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Molecular Mechanism of *Trichoderma harzianum* Secondary Metabolites in Inhibiting Cellulase Protein of *Colletotrichum capsici*

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Abstract: Fungal diseases in agriculture pose significant challenges to food security, necessitating sustainable biocontrol solutions. *Trichoderma harzianum*, a biocontrol agent, exhibits potent antifungal properties through its secondary metabolites. This study investigates the inhibitory mechanism of *T. harzianum* metabolites on the cellulase protein of *Colletotrichum capsici*, the causative agent of chili anthracnose, using molecular docking and dynamics simulations. The cellulase protein, crucial for plant cell wall degradation, was modeled through homology techniques, and its interactions with *T, harzianum* metabolites—cyanuric chloride, palmitinic acid, and massoia lactone—were analyzed. Massoia lactone demonstrated the highest inhibitory potential, with stable binding interactions confirmed through molecular dynamics. These findings provide insights into developing environmentally sustainable antifungal strategies. Further research is recommended to optimize the application of *T. harzianum* metabolites as biopesticides.

Keywords: Anthracnose, chili, homology modeling, molecular docking, molecular dynamics,

Introduction

The rising incidence of fungal diseases in agriculture presents an urgent threat to global food security, necessitating the development of sustainable and innovative biocontrol strategies. Among the most promising biological antagonists, *Trichoderma harzianum* has garnered significant attention for its robust antifungal properties, primarily attributed to its diverse arsenal of bioactive metabolites. This eco-friendly alternative to synthetic fungicides is increasingly favored over chemical treatments, addressing critical concerns such as fungicide resistance and environmental sustainability (Mallikarjunaswamy, 2018; Brauer et al., 2019).

Renowned for its mycoparasitic capabilities, *T. harzianum* deploys a sophisticated biochemical warfare strategy against phytopathogens, secreting an array of antifungal compounds, including metabolites synthesized by polyketide synthases (PKS) and non-ribosomal peptide synthetases (NRPS). These secondary metabolites not only exhibit potent inhibitory effects against notorious plant pathogens like *Fusarium solani* and *Alternaria solani* but also play a pivotal role in eliciting plant defense responses, underscoring their immense potential for agricultural applications (Lin et al., 2016; Uniyal & Singh, 2017; Lakhdari, 2023; Dania, 2019).

Despite substantial research on the pharmaceutical relevance of *Trichoderma* metabolites, their precise inhibitory mechanisms against plant pathogens remain insufficiently explored. Bridging this knowledge gap is crucial for harnessing their full potential as next-generation biocontrol agents in modern agriculture. This study specifically investigates *Colletotrichum capsici*, the etiological agent of chili anthracnose—one of the most economically

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devastating fungal diseases affecting chili production worldwide. With yield losses reaching up to 84% (Naziya et al., 2019), *C. capsici* poses a formidable challenge, manifesting as dark, sunken lesions on fruit, leading to severe pre-harvest and post-harvest deterioration (Saxena et al., 2014; Annad, 2020). Environmental factors, coupled with the pathogen's metabolic adaptability, further exacerbate its virulence and persistence in agricultural ecosystems (Puripunyavanich, 2024).

To unravel the molecular intricacies of *T. harzianum* metabolites in fungal suppression, this study employs molecular docking and dynamic simulations to examine their inhibitory potential against *C. capsici* cellulase a key enzymatic determinant in host invasion. The cellulase enzyme (EC 3.2.1.4) from *C. capsici* functions as a glycoside hydrolase (GH), catalyzing the endohydrolysis of (1→4)-β-D-glucosidic linkages in cellulose, lichenin, and cereal β-D-glucans. This enzymatic activity plays a fundamental role in degrading plant cell walls, facilitating fungal colonization and pathogenesis (Yan & Wu, 2013; Hassan et al., 2021). Given its indispensable function in fungal virulence, targeting cellulase represents a compelling strategy for disrupting *C. capsici* infections while maintaining environmental integrity. By elucidating the binding interactions and structural stability of *T. harzianum* metabolites with *C. capsici* cellulase, this research seeks to unveil novel molecular insights that could drive the development of precision-targeted, eco-sustainable biocontrol strategies for chili anthracnose and other agriculturally relevant fungal diseases.

Materials and method

Protein homology modeling and structure assessment

The protein sequence of the cellulase enzyme from *Colletotrichum capsici* was obtained from UniProt (https://www.uniprot.org/) by entering the relevant protein name and target organism as keywords. The target protein sequence, identified as UniProt Entry A0A023NB23 with the gene name GH45, was retrieved and downloaded for model construction. The protein model was generated and evaluated using templates provided by the SwissModel web server (https://swissmodel.expasy.org/) by inputting the previously obtained sequence. The best model, formatted as a Protein Data Bank (PDB) file, was downloaded for further analysis.

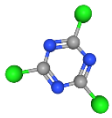
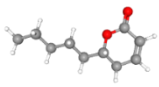
Hao's rule of pesticidelikeness assessment

The secondary metabolites of *Trichoderma harzianum* were obtained from the study by Lakhdari et al. (2023), as listed in Table 1. The properties of each compound, including logP, hydrogen bond donors, hydrogen bond acceptors, rotatable bonds, and water solubility, were analyzed using the SwissADME and ProTox platform. Accessible at http://www.swissadme.ch/ and https://tox.charite.de/protox3/, the platform was utilized by entering the compound's chemical structure in the form of canonical SMILES obtained from PubChem (https://pubchem.ncbi.nlm.nih.gov/). These properties were evaluated to ensure compliance with Hao's rules for pesticide-likeness, which include criteria such as a molecular weight (MW) ≤ 435, MLogP ≤ 6, hydrogen bond donors (HBD) ≤ 2, hydrogen bond acceptors (HBA) ≤ 6, and rotatable bonds (RB) ≤ 9 (Hao et al., 2011; Hao et al., 2015).

Molecular docking simulations

Molecular docking was performed using PyRx 08 and BIOVIA Discovery Studio to analyze protein–ligand interactions. The 3D structure of the *C. capsici* cellulase protein was prepared in BIOVIA Discovery Studio by adding hydrogen atoms, optimizing the structure, and removing water and ligands, then saved as a .pdb file. The 3D structures of *T. harzianum* metabolites were optimized using OpenBabel in PyRx. Receptor and ligand files were loaded into PyRx, and a maximum grid box was defined around the binding site for blind docking using AutoDock Vina. The best docking poses were selected based on binding scores and visual inspection. Further refinements and analyses were performed in BIOVIA Discovery Studio, and the results were documented.

Table 1. Chemical properties and identifiers of selected *T. harzianum* metabolites

Compound	Molecular Formula	Structure	PubChem CID	Canonical SMILES
Cyanuric chloride	C3C13N3		7954	C1(=NC(=NC(=N1)Cl)Cl)Cl
Palmitinic acid	C16H32O2		985	CCCCCCCCCCCCCCCC(=O)O
Massoia lactone	C10H16O2		39914	CCCCCC1CC=CC(=O)O1

Molecular dynamic simulations

Molecular dynamics simulations were performed using YASARA Dynamics version 24.10.5 to evaluate the behavior of the system, with *T. harzianum* secondary metabolites as ligands, and the *C. capsici* cellulase protein as the receptor. The initial structure was imported into the software, and energy minimization was conducted to optimize geometry and resolve steric clashes. The system was then solvated in a water box, and ions were added to neutralize the charge. Simulations were carried out under NPT ensemble conditions, maintaining a temperature of 300 K and a pressure of 1 bar, with an equilibration phase of 100 ps. Trajectory data from the simulations were analyzed to assess the system's stability and conformational changes, with results processed using Microsoft Excel.

Result and discussion

Cellulase protein homology model and structure

The homology modeling of *Colletotrichum capsici* cellulase protein, as sown in Figure 1, resulted in a high-quality structure that met standard benchmarks for structural validation. One of the key highlights of the model was the high percentage of residues positioned in favored Ramachandran regions, indicating good backbone geometry and structural integrity. Additionally, the absence of steric clashes, rotamer outliers, and bad bonds further supported the accuracy of the atomic-level representation of the protein.

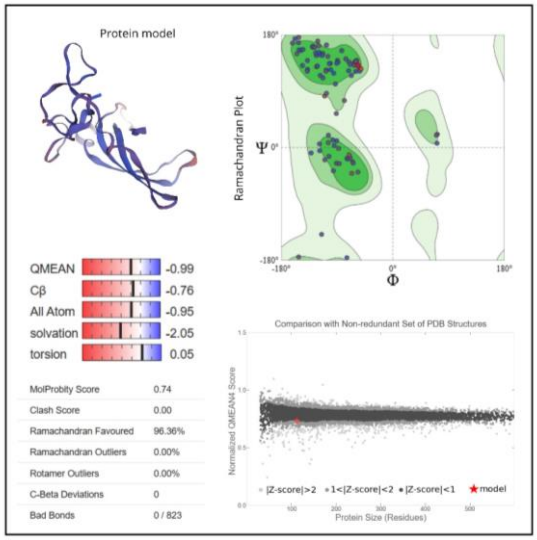


Figure 1. The homology modeling of *Colletotrichum capsici* cellulase protein

The model also exhibited a QMEAN score within the acceptable range for similar-sized proteins, validating its reliability as a predictive structure. However, despite these strengths, the slightly low solvation energy score suggested potential areas for refinement, particularly in flexible or loop regions. This could be addressed through further experimental validation, such as molecular dynamics simulations or comparison with functional data, to strengthen the model's applicability in structural and functional studies.

Given its reliability, the model was subsequently applied to various downstream applications, including receptor-ligand molecular docking and molecular dynamics simulations. These studies provided deeper

insights into the protein's interaction with ligands and its dynamic behavior, enhancing its functional characterization.

The evaluation of protein structures is indeed critical in the field of structural biology, particularly for understanding protein function and dynamics. The relationship between protein structure and function is foundational; as articulated in various studies, a protein's three-dimensional conformation directly influences its biological activity. For instance, Trellet et al. (2015) emphasize that "a protein's shape directly determines its function," underscoring the necessity of structural analysis in elucidating biological roles. This principle is further supported by the assertion that understanding protein dynamics how proteins change shape and interact with other molecules is essential for grasping their functional mechanisms (Wu et al., 2022).

Pesticide-likeness of volatile metabolites

The pesticide-likeness assessment of the secondary metabolites—cyanuric chloride, palmitinic acid, and massoia lactone—highlighted key differences in their physicochemical properties and structural characteristics. These differences provided insights into their potential as bioactive compounds, see Table 2.

Table 2. Evaluation of selected *T. harzianum* secondary metabolites based on Hao's rule of pesticide-likeness

Compound	Molecular weight (g/mol)	LogP (o/w)	H-bond Donor	H-bond Acceptor	Rotatable Bond	Hao's Rule Violation
Cyanuric chloride	184.41	0.6	0	3	0	0
Palmitinic acid	256.42	4.19	0	2	8	0
Massoia lactone	168.23	2.08	0	2	4	0

Cyanuric chloride exhibited the smallest molecular weight (184.41 g/mol) among the three metabolites and a LogP value of 0.6, indicating a balance between hydrophilicity and lipophilicity. It possessed no hydrogen bond donors, three hydrogen bond acceptors, and zero rotatable bonds, making it the most rigid compound. The absence of rotatable bonds contributed to its structural stability, which could favor its interaction with target molecules. Importantly, it did not violate Hao's rule, suggesting that it met essential criteria for pesticide-likeness.

In contrast, palmitinic acid displayed a higher molecular weight (256.42 g/mol) and a significantly higher LogP value (4.19), indicating greater lipophilicity. This suggested improved membrane permeability but potentially reduced solubility in aqueous environments. Unlike cyanuric chloride, palmitinic acid contained eight rotatable bonds, making it the most structurally flexible compound among the three. This flexibility might have enhanced its ability to interact with dynamic biological targets. Despite its higher lipophilicity and flexibility, it adhered to Hao's rule, supporting its potential for bioactivity.

Massoia lactone, the lightest compound (168.23 g/mol), occupied an intermediate position in terms of LogP (2.08), reflecting a well-balanced hydrophilic and lipophilic profile. Like cyanuric chloride, it possessed no hydrogen bond donors and two hydrogen bond acceptors. However, with four rotatable bonds, it was structurally more flexible than cyanuric chloride and less flexible than palmitinic acid. Its balance of properties made it a promising candidate for bioactivity, and its adherence to Hao's rule further supported its pesticide-likeness. All three metabolites complied with Hao's rule, signifying their potential as pesticide-like compounds. Cyanuric chloride appeared to excel in structural stability, palmitinic acid in flexibility and membrane interaction, and massoia lactone in achieving a balance between these characteristics. Further bioactivity testing is recommended to confirm their effectiveness as pesticides.

The foundational aspects of Hao's rule include specific thresholds for several key physicochemical properties. For instance, it stipulates that the molecular weight (MW) of a candidate compound should not exceed 435 Da, and the logarithm of the partition coefficient (Clog P) should be less than or equal to 6. Additionally, the number of hydrogen bond acceptors (HBA) should be limited to 6, while the number of hydrogen bond donors (HBD) should

not exceed 2. Furthermore, the rule allows for a maximum of 10 rotatable bonds in the molecular structure (Hao et al., 2015; Hao et al., 2011). These parameters are designed to ensure that the compounds maintain a balance between efficacy and safety, aligning with the characteristics of known pesticides.

Molecular docking

A comprehensive analysis of the molecular docking interactions between secondary metabolites from *Trichoderma harzianum* and the cellulase protein of *Colletotrichum capsici* visualized in Table 3 and Figure 2. Three metabolites—cyanuric chloride (Figure 2a), palmitinic acid (Figure 2b), and massoia lactone (Figure 2c)—were evaluated based on their binding affinity, interaction distance, binding categories, and interaction sites. The results highlighted the potential inhibitory effects of these metabolites on cellulase activity, offering valuable insights into controlling *C. capsici* infections.

Cyanuric chloride exhibited the lowest binding affinity among the three metabolites, with a value of -4.2 kcal/mol. Despite this, it demonstrated multiple interaction types, including hydrogen bonds, halogen bonds, and hydrophobic interactions. Notable interactions were observed at residues such as LEU27, THR19, and ALA6, which may have played roles in cellulase stability or function. The shorter interaction distances (e.g., 2.87 Å for a hydrogen bond with LEU27) suggested strong binding at these sites, although the overall affinity was weaker compared to the other ligands.

Palmitinic acid and massoia lactone both displayed a binding affinity of -4.8 kcal/mol, indicating stronger interactions with the cellulase protein than cyanuric chloride. Palmitinic acid exhibited an extensive array of interactions, including hydrogen bonds with THR83 and multiple hydrophobic contacts, particularly with ILE80 and PHE108. The predominance of π-alkyl interactions with aromatic residues like PHE108 underscored the importance of hydrophobic forces in stabilizing the ligand-protein complex. Such interactions were hypothesized to disrupt the enzymatic function of cellulase, making palmitinic acid a promising candidate for further investigation.

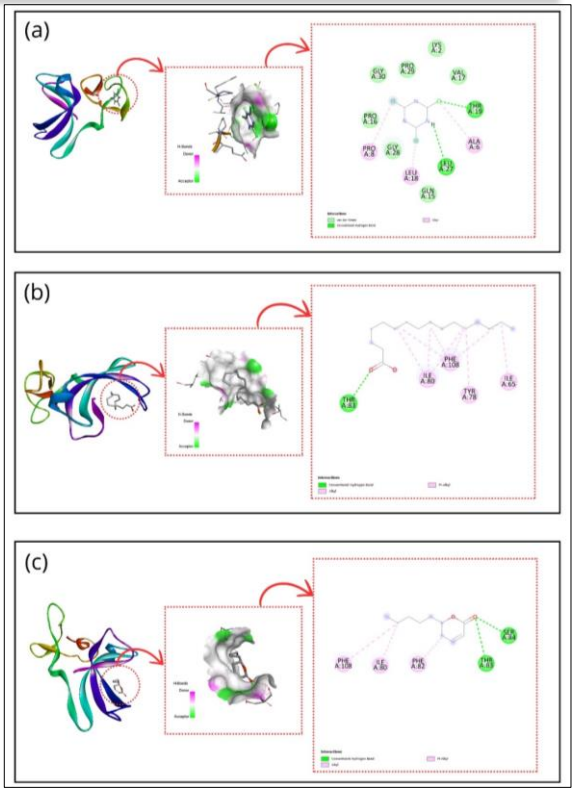


Figure 2. Molecular docking analysis of selected *T. harzianum* secondary metabolite compounds toward *C. capsici* cellulase protein

Massoia lactone, while sharing the same binding affinity as palmitinic acid, demonstrated a more specific interaction profile. It formed hydrogen bonds with residues THR83 and SER84, suggesting that these sites were critical for ligand binding. Additionally, hydrophobic interactions with residues such as PHE82 and PHE108 reinforced the stability of the complex. The relatively close distances (e.g., 2.97 Å for the hydrogen bond with SER84) further emphasized the strength of these interactions. This targeted binding pattern indicated that Massoia lactone might have been particularly effective as a cellulase inhibitor.

The cellulase protein of *Colletotrichum capsici* plays a crucial role in the pathogenicity of this fungus. Cellulases are enzymes that degrade cellulose, a major component of plant cell walls. This degradation is essential for the fungus to invade host tissues and establish infection. The cellulase activity facilitates the breakdown of plant cell wall components, allowing the pathogen to penetrate and colonize the plant tissues effectively (Padghan, 2023).

Table 3. Molecular docking simulation of selected *T. harzianum* secondary metabolite compounds toward *C. capsici* cellulase protein

Ligand	Binding affinity (kcal/mol)	Distance (Å)	Binding category	Binding type	Interaction site
Cyanuric chloride	-4.2	2.87	Hydrogen bond	Conventional Hydrogen Bond	LEU27
		3.11	Hydrogen bond; Halogen	Conventional Hydrogen Bond; Halogen (Cl, Br, I)	THR19
		3.87	Hydrophobic	Alkyl	ALA6
		4.16	Hydrophobic	Alkyl	PRO8
		4.93	Hydrophobic	Alkyl	LEU18
Palmitinic acid	-4.8	3.2	Hydrogen bond	Conventional Hydrogen Bond	THR83
		2.82	Hydrogen bond	Conventional Hydrogen Bond	THR83
		4.53	Hydrophobic	Alkyl	ILE80
		3.75	Hydrophobic	Alkyl	ILE80
		5.47	Hydrophobic	Alkyl	ILE80
		4.43	Hydrophobic	Alkyl	ILE65
		5.1	Hydrophobic	Pi-Alkyl	TYR78
		4.43	Hydrophobic	Pi-Alkyl	PHE108
		4.75	Hydrophobic	Pi-Alkyl	PHE108
		4.66	Hydrophobic	Pi-Alkyl	PHE108
		4.88	Hydrophobic	Pi-Alkyl	PHE108
Massoia lactone	-4.8	3.03	Hydrogen bond	Conventional Hydrogen Bond	THR83
		2.97	Hydrogen bond	Conventional Hydrogen Bond	SER84
		3.13	Hydrogen bond	Conventional Hydrogen Bond	SER84
		4.37	Hydrophobic	Alkyl	ILE80
		4.86	Hydrophobic	Pi-Alkyl	PHE82
		4.79	Hydrophobic	Pi-Alkyl	PHE108

Research has shown that *C. capsici* employs a range of cellulases to enhance its virulence. For instance, the production of cellulases is often upregulated in response to the plant cell wall components, indicating a sophisticated mechanism of host manipulation (Collier et al., 2020; Yanti, 2023). The enzymatic breakdown of cellulose not only aids in physical penetration but also releases oligosaccharides that can act as signaling molecules, further promoting fungal growth and pathogenicity (Sm et al., 2019).

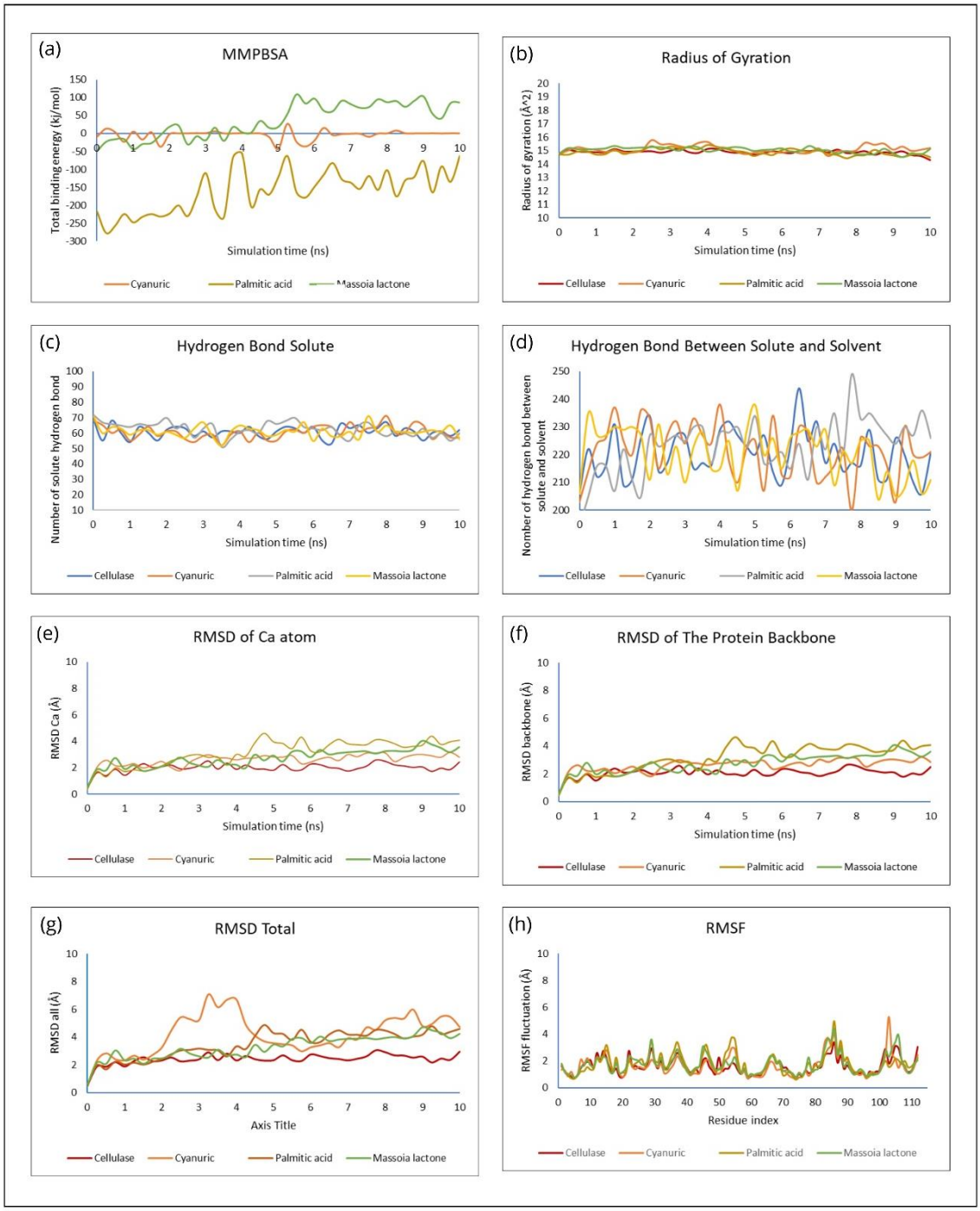


Figure 3. Molecular dynamics simulation of selected *T. harzianum* secondary metabolite compounds toward *C. capsici* cellulase protein

Molecular dynamics

The molecular dynamics simulation results align well with the molecular docking findings, providing a comprehensive evaluation of the interactions between cellulase, a protein from *Colletotrichum capsici*, and secondary metabolites from *Trichoderma harzianum*. The combination of these results allows for a more detailed comparison of the inhibitory potential of cyanuric acid, palmitinic acid, and massoia lactone.

Molecular docking, as can be seen in Figure 2, revealed that massoia lactone and palmitinic acid shared the strongest binding affinity (-4.8 cal/mol) compared to cyanuric chloride (-4.2 kcal/mol). Massoia lactone's docking profile indicated hydrogen bonds with key residues such as THR83 and SER84, as well as hydrophobic interactions with residues like PHE82 and PHE108, which were critical for stabilizing the ligand-protein complex. These observations were supported by the molecular dynamics simulation results, see Figure 3, where massoia lactone consistently demonstrated the most stable binding behavior, as evidenced by the lowest MM/PBSA binding free energy (Figure 3a) and minimal fluctuations in RMSD (Figure 3e-g) and RMSF (Figure 3h) values. The specific interactions and close distances, such as the hydrogen bond with SER84 (2.97 Å), reinforce the strong inhibitory potential of massoia lactone.

In contrast, palmitinic acid exhibited a similar binding affinity to massoia lactone in docking studies (-4.8 kcal/mol), forming hydrogen bonds with THR83 and several hydrophobic interactions, including significant contacts with ILE80 and PHE108. However, its performance in molecular dynamics simulations was less favorable, with higher RMSD fluctuations and weaker stability overall. While docking highlighted its reliance on hydrophobic interactions, these may not have translated into sustained stability during dynamic conditions, making it a less effective inhibitor compared to massoia lactone.

Combining the docking and simulation results clearly identifies massoia lactone as the best inhibitor of cellulase. Its strong binding affinity, targeted interaction profile, and dynamic stability make it the most promising candidate for further investigation. While palmitinic acid shows potential due to its binding affinity and hydrophobic contacts, its stability is inferior in dynamic conditions. Cyanuric chloride, with its diverse interaction types but weaker overall binding and stability, appears to be the least effective inhibitor among the three tested metabolites. These findings offer valuable insights into the potential of secondary metabolites from *Trichoderma harzianum* in managing *C. capsici* infections.

Massoia lactone, a compound derived from the bark of the *Cryptocarya massoy* tree, has garnered attention for its antimicrobial properties. Recent studies have demonstrated that massoia lactone exhibits significant antimicrobial activity against various pathogens, including bacteria and fungi. For instance, Hamzah et al. (2023)'s research highlights that C-10 massoia lactone shows robust inhibitory effects on biofilms formed by *Pseudomonas aeruginosa* and *Staphylococcus aureus*, suggesting its potential as an effective antimicrobial agent. This is further supported by findings from Wang et al. (2024), who noted that massoia lactone can disrupt microbial membranes, thereby inhibiting biofilm growth.

Moreover, massoia lactone has been shown to possess antifungal properties against a range of fungal pathogens. Lee's study indicates that C-10 massoia lactone effectively suppresses the growth of *Aspergillus flavus*, a known aflatoxin producer, by targeting biofilm formation rather than hyphal growth (Lee et al., 2023). Similarly, Zhang et al. (2021) reported that massoia lactone displays strong antifungal activity against various crop pathogens, reinforcing its potential application in agricultural settings. Additionally, the compound has been recognized for its broad-spectrum antifungal activity against human pathogens, making it a promising candidate for use in food industries as a natural bio-fungicide (Li et al., 2022).

Conclusion

This study unveils the antifungal potential of *Trichoderma harzianum* metabolites against the cellulase enzyme of *Colletotrichum capsici*, a devastating pathogen in chili production, through molecular docking and dynamics simulations. Massoia lactone emerged as the most potent inhibitor, exhibiting stable interactions with key enzymatic residues, providing a molecular foundation for its development as a biopesticide. By advancing our understanding of biocontrol mechanisms at the molecular level, this research supports the integration of eco-friendly biopesticides into sustainable agriculture, offering a viable alternative to chemical fungicides while enhancing crop yields and mitigating environmental and health risks.

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