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Biological and Molecular Studies on *Trichoderma Viride*-Plant Pathogenic Fungi Interactions

[1]Elsayed E. Hafez, [2]Asmaa Meghad, [3]Hassan A. Abd Elsalam and [1]Seham A. Ahmed

[1]Plant Protection and Biomolecular Diagnosis Department,
Arid Lands Cultivation Research Institute (ALCRI),
City for Scientific Research and Technology Applications, New Borg El Arab, 21934, Alexandria, Egypt
[2]Departmant of Genetic Engineering and Biotechnology Research Institute, City for Scientific Research and Technology Applications (CSAT), New Borg El Arab, 21934, Alexandria, Egypt
[3]Nucleic Acid Research Department, Arid Lands Cultivation Research Institute, City for Scientific Research and Technology Applications (CSAT), New Borg El Arab, 21934, Alexandria, Egypt

Abstract: Trichoderma is extensively used as bioagent against different plant pathogenic fungi. Different plant pathogens were isolated from rotted root and wilted tomato plants cultivated in Alexandria, Beni-Swiff, EL Dakahlya and El- Fayoum governorates in Egypt. From these plant pathogenic fungi; *Fusarium oxysporum, Rhizoctonia solani*, *Fusarium verticilloid, Alternaria alternata, Mucor racemosus* and *Aspergillus niger* were isolated and identified. Antagonistic test was performed between the *Trichoderma viride* and the above pathogenic fungi to study microbe-microbe interaction. Different inhibition zones were observed and the percentage of the fungal linear growth was ranged from 40 to 80 % compared with the control. The fungal hyphae were collected around the clear zones and subjected to Differential Display -PCR using 5 different arbitrary primers. The effects of the bioagent on the genetic profile of the examined pathogenic fungi were observed as up regulated and down regulated genes. Three of the up regulated genes were selected randomly and subjected to DNA sequence. The sequence analysis of the sequenced genes revealed that the three selected genes are; endogluconase (860 bp), polyketide synthas (508 bp) and FMK1(618 bp) respectively. It was observed that the function of the three analysed genes play an important role either in biocontrol or in fungal defense activity. *Trichoderma viride* could be used as biocontrol agent for a wide range of plant pathogens if the suitable conditions were serving for this valuable organism.

Key words: Polyketide Synthase · Differential Display-PCR · Defense System · Biological Control

INTRODUCTION

The suppression of plant diseases by biological control agents is an interaction between the plant, pathogen, biocontrol agent, microbial community around the plant [1]. Since long time ago, there has been a worldwide importance towards the use of non-chemical methods for protecting crops from pests and diseases [2]. Biological control of plant disease by microorganisms especially soil-borne plant pathogens, has been became a more natural and environmentally acceptable alternative to existing chemical treatment methods [3].

Researchers are interested by interactions between bioagent fungi and fungal plant pathogens, because fungi have much greater potential than bacteria to grow and spread through soil and in the rhizosphere [4]. Due to their ease of growth and their wide host ranges Trichoderma species are considered the dominant biocontrol agent among other bioagent fungi [4]. Biocontrol identified by scientists as "the ability of fungi to parasitize spores, sclerotia, hyphae and other fungal structures [5]. Moreover, most of the microscopical observations concerning mycoparasitism have come from *in vitro* studies or sterile systems [6].

Corresponding Author: Elsayed E. Hafez, Soil and Water Technology Department, Arid Lands Cultivation Research Institute, (ALCRI), City for Scientific Research and technology Applications, (CSAT), New Borg El Arab, 21934, Alexandria, Egypt.

It was reported that numerous strains of Trichoderma have biocontrol activity against several soil-borne plant pathogenic fungi [7-9], . This biocontrol activity was demonstrated as inhibition zones in dual culture without hyphal contact in treatments which assure that *T. viride* have the capability in secretion of some diffusible non-volatile antibiotics [10]. In addition, the inhibition in the radial growth of two interacting organisms in dual culture resulted in the secretion of extracellular hydrolytic enzymes [11].

Trichoderma can parasitize fungal pathogens and produce antibiotics. Weindling [12] described in detail the mycoparasitism of a fungal pathogen causing damping off disease *Rhizoctonia solani* by the hyphae of Trichoderma, including coiling around the hyphae, penetration and subsequent dissolution of the host cytoplasm. He also described an antibiotic which was toxic to both *R. solani* and *Sclerotinia americana* and named it gliotoxin. The production of antibiotics by Trichoderma was called antibiosis this mechanism was demonstrated in several studies. An antibiotic, gliovirin, from *Trichoderma virens* demonstrated strong inhibition of *Pythium ultimum* and the Phytophthora species [13]. More recent research indicated that certain strains of *Trichoderma* can induce systemic and localized resistance to several plant pathogens. Plants treated with Trichoderma in the root zone can produce higher levels of peroxidase, chitinase activity, deposition of callose-enriched wall appositions on the inner surface of cell walls and pathogenesis-related proteins

Among fungi which affected by *Trichoderma sp*; *Fusarium graminearum*, *Rhizoctoni Solani, Macrophomina phaseoli, Fusarium oxysporum* [14]. In addition to production of antibiotics; *Trichoderma sp.* are able to produce walls degrading enzymes such as chitinases, glucanases that break down polysaccharides, chitins and glucanase, there by destroying cell wall integrity[15]. Patale and Mukadam [16] reported the antagonistic effect of three Trichoderma species; *T. viride*, *T. harzianum* and *Trichoderma sp*. against seven pathogenic fungi, namely *Aspergillus niger*, *A. flavus, Phytophthora sp*. *Fusarium oxysporum*, *Rhizoctonia solani*, *Penicillium notatum* and *Alternaria solani*. Some biocontrol strains as *Trichoderma sp*. are used to strongly induce plant host defenses [17]. Where the Biological processes of living organisms occurs by changes in the levels and patterns of gene expression. The identification and analysis of these genes provide the key on how they influence and regulate biological processes and to understand the molecular mechanisms of the biological system [18].

Where there are different methods used to detect these induced or differentially expressed genes such as Differential Display (DDRT-PCR) which was developed by Liang and Pardee [19]. DDRT-PCR is a powerful tool in the analysis and identification of genes that are differentially expressed at the mRNA level [20]. these technique has been used in biological research to study differential gene expression in plant- pathogen interactions [21] or other developmental or physiological changes [22] in both plants and microorganisms.

Waniska and. Rooney [23] postulated that the use of *Trichoderma viride* as bio- control agent and the interaction with the other pathogenic fungi act as an evolutionary factor and cause induction of some important proteins, endogulcanase and polyketide and FMK1. Moreover, the antifangal proteins are large family of enzymes known as glycosyl hydrolases, enzymes that hydrolyze complex carbohydrate moieties and are comprised of cellulase, xylanase, pectinases, b-glucanase, exocellulase, mananase etc [24]. In addition to the polyketide synthases (PKSs) and FMK1are family of enzymes derived as a secondary metabolites due to plant diseases caused by Fusarium species, these fungal secondary metabolites can protect plants from other fungi [25].

The aim of this work is to study the *in vitro* microbe-microbe interactions between the *Trichoderma viride* as biocontrol agent against the plant pathogenic fungi; *Fusarium oxysporum, Rhizoctonia solani*, *Fusarium verticilloid, Alternaria alternata,* Mucor racemosus *a*nd Aspergillus niger. Studying the effects of the *Trichoderma viride* on transcriptome of these pathogenic fungi once they antigonsted with the *Trichoderma viride*. Identify some of the induced genes and studying their evolution compared with the other published ones.

MATERIALS AND METHODS

Isolation and Identification of Microbes Used in this Study: *Fusarium oxysporum, Rhizoctonia solani*, *Fusarium verticilloid, Alternaria alternata,* Mucor racemosus and Aspergillus niger were isolated from root rotted and wilted tomato plants cultivated in Alexandria,

Beni-Swiff, EL Dakahlya and El- Fayoum governorates in Egypt. It was microscopically identified on the basis of cultural and microscopic characteristics. Pathogenicity of these isolates toward tomato plant was estimated according to Sneh [26]. The isolates were maintained on PDA medium and stored at 4°C. Trichoderma was previously isolated from the rhizosphere of healthy tomato plants (from Alexandria governorate, Egypt) on PDA and identified by PCR in our lab as *Trichoderma viride* (HQ438699).

Screening the Efficacy of the Isolated Bioagent *Trichoderma* Against the Isolated Fungal Pathogens: The antagonistic effect of the tested biocontrol agent against *Fusarium oxysporum, Rhizoctonia solani*, *Fusarium verticilloid, Alternaria alternata,* Mucor racemosus *a*nd Aspergillus niger was examined. Trichoderma isolate and the fungal pathogens *were* cultured on PDA medium for 7 days at 28-30°C. Then, a disc (0.5 cm in-diam.) of the Trichoderma (antagonistic fungal) colony was cut and placed opposite to the colony of the pathogen. Three replicates were prepared in each pathogen. Then the inoculated plates incubated at 25°C and then the inhibition growth was calculated.

Fungal-Fungal Pathogen Interactions: To study the interaction between the bioagent Trichoderma isolate and the above listed pathogenic fungi, the hyphal samples were taken from intermediate region around the inhibition zones. We assumed that these inhibition zones are indicators for what is called microbe-microbe interaction between the bioagent and the pathogenic fungi.

Fungal-fungal Pathogen Interactions by Differential Display Technique.

RNA Isolation and cDNA Synthesis: total RNA was extracted from fungal isolates using GStract™ RNA isolation kit ıı Guanidium Thiocynate according to the manufacture method. Reverse transcription reaction was performed using oligo dT (USA). The 25 µL cDNA syntheis reaction consist of; 2.5 µL (5x) buffer with $MgCl_2$, 2.5 µL (2.5 mM) dNTPs, 1 µL (10 pmol) primer, 2.5 µL RNA (2mg/mL) and 0.5 unit reverse transcriptase enzyme. PCR amplification was performed in a thermal cycler (Applied Biosystems (ABI), USA) programmed at 42 °C for 1 hr, 72°C for 10 min (enzyme inactivation) and the product was stored at 4 °C until be used.

Table 1: Primers of used in differential display-PCR.

Primers	Primer sequence 35`→	Annealing (°C)
NS2	GGC TGC TGG CAC CAG ACT TGC	35
NAR52	GTA AAA CGA CGG CAA	36
RAPD1	TGCCGAGCTG	33
RAPD3	AGCCACCGAA	32
RAPD6	AAAGCTGCGG	34

Second PCR: Five primers were used in the differential display analysis and the primers sequence is illustrated in Table 1. The reaction mixture for differential display PCR was carried out in total volume 25µl contains; 2.5 µl 10x buffer with MgCl, 2 µl 2.5 mM dNTPS, 1 µl of 10 pmol primer, 1.5µl cDNA and 0.2 µl (5 units/µl) Taq DNA polymerase. PCR amplification was performed in a thermal cycler (Eppendorf, Germany) and programmed for one cycle at 95 °C for 5 min, then 40 cycles as follows: 30 sec at 95°C for denaturation, 1 min at 30-32°C for annealing for 1min and 1min at 72 °C for elongation. Reaction was then incubated at 72 °C for 10 min for final extension and stored at 4°C.

PCR Purification and Sequencing: The PCR products were purified using PCR clean up column kit (Maxim biotech INC, USA) according to manufacturer's instructions. The purified PCR product was subjected to DNA sequencing using forward primer in the sequence reaction. Sequencing was performed using BigDye® Terminator v3.1 Cycle Sequencing kit (Applied Biosystems, Foster City, CA, USA) and model 3130xl Genetic Analyzer (Applied Biosystems, Foster City, CA, USA).

RESULTS AND DISCUSSION

Screening of the Efficacy of the Isolated Bioagent *Trichoderma Viride* Against Isolated Fungal Pathogens: It will known that Trichoderma is a secondary opportunistic attacker for most of plant pathogenic fungi and this due to Trichoderma considered as source of cell wall degrading enzymes and antibiotic producers [27]. For that reasons, *Trichoderma viride* Egyptian isolate were used in this study. The antagonistic effect showed high activity of the *T. viride* against all the examined pathogenic fungi but with different levels (Table 2 and Figure 1). The activity was demonstrated as clear zone. The highest activity was demonstrated with the *Alternaria alternata* (80%) but the lowest activity was observed with *Mucor racemosus* (40%). On the other hand, the same antagonistic effect for the Trichoderma was gradually decreased with the other tested organisms,

Table 2: Antagonistic effect of the tested *Trichoderma viride* on the linear growth of the pathogenic fungi in dual culture test on PDA medium after seven days of incubation at 25°C.

	Linear Growth inhibition (%) of the pathogenic fungi					
	Pathogenic fungi					
Bioagent						
Trichoderma viride	*R. solani (R2)*	*F. oxysporum*	*F. verticilloid*	*A.alternata*	*M. racemosus*	*A. niger*
	79.6	76.6	65.6	80.0	40.0	55.0

Linear Growth inhibition (%) of the pathogenic fungi calculated according to the following equation: R= [(A-B)/A] x100 Where:R=percentage of growth reduction, A=Mycelial growth of the pathogenic fungus, B=Mycelial growth of the pathogenic fungus towards the antagonistic fungus.

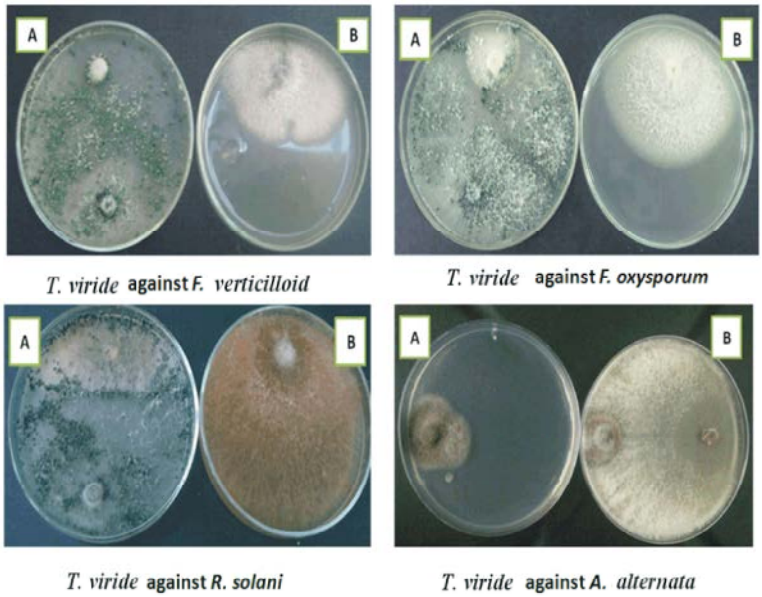

Fig. 1: Antagonistic effect of the tested *Trichoderma viride* on the linear growth of pathogenic fungi in dual culture test on PDA medium after 7 days of incubation at 25 °C. Where A: *Trichoderma viride* against pathogenic fungi B*:* The control (pathogenic fungus)

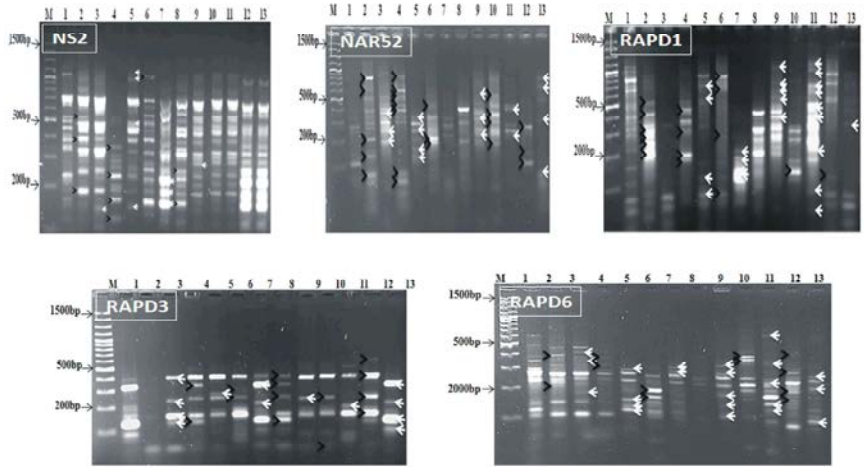

Fig. 2: Differential display (DD-PCR) using five different primers indicated on the right corner of each picture. M:1.5 Kbp DNA marker, 1:*Trichoderma viride* control, 2: *F. oxysporium c*ontrol, 3: *F. oxy.*+ *Trichoderma viride*, 4: *F. moniliforme* control, 5: F. moniliform+ Trichoderma, 6: *Rhizoctonia solani* control, 7: *R. solani*+ Trichoderma, 8: *Alternaria alternata* control, 9: *A. alternata*+ Trichoderma, 10: *Mucor racemosus* contro, 11: *Mucor racemosus*+ Trichoderma*, 12: Aspergillus niger, 13: asparagillus niger* +Trichoderma.

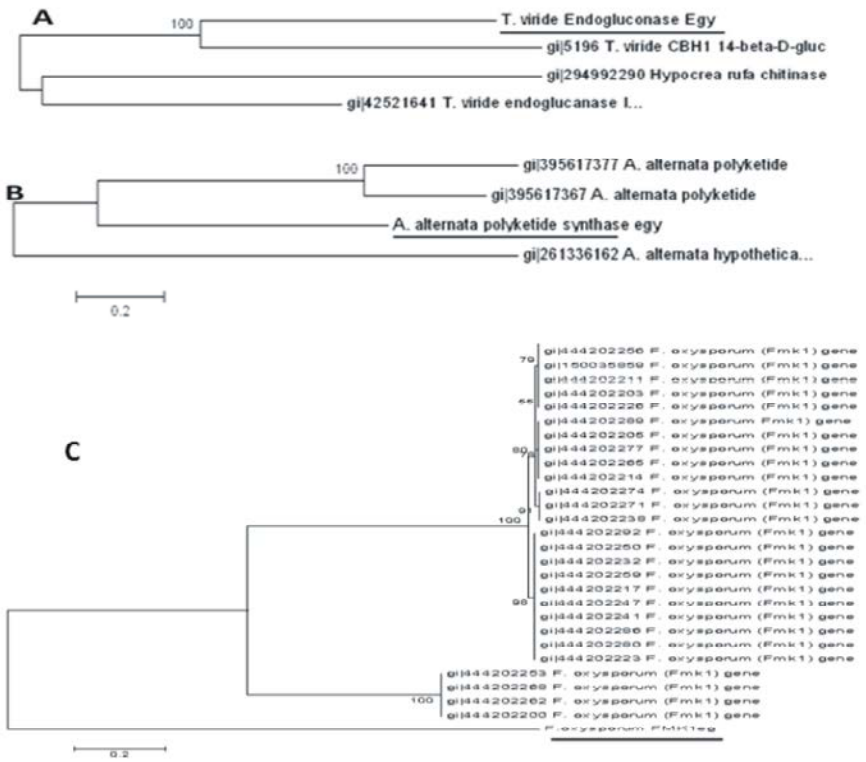

Fig. 3: Phylogentic tree for the isolated genes based on their DNA nucleotide sequence with the other genes listed on gene bank. The Neighbour-joining method Saitou [40] was used to construct the tree. The number on the branches represents bootstrap support for 1,000 replicates. A: endogluconase, B: Polyketide synthase gene and C: FMK1gene.

Rhizoctonia Solani, Fusarium oxysporum, *Fusarium verticilloid and Aspergillus niger* (79.6, 67.6, 65.6 and 55 %) respectively. Our results not agree with the results obtained by Kamal and Devi [28] they postulated that 80% of their Trichoderma isolates showed high activity against *Fusarium oxysporum* and 68% have strong activity against *Rhizoctonia solani* [28]. We assume that the biocontrol activity differed from isolate to another and that agree with the point of view of Sivan and Chet [29] that strains of *Trichoderma sp.* can vary in their biocontrol activity from pathogen to pathogen. Gajera and Vakharia [30] found that *T.viride* is the best biocontrol agent for the *A. niger* and the pathogen inhibition percentage ranged from 80 to 30% but it was 12.6 in case of *Mucor racemosus*.

Molecular Studies on Fungal-Fungal Pathogen Interactions by Differential Display PCR (DD-PCR): Differential display RT-PCR provides new approach to further inspect a microbe's response to experimental conditions which more closely similar to natural microbial associations and habitats [31]. Iakovlev [32] have used the differential display PCR to study the microbe-microbe interaction between the two fungal strains *Heterobasidion annosum* and *Physisporinus sanguinolentus*. They isolated 39 different induced genes and among them the *recA/RAD51* which is essential for homologous recombination, DNA repair and stress responses. Recently, it was suggested that the usefulness of messenger RAN (mRNA) differential display technique for the detection of differentially expressed genes in *D. nobile* whose growth could be promoted by mycorrhizal fungi [33]. We studied the effect of the Trichoderma on the transcriptome of other Results presented in figure 2 showed that the genome of all the antigonisted fungi was modified when compared with the control (non stressed). These modifications appeared as up-down regulated genes. About 73 down regulated genes and 84 up regulated genes were observed. The genetic profile of *F.oxysporum* was slightly modified 16 down regulated genes (ranged between 550 to 200 bp) and 11uprgulated gene (600-100bp). But in case of *F. verticilloid* 19 down regulated (300bp to 100) and 19 up regulated genes (1200 to 150 bp). In case of *Rhizoctonia solani* 10 up regulated genes (1100bp) and 11down regulated genes (250 to 100bp). In case of *Alternaria*

alternata 6 down regulated genes (250 to 100bp) and 15 up regulated gene (500-100bp). But identical profile was observed in case of Mucor racemosus 9 down regulated (500-100bp) and 17 up regulated genes (1000-100 bp). But in case of *Aspergillus niger* about 12 down regulated genes (600-100 bp) and 12 up regulated genes (700-100 bp).

Additionally, it was noticed that the majority of genes are the up regulated and most of them are high in molecular. The molecular basis of Trichoderma as biocontrol agent has not been completely known, but a synergistic mechanism between antibiosis, competition and mycoparasitism is hypothesized by Brožová [34]. Trichoderma species are also characterized by the production of antibiotic peptides [35]. Three different up regulated genes were randomly selected from *Trichoderma viride, Alternaria alternata* and *Fusarium oxysporum*. The sequence analysis of the selected genes revealed that the three selected genes are; polyketide synthases (PKS, 508 bp), endogluconase (860 bp) and FMK1 (618 bp) in respective manner. We suggest the mycoparastizm of the *Trichoderram viride* induced the defense genes (endogluconase and FMK1) both in Alternaria and Fusarium. It was reported that, mycoparasitism is an important mechanism of biological control of the Trichoderma and probably depends on the production of lytic enzymes including chitinases, β-1,3-glucanases and proteases [34]. It will know that polyketide synthase is produced by the fungus hyphae and used in the plant penetration, facilitating competition for substrates and communication between organisms [36]. Trichoderma genomes are rich in PKSs and Two PKS genes in *T. atroviride* are expressed during confrontation with *R. solani*, indicating a possible role in mycoparasitism [37]. In this study PKS was induced in the genome of *T. viride* which is agree with the previous suggestion by Mukherjee [37]. On the other hand Alternaria produced the endogluconase to resist the biocontrol effect of the Trichoderma and the same reaction was evolved by Fusarium when induce the FMK1 in its genome. These results agree with what postulated by Bhat [38] that most of the well identified fungi produce one or more cellulolytic enzymes including endoglucanase, exoglucanase and β-glucosidase. Moreover, Eshel [39] have proved that some of the species of Alternaria induce plant invasion by elaborating the cellulolytic enzymes and genes encoding endoglucanases. Di Pietro [24] have identified FMK1, encoding a mitogen-activated protein kinase (MAPK) of *F. oxysporum* and they proved that the mutated strains of *F. oxysporum* f. sp. lycopersici carrying an inactivated copy of fmk1 have lost pathogenicity on tomato plants but show normal vegetative growth. Finally, they concluded that FMK1 controls several key steps in the pathogenesis of *F. oxysporum* and suggested that FMK1 play an important role for the corresponding MAPK pathway in soil-borne and foliar plant pathogens. These finding confirm our suggestion that *F.oxysporum* induced such gene to stop or at least reduce the Trichoderma effects.

When the phylogenetic was constructed to study the relationship with isolated genes with their alleles presented in gene bank the endonuclease gene showed similarity with identity 100% with 14-Beta-D-gluconase gene of *T. viride* strain CHB1 (Fig. 3. A). But in case of polyketide synthases gene, ours showed less similarity with the other polyketide gene with identity 60% with the other polypeptide synthases genes isolated from *A. alternata* (Fig. 3B). On the other hand, the FMK1 gene was aligned with another 29 FMK1 genes presented on gene bank. The alignment analysis grouped the examined genes into two main clusters, cluster one included the other FMK1 genes presented on gene bank and the second cluster contains only the isolated FMK1 gene. Finally, all the examined FMK1 genes including ours have the same ancestor (Fig.3c).

CONCLUSION

It can be concluded that the bioagent (*Trichoderma viride*) suppressed a lot of genes in the treated pathogenic fungi and pathogenic fungi resist such effect by inducing some of their defense genes. These genes also play in important role in organism pathogenicity. The two selected induced genes extracted from the two pathogenic fungi (endguconase and FMK1) have the same mechanism; attacking the bioagnet cell wall. Future work should be focusing on; more screening for more genes and production of recombinant polyketide protein and find the suitable way to use it as biofungicide.

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