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Incidence of kernel smut caused by *Tilletia barclayana* in Egyptian rice cultivars

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The study was conducted to determine the effects of cultivars susceptibility to infection with the pathogenic isolates on the pathogenicity of rice kernel smut fungus (*Tilletia barclayana*). Rice (*Oryza sativa* L.) cultivars vary in susceptibility to kernel smut disease. The susceptibility of 18 commercial rice cultivars to *T. barclayana* was evaluated. Moreover, pathogenicity of 46 isolates of *T. barclayana* which had been isolated from six governorates in Egypt was studied using Giza 178 rice cultivar and Hybrid 1. The activities of certain oxidative enzymes, e.g. peroxidase (POX) and polyphenoloxidase (PPO) and total protein were determined in healthy and inoculated cultivars. Intron-exon splice junctions (ISJ) protocol was used to study the relationship among rice cultivars differing in their susceptibility to infection with the pathogen. Results showed that Giza 178 rice cultivar and Hybrid 1 were the most susceptible cultivars for rice kernel smut disease. Isolate no. 35 from the pathogenic fungus was the most aggressive isolate. Additionally, an increment in the defense-related enzymes and total protein was observed in rice cultivars as a result of inoculation with *T. barclayana.* ISJ technique showed the relationship between the studied rice cultivars and lines upon susceptibility to *T. barclayana*. It can also differentiate between the isolated *T. barclayana* isolates upon virulence on the studied rice cultivars and lines.

Key words: Rice kernel smut disease, intron-exon splice junctions, rice, enzymes activity.

INTRODUCTION

Rice kernel smut, known as grain smut (Biswas, 2003), is caused by the pathogen *Tilletia barclayana* which causes a partial bunt that affects both yield and quality. This disease was recorded in Egypt in 1999 (Ismail, 2003). *T. barclayana* causes the endosperm of the rice grain to be replaced partially or completely by a black mass of smut

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spores. In general, long-grain rice cultivars, the predominant type grown in the United States, are the most susceptible cultivars to infection with *T. barclayana* (Biswas, 2003). Even a low incidence of infection can cause substantial economic losses in hybrid rice seed production. In India only a maximum of 0.5% infection is allowed in certified seed (Biswas, 2003). Templeton et al. (1960) indicated that 1 to 10 rice grains per panicle may show partial or complete smut infection. This range of infection likely represents from 1 to 15% direct grain yield losses. Rice kernel smut epidemics on recently released cultivars increased the concern of rice growers because of the undesirable effects of kernel smut on both grain quality and yield (Cartwright et al., 1999). In 1982, Murty and Singh evaluated 31 rice varieties for infection with *T. barclayana*. They found that seven varieties had no infection while 24 cultivars suffered very slight infection, ranging from 0.04 to 0.8%. Additionally, Muthusamy and Ahmed (1997) studied reactions of 10 early maturing cultivars and 9 later maturing ones to natural infection by *T. barclayana*. They reported that the cultivars with a shorter growing season were infected more than those with a longer season. Misra et al. (1994) conducted disease surveys for 144 rice seed samples collected from 7 different regions in the Philippines during dry and wet seasons using the standard blotter method, 39 fungal species belonging to 30 genera were isolated. The percentage of infestation by different species varied with location. *T. barclayana* was evenly distributed irrespective of the season. The incidence of kernel smut *T. barclayana* was observed on cytoplasmic genetic male-sterile (CMS) lines and their respective maintainers. The disease was generally more severe on the CMS lines and hybrids, when compared with their maintainers, restorers and inbred rice cultivars (Chahal et al., 1993; Zheng, 2005).

It was reported that plants defend themselves from the invader pathogens by either their structural barriers or antimicrobial compound which prevent colonization of the tissues. The induced defense responses include hypersensitive responses, the production of reactive oxygen species (ROS), pathogensis-related protein and ion fluxes across the plasma membrane (Zhao et al., 2005; Elsharkawy et al., 2012a, b; Elsharkawy et al., 2013; Hassan et al., 2014).

The use of molecular markers has proven its value for a variety of purposes in molecular biology. DNA fingerprinting, gene mapping and phylogenetic studies have tremendously benefited from polymerase chain reaction (PCR) technology. Random amplified polymorphic DNA (RAPD) markers generate DNA fingerprints with a single synthetic nucleotide primer (Williams et al., 1990) which could efficiently detect polymorphism based on comparison throughout the genome. Molecular markers technology provides novel tools for DNA fingerprinting of rice cultivars to assess cultivar seed purity. Semi-random PCR primers targeting intron-exon splice Junctions (ISJ) were used to analyze the rice genome with the aim of evaluating potential of these markers for identification and classification of rice cultivars (El-Moghazy, 2007). DNA-based markers are highly heritable, available in high numbers and exhibit enough polymorphism, hence they can be used to discriminate closely related genotypes of plant (Yashitola et al., 1997). For this reasons, DNA fingerprinting for cultivar or varietal identification has become an important tool for genetic identification in plant breeding and germplasm management (McGregor et al., 2000). Weining and Langridge (1991) reported that the targeting of the intron-exon splice junctions in conjunction with primers of random and defined sequences provides a source of extensive variation in PCR products. The ISJ's primers were able to differentiate between studied varieties and species. El-Moghazy (2007) studied the genetic diversity among eleven rice genotypes using three ISJ primers. A total of twenty-six DNA fragments were amplified and high degree of polymorphism was observed. The objectives of this study are to evaluate rice cultivars with infection of rice kernel smut disease and to differentiate between rice cultivars in response to infection by rice kernel smut disease at molecular levels.

MATERIALS AND METHODS

Isolation and identification of rice kernel smut disease in Nile Delta

Infected rice grains showing typical kernel smut disease symptoms were collected from six governorates, that are, Kafr El-Sheikh, Dakahlia, Gharbia, Damietta, Beheira and Sharkia of Egypt. All samples were collected during rice maturity stage. Teliospores were collected from these infected rice grains and germinated after soaking in a solution of NaOH 1% for two days and centrifuged for 30 min (4,000 x g) then teliospores were soaked in distilled water for two days and transferred onto a 2% water agar (home-made) (Anil and Singh, 1987). The plates were incubated at 25°C±1 for 12 days till the germination of teliospores. Primary basidiospores were transferred onto potato-sucrose agar (PSA) (home-made) (Trione, 1964). Identification of *T. barclayana* isolates was carried out according to the morphological, microscopic characteristics and type of teliospore germination in Plant Pathology Laboratory, Rice Research and Training Center (RRTC), Sakha, Kafr El-Sheikh, Egypt using the key given by Fischer and Holton (1957) and Duran (1973). Rice cultivars and lines were obtained from RRTC.

Pathogenicity tests

Pathogenicity test of the isolated 46 isolates of *T. barclayana* was carried out using Giza 178 rice cultivar and Hybrid 1 (highly susceptible cultivars for rice kernel smut disease) grown in 30 x 25 cm diameter pots under greenhouse condition at RRTC. Plants at flowering stage were sprayed with 5 x 10^7 secondary sporidia/ml of each isolate using electrical spray gun (atomizer) in four replicates. 2.5 g/L of gelatin were added to spore suspension inocula to enhance infection (Bastiaans, 1993). The inoculated plants were held in a moist chamber with at least 90% RH and 25-28°C for 24 h and then moved to the greenhouse for maturity stage after inoculation. Disease severity was recorded using number of infected grains and total number of grains per 50 panicles (Slaton et

al., 2004).

Evaluation of certain rice cultivars and lines with infection of the causal fungus

The response of eighteen rice cultivars and two hybrids to infection with the most aggressive isolate of the causal fungus (isolate no. 35) was recorded. This experiment was conducted as mentioned above in pathogenicity test.

Disease assessment

To estimate severity and percentage of infection of rice plants in 50 panicles at maturity stage, the plants were examined to record the infected plants and calculate the infection percentage according to the following equations (Slaton et al., 2004):

$$\text{Disease severity} = \frac{\text{No. of infected grains}}{\text{Total no. of rice grains}} \times 100$$

$$\text{Percentage of infection} = \frac{\text{No. of infected grains}}{\text{Total no. of rice panicles}} \times 100$$

Randomized complete block design in plastic pots (30 x 25 cm diameter) with four replicates was used. The pots were kept in the greenhouse at 30-35°C and fertilized one time with urea 46.50% N at 3 g/pot.

Enzyme activities and total protein assay of rice cultivars

The activities of certain oxidative enzymes, e.g. peroxidase (POX) and polyphenoloxidase (PPO) and total protein were determined in healthy and inoculated cultivars. Samples were collected from rice hills at 3, 6, 9, 12 and 15 days after flowering stage. The tested cultivars were Giza 171, Giza 175, Giza 177, Giza 178, Giza 181, Giza 182, Egyptian yasmine, Sakha 101, Sakha 102, Sakha 103, Sakha 104, Sakha 105, Hybrid 1, Hybrid 2, BL 1, Doular, Giza 159, Giza 176, Rieho and Sakha 106. The experiment was repeated three times with three replicates per treatment.

Enzymes extracts

Enzymes extracts were prepared according to the methods recommended by Maxwell and Bateman (1967). 500 mg fresh weight of rice leaf samples were ground in a mortar and pestle containing liquid nitrogen. The resulting powder was macerated for 30 s. and homogenized with 3 ml of sodium phosphate buffer pH 6.8 (0.01 M). Triturated tissues were strained through 4 layers of cheese cloth and filtrates were centrifuged for 15 min at 6.000 rpm in a refrigerated centrifuge. The clear supernatant was taken as the enzymes source.

Peroxidase (POX) assay

Peroxidase enzyme activity was determined according to the method described by Allam and Hollis (1972) and Srivastava (1987) by measuring the oxidation of pyrogallol to pyrogallin in presence of H_2O_2. Peroxidase activity was expressed as changes in absorbance (optical density per 1 min/0.5g sample, OD/min/0.5g). The absorbance was measured at 425 nm and recorded at 0, 1, 2, 3, 4 till 5 min intervals using spectrophotometer (Milton Roy, Spectronic, 1201 Digital).

Polyphenoloxidase (PPO) assay

PPO was determined according to the method adopted by Matta and Dimond (1963). Polyphenoloxidase activity was expressed as changes in absorbance (optical density/ min/0.5 g), the absorbance was measured at 495 nm and recorded at 0, 1, 2, 3, 4 till 5 min intervals using spectrophotometer (Milton Roy, Spectronic, 1201 Digital).

Total protein assay

Determination of total protein using coomassie brilliant blue G-250 is based on the observation that coomassie brilliant blue G-250 exists in two different color forms, red and blue. The red form is converted to the blue form upon binding the dye with protein. The protein-dye complex has a high extinction coefficient, thus leading to great sensitivity in measurement of the protein. Protein contents were determined according to Bradford (1976).

Relationship between the tested rice cultivars as well as isolates of the causal pathogen of rice kernel smut using intron-exon splice junctions (ISJ) protocol

ISJ protocol was used in this work to study the relationship among rice cultivars differing in their susceptibility to infection with the pathogen. The tested rice cultivars were Sakha 101, Sakha 105, Hybrid 1, Giza 178, Giza 181 and Egyptian yasmine. Also, ISJ technique was used to differentiate between 13 isolates of such pathogenic fungus differing in their virulence to rice cultivars. Four primers, ISJ-5 (5'-CAG GGT CCC ACC TGC-3'), ISJ-6 (5'-GAC CGC TTG CAG GTA AGT-3'), ISJ-7 (5'-TGC AGG TCA GGA CCC T-3') and ISJ-9 (5'-AGG TGA CCG ACC TGC A-3') of intron splicing junction (ISJ) were used.

DNA isolation and quantification

DNA of the 6 selected rice genotypes and 13 isolates of the fungus were isolated using CTAB (Cetyl- Tetramethyl Ammonium Bromide) method (Murray and Thompson, 1988). For DNA isolation, 100 mg of fresh seedling leaves as well as fungal growth mycelia of different isolates were homogenized in chilled mortar and pestle using liquid nitrogen. 700 µl of 2X CTAB extraction buffer were added and homogenized as well. The samples were transferred to Eppendorf tubes and incubated at 65°C for 30-60 min with occasional gentile swirling. 700 µl of chloroform isoamyle alcohol (24:1) were added and mixed by inverting the tube several times. The sample was centrifuged at 15000 rpm for 15 min at 4°C. The aqueous was transferred to a new centrifuge tube with a wide bore tips to avoid DNA shearing. 0.6 volume of chilled isopropanol was added and followed by quick and gentle inversion and incubated at -20°C for 30 min. DNA pellet was precipitated at 10000 rpm for 10 min at 4°C. Pellet was washed three times with 70% ethanol, well dried and dissolved in 100 µl TE. DNA was quantified using gel quantification method in which the samples were loaded on 0.8% agarose gel in 0.5X TAE running buffer and using known concentrations of λ uncut genomic DNA as standard. After some cycles of dilutions, the concentration of DNA was approximately adjusted to 15 ng/ µl which is suitable for PCR reaction. PCR conditions were: initial denaturation at 94°C for 3 min, 45 cycles of amplification under the following parameters: template denaturation at 94°C for 1 min, primer annealing at 48°C for 1 min and extension at 72°C for 2.30 min by the end of the 45th cycle, final extension at

Table 1. Infection percent of Giza 178 rice cv. and Hybrid 1 by forty six isolates of *T. barclayana* under greenhouse conditions.

Governorate	Infection (%)							
	0-20		21-40		41-60		Total	
	Giza178	H 1	Giza178	H 1	Giza178	H 1	Giza178	H 1
Kafr El-heihk	10*	10	4	4	-	-	14	14
Beheira	-	-	1	1	-	-	1	1
Sharkia	-	-	1	1	-	-	1	1
Gharbia	3	6	4	1	-	-	7	7
Dakahlia	9	11	7	6	1	-	17	17
Damietta	3	4	3	2	-	-	6	6
Total	26	32	19	14	1	-	46	46

*= Number of isolates

72°C for 7 min followed by storage at 4°C.

Electrophoresis, staining and analysis

DNA amplified fragments were loaded in 1.2% agarose gel containing ethidium promide (2 µl/100 ml). The 0.5X TAE was used as a running buffer and 50 and 100 bp DNA ladders (0.5 µg/µl by fermentas) as molecular weight markers. Electrophoresis was conducted at 70 V for 3 h. Then, gels were photographed and analyzed using BioDoc Analysis software (Biometra, Germany).

Phylogenetic tree construction

The presence/absence of matrix for amplified DNA fragments of the four ISJ markers was used to study the phylogenic relationships among the studied genotypes. The statistical software NTSYS pc2.0 (Rohlf, 2000) was used to estimate the genetic relationships among the tested genotypes. Employing the computer package NTSYS pc2.0, Nei and Lei's similarity coefficients (Nei and Lei, 1979) were calculated and used to establish genetic relationships among the genotypes based on un-weighed pair group method of arithmetic means (UPGMA) and sequential agglomerative hierarchical nested (SAHN) clustering.

Statistical analysis

Data were statistically analyzed using analysis of variance (ANOVA). The completely randomized design was applied in laboratory and greenhouse experiments. Completely randomized block and split-plot designs were adopted according to Gomez and Gomez (1984). The treatment means were compared using the least significant difference (LSD) at 5%.

RESULTS

Isolation, identification and pathogenicity test of *T. barclayana*

Forty six fungal isolates were isolated from rice kernels showing typical symptoms of rice kernel smut collected from all the surveyed governorates. However, seventeen isolates was obtained from Dakahlia gov. (Table 1). Identification of these isolates were performed at Plant Pathology Laboratory, Rice Research & Training Center (RRTC), Sakha, Kafr El-Sheikh, Egypt. These isolates were identified according to their morphological characteristics as *T. barclayana* (Bref.) Sacc. and P. Syd., 1899. Pathogenicity test was carried out using Giza 178 rice cultivar and Hybrid 1 (the most susceptible cultivars). Data presented in Tables 1 and 2 indicated that the tested isolates varied in their virulence. All isolates were divided into three categories of infection percent, that are, 0.0 - 20, 21 – 40 and 41 – 60%. However, isolates numbers 40, 20, 13, 24 and 35 isolated from Damietta, Gharbia, Kafr El-Sheikh and Dakahlia governorates, respectively, proved to be the most aggressive isolates on Giza 178 rice cv. and Hybrid 1. At the same time isolate no. 31 isolated from Dakahlia gov. was the least virulent one which recorded the least disease severity among all the tested isolates.

Evaluation of certain rice cultivars and lines towards infection with *T. barclayana*

An aggressive isolate of *T. barclayana* (isolate no. 35, isolated from Giza 178 cv. in Dakhlia gov.) was used for evaluating the susceptibility of eighteen commercial rice cvs. and Hybrids. Results indicated that all the tested rice cultivars varied in their susceptibility toward the infection with the tested isolate (Table 3). However, Giza 178 was the most susceptible rice cultivar followed by Hybrid 1 since they were severely infected. On the other hand, Sakha 101 and Sakha 105 were the least susceptible cultivars. Concerning the tested lines, disease severity of GZ 6903-1-2-2-1 and GZ 8566-6-1-1-4-1 ranged from 0.028 to 1.71, respectively (Table 4). However, it is clear from data presented in Tables 3 and 4 that number and germinated spores increased with increasing the disease severity.

Enzyme activities and total protein assay

Data presented in Tables 5, 6 and 7 showed gradual

Table 2. Pathogenicity test of isolates of T. barclayana, isolated from rice cultivars grown in different governorates using Giza 178 cv. and Hybrid rice 1 under greenhouse conditions.

Number of isolate	Governorate	Source of isolate	Infection (%)		Disease severity	
			Giza178	H 1	Giza178	H 1
1	Kafr EL-Sheikh	Giza178	14.6	13.7	4.00	2.00
2		Giza178	15.0	11.4	1.36	1.30
3		Giza177	19.7	10.7	4.50	4.10
4		Sakha 104	11.3	8.00	6.64	4.25
5		Hybrid 1	12.0	12.0	4.88	2.43
6		BL1	19.0	9.00	3.30	0.64
7		Hybrid 1	30.7	20.6	2.30	1.81
8		Sakha 104	19.4	19.3	2.00	1.77
9		Hybrid 2	16.7	16.6	4.40	1.20
10		Gz1368-55-4	24.4	24.3	3.50	3.33
11		Sakha 101	8.00	7.20	2.88	1.10
12		Sakha 105	22.7	22.6	2.65	2.3
13		Giza 181	28.0	27.4	6.70	5.67
14		Egyptian Yasmine	17.6	15.7	2.60	1.32
15	Gharbia	Giza177	20.7	20.0	0.52	0.56
16		Hybrid 3	22.4	15.0	3.33	1.04
17		Hybrid 1	23.0	22.0	4.00	0.65
18		Giza 178	19.7	19.6	3.90	1.89
19		Hybrid 4	28.0	18.0	3.20	1.69
20		Giza177	18.7	18.6	6.13	5.92
21		Giza177	18.7	8.70	3.43	0.86
22	Dakahlia	Giza 178	16.7	6.70	3.20	0.34
23		Giza 177	17.7	11.0	3.30	0.53
24		Giza 178	31.0	32.7	8.10	7.86
25		Giza 178	22.6	9.00	3.82	1.64
26		Reiho	19.0	9.00	3.77	0.88
27		Giza 178	16.3	11.4	3.00	1.32
28		BL1	37.4	31.4	4.00	1.85
29		Giza 178	26.0	26.0	5.50	4.31
30		Hybrid 2	26.3	25.7	5.45	4.89
31		Gz7576-10-3-2-1	17.0	13.7	0.87	0.54
32		BL1	12.3	12.4	3.63	1.93
33		Sakha 102	14.0	13.4	5.00	2.60
34		BL1	12.0	11.7	3.26	0.50
35		Giza 178	41.0	36.7	9.66	8.70
36		Hybrid 2	32.4	32.4	4.42	2.20
37		Sakha 103	14.7	14.7	3.63	1.24
38		Giza 177	21.4	14.6	5.35	2.71
39	Damietta	Giza 178	19.0	19.0	3.63	1.25
40		Giza 178	37.0	27.0	6.50	6.25
41		Hybrid 2	29.0	15.3	3.90	1.78
42		Giza 177	18.0	11.4	4.27	2.55
43		Giza 178	26.4	26.3	4.01	2.03
44		Giza 177	11.0	10.3	3.00	1.93
45	Sharkia	Hybrid 1	26.6	26.70	3.27	1.20
46	Beheira	Giza 178	34.0	30.5	3.70	3.17
L.S.D. 5 %			7.34	6.80	0.74	0.85

Table 3. Evaluation of certain commercial rice cultivars and two rice hybrids for infection with isolate no. 35 of *T. barclayana* under greenhouse conditions.

Cultivar	Inoculated		After milling/ inoculated	
	Disease severity	Infection (%)	No. of spores/ gm	Germinated spores
Giza 159	0.43	24	2000	33
Giza 171	0.9	33.4	1600	50
Giza 175	1.38	61.5	1200	143
Giza 176	0.48	28.5	2000	177
Giza 177	0.09	4.67	2000	85
Giza 178	4	70	1000	394
Giza 181	0.35	19.2	2000	175
Giza 182	0.7	29	2000	94.33
Egyptian Yasmine	0.16	16	4000	101
Reiho	0.34	18	4000	100
Hybrid 1	1.22	38.1	8000	355
Hybrid 2	1.58	65	6000	335
Sakha 101	0.002	2.48	2000	85
Sakha 102	0.05	4.67	2000	95
Sakha 103	0.05	3	2000	115
Sakha 104	0.33	17.5	2000	90
Sakha 105	0.07	6	2000	225
Sakha 106	0.58	28.57	2000	100
BL 1	0.7	29	2000	121
Doular	0.4	22.8	4000	257
L.S.D. 5 %	0.273	9.96	3.55	15.8

Table 4. Evaluation of some rice lines for infection with isolate no. 35 of *T. barclayana* under greenhouse conditions.

Rice lines	Inoculation		After milling/inoculated	
	Disease severity	Infection (%)	No. of spores x10^3/g	Germinated spore
GZ 6903-1-2-2-1	0.040	0.40	2.00	53.0
GZ 8544-18-3-1-1-1	1.450	7.17	4.00	130.0
GZ 8566-6-1-1-4-1	1.717	7.17	2.00	154.0
GZ 7764-38-1-3-3	0.175	0.67	4.00	34.0
GZ 7769-2-1-1-2	0.028	0.28	6.00	100.0
L.S.D. 5%	0.123	0.96	1.34	11.57

increase in POX and PPO activities as well as total protein contents in both healthy and inoculated plants. However, the increment in the activity of POX and PPO and total protein levels were higher in inoculated plants than in healthy ones. Additionally, it was clear from our data that maximum increase in POX and PPO activities and total protein contents was recorded after 6 and 9 days from inoculation and then enzyme activities started to decrease. Also, results showed that POX, PPO and total protein levels in resistant cultivars (Sakh 105, Sakha 101 and Giza 177) and moderately resistant cultivars (Sakha 104 and Giza 182) were higher than those in highly susceptible cultivars (Giza 178 and Hybrid 2).

Relationship between the tested rice cultivars and isolates of *T. barclayana* using Intron-Exon Splice Junctions (ISJ) protocol

Results illustrated in Figure 1A, B, C and D showed that the phenogram constructed based on the molecular data of ISJ banding patterns was generated by using primers ISJ-5, ISJ-6, ISJ-7 and ISJ-9. The highest similarity percentage was observed in the most similar genotypes. The used primers detected reasonable levels of polymorphism among the tested genotypes. Hence, the overall similarity levels generated by these primers were 72 to 88%.

Table 5. Peroxidase activity in leaves of twenty rice cultivars in healthy or artificially inoculated rice plants with *T. barclayana*, the causal fungus of rice kernel smut disease.

Cultivar	POX activity/ minute (as optical density)									
	Healthy					inoculated				
	Time of measurement in days					Time after inoculation in days				
	3	6	9	12	15	3	6	9	12	15
Giza 159	0.86	0.91	1.10	1.00	0.95	0.88	0.92	1.20	1.12	0.98
Giza 171	0.45	0.54	0.9	0.80	0.77	0.59	0.64	1.18	0.87	0.85
Giza 175	0.69	0.81	1.10	0.90	0.85	0.79	0.91	1.20	1.02	0.95
Giza 176	0.33	0.64	0.90	0.85	0.67	0.43	0.73	1.05	0.93	0.75
Giza 177	1.00	1.25	1.26	1.26	1.10	1.14	1.38	1.39	1.38	1.20
Giza 178	0.35	0.53	0.75	0.73	0.57	0.38	0.63	0.86	0.84	0.62
Giza 181	0.86	1.15	1.16	1.16	1.10	0.95	1.17	1.28	1.20	1.05
Giza 182	0.86	1.00	1.25	1.20	1.15	0.96	1.25	1.26	1.27	1.10
Egyptian Yasmine	0.74	0.87	1.26	1.13	1.11	0.85	0.98	1.28	1.23	1.15
Reiho	0.47	0.56	0.55	0.64	0.42	0.57	0.64	0.70	0.65	0.64
Hybrid 1	0.45	0.54	0.62	0.58	0.57	0.55	0.65	0.65	0.68	0.59
Hybrid 2	0.37	0.57	0.95	0.83	0.56	0.45	0.68	1.05	0.93	0.67
Sakha 101	0.76	0.98	1.20	1.13	1.05	0.76	0.99	1.28	1.22	1.17
Sakha 102	0.78	0.96	1.15	1.03	0.92	0.88	1.06	1.18	1.17	0.98
Sakha 103	0.75	0.85	1.15	1.10	0.95	0.78	0.88	1.20	1.13	0.98
Sakha 104	0.65	0.87	1.00	0.90	0.80	0.69	0.88	1.10	0.95	0.97
Sakha 105	0.90	1.27	1.30	1.28	1.01	0.98	1.32	1.31	1.45	1.08
Sakha 106	0.53	0.60	0.82	0.8	0.65	0.54	0.68	0.83	0.84	0.73
BL 1	0.44	0.55	0.61	0.66	0.46	0.53	0.63	0.71	0.67	0.59
Doular	0.54	0.67	0.62	0.68	0.56	0.58	0.69	0.64	0.69	0.58
L.S.D. 5%	0.13	0.14	0.15	0.12	0.17	0.14	0.13	0.20	0.42	0.22

Table 6. Polyphenoloxidase activity in leaves of twenty rice cultivars in healthy or artificially inoculated rice plants with *T. barclayana*.

Cultivar	PPO activity/ minute (as optical density)									
	Healthy					inoculated				
	Time of measurement in days					Time after inoculation in days				
	3	6	9	12	15	3	6	9	12	15
Giza 159	0.27	0.28	0.3	0.29	0.27	0.53	0.54	0.55	0.54	0.50
Giza 171	0.44	0.44	0.47	0.46	0.44	0.58	0.63	0.57	0.56	0.56
Giza 175	0.4	0.42	0.5	0.42	0.45	0.59	0.6	0.64	0.6	0.52
Giza 176	0.18	0.22	0.21	0.21	0.2	0.23	0.27	0.29	0.28	0.24
Giza 177	0.56	0.59	0.62	0.61	0.58	0.61	0.72	0.83	0.81	0.75
Giza 178	0.24	0.25	0.24	0.24	0.23	0.26	0.29	0.36	0.28	0.25
Giza 181	0.25	0.25	0.32	0.27	0.26	0.25	0.27	0.28	0.26	0.25
Giza 182	0.22	0.22	0.24	0.26	0.22	0.39	0.49	0.49	0.48	0.47
Egyptian Yasmine	0.26	0.29	0.32	0.30	0.25	0.45	0.48	0.52	0.50	0.44
Reiho	0.25	0.40	0.50	0.40	0.40	0.35	0.41	0.59	0.45	0.49
Hybrid 1	0.43	0.43	0.59	0.43	0.33	0.56	0.56	0.68	0.63	0.46
Hybrid 2	0.43	0.46	0.45	0.41	0.41	0.58	0.59	0.60	0.59	0.58
Sakha 101	0.51	0.51	0.56	0.51	0.42	0.67	0.68	0.68	0.67	0.66
Sakha 102	0.42	0.43	0.43	0.42	0.42	0.48	0.49	0.52	0.51	0.43
Sakha 103	0.13	0.12	0.12	0.13	0.12	0.23	0.23	0.24	0.23	0.24
Sakha 104	0.45	0.45	0.49	0.44	0.42	0.49	0.49	0.52	0.51	0.45

Table 6. Contd

Sakha 105	0.69	0.72	0.74	0.69	0.68	0.79	0.80	0.81	0.79	0.78
Sakha 106	0.41	0.42	0.43	0.43	0.41	0.52	0.54	0.55	0.53	0.53
BL 1	0.17	0.18	0.19	0.18	0.14	0.29	0.31	0.35	0.34	0.32
Doular	0.24	0.25	0.26	0.25	0.24	0.26	0.28	0.31	0.27	0.24
L.S.D. 5%	0.13	0.1	0.12	0.12	0.08	0.16	0.17	0.16	0.25	0.16

Table 7. Total protein contents in leaves of twenty rice cultivars in healthy or artificially inoculated rice plants with *T. barclayana*, the causal fungus of rice kernel smut disease.

Cultivar	Total protein (mg/g fresh weight)									
	Healthy					Inoculated				
	Time of measurement in days					Time after inoculation in days				
	3	6	9	12	15	3	6	9	12	15
Giza 159	0.44	0.5	0.75	0.68	0.35	0.45	0.53	0.78	0.75	0.73
Giza 171	0.4	0.45	0.53	0.4	0.38	0.43	0.52	0.55	0.52	0.50
Giza 175	0.77	0.85	0.87	0.85	0.75	0.79	0.87	0.9	0.86	0.77
Giza 176	0.46	0.58	0.67	0.55	0.45	0.49	0.63	0.77	0.58	0.47
Giza 177	0.88	0.93	0.95	0.84	0.83	0.91	0.96	0.98	0.9	0.85
Giza 178	0.52	0.55	0.57	0.49	0.35	0.57	0.59	0.59	0.54	0.46
Giza 181	0.42	0.48	0.55	0.45	0.4	0.44	0.55	0.59	0.47	0.42
Giza 182	0.34	0.45	0.45	0.41	0.4	0.43	0.5	0.5	0.45	0.43
Egyptian Yasmine	0.41	0.46	0.47	0.42	0.32	0.49	0.47	0.49	0.49	0.36
Reiho	0.42	0.53	0.55	0.47	0.47	0.45	0.55	0.6	0.52	0.45
Hybrid 1	0.32	0.41	0.5	0.40	0.37	0.33	0.43	0.52	0.46	0.40
Hybrid 2	0.52	0.56	0.51	0.50	0.48	0.54	0.6	0.61	0.53	0.51
Sakha 101	0.89	0.98	1.17	1.20	0.95	0.9	1.02	1.21	1.21	0.99
Sakha 102	0.85	1.00	0.97	0.85	0.81	0.91	1.10	1.20	1.02	0.90
Sakha 103	0.94	1.10	1.10	0.94	0.85	0.98	1.12	1.17	0.95	0.88
Sakha 104	0.73	0.87	0.90	0.83	0.76	0.75	0.91	0.94	0.88	0.83
Sakha 105	1.01	1.17	1.19	1.03	0.93	1.15	1.23	1.24	1.14	0.97
Sakha 106	0.96	1.02	1.08	1.12	0.98	0.98	1.05	1.12	1.17	1.07
BL 1	0.25	0.35	0.42	0.50	0.17	0.27	0.36	0.46	0.53	0.42
Doular	0.79	0.93	0.93	0.85	0.78	0.87	0.94	0.96	0.85	0.82
L.S.D. 5%	0.1	0.17	0.12	0.14	0.15	0.10	0.28	0.39	0.2	0.16

Cluster analyses of six cultivars based on molecular data using the tested four primers resulted in two main groups (Figure 2). The first one included both Sakha 105 and Sakha 101 as moderately resistant cultivars, while the second one included four rice cultivars, namely, Hybrid 1, Giza 178, Giza 181 and Egyptian Yasmine as highly and moderately susceptible to rice kernel smut disease. However, the second group could be divided into another two subgroups according to similarity percentages. The first subgroup could be divided into another two sub-subgroups; namely, Giza 178 and Hybrid 1 as highly susceptible, and the second subgroup was Giza 181 as moderately susceptible, while the second one included Egyptian yasmine cv. as moderately susceptible.

Similarly, ISJ molecular marker technique was used to determine the relationship between 13 isolates differing in their virulence. The aforementioned four primers, namely, ISJ-5, ISJ-6, ISJ-7 and ISJ-9 were used in this study. It is clear from Figure 3A, B, C and D that the isolates were classified into three distinct groups. The first group included the highly virulent isolates no. 35 (4) and 24 (6) isolated from Dakahlia gov. The second group included the moderately virulent isolates no. 45 (2), 7 (9), 46 (3), 29 (5), 3 (8), 40 (10), 20 (13) and 13 (11) isolated from Sharkia, Kafr El- Sheikh, Behira, Dakahlia, kafr El-Seikh,

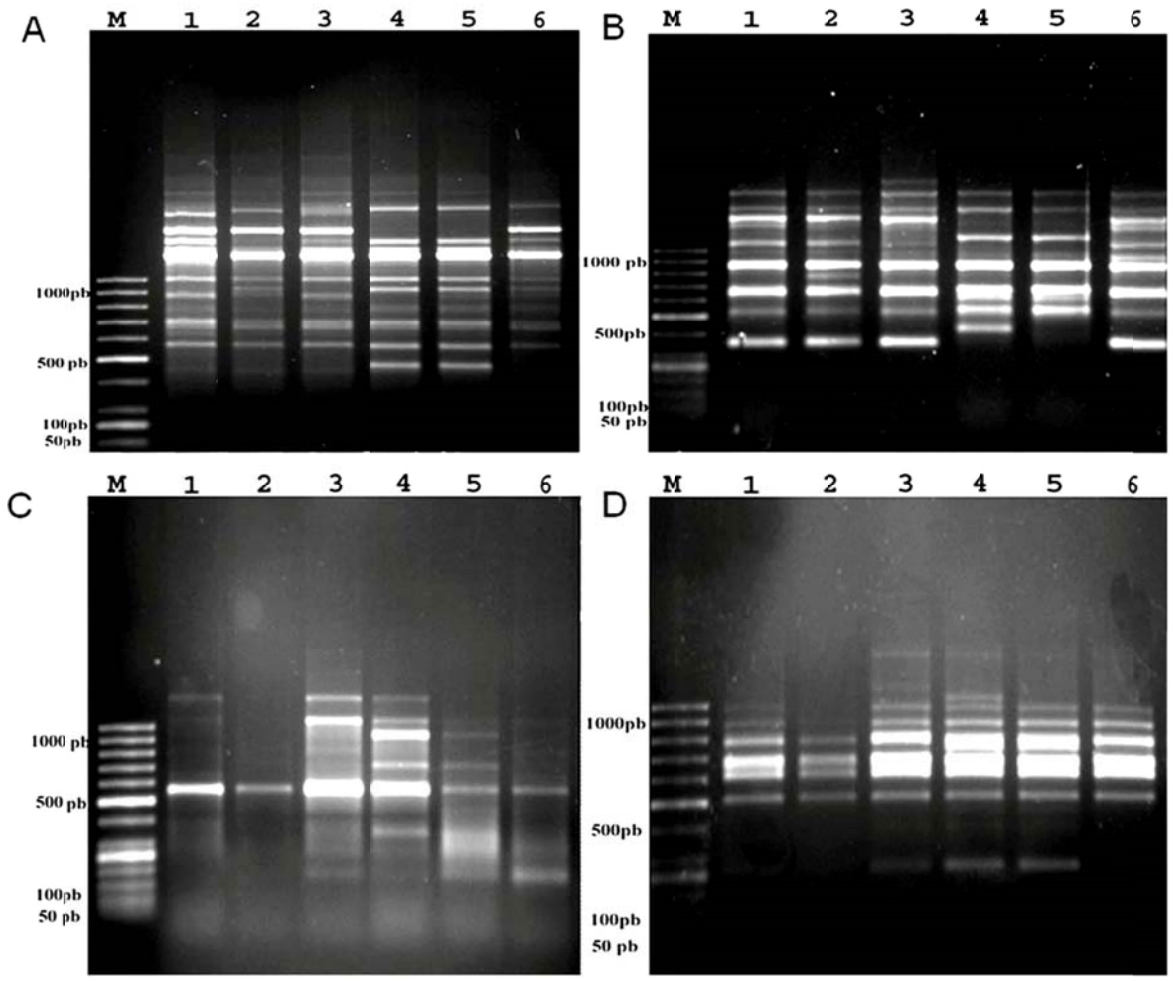

Figure 1. ISJ banding patterns of rice genotypes obtained by the primer, ISJ-5(A), ISJ-6(B), ISJ-7(C) and ISJ-9(D) electrophoresed gel. Lanes from left to right are Giza 178 (1), Egyptian Yasmine (2), Hybrid 1 (3), Sakha 105 (4), Sakha 101 (5) and Giza 181 (6). M = Molecular marker.

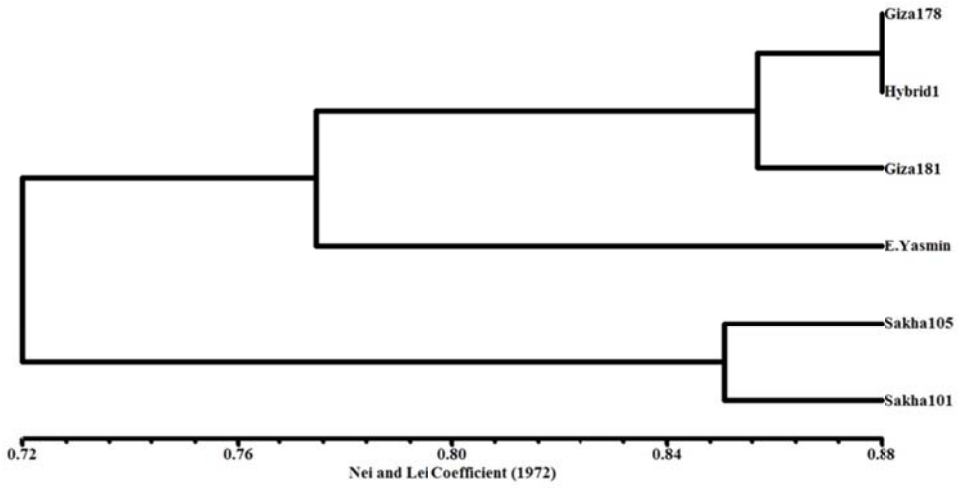

Figure 2. Phenogram based on molecular data of ISJ banding patterns of rice genotypes obtained with the four primers, ISJ-5, ISJ-6, ISJ-7 and ISJ-9.

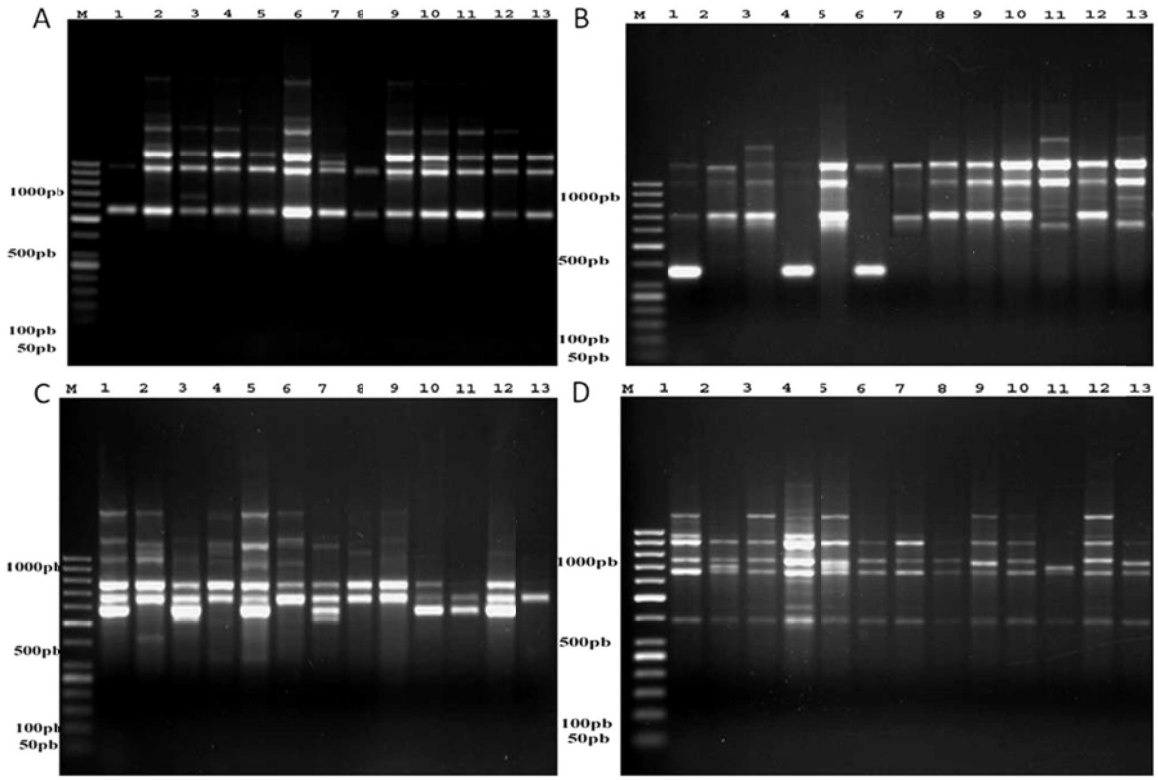

Figure 3. ISJ banding patterns of tested rice kernel smut isolates of *T. barclayana* obtained with the primer, ISJ-5(A), ISJ-6(B), ISJ-7(C) and ISJ-9(D) electrophoresed gel. Lanes from left to right are numbers of isolates 1 to 13. M = Molecular marker.

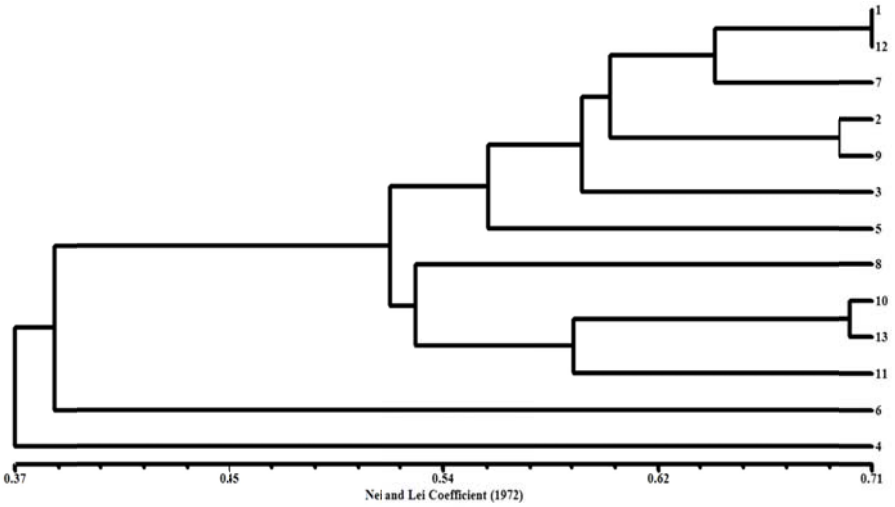

Figure 4. Phenogram based on molecular data of ISJ banding patterns of rice kernel smut isolates of *T. barclayana* obtained with the four primers, ISJ-5, ISJ-6, ISJ-7 and ISJ-9.

Damietta, Gharbia and Kafr El-Sheikh govs., respectively. The third group included the least virulent isolates no. 15 (1), 31 (12) and 2 (7) isolated from Gharbia, Dakahlia and Kafr El-Sheikh, respectively. In the present study, the isolates were screened inside the greenhouse under optimum conditions for disease development.

Clustering the thirteen isolates based on similarity of DNA fingerprint by using the aforementioned four primers resulted in two main groups (Figure 4). The first one included one isolate no. 35 (4) as highly virulent, while the

second one included twelve rice kernel smut isolates. However, the second group divided into other subgroups according to similarity percentages.

DISCUSSION

Rice kernel smut, caused by *T. barclayana*, causes a partial bunt that affects both yield and quality. Forty six fungal isolates of this fungus were isolated from diseased rice plants showing clear symptoms of black smut, purified and identified as *T. barclayana*. This fungus was previously reported to be the causal agent of rice kernel smut disease (Takahashi, 1896; Biswas, 2003). Pathogenecity tests revealed that these isolates were pathogenic to kernel rice with various degrees. Additionally, the evaluated rice cultivars and lines showed different degrees of susceptibility to *T. barclayana*. These findings were in agreement with the previous studies (Murty and Singh, 1982; Muthusamy and Ahmed, 1997). Since they reported that there were variations between rice cultivars in relation to infection with *T. barclayana*.

In the present study, an increment in the defense-related enzymes, that are, POX and PPO and total protein contents was observed in rice cultivars as a result of inoculation with *T. barclayana*. Among induced defense responses, production and accumulation of pathogensis related proteins are very important (Van Loon et al., 2006; Elsharkawy et al., 2012a, b; Elsharkawy et al., 2013; Hassan et al., 2014). Attempts have been made to exploit these anti-fungal proteins to develop disease resistant transgenic crop plants (Lin et al., 1995; Tabei et al., 1997). Peroxidases are involved in a broad range of physiological processes throughout the plant life cycle, probably due to the high number of enzymatic isoforms (isoenzymes) and to the versatility of their enzyme-catalyzed reactions (Passardi et al., 2005). Thus, plant peroxidases are involved in auxin metabolism, lignin and suberin formation, cross-linking of cell wall components, phytoalexin synthesis and the metabolism of reactive oxygen species (ROS) and reactive nitrogen species (RNS) (Gabr, 2010). Additionally, our results showed that total protein levels in resistant cultivars were higher than those in highly susceptible cultivars. Onifade and Agboola (2003) postulated that proliferation of microorganism synthesizes several enzyme proteins that cause rearrangement of nutritional composition of substrate due to formation of several degrading products thereby increasing its protein content.

Molecular markers technology provides novel tools for DNA fingerprinting of plant cultivars. Semi- random PCR primers targeting intron-exon splice junctions (ISJ) were used in this study to analyze the rice genome with the aim of evaluating potential of these markers for identification and classification of rice cultivars and also to study the relationship between isolates of *T. barclayana.* Results showed that ISJ technique can determine the relationship between studied rice cultivars and lines upon susceptibility to *T. barclayana*. Similarly, it differentiated the virulence between the isolated *T. barclayana* isolates upon virulence on studied rice cultivars and lines. Many authors used this technique and other techniques in this respect since they reported that conventional character-rization of cultivars based on specific morphological and agronomic data is time-consuming, restricted to a few characteristics and influenced by environmental conditions. In contrast, DNA-based markers are highly heritable, available in high numbers, and exhibit adequate polymorphism, hence they can be used to discriminate closely related genotypes of a plant as well as microbes isolates (Yashitola et al., 1997; Gawel et al., 2002; El-Malky, 2004; El-Wahsh and Ammar, 2007).

In conclusion, our results indicate that Giza 178 rice cultivar and Hybrid 1 were highly susceptible to rice kernel smut disease. The isolate no. 35 from the pathogenic fungus (*T. barclayana*) was the most virulent isolate. Additionally, increased levels of defense-related enzymes and total protein were observed in rice cultivars after inoculation with *T. barclayana*. ISJ protocol is very useful technique to investigate the susceptibility of rice cultivars and also to differentiate virulence among isolates of *T. barclayana*.

Conflict of Interests

The authors have not declared any conflict of interests.

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