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Impacts of *Trichoderma harazanium* Extract on *snf1, frp1, clc1* Gene Expression in *Fusarium oxysporum* f.sp *lycopersici*

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Abstract

Fusarium oxysporum f. sp. *lycopersici* is the causal agent of tomato wilt disease. The ability to infect particular plant species depends on specific genes that distinguish virulent fungi from their closely related nonvirulent relatives. These genes encode host-determining "virulence factors," including small, secreted proteins and enzymes involved in the synthesis of toxins. This research aims to study the impact of *Trichoderma harazanium* extract on the expression of *frp1, snf1*, and *clc1* genes expression on five severely pathogenic isolates of *Fusarium oxysporum* f.sp. *lycopersci* for the first time in Iraq. The fungal isolates used in this study were isolated from infected tomato plants in several regions of Iraq. The present results demonstrated the highest inhibition rates of growth for all isolates to be 100% at a 10 mg/ml concentration of *T. harazanium* extract. In contrast, the minimal inhibitory of 1.25 mg/ml caused inhibition rates of 28.12%, 30.37%, 36.2%, 38.59%, and 43.78%, consecutively. The results of qRT-PCR investigation showed a substantial downregulation of the expression of *clc1* gene in all *F. oxysporum* isolates in contrast to the other genes; the *snf1* gene showed a significant downregulation of transcription by 0.4697, 0.2552, and 0.1684 folds in three *F. oxysporum* isolates (Fox3, Fox 17, and Fox 20, respectively). The *frp1* gene showed an insignificant decrease in gene expression in all isolates. These results provide new insights into the molecular pathogenicity of *F. oxysporum* and demonstrate the possibility of employing these bioagents to influence the virulence genes of fungal phytopathogens.

Keywords: *Fusarium oxysporum,* gene expression, *Trichoderma harazanium,* virulence genes, wilt disease

تأثير مستخلص *Trichoderma harazanium* على التعبير الجيني لـ (*Snf1,frp1,clc1*) للفطر *Fusarium oxysporum* f.sp *lycopersici*

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الخلاصة

ان الفطر *Fusarium oxysporum f. sp. lycopersici* هو العامل المسبب لمرض الذبول على نبات الطماطم. . تعتمد القدرة على إصابة أنواع نباتية معينة بالعدوى على جينات محددة تميز الفطريات الضارة عن غيرها غير الضارة ذو الصلة الوثيقة في بعض الأحيان. تقوم هذه الجينات بتشفير "عوامل الفوعة" التي تحدد المضيف، بما في ذلك البروتينات الصغيرة المفرزة والإنزيمات المشاركة في تخليق السموم

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يهدف البحث الى دراسة تأثير مستخلص *Trichoderma harazanium* في التعبير عن جينات *frp1,snf1,clc1* لخمس عزلات شديدة الامراضية لفطر *Fusarium oxysporum* f.sp. *lycopersci* لأول مرة في العراق . تم عزل العزلات الفطرية المستخدمة في هذه الدراسة من نباتات الطماطم المصابة في عدة مناطق من العراق. أظهرت النتائج الحالية أن أعلى معدل لتثبيط النمو لجميع العزلات كان 100% عند التركيز 10 ملجم/مل من مستخلص *T.harazanium* . في المقابل، أقل معدل لتثبيط النمو كان عند تركيز عند 1.25 ملجم/مل منه 28.12%، 30.37%، 36.2%، 38.59%، و43.78% على التوالي. أظهرت نتائج قياس qRT-PCR انخفاضًا كبيرًا في تنظيم التعبير لجين *clc1* في جميع عزلات *F.oxysporum* مقارنة بالجينات الأخرى؛ يُظهرالتعبير لجين *snf1* انخفاضًا ملحوظًا (0.4697 ، 0.2552 , 0.1684) في ثلاث عزلات (Fox3، Fox 17 وFox 20) على التوالي. أظهر جين *frp1* انخفاضًا غير معنوي في التعبير الجيني في جميع العزلات المدروسة . توفر هذه النتائج رؤى جديدة حول القدرة المرضية الجزيئية لـ *F. oxysporum* وتوضح إمكانية استخدام هذه العوامل البايولوجية للتأثير على جينات الفوعة لمسببات الأمراض النباتية الفطرية.

1. Introduction

The Solanaceae family has around 3500 species, with the cultivated tomato (*Lycopersicon esculentum* Mill.) being one of the vegetable yields that is grown widely worldwide [1]. One of the most common and dangerous tomato diseases is Fusarium wilt, which is brought on by *Fusarium oxysporum* f. sp. *lycopersici* [2]. It is regarded as one of the serious systemic diseases that affect tomato plants, being transmitted by soil [3]. *Fusarium oxysporum* f. sp. *lycopersici* penetrates the root's epidermis, then travels into the vascular tissue and settles inside the xylem vessels of the plant, blocking the vessels and causing extreme water stress, which causes symptoms similar to wilting [4]. Chemical fungicides can eradicate plant pathogenic fungi, but their overuse has several negative effects, such as degrading soil quality, disrupting the natural balance of flora and fauna, increasing resistance to infections, and contaminating the surrounding area [5].

Biological control is highly recommended and endorsed as one of the world's most sustainable methods. Agents of biological control may inhibit the Fusarium crown and root rot disease [6]. *Trichoderma harzianum* Rifai is a common organism found in soil, root, and foliar ecosystems that has major potential for biological control; it is effective against a variety of soil-borne pathogenic fungi, such as strains of *Fusarium* [7]. Through mechanisms such as resource competition, colonization of root surfaces, mycoparasitism, and other strategic approaches, *T. harzianum* directly contributes to biocontrol. As a result, *T. harzianum* has emerged as one of the most widely used beneficial fungi, being essential in improving crops and decreasing fungal diseases that are transmitted through the soil [8].

The last decade has seen several scientific studies that provide more clarity on the molecular mechanisms underlying the pathogenesis of *Fusarium oxysporum* f. sp. *lycopersici.* Phytopathogenic fungi found in soil need to have the right signaling mechanisms to respond through changes in gene expression, which in turn allows them to recognize their host, penetrate the root and other tissues, haveing their hyphae proliferate inside the tissues to overcome the host defense system and cause disease [9]. A vast array of biological research employs transcriptase information, which offers an essential understanding of biological functions and processes. Through the advent of ribonucleic acid sequencing (RNA-seq.), next-generation sequencing methods have developed quickly and currently constitute a vital test for the investigation of fungal transcriptase, especially in terms of gene expression levels [10].

Our comprehension of the molecular mechanisms underlying diseases has improved because of the disciplines of forward and reverse genetics. The application of these methods enables the division of pathogenic genes into many groups, which include infection structure production, cell wall lysis, mycotoxins production, and signal coding. Pathogenicity is a process associated with specific genes and protein products that retards or damages the defensive mechanisms of the host. In *Fusarium oxysporum* f. sp. *lycopersici,* the *snf1* kinase of the glucose signaling pathway controls the response to nutritional and environmental stresses [11].

In phytopathogenic fungi, *snf1* acts as a global activator of plant cell wall degrading enzymes, which are major virulence factors for plant colonization. The secretion of a wide spectrum of plant cell wall degrading enzymes (CWDEs) is the first mechanism involved in the penetration and colonization of plant tissues. Cellulose, hemicelluloses, and pectin are the main polysaccharide components of the plant cell wall. CWDE encoding genes are repressed by glucose but derepressed by the function of the sucrose non-fermenting protein kinase 1 gene (*snf1*) [12].

frp1 (F-box protein required for pathogenicity) is required for growth on a broad variety of alternative carbon sources and the expression of several plant cell wall degradation enzyme genes. Studies have explained that mutants in the *frp1* gene can lead to a lack of expression of these CWDE genes and decrease their pathogenicity, reflecting the importance of the production of CWDEs for this root-invading fungus. The mutants in the *frp1* gene can lead to reduced absorption of organic acids, amino acids, and polysaccharides [13].

In *F. oxysporum*, the *clc1* gene is required for full laccase enzyme activity (probably through the facilitation of metal cofactor insertion into maturing laccases); laccases may be required for full pathogenicity. However, any mutant of the *clc1*gene may cause a decrease in virulence, a decrease in laccase activity, increased sensitivity to oxidative stress, and increased sensitivity to phenolic compounds such as chlorogenic acid, caffeic acid, and vanillic acid [14].

The present study aimed to evaluate the impacts of *T. harazanium* extract on gene expression of virulence genes (*Snf1, frp1, clc1*) of *F.oxysporum* f.sp. *lycopersici* for the first time in Iraq. The study also aimed to investigate the possibility of employing the bioagents to influence the virulence genes of fungal phytopathogens.

2. Materials and methods

❖ *Isolating and identifying of the pathogenic fungus*

F. oxysporum f.sp. *lycopersici* was the fungus chosen in the present research as an agent responsible for Fusarium wilt disease. From afflicted tomato plants, the fungus was isolated during the period from August to December of the year 2022 and January of the year 2023 from fields and greenhouses in Al-Rashidiya, Al-Zaafaraniya, Al-Mahmoudiya, Al-Nahrawan, Al-Taji, Al-Swayrah, and Karbala in Iraq. The collected samples were kept in individual plastic bags. For each infected plant, five portions (two for the root and three for the stem) were sterilized on the surface with a 1% hypochlorite solution, cultivated within Petri dishes with sterilized Potato Dextrose Agar (PDA), and kept at 25 ± 2 °C for five days in incubation. After obtaining the pure culture on the PDA medium by using the single-spore method, it was kept at 4 °C until needed [15]. According to [3], morphology of the colony, conidiophores, and spore forms were used to identify *Fusarium* isolates. Five pathogenic

isolates of *F. oxysporum* were chosen depending on the pathogenicity test; the isolates with the highest severity were chosen.

❖ *Preparation of T.harazanium crude extract*

To prepare *T. harazanium* extract, a 10mm-diameter mycelial agar disc was taken from the growing edge of a five-day-old culture of *T. harazanium* on a PDA. This disc was then transferred to a 500ml flask that contained 300 ml of autoclaved potato dextrose broth. The flask was then incubated for 10 days at 26±2˚C in a shaker incubator at 150 rpm. The fungal culture was filtered using Whatman filter paper number 1 and then centrifuged for 15 minutes at 10,000 rpm. The extract was concentrated using a rotatory evaporator [16].

❖ *Gas chromatography-mass spectroscopic analysis*

In the Ministry of Industry and Minerals laboratories, GC-MS analysis for *T. harzianum* extract was performed using a Shimadzu gas chromatograph. The column oven temperature was set to start at 60˚C for 2 minutes, followed by an increase at a rate of a10 ˚C /min. run to 300˚C with a final hold at 300˚C for 5 min.. The linear velocity of carrier gas was 46.3 cm/sec. Samples were injected by using the splitless mode. The ionization for MS detection was achieved with an ion source temperature of 200˚C and an interface temperature of 280˚C. The starting time after injection was 3 minutes, and the end time was 31 minutes. The compounds were identified by comparing the electron impact spectra to those in the National Institute of Standards and Technology (NIST) library [17].

❖ *Antifungal activity T. harzianum crude extract.*

The test was designed to investigate the *in vitro* antifungal efficiency of *T. harazanium* extract against *F. oxysporum* f.sp. *lycopersici*. The test occurred in Petri dishes containing sterilized PDA. Known quantities of *T. harazanium* extract were dissolved in distilled water under aseptic conditions. After adding the *T. harzianum* extract to PDA, the ultimate concentrations of 1.25, 2.5, 5, and 10 mg/ml were achieved. To prepare the fungal inoculum, *F.oxysporum* mycelia were transferred on PDA plates after having been taken out of a single colony. A fungal disc with a diameter of 5 mm was taken from the edge of the active growth and aseptically inserted into the center of the plates. A check-up was performed on the inoculation plates once a day until the negative control treatment reached the edge of the plates. The antifungal efficacy was compared with that of the commercial Topsin fungicide (active ingredient Thiophanate Methyl 70%) on the growth
F. oxysporum mycelium under the same conditions. Three replicates were used for each concentration, as well as for the positive and negative control treatments. The fungal growth inhibition was calculated using the formulation below:
Percentage inhibition of radial growth (PIRG) (٪) = [(A1-A2)/A1] × 100٪

where A1 represents the radial growth of *F.oxysporum* in the control plate, and A2 represents the radial growth of *F. oxysporum* in the treatment plate [18].

❖ *RNA extraction and cDNA Synthesis*

The manufacturer's instructions for the TransZol Up Plus RNA Kit (TransGen, Biotech-China) were followed to extract total RNA. The nanodrop spectrophotometer was utilized to quantify both the quantity and the purity of the isolated RNA. The total RNA extract is converted to cDNA by using the EasyScript® One-Step gDNA Removal and cDNA Synthesis SuperMix reagent (TransGen Biotech-China) according to the manufacturer's instructions. cDNA was preserved until being used as an RT-PCR template. The primers were supplied by the Macrogen Company , Korea. The primer sequences for the *Tub* (housekeeping) and the virulence (*clc1, snf1, and frp1*) genes are displayed in Table 1.

Table1: Primers used for testing the gene expression of the virulence (*clc1, snf1, frp1)* and Housekeeping (*Tub*) genes**.**

Primer	Sequence (5′→3′ direction)	primer size bp	Amplicon size bp	Annealing Temp. (°C)	Reference
Snf1 gene					
Forward	GGTCGGTATCTTGCCTTCAA	20	115	57	[12]
Reverse	GGGAGGTTCGTCGTTGATAA	20			
frp1 gene					
Forward	CCTCCAAATCGTGGCATACT	20	143	57	[12]
Reverse	CCCGCATAGATGTTGGAAGT	20			
clc1 gene					
Forward	ACCATATCCGTGGTGGTCAT	20	101	57	[12]
Reverse	AATTCGCTGACAGCTTTGGT	20			
Tub (Housekeeping gene)					
Forward	CCCCGAGGACTTACGATGTC	20	68	57	[12]
Reverse	CGCTTGAAGAGCTCCTGGAT	20			

➢ *Quantitative Real-Time PCR (qRT-PCR)*

The experiment was designed by using five severe pathogenic isolates of *F. oxysporum* f.sp. *lycopersici* that had the *clc1.frp1.and snf1*genes . RT-PCR reaction mixtures were made using *2xTransStart*® Top Green qPCR Super Mix and run on the Qiagen Rotor Gene Real Time PCR System in order to determine the cycle threshold (Ct). The housekeeping gene utilized was the *Tub* gene. The following circumstances were followed for the gene expression reactions: Enzyme activation was performed at 94 oC for 30 sec. in one cycle, fluorescence was measured after 40 cycles of denaturation at 95 oC for 5 sec., annealing at 57 oC for 15 sec., and extension at 72 oC for 20 sec.

The minimum inhibitory concentration of *T. harazanium* extract was determined as the lowest concentration of an antifungal that will inhibit the visible growth of fungi after a defined period of incubation. The three genes' expression in the pathogenic isolates was assessed in the control group (without *T. harazanium* extract) and after they were treated with *T. harazanium* extract. The Livak method was utilized for analyzing the information after normalizing the mRNA expression levels to those of the housekeeping gene [19].

➢ *Statistical Analysis*:

To evaluate the effect of different elements on study parameters, the Statistical Analysis System (SAS) software was used [20]. In the current research, the analysis of variance (ANOVA) test, known as the least significant difference (LSD), and the T-test were employed to contrast the means.

3. Results& Discussion

➢ *Gas chromatography-mass spectroscopic analysis*

The content of all antifungal compounds in the *T. harzianum* extract was analyzed using GC-MS, which showed 15 peaks (Table 2, Figure 1). The main active component of *T. harzianum* extract was Decanoic acid (39.41 %), followed by 2H-Pyran-2-one (13.67%), Palmitic acid (7.53%),Spirohexan-4-one (6.14%),Hexadecanoic acid (6.12%),Hydrazinecarboxylic acid (5.57%),2-Pyrrolidine thione(3.65%),butyn-1-ol(3.27%),4-Trifluoroacetoxytetradecane(2.66%),Oleic acid (2.30 %), Thiophene, 2,3-dihydro(2.01%), 4H-Pyran-4-one (2%),Cis-aconitic anhydride (1.96 %) , Acetic acid

(1.94%),N-Trifluoroacetylimidazole (1.76 %). The antifungal activity of *T. harzianum* extract may be attributable to the presence of many bioactive ingredients. The antifungal potency of the extract may also be referred to as synergism between different bioactive components [21]. On the other hand, the bioactive alkyl pyrones produced by *T. harzianum* strain, such as 2H-pyran-2-one and4H-Pyran-4-one , were reported to possess antifungal properties[22]. Furthermore, Decanoic acid, Palmitic acid, Hexadecanoic acid, and Oleic acid compounds were reported to have antifungal activities against different fungal strains [23].

Table2: GC–MS analysis of *T. harzianum* extract

Peak No.	Compound Name	Retention Time	Area %
1.	Acetic acid	4.139	1.94
2.	butyn-1-ol	4.442	3.27
3.	4H-Pyran-4-one	5.635	2.00
4.	Hydrazinecarboxylic acid	6.480	5.57
5.	Palmitic acid	7.597	7.53
6.	Thiophene, 2,3-dihydro	8.877	2.01
7.	2H-Pyran-2-one	12.801	13.67
8.	Cis-aconitic anhydride	19.307	1.96
9.	2-Pyrrolidine thione	21.432	3.65
10.	Oleic acid	21.974	2.30
11.	4-Trifluoroacetoxytetradecane	22.451	2.66
12.	N-Trifluoroacetylimidazole	23.069	1.76
13.	Decanoic acid	23.600	39.41
14.	Spirohexan-4-one	24.250	6.14
15.	Hexadecanoic acid	25.996	6.12

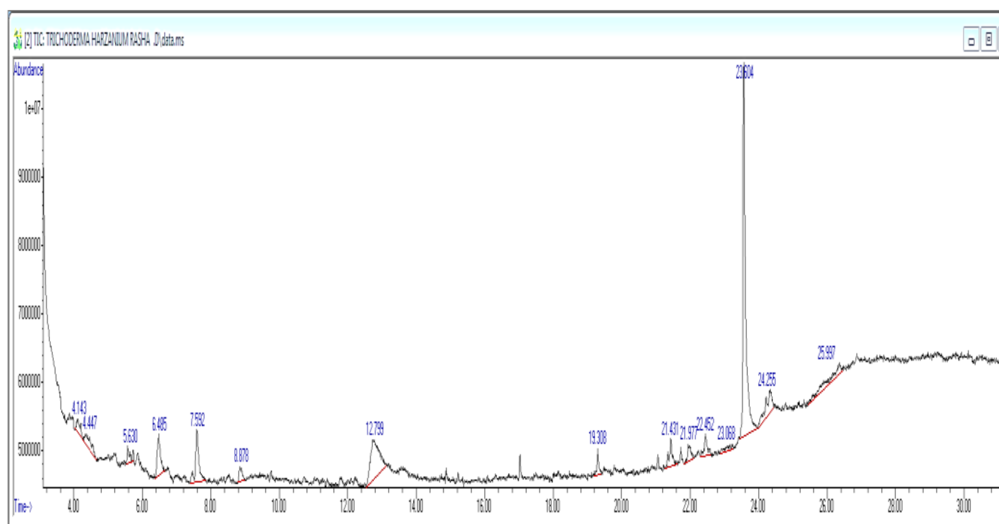

Figure 1:GC-MS analysis chromatogram of antifungal compounds identified from *T. harzianum* extract

➢ *Antifungal Activity of T. harzianum crude Extract*

The *in vitro* antifungal efficacy of *T. harzianum* extract demonstrated a significant inhibition against *F. oxysporum* f.sp. *lycopersci* growth in the PDA medium. Previous research showed that the growth of mycelium for certain phytopathogenic fungal strains was inhibited by secondary metabolites for *Trichoderma* spp. [24]. Each experiment was conducted in a PDA medium with varying *T. harzianum* extract concentrations, ranging from 1.25 to 10 mg/ml, with incubation duration of 7 days at 25 °C. Figure 2 and Table 3 illustrate

the impact of *T. harzianum* extract on the mycelium radial growth of five isolates of *F. oxysporum* f.sp.*lycopersci*. The results indicate that elevating *T. harzianum* extract concentration correlates with increased inhibition percentages for all isolates.

The highest inhibition rates for all isolates were observed at 100% for a *T. harzianum* extract concentration of 10 mg/ml, while the minimum inhibition rates for the five isolates were 28.12%, 30.37%, 36.2%, 38.59%, and 43.78% , respectively, at a *T. harzianum* extract concentration of 1.25 mg/ml. In comparison, the recommended dosage of Topsin fungicide (positive control) resulted in nearly 100% inhibition of fungal growth. Our findings are inconsistent with those of [7], who reported the antifungal efficiency of culture filtrates of *T. harzianum* strains at a concentration of 5% v/v against *F. oxysporum* strain, recording a mycelial inhibition rate of 24.71%. It is crucial to note that variations in experimental conditions and fungal types may contribute to discrepancies between the results of the present study and those reported in other studies.

F. oxysporum f.sp. *lycopersci* was effectively inhibited by *T. harzianum* extract, suggesting potential strategies employing antagonistic substances to prevent infections as well as reduce diseases [25]. As a result, *Trichoderma* spp. may employ a variety of mechanisms to suppress the pathogen, and the antifungal substances it secretes may have been a significant factor in suppressing the pathogen and reducing the frequency of Fusarium wilt on tomatoes [26].Therefore, in this study, the opportunity to use innovative and safe bio fungicides to mitigate the risks of chemical fungicides on health of human as well as the environment is highlighted by the crude extract's antifungal efficacy against Fusarium wilt.

Table3: Effect of *T. harazanium* extract concentration on the inhibition percentage of the selected isolates

***T. harazanium* extract conc. (mg/ml)**	**Mean ±SE of Inhibition (%)**				
	F.oxysporum 3	*F.oxysporum* 5	*F.oxysporum* 9	*F.oxysporum* 17	*F.oxysporum* 20
C: 1.25	43.78 ±2.65c	36.2 ±1.38d	30.37 ±1.41d	28.12 ±1.26d	38.59 ±2.05d
C: 2.5	63. 76 ±3.08b	50.0 ±2.77c	60.75 ±3.05c	56.25 ±2.74c	64.91 ±3.54c
C: 5	72.46 ±3.98b	77.58 ±3.73b	74.68 ±3.56b	71.87 ±3.91b	77.19 ±4.03b
C: 10	100 ±0.00a	100 ±0.00a	100 ±0.00a	100 ±0.00a	100 ±0.00a
Control (+ve)	100 ±0.00a	100 ±0.00a	100 ±0.00a	100 ±0.00a	100 ±0.00a
LSD value	9.41 **	9.07 **	8.67 **	7.42 **	8.07 **

Means posses with the various letters in same column vary significantly.
** (P≤0.01)

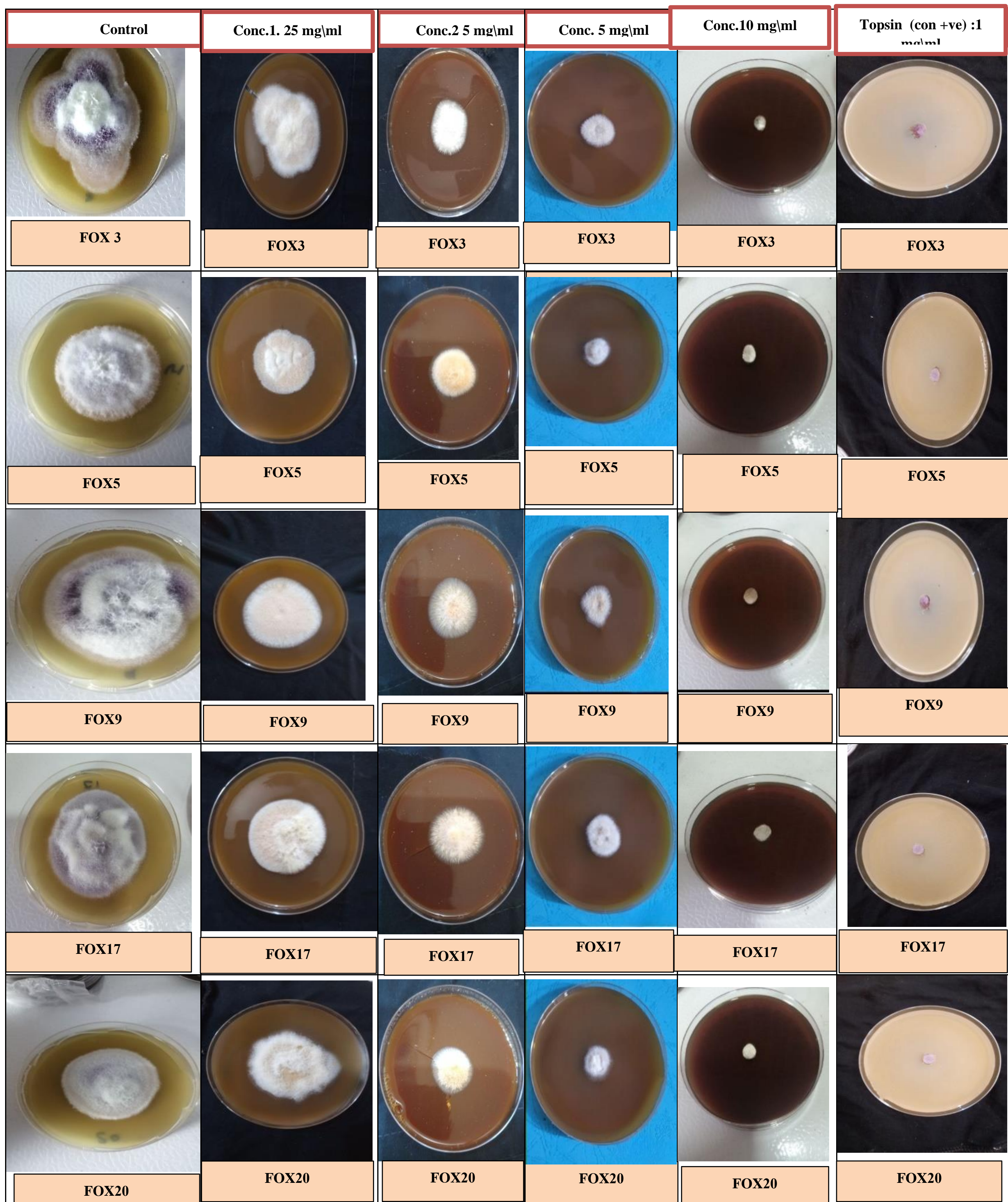

Figure2: Antifungal activity of *T.harazanium* crude extract against *F.oxysporum* f.sp.*lycopersc*

➢ *Expression of virulence genes of F.oxysporum f. sp .lycoperscici isolates*

The RNA, whose concentration ranged from 69 to 146 ng/µl, was then converted to complementary DNA by reverse transcription. All of the steps were linked to a perfect yield, indicating effective reverse transcription (17, 18). In the present research, the study of *clc1* gene expression exhibits a downregulation of transcription in the five isolates of *F. oxysporum* by about 0.3056 ±0.08, 0.1425 ±0.02, 0.2773 ±0.04, 0.2268 ±0.04, and 0.2030 ±0.01 folds change, respectively, compared with the untreated control with significant differences p-value ≤0.05 as shown in Table 4. The range of the Ct value for the *clc1* gene in

the FOX3 isolate was 19.71 to 21.08; however, the value in the FOX5 isolate was 17.43 to 19.63, while in the isolate (FOX9) it was 17.5 to 20.02. The range value of Ct for the *clc1* gene was 19.79 to 21.34 in the FOX17 isolate; however, it was 21.63 to 23.53 in the FOX20 isolate (Table4).

The results of the qRT-PCR investigation showed a significant reduction in the expression of the *clc1* gene in all *F. oxysporum* isolates in contrast to the other genes. The chloride channel (*clc1*) gene is primarily known as being required for full laccase enzymes activity (probably through the facilitation of metal cofactor insertion into maturing laccases). The *clc1* gene contributes to virulence through its roles in pigmentation, the production of aspersoriums, and defense against poisonous phytoalexins and laccases, which collectively are required for full pathogenicity. Several studies have provided circumstantial evidence linking the *clc1* gene to plant pathogenicity; the decrease in the expression of the *clc1* gene may lead to a decrease in virulence [14].

The result of the study shows a significant downregulation of the transcription of the *snf1* gene by about 0.4697±0.11, 0.2552±0.07, and 0.1684±0.03 folds in three isolates (Fox3, Fox 17, and Fox 20, respectively) compared with untreated control, with significant differences at p-value ≤0.05, as shown in the Table 5 .The *snf1* gene acts as a global activator of plant cell wall degrading enzymes, which are major virulence factors for plant colonization. In plant-pathogenic fungi, the secretion of a wide spectrum of plant cell wall degrading enzymes (CWDEs) is the first and most studied mechanism involved in the penetration and colonization of plant tissue. Cellulose, hemicelluloses, and pectin are the main polysaccharide components of the plant cell wall .CWDE genes are derepressed by the function of the sucrose non-fermenting protein kinase 1 gene (*snf1*). The decrease their expression may reduce the virulence of the pathogen.

The *frp1* gene shows a decrease in gene expression in all isolates, but this difference was not significant (Table 6). The extract of *T. harazanium* increased the CT value, and this is a good indication of the low expression of the *snf1, frp1*, and *clc1* genes (folding).These findings demonstrated that the samples treated with *T. harazanium* extract reduced their virulence. The reason for the decrease in gene expression is the numerous bioactive components in the *T. harzianum* extract, which, as determined by GC-Mas, may be responsible for its antifungal activity. The prior extract's antifungal activity could be attributed to the synergy between various bioactive components. In addition to that, *T. harazanium* is known to generate extracellular pectinase and cellulase, which can hydrolyze pathogenic fungus' cell walls and possess glucose-methanol-choline oxidoreductases, which are important for both the biological management of plant diseases and the antibiosis of plant pathogenic fungi [27].

The precise, sensitive, and expeditious results of reverse transcription quantitative PCR (RT-qPCR) set it apart from other methods for analyzing gene expression. This method has already proven itself to be a highly effective standard in the field of analysis of gene expression. It is important to understand that, in studies involving comparative quantification, the primary goal of each experiment is to compare the degree of gene expression in different samples [28-30].

Table 4: Fold of *clc1* gene expression in five fungal isolates based on the 2- $^{\Delta\Delta Ct}$ Method

Groups	Sample	*Means Ct of *clc1*	Means Ct of *Tub*	Δct	ΔΔct	Folding	LSD (P-value)
***F.oxysporum* f.sp.*lycopersci* 3**	Without treatment	19.71	13.8	5.91	0	1 ±0.00	0.490 * (0.0375)
	*THE	21.08	13.46	7.62	1.71	0.3056 ±0.08	
***F.oxysporum* f.sp.*lycopersci*5**	Without treatment	17.43	13.76	3.67	0	1 ±0.00	0.561 * (0.0385)
	THE	19.63	13.15	6.48	2.81	0.1425 ±0.02	
***F.oxysporum* f.sp.*lycopersci* 9**	Without treatment	17.5	13.3	4.2	0	1 ±0.00	0.583 * (0.0351)
	THE	20.02	13.97	6.05	1.85	0.2773 ±0.04	
***F.oxysporum* f.sp.*lycopersci* 17**	Without treatment	19.79	13.91	5.88	0	1 ±0.00	0.529 * (0.0393)
	THE	21.34	13.32	8.02	2.14	0.2268 ±0.04	
***F.oxysporum* f.sp.*lycopersci* 20**	Without treatment	21.36	13.39	7.97	0	1 ±0.00	0.576 * (0.0367)
	THE	23.53	13.26	10.27	2.3	0.2030 ±0.01	
*** (P≤0.05).**							

*THE mean *T.harazanium* extract
*Mean of Ct value for each gene represent three replicate

Table 5. Fold of *snf1* gene expression in five fungal isolates based on the 2- $^{\Delta\Delta Ct}$Method

Groups	Sample	Means Ct of *snf1*	Means Ct of *Tub*	Δct	ΔΔct	Folding	LSD (P-value)
***F.oxysporum* f.sp.*lycopersci*3**	Without treatment	18.23	13.8	4.43	0	1 ±0.00	0.407 * (0.0467)
	THE	18.98	13.46	5.52	1.09	0.4697 ±0.11	
***F.oxysporum* f.sp.*lycopersci* 5**	Without treatment	17.75	13.76	3.99	0	1 ±0.00	0.398 NS (0.302)
	THE	17.55	13.15	4.4	0.41	0.7526 ±0.19	
***F.oxysporum* f.sp.*lycopersci* 9**	Without treatment	16.56	13.3	3.26	0	1 ±0.00	0.267 NS (0.906)
	THE	17.26	13.97	3.29	0.03	0.9794 ±0.26	
***F.oxysporum* f.sp.*lycopersci* 17**	Without treatment	18.84	13.91	4.93	0	1 ±0.00	0.502 * (0.0356)
	THE	20.22	13.32	6.9	1.97	0.2552 ±0.07	
***F.oxysporum* f.sp.*lycopersci*20**	Without treatment	18.81	13.39	5.42	0	1 ±0.00	0.503 * (0.0271)
	THE	21.25	13.26	7.99	2.57	0.1684 ±0.03	
*** (P≤0.05).**							

Table 6. Fold of *frp1* gene expression in the five fungal isolates based on the $2^{-\Delta\Delta Ct}$ Method

Groups	Sample	Means Ct of *frp 1*	Means Ct of *Tub*	Δct	ΔΔct	Folding	LSD (P-value)
***F.oxysporum* f.sp.*lycopersci* 3**	Without treatment	17.11	13.8	3.31	0	1 ±0.00	0.381 NS (0.308)
	THE	17.1	13.46	3.64	0.33	0.7955 ±0.28	
***F.oxysporum* f.sp.*lycopersci* 5**	Without treatment	15.96	13.76	2.2	0	1 ±0.00	0.573 NS (0.078)
	THE	16.51	13.15	3.36	1.16	0.4475 ±0.10	
***F.oxysporum* f.sp.*lycopersci* 9**	Without treatment	15.7	13.3	2.4	0	1 ±0.00	0.397 NS (0.418)
	THE	16.79	13.97	2.82	0.42	0.7474 ±0.19	
***F.oxysporum* f.sp.*lycopersci*17**	Without treatment	16.8	13.91	2.89	0	1 ±0.00	0.502 NS (0.266)
	THE	16.95	13.32	3.63	0.74	0.5987 ±0.16	
***F.oxysporum* f.sp.*lycopersci* 20**	Without treatment	16.48	13.39	3.09	0	1 ±0.00	0.429 NS (0.205)
	THE	17.09	13.26	3.83	0.74	0.5987 ±0.16	
NS: Non-Significant.							

4. Conclusions

The findings demonstrate that the *snf1, frp1*, and *clc1* genes are recognized as being linked to the *F. oxysporum* virulence that infects tomato plants. The virulence of the tomato Fusarium wilt pathogen might be significantly influenced by decreased *clc1, snf1*, and *frp1* expression; *clc1* gene affects the function of laccase enzymes and chloride transport, while the *snf1* and *frp1* genes are necessary for the Fusarium wilt pathogen to penetrate the xylem tissue of the host. *T. harazanium* is crucial for the biological management of plant diseases and the antibiosis of plant pathogenic fungi, in addition to the development of new, safe bio fungicides that can be used to prevent the passive effects of chemical fungicides on human health and the environment. These results provide new insights into the molecular pathogenicity of *F. oxysporum* and demonstrate the possibility of employing these bioagents to influence the virulence genes of fungal phytopathogens.

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