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Down Regulation of Two Pectinases Encoding Genes in *Aspergillus flavus* Treated with Clove and Tea tree oils

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ABSTRACT

Inhibition effect of two essential oils, clove and tea tree oils, on Aspergillus flavus were investigated. Agar disk diffusion, agar plate diffusion plate and fungi –substrate contact methods are three different plating methods for applying tested materials. All used methods confirmed the inhibition effect of clove and tea tree oils on A. flavus growth. Minimum inhibitor (MIC) concentration analysis revealed that 10µl/ml of clove oil and 4 µl/ml of tea tree oil induced a complete inhibition of A. flavus growth. Reduction in the production of reduced sugar indicates to the reduction of analytical activity of A. flavus treated with both used oils. Quantitative analysis of two pectinases encoded genes (Pec-A and Pec-B) indicated to the reduction in the transcript amount of both genes as a result of treatment with both oils. Obtained results pointed to the promising role of clove and tea tree oils as good strategy in controlling A. flavus infection.

1. INTRODUCTION

Aspergillus flavus is a fungus that principally obtains resources for growth in a saprophytic mode and possesses the characteristics of an opportunistic pathogen with a wide and non-specific host range (plants, animals, and insects) **(Mellon, et al., 2007).** It causes hazardous effects on the human respiration system **(Hedayati, et al., 2007).**

A. flavus is widely distributed in tropical and subtropical zones around the world. It has taken on significant agricultural importance because it produces the potent mycotoxin aflatoxin B1, the most carcinogenic naturally occurring compounds during infection of agronomically important crops such as corn, peanuts, and cotton **(Sheieh et al., 1997).** Thus, it significantly reduces the value of contaminated crops **(Cotty et al. 1994).** Aflatoxins are active at very low levels; its content in agricultural commodities is highly regulated with respect to the maximum allowable levels permitted by the US Food and Drug Administration (FDA) ranging from 0.5 to 300 ppb, depending on both product and use **(Fouad et al., 2019).** The ability to degrade plant tissues is important for many plants pathogenic and saprophytic organisms **(Osmolovskiy et al., 2021).** To survive, saprophytic microorganisms produce a battery of plant cell wall degrading enzymes **(Hahn, et al., 1989).** A. flavus possesses the capacity to produce numerous extracellular hydrolases which assist in the degradation of complex substrates subsequently, access a large variety of nutrient substrates and penetrate host tissues **(Osmolovskiy et al., 2021; St. Leger et al., 1997).** A. flavus was found to have a greater capacity for growth on complex protein substrates (e.g., elastin and mucin) and complex carbohydrate substrates (e.g., cellulose, chitin, pectin, and xylan **(St. Leger et al., 1997).**

Pectin is a plant-produced polymer of galacturonic acid that is usually highly methylated. Pectin constituting middle lamella found between the primary cell walls of adjacent young plant cells **(Hoondal, et al., 2002; Jabeen, et al., 2015).** Endopolygalacturonases or pectinases are a class of enzymes that hydrolyze long-chain pectin's the major constituent of plant cell walls at internal locations, producing a mixture of molecular sizes. Many plant pathogens secrete pectinases to help in soften host tissues, subsequently assist in pathogen entry hosts, and for nutrient capture. A positive correlation has been established between the production of analytical enzymes, virulence and disease symptoms in several pathosystems **(Ramos et al., 2016).**

Endopolygalacturonases (EC 3.2.1.15) are important in some plant-pathogen interactions **(Brown et al., 1992; Rodriguez-Palenzuela et al., 1991)**. They are the first cell wall-degrading enzymes produced by fungal pathogens when cultured on isolated plant cell walls or during infection **(Anderson 1978)**. The ability of A. flavus isolates to spread between locules of developing cotton bolls (fruits) strongly correlates with the production of specific pectinases **(Cleveland and Cotty 1991)**. The expression level of pectinase enzymes is a limiting factor determining virulence and infection ability in A. flavus **(Liu et al., 2017; Pradal et al., 2018).**

One of the most effective aflatoxin control strategies is the application of biocontrol of fungus growth to reduce their ability for host penetration as a starvation strategy.

Many studies suggest that some plant extracts' overall antifungal activity is due to their various components' synergistic effects **(Moon et al., 2011; Pinto et al., 2014)**. The inhibition effects of clove and tea oil were reported **(Bentayeb, et al., 2014; Kwieciński**

et al., 2009; Hammer et al. 2012). The inhibitory effects of these extracts on the expression level of the analytical fungal enzymes are less documented. Our study aimed to investigate the inhibitory effect of clove and tea tree oils on the growth and analytical activity of A.flavus considering their effect on the expression level of two A. flavus's pectinases.

2. MATERIALS AND METHODS

2.1. Fungal strains

Aspergillus flavus isolate was kindly provided by Prof. Yousef Sultan, The National Research Center, Giza, Egypt. The fungal isolate was inoculated on potato dextrose agar (PDA) and incubated at 28 °C for further use.

2.2. Characterization of Aspergillus strain

The fungus used in the present study was characterized using ITS rRNA encoded gene. DNA of the fungi isolates was extracted from hyphea using DNA extraction kit (i-genomic plant cat No.17371) according to the attached procedure. DNA concentration was determined using Nano drop spectrophotometer (BioDrop µLITE.UK). DNA quality was assessed on 1% agarose gel stained with ethidium promide. The amplified PCR product of the ITS rRNA gene was accomplished using a thermal cycler (SENSO Quest) using forward primer (F:5′-TCCGTAGGTGAACCTGCGG-3′) and reverse primer R:5′-(TCCTCCGCTTATTGATATGC) -3′ in 25µl reaction volume containing 50 ng of isolated DNA using PCR master mix (2XTopsimple TM DyeMIx-nTag, enzynomics) The PCR amplification condition was adjusted for an initial denaturation for 3 min at 95°C, followed by 35 cycles of denaturation for 30 s at 95°C, annealing for 30 s at 53°C, extension for 90 s at 72°C, followed by a final extension step for 7 min at 72 °C. PCR purification kit (Easy Pure Quick gel Extraction kit, code#EG101-01) was used to purify PCR products from the gel following manufacturer's protocol. Purified PCR products were sequenced with forward 16S rRNA primer using Macrogene, Inc., (Seoul, South Korea). The partial sequences of ITS rRNA gene were BLAST searched with NCBI database **(Altschul et al., 1990).**

2.3. Characterization of used materials

Recent study used two plant oils (clove and tea tree oil). Commercial products with 100 % concentrated of used oil were used after dilution. Clove and tea tree oils treatments were carried out with 5µl/ml media and 2µl/ml for clove and tea tree oils, respectively.

2.4. Minimum inhibitory concentration.

The minimum inhibitory concentration (MIC) for all used materials was determined by the culture panel using gradually increased concentration from each material (Table 1) on Potato dextrose agar (PDA) according to **Macro et al. (2020).**

Table 1: concentrations of different tested materials used for MIC calculation

Tested substrates	Concentrations (µl/ml media)				
Clove Oil	6	7	8	9	10
Tea Tree Oil	0.5	1	2	3	4

2.5. Antifungal activity methods

Three different methods were used to determine the antifungal activity of the tested material on A. flavus.

2.5.1. Agar disk diffusion method

The Agar disk diffusion method was used according to **(Fu et al., 2007).** Agar plates were inoculated with 100 µl of suspended spores (1×106 CFU/ml). Filter paper discs (6 mm in diameter) were saturated (50 µL) with tested compound (clove or tea oil) and placed on the agar surface. The Petri dishes are incubated at 28 ºC. The average diameter of the clear zone was measured after 48 h and seven days of incubation.

2.5.2. Agar plate diffusion plate method

Agar plate diffusion plate method was carried out according to **(Sitara, et al., 2011).** Not solidified autoclaved potato dextrose agar medium (PDA) was mixed with tested concentration for each material (clove oil or tea tree oil). Three replicates for control and each material were carried out. Plates were inoculated centrally with a mycelial disk (5 mm in diameter) of A. flavus after solidification of the medium. All Petri dishes were incubated at 28°C. The average diameter of the growth zone was measured after 48 h and seven days of incubation.

2.5.3. Fungi-substrate contact method

Fungi-substrate contact method was developed to examine the effect of direct contact of the tested material with the tested fungi. The autoclaved PDA medium was poured into Petri dishes and left to stiffen. 100 µl of suspended spores (1×106 CFU/ml) was placed on the plate and topped with tested material (clove or tea oil). Spores suspension and tested material were mixed and spread on the surface of the medium by the spreader. Three replicates for control and each material were used. All Petri dishes were incubated at 28 ºC. The intensity of the growth was monitored after 48 h and again after seven days of incubation and compared with the control.

2.6. Reducing sugar assay:

Cellulases activity was primarily determined using filter paper assay saccharifying cellulose (FPU Assay) according to **Ghose (1987)** with some modification **(Legodi et al., 2019).**

2.7 Analysis of pectinases expression

2.7.1. RNA extraction and cDNA synthesis

About 100 mg of mycelium were used for total RNA extraction using IQeasy TM plus plant RNA Extraction kit according to the manufacturer procedures. RNA concentration and purity were determined using nano-drop spectrophotometer (BioDrop µLITE.UK). Samples with RNA purity of more than 1.9 were considered for gene expression analysis. One µg of total extracted RNA for each sample was used for cDNA synthesis using TopscriptTMcDNA Synthesis kit (cat.# EZ005S) according to the manufacturer procedures.

2.7.2 Quantitative real-time PCR analysis (qRT-PCR)

Changes in the transcript amount of two pectinase genes (Pec-A and Pec-B) related genes were determined using quantitative real-time PCR (qRT-PCR). Syper green with low ROX (WizPureTm qPCR Master) was used for qRT-PCR reactions in 20 µL reaction volume. The reactions were run on Applied Biosystem™ Step One Plus™ Real-Time PCR system. The endogenous control for all reactions was β-actin gene (accession no. XM_026746287.1) from A. flavus. Gene's specific primers were designed according to available data of Aspergillus sp on National center on biological information (NCBI) using primer 3 and blast online

software (Table 2). The reaction was conducted under standard conditions using variflex option to adjust different annealing temperature for different genes on the same plate. Relative expression (RQ) was calculated as 2-ΔΔct calibrated with the endogenous gene and control treatment according to **Livak and Schmittgen (2001)**. Means ±SE of RQ was calculated for three biological replicates for each cDNA sample.

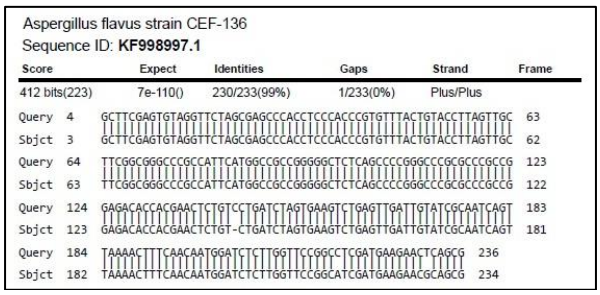
Table 2: list of used primers

Gene	Gene function	Accession no.	Primer sequence 5'--------3'	Ta
Pec-A	Endopolyglacturonases	U05015.1	5- TCCATCACCGGTCTCCAGAT-3 5- CGATCCAGAGTTGATGGCCA-3	57
Pec-B	Endopolyglac-turonases	CP051061.1	5- TGCACGTTAAGAACTCCCCC-3 5- CGATGGAGATACCGTGACCG-3	58
Actin	Reference gene	XM_026746287.1	5-AGCGTGGTATCCTCACGCTC -3 5-CTTCATGATGGAGTTGAACG -3	58

3-Results and discussions

3.1. Strain characterization

Similarity analysis of flavus strain was found to be very closely related to A. flavus CEF-136, with an ITS gene sequence similarity of 98.71% (Fig.1)


Aspergillus flavus strain CEF-136
Sequence ID: **KF998997.1**

Score	Expect	Identities	Gaps	Strand	Frame
412 bits(223)	7e-110()	230/233(99%)	1/233(0%)	Plus/Plus	

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Query  4    GCTTCGAGTGTAGGTTCTAGCGAGCCCACCTCCCACCCGTGTTTACTGTACCTTAGTTGC  63
Sbjct  3    GCTTCGAGTGTAGGTTCTAGCGAGCCCACCTCCCACCCGTGTTTACTGTACCTTAGTTGC  62

Query  64   TTCGGCGGGCCCCGCCATTCATGGCCGCCGGGGGCTCTCAGCCCCGGGCCCGCGCCCGCCG  123
Sbjct  63   TTCGGCGGGCCCCGCCATTCATGGCCGCCGGGGGCTCTCAGCCCCGGGCCCGCGCCCGCCG  122

Query  124  GAGACACCACGAACTCTGTCCTGATCTAGTGAAGTCTGAGTTGATTGTATCGCAATCAGT  183
Sbjct  123  GAGACACCACGAACTCTGT-CTGATCTAGTGAAGTCTGAGTTGATTGTATCGCAATCAGT  181

Query  184  TAAAACTTTCAACAATGGATCTCTTGGTTCCGGCCTCGATGAAGAACTCAGCG  236
Sbjct  182  TAAAACTTTCAACAATGGATCTCTTGGTTCCGGCATCGATGAAGAACGCAGCG  234
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Figure 1: Alignment results for the amplified PCR product of the partial sequence of ITS rRNA encoded gene on NCBI data base. Query ID; 57973.

3.2. MIC analysis of studied substrates

MIC analysis of used substrates (clove and tea oils) indicated that both oils inhibited the growth of A. flavus (Fig. 2). Inhibition effect of both clove and tea oil increased along with increasing the concentration of used material. It was found that complete inhibition of A. flavus was induced at a concentration of 10µl/ml clove oil, where no growth

was shown. Growths began to appear at a concentration of 9µl/ml and increased with a reduction of clove oil concentration (6, 7, 8 µl/ml) (Fig. 2). Inhibition effect of clove oil was reported as it used as fungicide with antifungal activities against A. flavus, Penicillium citrinum, and Rhizopus nigricans **(Xing et al., 2012; Sinha et al., 1993**). Complete control of A. flavus was recorded at concentration of 3% of clove oil **(Xing et al., 2012). Achar et al. (2020**) reported the complete inhibition of A. flavus on agar treated with 500 ppm clove oil. 84.7% inhibition of the conidial germination of A. flavus PSRDC-2 occurred at 100 µL/L, while the complete inhibition of disease infection on maize seeds occurred at 10µl/L. **(Boukaew et al., 2017). Sukatta et al. (2008**) reported the antifungal activity of clove oil against A. niger, Alternaria alternata, Colletotrichum gloeosporioides, Lasiodiplodia theobromae, Phomopsis viticola and Rhizopus stolonifer. Treatement with Tea tree oil at a 2.0% v/v concentration showed the strongest mycelium growth inhibition of A. flavus (37.03%) **(Thobunluepop et al., 2009**)

For tea tree oil, it was found that at a concentration of 4µl/ml, there was complete inhibition of *A. flavus*, where no growth has appeared. The growth began to appear at a concentration of 3µl/ml. Growths increased with decreasing the concentration i.e. at concentrations of (0.5, 1 and 2 µl/ml). Previous studies agreed with this study that tea tree oil is an inhibitor for *Trichoderma viride* and *Fusarium oxysporum* **(Sahab Ahmed *et al.*, 2014).** Effective antifungal activity of tea tree oil was reported against *Candida albicans, A. niger, A. flavus* **(Li *et al.*, 2016; Yasin *et al.*, 2021 ;Thobunluepop *et al.*, 2009; Sevik *et al.*, 2021)**.

The MIC values of tea tree oil against *A. flavus* and *A. niger* varied between 0.032 and 0.376 mg/L (**Sevik, *et al.*, 2021)**. **Christoph *et al.* (2000)** reported that the concentrations needed to control *A. flavus* effectively varied between 0.3 and 0.7% (v/v).

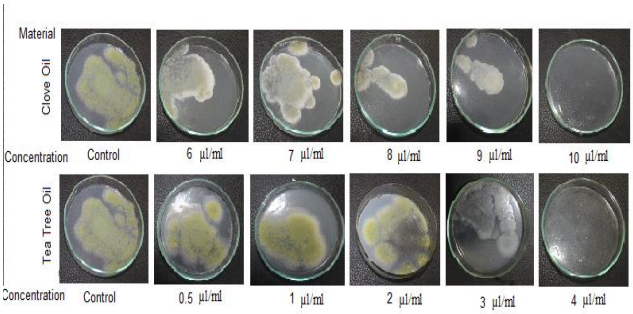

Figure 2: Minimum inhibitory concentration (MIC) of clove oil and tea tree oil for A. flavus strain.

3.3. Methods of applying treatments

Three methods for applying the treatment for clove and tea oils with *A. Flavus* were conducted. The obtained results showed that all used methods confirmed the inhibition effect of clove oil on *A. flavus* growth (figure 3A). The agar plate diffusion plate method (Figure 3) showed that treatment with clove oil reduced the diameter of the growth zone significantly compared to the control treatment after 48h and seven days of incubation (figure 3Aa, B). In the agar disk diffusion method, the inhibtion effect of clove oil was shown as a clear zone (figure 3A: b,C). Diameters of the clear zone increased significantly with the treatment of clove oil after 48h and seven days comparing with the control (Figure 3C). At the fungi-substrate contact method, the inhibition effect was shown as a decrease in growth density (Figure 3Ac).

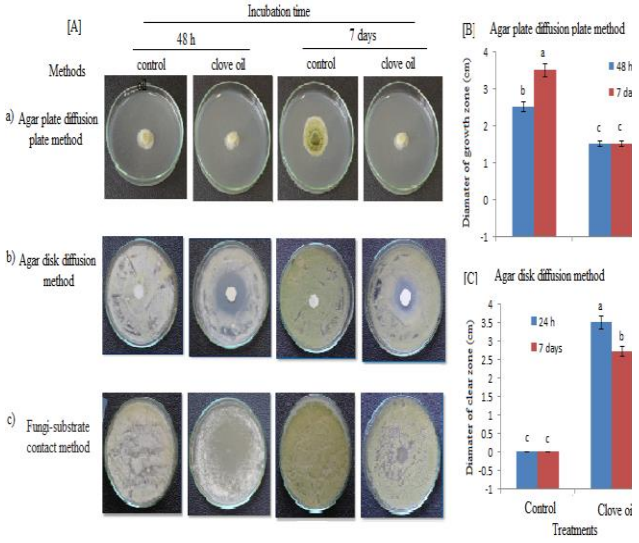

Figure 3: Methods of clove oil applying on A. flavus. Growth shape on plates[A], changes in the diameter of growth zone in agar plate diffusion plate method [B], changes in the diameter of a clear zone in agar disk diffusion method [C].

For tea tree oil, the obtained results showed that all used methods confirmed the inhibition effect of tea tree oil on the growth of A. flavus fungus (Fig. 4A). The agar plate diffusion plate method (Fig. 4) showed that treatment with tea tree oil completely inhibited the growth zone compared to control treatment after 48h and seven days of incubation (Fig. 4Aa, B). In the agar disc diffusion method, the inhibition effect of tea tree oil was shown as a clear area (Fig. 4Ab, C). Inhibition of the whole plate was clearly marked as it was completely free of growth with tea tree oil treatment after 48 h and seven days compared with the untreated plate (Fig. 4C). In the fungal substrate contact method, the inhibition effect was shown to be complete inhibition of the fungus growth (Figure 4Ac).

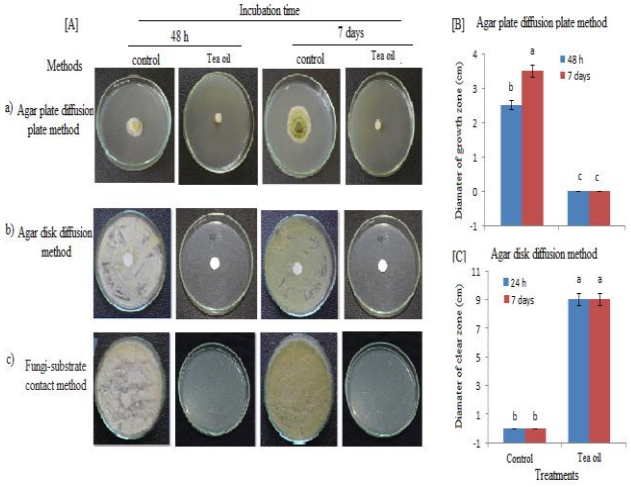

Figure 4: Methods of tea tree oil applying on A. flavus. Growth shape on plates[A], changes in the diameter of growth zone in agar plate diffusion plate method [B], changes in the diameter of clear zone in agar disk diffusion method [C].

The antifungal activity of essential oil is associated with their phytochemical components and depends on the tested concentration and examined fungal species

(Moghaddam and Mehdizadeh 2016). The antifungal activity of essential oils is due to the synergistic effects of their various components **(Moon et al. 2011; Pinto et al., 2014)**. Clove oil was used for biocontrol of A. spp (**Passone et al., 2012**). The major component of clove oil is phenylpropanoid eugenol (C10H12O2; 4-alyl-2-methyoxyphenol), a powerful antimicrobial and antioxidant agent (**Bentayeb, et al, 2014).**

Its antimicrobial activity is attributed to the phenyl group capable of denaturing proteins and interacting with the lipids from the cell membrane of the microorganisms, altering the permeability and causing subsequent cell death **(Hossain et al., 2012; Sharif et al., 2017)**. Hydroxyl group in the eugenol molecule is credited for its strong antioxidant activity, which makes it comparable to some commercial antioxidants currently used in foods like butylated hydroxyanisole and butylated hydroxytoluene **(Gülçin et al., 2012)**. Our results confirmed the antifungal activities of clove oil on A. flavus

Clove oil was evaluated for its antifungal activities against ten isolates of A. flavus. All strains, except A. flavus PSRDC-2 were strongly inhibited by clove oil **(Boukaew et al., 2017).** It was also recorded for its effective and strong inhibition for A. niger **(Hu et al., 2019).** Clove oil was used to control pathogenic fungi in food and agricultural products by direct contact (**Passone et al., 2012; Alamene 2015).** Our results and the previous **(Achar et al., 2020; Christoph et al., 2000)** demonstrate that clove oil could be a promising natural fungicide for an effective bio-control, non-toxic bio-preservative, and an eco-friendly alternative to synthetic additives against A. flavus.

Tea tree oil consists of about 100 different compounds, and the major component is terpenes and sesquiterpenes, such as terpinen-4-ol, α- and γ-terpinene, 1,8-cineole, and terpinolene (**Kwieciński et al., 2009; Hammer et al., 2012)**.

The antifungal activity of tea tree oil was confirmed in vivo assay against Trichophyton equinum **(Pisseri et al., 2009).** Tea tree oil vapour exhibited high activity against spore germination and mycelial growth of the main post-harvest pathogens Botrytis cinerea and Rhizopus stolonifer in vitro experiments (Shao et al., 2013). The effectiveness of tea tree oil against fungal strains of Fusarium, Aspergillus (flavus and niger), and Candida species were recorded (Yasin, et al., 2021). A

previous study reported the antifungal properties of tea tree oil extracted from M.

alternifolia against Aspergillus flavus (**Christoph, et al., 2000).**

3.4. Reducing Sugar

The effect of the used materials on analytical activity was determined by estimating the reduced sugar production. Obtained data showed that treatment with both clove and tea tree oil induced a significant reduction in the production of reduced sugars (Fig. 5). Reduction in the reduced sugar values is related to the inhibition effect of clove oil on the production of cellulolytic enzymes in A. flavus **(Montes-Belmont and Carvajal 1998; Pratha et al., 2016; Achar et al., 2020)** as well as tea tree oil **(Hammer et al., 2012; Sevik et al., 2021).**

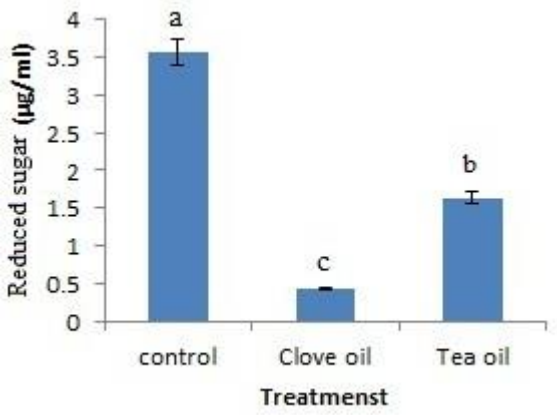

Figure 5: Effect of clove and tea tree oils on the production of reduced sugar

3.5- Analysis of gene expression (qRT-PCR)

Quantitative Real time-PCR analysis was used to investigate the changes in the expression levels of two polygalacerturonase enzymes encoded genes (Pec-A and Pec-B) (Fig. 6) as members of the pectinases which is the main enzyme responsible for the degradation of pectin. Our results showed that the transcript amount of both studied pectinases (Pec-A and Pec-B) in treated fungi was reduced significantly after treatment with clove oil (Fig. 6A) and tea tree oil (Fig.6 B) compared with un-treated fungi. The reduction in transcript amount is pointed to the down regulated of both pectenases encoded genes due to treatment with clove and tea tree oils. Pectinases are the first cell wall-degrading enzymes produced by bacterial or fungal pathogens during infection, and it has been proposed that they are important in some plant-pathogen interactions **(Anderson 1978; Brown et al., 1992; Rodriguez-Palenzuela et al., 1991).** Most studies of pectinase enzymes have revealed that gene expression is substrate-stimulated and catabolite-repressed **(Dean and Timberlake, 1989)**. Expression of pectinase (P2c) contributes to the invasion and proliferation of A. flavus during the infection of cotton bolls **(Shieh et al., 1997).** Considering previous and our results, controlling of pectinases activity is a good strategy to control and inhibit A. flavus, which is considered one of the most harmful and dangerous fungi and control the production of a contaminated product with aflatoxins which affect poultry, meat, egg, and crop production subsequently human health.

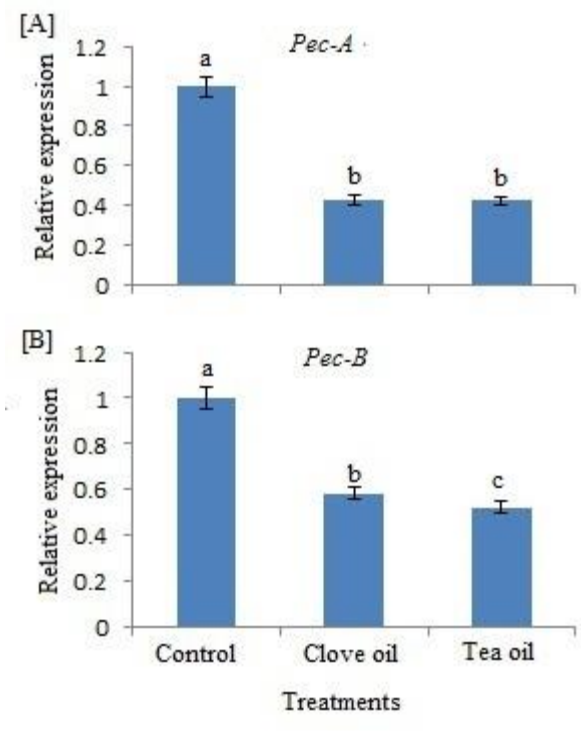

Figure 6: The changes in the expression levels of two polygalacerturonase enzymes encoded genes (Pec-A and Pec-B) by quantitative real time-PCR analysis. The expression was determined as changes in the transcript amount using qRT-PCR. Relative expression (RQ) was calculated as 2-ΔΔct calibrated with the endogenous gene (XM_026746287.1) from A. flavus and control treatment as endogenous control. Data are given as means ± SE of relative expression for three biological replicates for each cDNA sample. Values with different letters are significant.

4.References

Achar, P. N., Quyen, P., Adukwu, E. C., Sharma, A., Msimanga, H. Z., Nagaraja, H., & Sreenivasa, M. Y. (2020). Investigation of the antifungal and anti-aflatoxigenic potential of plant-based essential oils against Aspergillus flavus in peanuts. Journal of Fungi, 6(4), 383. .https://doi.org/10.3390/jof6040383

Alamene, A., 2015. Effects of plant essential oils and biocontrol agents on the growth of and mycotoxin production by Aspergillus spp. on groundnut (Doctoral dissertation, University of Nottingham).

Altschul, S. F., Gish, W., Miller, W., Myers, E. W., & Lipman, D. J. (1990). Basic local alignment search tool. Journal of molecular biology, 215(3), 403-410. https://doi.org/10.1016/S0022-2836(05)80360-2.

Anderson, A. J. (1978). Extracellular enzymes produced by Colletotrichum lindemuthianum and Helminthosporium maydis during growth on isolated bean and corn cell walls. Phytopathology, 68(1), 585-1589.

Bentayeb, K., Vera, P., Rubio, C., & Nerín, C. (2014). The additive properties of Oxygen Radical Absorbance Capacity (ORAC) assay: The case of essential oils. Food chemistry, 148, 204-208. https://doi.org/10.1016/j.foodchem.2013.10.037

Brown, R. L., Cleveland, T. E., Cotty, P. J., & Mellon, J. E. (1992). Spread of Aspergillus flavus in cotton bolls, decay of intercarpellary membranes, and production of fungal pectinases. Phytopathology, 82(4), 462-467.

Boukaew, S., Prasertsan, P., & Sattayasamitsathit, S. (2017). Evaluation of antifungal activity of essential oils against aflatoxigenic Aspergillus flavus and their allelopathic activity from fumigation to protect maize seeds during storage. Industrial Crops and Products, 97, 558-566. https://doi.org/10.1016/j.indcrop.2017.01.005

Christoph, F., Kaulfers, P. M., & Stahl-Biskup, E. (2000). A comparative study of the in vitro antimicrobial activity of tea tree oils sl with special reference to the activity of β-triketones. Planta medica, 66(06), 556-560. DOI: 10.1055/s-2000-8604

Cleveland, T. E., Cotty, P. J. (1991). Invasiveness of Aspergillus flavus isolates in wounded cotton bolls is associated with production of a specific fungal polygalacturonase. Phytopathology, 81(2), 155-158.

Cotty, P. J., Bayman, P., Egel, D. S., & Elias, K. S. (1994). Agriculture, aflatoxins and Aspergillus. In The Genus Aspergillus (pp. 1-27). Springer, Boston, MA. DOI: 10.1007/978-1-4899-0981-7_1

Dean, R. A., and W. E. Timberlake. 1989. Regulation of the Aspergillus nidulans pectate lyase gene (pelA). Plant Cell 1:275–284.

Fouad, A. M., Ruan, D., El-Senousey, H. K., Chen, W., Jiang, S., & Zheng, C. (2019). Harmful effects and control strategies of aflatoxin b1 produced by Aspergillus flavus and Aspergillus parasiticus strains on poultry. Toxins, 11(3), 176. doi:10.3390/toxins11030176

Fu, Y., Zu, Y., Chen, L., Shi, X., Wang, Z., Sun, S., & Efferth, T. (2007). Antimicrobial activity of clove and rosemary essential oils alone and in combination. Phytotherapy research, 21(10), 989-994. https://doi.org/10.1002/ptr.2179

Ghose, T. K. (1987). Measurement of cellulase activities. Pure and applied Chemistry, 59(2), 257-268 https://doi.org/10.1351/pac198759020257

Gülçin, İ., Elmastaş, M., & Aboul-Enein, H. Y. (2012). Antioxidant activity of clove oil–A powerful antioxidant source. Arabian Journal of chemistry, 5(4), 489-499. https://doi.org/10.1016/j.arabjc.2010.09.016

Hahn, M. G. (1989). Roles of cell wall constituents in plant-pathogen interactions. Plant-microbe interactions: molecular and genetic perspectives, 3, 131-181.

Hammer, K. A., Carson, C. F., & Riley, T. V. (2012). Effects of Melaleuca alternifolia (tea tree) essential oil and the major monoterpene component terpinen-4-ol on the development of single-and multistep antibiotic resistance and antimicrobial susceptibility. Antimicrobial Agents and Chemotherapy, 56(2), 909-915. https://doi.org/10.1016/j.ijantimicag.2008.08.028

Hedayati, M. T., Pasqualotto, A. C., Warn, P. A., Bowyer, P., & Denning, D. W. (2007). Aspergillus flavus: human pathogen, allergen and mycotoxin producer. Microbiology, 153(6), 1677-1692 DOI 10.1099/mic.0.2007/007641-0

Hoondal, G., Tiwari, R., Dahiya, N., Beg, Q. 2002. Microbial alkaline pectinases and their industrial applications: a review. Appl Microbiol Biotechol., 59:409-18. https://doi.org/10.1007/s00253-002-1061-1

Hossain, M. A., Al-Hashmi, R. A., Weli, A. M., Al-Riyami, Q., & Al-Sabahib, J. N. (2012). Constituents of the essential oil from different brands of Syzigium caryophyllatum L by gas chromatography–mass spectrometry. Asian Pacific Journal of Tropical Biomedicine, 2(3), S1446-S1449. https://doi.org/10.1016/S2221-1691(12)60435-3

Hu, F., Tu, X.F., Thakur, K., Hu, F., Li, X.L., Zhang, Y.S., Zhang, J.G. and Wei, Z.J., 2019. Comparison of antifungal activity of essential oils from different plants against three fungi. Food and Chemical Toxicology, 134,

p.110821. https://doi.org/10.1016/j.fct.2019.110821

Jabeen, A., Hanif, Q.A., Hussain, M., Munawar, A., Farooq, N., Bano, S. 2015. Screening, isolation and identification of pectinase producing bacterial strains from rotting fruits and determination of their pectinolytic activity. Science Letters., 3(2):42-45.

Kwieciński, J., Eick, S., & Wójcik, K. (2009). Effects of tea tree (Melaleuca alternifolia) oil on Staphylococcus aureus in biofilms and stationary growth phase. International journal of antimicrobial agents, 33(4), 343-347. https://doi.org/10.1016/j.ijantimicag.2008.08.028

Legodi, L. M., La Grange, D., Van Rensburg, E. L., & Ncube, I. (2019). Isolation of cellulose degrading fungi from decaying banana pseudostem and Strelitzia alba. Enzyme research, 2019

Li, W. R., Li, H. L., Shi, Q. S., Sun, T. L., Xie, X. B., Song, B., & Huang, X. M. (2016). The dynamics and mechanism of the antimicrobial activity of tea tree oil against bacteria and fungi. Applied microbiology and biotechnology, 100(20), 8865-8875.

Liu, C. Q., Hu, K. D., Li, T. T., Yang, Y., Yang, F., Li, Y. H., ... & Zhang, H. (2017). polygalacturonase gene pgxB in Aspergillus niger is a virulence factor in apple fruit.PloS one 12, e0173277.DOI:10.1371/ journal.pone.0173277

Mellon, J. E., Cotty, P. J., & Dowd, M. K. (2007). Aspergillus flavus hydrolases: their roles in pathogenesis and substrate utilization. Applied Microbiology and Biotechnology, 77(3), 497-504. DOI 10.1007/s00253-007-1201-8

Moghaddam, M., & Mehdizadeh, L. (2016). Essential oil and antifungal therapy. Recent trends in antifungal agents and antifungal therapy, 29-74. DOI 10.1007/978-81-322-2782-3

Montes-Belmont, R. and Carvajal, M., 1998. Control of Aspergillus flavus in maize with plant essential oils and their components. Journal of Food Protection, 61(5), pp.616-619. https://doi.org/10.4315/0362-028X-61.5.616

Moon, S. E., Kim, H. Y., & Cha, J. D. (2011). Synergistic effect between clove oil and its major compounds and antibiotics against oral bacteria. Archives of oral biology, 56(9), 907-916. https://doi.org/10.1016/j.archoralbio.2011.02.005

Osmolovskiy, A. A., Schmidt, L., Orekhova, A. V., Komarevtsev, S. K., Kreyer, V. G., Shabunin, S. V., & Egorov, N. S. (2021). Action of Extracellular Proteases of Aspergillus flavus and Aspergillus ochraceus Micromycetes on Plasma Hemostasis Proteins. Life, 11(8), 782. https://doi.org/10.3390/life11080782

Passone, M. A., Girardi, N. S., & Etcheverry, M. (2012). Evaluation of the control ability of five essential oils against Aspergillus section Nigri growth and ochratoxin A accumulation in peanut meal extract agar conditioned at different water activities levels. International Journal of Food Microbiology, 159(3), 198-206. https://doi.org/10.1016/j.ijfoodmicro.2012.08.019

Pradal-Velázquez, M., Martínez-Trujillo, M. A., & Aguilar-Osorio, G. (2018). Xylanases and pectinases of Aspergillus flavus cect-2687 on different carbon sources and initial pH values. Revista Mexicana de Ingeniería Química, 17(2), 421-431.

Pinto, E., Gonçalves, M. J., Oliveira, P., Coelho, J., Cavaleiro, C., & Salgueiro, L.

(2014). Activity of Thymus caespititius essential oil and α-terpineol against yeasts and filamentous fungi. Industrial Crops and Products, 62, 107-112. https://doi.org/10.1016/j.indcrop.2014.08.004

Pisseri, F., Bertoli, A., Nardoni, S., Pinto, L., Pistelli, L., Guidi, G., & Mancianti, F. (2009). Antifungal activity of tea tree oil from Melaleuca alternifolia against Trichophyton equinum: an in vivo assay. Phytomedicine, 16(11), 1056-1058. https://doi.org/10.1016/j.phymed.2009.03.013

Pratha, A. A., Priya, V. V., & Gayathri, R. (2016). Comparative analysis of Antifungal activity of Tea Tree and Clove Oil. Research Journal of Pharmacy and Technology, 9(8), 1128-1132 . 10.5958/0974-360X.2016.00214.6 .

Ramos AM, Gally M, Szapiro G, Itzcovich T, Carabajal M, LevinaIn L (2016). In vitrogrowth and cell wall degrad-ing enzyme production by Argentinean isolates of Mac-rophomina phaseolina, the causative agent of charcoal rot in corn. Revista Argentina de microbiologia 48(4):267-273. http://doi.org/10.47440/JAFE.2020.1101

Rodriguez-Palenzuela, P., T. J. Burr, and A. Collmer. 1991. Polygalacturonase is a virulence factor in Agrobacterium tumefaciens biovar 3. J. Bacteriol 173:6547–6552.

Sahab Ahmed, F., Sidkey Nagwa, M., Abed Nermine, N., & Mounir, A. (2014). Studies on indoor air quality in the repositories of the National Library and Archives of Egypt. Int J Sci Res, 3(11), 2122-2128.

Sevik, R., Akarca, G., Kilinc, M., & Ascioglu, Ç. (2021). Chemical composition of tea tree (Melaleuca alternifolia)(Maiden & Betche) cheel essential oil and its antifungal effect on foodborne molds isolated from meat products. Journal of Essential Oil Bearing Plants, 24(3), 561-570. https://doi.org/10.1080/0972060X.2021.1942232

Shao, X., Wang, H., Xu, F., & Cheng, S. (2013). Effects and possible mechanisms of tea tree oil vapor treatment on the main disease in postharvest strawberry fruit. Postharvest Biology and Technology, 77, 94-101. https://doi.org/10.1016/j.postharvbio.2012.11.010

Sharif, H. R., Abbas, S., Majeed, H., Safdar, W., Shamoon, M., Khan, M. A., ... & Haider, J. (2017). Formulation, characterization and antimicrobial properties of black cumin essential oil nanoemulsions stabilized by OSA starch. Journal of food science and technology, 54(10), 3358-3365. https://doi.org/10.1007/ s13197-017-2800-8

Shieh, M. T., Brown, R. L., Whitehead, M. P., Cary, J. W., Cotty, P. J., Cleveland, T. E., & Dean, R. A. (1997). Molecular genetic evidence for the involvement of a specific polygalacturonase, P2c, in the invasion and spread of Aspergillus flavus in cotton bolls. Applied and Environmental Microbiology, 63(9),3548-3552.. https://doi.org/10.1128/aem.63.9.3548-3552.1997

Sinha, K. K., Sinha, A. K., & Prasad, G. (1993). The effect of clove and cinnamon oils on growth of and aflatoxin production by Aspergillus flavus. Letters in Applied Microbiology, 16(3), 114-117.

Sitara, U., Hassan, N., & Naseem, J. (2011). Antifungal activity of Aloe vera gel against plant pathogenic fungi. Pakistan Journal of Botany, 43(4), 2231-2233. https://doi.org/10.1016/j.biocontrol.2009.04.015

St Leger, R. J., Joshi, L., & Roberts, D. W. (1997). Adaptation of proteases and carbohydrases of saprophytic, phytopathogenic and entomopathogenic fungi to the requirements of their ecological niches. Microbiology, 143(6), 1983-1992.

Sukatta, U., Haruthaithanasan, V., Chantarapanont, W., Dilokkunanant, U., & Suppakul, P. (2008). Antifungal activity of clove and cinnamon oil and their synergistic against postharvest decay fungi of grape in vitro. Agriculture and Natural Resources, 42(5), 169-174.

Thobunluepop, P., Udomsilp, J., Piyo, A., & Khengkhan, P. (2009). Screening for the antifungal activity of essential oils from bergamot oil (Citrus hystrix DC.) and tea tree oil (Melaleuca alternifolia) against economically rice pathogenic fungi: A driving force of organic rice cv. KDML 105 production. As. J. Food Ag-Ind. Special, (S374), S380.

Xing, Y., Xu, Q., Li, X., Che, Z., & Yun, J. (2012). Antifungal activities of clove oil against Rhizopus nigricans, Aspergillus flavus and Penicillium citrinum in vitro and in wounded fruit test. Journal of Food Safety, 32(1), 84-93. HYPERLINK "https://doi.org/10.1111/j.1745-4565.2011.00347.x"

Yasin, M., Younis, A., Javed, T., Akram, A., Ahsan, M., Shabbir, R., ... & El-Esawi, M. A. (2021). River Tea Tree Oil: Composition, Antimicrobial and Antioxidant Activities, and Potential Applications in Agriculture. Plants, 10(10), 2105. https://doi.org/10.3390/plants10102105