E. Yasmine rice leaves at 2 dpi (Fig. 5A1). In the case of leaves of the E. Yasmine the hyphal growth was comparatively less at 3 dpi (Fig. 5A2). Conversely, on the susceptible SK101 leaves growth the fungal hypha was more profuse at 3 dpi (Fig. 5B2). Hyphae approaching stomata directly and was showing penetration on E. Yasmine (5A1). Hyphae approaching stomata using papillae as props and showing penetration of stomata by hyphae on SK101 (Fig. 5B1), and within 3 dpi the hyphal colony were covered SK101 leaf tissues (Fig. 5B2). *R. solani* developed sclerotia in the vicinity of the surface of leaves of both cultivars. However, major differences were observed between developments of sclerotia on the surface of leaves for SK101 and E. Yasmine (Fig. 5A3 and 5B3). Sclerotial development on the leaves did not begin until 4 dpi. At 5 dpi, the number of fungal sclerotia was considerably higher in the surface of SK 101 susceptible rice leaves (Fig.5B4), whereas in the E. Yasmine sclerotial development on the leaves did not begin until 7 dpi (Fig . 5A4). All of the sclerotia observed in the vicinity of the E. Yasmine rice leaves at this time point were in the initial stages of maturation (Fig. 5A). Infected leaves after 2, 3, 4, 5, 6 and 7 dpi with *R. solani* (RS13) were shown on (Fig. 6). Infected leaves on E. Yasmine were started after 2 dpi (Fig.6A) as well as SK101 (Fig. 6B). While, after 7dpi the leaf area was completely infected in the case of SK101 compare with E.Yamine only a part of leaf area was infected.

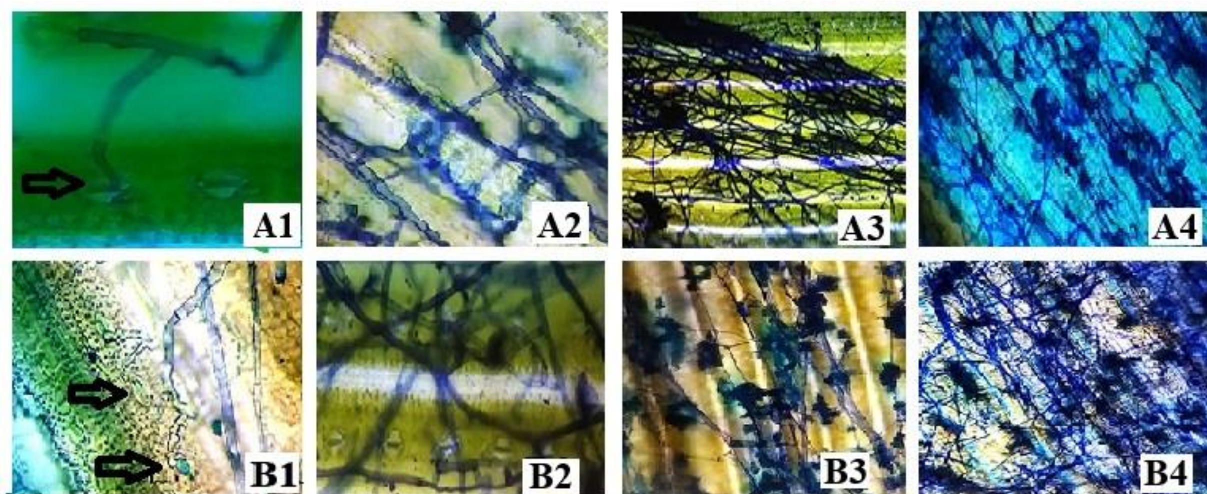

Fig. 5: *Rhizoctonia solani* on the leaves surface of Egyptian Yasmine (A) and Sakha 101 (B) rice cultivar. Hyphal growth at 2 dpi (days post-infection) (A1) and (B1), Hyphae growing on stoma at 2 dpi arrow (A1), 2 dpi hyphae growing in branches on stoma arrow (B1). Developed of hyphae growth and making more dispersed onto the leaf surface on (A2). Hyphae making more dispersed and closely entrenched onto the leaf surface on (B2 and A3), Sclerotial development on the leaf surface on (A4 and B3), Sclerotial more dispersed and closely entrenched onto the leaf surface on (B4).

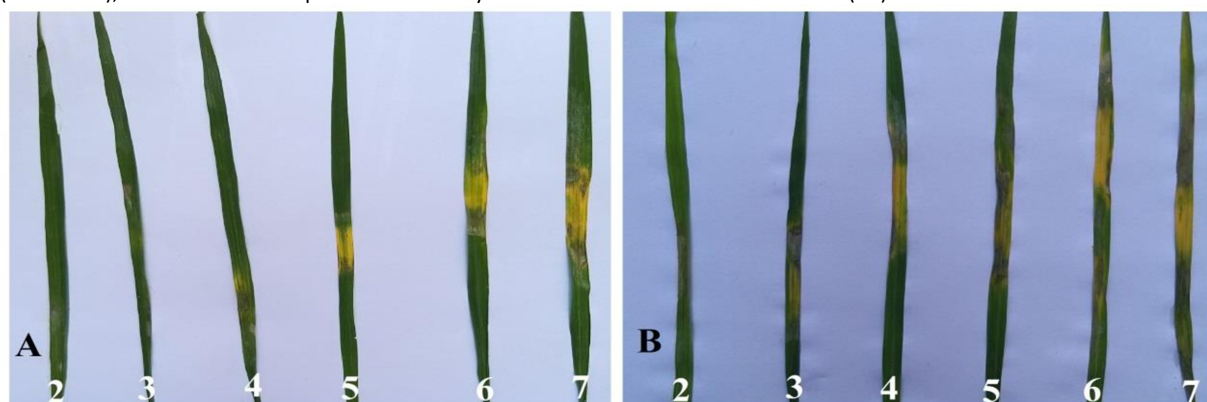

Fig.6: Infected leaves developments after 2,3,4,5, 6 and 7 dpi of E. Yasmine (A) and of Sakha 101(B).

Interception of stomata and trichomes of rice leaves by *R. solani* hyphae:

To investigate the influence of surface structures of leaves on the direction of growth of hyphae, the interaction of the hyphae with trichomes of leaves was observed by SEM. One interesting observation was that the hyphae could distinctly locate nearby protrusions and openings when growing over the surface of rice leaves (Fig. 7). The hyphae branches were growing more than in the case of SK101 after 2dpi (Fig. 7D) compare with E.Yasmine at 2dpi (Fig. 7A). SEM showed that the

hyphae sometimes grew around the papillae and used these as a support before penetration in E.Yasmine and SK 101 cv on (Fig. 7B and E). Also, the observation in the SK 101 leaf surface, the hyphae were seen to wrap around the trichomes to ensure secure anchorage (Fig. 7E). Although the interaction of hyphae with individual surface structures in SK 101 and E. Yasmine leaves were similar, they were fewer in the E. Yasmine as the amount of hyphae infestation was visibly less. Also, the present results found that the infection cushions on leaves to be structurally similar and the pathogen produced more than 10 times the number of infection cushions on SK101 than on the E. Yasmine (Fig. 7F and C).

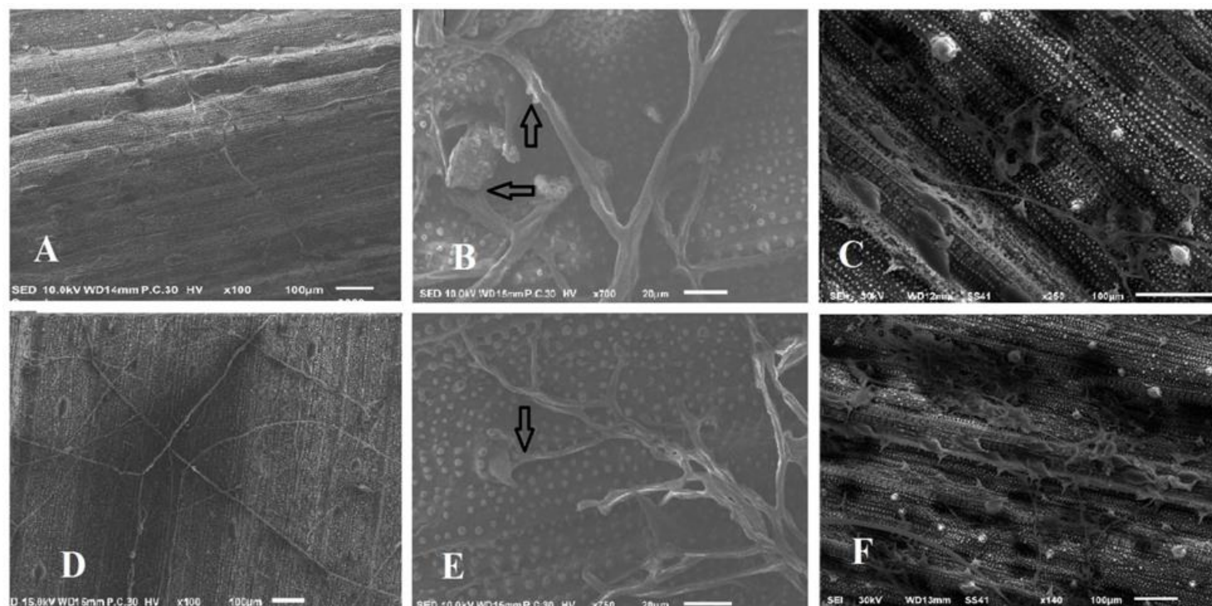

Fig. 7: Scanning electron microscopy (SEM) on the leaves surface of Egyptian yasmine (A, B and C) and Sakha 101 (D, E and F) rice cultivar inoculated with *Rhizoctonia solani* (isolate RS13), Hyphae growth at 2-day post-inoculation of E. Yasmine (A) and S.101 (D). (B), fungal hyphae wrapping around a prickled trichome of E. Yasmine (B) and S.101 (E). the hyphae growing in bunches, many contacts with surface of the E.Yasmine at 4 dpi (C), hyphae making more dispersed and closely entrenched onto the leaf surface at 5 dpi on S.101 (F).Bar =100µm (A,C,D and F) and 20µm (B and E).

DISCUSSION

R. solani is one of the soilborne fungal pathogens causing the rice sheath blight, which is one of the most serious diseases of rice worldwide (Wamishe *et al*, 2007). Under Egyptian conditions, 17 samples with typical symptoms for sheath blight were collected from different regions. The isolates were identified based on their morphological and molecular characterization. Variation between *R. solani* AG-1 IA isolates using morphology, physiology and pathogenicity tests have previously been studied Moni *et al*., (2016). The morphological parameters of the isolates showed a wide range of diversity in the results. By using a compound microscope, characteristic right angles can be detected at the point of branching in *R. solani* hyphal constrictions. The mycelium is made up of hyphae that have been partitioned into separate cells by dolipore septa, which have significant taxonomical features. The results agree with Moni, *et al*. (2016) using a compound microscope, the 18 isolates with hyphal branching at a right angle, constriction at the point of branching of the mycelium, and the presence of a septum near the branching junction were identified as *R. solani*.

Sequence analysis was used to classify Rhizoctonia species into genetic groups rather than morphological characters (González *et al*., 2006). For determination of the anastomosis groups (AG) using ITS-rDNA primer is a convenient sequence analysis of the genomic regions of the fungus (Lehtonen *et al*., 2008). Two isolates; RS10 and RS13 were selected using ITS1/ITS4-specific primer pair for molecular identification. The ITS1/ITS4 sequences of the tested two Egyptian Rhizoctonia isolates were got accession numbers and submitted as follows MZ357689 for RS10 and MZ040497 for (AB5) RS13 in (NCBI) Genebank. Phylogenetic trees revealed high similarity between isolates RS10 (MZ357689) and RS13 AB5 (MZ040497) and other isolates. The most closely related sequence to our isolate's sequences was *R. solani* AG1-IA isolates from Egypt and China. This indicated that there is a high similarity percentage between our isolates and other isolates under the same species and different species as well. These results indicated that RS10 and RS13 were both *R. solani* and belong to anastomosis group AG1-IA.

The results are in agreement with those of Nadarajah *et al*. (2014) who screened Malaysian isolates of *R. solani* from rice and showed that these strains were closely related to *R. solani* AG1-1A (with 99-100% identity). El-Shafey, *et al*. (2019) identified five Egyptian isolates from *R. solani* isolated from rice sheath blight of Egypt was submitted recive accession

numbers as follows; KY570520 for R01, R02 KY884017 for R05, KY884016 for, KY884015 for R12 and KY884014 for R18 in NCBI Genebank. The Egyptian isolates had a sequence similarity to *R. solani* AG1-1A of 85–100% El-Shafey, *et al*. (2019).

Enzymes play an important role to investigate the interactions between the host and the pathogen, as well as the mechanisms involved in the degradation of the host cell wall. Fungal endophytes produce many extracellular enzymes such as cellulase, pectinase and amylase which can depredate the major polymeric components of the host cell wall. In vitro all isolate can produce extracellular enzymes such as cellulase, pectinase and amylase. The results agree with Mondal, *et al*. (2013) who's studied the ability of 20 *R. solani* isolates recovered from symptoms of sheath blight disease of rice to produce cellulase and pectinase enzymes. There was a link between biochemical ingredients and enzyme activity and *R. solani* isolate virulence, with pectinase activity being the most relevant determinant in *R. solani* strain virulence. Cell-wall-degrading enzymes are playing important factors in host-pathogen interactions determining. Cellulase and PME enzyme production were less in E. Yasmine compared with SK101 and the results of two enzymes positive correlation with virulence of the test isolates *R. solani*. The cellulase and PME enzyme are relating to development of rice sheath blight *R. solani*. However, The regulation of these enzymes in obligate biotrophic fungi is poorly understood. A cell wall is composed of polysaccharides, pectin, cellulose, lipids, hemicellulose, and proteins. Virulent fungi such as Rhizoctonia isolates were produced enzymes which degraded of cell wall (Gawade *et al*., 2017). Production of pectinase and cellulase activity plays an active role in disease development of soybean root rot *R. bataticola* (Gawade *et al*., 2017).

In rice germplasm, no source of complete resistance has been identified for sheath blight. The likelihood of finding such a resistance source of rice genotypes is also low. Given the low prevalence of the sheath blight fungus, partial resistance may provide a path to reducing epidemics (Srinivasachary *et al*., 2011). In a greenhouse, the artificially inoculated rice plants (SK101 and E. Yasmine) with 17 *R. solani* isolates exhibited typical lesions of sheath blight. All the tested isolates varied in their virulence. Sakha 101 (Japonica type) rice cv proved the most susceptible for infecting with sheath blight pathogen compare with E. Yasmine (Indica type). This main E. Yasmine maybe has partial resistance to infection with *R. solani* pathogen. This partial resistance maybe due to different morphological characters of E. Yasmine and S.101 such as leaf area, leaf length, plant duration and plant high in E. Yasmine more than SK 101 so the pathogen want more time to occupy the leaf area and plant high. Anis *et al.* (2016) studies different morphological characters for 20 genotypes of rice and found that plant height of Egyptian Yasmine (111.3 cm) was the highest compare with SK 101 (90.3cm). Hossain *et al*. (2016) found morphological characters like flag leaf angle, flag leaf length, and plant compactness significantly correlated with sheath blight resistance. Willocquet, *et al*. (2011) studied three types of rice (Indica, tropical japonica and aromatic) against sheath blight infection and found that Indica genetic groups had consistently lower disease levels than japonica genetic groups, but the difference was not significant. Also, the results agree with those of El-Shafey, *et al*. (2019) who inoculated Sakha 101rice cultivar under greenhouse with *R. solani* Egyptian isolates and showed that most of 19 isolates in both seedling and adult stages, had a high disease score (9). SK101 was highly susceptible to sheath blight isolates under the greenhouse conditions of El-Shafey, *et al*. (2019). Wild relatives of rice were used as sources of resistance for sheath blight but none of which was identified to have immunity against this disease. Those genotypes identified as having moderate to high levels of resistance should be used as resistance donors in breeding efforts. Tetep, Jasmine 85, and Teqing from *O. sativa* germplasm have sources of resistance for sheath blight (Srinivasachary *et al*., 2011).

An earlier reports study that the pre-penetration stage of *R. solani* and showed forms typical infection cushions (Singh *et al*., 2003) by aggregation of branched hyphal tips the infection cushions were formed, as reported by Łaźniewska, *et al*. (2012). The growth behavior of *R. solani* isolate was studied using two rice cultivars Sakha 101 and E. Yasmine. Hyphal growth of *R*. *solani* in SK101 cultivar was more than in the E. Yasmine cultivar. Furthermore, we examined the morphological developments of sclerotial on both cultivars using light microscopy and (SEM). It was observed that the fungal pathogen could intercept host surface structures for improving cell penetration and anchorage. These findings agree with those of Basu, *et al*. (2016) who observed that one of the many openings on the surface of the host leaf, such as trichomes, stoma, or papillae, and the direct development of *R. solani* hyphae. Chemotaxis and/or touch sensing may be used by the hyphae to detect substances released by the host cells, allowing for precise interception.

CONCLUSION

In the present work it could be concluded that sheath blight caused by *R. solani* a serious disease of rice in Egypt. *R. solani* isolates showed a wide range of variability of the morphological and pathogenicity properties of isolates. Also, two isolates of *R. solani* were found aligned with Egyptian and Chinese isolates. A strong association between the ability of isolates to produce extracellular enzymes in vitro or infected rice tissue samples to developments the sheath blight disease of rice. Sakha 101 (Japonica type) is more susceptible to sheath blight disease as compared to (indica type) Egyptian Yasmine.

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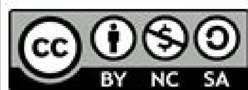

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الدراسات الجزيئيه، الفسيولوجيه و سلوك الاصابه لفطر الريزوكتونيا سولاني المسبب لمرض لفحة غمد الارز

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الملخص

يعتبر مرض لفحه الغمد فى الأرز المتسبب عن الريزوكتونيا سولاني واحد من أخطرالآمراض التى تؤثر على إنتاج الأرز في جميع أنحاء العالم .فى هذه الدراسه استخدمت الخصائص المورفولوجيه و الجزيئيه لتعريف سبعة عشرعزلة مصرية من المسبب المرضى للفحة الغمد بواسطة تتابع النسخ الداخلي البيني. قدرت الانزيمات المفرزه خارجيا في المعمل وعينات الارز المصابه. ايضا اجريت اختبارات الشده المرضيه لكل العزلات علي صنفين ارز تحت ظروف الصوبه الزجاجيه. تمت دراسه سلوك نمو هيفات الفطر بواسطة العزله ار اس 13على اثنين من أصناف الأرز وهما سخا 101 (الطراز الياباني) والياسمين المصري (الطراز الهندي).اوضحت النتائج المتحصل عليها وجود مدى واسع من التباين في الصفات المورفولوجية والمرضيه للعزلات. تم تسلسل اثنين من العزلات ار اس 10 و ار اس 13 ووجدتا متماثله مع العزلات المصرية والصينية. في زراعة الاطباق اظهرت العزلتين ار اس 10 و ار اس 13 اعلي انتاج لانزيمات السليلوز، البكتين والاميليوز معمليا. بينما في فحص انسجه العينات النباتيه كان نشاط انزيم السليلوز والبكتين ميثيل استيريز مرتبطا مع تطور مرض لفحة الغمد. تحت ظروف العدوي الصناعيه برهن الصنف الارز سخا 101 انه الاكثر حساسيه للاصابه بمسبب لفحة الغمد مقارنه بالصنف الياسمين المصري. نمو الهيفات على الصنف سخا 101 أكثر من الهيفات المتكونه على الصنف الياسمين المصرى . علاوه على ذلك تم فحص التطورات المورفولوجيه للهيفات و الاجسام الحجريه على كلا الصنفين باستخدام الفحص المجهرى الضوئى والماسح الالكترونى وقد لوحظ أن المسبب الفطري يمكنه اختراق الهياكل السطحية للعائل للتمكن من اختراق الخلايا و الإرساء.

الكلمات المفتاحية : لفحة الغمد - الارز - ريزوكتونيا سولاني - النسخ الداخلى البينى- الانزيمات الخارجية