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The Effect of Clove Oil on the Expression of Xly Gene in Tomato Infected with *Fusarium oxysporum*

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ABSTRACT

Background: *Fusarium oxysporum* is a soil-borne pathogen and a very common cause of tomato root rot. This pathogen infects the plant's vascular system, contributing to wilting, stunted growth and stunted growth.

Methods: The determination of the minimum inhibitory concentration (MIC) of clove extract against *Fusarium oxysporum* was used to assess test its antimicrobial activity. The results indicated that the clove extract was effective at low concentrations, inhibiting the growth of a significant proportion of fungal isolates. However, higher concentrations showed a low inhibitory effect. Although these concentrations were sufficient to inhibit growth, they did not lead to complete cell death. Therefore, there is an urgent need to find alternative solutions to combat Fusarium wilt in tomato plants. Clove oil, extracted from the buds of the aromatic Syzygium tree, is known for its antimicrobial and antifungal properties. It contains eugenol, a natural compound that exhibits strong fungicidal activity against various plant pathogens. Previous research has indicated that gene expression of this gene is increased in response to infection with the fungus *Fusarium oxysporum*.

Rusult: In this study, we aimed to investigate the effect of clove oil treatment on the expression of the Xly gene in tomato plants infected with Fusarium wilt. The plants were treated and the expression levels of the Xly gene were measured using quantitative polymerase chain reaction (qPCR) at different time points after treatment. Preliminary results indicate a significant increase in the expression of the Xly gene in tomato plants infected with *Fusarium oxporum*. However, when the Fusarium-infected tomato plants were treated with clove oil, the expression levels of the Xly gene were found to be significantly lower than in the control group. This finding suggests that clove oil treatment may help modify the defense response of tomato plants and possibly mitigate the effects of Fusarium wilt. The results of this study provide valuable insights into the potential use of clove oil as a natural and environmentally friendly alternative to chemical fungicides in preventing Fusarium wilt in tomato crops. The decreased expression of the Xly gene suggests that treating plants with clove oil may combat the spread of the disease and help them resist *Fusarium oxysporum*.

Key words: Clove oil, *Fusarium oxysporum*, Xly gene.

INTRODUCTION

Compared to the control group, expression of the Xly gene persisted for five days post-inoculation in chitosan-coated tomatoes treated with clove oil. The Xly gene plays a role in the mechanism by which clove oil-treated tomatoes exhibit higher expression of the Xly gene. Because xylem obstruction results from a severe attack and because plant defenses were ineffective, the evidence suggests that the effect of clove oil activated the plant's defense mechanism, but was not selective for the type of defense mechanism used to prevent *F. oxysporum* attack (Khan *et al.,* 2022; Yahya *et al*., 2024). *Fusarium oxysporum* is a soil-borne fungus that often causes tomato root rot. One of its genes, the xly gene, induces the plant to develop disease resistance through gene overexpression. Fusarium has been observed to degrade and enter the plant cell wall using an enzyme called xylanase. It degrades xylan, the main component of the plant cell wall, to remove xylose residues. This action will lead the plant to develop systemic acquired resistance (Deblais *et al.,* 2023; Yaseen *et al*., 2025; Al-Maliki *et al.,* 2025). Eugenol is found in clove oil, also known as *Eugenia caryophyllata*. Previous research has shown that eugenol inhibits the production of xylanase by the fungus *Fusarium oxporum* and inhibits spore germination. Based on this information, eugenol may protect tomato plants from *Fusarium oxporum* infection by disrupting xylanase activity (Dong *et al*., 2021: Alyasiri *et al*., 2025). As plants grow, the xylose isomerase (Xly) gene converts d-xylose to d-xylulose. This is a specific, irreversible process that occurs in the xylem vessels. The product of this reaction is important for maintaining vessel function and is a source of carbon. Researchers have also suggested

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that the xly gene plays a role in plant defense mechanisms, contributing to a deeper understanding of its function (Li *et al*., 2022; Yaseen *et al*., 2024). *Fusarium oxysporum* lycopersici is a host-specific pathogen that causes tomato wilt. When tomatoes are infected with this pathogen, it disrupts the plant's xylem function, ranging from browning to clogging with spores and fungi. Symptoms of infected plants include prolonged wilting, starting from the lowest leaf and extending up the entire stem, followed by yellowing and necrosis (Jamil *et al*., 2021; Obaid *et al.,* 2025).

MATERIALS AND METHODS

Extraction of clove oil extract

Extract preparation (oil extract): A clevenger apparatus is used to extract the volatile clove oil from the clove plant (*Syzygium aromaticum*). 10 grams of ground cloves are mixed with 100 ml of ethanol, the mixture is distilled and the distillate is transferred quantitatively to a separating funnel. The mixture is gently evaporated to obtain eugenol as a pale-yellow oil.

Isolation and identification of fusarium oxysporum

All samples were cultured in B.H.I. broth medium and incubated at 28°C for 48-72 h to promote fungal growth and re-cultured on fresh agar plates to obtain well-isolated pure colonies.

Minimum inhibitory concentration (MIC)

The lowest concentration of an antimicrobial agent that can inhibit visible fungal growth without killing it is defined as the MIC. The broth microdilution method in a 96-well polystyrene plate is most suitable for determining MIC values and was performed to quantitatively study the antimicrobial activity of clove extract against fungal isolates *in vitro,* according to Clinical and Laboratory Standards Institute (CLSI) guidelines.

The primers preparation

The primers were freeze-dried, then dissolved in free ddH_2O to give a final concentration of 100 pmol/µL as a stock solution and maintained at -20°C. To prepare a 10 pmol/µL concentration as a working suspension, 10 µL of the stock solution was dissolved in 90 µL of free ddH_2O to reach a final volume of 100 µL.

I. Preparation of antimicrobial agents

A stock solution of clove was prepared in 1.5 ml microcentrifuge tubes (Eppendorf) by dissolving it in 10% DMSO to a final concentration of 50 mg/ml. Serial dilutions of the stock solution were made to concentrations ranging from 25 mg/ml to 0.09 mg/ml using potato dextrose medium in a 96-well plate. 100 µL of the sterile base solution was transferred to plate A1 of a microtiter plate, which contained 100 µL of sterile MHB microfractions, producing a 50% dilution of the base solution to 25 mg/ml. After thoroughly mixing the contents of each well, the aliquots from A1 were transferred to the corresponding wells in B1 (which also contained 100 µL of MHB microfractions), followed by mixing, producing another 50% dilution of the antibiotic (to 12.5 mg/ml). The previous process was repeated for each row to obtain the following dilutions: 6.25 µg/ml, 3.1 mg/ml, 1.5 mg/ml, 0.7 mg/ml, 0.3 mg/ml, 0.19 mg/ml and finally 0.09 mg.

II. Preparation of inoculums

Isolates were obtained from fungal agar cultures. For the minimum inhibitory concentration (MIC) test, 3 to 5 well-isolated colonies were selected from the agar plate. A sterile loop was used to transfer the culture to a tube containing 4-5 ml of palladium broth, which was then incubated at 28°C for 4 h until its turbidity reached 0SS.5 McFarland's standard. The turbidity of the broth culture was adjusted with sterile normal saline (MHB) or normal saline and then diluted 1:100 by adding 100 µL of the fungal suspension to 9900 µL of MHB. Next, 100 µL of the standard fungal suspension was added to each well containing 100 µL of the diluted antimicrobial agents, bringing the total volume to 200 µL per well. Column 11 of the microplate was used as a positive control, containing broth, dimethyl sulfoxide (DMSO) (the solvent used in antimicrobials) and a fungal sample. Column 12 was used as a negative control, containing broth and DMSO without a fungal sample. Both microplates were incubated at 28°C for 24-48 hours. MIC values were determined visually by adding 30 µL of Alamar Blue dye to each well and incubating at 37°C for 1 hour. Alamar Blue, a resazurin-based indicator, changes color from blue (non-fluorescent) to red (highly fluorescent) in the presence of live cells. The MIC was recorded as the lowest concentration at which no visible growth was observed. All MIC values were determined, at least in duplicate, to confirm activity.

Gene expression analysis

Reverse transcriptase polymerase chain reaction (RT-PCR) was performed to verify the gene expression of the Xly gene in tomato root tissues. Xly gene-specific nested primers were used to amplify complementary DNA (cDNA). The first round of PCR was performed using primers for the disease-associated PR1 gene (5′-GCAGCTCGTAG ACAAGT TGGAGTCG-3′) and (5′-TGTTGCATCCTGC AGTCCCC-3′). The second round of PCR was performed using primers for the reference GAPDH gene (5′-CTGCTCTCTCA GTAGC CAACAC-3′) and (5′-CTTTCCTCC AATAGCAGAGG TTT-3′). This nested primer strategy was also used on the xylanase gene specific for strain 1 to ensure that the Xly gene cloned from FOL was not a pseudogene for tomato xylanase. Control genes for tomato actin and 18s rRNA were also amplified to check sample loading and RNA content variation. All PCR reactions were performed using a PTC-200 Peltier Thermal Cycler (MJ Research), with the following reaction conditions: initial denaturation at 94°C for 3 min, 30 cycles at 94°C for 1 min, 30 s at an annealing temperature between 55 and 65°C and final extension at 72°C for 1 min. Total RNA was isolated and polymerase chain reaction (PCR) performed as

previously described. Tomato roots were collected 2 days post-treatment (2DAT) with clove oil following infection with *Fusarium oxysporum* f. sp. lycopersici (FOL). A 100 mg sample of tomato roots was ground in liquid nitrogen using a mortar and pestle. The frozen powder was suspended in phosphate buffer and centrifuged at 5,000 g. The upper liquid was discarded and the pellets were resuspended in 1 ml of Trizol reagent (Gibco BRL). The sample was processed and RNA was collected following the manufacturer's instructions for the Trizol reagent. RNA was cleaned up using the RNeasy Plant Mini Kit (Qiagen) following the manufacturer's instructions. Two micrograms of RNA were used for complementary RNA synthesis using oligo-dT primers and Superscript II reverse transcriptase (Gibco BRL). An equal volume of reaction mixture containing identical components, but lacking reverse transcriptase, was prepared for each sample to be used as a control for genomic DNA contamination.

Statistical analysis

Statistical analysis was performed using SPSS version 24. The results were presented as mean±standard error. To compare the means, a one-way ANOVA was conducted, followed by Duncan's multiple range test for post-hoc analysis. A significance level of $P \leq 0.05$ was considered to indicate a statistically significant difference.

***Fusarium oxysporum* infection in tomato plants**

Tomato plants are infected with the fungus Fusarium oxysporum. *Fusarium oxysporum* lycopersici is a host-specific pathogen that causes tomato wilt (Wang *et al*., 2022). When tomatoes are infected with this pathogen, the plant's xylem functions are disrupted, ranging from browning of the wood to clogging with spores and fungi. Symptoms of infected plants include prolonged wilting, starting from the lowest leaf and extending to the entire stem, followed by yellowing and necrosis (Yoo *et al*., 2021). The aim of this work was to determine whether clove oil has a significant effect on the expression of the Xly gene in tomato plants infected with the fungus *Fusarium oxyporum*. Recent studies have shown a significant effect compared to tomato plants not treated with clove oil (Bastas *et al*., 2020). *Fusarium oxysporum* is a plant pathogen that infects roots and stems, then spreads to the leaves, causing wilting and death. Clove oil has been shown to reduce infection in tomato plants by forming a thick cuticle layer and cell wall in the epidermal tissue, preventing the organism from invading the plant. This means that treating the disease can be expensive and less effective than taking preventative measures, such as using clove oil as a safe and natural fungicide (Michalak *et al.,* 2022).

Xly gene and its role in tomato defense mechanism

In 1987, Murray and colleagues discovered a unique complementary DNA (cDNA) fragment in tomato leaves infected with pathogens, but absent in healthy leaves. Saidani's Xly clone and later XDH, was used in a wave of research by 1989 (Chen *et al.,* 2023). EcoR1 digestion of tomato genomic DNA produced six bands when the clone was used as a probe in a DNA gel blot assay. It was noted that while the last three bands were four times more prevalent in infected leaves, the first three bands were similarly distributed in healthy and pathogen-resistant leaves (Liu *et al*., 2022). The Xly gene initiates the use of NAD+ as a hydride acceptor, transferring electrons from NADH to oxygen without wasting O2. A new enzyme called xylose reductase (XR) has also been found in cells. This enzyme is made from NADPH and is said to be essential for pathogen growth in the host plant, but little is known about this yet. This action may eliminate the Xly pathway and reduce disease symptoms in a particular plant. Considering all factors, the discovery of increased domain, protein and enzyme activity is believed to be a good indicator that the plant can develop a defense mechanism. Several studies have documented increased expression of Xly gene products in a wide range of pathogen species (Bhuyan *et al*., 2020).

Clove oil as a potential antifungal agent

Clove oil has been observed to act as an antifungal agent. Therefore, the use of clove oil and its components, such as eugenol, as antifungal agents may lead to new ways of using genetic engineering to treat plant diseases in an environmentally friendly manner. While eugenol is generally considered a harmless substance, genetically modified plants that overexpress the Xly gene and PR proteins may exhibit enhanced disease resistance (Milićević *et al*., 2022). Previous research has also shown that postharvest application of eugenol helps inhibit banana anthracnose, apple scab caused by Elsinoe fawcetti or Venturia inaequalis and tomato surface rot caused by various fungi (Ju *et al*., 2020). Clove oil is found in plants such as *Eugenia caryophyllata* and *Syzygium aromaticum*. Clove oil also contains eugenol, an antifungal substance (Ulanowska *et al*., 2021). Previous research has demonstrated the high antifungal activity of eugenol, but the exact mechanism behind this activity remains unknown. By studying its effect on gene expression of the Xly gene, we aim to shed more light on how eugenol inhibits fungi and its potential as an antifungal (Hiwandika *et al*., 2021).

RESULTS AND DISCUSSION

Determination of minimum inhibitory concentrations (MIC) for clove extract

Fig 1 shows the results of the antimicrobial activity of cloves against *Fusarium oxporum in vitro*. The minimum inhibitory concentrations (MICs) of the clove extract ranged from 0.09 to 50 mg/ml. A 62.5% (5/8) growth inhibition of *Fusarium oxporum* isolates was observed at a concentration of 3.1 mg/ml, but a 37.5% (3/8) growth inhibition of *Fusarium oxporum* isolates was observed at a concentration of 6.2 mg/ml. It should be noted that high MICs are only required to inhibit the growth of the isolates, not to kill them, as shown in Fig 1 and 2.

The gene expression of the Xly gene in the quantitative polymerase chain reaction (qPCR) is shown in Fig 3. This is based on the fluorescence of the FAM channel at the cycle number. The $2^{-\Delta\Delta Ct}$ value, which represents the relative change in gene expression, decreased significantly from 1.2384 before treatment to 0.0361 after treatment. This value indicates a significant decrease in the gene expression of the Xly gene after treatment. By looking at the 2-ΓCt values statistically, we saw that the gene expression of the Xly gene decreased significantly ($p \leq 0.05$), supporting the idea that the treatment significantly suppressed the expression of the Xly gene.

Comparison of Xly gene expression levels

One way to analyze the effect of clove oil on Xly gene expression in tomatoes infected with *Fusarium oxsporum* is to compare gene expression levels before and after treatment with clove oil. This can be done using semi-quantitative reverse transcriptase PCR (RT-PCR). The results indicated that Xly gene expression was absent in the positive control group, while it was present in plants inoculated with *Fusarium oxsporum*. This may be due to the genetic resistance of tomato plants to *Fusarium oxsporum*. Clove oil treatment subsequently affects the expression of this Xly gene, as demonstrated by the undissolved clove oil treatment. In plants inoculated with *Fusarium oxysporum*, expression of the Xly gene could still be detected, albeit at a lower intensity than in the control group. This situation was also observed with the dissolved clove oil treatment, but in this case, we observed a decrease in Xly gene expression compared to the control group. Unlike these two treatments, the Xly gene was not found in plants that were infected with *Fusarium oxysporum* and then given clove oil along with the carrier treatment. This result can be interpreted as indicating that clove oil is indeed capable of inhibiting the expression of the Xly gene in infected plants.

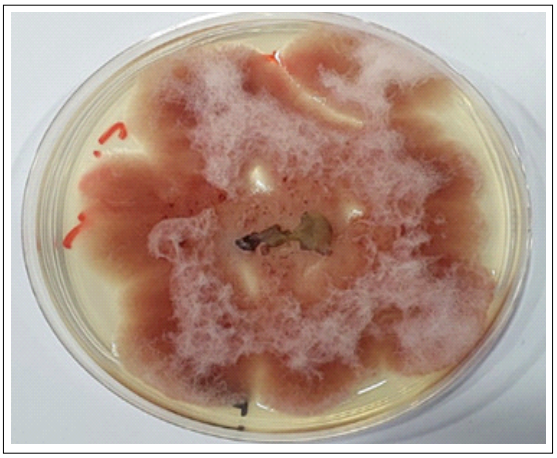


Fig 1: *Fusarium oxporum* isolated from tomatoes grown on Sabouraud dextrose agar at 28°C for 72 hours.

In vitro studies have shown that clove oil stops the germination of several types of fungal spores. In our study, it stopped *Fusarium oxysporum* spores from germination 100% of the time. In addition to inhibiting spore germination, our results demonstrate that clove oil is also highly effective at inhibiting *Fusarium* growth within the tomato plant itself, even at very low concentrations (Makhlouf *et al*., 2021; Hasan *et al*., 2024). RT-qPCR showed that the glyceraldehyde 3-phosphate dehydrogenase (G3PDH) gene was 14 times lower in infected plants treated with a 2% clove oil solution compared to healthy plants that were not infected. The G3PDH gene is a housekeeping gene that is always expressed and was used as a control. There was a greater variability in gene expression in infected plants compared to healthy plants. This decrease in G3PDH gene expression is probably due to the plant's stress response in an attempt to maintain normal physiological function; altered gene expression could also be due to the effect of clove oil (Soliman *et al*., 2022; Hameed *et al*., 2025).

The spores and culture of *F. oxysporum f. sp. lycopersici* were grown on PDA. The effects of clove oil on *F. oxysporum* were seen under a microscope. The growth of mycelial and

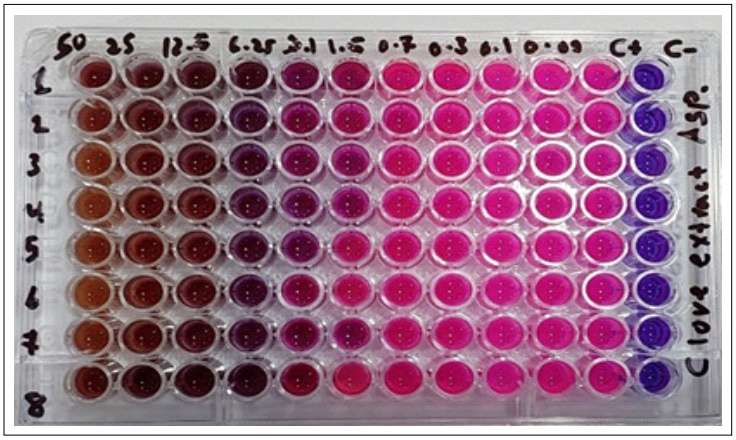


Fig 2: Shows the results of the broth microdilution method used to determine the minimum inhibitory concentrations (MICs) of the clove extract. (C-) Negative control (broth only), (C+) Positive control (fungus only, broth).

aerial hyphae was slowed down, the turgor of the mycelial and spores was lost and fewer spores were produced. Microscopic observations of hyphal cells revealed that 0.04% clove oil caused time-dependent damage to morphological characteristics (Valente *et al*., 2023; Hasan *et al*., 2023). After 24 h, hyphal cells treated with clove oil showed swollen spherical hyphae with thickening and vesicle formation in the cell wall, whereas cells that were untreated with clove oil maintained their normal morphology. After 48 hours, the treated cells displayed an irregular cytoplasmic mass, with occasional observations of cytoplasmic granulation (Saleh *et al*., 2023). After 72 hours, only fragmented cells and debris were observed and the majority of cells had undergone plasmolysis, leaving an empty and collapsed cell wall. These changes in both growth and lysis show how strong clove oil is on cells. Our observations of cell damage followed by progressive cytoplasmic changes and cell wall collapse show that the killing effect starts with damage to the cell wall and membrane, which lets cytoplasm leak out. Cellular changes caused by oil are common ones that happen when lipophilic cell wall components interact with each other and mess up osmoregulation (Pandey *et al*., 2020; Hasan *et al*., 2024). Using reverse transcriptase polymerase chain reaction (RT-PCR), we analyzed the effect of clove oil treatment on the Xly wood hydrolase gene in infected tomato plants. We used primers that were specific for the Xly gene and when we amplified RNA from both control and treated plants, we got a band that was the right size. The densitometric analysis of the gel showed that infected control plants had lower levels of Xly mRNA than healthy control plants (Jamil *et al*., 2021; Hasan *et al*., 2024). Infection of tomato plants with *F. oxysporum* suppresses gene expression of the Xly gene (Table 1). To amplify RNA isolated from control and treated plants, primers specific for amplifying RNA isolated from control and treated plants produced a band of the expected size. The resulting gel underwent densitometric analysis (Kumar *et al*., 2021; Abd El-Rahmana *et al*., 2024; Ghiath *et al.,* 2025). The effect of clove oil on xylan gene expression in infected tomato plants is significant. This study shows that clove oil might be able to stop *F. oxysporum* from growing by slowing down the transcription of xylan genes. There is less xylan gene expression in infected plants that were treated with clove oil at 750 and 1000 ppm concentrations compared to 500 ppm. This shows the pattern of xyl gene expression. The decrease in gene expression is dose-dependent, with the lowest level at 1000 ppm clove oil. Several potential mechanisms exist for the decreased gene expression; however, the damage caused by clove oil results in the leakage of cell contents (Yarra *et al.,* 2021; Al-Khuzaay *et al*., 2024). Clove oil has been shown to inhibit the germination and growth of several plant pathogenic fungi. 100% inhibition of *F. oxysporum* was demonstrated at a concentration of 500 ppm clove oil. Clove oil also reduced the biomass and growth rate of *F. oxysporum* in a dose-dependent manner. The results showed significant inhibition of the growth and germination of *F. oxysporum* in a CCGE medium containing 250 ppm clove oil (Sharmin *et al*., 2020; Al-Maliki *et al*., 2025: Obaid *et al.,* 2025).

Table 1: Gene expression of the Xly gene; values are given by the mean±SE.

Xly gene	ΔCt	ΔΔCt	Fold
Before	3.8±1.1	0.44±0.07	1.023
After	8.4±1.2	5.04±0.7	0.0361
P. value	0.04	0.012	0.001

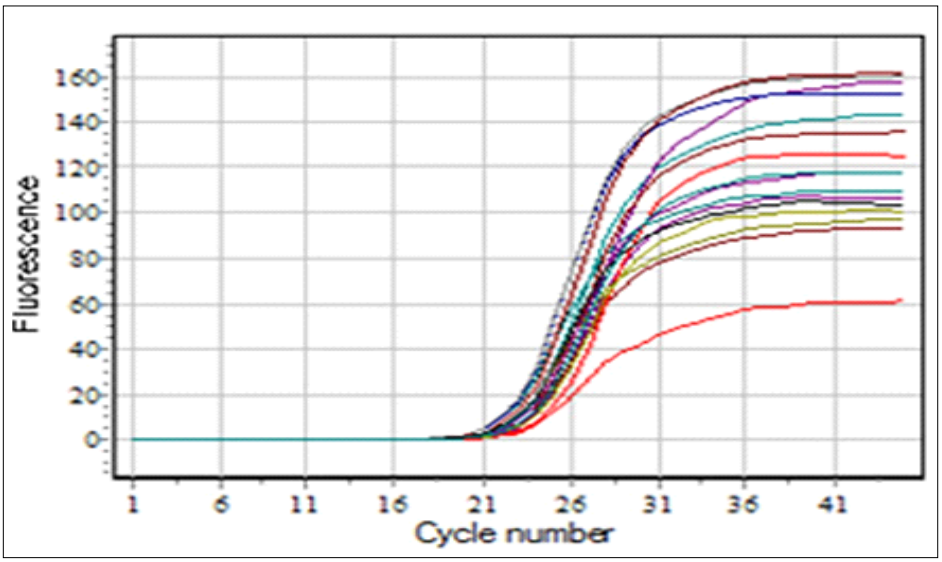


Fig 3: Dependence of FAM channel fluorescence on cycle number.

CONCLUSION

This study investigates the effect of clove oil on gene expression of the Xly gene in tomatoes infected with *F. oxysporum.* Clove oil can be used to eliminate fungi in plants, but it does not directly kill them. Rather, it inhibits their growth, particularly in *F. oxysporum.* It is evident that high concentrations of clove oil inhibit fungal growth more rapidly. Due to the polygenic nature of the xyl protein gene, real-time polymerase chain reaction (PCR) should be repeated using a common primer and probe. Further research is needed to find alternative methods to inhibit gene expression and identify compounds that can more effectively replace its function. Investigating the inhibitory mechanism of *F. clovipodium* through xyl protein gene inhibition is of paramount importance. This requires advanced molecular techniques and technological approaches that can efficiently inhibit gene expression. Modified real-time polymerase chain reaction (RT-PCR), along with improved primer and probe design, provides promising insights. This research contributes to improved prevention of vascular diseases and expands the horizons of gene inhibition beyond previous studies. Collaboration between scientific institutes and universities around the world is crucial for advancing this field.

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Additional information

Dominion codes

There are no data deposits associated with this work.

Conflict of interest

The authors warrant that there is no conflict of interest between the authors.

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