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Molecular characterization and biological control of some rice seed-borne fungal pathogens

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Abstract

Seed-borne fungi cause enormous losses in rice production in Egypt. Ten different fungal species were isolated from four rice seed cultivars (Giza 177, Giza 179, Sakha 101 and Sakha 106) showing grain discoloration symptoms. Ten fungal species were initially identified as *Fusarium* spp. and *Bipolaris* spp. based on conidial morphology, colony appearance, pigmentation and growth rate. Molecular identification of these fungal isolates via PCR utilizing Internal transcribed spacer (ITS) region universal primers was carried out. ITS region was amplified to confirm the species identification. DNA sequence of PCR products and analysis via BLAST and data of the Genbank showed that four isolates belonging to *F. graminearum*, four isolates belonging to *F. verticilliodies* and two isolates identified as *Bipolaris oryzae*. The phylogenetic tree revealed different levels of molecular variation among the fungal species isolates compared to the international isolates deposited in the Genbank. Two biological control isolates of *T. harzianum* (Tr1 and Tr2) were used against *F. graminearum*, *F. verticilliodies* and *B. oryzae* isolates. The highest growth inhibition was exhibited by both *T. harzianum* isolates against *F. graminearum* (76.77% and 76.74% respectively), followed by *F. verticilliodies*. The least growth inhibition was observed on *Bipolaris oryzae* using Tr2 and Tr1 isolates (53.33% and 50.0% respectively). Moreover the naturally infected rice grains treated with both isolates Tr1 and Tr2 showed 100% inhibition of fungal pathogens associated with rice grains compared to untraded naturally infected grains.

Keywords: rice, biological control, grain discoloration symptoms.

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1. Introduction

Egypt is the largest rice producer in the Near East region. Rice (*Oryza sativa*) production was probably introduced into Egypt in the 7th Century. On the basis of nutrition value, rice is rated as second important cereals after wheat (RRTC, 2014). In 2018, the rice harvest declined by over 20 percent compared to last year and the average, mostly due to a decline in the planted area from 850,000 hectares in 2017 to 762,000 hectares. Further declines in rice planted area are projected in 2019 as farmers are expected to continue shifting to crops with more competitive government procurement prices such as cotton and maize (FAO, 2018). The grain yield/unit area of rice is reducing due to various factors among which diseases are one of the major factors. Many diseases and disorders can affect rice plants during the growing season under the local Egyptian conditions which affect the production of grain yield and quality. Grain discoloration of rice is a complex disease due to infection by certain microorganisms on the glumes, kernels, or both. The fungi that are reported to be associated with discoloration of grains are *Bipolaris oryzae, Alternaria padwickii, Pyricularia oryzae, Fusarium verticillioidies, Fusarium grminearum, Nigrospora oryzae, Epicoccum nigrum, Curvularia spp* and *Phoma sorghina* (Ahmed et al., 2013; Phat et al., 2005; Ou, 1985). One of the most important rice disease is brown spot caused by the fungus *Helminthosporium oryzae* Breda de Hann = *Cochiobolus miyabeanus* (also known as *Bipolaris oryzae*) Breda de Hann (Kumar et al., 2011; Harish *et al*., (2008). The pathogen causes infection on all growth stages of rice plant from nursery to field and results in significant yield and grain quality losses. In Egypt, the second important disease after blast disease is brown spot; it can cause yield loss and affects the quality and the number of grains (El-shafey et al., 2018; Elshenawy et al., 2018). *Fusarium moniliforme* which is later identified as *F. fujikuroi*, the anamorph stage is *F. verticillioidies* caused Bakanae disease, observed for the first time at 2001 season on Giza 177 and Sakha 101 at Kafr ElSheik governorate (Gabr, 2010). *F. verticillioidies* was isolated from Egyptian rice grains cultivars and root rots (Makhlouf & Gabr, 2015). *Gibberella zeae* as a teleomorph of *Fusarium graminearum*, it causes head blight of small grains including rice, wheat and barley. In rice it can turn affected grains red and cause brown discoloration in certain areas on the grain or the entire grain surface. Infected grains are light, shrunken and brittle (Lee et al., 2009). The differentiation among the rice fungal pathogens by traditional methods, involve cultural characters, physiological and microstructure measurement are labor-intensive and time-consuming but still most accurate too. For example, *Fusarium* isolates from different plant species were identified on the basis of morphological characteristics (Mandhare et al., 2011; Nath, 2011; El-Kazzaz et al., 2008). Ribosomal DNA (rDNA) regions have been used for taxonomic and phylogenetic studies because sequence data are available and because they contain both variable and conserved regions, allowing discrimination at the genus, species, or intra-specific levels. The non-coding regions of rDNA have been used as variable region. The internal transcribed spacers (ITS) of the rDNA can display variation within genera and used in the differentiation of species (Moore et al., 2011; O'Donnell et al., 1998, 2000). A reduction or elimination of synthetic pesticide applications in agriculture is highly desirable. One of the most promising means to achieve this

goal is by the use of biocontrol agents for disease control (Chet & Inbar, 1994). Antagonists belonging to the genus *Trichoderma* are among the most commonly isolated soil fungi. Due to their ability to protect plants and contain pathogen populations under different soil conditions, these fungi have been widely studied and commercially marketed as biopesticides, biofertilizers and soil amendments (Vinale *et al*., 2008). Screening studies *in-vitro* showed that *Trichoderma* spp. had high antagonistic effect against mycelia growth such as *F. moniliforme*, *F. oxysporum*, *Rhizoctonia solani*, *Alternaria alternate* (Bhattacharjee & Dey, 2015; Mustafa et al., 2009; Adams, 1989; Harman et al., 1980). In Iran, Khalili et al. (2012) found that two strains of *T. harzianum* significantly controlled the brown spot disease caused by *Bipolaris oryzae* in rice and increase of seedling growth. The objectives of the present investigation were (i) to isolate and identify fungal pathogens from rice grains varieties showing grain discoloration symptoms based on morphological characteristics, (ii) to characterize and confirm identification of isolated fungal pathogens through molecular PCR based methods using DNA nucleotide sequence of internal transcribed spacer (ITS) region, (iii) to examine the biocontrol activities of *T. harzianum* isolates against isolated fungal pathogens, (IV) to study the effect of *T. harzianum* isolates on naturally infected rice grains and enhance grain germination.

2. Materials and methods

2.1 Isolation and morphological identification

One hundred of rice grain samples were obtained from Rice Research and Training Center (RRTC), Sakha, Kafr El-Sheikh, Egypt. The collected grains from four cultivars (Giza 177, Giza 179, Sakha 101 and Sakha 106) were washed in running tap water, then surface sterilized in 1% sodium hypochlorite for 2 minutes, then rinsed in sterile water. The surface sterilized grains were dried on sterilized filter paper, then plated on potato dextrose agar medium (PDA), plates were incubated at 25°C for 5 days. The developed fungi were purified by single spore isolation, and then sub cultured on PDA slants, kept at 4°C (Ilyas & Javaid, 1995). The different fungal isolates were identified based on the morphological characteristics and microscopic examination. Temporary slides were also prepared and observed under compound light microscope for proper identification. The fungal isolates were identified to species level, wherever possible, following the appropriate Keys (Manamgoda et al., 2011; Mew & Gonzales, 2002; Mew & Misra, 1994; Agarwal, 1989; Ellis, 1980; Booth, 1971; Barnet, 1962).

2.2 Molecular identification

2.2.1 Isolation of genomic DNA from fungal isolates

Genomic DNA was extracted using a rapid mini preparation procedure (Shahda Wafaa et al., 2015; Edel et al., 2001). Isolates were grown for 5-15 days on PDA plates. 1 mL of lysis buffer (50 mM Tris-HCl (pH 7.5), 50 mM EDTA and 3% SDS) was added to the plate and the mycelium was scraped with a spatula. 500 µL of buffer mixed with mycelium was recovered in a microtube and mixed using a vortex shaker. The microtubes

tubes were incubated at 65ºC for 10 min and centrifuged at 16099 *xg* for 10 min at 4°C. The supernatants were transferred to new microtubes and the DNA was precipitated by adding 0.5 volume of 3M sodium acetate and one volume of ice-cold isopropanol. Microtubes were gently inverted three times and centrifuged at 16099 *xg* for 15 min at 4ºC. The supernatant was discarded and the pellet was rinsed with 300 µL of 70% ethanol. After centrifugation at 16099 *xg* for 5 min at 4ºC, the ethanol was discarded. The DNA pellet was air-dried, dissolved in 100 µL of TE buffer (10 mM Tris-HCl, pH 8.0 and 1 mM EDTA) and stored at 4ºC until use.

2.2.2 Molecular characterization based on internal transcribed spacer

Molecular identification of fungal cultures were carried out based on conserved ribosomal internal transcribed spacer (ITS) region (Moore, *et al.*, 2011). We amplified the ITS regions between the small nuclear 18S rDNA and large nuclear 28S rDNA, including 5.8S rDNA using universal primer pairs ITS1 (5-TCCGTAGGTGAACCTGCGG-3) and ITS4 (5-TCCTCCGCTTATTGA TATGC-3). The PCR amplification was carried out in a total volume of 25 µL containing 3 µL of template DNA, 12.5 µL PCR Green Master Mix (Thermo Scientific™), 0.5 µL of each primer (10 pmol) and 8.5 µL molecular grade water. The amplification cycle consists of an initial denaturation at 95°C for 1 min followed by 35 cycles at 94°C for 30s, 55°C for 2 min, and 72°C for 1 min and a final extension at 72°C for 10 min. Amplified PCR products were separated on 1.5% agarose gel, in 1X TAE buffer at 65 V for 15 min.

2.2.3 Sequencing of amplified ITS region, alignment and phylogenetic analysis

The amplified fragment of ITS1-5.8s and ITS2 region (500-700 bp) of 10 selected isolates were sent for sequencing (Macrogen, Scientific Services Company, Korea). Identification of isolates were confirmed by applying Basic Local Alignment Search Tool (BLAST search) on National Center for Biotechnology information (NCBI) site (http://www.ncbi.nlm.nih.gov) using the obtained sequences of the amplified regions. Alignments were done by using Molecular Evolutionary Genetics Analysis version 7 (MEGA 7) software. Phylogenetic tree was constructed using neighbor-joining (NJ) method from the CLUSTALW alignment (Kumar *et al*., 2016). The obtained sequences were compared with different international fungal strains obtained from Genbank with accession numbers (MK409313), (MK409312), (MK409311) and (MG182681) for *F. graminearum*, (MG820082), (MH591464), (KT211540) and (KJ598858) for *F. verticilliodies* and (GU373634), (JX256415), (MF185132), (KU499544) and (MK051170) for *B. oryza*e. Sequences obtained in this study were deposited in European nucleotide archive for accession numbers.

2.3 Biological control

2.3.1 Antifungal activity of *T. harzianum* against isolated rice fungi

Two isolates of *T. harzianum* (Tr1) and (Tr2) were used in this study. The isolate

(Tr1) was obtained from soil collected from Kafr ElSheik Governorate and the isolate (Tr2) was isolated from rice grain samples (Sakha 101) cultivar. These two isolates of *T. harzianum* were used against fungal isolates *F. graminearum* (F.g.101), *F. verticilliodies* (F.v.101) and *B. oryzae* isolates (B.o.101). Plates of PDA medium were prepared; each plate was inoculated with a 0.5 cm PDA disk of each *T. harzianum* isolates on the periphery of the plate. One day after, each of these plates were inoculated, on the other side with a si`milar PDA disk of ten days old culture of the tested fungi *F. graminearum* (F.g.101), *F. verticilliodies* (F.v.101) and *B. oryzae* isolates (B.o.101). The plates were incubated at 25°C. three replicates were used for each treatment. Plates inoculated with the tested pathogenic fungus served as control. Growth diameters of the tested fungi growing with *T. harzianum* were measured compared with the control (the treated fungi only) (Desai et. al., 2002). The percent growth inhibition of a rice pathogen was calculated according to Abdel-Fattah et al. (2007) by using the following equation:

$$X = 100 - [(G2/G1) \times 100]$$

Where, X was the percentage of reduction in mycelia growth, G1 was the averaged growth of pathogenic fungus in control plates, and G2 was the averaged growth of pathogenic fungus in treated plates.

2.3.2 Effect of *T. harzianum* isolates on naturally infected rice grains

Spore suspension of the two *T. harzianum* isolates was prepared from 8 days old culture of the isolates on PDA. The plate (9 cm diameter) was flooded with 10 mL of sterilized distilled water and shaken for a few minutes**.** Two hundred rice grains of each cultivar were sterilized in 1% sodium hypochlorite for 2 min., rinsed in distilled water then surface dried, and then soaked in a conidial suspension of *T. harzianum* isolates. The soaked grains were dried and placed on PDA in Petri dishes and kept at room temperature (20-25°C) for 8 days. Sterilized grains only served as a control (Abdel-Fattah et al., 2007).

3. Results

3.1 Isolation and identification of fungal isolates

During the present investigation, two different genera of fungi were isolated from grains of four rice varieties (Giza 177, Giza 179, Sakha 101 and Sakha 106). The different fungal species were showing grain discoloration symptoms. Ten fungal isolates were initially identified as *Fusarium* spp. and *Bipolaris* spp. (Table 1). Based on their morphological characteristics; colony morphology, conidial, conidiophores and growth pattern, the isolates were identified as *Bipolaris oryzae*, *Fusarium verticilliodies* and *Fusarium graminearum* (Figure 1).

3.2 Molecular identification

3.2.1 Molecular characterization through ITS region and sequence analysis

The ITS region from the ten fungal isolates were sequenced using ITS1 and ITS4 universal primers. The resulting

partial DNA sequences were analyzed using BLAST tool at the National Center of Biotechnology and Information site (http://www.ncbi.nlm.nih.gov). The search revealed that, the nucleotide sequences of the ten fungal isolates were identical to those of *B. oryzae* (2 isolates), *F. graminearum* (4 isolates) and *F. verticilliodies* (4 isolates). The homology of *B. oryza*e, *F. graminearum* and *F. verticilliodies* isolates to the Genbank strains reached 99%. Sequences were submitted to Genbank and given accession numbers stated in Table (2).

Table 1: Pathogens isolated from discolored grain of rice cultivars.

Isolate code	Cultivar	Isolated pathogens
F.g.177	Giza 177	*Fusarium graminearum*
F.v.177		*Fusarium verticilliodies*
B.o.177		*Bipolaris oryzae*
F.g.179	Giza 179	*F. graminearum*
F.v.179		*F. verticilliodies*
F.g.101	Sakha 101	*F. graminearum*
F.v.101		*F. verticilliodies*
F.g.106	Sakha 106	*F. graminearum*
F.v.106		*F. verticilliodies*
B.o.106		*B. oryzae*

Table 2: GenBank accession numbers and laboratory code of partial ITS region of ten isolates.

Laboratory code	Accession number
F.g.177	MK450464
F.v.177	MK450465
B.o.177	MK450466
F.g.179	MK450467
F.v.179	MK450468
F.g.101	MK450469
F.v.101	MK450470
F.g.106	MK450471
F.v.106	MK450472
B.o.106	MK450473

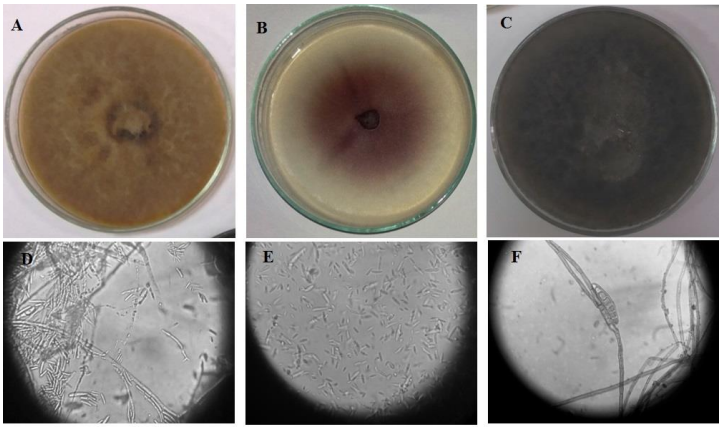

Figure 1: Morphological characteristics of seed born fungi from rice grains (A, D) *Fusarium graminearum*, (B, E) *Fusarium verticilliodies* and (C, F) *Bipolaris oryzae*.

3.2.2 Alignment and phylogenetic analysis

Alignment of the ITS region nucleotide sequences of ten fungal isolates with the ITS region nucleotide sequences of other fungal isolates collected from the GenBank was carried out utilizing CLUSTAL W (1.82) (http://www2.ebi.ac.uk/clustalw; Thompson et al., 1994) at which MEGA version 7 (Kumar et al., 2016) was used to generate the Bootstrap neighbor-joining tree. Data illustrate that, there are interferences among our fungal isolates *F. graminearum*, *F. verticilliodies*, *B. oryzae* which isolated from different rice varieties and the identified fungal strains collected from GenBank except the isolate *F. verticilliodies* MK450472 (Sakha 106) which had a unique cluster based on constructed phylogenetic analysis (Figures 2 and 3).

3.3 Biological control

3.3.1 Antifungal activity of *T. harzianum* against isolated rice fungi

Data from the dual culture test in Table 3 showed that although the linear growth of both tested fungal isolates (*B. oryzae*, *F. verticilliodies* and *F. graminearum*) and *T. harzianum* Tr1 and Tr2 on single culture plates increased after inoculation, the linear growth of the two isolates of *T. harzianum* were more rapid than that of tested rice fungi. The highest growth inhibition was exhibited by *T. harzianum* Tr2 and Tr1 isolates against *F. graminearum* (76.77% and 76.74% respectively), followed by *F. verticilliodies*. The least growth inhibition was observed by *B. oryzae* using Tr2 and Tr1 isolates (53.33% and 50.0% respectively) compared to the control (Figure 4).

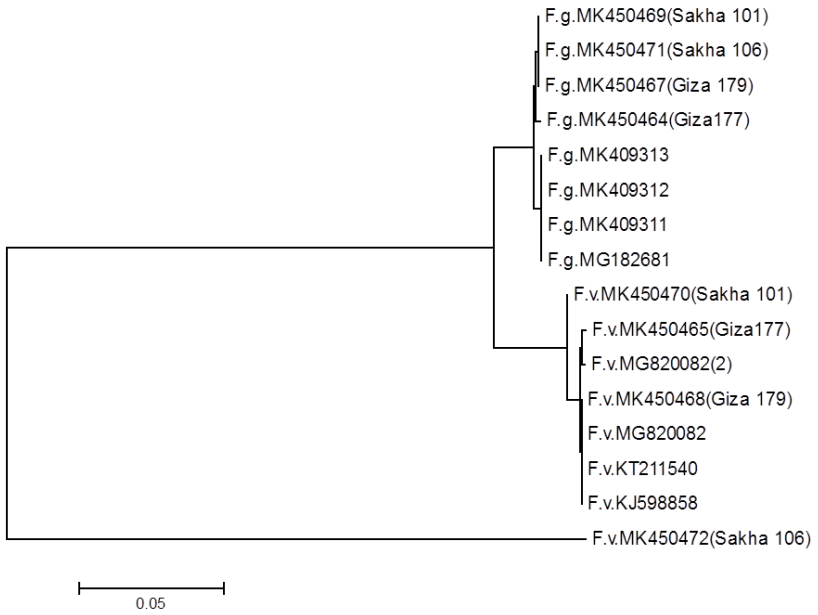

Figure 2: Phylogenetic tree of *Fusarium graminearum* and *Fusarium verticilliodies* isolates obtained in this study compared with three ITS sequences collected from Genbank (MK409313), (MK409312), (MK409311), (MG182681), (MG820082), (MH591464), (KT211540) and (KJ598858).

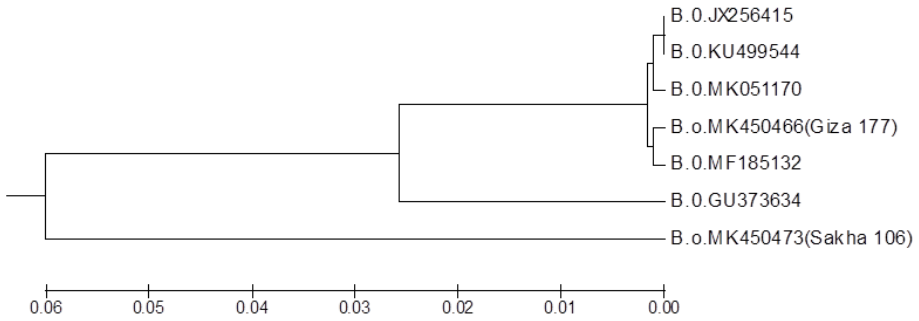

Figure 3: Phylogenetic tree of *Bipolaris oryzae* isolates obtained in this study compared with three ITS sequences collected from GenBank (GU373634), (JX256415), (MF185132), (KU499544) and (MK051170).

Table 3: Antagonistic effect of *T. harzianum* against *B. oryzae*, *F. verticilliodies*, *F. graminearum* on PDA[a].

Isolates	linear growth[b] (cm)			[*]Growth inhibition%	
	Control	*T. harzianum* Tr1	*T. harzianum* Tr2	*T. harzianum* Tr1	*T. harzianum* Tr2
B. oryzae	3.0	1.5	1.4	50.0	53.33
F. verticilliodies	5.6	1.7	1.6	69.64	71.42
F. graminearum	4.3	1.0	0.9	76.74	76.77

[a]Each value represents the mean of 3 replicates. [b] Distance between disk's center and the margin of the colony. [*]Growth inhibition of three major seed-borne fungal pathogen on PDA medium.

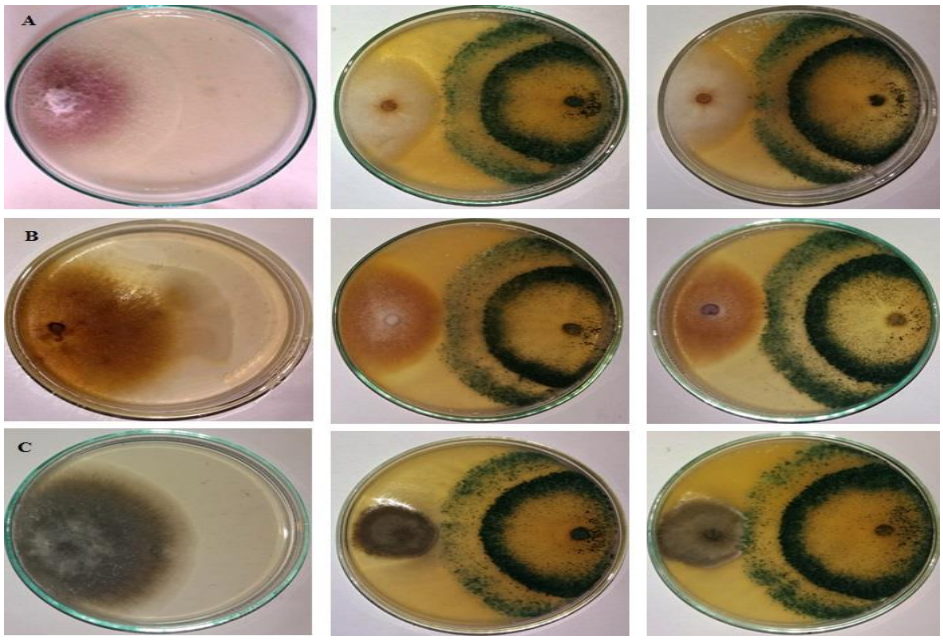

Figure 4: The antagonistic effect of *T. harzianum* Tr1 represented in the middle column and *T. harzianum* Tr2 represented in the right column against three isolates of *F. verticilliodies* (A), *F. graminearum* (B) and *B. oryzae* (C) represented in the left column.

3.3.2 Effect of *T. harzianum* isolates on naturally infected rice grains

The naturally infected rice grains treated with *Trichoderma* Tr1 and Tr2 isolates

exhibited 100% inhibition of fungal pathogens associated with rice grains compared to untreated naturally infected grains (Figure 5).

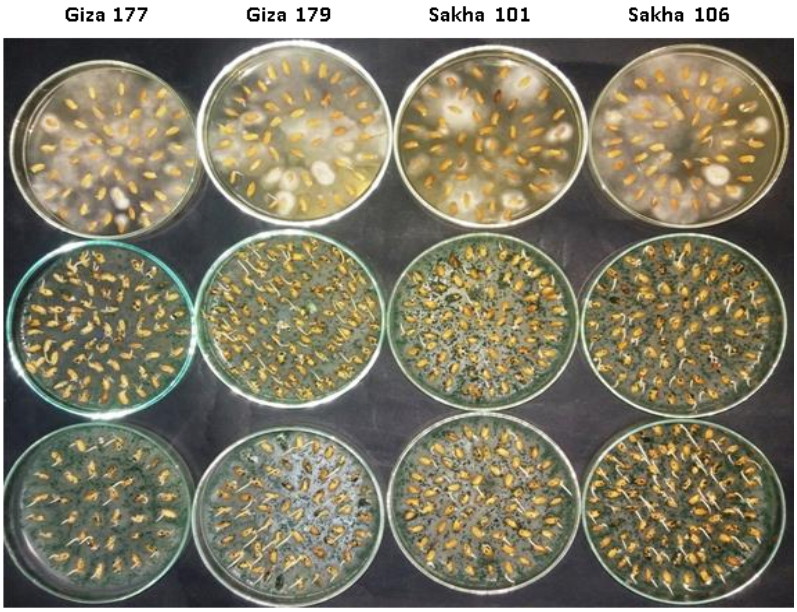

Figure 5: The antagonistic effect of *T. harzianum* Tr1represented in the middle row and *T. harzianum* Tr2 represented in the last row against rice fungi compared to represent in the first row.

4. Discussion

In the present study, ten fungal isolates were isolated from four rice seed cultivars (Giza 177, Giza 179, Sakha 101 and Sakha 106) showing grain discoloration symptoms. These fungal isolates were belonged to *Fusarium* spp. and *Bipolaris* spp. The pathogens caused discoloration of grains has also been reported by Javaid et al. (2002), Khan et al. (2000) and Ilyas and Javaid (1995). The transmission of pathogens through seeds caused pre & pest emergence of seeds and seedling. In addition to inflorescence abnormality at later stage (Ou, 1985; Neergaard, 1970). Khalid et al. (2001) demonstrated that the association of fungal pathogens decreased the percentage of seed germination and consequently poor crop stand. The cultural characteristics of *B. oryzae*, *F. graminearum* and *F. verticilliodies* isolates from Egypt were in agreement with those in a previous study (Manamgoda et al., 2011; Misra, 1994; Ellis, 1980) and thus their identification was confirmed. Molecular markers as a genetic variation assessment of fungal species were importance became clear in many modern studies (Motlagh & Anvari, 2010; de Oliveira et al., 2002). The ITS region as a marker for phylogenetic analyses in eukaryotes. It has been also used a DNA barcode to identify fungal

species (Keller et al., 2015; Schoch et al., 2012). ITS is very heterogeneous in both of size and nucleotide sequences, so become a good tool using in different levels of taxa especially in species level taxa (Sickel et al., 2015). In the present study we used the ITS region as a DNA molecular tool for identification and variation analysis of rice fungal species. The findings from this study that *T. harzianum* antagonizes *B. oryzae*, *F. graminearum* and *F. verticilliodies in vitro* by a combination of reducing the linear growth of *B. oryzae*, *F. graminearum* and *F. verticilliodies* through the creation of an inhibition zone and eventual overgrowth are consistent with those of Xu et al. (1999) and Rasmy (1991). Seed dressing with *T. harzianum* showed maximum growth inhibition of tested fungal pathogens indicating significant reduction in the population of fungi present on naturally infected discolored grains. Antagonistic *Trichoderma* isolates produce structure for attachment, infection and kill plant pathogenic hosts by cell wall degrading enzymes (CWDEs) (Nygren et al., 2018; Karlsson et al., 2017; Mukherjee et al., 2012; Harman, 2006; Harman et al., 2004). Karlsson et al. (2017) reported that *Trichoderma* has mycoparasitism related gene such as ech42 and prb1 are unregulated during mycoparasitism. These genes have a role in the initial activities of CWDEs. With *Trichoderma atroviride*, Reithner et al. (2011) reported that a synergistic transcription of various genes involved in cell wall degradation in interaction with *B. cinerea* and *Phytophthora capsici* (Reithner et al., 2011). Our results were in accordance with the studies of Abdel-Fattah et al. (2007) and Ahmed et al. (2013) in using biotic control as a seed treatment before saving of rice nurseries will enhance the seed germination, avoid fungal infection and ultimately crop yield.

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