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Finger Printing of Certain Seed-Borne Fungi on Imported Tomato Seeds in Egypt

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Abstract: Egypt imported different cultivars of tomato seeds from different companies. Different microorganisms associated these seeds, among them the seed borne fungi. Seeds of five different companies were collected and subjected to fungal isolation using Standard blotter Technique and agar plate technique. Results revealed that five species were observed. The observed fungi were morphologically identified under microscope and the results were confirmed using specific PCR with primers (ITS-1 & ITS-4). Amplicones with molecular size 600 bp was observed. The bands were purified and subjected to DNA sequence. The sequence analysis revealed that the five fungi are *Trichoderma longibrachiatum, Aspergillus fumigatus, A. fumigatus* strain TFR-8, *A. flavus* and *A. oryzae.* The sequences were submitted into the Gene Bank and were deposited under accession no. KJ424352, KJ424353, KJ734707, KJ734708 and KJ742820. *T. longibrachiatum* was used as biocontrol agent against several plant pathogenic fungi and the results showed significant growth inhibition for the treated fungi with percentage ranging from (49.7 % to 73.4%). Using *T. longibrachiatum* strain as abiological control agent is effective, applicable, safe and commercial.

Key words: Biological control · Egypt · Seed borne fungi · Specific PCR · Tomato · *Trichoderma longibrachiatum*

INTRODUCTION

Tomato (*Lycopersioon esoulentum* Mill.) is an important vegetable crop cultivated over an (216,400,000) hectare area in Egypt. Tomato seeds such as all the seeds play an important role in crop production, however the seeds are attacked by different fungi either saprophytically or within the tissue of embryo [1]. The disease caused by fungi may lead to seed abortion or elimination of germination capacity [2]. At least 10 different fungi have been reported as seed-borne fungi on tomato from different parts of the world, which include *Alternaria alternate, A. solani*, *Aspergillus* spp., *Botrytis cinerea*, *Cheatomium globosum, Cladosporium* spp., *Colletotrichum dematium*, *Curvularia lunata*, *F. equiseti*, *F. moniliforme*, *F. oxysporum, F. solani*, *Macrophomina phaseolina*, *Penicillium* spp., *Phoma destouctiva, Pythium aphanidermatum*, *Rhizoctonia solani*, *Verticillium* spp. [3-8]. Biological control offers an alternative to the use of synthetic fungicides and has become a well established principle over the last few decades [9]. Various fungi (*e.g. Trichoderma* have shown good biocontrol potential [10, 11]. Biological control is considered a sustainable and safe [12]. It is relative free from problems of pest resistance and is extremely safe to human health [12]. Biopesticides often are effective in very small quantities and often decompose quickely, thereby resulting in lower exposure and largely avoiding the pollution problems caused by conventional pesticides [13]. In general, cultural practices are less likely to be in conflict with natural enemies than pesticide [13]. In contrast to chemical control, biological control lessens long-term damage to the environment by persistent chemicals [13]. It may be a more economical alternative to some insecticide and fungicides applications [13, 14]. Unlike most fungicides biological control is often very specific for a particular pathogen, thus other beneficial microorganisms can go completely

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unaffected or undistributed by their use [13]. Fungicides are typically used as protectants against foliar disease [13]. However, Pathogens in the soil are very difficult to control by fungicides, because the active ingredient must be delivered through the soil to contact the inoculums and because of losses from the soil leaching are microbial breakdown [14]. Biological control is cost-effective for specialized applications and where no chemical control exists [12]. Biological control would seem to be ideally suited for controlling root diseases, especially for soil-less systems in closed structures [12].

This study aimed to isolation and identification of seed-borne fungi in imported tomato seeds in Egypt as well as examination of a biocontrol method to control these fungi.

MATERIALS AND METHODS

Samples Collection and Fungal Isolation: Seeds samples were collected from different companies located in Egypt as explained in Table 1. Fungal isolation was performed using two different methods Standard blotter method (SBM) and agar plate method (APM) according to Anonymous [15].

Standard Blotter Method (SBM): Under aseptic condition, non-sterilized, surface-sterilized (with 1% sodium hypochlorite solution for 1 min and grinded seeds plated in 9-cm diameter sterile Petri-dishes containing three layers of sterile blotter (filter) paper moistened with sterilized tap water, 10 seeds per Petri dish. Then the plates incubated at 28±2°C for 5 days under cool white fluorescent light with alternating cycles of 12 hr light/darkness.

Agar Plate Method (APM): Under aseptic condition, non-sterilized, surface-sterilized and grinded seeds will be plated on potato dextrose agar (PDA), 10 seeds per Petri dish. The dishes incubated at 28 ±2°C for 5 days under cool white fluorescent light with alternating cycles of 12 hr light and 12 hr darkness.

Identification: The isolated fungi were purified by using single spore technique [16]. The purified fungi were identified morphologically under microscope at the Regional Center for Mycology and Biotechnology (RCMB) at Al-Azhar University, Cairo. Then confirmed by Specific-PCR by International Transcribed Spacer technique (ITS).

Genomic DNA Extraction: For DNA extraction, liquid culture comprising 100 ml of potato dextrose liquid medium in 250 ml Erlenmeyer flasks were inoculated with 7 mm mycelia disks derived from colony margin of six days old cultures of the isolated fungi. Mycelia mates were harvested by filtration through apiece of filter paper (0.45) in a Buchnur funnel, washed with sterile distilled water and frozen at -80°C. To extract genomic DNA100 mg of freeze-dried mycelium was used to extract genomic DNA using Wizard ® DNA purification Kit (Promega, USA). The frozen mycelia were ground to a fine powder in liquid nitrogen.DNA samples were diluted to approximate concentration of 50 ng/µl for PCR reaction.

Amplification of ITS Region (600 bp): Nuclear ribosomal international transcribed spacer (ITS) rDNA was amplified by using primer ITS 1 and ITS 4 illustrated in Table 2 to amplify 600 bp of 18S rDNA gene from genomic DNA of all fungal isolates. The PCR Program was applied as following by initial denaturation at 95°C for 5 min, 30 cycles of 95°C for 1 min, annealing at 54°C for 1 min and extension at 72°C for 1 min. A final extension step at 72°C for 10 min [17]. Two µl of loading dye was added prior to loading of 10 µl of PCR product per gel slot. PCR products were separated on agarose gel electrophoresis using 2% (w/v) agarose in 0.5x TBE buffer. The size of each band was estimated by using DNA molecular weight marker. Finally, the gel was photographed by using gel documentation system.

Gene Bank Accession Number: The isolated strains were identified at molecular level using PCR amplification of the specific gene sequence with universal primers ITS1and ITS4 according to Mullis [18, 19]. The DNA sequences were deposited in Gene Bank under accession numbers.

Antagonistic Effect of *Trichoderma longibrachiatum* Against Growth of Seed-Borne Fungi *in vitro* Using Dual Culture Method: Antagonistic effect of *Trichoderma longbrachitum* as a biocontrol agent, on the growth of seed borne fungi were tested *in vitro* using dual culture technique described by Lorito [11]. This method used to study the efficacy of biocontrol agent, against plant pathogens under laboratory conditions. Prepared sterilized petri dishes 9 cm in diameter containing 20 ml sterilized PDA was used. Disc 5 mm from 7 day old culture taken from the bio-agent and pathogen separately with the help of sterilized cork bores. The culture discs of the pathogen and bioagent transfered aseptically and

Table 1: Tomato seeds samples collected from different sources

Sample	Origin	Purity %	Germination %	Lot. No	Inert
SUPER STRAIN (B)	USA	99.99	85	361705X000.0	0.001%
JACALF1	France	99.90	85	481p314	0.1%
NEMAGUARD	Japan	99.00	85	211261	1%
RED SUN NO.1	USA	99.00	90	Ms/QT8/10	1%
HYBIRD SUPER STRAIN (B)	USA	99.90	85	Ms/QT8/10	0.001%

Table 2: Primers sequence used in the amplification of International Transcribed Spacer regions (ITS)

Primer	Primer sequence (5'→'3)	Size of amplified product (bp)	Annealing (°C)	Reference
ITS(1)	TCCGTAGGTGGAACCTGCGG	600 bp	30	[17]
ITS(4)	TCCTCCGCTTATTGATATGC	600 bp	28	

placed at periphery of the Petri plate containing the medium. The both discs of pathogen and bioagent placed at equidistance in opposite direction, three replicates were used for each organism. Inoculated plate with culture disc of the pathogen alone served as control. Inoculated Petri-plates then transfered to an incubator and incubate at 25±1°C.

Growth of the pathogen observed periodically and antagonist in Petri plates and the colony growth diameter in each Petri plate measured. The per cent inhibition of the pathogen by the bio agent when the growth of the pathogen is full in the control plates calculated using the following formula:

$$\text{Percent inhibition} = \frac{\text{Radial growth in the Control (C)} - \text{Radial growth in treatment (T)}}{\text{Radial growth in control (C)}}$$

RESULT

Rapid identification of microorganisms is necessary in the pathological laboratory in an attempt to proceed for the preparation of a bioformulation that can be useful for the farmers. The experimental results of isolation of fungi by using Standard blotter method (SBM) and agar plate method (APM) showed that agar plate method is more effective in isolation of seed borne fungi as shown in Tables 3 and 4. The results revealed that total fungal count isolated by APM is 74 species, while the total fungal count isolated by SBM is 56 species. The results of isolation also revealed that grinded tomato seeds of super strain B and JACALF1 have no growth of fungal species by using two methods. Morphologically identified species were five species of two genera *Trichoderma longibrachiatum, Aspergillus fumigatus, Aspergillus fumigatus* strain TFR-8, *Aspergillus flavus* and *Aspergillus oryzae* agar plate method is also more effective in isolation of seed borne fungi as the result obtained where the total fungal count of each species by APM is greater than the count recorded by using SBM as shown in Table 5.

Molecular Identification by Using Universal Primers (ITS): The DNA templates of ITS regions (ITS1, ITS4) in rDNA of the fungi were amplified by PCR using the universal primers ITS1/ITS4. ITS genes were amplified for *Trichoderma longibrachiatum, Aspergillus fumigatus* E1, *Aspergillus fumigatus* strain TFR-8, *Aspergillus flavus* and *Aspergillus oryzae.* Approximately 600 bp were amplified Fig.1. PCR amplicons with specific forward ITS (600) primers of the fungal species were subjected to DNA sequence analysis and the resulting nucleotide sequencing deposited in Gene bank and accession no. obtained recorded in Table 6.

Antagonistic Effect of *T. longbrachiatum* Against Growth of Seed-Borne Fungi *in vitro* Using Dual Culture Method: In this experiment isolated *T. longbrachiatum* used as a biocontrol agent. The result show that *T. longbrachiatum* has great effect on seed borne fungi, the maximum growth inhibition was recorded for *A. flavus* but the minimum growth inhibition was for *A. fumigatus* strainTFR-8 (Table 7).

The interaction between *T. longbrachiatum* as a biocontrol agent against growth of isolated fungi *A. fumigatus, A. fumigatus strain* TFR-8*, A. flavus and A. oryzae in vitro* using dual culture method was examined. The results revealed that *A. flavus* is the most affected strain with growth inhibtion reaced to (73.4 %) followed by *A.oryzae* with growth inhibtion (55.2 %) followed by *A. fumigatus isolate E1* with growth inhibtion(51.1 %) and the most resistant strain which give the lowest growth inhibtion is *A.fumigatus strain TFR-8* with growth inhibtion reached to (49.7 %) as shown in Fig. 2.

Table 3: Isolation of seed-borne fungi by using plate agar method

	Super strain (B)			JACALF1			NEMAGUARD			RED SUN NO.1			HYBIRD SUPER STRAIN (B)		
Fungal species	N	S	G	N	S	G	N	S	G	N	S	G	N	S	G
A. fumigatus isolate E1	7	5	0	0	0	0	0	0	0	0	0	0	0	0	0
A. oryzae	0	0	0	8	6	0	0	0	0	0	0	0	0	0	0
T. longibrachiatum	0	0	0	0	0	0	6	4	3	0	0	0	0	0	0
A. fumigatus strain TFR-8	0	0	0	0	0	0	0	0	0	7	4	4	0	0	0
Aspergillus flavus	0	0	0	0	0	0	0	0	0	0	0	0	9	5	6
Total count	12			14			13			15			20		

(N) = Non sterilized, (S) = Sterilized, (G) = Grinded

Table 4: Isolation of seed-borne fungi by using Standard blotter method (SBM).

	Super strain (B)			JACALF1			NEMAGUARD			RED SUN NO.1			HYBIRD SUPERSTRAIN (B)		
Fungal species	N	S	G	N	S	G	N	S	G	N	S	G	N	S	G
A. fumigatus isolate E1	6	3	0	0	0	0	0	0	0	0	0	0	0	0	0
A. oryzae	0	0	0	5	3	0	0	0	0	0	0	0	0	0	0
T.longibrachiatum	0	0	0	0	0	0	6	2	3	0	0	0	0	0	0
A. fumigatus strain TFR-8	0	0	0	0	0	0	0	0	0	5	2	4	0	0	0
A. flavus	0	0	0	0	0	0	0	0	0	0	0	0	9	3	4
Total count	9			8			11			11			17		

(N) = Non sterilized, (S) = Sterilized, (G) = Grinded

Table 5: Isolation of seed borne fungi by using two methods

		Total fungal count	
Code	Fungal species	SBM	APM
M1	*Aspergillus fumigatus* isolate E1	9	12
M2	*Aspergillus oryzae*	8	14
M3	*Trichoderma longibrachiatum*	11	13
M4	*Aspergillus fumigatus* strain TFR-8	11	15
M5	*Aspergillus flavus*	17	20

Table 6: Accession numbers of fungal species recorded in Gene bank

Organism	Accession number
Aspergillus fumigatus isolate E1	KJ424352
Aspergillus oryzae	KJ742820
Trichoderma longibrachiatum	KJ424353
Aspergillus fumigatus strain TFR-8	KJ734707
Aspergillus flavus	KJ734708

Table 7: Antagonistic effect of *T. longibrachiatum* as a biocontrol agent against isolated fungi using dual culture method

Name of fungi	Biocontrol agent	Linear growth (cm)	Growth inhibition (%)
A. fumigatus isolate E1(KJ424352)	*T. longibrachiatum*	4.2± 0.47	51.1
A. oryzae (KJ742820)		3.85±0.20	55.2
A. fumigatus strain TFR-8(KJ734707)		4.32±0.26	49.7
A. flavus(KJ734708)		2.28±0.31	73.4
P- Value		0.001	

Each value is the mean of three replicate ± Standard deviation. All the results are highly significant at P = 0.001 using one way analysis of variance (ANOVA).

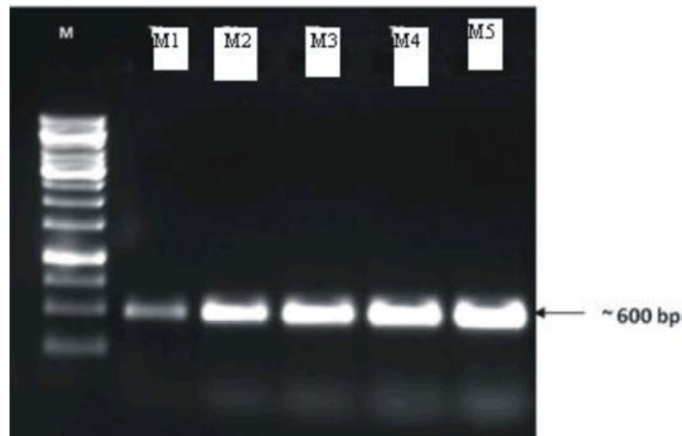

Fig. 1: Show the amplified PCR Product of isolated fungi where M at the far left–hand side of the gel loaded with 3 K bp DNA marker and M1, M2, M3, M4 and M5 are fungal species

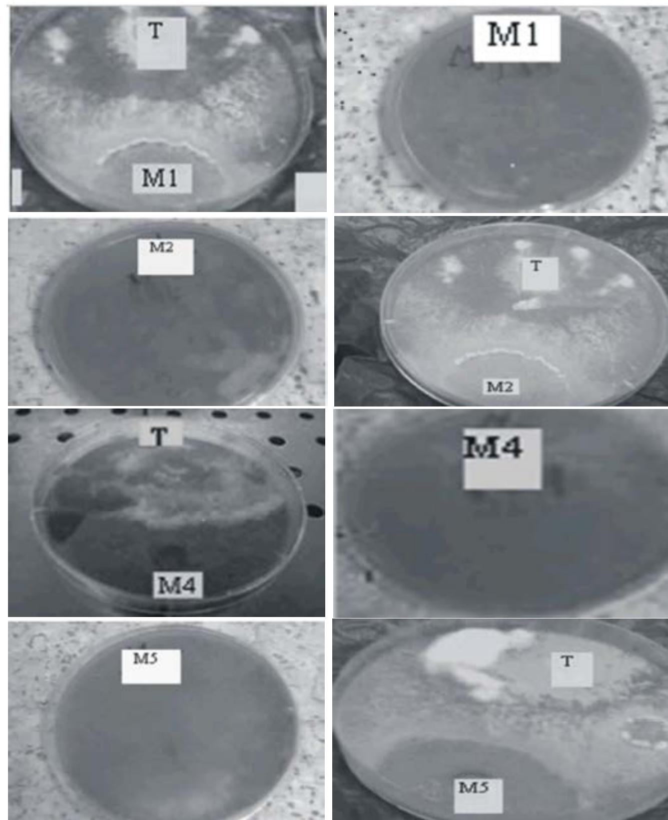

Fig. 2: Antagonistic effect of *T. longibrachiatum* against growth of isolated fungi *in vitro* using dual culture method where (T) refer to *T. longbrachiatum and* M1: *A. fumigatus* isolate E1 (KJ424352), M2: A. oryzae (KJ742820), M4: *A. fumigatus strain* TFR-8(KJ734707) and M5: *A. flavus* (KJ734708)

DISCUSSION

Five species of two genera isolated from collected seeds samples from different origins U.S.A., France and Japan. Isolated strains are defined as *A. fumigatus isolate E1, A. oryzae, T. longibrachiatum, A. fumigatus* strain TFR-8*, A. flavus.* All the imported tomato seeds have seed–borne fungi. However, the tomato cultivar Nemaguard has *T. longbrachiatum* which defined as a biocontrol agent against fungi that imported from Japan which contain *T. longbrachiatum*. Mould contamination can occur on a crop during development, harvest, storing,

terminal shipment and processing. Many investigations have been reported on mycoflora [20, 21, 22, 23] and pathogenic fungi on tomato plants [3, 24]. In Egypt, Saber *et al.* [21] identified 22 species contributed to 7 genera from 21 tomato samples as seed borne fungi. They found that *Aspergillus fumigatus, A. flavus, A. niger* and *Penicillium oxalicum* were the predominant on glucose-Czapek's agar medium. While, *Aspergillus niger*, *A. flavus, A. sydowii* and *Eurotium montevidensis* were the most common species on 10% NaCl glucose-Czapek's agar medium. Perveen [23] isolated 37 species of fungi belonging to 20 genera from 24 samples of tomato seeds collected from different parts of Pakistan. The most common fungi *Fusarium solani*, *F. moniliforme*, *A. flavus, Alternaria alternata* and *Drechslera australiensis* were predominant. The occurrence and distribution of fungal flora on different plant species were also studied in Upper Egypt [25-28]. Rathod [29] indicated that Legume. TLG – 45 was associated with sixteen fungi such as *A. flavus, A. niger, Aspergillus ustus, A. fumigatus, A. nidulans, A. terrus, Alternaria tenuis, F. oxysporum, F. semitectum, Macrophomina phaseolina, Penicillium citrinum, Sclerotium rolfsii, Cephalosporium acromonium, R. solani, Rhizoctonia batatiocola* and *Rhizopus nigricans.* In the present study the results of fungal isolation showed that APM is more effective in the isolation of seed-borne fungi than SBM as shown in Table 5. These results are in agreement with those obtained by Rathod [29]. The results of the interaction between the tested *T. longibrachiatum* against growth of isolated fungi *Aspergillus fumigatus, A. fumigatus strain TFR-8, A. flavus and A. oryzae* in vitro using dual culture method the results indicated that *Aspergillus flavus* is the most affected isolate with growth inhibition reached to (73.4%) followed by *Aspergillus oryzae* with growth inhibtion (55.2 %) followed by *Aspergillus fumigatus isolate* E1 with growth inhibition (51.1%) and the most resistant strain which give the lowest growth inhibtion is *Aspergillus fumigatus* strain TFR-8 with growth inhibition reached to (49.7%).

Fungal species belonging to the genus *Trichoderma* are worldwide in occurrence and easily isolated from the soil.The potential of *Trichoderma* species as bioconrol agents against various plant diseases has been reported by Wells *et al.* [30] and Sharon *et al.* [31]. The antagonist *T. harzianum* (ANR-1) is chosen to be the most promising bio-control agent for *F. oxysporum* f.sp. *Lycopersici*. The bioagents of fungi might be exploited for sustainable disease management programs to save environmental risk [32]. Prospects of biological control of soil-borne plant pathogens using most promising bio-control agent, the genus *Trichoderma* has been described [33]. Successful reductions of *Fusarium* wilt in many crops with application of different species of *Trichoderma* have been found [34, 35, 36]. However, it is also reported that all the isolates of *Trichoderma* spp. are not equally effective in control of pathogen *in vitro* and *in vivo* conditions to control diseases [36]. Therefore, specific isolates are needed for successful control of a particular pathogen [36]. This study reported that *T. Longbrachiatum* has antagonistic effect on *A. fumigatus, A. fumigatus* strain TFR-8*, A. flavus* and *A. oryzae in vitro.* and this effect lead us to think in a suitable method for treatment seeds by *T. longbrachiatum* before reached to the farmer to avoid hazards produced from seed contamination.

CONCLUSION

From the present study it could be concluded that:

- Imported tomato seeds in Egypt must subjected to more investigations before reaching to farmers.
- Nuclear ribosomal international transcribed spacer (ITS) rDNA was valuable for identification and fast differentiation of strains and show genetic variation among the tested isolated fungi
- Potato-dextrose agar was the most suitable growth medium for morphological character (mycelial growth, sporulation and pigmentation) assessment of tested fungi.
- Dual culture test indicate that tested *T. longbrachiatum* was effective in inhibtion in the growth of seed borne fungi (*A. fumigatus* E1, *A. fumigatus strain TFR-8, A. flavus and A. oryzae*).

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