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APPLYING AN ORTHOGONAL ARRAY TAGUCHI DESIGN FOR CHITINASE OPTIMIZATION BY *Trichoderma longibrachiatum* MW433820 AND ITS EFFICACY IN GREEN BIOCONTROL FOR SUSTAINABLE AGRICULTURE

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ABSTRACT

Increasing efforts are aimed at utilizing rhizospheric saprophytic fungi as biological control agents against phytopathogens to improve crop production. In this study, an unconventional fungal isolate "genetically identified as *Trichoderma longibrachiatum"* and given the GenBank accession no. MW433820, has shown the potential to produce chitinase enzyme, using liquid and solid wastes from the olive oil-processing industry. Olive mill solid waste and olive mill waste water demonstrated high nutritional value and strong physical support for fungal growth and enzyme production. Value of 417.60 U/ml was the highest chitinase activity achieved after 7 days of submerged cultivation in both solid and liquid olive wastes without any additives. The orthogonal array Taguchi design used for further optimization of the production process. The highest significant optimization of chitinase production (3184.69 U/ml), with ~7.63-fold increase was obtained by supplementation of basal medium with the organic nitrogen source (soy bean), NaCl and K_2HPO_4. The high antagonistic activity of *Trichoderma longibrachiatum* was observed against the soil-borne pathogens *Fusarium oxysporum* and *Fusarium solani*, depending on fungal parasitism, by coiling around the pathogens and producing the cell wall-degrading enzyme chitinase. From the obtained results, we can deduce, the potential application of *Trichoderma longibrachiatum* as a biocontrol agent with no harm to the host plant.

Keywords: Taguchi design,*Trichoderma longibrachiatum*, Chitinase, Soil-Borne Pathogens, Bio-control

INTRODUCTION

Plant diseases in the field are primarily caused by pathogens such as bacteria, fungi, viruses, and nematodes. (**Kumar *et al.*, 2022**). Pathogenic fungi are a major concern in agriculture, necessitating comprehensive strategies to mitigate their impact on crop production and food security. Fungi can cause extensive damage to crops, such as, leaf mold, basal stem rot, *Fusarium* wilt, rusts, etc. (**Chawade *et al.*, 2018**). The most common pathogens of crop plants worldwide were the fungal species of *Fusarium spp*. (**Burgess *et al.*, 2009**). The chemical fungicides have succeeded to some extent to overcome these phytopathogens, therefore, effective management of these fungal plant diseases is still necessary. Applying the enzymes chitinases [EC 3.2.1.29], as a bio-fungicide agent serve as effective biofungicides by targeting the structural integrity of fungal pathogens without harming the host plant (**Maharana *et al.*, 2022**). Micro-organisms are the primary producers of chitinases owing to their ubiquitous presence in nature, and the cost-effectiveness of cultivating them with readily available raw materials (**Kuranda and Robbins 1991; Abd El Aty, 2023**). Fungi such as *Trichoderma harzianum* (**Sandhya *et al.*, 2004**), *Trichoderma koningiopsis* (**Baldoni *et al.*, 2020**), *Trichoderma harzianum* (**Urbina-Salazar *et al.*, 2019**) and *Trichoderma longibrachiatum* KT693225 (**Abdel Wahab *et al.*, 2018**) have shown the potential to produce chitinases, which have been studied as anti-fungal agents for the biological control of numerous plant diseases(**Sharma *et al.*, 2018**).
A variety of agricultural and shellfish wastes were applied as natural substrates for the growth of different fungal strains for chitinase production (**Ashour *et al.*, 2016; Shehata *et al.*, 2018; Abd El Aty, 2024**). The industry of olive oil production discharge large quantities of solid and liquid wastes, include the crude olive oil cake (OOC), and olive mill wastewater (OMW). **Di Giacomo and Romano (2022)** estimated that the total waste biomass produced from olive oil extraction was at least 40 Mt/year, with over 20 Mt/year being dry biomass. And the annual production of (OMW) was estimated to be between 10 and 30 million m³/year **(Annab *et al.*, 2019).** Waste substances have high organic load which can be used as substrate materials for the obtaining metabolites of high value, such as microbial lipids, organic acids, bio-surfactants, single cell proteins, and enzymes (**Mafakher *et al.*, 2010, Enaime *et al.*, 2024).**
Statistical experimental designs such as, "Taguchi" can be applied as a simple systematizes experimentation, for obtaining maximum enzymes production. The analysis of variance ANOVA are applied for all obtained results in the"Taguchi" experiment (**Abd El Aty *et al.*, 2014**).

This study focused on evaluating the newly isolated rhizospheric strain of *Trichoderma longibrachiatum* for the production of a constitutive exochitinase enzyme using low-cost olive oil processing wastes as a culture medium. Optimal ratios were determined based on a statistical experimental design (Taguchi). Additionally, the study assessed its potential application in controlling the phytopathogenic fungi *Fusarium solani* and *Fusarium oxysporum*, which enhancing plant growth, defense, and yield crucial for sustainable agriculture

MATERIALS AND METHODS

Microorganism isolation and purification

For fungal isolation, The roots of the medicinal plant basil (*Ocimum basilicum*) were applied **(Zohair *et al.*, 2018).** The roots were prepared by fragmentation into about (~1-1.5 cm) length , the root samples from different places were spread over the surface of Potato Dextrose Agar medium (PDA). Colonies of fungal culture obtained after incubation for 72 h at 28 °C. A regular inoculation on PDA slants has been done for obtaining stock culture, and kept at 4 °C.

Morphological and molecular identification

Fungal characterizations were preliminary done according **to Hanlin (2000) and Ahmed *et al.*, (2016).** Depending on the cultural and morphological characteristics**.** 18S rDNA were applied to confirm the morphological identification and to study the fungal phylogeny. The fungal Inter Transcribed Spacer (ITS) fragments were amplified by PCR using the universal primers ITS1 and ITS4. The sequences of the ITS1 and ITS4 primers were 5′-TCCGTAG GTGAACCTGCGG-3′ and 5′-TCCTCCGCTTATTGATATGC-3′ **(White *et al.*, 1990),** respectively. Sequence data was analyzed in the GenBank database by using the BLAST program and assigned specific accession number.

Evaluation of *T. longibrachiatum* MW433820 chitinase enzyme production

Three different media were prepared from solid and liquid olive oil industry wastes, the wastes used to evaluate *T. longibrachiatum* chitinase production include, Olive mill waste (OMW) medium, containing 2g solid waste (pomace), 50ml distilled water in 250ml Erlenmeyer flask. Olive mill wastewater (OMWW) medium, containing 50 ml of 10% liquid waste concentration. Mixed medium, containing 2g solid waste and 50ml of 10% liquid waste. Flasks were covered with hydrophobic cotton and autoclaved at 121°C for 20 min. After cooling, it was inoculated with 1.0 ml of (5×10^8 spores/mL) of 6 days old culture. All flasks were incubated for 7 days at 28-30 °C under static conditions.

Assay of chitinase enzyme

Chitinase assay was performed using colorimetric method for the estimation of N-acetyl amino sugar using the modified dinitros-alicyclic acid (DNS) method (**Miller, 1959**). The unit (U) of chitinase activityis defined as the amount of enzyme required to produce 1.0 mol of N- Acetyl glucosamine per minute.

Statistical optimization of *T. longibrachiatum* MW433820 chitinase enzyme

Taguchi method of five-factors, four-level design L16 $4^{\wedge 5}$ was applied. Five variables of (olive mill waste (OMW) solid pomace, olive mill wastewater (OMWW), Soy bean, NaCl and K_2HPO_4) were used during the study as shown in **Table 1**. Chitinase enzyme activities were recorded in all sixteen different experiments obtained in the design. In addition, The optimization conditions were confirmed by a verification trial. ANOVA of the results was performed using Design-Expert 8 software from Stat-Ease, Inc. **(Abd El Aty *et al.*, 2014; Abd El Aty and Zohair, 2020).**

Table 1 Factors and their levels which were studied by Taguchi -Method.

Factors	L- 1	L- 2	L- 3	L- 4
A-OMW (g/l)	10	20	30	40
B- OMWW (%)	5	10	15	20
C-Soy bean (g/l)	2	4	6	8
D-NaCl (g/l)	1	2	3	4
E-K_2HPO_4 (g/l)	1	1.5	2	2.5

Evaluation of *T. longibrachiatum* MW433820 bio-control potential

T. longibrachiatum was tested for their bio-control effect against the phytopathogens, *F. solani* NRC15 and *F. oxysporum* NRC 25 obtained from the culture collection of the Chemistry of Natural and Microbial Products Department, NRC, Egypt. 6mm diameter agar discs of *T. longibrachiatum* isolate and the pathogen were placed along a diametrical axis 3 cm away, on the same Petri-dish filled with 15 ml PDA medium. Incubation is done in dark at 28 °C for 7 days **(Zohair *et al.*, 2018; Abd El Aty, 2025).**

Microscopic examination

The mycoparasitism of *T. longibrachiatum* was studied by harvesting the hyphae from the contact zone between *T. longibrachiatum* and the phytopathogens. This zone was where the parasitic interaction, between *T. longibrachiatum* and the pathogenic fungi occurs .The examination was done under light microscope Olympus CX40/ RF100 150 with a Canon A620 digital camera.

RESULTS AND DISCUSSION

Identification of rhizospheric fungus

The soil inhabiting filamentous fungus was first identified on bases of morphological features, like colony colour, appearance and sporulation pattern. The fungal examination indicated that, conidia are mostly green, sometimes hyaline, with smooth or rough walls and are formed in slimy conidial heads (gloiospora) clustered at the tips of flask-shaped phialides. Conidiophores are branched, bearing clusters of divergent, often irregularly bent phialides. The second step for the fungal identification depends on, the most reliable molecular typing technique of partial sequencing with analysis of the 18S rDNA. The sequence analysis indicated that the rhizosphere isolated fungus revealed high identity (99%) to *Trichoderma longibrachiatum* isolate Tlongi12 (Gene Bank accession number KU317858) and revealed a close similarity (99%) with species *Trichoderma longibrachiatum* isolate Tlong1 (Gene Bank accession number KT426898) and *Trichoderma longibrachiatum* strain LC (Gene Bank accession number MW193401) according to the available NCBI Gen Bank database. The phylogenetic tree was displayed using the TREEVIEW Program that showed the sequences of close relatives obtained from Gen Bank to resolve the phylogenetic relations with ancestor **(Figure 1)**. Other studies showed the isolation of *Trichoderma longibrachiatum*KT693225 with chitinase activity from decayed wood of Port-Said Governorate, Egypt **(Abdel Wahab *et al.*, 2018),** and **Anwar et al., (2023)** indicated the ability of *Trichoderma longibrachiatum* chitinase to control *Aphis gossypii*.

Last results obtained from molecular identification using partial sequencing of 18S rDNA gene confirmed our morphological analysis of *Trichoderma longibrachiatum* strain, and the nucleotide sequence of the fungus was deposited in the Gen Bank under the accession number MW433820.

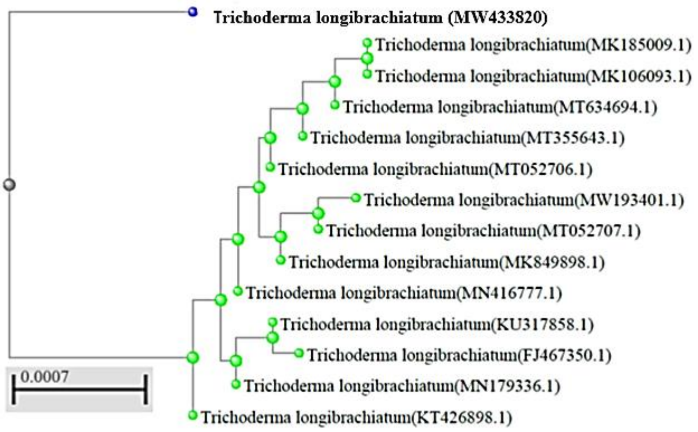


Figure 1 The phylogenetic tree based on partial sequencing of 18S rDNA showing relationship neighbor-joining between the fungal isolate *Trichoderma longibrachiatum* MW433820 and other closely related sequences on NCBI Gen Bank reference taxa.

Chitinase production by utilizing olive oil processing wastes

The OMW and OMWW samples were obtained from the Oil Unit at the National Research Centre, Egypt, and were used as fermentation media for the fungal cultivation. In this study, the fungus *T. longibrachiatum* was tested for its ability to grow and produce chitinase enzyme in such medium without any nutrient supplement or inducers. The three different cultivation media of OMW, OMWW and mixed one were inoculated with the strain for a preliminary study to estimate the suitability of solid and liquid olive oil wastes as a substrate for growth and chitinase production.

Results obtained in (**Figure 2**) showed the ability of the fungus to grow on three different fermentation conditions with different degree after 7 days incubation. *T. longibrachiatum* able to grow in un-supplemented OMW medium and use it as the sole carbon source, yielding about 295.33 U/ml chitinase activity, and showed 323.55 U/ml using olive mill wastewater (OMWW-10%) medium. Where, the maximum chitinase activity of 417.60 U/ml obtained when both solid and liquid olive oil processing wastes were mixed.

Kovacs *et al.*, (2004) Reported the best extracellular chitinase production of *T. longibrachiatum* IMI 92027 (ATCC 36838) using wheat bran–chitin mixture wetted with salt solution to a moisture contentof 66.7%.

Results indicated the good ability of *T. longibrachiatum* strain to overcome the high concentration of phenolic compounds in OMW and produce a constitutive chitinase enzyme without induction. Where other researchers have found that phenolic compounds can inhibit *R. necatrix* **(Lee, 2000),** *Alternaria spp.* **(Brenes et al., 2011)**; *F. oxysporum* **(Patel and Saraf, 2017).** On the other hand, suitability of the OMW and OMWW media for growth of various *Yarrowia lipolytica* strains for lipase enzyme production was demonstrated by several researchers **(Bankar *et al.*, 2009; Fickers *et al.*, 2011).**

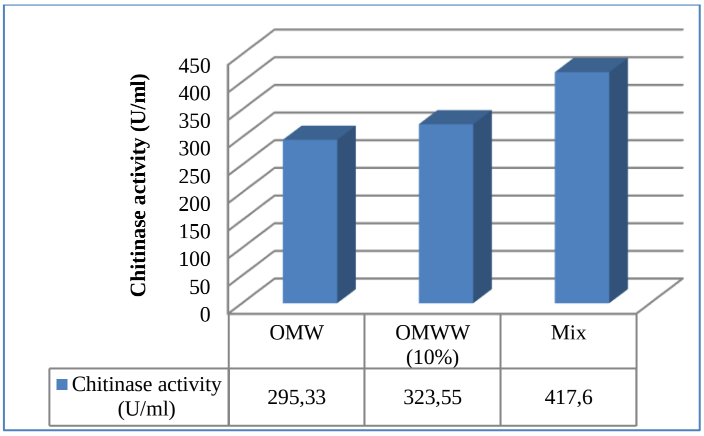


Figure 2 Fungal cultivation on olive oil processing wastes for chitinase enzyme production.

Five factors–four level (L16 4^5) design

To increase chitinase enzyme production by the fungus *T. longibrachiatum*, the composition medium of (OMW and OMWW) was supplemented by the addition of Soy bean (2-8 g/l), NaCl (1-4 g/l) and K_2HPO_4 (1-2.5 g/l) in 16 trails of an orthogonal array design. **Table 1.**

The statistical design and chitinase activity of 16-experiments together with the predicted activity from the regression equation for the combinations are shown in

Table 2, (Figures 3&4). The results showed that the maximum average yield of chitinase activity (3184.69 U/ml) was obtained in trail 12, at the following nutrient concentrations: OMW (40 g/l), OMWW (20 %), Soy bean (2 g/l), NaCl (3 g/l) and K2HPO4 (1.5 g/l). The supplementation of basal medium with the organic nitrogen source (soy bean) and minerals (NaCl, K_2HPO_4) improved the enzyme production to ~7.63-fold. These predicted parameters were tested in the laboratory and the final chitinase activity obtained was 3184.69 U/ml, which is similar to the predicted value 2882.51U/ml.

Final equation in terms of coded factors:

$$\text{Chitinase activity} = +968.40 - 591.57 * A[1] - 424.94 * A[2] + 363.46 * A[3] - 346.61 * B[1] - 383.67 * B[2] + 58.74 * B[3] + 589.52 * C[1] + 207.04 * C[2] - 450.16 * C[3].$$

Narayana and Vijayalakshmthere (2019) and Vaidya *et al.*, (2001) reported significant increase in chitinase yield in CYS medium amended with organic nitrogen source of soybean meal (0.6%). Other studies indicated that, OMW can be used as substrate for enzyme production from different fungal strains in the fermentation media inoculated with *Cryptococcus albidus* var. *albidus* IMAT-4735. Also *Gonatorrhodiella parasitica* showed the ability to produce protease, pectinase and chitinase enzymes using the cultivation media containing OMW as a sole carbon source under different pH values **(Atallaa *et al.*, 2020).**

Table 2 Five factors–four level (L16 4^5) design.

Trial number	Factor 1 A: OMW (g/l)	Factor 2 B: OMWW (%)	Factor 3 C: Soy bean (g/l)	Factor 4 D: NaCl (g/l)	Factor 5 E: K_2HPO_4 (g/l)	Chitinase activity U/ml	
						Experimental	Predicted
1	3	4	2	1	3	2240	2210
2	1	3	3	3	3	267.12	144.58
3	2	3	4	1	2	306.62	255.81
4	3	1	3	4	2	592.55	535.09
5	3	2	4	3	1	607.59	601.79
6	2	2	1	4	3	786.30	749.31
7	1	2	2	2	2	288.55	200.21
8	1	1	1	1	1	374.17	619.74
9	4	3	2	4	1	1648.31	1887.23
10	2	1	2	3	4	524.13	403.89
11	3	3	1	2	4	1886.53	1980.12
12	4	4	1	3	2	3184.69	2882.51
13	4	1	4	2	3	996.31	928.44
14	4	2	3	1	4	656.50	787.62
15	2	4	3	2	1	556.80	764.84
16	1	4	4	4	4	577.50	701.98

*The numbers under factors refer to their levels according to **Table 1**.

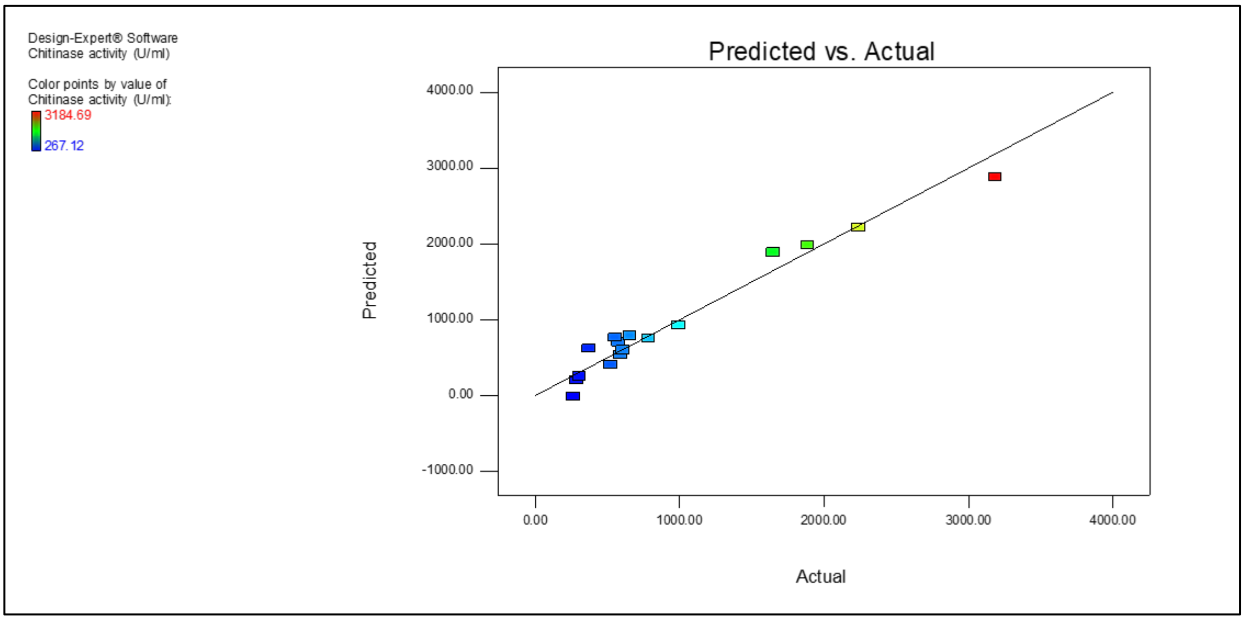


Figure 3 Predicted and actual values of chitinase activity (U/ml).

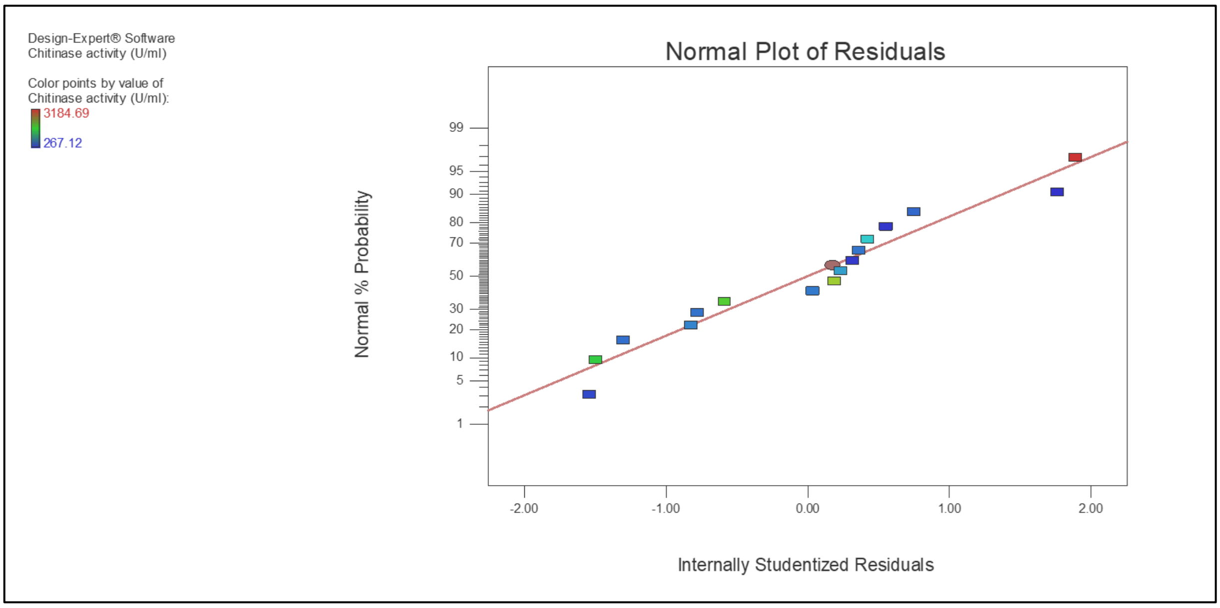


Figure 4 Normal plot of residuals.

ANOVA results in **Table 3** indicated the main effects of all tested factors. The Model F-value of 16.50 implies the model is significant. Values of "Prob > F" less than 0.0500 indicate model terms are significant, In this case A: OMW, B: OMWW, C:Soy bean, are significant model terms (**Figure 5)**. On the other hand, values greater than 0.1000 indicate the model terms are not significant as the factor D: NaCl, E: K_2HPO_4. Results also emphasized that, The "Pred R-Squared" of 0.7239 is in reasonable agreement with the "Adj R-Squared" of 0.9029. Adeq Precision" measures the signal to noise ratio. The obtained ratio of 14.055 indicates an adequate signal, where a ratio greater than 4 is desirable.

Table 3 ANOVA for selected factorial model.

Source	Sum of squares	Df	Mean square	F Value	p-value Prob>F
Model	1.010E+007	9	1.122E+006	16.50	0.0014 significant
A- OMW	4.356E+006	3	1.452E+006	21.36	0.0013
B- OMWW	2.887E+006	3	9.623E+005	14.16	0.0040
C- Soy bean	2.852E+006	3	9.507E+005	13.99	0.0041
Residual	4.079E+005	6	67977.01		
Cor Total	1.050E+007	15			

*R^2 0.9612, Adj-R^2 0.9029, Std. Dev. 260.72, Mean 968.40. The factors (D- NaCl and E- K2HPO4 were removed where p-value Prob>F greater than 0.1000

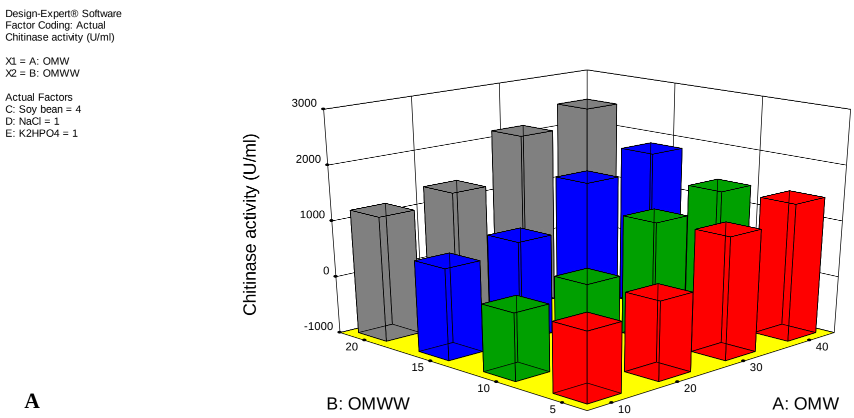
In-vitro biocontrol properties of *T. longibrachiatum* MW433820

Antagonistic properties of *Trichoderma* strains mainly based on the activation of multiple mechanisms which affect the plant pathogenic strains either directly, by mechanisms such as myco-parasitism or in-directly, by competition for nutrients, and changing the environmental conditions **(Benítez *et al.*, 2004).** The difference in the growth rate of both *T. longibrachiatum* and the phytopathogens *Fusarium* spp. indicating the interaction effect was first observed after incubation for 7 days. **Figure (6 A &B)** showed the high growth rate of *T. longibrachiatum* over the surface of PDA plates, compared to the pathogenic strains, and it was strongly controlled the growth of *F. solani* and *F. oxysporum.*

The obtained results emphesized good ability of the fungal isolate to secrete extracellular chitinolytic enzyme able to control the pathogens spread, and finally led to inhibition of the pathogen growth, that was in agree with **Pates *et al*., (1999).** *T. longibrachiatum* Showed the ability to exert direct biocontrol by coiling around the pathogen and directly invade it by secreting hydrolytic enzymes that degrade the pathogen's cell wall, leading to its lysis. *Trichoderma* parasitizing a range of pathogenic fungi, with secretion of exochitinases constitutively at low levels, and when chitinases degrade the fungal cell walls, they release oligomers that induce exochitinases, and attack begins, in accordance with **Harman *et al.*, (2004)** and **Zohair *et al.*, (2018)**. In addition, the study of **Anwar *et al.*, (2023)** showed promising results of *T. longibrachiatum* in controlling aphids by producing fungal chitinase in cotton plants.

The contact zone between the *T. longibrachiatum* and the phytopathogens was examined under light-microscope. The examination photos demonstrated the hyphal interactions, which could explain the parasitic effect of *T. longibrachiatum* on *F. oxysporum* and *F. solani*. **Figure 7** showed that, *T. longibrachiatum* hyphae were able to grow arround the mycelia of both phytopathogens and wrapping them strongly**.** As reported by **Howell (2003)** who indicated, the attachment between *Trichoderma* and pathogen by binding the cell-wall carbohydrates to pathogen lectins. After that *Trichoderma* coiled around the pathogen and forms the appresoria. The following step consists of the production of cell wall degrading enzymes.

Photos also showed a disorganization in the cytoplasm of *F. oxysporum* mycelia, and the disappearance of pathogen spores (**Figure 7C)**. The morphological effects caused by *T. longibrachiatum* on the structure of pathogenic fungi were similar to the parasitism caused by *Trichoderma harzianum* according to **Cuervo-parra *et al.*, (2011).**



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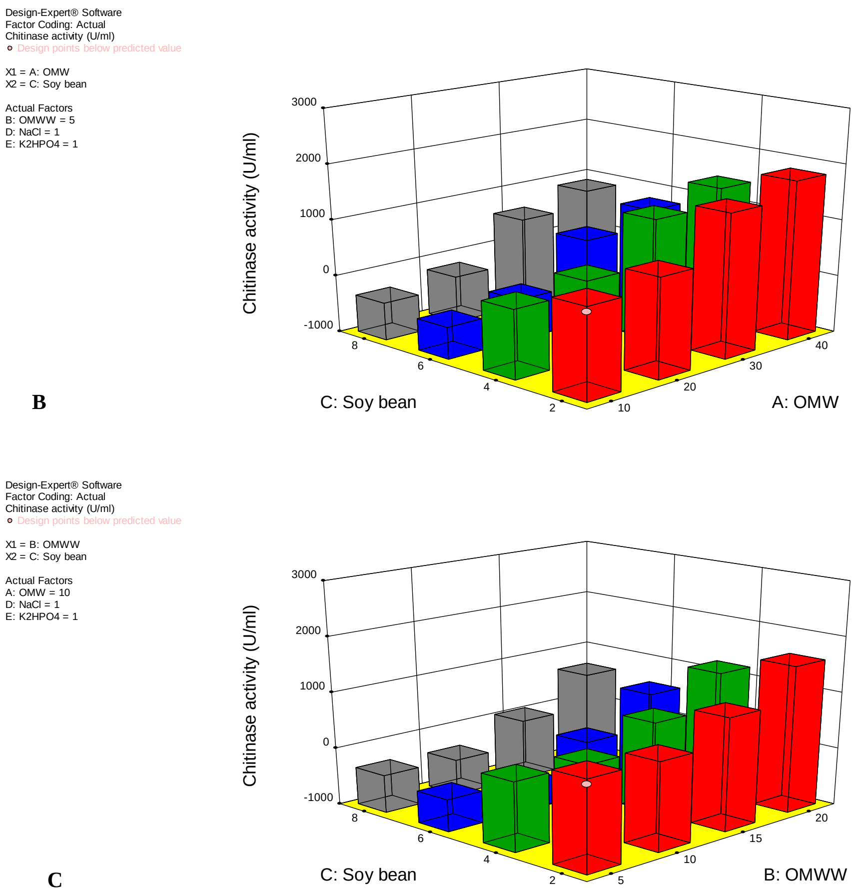

Figure 5 Three D/surface plot for the effect of (A) OMW and OMWW, (B) OMW and soy bean, (c) OMWW and soy bean.

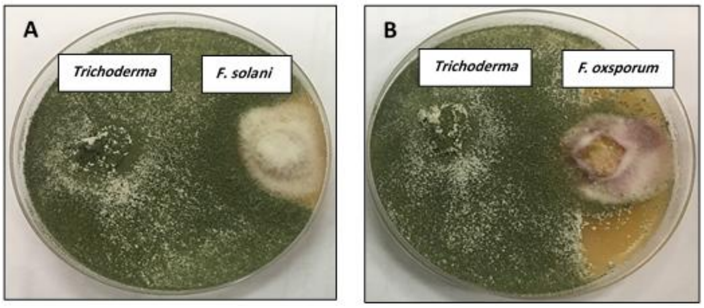

Figure 6 Antagonistic potential of *T. longibrachiatum* and growth inhibition of *F. solani* (A). and *F. oxysporum* (B).

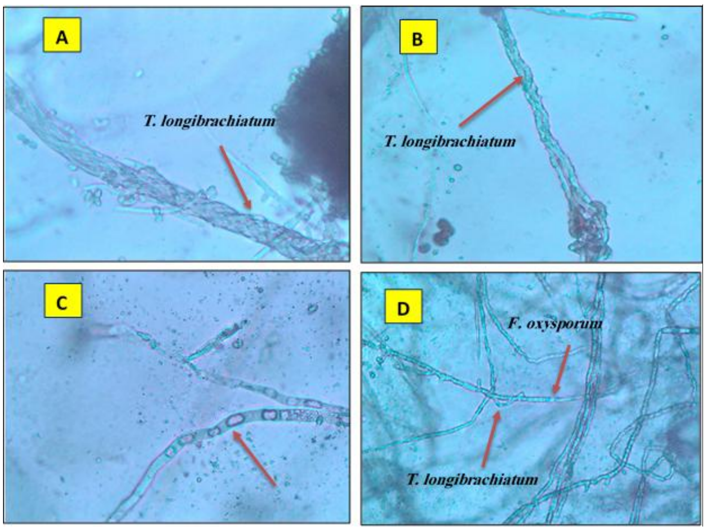

Figure 7 Microscopic examination of *T. longibrachiatum* antagonistic potential against *F. solani* (A, B). and against *F. oxysporum* (C, D). (light microscope Olympus CX40/ RF100 150 with a Canon A620 digital camera).

CONCLUSION

In the following study, *T. longibrachiatum* strain could be successfully utilized the olive oil processing wastes. OMWW and OMW used as a sole carbon source providing the necessary nutrients for fungus growth and constitutive exochitinase production. Chitinase production was optimized to ~7.63-fold by media supplementation with soy bean, NaCl and K_2HPO_4 in a statistical experimental Taguchi design. The antagonistic properties of *Trichoderma* strain was evaluated against the root-rot *F. solani* and *F. oxysporum.*

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