

Inhibition of *Rhizopus stolonifer* Growth and Pectin Degrading Enzymes by Tannic Acid with an *in vitro*, *in vivo*, and *in silico* Assessment

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Tannic acid has attracted considerable attention as a natural inhibitor of fungi. The antifungal activity of tannic acid was evaluated by assessing fungal growth and the activities of polygalacturonase and pectin methylesterase. Fungal growth was stimulated at 200 $\mu\text{g mL}^{-1}$ (112.6%), but significantly reduced at higher concentrations, reaching 12.4% at 1000 $\mu\text{g mL}^{-1}$ (87.6% inhibition) of tannic acid. *In vivo*, tannic acid effectively controlled postharvest soft rot in muskmelon, achieving up to 81.4% disease control. Enzyme activities in the culture medium declined markedly, reduced to 15.3% for polygalacturonase and 20.1% for pectin methylesterase. However, it should be noted that this reduction may result from either direct inhibition of enzyme catalytic activity or reduced enzyme secretion due to suppressed fungal growth, or a combination of both. Molecular docking was performed using heterologous protein structures (endopolygalacturonase from *Fusarium verticillioides*, PDB: 1HG8; pectin methylesterase from *Daucus carota*, PDB: 1GQ8) as representative models. Because these proteins are not from *R. stolonifer*, the docking results have no direct biological relevance to the target pathogen and cannot be used to infer binding to *R. stolonifer* enzymes. Thus, the experimental data demonstrate that tannic acid reduces extracellular pectin-degrading enzyme activity in fungal cultures, but the mechanism remains unresolved.

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INTRODUCTION

Postharvest losses of fruits and vegetables remain a major concern worldwide, with fungal pathogens responsible for approximately 20% to 25% of total losses occurring along the supply chain each year (Droby *et al.* 1992). Such deterioration not only reduces economic returns, but it also compromises fruit quality, shelf life, and consumer acceptance. Among postharvest fungal pathogens, a fungus tentatively identified

as *Rhizopus stolonifer*, a member of the Zygomycetes, is recognized as one of the most aggressive and rapidly growing species, causing severe decay in a wide range of horticultural commodities (Al-Rajhi *et al.* 2023; Liu *et al.* 2024). This pathogen affects more than 100 fruit species, including tomatoes, strawberries, and melons, and is considered one of the most destructive fungi during postharvest storage (Du *et al.* 2023). Diseases caused by *R. stolonifer* are commonly referred to as *Rhizopus* rot, soft rot, or black mold. Under favorable temperature and humidity conditions, visible symptoms can develop within 24 h, accompanied by vigorous mycelial growth and the formation of dense gray sporangia on the fruit surface. Excessive softening is a critical factor contributing to quality loss, as it results in tissue collapse and increased defenselessness to secondary infections. These textural deviations are primarily linked with the dissolution of the middle lamella, reduced cell-to-cell adhesion, and weakening of parenchyma cell walls due to the action of cell wall-modifying enzymes, including those secreted by the fungus during infection as well as endogenous fruit ripening enzymes (Yan *et al.* 2022). In the context of fungal infection, pathogen-secreted enzymes are primarily responsible for the rapid tissue maceration characteristic of soft rot diseases.

Pectin is a major structural polysaccharide of the primary cell wall and middle lamella and plays a central role in maintaining fruit firmness. Alterations in pectin structure are closely linked to fruit softening during ripening and pathogen attack (Zhang *et al.* 2018). Softening is generally accompanied by pectin solubilization and depolymerization. These processes are largely regulated by alterations in the activities of pectin methylesterase (PME) and polygalacturonase (PG) (Zhang *et al.* 2021). During storage, fruits and vegetables become increasingly vulnerable to fungal invasion due to their high water content, acidic pH, nutrient-rich composition, and declining natural resistance mechanisms. The degradation of the pectin backbone involves the coordinated action of multiple enzymes, including PME, endo- and exo-polygalacturonases, pectate lyase, exopectate lyase, and endo-pectin lyase (Siddiqui *et al.* 2012).

During spore germination, *R. stolonifer* secretes a range of extracellular proteins, enzymes, and amino acids that facilitate host tissue invasion. Among these, the fungus produces polygalacturonase (PG) and pectin methylesterase (PME), which play key roles in degrading pectic substances, enabling rapid digestion of host cell walls, electrolyte leakage, tissue maceration, and subsequent decay (Petrasch *et al.* 2019). While melon fruits also produce endogenous PG and PME during natural ripening, the focus of this study is on the fungal-derived enzymes that accelerate decay during infection. Traditionally, *R. stolonifer* has been considered a wound-dependent pathogen, entering host tissues through mechanical injuries incurred during harvesting and postharvest handling. However, evidence has shown that the fungus can infect intact tomato and peach fruits when spores germinate in the presence of external nutrient sources, indicating that infection may occur even in the absence of visible wounds (Baggio *et al.* 2016).

Given the central role of cell wall-degrading enzymes in fruit softening and decay, various strategies have been explored to suppress their activity and extend postharvest life. Calcium chloride treatments have been shown to delay fruit softening by reducing the activities of PG, PME, and cellulase while maintaining higher protopectin levels (Lv *et al.* 2020). Similarly, 1-methylcyclopropene (1-MCP) treatment significantly delays firmness loss and disease development in harvested strawberries. This effect has been attributed to the inhibition of several cell wall-degrading enzymes, including α -arabinofuranosidase,

PG, β -galactosidase, and β -xylosidase, along with a reduction in the growth and severity of fungal pathogens such as *Botrytis cinerea* and *R. stolonifer* (Langer *et al.* 2022). Alkaline treatments, such as sodium bicarbonate, have also been reported to suppress PG activity during fungal pathogenesis, although PME activity may increase under elevated pH conditions (Venditti *et al.* 2018).

Natural polyphenolic compounds, particularly tannins, have gained increasing attention as eco-friendly alternatives for postharvest disease control. Tannins exhibit broad antimicrobial activity and contribute to disease suppression through enzyme inhibition, oxidative stress reduction, and interference with microbial metabolism (Iqbal and Poór 2025). Tannic acid, a widely distributed plant-derived polyphenol composed of gallic acid esterified to glucose, is especially attractive because of its low cost, abundance, and biocompatibility. Antifungal activity of tannic acid against postharvest fungal pathogens has been increasingly documented. Previous studies have shown that tannic acid effectively inhibits *Penicillium digitatum* and *Alternaria alternata* via mitochondrial dysfunction, glycolytic enzyme interference, and oxidative stress induction (Zhu *et al.* 2019; Jiang *et al.* 2023; Wang *et al.* 2023). Regarding *R. stolonifer*, Liu *et al.* (2024) comprehensively reviewed its pathogenicity, while Du *et al.* (2023) developed controlled-release systems for its control. However, systematic investigations on the direct inhibitory effects of tannic acid on *R. stolonifer* growth and pectinolytic enzyme activities remain limited. Furthermore, the molecular basis of such inhibition—specifically the binding interactions between tannic acid and catalytic residues of polygalacturonase and pectin methylesterase—has not been elucidated through structural approaches such as molecular docking. Recent studies have demonstrated that chitosan-based edible coatings reinforced with tannic acid and ferric ions effectively preserve postharvest quality by reducing respiration rates, minimizing weight loss, and delaying decay progression in fruits such as wax apple (Guo *et al.* 2025). Moreover, tannins have been reported to inhibit a wide range of microorganisms *in vitro*, with effective concentrations ranging from 0.012 g L⁻¹ for pathogenic fungi to the range 0.5 to 20 g L⁻¹ for bacteria (Njokuocha 2020). Nevertheless, the antimicrobial efficacy of tannins is highly dependent on their chemical structure and the susceptibility of the target microbial species (Anderson *et al.* 2012). Molecular docking is an *in silico* technique used to predict the binding orientation and interaction strength between bioactive compounds and target proteins, providing insight into their possible mechanisms of action (Alsalamah *et al.* 2023; Qanash *et al.* 2023). In antifungal and antimicrobial studies, docking analysis complements experimental data by identifying key molecular interactions that may underlie growth inhibition and enzyme suppression (Yahya *et al.* 2022; Alsalamah *et al.* 2025; Adelskareema *et al.* 2026).

Despite the increasing interest in tannic acid as a natural antifungal compound, its inhibitory effects on *R. stolonifer* and the associated pectin-degrading enzymes remain insufficiently understood. In particular, comprehensive studies integrating biological evaluation with molecular-level mechanistic analysis are still lacking. Therefore, the present study was designed to investigate the antifungal activity of tannic acid against *R. stolonifer* and to evaluate its effects on the two major pectinolytic enzymes involved in fruit tissue maceration, polygalacturonase and pectin methylesterase, using *in vitro* assays complemented by *in silico* molecular docking analysis.

The novelty of this work lies in main aspects. First, unlike previous studies that mainly focused on other phytopathogenic fungi, this study systematically investigates the

concentration-dependent effects of tannic acid on *R. stolonifer* growth while simultaneously evaluating its influence on both polygalacturonase and pectin methylesterase within the same experimental framework. Second, this study provides the first molecular docking analysis exploring the interactions between tannic acid and the catalytic sites of pectinolytic enzymes, offering structural insights into the possible mechanism underlying enzyme inhibition.

It should be emphasized that the present work was conducted exclusively under *in vitro* and *in vivo* conditions on melon fruits. Accordingly, the findings should be considered preliminary and require confirmation through fruit-based efficacy studies before practical postharvest applications can be recommended.

EXPERIMENTAL

Isolation of the Causal Pathogen

Melon fruits (*Cucumis melo* L.) exhibiting typical soft rot symptoms were collected during postharvest storage. Small tissue segments (5 mm²) were excised from the margin between healthy and infected tissues, surface-sterilized with 1% sodium hypochlorite for 1.0 min, rinsed three times with sterile distilled water, and air-dried under aseptic conditions. The sterilized tissues were placed on potato dextrose agar (PDA) plates supplemented with streptomycin (50 mg L⁻¹) to suppress bacterial growth. It should be noted that sodium hypochlorite treatment, while effective for eliminating surface contaminants, may introduce selection bias by preferentially affecting certain fungal taxa due to differential tolerance to oxidizing agents [6†L26-L33]. However, because the target pathogen (*R. stolonifer*) was consistently isolated from symptomatic tissues and identified based on its characteristic morphological features, this limitation does not compromise the primary identification. Plates were incubated at 25±2 °C for 3 to 5 days. Emerging fungal colonies were sub-cultured repeatedly to obtain pure isolates. Identification of the fungal isolate was performed based on cultural and microscopic characteristics (rate of growth, initial and final color, hyphae septation, sporangia and sporangiospores shape, and presence of stolons and rhizoids) according to Schipper (1984). It should be noted that molecular identification (*e.g.*, ITS sequencing) was not performed; therefore, the isolate is tentatively identified as *R. stolonifer* based on morphological characteristics.

Effect of Tannic Acid on Growth and Pectinolytic Enzyme Activities of *Rhizopus stolonifer* in Substrate-Supplemented Broth

Tannic acid (Sigma-Aldrich, USA) was dissolved in sterile distilled water to prepare stock solutions. Working concentrations of 200, 400, 600, 800, and 1000 µg mL⁻¹ were prepared by serial dilution. *R. stolonifer* was maintained on PDA at 28 ± 2 °C. For experiments, a 5 mm mycelial disc from a 7-day-old culture was inoculated into 50 mL of liquid minimal medium supplemented with 1% (w/v) pectin (for polygalacturonase) or 1% citrus pectin (for pectin methylesterase) in 250 mL Erlenmeyer flasks (Bakri *et al.* 2022). Tannic acid was added at the desired concentrations. Flasks without tannic acid served as controls. Cultures were incubated at 28 °C with continuous shaking at 120 rpm for 5 days. Fungal biomass was determined by harvesting mycelial mats *via* filtration through pre-

weighed filter paper, followed by drying at 60 °C until constant weight. Fungal growth (%) was calculated relative to the control:

$$\text{Fungal growth (\%)} = \frac{\text{Dry weight of treated mycelium}}{\text{Dry weight of control mycelium}} \times 100 \quad (1)$$

Fungal inhibition (%) was calculated as:

$$\text{Fungal inhibition (\%)} = 100 - \text{Fungal growth (\%)} \quad (2)$$

Enzyme Extraction

The culture filtrate was collected and centrifuged at $10,000 \times g$ for 15 min at 4 °C to remove residual mycelium. The supernatant was used as the crude enzyme extract.

Polygalacturonase and Pectin Methylesterase Assays

Polygalacturonase activity was measured by quantifying the reducing sugars released from 1% (w/v) polygalacturonic acid using the DNS method at 540 nm (Miller 1959; Carmona *et al.* 1998). Pectin methylesterase activity was determined by titrating the released carboxyl groups from 0.5% (w/v) citrus pectin with 0.01 M NaOH (L'Enfant *et al.* 2015). One unit of enzyme activity was defined as the amount of enzyme releasing 1.0 μmol of product per min under the assay conditions.

In vivo Evaluation of Tannic Acid against *Rhizopus stolonifer*-induced Soft Rot in Melon Fruits

Ten healthy muskmelon fruits of uniform size, maturity, and free from visible defects of the same source of infected fruits were obtained from a local commercial orchard. Fruits were washed with running tap water, surface-disinfected by immersion in 1% (v/v) sodium hypochlorite solution for 2 min, rinsed three times with sterile distilled water, and air-dried under aseptic conditions. A single wound (approximately 4 mm in diameter and 3 mm deep) was made at the equatorial region of each fruit using a sterile cork borer. Tannic acid solutions (0.25, 0.50, and 1.00 mg mL⁻¹) were applied by pipetting 20 μL into each wound. Control fruits received sterile distilled water. After 2 h at room temperature, each wound was inoculated with 20 μL of a *R. stolonifer* spore suspension (1×10^6 spores mL⁻¹). The fruits were placed in plastic containers lined with moist filter paper to maintain approximately 95% relative humidity and incubated at 25 °C for 7 days (Zhu *et al.* 2025). Disease development was assessed after seven days by recording disease incidence, lesion diameter, and control efficacy according to the following equations:

$$\text{Disease incidence (\%)} = \frac{\text{Number of infected fruits}}{\text{Total number of fruits}} \times 100 \quad (3)$$

$$\text{Control efficacy (\%)} = \frac{\text{Lesion diameter of control} - \text{Lesion diameter of treatment}}{\text{Lesion diameter of control}} \times 100 \quad (4)$$

Lesion diameter (mm) was measured using a digital caliper as the average of two perpendicular measurements.

Docking Investigation

Protein preparation

The three-dimensional crystal structures of endopolygalacturonase (PDB ID: 1HG8, from *Fusarium verticillioides*) and pectin methylesterase (PDB ID: 1GQ8, from *Daucus carota*) were obtained from the Protein Data Bank (<http://www.rcsb.org>). It should be noted that these structures are not from *R. stolonifer*; however, they serve as representative structural models for the respective enzyme families, providing a framework for predicting potential binding interactions. The absence of a crystal structure of *R. stolonifer* homologs represents a limitation of this *in silico* approach.

Ligand preparation

Tannic acid was sketched in MOE and protonated, then energy-minimized using the MMFF94x force field to generate a stable, low-energy conformation suitable for docking.

Active site identification and docking protocol

The active sites of both enzymes were defined using dummy atoms to accurately delineate them. Molecular docking was performed using the MOE docking module, applying the triangle matcher placement method and London dG scoring for initial ranking, followed by refinement using the GBVI/WSA dG scoring function. Multiple docking poses were generated, and the best-ranked conformations were selected based on docking score, RMSD, and interaction pattern.

A limitation of the present docking analysis is the absence of crystallographic structures of polygalacturonase and pectin methylesterase from *R. stolonifer*. The structures used (1HG8 and 1GQ8) are derived from *F. verticillioides* and *Daucus carota*, respectively. While these proteins belong to the same enzyme families and share conserved catalytic domains and active site architectures, the binding interactions predicted may not fully reflect those in the *R. stolonifer* enzymes. Future studies should aim to obtain or model the *R. stolonifer* homologs (e.g., via homology modeling) once their sequences become available to enable more accurate docking predictions.

Statistical Analysis

All experiments were conducted in triplicate, and the results are presented as mean $\pm$ standard deviation (SD).

RESULTS AND DISCUSSION

Melon fruits (*Cucumis melo* L.) showed typical symptoms of soft rot caused by *R. stolonifer* during postharvest storage. Early symptoms appeared as water-soaked lesions on the fruit surface that rapidly expanded, leading to extensive tissue maceration and softening. Infected areas became translucent and collapsed, accompanied by juice leakage. Profuse cottony mycelial growth developed on the affected tissues, followed by the formation of dark gray to black sporangia. Disease development was strongly associated with wounded fruit surfaces, and advanced infection resulted in severe decay and complete loss of fruit marketability. Colonies of isolates on PDA were fast-growing, cottony, and

white at early stages, later turning gray to black due to abundant sporangia formation. Microscopic examination revealed broad, aseptate hyphae, stolons with rhizoids formed opposite sporangiophores, spherical sporangia, and oval to subglobose sporangiospores, which are characteristic features of *R. stolonifer*. Based on these morphological characteristics, the isolate was tentatively identified as *R. stolonifer*. However, molecular identification (e.g., ITS sequencing) was not conducted, and therefore the identification remains provisional. *R. stolonifer* is recognized as a highly damaging postharvest pathogen, causing significant losses in a wide range of important horticultural crops (Yan *et al.* 2022; Du *et al.* 2023).



Fig. 1. Soft rot symptoms of melon (*Cucumis melo* L.) caused by *Rhizopus stolonifer* during postharvest storage

Tannic acid exhibited a concentration-dependent effect on the growth of *R. stolonifer* and its pectinolytic enzymes (Table 1). At 200 $\mu\text{g mL}^{-1}$, fungal growth was slightly stimulated ($112.6 \pm 3.4\%$), reflecting a hormetic response at low concentrations. However, increasing tannic acid levels progressively reduced growth, with the most pronounced inhibition observed at 1000 $\mu\text{g mL}^{-1}$ ($12.4 \pm 1.9\%$). A similar trend was noted for polygalacturonase and pectin methylesterase activities in the culture medium, which were marginally higher at 200 $\mu\text{g mL}^{-1}$ but declined markedly at higher concentrations. This reduction in extracellular enzyme activity could be attributed to two possible mechanisms: (i) direct inhibition of the catalytic activity of the enzymes by tannic acid, and/or (ii) indirect reduction in enzyme secretion resulting from suppressed fungal growth at higher tannic acid concentrations. The current experimental design, which measured enzyme activity after co-cultivation of tannic acid with the fungus, does not allow these mechanisms to be distinguished. The stimulatory effect at low concentration aligns with previous reports on fungi such as *Pleurotus sajor-caju*, where sub-inhibitory levels of tannins promoted mycelial growth, while higher concentrations exerted negative effects (Fan *et al.* 2006). Strong inhibition at higher tannic acid levels is consistent with findings for phytopathogenic fungi, including *Penicillium digitatum* and *Alternaria alternata*, where mycelial growth and spore germination were significantly suppressed in a concentration-dependent manner (Zhu *et al.* 2019; Wang *et al.* 2023). Mechanistically, tannic acid may disrupt fungal growth by affecting multiple cellular processes. Previous studies suggest interference with mitochondrial function, glycolytic enzymes, and

antioxidant defense systems, leading to impaired energy metabolism and increased oxidative stress (Jiang *et al.* 2023; Hu *et al.* 2025).

The observed decrease in polygalacturonase and pectin methylesterase activities in the culture medium parallels the reduction in fungal biomass at higher tannic acid concentrations, raising the possibility that the decline in enzyme activity may be partially or wholly due to reduced enzyme secretion rather than direct enzyme inhibition. Direct assessment of enzyme inhibition would require assays using cell-free enzyme preparations treated with tannic acid independently of fungal growth (Rodriguez *et al.* 2017). Such experiments were beyond the scope of this study but would be necessary to definitively establish direct enzyme inhibition. It is essential to acknowledge that the present study did not include a direct enzyme inhibition assay, where cell-free crude enzyme extracts are incubated with tannic acid independently of fungal growth. Without such an experiment, the claim that tannic acid directly inhibits polygalacturonase and pectin methylesterase cannot be substantiated. The reduction in enzyme activity observed in co-culture experiments could be entirely explained by reduced enzyme secretion resulting from fungal growth inhibition, rather than any direct interaction between tannic acid and the enzymes themselves. This represents a significant gap in the logical chain of evidence. To unequivocally demonstrate direct enzyme inhibition, future studies must perform cell-free assays where tannic acid is added directly to pre-extracted enzymes, followed by measurement of residual catalytic activity. Such experiments are essential before any mechanism of direct enzyme inhibition can be conclusively established. The concentration-dependent inhibition observed in this study is consistent with previous reports. Zhu *et al.* (2019) reported ~70% inhibition of *P. digitatum* at 800 $\mu\text{g mL}^{-1}$, while Wang *et al.* (2023) observed >80% inhibition of *A. alternata* at similar concentrations. The 87.6% inhibition achieved at 1000 $\mu\text{g mL}^{-1}$ against *R. stolonifer* is comparable to or higher than these values. Regarding enzyme inhibition, the reduction of polygalacturonase to 15.3% and pectin methylesterase to 20.1% at 1000 $\mu\text{g mL}^{-1}$ exceeds the inhibitory effects reported for other phenolic compounds against fungal pectinases (Siddiqui *et al.* 2012; Rodriguez *et al.* 2017), indicating the potent anti-virulence potential of tannic acid.

Table 1. Effect of Tannic Acid on Fungal Growth and Enzymes Activity

Concentration $\mu\text{g mL}^{-1}$	Fungal Growth (%)	Fungal Inhibition (%)	Enzymes Activity (U mg^{-1} protein)	
			Polygalacturonase	Pectin Methylesterase
0	100.0 $\pm$ 2.8	0.0 $\pm$ 0.0	100.0 $\pm$ 3.1	100.0 $\pm$ 2.9
200	112.6 $\pm$ 3.4	-12.6 $\pm$ 3.4	108.4 $\pm$ 4.2	110.9 $\pm$ 3.8
400	85.3 $\pm$ 2.9	14.7 $\pm$ 2.9	79.6 $\pm$ 3.6	82.1 $\pm$ 3.4
600	56.7 $\pm$ 3.1	43.3 $\pm$ 3.1	48.9 $\pm$ 2.8	52.4 $\pm$ 3.0
800	31.8 $\pm$ 2.4	68.2 $\pm$ 2.4	29.7 $\pm$ 2.1	34.2 $\pm$ 2.6
1000	12.4 $\pm$ 1.9	87.6 $\pm$ 1.9	15.3 $\pm$ 1.7	20.1 $\pm$ 1.8

***In vivo* Effect of Tannic Acid on Postharvest Soft Rot Development in Melon Fruits**

Tannic acid significantly suppressed the development of soft rot caused by *R. stolonifer* on melon fruits in a concentration-dependent manner (Table 2). All inoculated untreated fruits developed severe soft rot symptoms within seven days, resulting in 100% disease incidence, and lesion diameter of 46.2 mm. Application of tannic acid markedly

reduced disease development compared with the pathogen-inoculated control. Treatment with 0.25 mg mL⁻¹ tannic acid decreased disease incidence to 80% and reduced lesion diameter by approximately 35.5%. Increasing the tannic acid concentration to 0.50 mg mL⁻¹ further reduced disease incidence to 55%, with a corresponding control efficacy of 60.4%. The highest tannic acid concentration (1.00 mg mL⁻¹) exhibited the greatest protective effect, reducing disease incidence to only 25%, while lesion diameter decreased to 8.6 mm. The calculated control efficacy reached 81.4%. These findings demonstrate that tannic acid effectively suppresses the progression of *R. stolonifer*-induced soft rot under postharvest conditions and indicate that its protective activity increases with concentration. The substantial reduction in lesion development confirms that the strong antifungal activity observed *in vitro* can be translated into practical disease control on melon fruits. Under *in vivo* conditions, *A. alternata* was inhibited by tannic acid on apple fruit (Wang *et al.* 2023).

Table 2. Effect of Tannic Acid on Soft Rot Development Caused by *R. stolonifer* in Melon Fruits

Treatment	Disease Incidence (%)	Lesion Diameter (mm)	Control Efficacy (%)
Healthy control	0.0 ± 0.0	0.0 ± 0.0	—
Pathogen control	100.0 ± 0.0	46.2 ± 2.1	0.0
Tannic acid (0.25 mg mL ⁻¹)	80.0 ± 4.5	29.8 ± 1.8	35.5
Tannic acid (0.5 mg mL ⁻¹)	55.0 ± 5.0	18.3 ± 1.2	60.4
Tannic acid (1.0 mg mL ⁻¹)	25.0 ± 3.8	8.6 ± 0.9	81.4

Docking Investigation

Docking analysis, using the available crystal structures of endopolygalacturonase from *F. verticillioides* (1HG8) and pectin methylesterase from *Daucus carota* (1GQ8) as representative models, predicted favorable binding of tannic acid within the active sites of both enzymes. However, it must be emphasized that these structures are not from *R. stolonifer*; therefore, the docking results should be interpreted as indicative of potential interactions within conserved enzyme families rather than definitive predictions for the *R. stolonifer* enzymes. For polygalacturonase (1HG8), tannic acid showed docking scores ranging from −10.77 to −12.37 kcal/mol, with the best pose exhibiting an RMSD of 2.29 Å (Table 3). The ligand formed multiple hydrogen bonds with key catalytic residues, including ASP194, ASP301, GLN299, and ASN304, with bond distances ranging from 2.93 Å to 3.16 Å (Table 4). In the case of pectin methylesterase (1GQ8), tannic acid displayed docking scores between −8.15 and −9.43 kcal/mol, with RMSD values around 3.15 Å to 3.97 Å (Table 5). Hydrogen bond interactions were observed with residues THR67, THR72, GLN104, THR106, and VAL45 (Table 6). The 2D and 3D interaction diagrams confirmed stable positioning of tannic acid within the catalytic groove of both enzymes (Figs. 2 and 3).

A notable observation from this study is the differential response of polygalacturonase (PG) and pectin methylesterase (PME) to tannic acid treatment. At 1000 µg mL⁻¹, PG activity was reduced to 15.3% of the control, while PME activity was reduced to 20.1%. Although both enzymes were significantly inhibited, PG appeared slightly more susceptible to tannic acid than PME. This difference may be attributed to several factors. First, PG and PME have distinct catalytic mechanisms and active site architectures. PG

catalyzes the hydrolysis of glycosidic bonds in the pectin backbone, requiring acidic residues (e.g., Asp) for proton donation and nucleophilic attack (van Santen *et al.* 1999). PME, in contrast, catalyzes the demethylation of methylesterified galacturonic acid residues, relying on a different catalytic dyad (Johansson *et al.* 2002). The polyphenolic structure of tannic acid, with multiple hydroxyl groups, may form stronger hydrogen bonds with the Asp-rich active site of PG compared to the Thr/Gln-rich environment of PME.

Second, the differential binding affinities predicted by docking (PG: -12.36 kcal/mol vs. PME: -9.43 kcal/mol) support this possibility, although, as noted above, these results are based on heterologous proteins and require confirmation.

Third, the higher molecular weight and complexity of tannic acid may create greater steric hindrance within the PG active site cleft compared to PME. These factors collectively suggest that tannic acid may have a preferential inhibitory effect on PG over PME, which could have implications for its application in postharvest preservation, as PG is considered the primary enzyme responsible for pectin depolymerization and fruit softening (Zhang *et al.* 2021). However, direct experimental validation using purified enzymes is required to confirm this differential sensitivity.

The findings of this study can be contextualized within the broader literature on tannic acid as an antifungal agent. The concentration-dependent inhibition of *R. stolonifer* growth observed here (87.6% at $1000\text{ }\mu\text{g mL}^{-1}$) is consistent with, and in some cases exceeds, previously reported effects on other postharvest fungal pathogens. Zhu *et al.* (2019) reported approximately 70% inhibition of *P. digitatum* mycelial growth at $800\text{ }\mu\text{g mL}^{-1}$, while Wang *et al.* (2023) observed $>80\%$ inhibition of *A. alternata* at similar concentrations. The higher susceptibility of *R. stolonifer* in the present study may reflect species-specific differences in cell wall composition, membrane permeability, or metabolic sensitivity to tannic acid.

Regarding enzyme inhibition, the present study demonstrates that tannic acid reduces extracellular PG and PME activity in *R. stolonifer* cultures. This finding aligns with previous reports on other phenolic compounds, where polyphenols have been shown to inhibit fungal pectinases (Siddiqui *et al.* 2012; Rodriguez *et al.* 2017). However, direct quantitative comparison is challenging due to differences in experimental conditions, enzyme sources, and assay methods. The present study extends previous work by simultaneously evaluating fungal growth and two key pectinolytic enzymes within the same experimental framework, providing a more integrated assessment of tannic acid's anti-virulence potential.

The novelty of this study lies in three key contributions. First, while tannic acid has been studied against several postharvest pathogens, systematic investigation of its effects on *R. stolonifer* growth and pectinolytic enzymes is limited. This study provides the first dose-response data for tannic acid against this pathogen, demonstrating potent growth inhibition at concentrations comparable to or lower than those effective against other fungi. Second, the simultaneous quantification of PG and PME activities reveal differential sensitivity that has not been previously reported, suggesting potential preferential targeting of PG. Third, the integration of *in vitro* assays with *in silico* docking (despite the limitation of heterologous proteins) provides a framework for understanding the molecular basis of tannic acid's effects, although further validation is required. Collectively, these findings advance the understanding of tannic acid as a potential natural antifungal agent and provide a foundation for future research on its application in postharvest preservation.

This study makes several contributions to the field of postharvest pathology and natural antifungal agents. First, it provides the first comprehensive evaluation of tannic acid's antifungal activity against *R. stolonifer*, including both growth inhibition and enzyme activity suppression. Previous studies have focused on other fungal species (Zhu *et al.* 2019; Wang *et al.* 2023), and this work extends the knowledge base to one of the most destructive postharvest pathogens. Second, the simultaneous assessment of PG and PME activities within the same experimental framework allows direct comparison of tannic acid's effects on two key virulence factors, revealing differential sensitivity that may inform future application strategies. Third, the study identifies a concentration range (600 to 1000 $\mu\text{g mL}^{-1}$) that is effective for both growth inhibition and enzyme suppression, providing practical guidance for future formulation development. Fourth, the integration of experimental and computational approaches, despite the limitations of heterologous proteins, establishes a methodological framework that can be refined in future studies. However, it is important to emphasize that these findings are preliminary and require validation through fruit-based efficacy trials and direct enzyme inhibition assays before any practical recommendations can be made.

It is important to clarify that the experimental design used in this study—measuring extracellular enzyme activity after co-cultivation of the fungus with tannic acid—does not distinguish between two possible mechanisms for the observed reduction in enzyme activity. The decrease could result from: (i) direct inhibition of the catalytic activity of polygalacturonase and pectin methylesterase by tannic acid, as suggested by the docking analysis; and/or (ii) indirect reduction in total enzyme secretion due to suppressed fungal growth and metabolic activity at higher tannic acid concentrations. To resolve this ambiguity, future studies should employ cell-free enzyme activity assays, where tannic acid is added directly to pre-extracted enzymes, independent of fungal growth. Such experiments would clarify whether tannic acid acts as a direct enzyme inhibitor or primarily reduces enzyme levels through growth suppression. Until such experiments are conducted, the observed reduction in enzyme activity should be interpreted with caution, as it may reflect a combination of both mechanisms.

The molecular docking analysis presented in this study provides predictive insights into the potential binding interactions between tannic acid and representative pectinolytic enzyme structures (endopolygalacturonase from *F. verticillioides* and pectin methylesterase from *D. carota*). However, because these structures are not from *R. stolonifer*, the predicted interactions should be considered preliminary and hypothesis-generating rather than definitive. The conserved nature of catalytic residues (*e.g.*, Asp, Gln, Thr) across the respective enzyme families supports the plausibility of similar interactions in *R. stolonifer* homologs, but direct confirmation would require structural data or homology models of the *R. stolonifer* enzymes.

The strong predicted binding affinity of tannic acid toward polygalacturonase can be attributed to its polyphenolic structure, which may enable extensive hydrogen bonding with acidic residues such as Asp and polar residues such as Gln. These residues are known to play critical roles in the catalytic mechanism of polygalacturonases, particularly in substrate binding and proton donation (van Santen *et al.* 1999). For pectin methylesterase, tannic acid demonstrated moderate yet significant predicted binding affinity, forming potential hydrogen bonds with residues lining the active site cleft. The observed interactions with Thr and Gln residues are consistent with previously reported inhibitor–

PME complexes, suggesting that tannic acid may sterically hinder substrate access or disrupt catalytic efficiency (Johansson *et al.*, 2002; Di Matteo *et al.* 2005). However, it is important to acknowledge that docking results are computational predictions and do not constitute direct experimental evidence of the inhibition mechanism.

The binding affinities (docking scores: −12.36 kcal/mol for polygalacturonase and −9.43 kcal/mol for pectin methylesterase) and hydrogen bond interactions with residues such as Asp194, Asp301, Gln299, Thr67, Thr72, and Gln104 suggest a plausible molecular basis for the observed enzyme inhibition. Nevertheless, complementary experimental validation—including cell-free enzyme inhibition assays to confirm direct enzyme inhibition, enzyme kinetics to determine inhibition type and inhibition constant (K_i), and site-directed mutagenesis to verify the functional relevance of the predicted key residues—is required to conclusively establish the mechanism of action. Without cell-free assays, the hypothesis of direct enzyme inhibition remains unproven. Such investigations are beyond the scope of the present study but represent important directions for future research.

Despite these limitations, the integration of *in vitro* enzyme activity data with *in silico* docking analysis provides a useful framework for understanding the potential inhibitory effects of tannic acid against *R. stolonifer* virulence factors. The use of dummy atoms in defining the active sites enhanced the accuracy of docking by restricting ligand placement to biologically relevant regions.

Table 3. Docking Scores and Energies of Tannic Acid with the Structure of Endopolygalacturonase (PDB ID: 1HG8)

Poses Mol	rmsd_refine	E_conf	E_place	E_score1	E_refine	E_score2
Tannic acid	2.2976913	-78.70307	-66.8581	-13.7077	-86.8689	-12.3659
Tannic acid	2.767256	-46.38141	-47.4587	-10.2807	-82.2302	-11.420
Tannic acid	2.2595255	-54.26448	-38.3288	-9.74474	-69.8089	-10.8635
Tannic acid	2.229856	-66.41643	-55.5874	-11.6772	-77.9553	-10.8244
Tannic acid	2.20348	-87.0658	-33.0242	-9.29285	-75.132	-10.7693

Table 4. Interaction of Tannic Acid with the Structure of Endopolygalacturonase (PDB ID: 1HG8)

Mol	Ligand	Receptor	Interaction	Distance	E (kcal/mol)
Tannic acid	O 35	OD2 ASP 301 (A)	H-donor	2.95	-2.0
	O 36	OD2 ASP 194 (A)	H-donor	2.96	-3.3
	O 38	OE1 GLN 299 (A)	H-donor	3.16	-1.1
	O 40	O ASN 304 (A)	H-donor	2.96	-3.6
	O 42	OD1 ASP 194 (A)	H-donor	2.93	-3.7

Table 5. Docking Scores and Energies of Tannic Acid with the Structure of Pectin Methylesterase (PDB ID: 1GQ8)

Poses Mol	rmsd_refine	E_conf	E_place	E_score1	E_refine	E_score2
Tannic acid	3.8440986	-40.81713	-41.8605	-11.9995	-65.2413	-9.43312
Tannic acid	3.967473	-51.93696	-41.9944	-10.418	-58.2845	-8.83363
Tannic acid	3.1521864	-32.11738	-62.4105	-11.1402	-55.1299	-8.50701
Tannic acid	3.5756979	-49.47376	-39.2811	-10.5485	-55.0215	-8.16452
Tannic acid	3.4631326	-49.37666	-71.1484	-11.4306	-51.1434	-8.15197

Table 6. Interaction of Tannic Acid with the Structure of Pectin Methylesterase (PDB ID: 1GQ8).

Mol	Ligand	Receptor	Interaction	Distance	E (kcal/mol)
Tannic acid	O 15	OG1 THR 72 (A)	H-donor	3.13	-1.2
	O 17	OE1 GLN 104 (A)	H-donor	3.00	-1.1
	O 25	O THR 67 (A)	H-donor	2.96	-0.8
	O 38	O THR 106 (A)	H-donor	3.00	-1.2
	O 28	N VAL 45 (A)	H-acceptor	3.07	-0.8

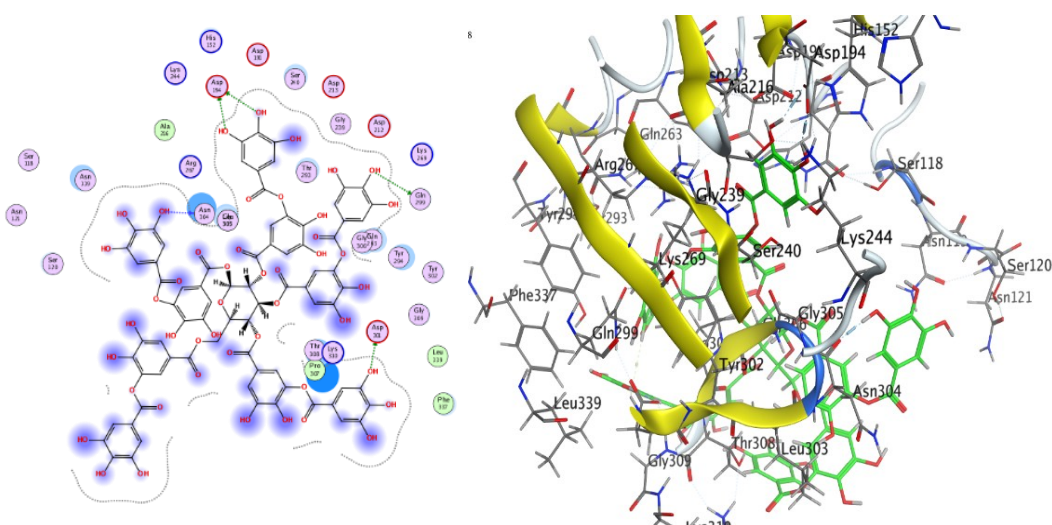


Fig. 2. 2D and 3D diagrams show the interaction between tannic acid and the active sites of Endopolygalacturonase 1HG8 protein

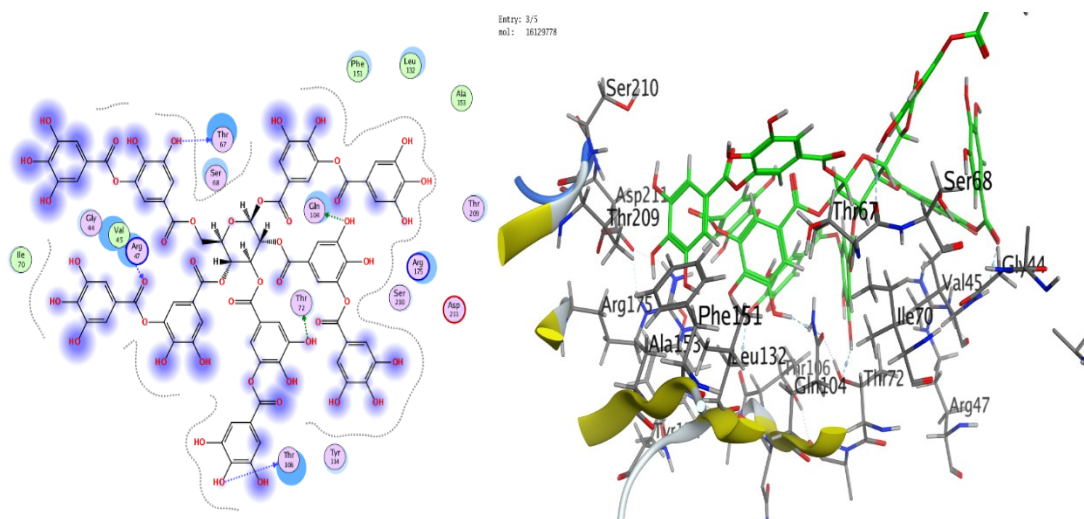


Fig. 3. 2D and 3D diagrams show the interaction between Tannic acid and the active sites of Pectin methylesterase 1GQ8 protein

It is important to acknowledge that the identification of the fungal isolate in this study was based solely on morphological characteristics. While the observed features (aseptate hyphae, stolons, rhizoids, sporangia, and sporangiospores) are consistent with *R. stolonifer* as described by Schipper (1984), morphological identification alone has inherent limitations due to variability and subjectivity. Molecular identification methods, particularly ITS sequencing and phylogenetic analysis, are the current standard for accurate species-level identification of fungi. The absence of molecular confirmation in this study represents a limitation that should be addressed in future investigations to definitively confirm the species identity.

An additional methodological consideration is the use of sodium hypochlorite for surface sterilization during pathogen isolation. While this is a standard procedure for eliminating surface contaminants in plant pathology (Zhu *et al.* 2025), hypochlorite treatment can introduce bias by selectively eliminating certain fungi while allowing others with greater tolerance to oxidizing agents to survive (Copes and Ojiambo 2021). The composition of isolated fungi has been shown to vary depending on the type and concentration of sterilizer as mentioned (Thomas *et al.* 2024). In the present study, this bias is unlikely to affect the primary identification of *R. stolonifer*, as this fungus was consistently isolated from symptomatic tissues and identified based on its characteristic morphological features. Nevertheless, based on our findings, we recommend that future studies investigating fungi associated with soft rot lesions consider comparing different surface sterilization protocols and incorporating culture-independent molecular techniques to achieve a more comprehensive assessment of fungal diversity. This recommendation is supported by evidence that surface sterilization methods can substantially influence the composition of fungal communities recovered from plant tissues (Yu *et al.* 2022).

The mechanistic insights provided in this study are primarily based on computational docking predictions and enzyme activity assays. While these approaches offer valuable preliminary evidence, they have inherent limitations. Molecular docking predicts possible binding conformations but cannot confirm the actual biological relevance of the identified interactions. Enzyme kinetics experiments—including determination of

inhibition type (competitive, uncompetitive, or mixed) and measurement of inhibition constant (K_i)—are needed to characterize the mode of inhibition. Furthermore, site-directed mutagenesis of the predicted key residues (e.g., Asp194, Asp301, Gln299 in polygalacturonase; Thr67, Thr72, Gln104 in pectin methylesterase) would be required to verify their functional roles in tannic acid binding and enzyme inhibition. These experiments are beyond the scope of the current study but are essential for definitive mechanistic elucidation and should be addressed in future investigations. It is important to acknowledge that all experiments in this study were conducted under *in vitro* conditions using liquid medium. While the results demonstrate concentration-dependent inhibition of fungal growth and pectinolytic enzyme activities in a controlled laboratory setting, these findings do not directly translate to efficacy on intact melon fruits.

Although the present study demonstrated that tannic acid effectively suppresses *R. stolonifer* growth under both *in vitro* and *in vivo* conditions, further studies are warranted before commercial application can be recommended. Future investigations should optimize treatment concentration and application methods (e.g., dipping, spraying, or incorporation into edible coatings), evaluate treatment efficacy during long-term storage under commercial postharvest conditions, and assess its effects on fruit quality attributes, including firmness, color, flavor, weight loss, and consumer acceptability. In addition, studies on residue persistence, regulatory safety, compatibility with existing postharvest handling practices, and economic feasibility would facilitate the translation of the present findings into practical disease management strategies for muskmelon and other susceptible fruits.

CONCLUSIONS

1. Tannic acid was found to effectively suppress the growth of the post-harvest fungal pathogen *R. stolonifer* in a concentration-dependent manner under *in vitro* conditions, with strong inhibition (87.6%) at 1000 $\mu\text{g mL}^{-1}$.
2. Polygalacturonase and pectin methylesterase activities in the culture medium were significantly reduced by tannic acid. However, this reduction may have resulted from direct enzyme inhibition, reduced enzyme secretion due to suppressed fungal growth, or a combination of both. The current experimental design does not distinguish between these mechanisms. Critically, the study did not include direct cell-free enzyme inhibition assays, which are essential to prove that tannic acid directly inhibits the catalytic activity of these enzymes. Therefore, claims of direct enzyme inhibition remain speculative and require experimental confirmation.
3. Molecular docking analysis, using representative structures of endopolygalacturonase from *F. verticillioides* (1HG8) and pectin methylesterase from *Daucus carota* (1GQ8), suggests that tannic acid exhibits potential binding affinity toward these enzyme models (docking scores: -12.36 kcal/mol and -9.43 kcal/mol, respectively), with predicted hydrogen bonding interactions involving key catalytic residues including Asp194, Asp301, and Gln299 in polygalacturonase, and Thr67, Thr72, and Gln104 in pectin methylesterase. However, because these structures are not from *R. stolonifer*, the results should be interpreted as indicative rather than definitive. These computational findings provide a preliminary predictive basis for the observed enzyme inhibition,

warranting further experimental validation using *R. stolonifer*-specific enzymes and enzyme kinetics studies.

4. The absence of direct enzyme inhibition assays (cell-free enzyme + tannic acid) represents a significant experimental gap. Without such experiments, the mechanism of enzyme activity reduction cannot be conclusively assigned to direct inhibition. Future studies should prioritize cell-free assays to establish whether tannic acid directly inhibits polygalacturonase and pectin methylesterase catalytic activity, independent of fungal growth effects.

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