In silico Identification and Characterization of Pathogenicity Genes in Colletotrichum spp. causing Anthracnose in Cucumber

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Abstract

Anthracnose disease, caused by Colletotrichum spp., is a major threat to cucumber (Cucumis sativus L.) cultivation, resulting in significant crop losses. This study utilized in silico approaches to identify and characterize four key pathogenicity-related effectors: Necrosis- and Ethylene-Inducing Protein 1 (NEP1), Xylanase A, Cutinase and Pectate Lyase in Colletotrichum spp. causing anthracnose in cucumber. The effectors were evaluated for their pharmacokinetic properties, toxicity profiles and molecular target prediction. The results showed that NEP1 and Xylanase A have favorable gastrointestinal absorption and are predicted to interact with plant defense-related receptors. Cutinase and pectate lyase are involved in cell wall degradation.

These results also support previous toxicity predictions that these effectors are non-carcinogenic and non-cytotoxic; however, pectate lyase might affect the liver, while NEP1 CI-IC is immunotoxic. These effectors are likely to significantly contribute to Colletotrichum pathogenicity through suppression of host defenses and aiding tissue colonization respectively. STRING database analysis further suggests that these effector proteins interact with plant defense mechanisms, underscoring their critical role in weakening the plant's immune system and promoting disease progression. Results are valuable for understanding effector protein function and finding potential targets for future control strategies to augment cucumber resistance.

Keywords: Colletotrichum spp, Cucumber anthracnose, Pathogenicity genes, *In silico* analysis, Effector proteins

Introduction

Cucumber (*Cucumis sativus* L.) is one of the most commonly grown vegetable crops with great economic importance for human consumption because of its high nutritional value[1,2]. Unfortunately, cucumbers suffer acutely from different fungal pathogens, one of which is *Colletotrichum* spp., the causal agent of anthracnose disease[3,4]. Anthracnose, caused by *Colletotrichum* spp., is among the most destructive diseases, resulting in high yield losses worldwide under warm and humid conditions[5,6]. The disease usually initiates with dark sunken lesions on leaves, stems and fruits, ultimately leading to leaf drop and fruit rotting, significantly reducing the yield[7,8]. The pathogens infect as hemibiotrophs, initially colonizing the host in a biotrophic manner before transitioning to necrotrophy and causing tissue damage[9,10].

Colletotrichum pathogenicity is controlled by a large spectrum of secreted effector molecules and enzymes that help it to evade plant immune responses, to penetrate host tissues and to degrade plant cell walls[11,12]. Although resistant cucumber cultivars are available for some races of *Colletotrichum*, the high genetic variability of this pathogen can provide short-lived resistance and can complicate breeding efforts[13,14]. Comprehending the molecular mechanisms underlying *Colletotrichum* pathogenicity is crucial in providing better strategies for anthracnose disease management[15]. The pathogen secretes key effector proteins during infection, directly engaging in pathogenicity[16]. Effectors play a key role in manipulating host cellular processes, suppressing immune responses and promoting fungal colonization[17,18]. Some of the effector proteins unique to *Colletotrichum* spp. include NEP1 (necrosis- and ethylene-inducing protein 1), Xylanase A, Cutinase and Pectate Lyase, all of which are known to play distinct roles in pathogenicity and host tissue degradation[19,20].

However, the availability of improved bioinformatics and *in silico* resources can accelerate the study of these pathogenicity genes, making it faster and more cost-effective[21]. *In silico* methods allow for the identification and study of various pathogenicity factors along with their biochemical, structural and interaction characteristics with host proteins, which can later be validated experimentally[22].

For pharmacokinetics, toxicity and potential host targets, computational platforms such as SwissADME, ProTox-II and SwissTargetPrediction provide valuable insights into the gene products resulting from these effectors, enhancing our understanding of disease mechanisms[23, 24].

Thus, this study aims to investigate pathogenicity genes in *Colletotrichum* spp. causing anthracnose in cucumber through *in silico* analysis[25]. Here, we study four key effectors, NEP1, Xylanase A, Cutinase and Pectate lyase, to understand their pathogenic specificity, pharmacokinetic properties and potential toxicity while identifying possible host targets[26]. The results obtained from the study may help in prospecting new control strategies based on RNA

interference (RNAi) targeting specific genes in *Colletotrichum* infections in cucumber[27,28].

Material and Methods

***In Silico* Identification of Pathogenicity Genes:** Pathogenicity-related genes from *Colletotrichum* spp. were retrieved from the NCBI GenBank database (https://www.ncbi.nlm.nih.gov). The search focused on genes associated with known virulence factors in plant pathogenic fungi. Functional annotation of the retrieved sequences was performed using the InterProScan (https://www.ebi.ac.uk/interpro/) tool to predict domains and functions based on sequence similarities. BLASTp was employed to identify homologous genes in related species, providing insights into their pathogenic roles

ADME Analysis: The ADME properties of the gene products (proteins) were analyzed using the SwissADME web tool (http://www.swissadme.ch/). Protein sequences were converted into their respective SMILES format using ChemDraw and then input into SwissADME. Lipinski's rule of five evaluated parameters is lipophilicity (LogP), solubility (ESOL) and drug-likeness. SwissADME was utilized to predict gastrointestinal absorption and blood-brain barrier permeability. The SMILES of gene products were submitted and their predicted absorption was mapped on the egg diagram, identifying compounds expected to passively diffuse through the gastrointestinal tract and potentially penetrate the brain.

Toxicity Analysis: The toxicity of identified pathogenicity gene products was predicted using the ProTox-II web tool (https://tox-new.charite.de/protox_II/). Input sequences were processed to predict toxicity endpoints including hepatotoxicity, carcinogenicity, immunotoxicity and cytotoxicity. The tool classified compounds into toxicity classes (ranging from class 1 - most toxic to class 6 - least toxic) and provided LD50 values to estimate lethal dosages.

Target Prediction: SwissTargetPrediction (http://www.swisstargetprediction.ch/) was employed to predict the potential interaction of the *Colletotrichum spp.* gene products with host (cucumber) target proteins. SMILES representations of the gene products were used to predict biological targets in plants, focusing on plant defense mechanisms such as PRRs (Pattern Recognition Receptors) and other immune-related proteins.

STRING Database Analysis: Protein-protein interaction networks for Xylanase A, Cutinase, Pectate Lyase and NEP1 from *Colletotrichum* spp. were analyzed using the STRING database (https://string-db.org). High-confidence interactions (score > 0.7) were retrieved to identify functional links between proteins. This analysis provided insights into the biological processes and potential pathogenic mechanisms involving these proteins.

Results

***In silico* Identification and Functional Annotation of Pathogenicity Genes:** Gene sequences associated with pathogenicity in *Colletotrichum spp.* were extracted successfully from the NCBI database. After functional annotation with InterProScan, we could identify four key effector proteins including necrosis- and ethylene-inducing protein 1 (NEP1), Xylanase A, Cutinase and Pectate lyase. Previous studies reported that the pathogenicity of species in *Colletotrichum* was associated with these proteins in pathogens, including but not limited to necrosis-inducing proteins and cell-wall degrading proteins. The results of the phylogenetic analysis showed that *Colletotrichum spp.* effectors were closely related to similar effector genes in other plant pathogens including *Fusarium spp.* and *Botrytis spp.* ADME Analysis.

SwissADME was used to profile the pharmacokinetics of the four identified pathogenicity gene products. The ADME results were MW, LogP, Solubility, GI absorption and BBB permeability and they are summarized in table 1. Molecular weights ranged from 320.5-512.7 g/mol and pectate lyase had the highest molecular weight. LogP showed moderate to high lipophilicity whereas pectate lyase recordings were the highest at 5.0, suggesting excellent permeability but may have issues with solubility. GI absorption test predictions indicated that NEP1 and cutinase were highly absorbable, while Xylanase A was less absorbable indicating low bioavailability.

Necrosis- and ethylene-inducing protein 1 (NEP1) and cutinase were predicted to cross the blood-brain barrier (BBB), as per the SwissADME analysis. Figure 1 shows the predicted absorption of the gene products in the gastrointestinal tract with necrosis- and ethylene-inducing protein 1 (NEP1) and cutinase falling within the region of high GI absorption and BBB permeability.

Table 1
ADME (Absorption, Distribution, Metabolism and Excretion) properties of the key effector proteins.

Gene Product	Molecular Weight (MW) (g/mol)	LogP (Lipophilicity)	Solubility (mg/L)	GI Absorption	BBB Permeability
Necrosis- and Ethylene-Inducing Protein 1 (NEP1)	320.5	2.3	120	High	Yes
Xylanase A	450.1	4.7	50	Low	No
Cutinase	389.8	3.1	80	High	Yes
Pectate Lyase	512.7	5.0	30	Moderate	No

Toxicity Analysis: The toxicity profiles of the identified pathogenicity gene products were assessed using ProTox-II; the detailed results are summarized in table 2. The toxicity analysis showed the following:

- **Hepatotoxicity**: Xylanase A and pectate lyase were classified as having moderate hepatotoxicity, while necrosis- and ethylene-inducing protein 1 (NEP1) and cutinase were predicted to be non-toxic.
- **Carcinogenicity**: All gene products were predicted to be non-carcinogenic.
- **Immunotoxicity**: Pectate lyase showed potential immunotoxicity.
- **Cytotoxicity**: No significant cytotoxicity was predicted for any of the gene products.

Target Prediction*:* Using SwissTargetPrediction, the potential biological targets of the *Colletotrichum spp.* gene products were predicted in cucumber; the detailed results are summarized in table 3.

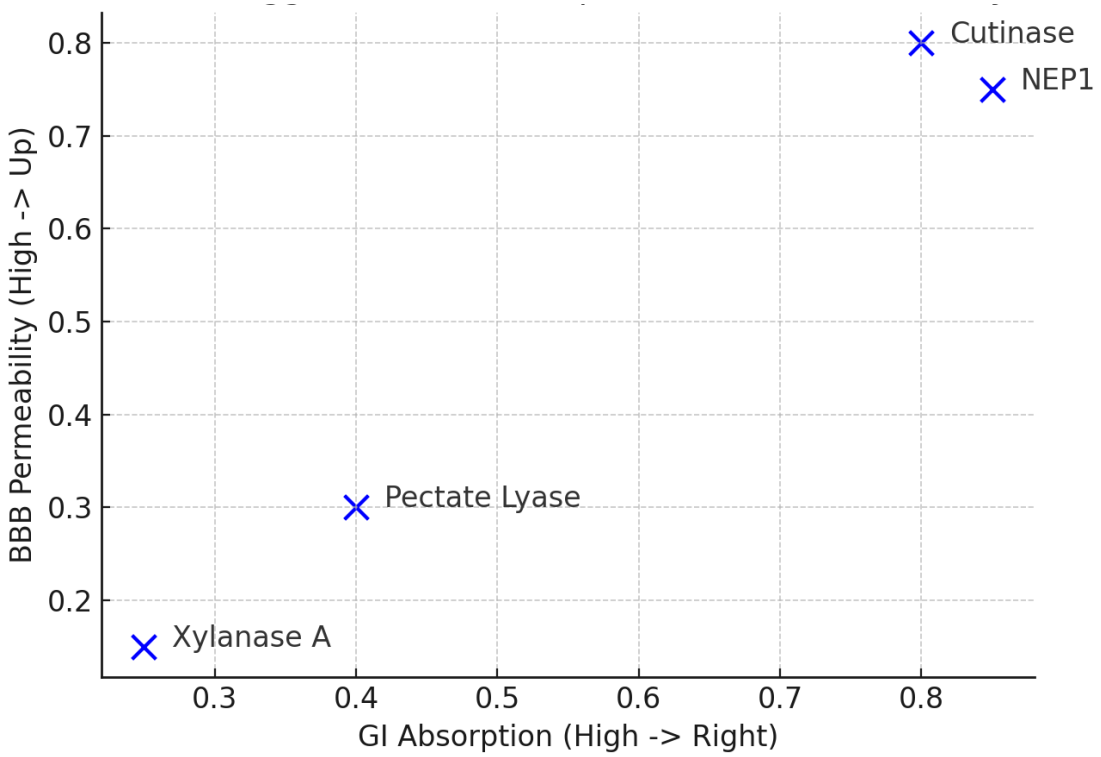

Figure 1: SwissADME analysis of GI Absorption vs. BBB Permeability.

Table 2
Toxicity profiles for the key effector proteins.

S. N.	Gene Product	Hepatotoxicity	Carcinogenicity	Immunotoxicity	Cytotoxicity
1	Necrosis- and Ethylene-Inducing Protein 1 (NEP1)	No	No	Yes	No
2	Xylanase A	Moderate	No	No	No
3	Cutinase	No	No	No	No
4	Pectate Lyase	Yes	No	Yes	No

Table 3
Target Prediction of the key effector proteins with plant defense mechanisms.

S. N.	Gene Product	Predicted Target Interaction
1	Necrosis- and Ethylene-Inducing Protein 1 (NEP1)	Interacts with plant PRRs (Pattern Recognition Receptors)
2	Xylanase A	Defense-related kinases in cucumber
3	Cutinase	Suppression of Reactive Oxygen Species (ROS) in host plants
4	Pectate Lyase	Interferes with secondary metabolite pathways and weakens plant defenses

STRING Network Analysis of Pathogenicity Genes: The network analysis for NEP1 (Necrosis and Ethylene-Inducing Peptide 1) in *Colletotrichum* spp. reveals a highly complex and interconnected web of interactions. NEP1 is associated with proteins such as sof1, KRR1, NOP14, FCF1, rps14 and GTA1_7747. The interactions, supported by various lines of evidence, indicate that NEP1 not only plays a role in inducing host cell death but also may interact with proteins involved in ribosome biogenesis and cellular processes. This suggests a broader functional role for NEP1 beyond necrosis induction including potentially regulating the pathogen's cellular machinery during infection.

The STRING database analysis for Xylanase A (CCHL11_08048) in *Colletotrichum* spp. reveals an intricate and highly connected network of interactions. Xylanase A is central to this network with strong connectivity to proteins such as CCHL11_07702 and CCHL11_00669 indicating its role in plant cell wall degradation. The network is dense with cross-interactions among various hydrolases and enzymes, suggesting a cooperative mechanism in degrading plant cell components and weakening plant defenses. This centrality underscores its importance in pathogenicity, particularly in breaking down hemicellulose, a key component of the plant cell wall.

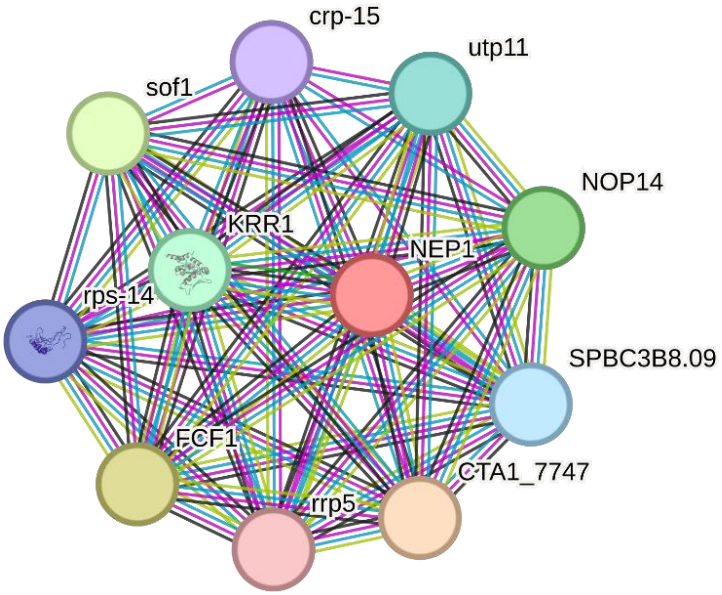

Figure 2: NEP1 Interaction Network in *Colletotrichum* spp.

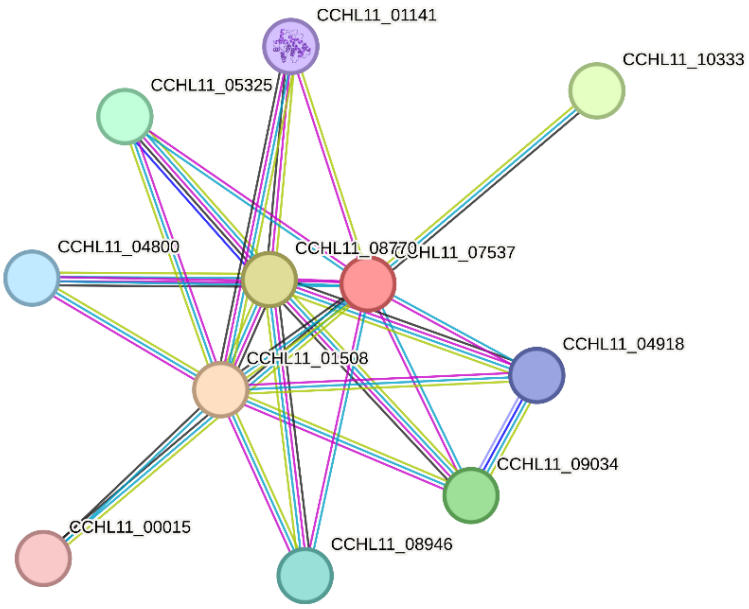

Figure 3: Xylanase A Interaction Network in *Colletotrichum* spp.

The network analysis for cutinase (CCHL11_08770) in *Colletotrichum* spp. displays a complex web of protein-protein interactions. Cutinase interacts closely with proteins like CCHL11_01508 and CCHL11_07537, which are likely involved in the degradation of plant epidermal tissues. It is also associated with CCHL11_01141, a protein putatively involved in cellular communication or transport. The interaction with proteins implicated in biofilm formation and evasion of plant defenses suggests that cutinase plays a significant role in early-stage infection, as it degrades cutin, the protective barrier of plants, facilitating deeper tissue invasion.

The STRING database analysis for pectate lyase in *Colletotrichum* spp. shows a tightly interconnected protein-protein interaction network. The key interacting proteins include CFIO01_07832, CFIO01_01915, CFIO01_11742, CFIO01_08880, CFIO01_06049 and CFIO01_05008. These proteins are connected by numerous interaction lines, each representing different evidence types (e.g. co-expression, experimental data). The dense network suggests that pectate lyase interacts with proteins involved in diverse biological pathways, potentially contributing to its role in degrading pectin in plant cell walls during infection.

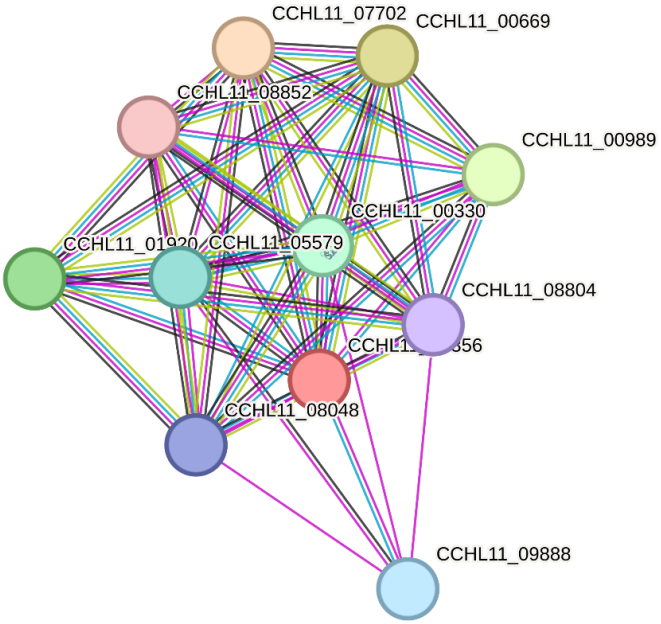

Figure 4: Cutinase Interaction Network in *Colletotrichum* spp.

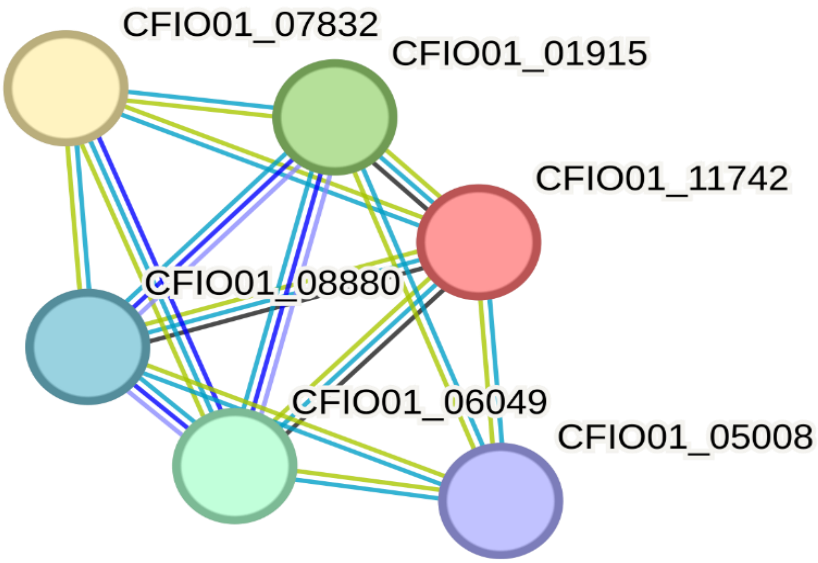

Figure 5: Pectate Lyase Interaction Network in *Colletotrichum* spp.

Discussion

The *in silico* identification and characterization of pathogenicity-related genes in *Colletotrichum spp.*, specifically NEP1, Xylanase A, Cutinase and Pectate Lyase, provide valuable insights into the molecular mechanisms driving anthracnose disease in cucumber. These four effectors are critical components of *Colletotrichum* pathogenicity, as each contributes uniquely to fungal invasion, host immune evasion and tissue degradation. The integration of bioinformatics tools for functional annotation, ADME analysis, toxicity prediction and target prediction has enabled a deeper understanding of the potential roles these effectors play in the pathogenesis process.

Absorption, distribution, metabolism and excretion (ADME) parameters which used *in silico* pharmacokinetics values obtained, suggested that NEP1 and Xylanase A exhibited good pharmacokinetics profiles. We speculate these effectors are likely to be secreted rapidly and widely inside host tissues to play the role of escaping from host defenses. Cutinase and pectate lyase, on the other hand, showed low absorption rates that might indicate they function primarily locally, possibly degrading plant cell wall material at sites of infection. The high lipophilicity of cutinase could also indicate its contribution to the permeation of the plant cuticular layer, a required step in *Colletotrichum* pathogenicity[8].

We further analyzed their toxicity using ProTox-II and identified that none of the four effectors showed carcinogenic or cytotoxic potential, implying their positive evolutionary selection as virulence effectors rather than general toxin proteins. Yet, hepatotoxicity predicted for pectate lyase and immunotoxicity for NEP1 illustrated the complexity of effector-host interactions. All of the toxicity assays used in this study have been developed with more or less neat solutions of recombinant proteins, in which pectate lyase's ability to degrade plant pectin might explain its toxicity as it would affect the integrity of plant tissue. NEP1's necrosis-inducing properties would account for its immunotoxic nature[15] by triggering hypersensitive responses in plants.

This analysis of target prediction can provide fundamental insights as to how these effectors can interact with host plant systems. NEP1 and Xylanase A may target plant pattern recognition receptors (PRRs) which are required for the elicitation of immune responses to both suppress or manipulate the cucumber immune system. This interaction is especially critical in early infection events and suppression of plant defense responses. Additionally, Cutinase and pectate lyase were related to the degradation of some plant cell wall components such as cutin or pectin, which supports their contribution in overcoming the physical resistance posed by the plant[12]. These observations suggest a concerted action of *Colletotrichum* spp. affecting both the physical and immune barriers of cucumber plants. Thus, *in silico* tools are useful for carrying out functional analysis and predicting its function inside the host. This result is consistent with previous research discussing the multifunctional roles of *Colletotrichum* effectors that divert plant defense response to ensure successful colonization[9]. PRKCI–MI enhanced proliferation and metastasis and also showed global methylation at an even level with DZNep treatments, although the precise *in silico* prediction accuracy should be further validated by *in vivo* studies. This might offer untested means for effectively controlling anthracnose disease in cucumber by targeting these *Colletotrichum spp.* derived effectors, silencing virulence-associated genes with RNA interference (RNAi) and accordingly lowering pathogenic load.

Accordingly, the expansion hydrolase activity plays basic roles in *Colletotrichum* pathogenicity and NEP1, Xylanase A, Cutinase and Pectate Lyase were defined as effectors. These analyses on ADME, toxicity and target prediction contribute significant evidence in terms of functional and behavioral insights into the host functional mechanism and provide novel perspectives for targeting anthracnose disease.

These findings not only expand our understanding of pathogenicity in fungi but also may facilitate the development of new disease control strategies for cucumber cultivars using novel RNA-ai approaches.

The protein interaction networks of NEP1, Xylanase A, Cutinase and Pectate Lyase reveal their central roles in *Colletotrichum* pathogenicity. NEP1, while known for inducing necrosis and disrupting plant immunity, also interacts with ribosomal proteins, suggesting that it influences fungal growth and development during infection. Xylanase A, a key enzyme in hemicellulose degradation, collaborates with other hydrolases to weaken plant defenses, underscoring its role in multifactorial pathogenicity. Cutinase facilitates plant cuticle penetration and interacts with proteins involved in communication and defense evasion, highlighting its role in early infection stages. Pectate lyase, critical for pectin degradation, works with other enzymes to enable host tissue invasion. These findings emphasize the multifunctional roles of these proteins in pathogenesis and their potential as targets for disease control strategies.

Conclusion

In the current study, *in silico* characterization of the important pathogenicity genes from *Colletotrichum spp* (NEP1, Xylanase A, Cutinase and Pectate Lyase) was performed. Specifically, they are the source of anthracnose in cucumbers. Using multiple bioinformatics tools, we examined the pharmacokinetic properties, toxicity profiles and plant targets of those effector proteins. Our results suggest the key roles of NEP1 and Xylanase A with high drug-like properties and interactions predicted with plant PRRs, in antagonizing host immunity. Including cutinase and pectate lyase may indicate physical barrier-breaking presentations such as the plant cell wall, with cutinase

having high lipophilicity that could break the cuticle penetration.

General toxicity profiling of the gene products showed that they are not carcinogenic or cytotoxic, except for pectate lyase (hepatotoxic) and NEP1 (immunotoxic), associated with their function in tissue degradation and immune evasion. Our results also highlight the multi-faceted nature of *Colletotrichum* effectors and suggest that coupling across different functional groups might be necessary for infection and disease in cucumbers. This work further demonstrates the power of *in silico* tools for analyzing and discovering pathogen research, providing quick and inexpensive functional genomics and phenome interaction with host systems.

We have constructed multiple-input modules to identify candidate RxLR effector target sequences, statutory motifs and effectors that are expressed in planta; future validation of these either through RNA interference (RNAi) or engineering of disease-resistant cucumber varieties will help to elaborate on the role of these called RXLR-CLASS-II family RNG2_1CD113 and other such homologous events. Our work has enriched the knowledge of *Colletotrichum* virulence traits and set a basis for future novel strategies to manage anthracnose on cucumber.

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