POTENTIAL OF ANTIMICROBIAL PEPTIDES, NATURAL AND BIOINSPIRED, FROM PLANTS OF THE GENUS *Capsicum* IN THE CONTROL OF PHYTOPATHOGENS *Colletotrichum scovillei* AND *Xanthomonas euvesicatoria*

LARISSA MAXIMIANO RESENDE

UNIVERSIDADE ESTADUAL DO NORTE FLUMINENSE DARCY RIBEIRO - UENF

CAMPOS DOS GOYTACAZES - RJ
MARCH - 2024

POTENTIAL OF ANTIMICROBIAL PEPTIDES, NATURAL AND BIOINSPIRED, FROM PLANTS OF THE GENUS *Capsicum* IN THE CONTROL OF PHYTOPATHOGENS *Colletotrichum scovillei* AND *Xanthomonas euvesicatoria*

LARISSA MAXIMIANO RESENDE

"Thesis presented to the Centro de Ciências e Tecnologias Agropecuárias of the Universidade Estadual do Norte Fluminense Darcy Ribeiro, as part of the requirements for obtaining the title of Doctor of Science in Genetics and Plant Breeding."

Advisor: Prof.ª Valdirene Moreira Gomes
Co-advisor: Prof.ª Érica de Oliveira Mello

CAMPOS DOS GOYTACAZES - RJ
MARCH - 2024

FICHA CATALOGRÁFICA
UENF - Bibliotecas
Elaborada com os dados fornecidos pela autora.

R433 Resende, Larissa Maximiano.

Potential of antimicrobial peptides, natural and bioinspired, from plants of the genus *Capsicum* in the control of phytopathogens *Colletotrichum scovillei* and *Xanthomonas euvesicatoria* / Larissa Maximiano Resende. - Campos dos Goytacazes, RJ, 2024.

125 f. : il.
Bibliografia: 89 - 114.

Tese (Doutorado em Genética e Melhoramento de Plantas) - Universidade Estadual do Norte Fluminense Darcy Ribeiro, Centro de Ciências e Tecnologias Agropecuárias, 2024.
Orientadora: Valdirene Moreira Gomes.

1. Antimicrobial potential. 2. Amps gene expression. 3. *Capsicum*. 4. *Colletotrichum scovillei*. 5. *Xanthomonas euvesicatoria*. I. Universidade Estadual do Norte Fluminense Darcy Ribeiro. II. Título.

CDD - 631.5233

POTENTIAL OF ANTIMICROBIAL PEPTIDES, NATURAL AND BIOINSPIRED, FROM PLANTS OF THE GENUS *Capsicum* IN THE CONTROL OF PHYTOPATHOGENS *Colletotrichum scovillei* AND *Xanthomonas euvesicatoria*

LARISSA MAXIMIANO RESENDE

"Thesis presented to the Centro de Ciências e Tecnologias Agropecuárias of the Universidade Estadual do Norte Fluminense Darcy Ribeiro, as part of the requirements for obtaining the title of Doctor of Science in Genetics and Plant Breeding."

Approved on March 19, 2024

Examining Committee:

Documento assinado digitalmente
gov.br
ADRIANA FERREIRA UCHOA
Data: 04/06/2024 16:25:48-0300
Verifique em https://validar.iti.gov.br

Prof.ª. Adriana Ferreira Uchôa (D.Sc., Biosciences and Biotechnology) - UFRN

Ana Eliza Zeraik

Prof.ª Ana Eliza Zeraik (D. Sc. Applied Physics) - UENF

Vitor Batista Pinto

Prof. Vitor Batista Pinto (D.Sc., Genetics and Breeding) - UENF

Prof.ª Érica de Oliveira Mello (D.Sc., Biosciences and Biotechnology) - UENF (Co-advisor)

Prof.ª. Valdirene Moreira Gomes (D.Sc., Biological Sciences) - UENF (Advisor)

ACKNOWLEDGMENTS

To the Universidade Estadual do Norte Fluminense Darcy Ribeiro and the Postgraduate Program in Genetics and Plant Breeding, for competent academic training.

To Coordenação de Aperfeiçoamento de Pessoal de Nível Superior – CAPES-, to Fundação Carlos Chagas Filho de Amparo à Pesquisa do Estado do Rio de Janeiro – FAPERJ-, and the Conselho Nacional de Desenvolvimento Científico e Tecnológico- CNPq.

To professors Adriana Ferreira Uchôa, Ana Eliza Zeraik and Vitor Batista Pinto for participating in the evaluation panel of this work.

To Advisor Valdirene Moreira Gomes, for her dedicated guidance during my eight years of scientific research.

To co-Advisor Érica de Oliveira Mello, for her co-guidance during this journey.

To Professor André de Oliveira Carvalho, and Dr. Gabriel Bonan Taveira, for all their contributions and clarified doubts during the research process.

To the team at the Plant Genetic Improvement Laboratory, especially doctors Anderson Yusei Suzuki Fukuji, Antonio André da Silva Alencar, and Claúdia Pombo Sudré, who helped me with the greenhouse experiments.

To Dr. Viviane Yumi Baba, Dr. Juarez Pires Tomaz, and Professor Dr. Leandro Simões Azeredo Gonçalves for developing the gene expression analysis that took place in Londrina/PR.

To my colleagues in the laboratory and research group, I thank you for the coexistence, exchange of knowledge and moments of relaxation, which made the routine easier.

To my family, for the encouragement, understanding and constant love that were my driving force.

Finally, I thank God for blessing me and taking care of my life.

This study was financed in part by the Coordination for the Improvement of Higher Education Personnel - Brazil (CAPES) – Finance Code 001.

SUMMARY

ABSTRACT vi
RESUMO..viii
1. INTRODUCTION..1
2. OBJECTIVES 3
2.1. Objectives of the First Chapter 3
2.2. Objectives of the Second Chapter..3
2.3. Objectives of the Third Chapter...4
3. CHAPTERS..5
3.1. DEFENSIN -LIKE PEPTIDES FROM *Capsicum chinense* INDUCE INCREASED ROS , LOSS OF MITOCHONDRIAL FUNCTIONALITY, AND REDUCED GROWTH OF THE FUNGUS *Colletotrichum scovillei*........................5
3.1.1. INTRODUCTION..5
3.1.2. LITERATURE REVIEW 7
3.1.3. MATERIAL AND METHODS 14
3.1.4. RESULTS..20
3.1.5. DISCUSSION...28
3.1.6. CONCLUSIONS...31
3.2. CONTROL OF PEPPER PLANT PATHOGENS, *IN VITRO* AND *IN VIVO*, BY A PEPTIDE BIOINSPIRED FROM *Capsicum annuum* DEFENSIN....................32
3.2.1. INTRODUCTION..32
3.2.2. LITERATURE REVIEW 34

3.2.3. MATERIAL 40
3.2.4. RESULTS...47
3.2.5. DISCUSSION..60
3.2.6. CONCLUSION 62
3.3. DIFFERENTIAL EXPRESSION OF ANTIMICROBIAL PEPTIDES IN *Capsicum annuum* FRUITS, RESISTANT AND SUSCEPTIBLE TO ANTHRACNOSE, IN RESPONSE TO INFECTION WITH *Colletotrichum scovillei* 63
3.3.1. INTRODUTION 63
3.3.2. LITERATURE REVIEW 65
3.3.3. MATERIAL AND METHODS 77
3.3.4. RESULTS...80
3.3.5. DISCUSSÃO 83
3.3.6. CONCLUSÃO 86
4. FINAL CONSIDERATIONS 87
REFERENCES..89

ABSTRACT

RESENDE, Larissa Maximiano; D.Sc. Universidade Estadual do Norte Fluminense Darcy Ribeiro; march de 2024; POTENTIAL OF ANTIMICROBIAL PEPTIDES, NATURAL AND BIOINSPIRED, FROM PLANTS OF THE GENUS *Capsicum* IN THE CONTROL OF PHYTOPATHOGENS *Colletotrichum scovillei* AND *Xanthomonas euvesicatoria*; Advisor: Prof.ª Valdirene Moreira Gomes; Co-advisor: Prof.ª Érica de Oliveira Mello; Counselors: Prof.ª Rosana Rodrigues.

Antimicrobial peptides (AMPs) are peptides found naturally in all groups of organisms. In plants, these proteins play important roles in physiological functions and in the innate defense system and can control the growth and development of a wide range of microorganisms. Due to their agile mode of action in eliminating pathogens, low cytotoxicity to mammalian cells, broad spectrum of action and great diversity of structures and amino acid sequences, AMPs are of interest in the development of new antimicrobial agent candidates, such as those that guarantee well-being and food security in the face of the threat of antimicrobial resistance and lack of control over certain rules in agriculture. Peppers of the *Capsicum* genus are known as abundant reservoirs of compounds with antimicrobial properties, especially in the presence of AMPs. Due to this potential, AMPs have stood out in research dedicated to the exploration and understanding of new molecules and their mechanisms of action. Therefore, the objective of this work was to investigate the potential of AMPs from both natural and synthetic pepper plants for controlling *Xanthomonas euvesicatoria* and C*olletotrichum scovillei*. We also analyzed the expression profiles of AMP genes during *C. scovillei* infection in pepper fruits to

better understand the roles of these molecules in plant defense. In the first chapter, we address the purification of natural AMPs using the ion exchange chromatography method, molecular exclusion, and phase reversal in a high-performance liquid chromatography system. A fraction rich in plant defensins was isolated from *C. chinense* fruits, which inhibited 90% of the growth of the fungus *C. scovillei* at a concentration of 100 µg/mL. This fraction involved an endogenous increase in reactive oxygen species (ROS), loss of mitochondrial functionality, and morphological alteration of hyphae. A fraction of the fungus also inhibited the activity of insect alpha-amylases. In the second chapter, we address the activity of synthetic peptides with a design inspired by natural AMPs. Therefore, we tested the antimicrobial activity of the synthetic peptide *Ca*Def2.1$_{G27\text{-}K44}$, which is bioinspired from a *C. annuum* defense agent, against *C. scovillei* and *X. euvesicatoria*. The strategies of increasing the hydrophobicity and net charge of *Ca*Def2.1$_{G27\text{-}K44}$ increased the inhibitory effect on microorganisms. A reduction in symptoms of common bacterial growth was also observed in *C. annuum* leaves treated with *Ca*Def2.1$_{G27\text{-}K44}$. In the third chapter, we investigated the gene expression profiles of three families of AMPs, CA07g03740 (defensin), CA03g33250 (LTP) and the Kunitz-type protease inhibitor CA05g17340 (KTI), in response to *C. scoviilei* infection in fruit pepper (*C. annuum*). The genes were analyzed by RT–qPCR, which revealed differential expression. The LTP gene is regulated late, KTI responds within the first 24 hours after inoculation, and defensin is repressed first, followed by induction. The difference in transcriptional behavior between the genes suggested specific roles for peptides during the interaction between *C. annuum* and *C. scoviilei.* These findings provide promising information about the role of AMPs in plant defense and control of agriculturally important phytopathogens.

Keywords: Antimicrobial potential; Amps gene expression; *Capsicum; Colletotrichum scovillei;* X*anthomonas euvesicatoria*.

RESUMO

RESENDE, Larissa Maximiano; D.Sc. Universidade Estadual do Norte Fluminense Darcy Ribeiro; março de 2024; POTENCIAL DE PEPTÍDEOS ANTIMICROBIANOS, NATURAIS E BIOINSPIRADOS, DE PLANTAS DO GÊNERO *Capsicum* NO CONTROLE DE FITOPATOGÊNIOS *Colletotrichum scovillei* E *Xanthomonas euvesicatoria*; Orientadora: Prof.ª Valdirene Moreira Gomes; Coorientadora: Prof.ª Érica de Oliveira Mello; Conselheira: Prof.ª Rosana Rodrigues.

Os peptídeos antimicrobianos (AMPs, do inglês *antimicrobial peptides*) são peptídeos encontrados naturalmente em todos os grupos de organismos. Nas plantas, desempenham um papel importante em funções fisiológicas e no sistema de defesa, com capacidade de controlar o crescimento e desenvolvimento de uma ampla gama de microrganismos. Devido ao seu ágil modo de ação na eliminação de patógenos, sua baixa citotoxicidade para células de mamíferos, ter amplo espectro de ação e uma grande diversidade de estruturas e sequências de aminoácidos, os AMPs têm atraído interesse no desenvolvimento como novos candidatos a agentes antimicrobianos, com o objetivo de garantir o bem-estar e a segurança alimentar diante da ameaça de resistência antimicrobiana e descontrole de pragas na agricultura. As pimentas do gênero *Capsicum* são conhecidas como um reservatório abundante de compostos com propriedades antimicrobianas, em especial a presença de AMPs. Devido a esse potencial, os AMPs têm se destacado em pesquisas dedicadas à exploração e compreensão de novas moléculas e de seus mecanismos de ação. Dessa forma, o objetivo deste trabalho foi investigar o potencial de AMPs de plantas de pimenta, naturais e sintéticos, no controle de

Xanthomonas euvesicatoria e *Colletotrichum scovillei*. Também foi analisado o perfil de expressão de genes de AMPs diante a infecção de *C. scovillei* em frutos de pimenta, podendo assim compreender melhor o papel dessas moléculas na defesa da planta. No primeiro capítulo, abordou-se a purificação de AMPs naturais pelo método de cromatografias de troca iônica, exclusão molecular e fase reversa em sistema de cromatografia líquida de alta eficiência. Foi isolada uma fração rica em defensinas de plantas a partir de frutos de *C. chinense* a qual inibiu 90% de crescimento do fungo *C. scovillei* na concentração de 100 µg/mL. Essa fração causou aumento endógeno de espécies reativas de oxigênio, perda da funcionalidade mitocondrial e alteração morfológica das hifas. A fração contendo a defensina também inibiu a atividade das α-amilases de insetos. No segundo capítulo, abordamos a atividade de peptídeos sintéticos com *design* inspirado em AMPs naturais. Dessa forma, testamos a atividade antimicrobiana do peptídeo sintético *Ca*Def2.1$_{G27\text{-}K44}$, bioinspirado a partir de uma defensina de *C. annuum*, contra *C. scovillei* e *X. euvesicatoria.* Verificou-se que estratégias do aumento da hidrofobicidade e de carga líquida de *Ca*Def2.1$_{G27\text{-}K44}$ proporcionou um aumento na capacidade de inibição dos microrganismos. Foi observado também uma redução dos sintomas de crestamento bacteriano comum nas folhas de *C. annuum* tratadas com *Ca*Def2.1$_{G27\text{-}K44}$. No terceiro capítulo, investigou-se o perfil de expressão de genes de três famílias de AMPs, *CA07g03740* (defensina), *CA03g33250* (LTP) e inibidores de proteases do tipo kunitz *CA05g17340* (KTI) em resposta à infecção por *C. scoviilei* em frutos de pimenta (*C. annuum*). Os genes foram analisados por RT-qPCR e apresentaram expressão diferencial. O gene da LTP apresentou regulação tardia, o KTI responde já nas primeiras 24 horas após a inoculação e a defensina apresenta repressão inicial, seguida de indução. A diferença de comportamento transcricional entre os genes sugere papéis específicos dos peptídeos durante a interação entre *C. annuum e C. scoviilei*. Esses achados fornecem informações promissoras sobre o papel de AMPs na defesa das plantas e controle de fitopatógenos importantes para a agricultura.

Palavras-chave: Potencial antimicrobiano; Expressão gênica de AMPs; *Capsicum; Colletotrichum scovillei;* X*anthomonas euvesicatoria*.

1. INTRODUCTION

Antimicrobial peptides (AMPs) are a group of naturally occurring polypeptides that constitute part of the innate defense system found in virtually all groups of living organisms. These peptides have the ability to control the growth of various microorganisms, including fungi, bacteria, viruses, protozoa, and other parasites. They exert their biocidal or biostatic properties, either directly or indirectly, in the host's immune response (Yuan et al., 2023).

AMPs have attracted significant attention in research aimed at developing new microbial control agents, forming part of global strategies for health promotion and sustainable development. This recognition is attributed to their broad-spectrum antimicrobial properties, unique and rapid mechanisms of action, and their ability to induce low resistance compared to traditional antibiotics (Boto et al., 2018; Răileanu et al., 2023). This prominence arises amid growing concerns about the safety of human health, livestock, and agriculture due to the increasing cases of microorganisms resistant to multiple drugs and agrochemicals (Mendelson et al., 2024).

Moreover, AMPs are of natural origin and, as such, are considered less harmful to the environment. They are biodegradable and their degradation does not produce toxic or accumulative residues. These characteristics make them promising components in strategies to address challenges related to antimicrobial resistance while maintaining a commitment to sustainability (Gupta e Mahajan, 2015; Sarkar et al., 2021).

AMPs exhibit a wide diversity of characteristics, encompassing differences in biological source, physicochemical attributes such as hydrophobicity and charge, target of action, and secondary structure arrangements, which enables their classification and biological activities (Zhang et al., 2022). Regarding structure, these peptides can be grouped into four main categories based on the conformation of their secondary structures. These categories include peptides with linear and disordered structures in solution that may undergo conformational changes upon interaction with target membranes, α-helix structures, β-sheet structures, and proteins that combine both α-helix and β-sheet structures in a single organized chain (Bin Hafeez et al., 2021).

AMPs, which are present in all groups of living organisms, can also be classified according to their origin. They have been identified in insects, mammals, amphibians, fish, and other marine animals, as well as in bacteria, fungi, and plants (Zhang et al., 2022). The objective of this study is to investigate the role of natural and bioinspired plant-derived antimicrobial peptides (AMPs) in controlling pepper phytopathogens.

2. OBJECTIVES

2.1. Objectives of the First Chapter

- Separate and characterize AMPs from *C. chinense* pepper fruits present in fraction G3, obtained via molecular exclusion chromatography, using high-performance liquid chromatography (HPLC).
- Evaluate the in vitro antifungal potential of the fractions on the growth of the fungus *C. scovillei*.
- Investigate the toxic effect of the fraction that exhibited antifungal activity on cellular structures.

2.2. Objectives of the Second Chapter

- Investigate the antimicrobial potential of the synthetic peptides *Ca*Def2.1 and *Ca*Def2.1$_{G27\text{-}K44}$ on the in vitro inhibition of the fungus *C. scovillei* and the bacterium *X. euvesicatoria*.
- Understand the toxic effects of the synthetic peptide *Ca*Def2.1$_{G27\text{-}K44}$ on cellular structures of *C. scovillei* and the bacterium *X. euvesicatoria*.
- Assess the potential of the synthetic peptide *Ca*Def2.1$_{G27\text{-}K44}$ in controlling the development of the bacterium *X. euvesicatoria* in vivo in pepper leaves.

2.3. Objectives of the Third Chapter

Identify transcribed AMP genes in *C. annuum* pepper fruits inoculated with the fungus *C. scovillei*.

Analyze the expression profile of AMPs in *C. annuum* fruits inoculated with the fungus *C. scovillei*.

3. CHAPTERS

3.1. DEFENSIN-LIKE PEPTIDES FROM *Capsicum chinense* INDUCE INCREASED ROS, LOSS OF MITOCHONDRIAL FUNCTIONALITY, AND REDUCED GROWTH OF THE FUNGUS *Colletotrichum scovillei* (Manuscritpt accepted in Pest Management Science - PM-23-1410)

3.1.1. INTRODUCTION

Fungi and oomycetes are two of the most common families of pathogens capable of destroying crops (Fones et al., 2020). On average, fungal phytopathogens have the potential to cause a 30% reduction in agricultural yields (Avery et al., 2019). In addition to losses during planting and harvesting, mycotoxins produced by fungi in postharvest fruits not only compromise their nutritional quality but also pose long-term health risks for those who consume them (Kępińska-Pacelik e Biel, 2021). In this context, controlling phytopathogenic fungi that threaten food safety, human health, and biodiversity is an essential socioeconomic goal. Currently, synthetic chemical fungicides are used to control phytopathogenic fungi. Fungicides, on the other hand, can be hazardous and accumulate in the environment if applied widely to crops. As a result of the amplification of selective

pressures, fungal resistance to chemicals or cross-resistance grows (Fones et al., 2020; Bastos et al., 2021).

Therefore, new strategies for mitigating the impacts of phytopathogenic fungi have been intensively investigated. Natural plant defense chemicals are interesting alternative biotechnological strategies for microorganism growth control (Parthasarathy et al., 2021). The natural defense components of plants include protein inhibitors, chitinases, β-1,3 glucanases, secondary metabolites, and antimicrobial peptides (AMPs) (Zaynab et al., 2018, 2019).

AMPs are amino acid polymers with up to 100 residues that play an important role in plant defense. They are synthesized by ribosomes and become mature peptides by undergoing cleavage from larger precursor proteins or other posttranslational modifications. AMPs have already been found in several botanical families, including Violaceae, Fabaceae, Rubiaceae, and Solanaceae, as well as in diverse plant parts, including leaves, flowers, fruits, and roots (Tam et al., 2015). AMPs have an important role in plant defense pathways in response to biotic and abiotic stressors, in addition to physiological tasks such as growth and development regulation (Maximiano e Franco, 2021; Rodrigues et al., 2021). Because of their diverse architectures, AMPs are classified as lipid transfer proteins, heveins, thionines, protease inhibitors, cyclotides, defensins, and others (Tam et al., 2015).

Defensins were among the first AMPs to be discovered and researched. They are an important component of plants' innate immune system. Most are cationic, have little toxicity to mammalian cells and have a wide range of amino acid sequence variations (45-54). The three-dimensional structure, on the other hand, is substantially conserved in three antiparallel beta sheets joined by an alpha helix. Disulfide linkages between the eight cysteine residues (C1-C8/C2-C5/C3-C6/C4-C7) offer great stability to chemical proteolysis and high temperatures and a conserved tertiary structure (Parisi et al., 2019). They have a broad spectrum of activity and are capable of targeting various pathogens, including fungi (both yeast and filamentous), protozoa, enveloped viruses, gram-positive bacteria, and gram-negative bacteria (Nascimento et al., 2015; Gebara et al., 2020; Mello et al., 2011).

The pathogen-killing mechanisms of defensins are highly dependent on the interaction between the AMP structure and the pathogen. These mechanisms can be membrane-disruptive when they interact with and disrupt membrane phospholipids, leading to cell lysis and death (Benfield e Henriques, 2020).

Furthermore, defensins can transiently cross cell membranes and act on specific intracellular targets(Corrêa et al., 2019). AMPs that act on intracellular targets can induce increased reactive oxygen species (ROS) production, apoptosis, inhibition of DNA, RNA, and protein synthesis, inhibition of enzymatic activities, and mitochondrial dysfunction (Khani et al., 2020).

The pepper genus *Capsicum* is a promising source of AMPs, including defensins, with a wide range of inhibitory actions (Maracahipes et al., 2019b; Santos et al., 2020; Oliveira et al., 2022). However, few investigations have been conducted to identify and analyze defensins in *Capsicum chinense*. In previous studies, Resende et al. (2021) obtained a fraction rich in peptides called the G3 fraction, which inhibited the growth of fungi of the genera *Colletotrichum* and *Fusarium*. The mechanism of action of the peptides on *F. oxysporum* growth inhibition was investigated, and damage to the membrane and increased ROS levels were observed. However, characterization of the proteins found in this fraction is still unknown. Therefore, the present study aimed to identify the peptides present in the G3-Fraction from *Capsicum chinense*, evaluate their inhibition potential, and elucidate the mechanism of action of the peptides on the in vitro growth of the fungus *Colletotrichum scovillei*.

3.1.2. LITERATURE REVIEW

3.1.2.1. Plant antimicrobial peptides

Plant AMPs are polypeptides synthesized by ribosomes, where their mature forms are cleaved from protein precursors and undergo additional post-translational modifications to reach their active conformation (Li e Rebuffat, 2020). They are constitutively found in various plant organs, including fruits, flowers, seeds, roots, and leaves (Zottich et al., 2011; Mulla et al., 2021; Resende et al., 2021; Cherene et al., 2023b).

This distribution suggests that, in addition to essential physiological functions, these peptides are immediately available in any tissue where an infection occurs. It is also possible that others may be induced only under specific conditions.

This hypothesis is supported by the observation that AMP-mediated defense is not limited to the presence of a single AMP at the infection site. Rather, it is the coordinated action of a mixture of peptides, each with distinct characteristics and mechanisms of action, which is responsible for this phenomenon. Furthermore, AMPs are considered versatile as they can exhibit multi-target effects, allowing them to act simultaneously on multiple biological processes and cellular structures (Bakare et al., 2022; Lima et al., 2022).

The majority of plant AMPs exhibit conserved characteristics, being predominantly cationic with a net charge of +2 and amphipathic, where the amino acid residues are arranged in the structure to form a surface with one hydrophilic and one hydrophobic face, and with a molecular mass between 2 and 10 kDa. These molecules have short amino acid sequences, not exceeding 100 residues, and generally contain 4 to 12 cysteine residues, establishing 2 to 6 intramolecular disulfide bonds. These specific disulfide bond patterns are crucial for the tertiary structure conformation, giving them a compact structural nature with high thermal, chemical, and enzymatic stability (Tang et al., 2018).

Although they exhibit conserved characteristics, AMPs display a diversity of amino acid sequences, structures, functionalities, expression patterns, and specific targets. This indicates the complexity of this group. To date, the AMP Data Repository of Antimicrobial Peptides (DRAMP), accessible at http://dramp.cpu-bioinfor.org/, describes 3,791 AMPs from six kingdoms, including 431 from bacteria, 4 from archaea, 7 from protozoa, 6 from fungi, 824 from plants, and 2,519 from animals (Zhang et al., 2021).

The classification of plant AMPs is often based on their amino acid sequences, as well as the number and position of cysteines that form disulfide bonds and the tertiary structure. The main families of AMPs described include thionins, defensins, lipid transfer proteins, snakins, cyclotides, protease inhibitors, and hevein-like peptides (Tam et al., 2015).

3.1.2.2. Mode of action

The mechanism of action of AMPs remains incompletely elucidated. The diversity of this group of proteins gives rise to several peculiarities in the manner by which they lead to the death of microorganisms. Consequently, this area continues

to be extensively studied, and the understanding of these mechanisms has gradually expanded.

The mechanisms of action may involve both the induction of immediate death and the modulation of late apoptotic events. Direct death can be categorized into two distinct events: those that result in the direct activation of AMP under the cytoplasmic membrane, causing cell lysis, and those that result in a transient passage through the membrane, acting on intracellular targets, compromising the structural integrity and physiology of the cell (Yuan et al., 2023).

Due to their cationic characteristics, most AMPs approach the cytoplasmic membrane of microorganisms, interacting through electrostatic attraction. The cytoplasmic membrane of these microorganisms is predominantly composed of negatively charged phospholipids, such as phosphatidylglycerol and cardiolipin, which gives the surface an electronegative charge. This electronegativity makes the microorganism's cell membrane susceptible to the selectivity of AMPs. Furthermore, the hydrophobicity of AMPs allows the lipophilic portion to insert into the lipid bilayer through hydrophobic interactions. This process can compromise the integrity of the membrane (Malmsten, 2015; Yuan et al., 2023).

Some interaction models between antimicrobial peptides (AMPs) and membrane lipids have been described in order to explain how membrane permeabilization occurs (Figure 1). The proposed modes of action for subsequent pore formation include the barrel stave, mat type, toroidal pore, aggregate channel, and anionic lipid template cluster (Corrêa et al., 2019; Bin Hafeez et al., 2021).

The initial interaction between AMPs and microorganisms occurs through an electrostatic attraction (Figure 1A). The barrel stave model can be described as a peptide aggregate with its hydrophobic face oriented towards the central lipid region of the membrane (Figure 1B). The hydrophilic portions of these AMPs then organize themselves to cover the internal region of the pore, which allows for cytoplasmic flow. This is in line with the findings of Shenkarev et al. (2011). The toroidal pore mechanism is analogous to the barrel stave model, yet the peptides remain aligned with the lipid head groups even when they are inserted perpendicularly into the lipid bilayer, forming transient pores, as illustrated in Figure 1C (Allende et al., 2005). In the carpet model, as illustrated in Figures 1D and E, the peptides initially aggregate, forming a layer that covers the membrane like a carpet. This process continues until a threshold concentration of AMPs is reached, at which point membrane disruption

occurs. In this context, the hydrophobic regions of AMPs interact with the hydrophobic portions of lipids, while the hydrophilic ends remain in contact with the solution (Pouny et al., 1992). The anionic lipid clustering mechanism is initiated by the interaction of cationic AMPs with anionic charged lipids, which results in the lateral segregation of these lipids from zwitterionic lipids. This process ultimately leads to the disruption of lipid domains and the complete rupture of the membrane, as illustrated in Figure 1F (Sengupta et al., 2008).

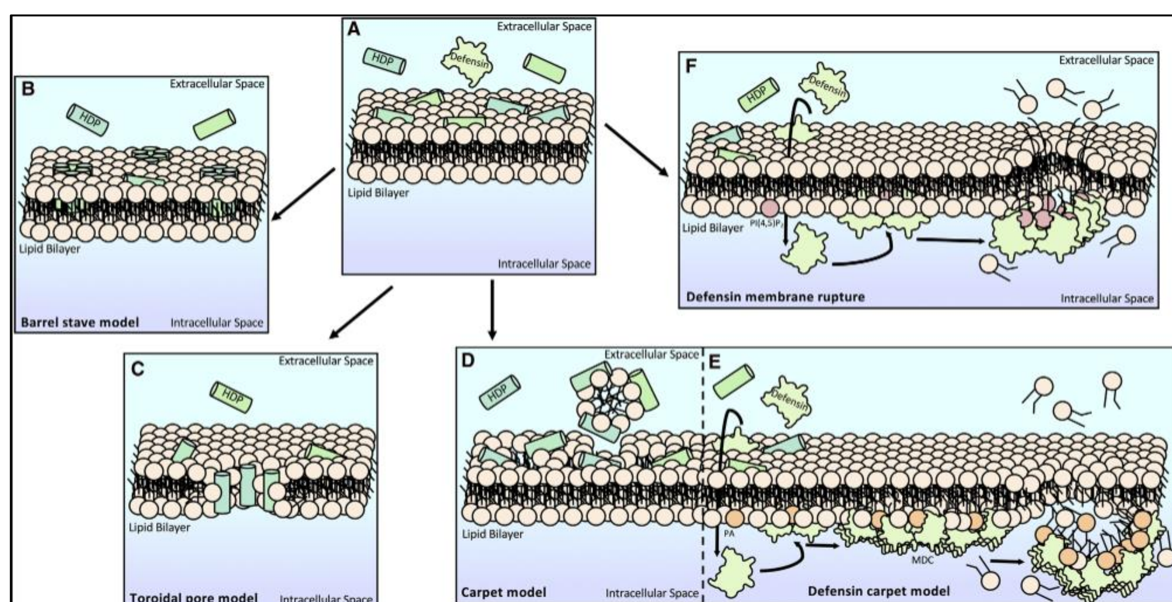


Figure 1: Membrane Permeabilization Models. (A) Approach of AMPs by electrostatic attraction to the cytoplasmic membrane of microorganisms. (B) Positioning of AMPs in the barrel stave model. (C) Organization of AMPs in the lipid bilayer in the toroidal pore model. (D, E) Interaction of AMPs with membrane lipids in the carpet model. (F) Membrane rupture. Extracted and translated from Hein et al. (2022).

However, AMPs can exert their biocidal activity without directly affecting the membrane. In this situation, AMPs cross the membrane without causing disturbances and act on intracellular targets, such as DNA and proteins. Their actions involve interference with DNA replication, transcription, translation, post-translational modifications, and cell division. Furthermore, they can impact the inhibition of proteases, opening of ion channels with cellular extravasation, inhibition of cell wall synthesis, loss of mitochondrial functionality, among others (Bin Hafeez et al., 2021; Bakare et al., 2022).

3.1.2.3. Peppers and chillies of the genus *Capsicum*

Peppers and peppers of the genus *Capsicum* belong to the Solanaceae family and are facultative autogamous, although the cross-fertilization rate can be high, reaching up to 10%, due to the activity of pollinating insects. Species of the genus are diploid and have a chromosome number of 12 or 13 (Slavokhotova et al., 2017; Moscone et al., 2007).

The center of origin for this species is located in Central and South America. The oldest archaeological records, approximately 9,000 years old, that support this theory were identified in Tehuacán, Mexico. Following the colonization of the Americas, expeditions transported *Capsicum* seeds throughout Europe, and then they spread throughout Africa and Asia (Saxena et al., 2016).

Currently, forty species of peppers have been classified into three categories according to the degree of domestication: domesticated, semi-domesticated, and wild. Of these, only five species are domesticated: *C. annuum* var. *annuum* L., *C. baccatum* var. *pendulum* L., *C. chinense* Jacq., *C. frutescens* L. *e C. pubescens* Ruiz et Pavon (LIMA et al., 2017).

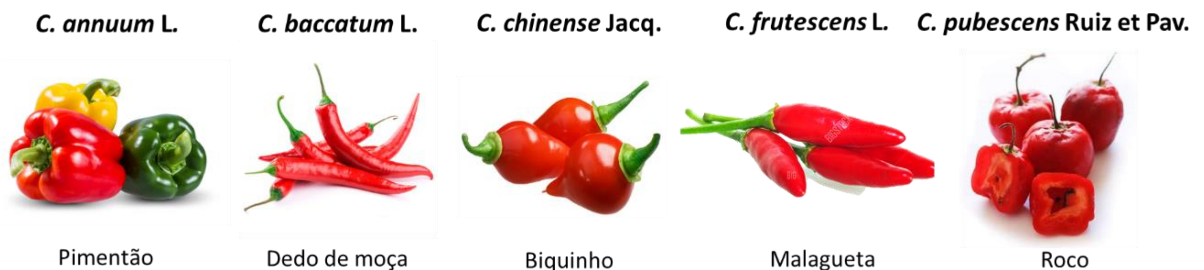


Figure 2: Domesticated pepper species. (Image source: Google photos).

Capsicum species are also divided by gene complexes based on cytogenetic and cross-fertility analyses: The *C. annuum* complex (*C. annuum, C. chinense, C. frutescens,* and *C. galapagoense*), the *C. baccatum* complex, and the *C. pubescens* (Walsh e Hoot, 2001; Onus e Pickersgill, 2004).

Brazil is an important center of diversity for several species, evident mainly in the fruits, which can have different shapes, colors, sizes, and pungency levels. The species *C. chinense* is considered the most brazilian among domesticated

species, as it has the Amazonia Bay region as its center of diversity (Moura e Ryan, 2001; Perry et al., 2007).

Peppers have long been regarded as having cultural significance in many societies around the world. They have been incorporated into cuisine, religious traditions, and cultural festivals. Furthermore, peppers play a crucial role in the global economy, with an expanding market that ranges from small local producers to large agricultural companies. This contributes to international trade and the economic development of exporting countries (Ribeiro et al., 2008).

Despite its relevance, statistical data on the production and marketing of peppers in Brazil are incomplete. But it is known that the brazilian pepper agrobusiness occupies an area of approximately 12 thousand hectares, producing more than 348 thousand tons of fruit per year. Pepper cultivation occurs in practically all regions of Brazil and fits into the family farming model (Ribeiro et al., 2008)

The popularity of peppers is also reflected in the food and beverage industry, where they are used as essential ingredients in a wide range of products. These include sauces and condiments, alcoholic beverages, and confectionery (Sudré et al., 2010).

Peppers are widely recognized for their potential therapeutic effects, which include anti-inflammatory, antioxidant, antictancer, and analgesic properties. These benefits are attributed to the presence of vitamins, carotenoids, capsaicinoids, flavonoids, and proteins in the seeds, fruits, and leaves (Ghiasi et al., 2019; Zheng et al., 2016; Neitzke et al., 2015).

Moreover, these molecules are also associated with the plant's defense against microbial threats. Consequently, scientific studies on the role of metabolites and proteins have demonstrated the antimicrobial potential of peppers (Aguieiras et al., 2021; Baba et al., 2020; Cherene et al., 2023; Giacomin et al., 2021; Von Borowski et al., 2019).

In recent years, a diversity of proteins and peptides from the *Capsicum* genus with antimicrobial activity have been isolated and characterized (Oliveira et al., 2022). As examples, we may cite defensins (Silva et al., 2021; Anaya-López et al., 2006), LTPs (Resende et al., 2023; Cherene et al., 2023; Diz *et. al*, 2003;), protease inhibitors (Pereira et al., 2018; Ribeiro et al., 2007), thionins (Taveira et al., 2014), vicilins (Bard et al., 2014), and hevein-like peptides (Games et al., 2016).

This diversity serves to reinforce the potential of the genus in research aimed at the development of new pathogen control compounds. It demonstrates the richness and versatility of the substances present in peppers, offering a wide range of possibilities for the development of effective antimicrobial agents.

The genome of several pepper species has already been sequenced. This includes the genome of species such as *C. annuum, C. baccatum, C. chinense* and, *C. frutescens*. Genome sequencing is an important step in understanding the biology of peppers, including their genetic diversity, development, disease resistance, metabolism of bioactive compounds, and other related aspects (Kim et al., 2014; Lee et al., 2022; Liu et al., 2023a). This information is valuable for improving the cultivation, quality, and resistance of peppers, in addition to enabling advances in assisted genomic selection and genetic improvement of these plants.

Despite the significance of pepper cultivation, the occurrence of factors such as deficiencies or excesses of nutrients, lack of water and inadequate light can facilitate the development of diseases caused by various pathogenic agents, including fungi, oomycetes, bacteria, viruses, and nematodes. This can result in adverse impacts on pepper production (Babu et al., 2011). Among the most significant diseases are anthracnose, caused by a complex of fungi of the genus *Colletotrichum* (Ali et al., 2016), and bacterial spot, caused by the bacteria *Xanthomonas* (Hernández-Huerta et al., 2021)

Bacterial leaf spot, caused by the genus *Xanthomonas*, is one of the most prevalent and destructive diseases of peppers and other nightshades. The lesions, which are small, brown, angular, and water-soaked, appear on leaves, stems, and fruit. They result in defoliation and direct damage to the fruit. Severe infection can result in extensive crop damage with significant yield losses. Bacterial, primarily through the stomata, colonizing mesophyll cells and causing leaf spots and, in some cases, chlorosis. The bacteria can be spread through seeds and in the field by rain splashes. Disease control can be achieved through the use of resistant cultivars, the application of antibiotics, and the use of copper sprays (Roach et al., 2018).

The incidence of anthracnose in post-harvest fruits is typically attributed to the dissemination of conidia of acervuli or microsclerotia by irrigation or rain, while the fruits are still in the field. The occurrence of hot and humid conditions is known to increase the rate of infection, thereby facilitating the development of the fungus. The conidia adhere to the surface of the fruits and germinate, forming appressoria

that penetrate the fruit's epidermis. The symptoms are characterized by depressed and waterlogged lesions with translucent edges and a light brown color. In advanced stages, the lesions are depressed and covered by concentric rings of moist, gelatinous spores with a salmon color (Ali et al., 2016).

3.1.3. MATERIAL AND METHODS

3.1.3.1. Biological material

The fungus *Colletotrichum scovillei* isolate 8.1 was donated by the Laboratório de Melhoramento Genético Vegetal of the Centro de Ciências e Tecnologias Agropecuárias, Universidade Estadual do Norte Fluminense Darcy Ribeiro (UENF), Campos dos Goytacazes, RJ, Brasil. The fungus was cultured at 30°C in a 2% Sabouraud agar culture medium (10 g/L peptone, 20 g/L glucose, and 17 g/L agar; Merck KGaA, Darmstadt, Germany) in the Laboratório de Fisiologia e Bioquímica de Microrganismos LFBM), Centro de Biociências e Biotecnoloia, UENF, RJ, Brasil. *Capsicum chinense* accession UENF 1751 fruits were produced in a greenhouse at UENF. Harvesting was performed on mature fruits approximately 50 days following anthesis.

3.1.3.2. Peptide extraction and purification

G3, the peptide-rich fraction of *C. chinense* of the fruit pericarp, was prepared using molecular exclusion chromatography with a glass column packed with Sephadex G-50 resin (Sigma–Aldrich Co.) solubilized in 8 mM trifluoroacetic acid (TFA) (Sigma–Aldrich Co.), as detailed by Resende et al.(2021) in a high-performance liquid chromatography (HPLC) system (Shimadzu, Kyoto, Japan), the peptide G3 fraction was separated using reverse-phase chromatography. A LiChrosorb® RP-18 column (5 m), Hibar® RT 250-4 (Merck KGaA, Darmstadt, Germany), and an RPC C18 guard column were employed. Approximately 1 mg/mL of protein was solubilized in 500 µL of 8 mM TFA and filtered through a 0.2 µM Millex-GV filter (Millipore). Subsequently, the sample was introduced into the HPLC

system, and chromatography was conducted at a temperature of 40°C, with a flow rate set at 0.3 mL/min. As the mobile phase, two solvents were used: ultrapure water (solution A) and propanol (solution B), both acidified with 8 μM TFA. The mobile phase gradient conditions were as follows: from 0'01" to 14'59" 100% solvent A, 15'00" to 79'59" linear increase in solvent B concentration up to 40%, 80'00" to 82'59" linear increase in solvent B concentration up to 50%, 83'00" to 84'59" maintenance of solvent B concentration at 50%, and 85'00" solution B pumping ceased, and only solution A was supplied to the system until the final time of 90 minutes. The elution of proteins on the column was monitored by absorbance measurements at 220 ±4 nm with a diode array detector (SPD-M20A, Shimadzu). Protein quantification was carried out using the bicinchoninic acid method published by Smith et al.(1985), with ovalbumin (Sigma–Aldrich Co.) used as the reference standard.

3.1.3.3. Fungal growth inhibition

To obtain conidia, a disk of approximately 1 cm of *C. scovillei* mycelium maintained in stock culture in the LFBM (UENF, Rio de Janeiro, Brazil) was transferred to 20 mL of Sabouraud broth (Merck KGaA) under constant stirring at 180 rpm for 48 h at 30°C. Following this interval, the fluid was filtered through sterile gauze to remove mycelial fragments and hyphae. Afterward, the conidia were cultured in 100 μL of Sabouraud broth containing protein fractions (F1-F5) at concentrations ranging from 25 to 400 μg/mL. The experiment was carried out in 96-well microplates (Thermo Fisher Scientific Inc., Waltham, MA, USA) that were incubated at 30°C for 48 h. Every 6 h, optical density readings at 620 nm (EZ Read 400, Biochrom Ltd, Cambridge, UK) were taken. Wells containing only Sabouraud broth were considered blank. The experiments were carried out in triplicate under aseptic conditions as described by Broekaert et al.(1990). Inhibition percentages were calculated using the formula $[100 - (tABS_{620} \times 100/cABS_{620})]$, where $tABS_{620}$ is the average absorbance at 620 nm of the sample treated with the fractions for 48 h and $cABS_{620}$ is the average absorbance at 620 nm of the control sample at 48 h. Inhibition percentages were calculated assuming that the control represented 100% growth according to the formula $[100 - (tABS_{620} \times 100/cABS_{620})]$, where $tABS_{620}$ is the average absorbance at 620 nm of the sample treated with the fractions for 48 h,

and $cABS_{620}$ is the average absorbance at 620 nm of the control sample at 48 h. Using a linear regression curve, the IC_{50} (50% growth inhibition) was derived from the absorbance data.

3.1.3.4. Assessment of intracellular ROS generation

The inhibitory mechanism of action involving the endogenous increase in ROS levels was investigated using the fluorescent probe dichlorofluorescein 2,7-diacetate (H_2DCFDA) (Calbiochem - EMD, San Diego, CA, USA) following the methodology described by Mello et al.(2011). After 24 h of *C. scovillei* growth inhibition with 21.5 µg/mL F1-Fraction, the total well volume (100 µL) was treated with 20 µM H_2DCFDA for 30 min at 30°C. Under the same conditions, control cells were incubated with the H_2DCFDA probe, and for the positive control, 1 mM acetic acid was added along with the probe. Cells were examined using a DIC light microscope (Axioplan. A2, Zeiss) with a 63X magnification oil immersion objective and a fluorescent filter set for fluorescein detection (excitation: 450-490 nm; emission: 500 nm). The fluorescence intensity and exposure time parameters for fluorescence imaging were adjusted using the positive control, and these values were used for all subsequent treatments.

3.1.3.5. Mitochondrial functionality

The membrane potential of hyphal mitochondria was measured using a MitoTracker™ Deep Red FM probe (Thermo Fisher Scientific Inc., Waltham, MA, USA) as directed by the manufacturer. After a 24 h growth inhibition experiment with the F1 fraction, a total well volume of 100 µL was incubated with 50 nM of the probe at 30°C in the dark for 40 min. The cells were then rinsed twice with HEPES buffer, placed on a slide, covered with coverslips, and viewed under DIC and fluorescence (581 nm excitation, 644 nm emission).

The activity of mitochondrial dehydrogenase enzymes was also investigated. The *C. scovillei* fungus was incubated with 10 µL of 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt (WST-1) (Sigma–Aldrich Co.) and 0.24 mM duraquinone (Sigma–Aldrich Co.) after being treated with 21.5 µg/mL F1-Fraction for 24 h (as explained in section 2.3). As a

positive control, 174.8 mM acetic acid was used. Acetic acid was added to the control (untreated) cells 30 min before WST-1 incubation. Under aseptic circumstances, the tests were carried out in triplicate. Using a microplate reader (EZ Read 400), the absorbance of the formazan crystals in the mixture was measured at 450 nm (Tsukatani et al., 2003).

3.1.3.6. Transmission electronic microscopy

The F1-treated and control samples were fixed for 1 h in a Karnovsky solution (glutaraldehyde at 2.5%, formaldehyde at 4% in 0.1% sodium cacodylate buffer, pH 7.2). After fixation, hyphae were washed twice with 100 mM sodium cacodylate buffer for 10 min. Postfixation was performed in 1% osmium tetroxide and 1.6% ferrocyanide solution in 100 mM sodium cacodylate buffer for 1 h in an environment protected from light. Subsequently, hyphae were washed twice with 100 mM sodium cacodylate for 10 min. The samples were dehydrated in different concentrations of acetone (30%–100%), followed by infiltration in epoxy resin. After this process, the material was incubated for 48 h at 60°C for polymerization. Ultrathin sections of samples were obtained and stained with uranyl acetate and lead citrate. Thereafter, the samples were observed, and images were obtained using a transmission electron microscope (Joel JEM 1400 Plus).

3.1.3.7. Identification of peptides by mass spectrometry

An aliquot of 70 µg of F1-Fraction protein was evaluated using mass spectrometry (León et al., 2007). The sample was first digested with trypsin and then cocrystallized with a large molar excess of the α-cyano-4-hydroxycinnamic acid matrix before being analyzed via matrix-assisted laser desorption time-of-flight mass spectrometry. An AB SCIEX TOF/TOF^{TM} 5800 System spectrometer (AB SCIEX) in reflection mode was employed. Sequences were compared with databases to identify similar proteins in the NCBI databases using BLASTp (Altschul et al., 1990). Sequences with similarity were aligned using the Clustal W program (Larkin et al., 2007).

3.1.3.8. Cloning of defensins expressed in the pericarp of fruit *C. chinense* peppers

To identify the complete sequences of defensins expressed in our extract, total RNA was isolated from pericarp of frozen ripe fruits (50 days after anthesis) using TRIzol® reagent (Invitrogen) according to the manufacturer's instructions. The quality of RNA was assessed using a Thermo Scientific NanoDrop 2000c Spectrophotometer. cDNA was synthesized using 1 μg of RNA and specific reverse primers (Table 1) using the cDNA Synthesis Kit (Cellco Biotec, São Paulo, BR) in accordance with the manufacturer's provided instructions. PCR amplification was performed with Taq High Fidelity Pol Master Mix 2X (Cellco Biotec, São Paulo, BR) using cDNA as a template and the primers described in Table 1 at an annealing temperature of 55°C. PCR products were confirmed by 0.80% agarose electrophoresis and extracted from the gel using the Wizard® SV Gel and PCR Clean-Up System (Promega). Inserts were cloned and inserted into the pGEM-T easy vector (Promega, Madison, EUA). The resulting plasmid was transformed into *Escherichia coli* DH5α competent cells, extracted with the PureYield™ Plasmid Midiprep System (Promega, Madison, EUA), and sequenced by the PSEQDNA-UFRJ, Instituto de Biofísica, Universidade Federal do Rio de Janeiro.

Table 1: Primers used in PCR.

Amplification target defensin	Oligo name	Sequence 5'-3'
CcDef1	Primer 1	
	PHT96429.1	F: GGATCCAAAGTATGCCAACGGCGC
	PHU20899.1	R: CTCGAGTTAGCAGTTGAAGTAGCAGAAGC
CcDef2	Primer 2	F: GGATCCAGAACTTGCGAGTCGCAGA
	PHU11333.1	R: CTCGAGTTAACATGGCCTAGTGCAGAAG
	Primer 3	F: GGATCCAGAGTGTGCATTTCGCAGAG
	PHU17080.1	R: CTCGAGTTAACAATTCTTAGTGCAGTAACACT
	Primer 4	F: GGATCCAGGCATTGCGAGTCGCAG
	PHU11335.1	R: CTCGAGTTAGCATGGCCTAGTGCAGAAG

3.1.3.9. Enzyme inhibitor activity assay

The ability of the F1-Fraction to inhibit the activity of trypsin (Sigma–Aldrich Co.) on the substrate N-benzoyl-DL-arginyl-p-nitroanilide (BA*p*NA) (Sigma–Aldrich Co.) was evaluated. We incubated 50 µg/mL F1-Fraction for 30 min at 37°C with 25 µL of 5 mM BA*p*NA, 10 µL of trypsin (1 mg/mL), and 50 mM Tris-HCl buffer (pH 8.0) to adjust the final volume of the reaction to 200 µL. As a negative control, we used 1 µL Protease Inhibitor Cocktail Power (PICP) (Sigma–Aldrich Co.). The reaction was stopped with 100 µL of 5 M acetic acid (Merck KGaA). The amount of substrate hydrolysis was measured by detecting the p-nitroaniline emission at 405 nm using a 96-well plate-reading spectrophotometer (EZ Read 400, Biochrom Ltd, Cambridge, UK) (Ribeiro et al., 2013).

To evaluate the ability of peptides in the F1-Fraction to inhibit the activity of α-amylases, we used an extract rich in intestinal α-amylases extracted from *Tenebrio molitor* as described by Resende et. Al. (2021) We incubated 50 µg/mL of the F1-Fraction for 30 min at 30°C with 10 units (defined below) of extract rich in α-amylases and 25 µL of 30 mM starch solution (Sigma–Aldrich Co.), adjusting the final assay volume to 100 µL with ultrapure water. Thereafter, we added 400 µL of a 3,5-dinitrosalicylic acid solution and heated it at 100°C for 5 min. As a negative control, 5 mM ethylenediaminetetraacetic acid was used. The absorbance of the samples was measured at 540 nm (EZ Read 400, Biochrom Ltd, Cambridge, UK). One unit of α-amylase was defined as the amount of enzyme (µg) that increased the absorbance at 540 nm by 0.1 absorbance units at 30 min intervals, as described by Franco et al.(2000). Assays were conducted in triplicate in two replicates.

3.1.3.10. Statistical analysis

Data on absorbance were collected from three replicates in three separate tests. Measurements of fluorescence intensity in microscopy were obtained using ImageJ software with the pixel intensity of five fields for each treatment. Data were submitted to analysis of normality through the Shapiro–Wilk test, analysis of variance, and coefficient of variation. Mean values were normalized and compared using Dunnett's and Tukey's tests, with $p < 0.05$. All analyses were performed using GraphPad Prism software (version 8.0).

3.1.4. RESULTS

3.1.4.1. Reverse-phase chromatography on HPLC from the G3 fraction

Based on the column elution profile, we divided the G3 fraction into five distinct protein fractions, labeled F1, F2, F3, F4, and F5 (Figure 3). F1 was eluted only with solution A, demonstrating a lower affinity for the hydrophobic stationary phase. Fractions F2 to F5 were eluted as the propanol concentration increased, indicating that these proteins are more hydrophobic, resulting in higher contact with the C18 stationary phase.

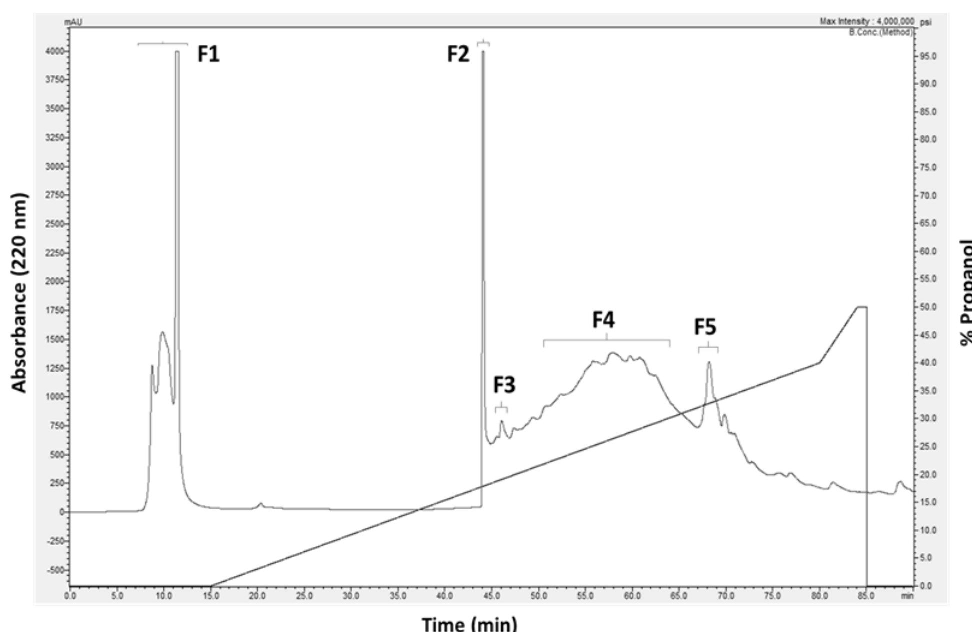
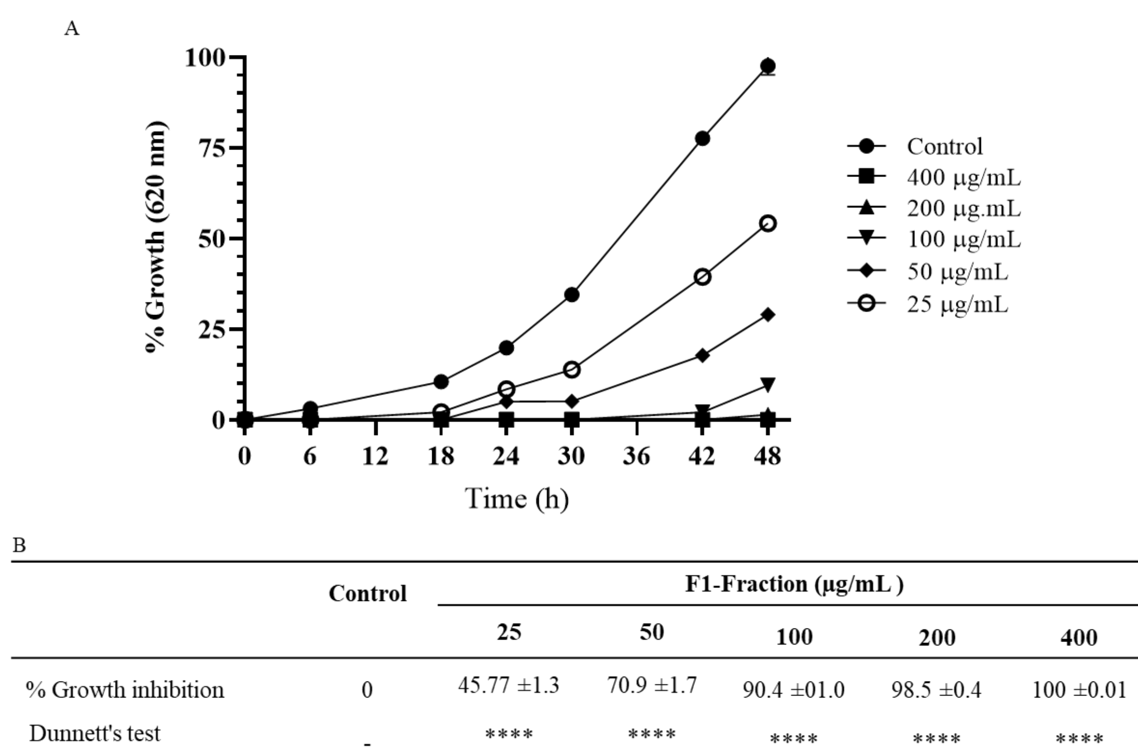


Figure 3: Separation of peptides from the G3 fraction of *Capsicum chinense* fruit accession UENF1751 by reversed-phase chromatography. A C18/C18 column was used within an HPLC system with a 0-50% propanol gradient at a flow rate of 0.300 µL/min at 40°C. The resulting fractions were named F1 to F5 according to the elution order.

3.1.4.2. Inhibitory effect of the fractions on mycelial growth

The *C. scovillei* growth inhibition test was performed with all fractions (F1-F5), but fractions F2, F3, F4, and F5 did not demonstrate inhibitory potential, even at high doses such as 400 µg/mL (results not shown). In contrast, the F1-fraction

showed strong inhibitory potential at all tested concentrations, showing its antifungal activity. Mycelial growth was monitored for 48 h (Figure 4A). At the highest concentrations (400 and 200 µg/mL), no signs of absorbance were observed. The MIC (minimum inhibitory concentration) was set at 200 µg/mL. The growth inhibition percentages were determined to be 90%, 70.4%, and 44% at doses of 100, 50, and 25 µg/mL, respectively, as depicted in Figure 4B. Based on these findings, the IC_{50} was predicted to be 21.5 µg/mL, and a 24 h incubation period was chosen to enhance the visibility of cells under light microscopy.

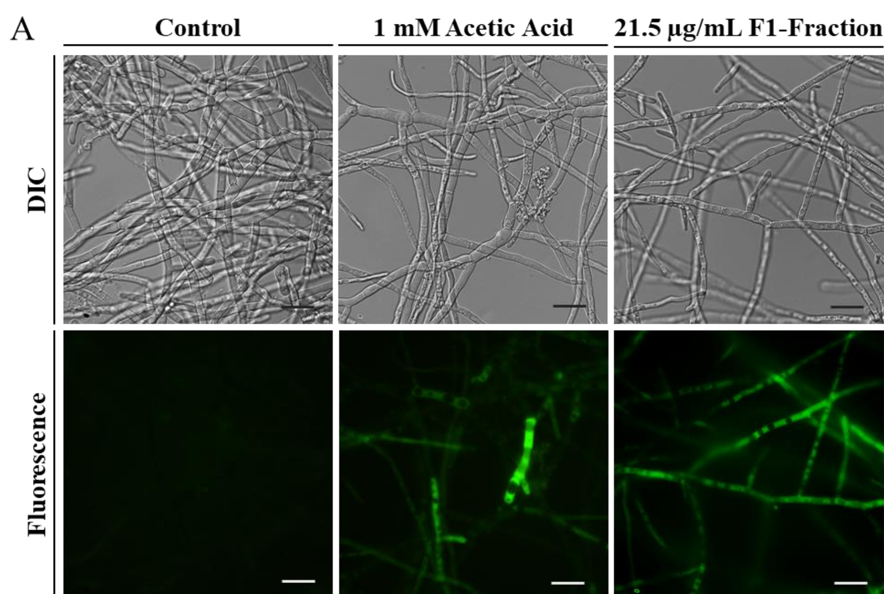


	Control	F1-Fraction (µg/mL)				
		25	50	100	200	400
% Growth inhibition	0	45.77 ±1.3	70.9 ±1.7	90.4 ±01.0	98.5 ±0.4	100 ±0.01
Dunnett's test	-	****	****	****	****	****

Figure 4: Effect of F1-Fraction on the growth of *Colletotrichum scovillei*. The fungal conidia (1 × 10^4 conidia/mL) were treated with 0-400 µg/mL of the F1-Fraction. (A) The growth curve in 2% Sauboraud medium was monitored every 6 h for up to 48 h by optical density at 620 nm. Data are mean values obtained in three experiments performed in triplicate and are presented as the percentage of growth. Control (untreated) is assumed to be 100% compared to cultures treated with different concentrations of the F1-Fraction. (B) Statistical analysis of the percentage of inhibition of fungal growth with the variation in F1-Fraction concentrations. The asterisk indicates a significant difference from the control (untreated hyphae) according to Dunnett's test **** $p < 0.001$.

3.1.4.3. Endogenous increase in ROS

The fungus treated with the F1-Fraction was stained with fluorescence dye (Figure 5A), demonstrating the presence of ROS. ROS levels in the F1-Fraction were significantly ($p < 0.05$) increased (180.9%) compared to those in the positive control, which was regarded as a 100% increase (Figure 5B). Because of basal ROS levels from cellular metabolism, control cells produced a modest fluorescence signal. The control cells also had a 28% fluorescence signal, but this was not significant because the cells have basal ROS levels due to biological metabolism.



B

Measurement of endogenous increase in reactive oxygen species (ROS) by the H_2DCFDA probe.

H_2DCFDA	Intensity fluorescence in pixels [(a)]	% Fluorescence	Dunnett's test multiple comparisons test
Control	4.30 ±1.4	28.0	A'
1 mM Acid acetic	12.44 ±3.7	100[(b)]	B'
21.5 µg/mL F1-Fraction	21.32 ±2.1	180.9	C'

Figure 5: The F1-Fraction causes an endogenous increase in reactive oxygen species in *Colletotrichum scovillei*. (A) Fluorescence microscopy of the fungus treated with 21.5 µg/mL F1-Fraction for 24 h, Control (untreated) and positive control (treated with 174.8 mM acetic acid) incubated with 20 mM H_2DCFDA. DIC: Differential interface contrast. Scale bars = 20 µm. (B) Statistical analysis of the fluorescence intensity measurements of micrograph pixels. ([a]) Fluorescence intensity count in a given number of pixels from five fields of fluorescence microscopy images by ImageJ software. ([b]) The mean number of fluorescent pixels from cells treated with 1 mM acetic acid (positive control) was assumed to be 100%. A', B' and C' indicate significant differences from the control (untreated hyphae) according to Dunnett's multiple comparisons test ($p < 0.05$). Overlapping significance was assigned the same letter, and values with different letters were significantly different.

3.1.4.4. Loss of mitochondrial functionality

The loss of mitochondrial functionality after treatment with the F1-Fraction was qualitatively monitored from the perspective of membrane potential using a MitoTracker red probe. As shown in Figure 6A, the control hyphae, which were found near the periphery of the mycelium, were the youngest and required more intensive energetic activity, resulting in more intense and distinct fluorescent labeling. In contrast, hyphae treated with the F1-Fraction showed weak and diffuse fluorescent markings. We quantified mitochondrial activity by measuring the activity of mitochondrial dehydrogenase enzymes that convert WST-1 to formozam. F1-Fraction decreased dehydrogenase activity in *C. scovillei* cells by 79% (Figure 6B). Acetic acid, which is recognized for its toxicity, was utilized as a positive control and inhibited enzyme activity very similarly to the F1- Fraction. As a result, we propose that F1-Fraction has a mode of action that involves a reduction in mitochondrial function.

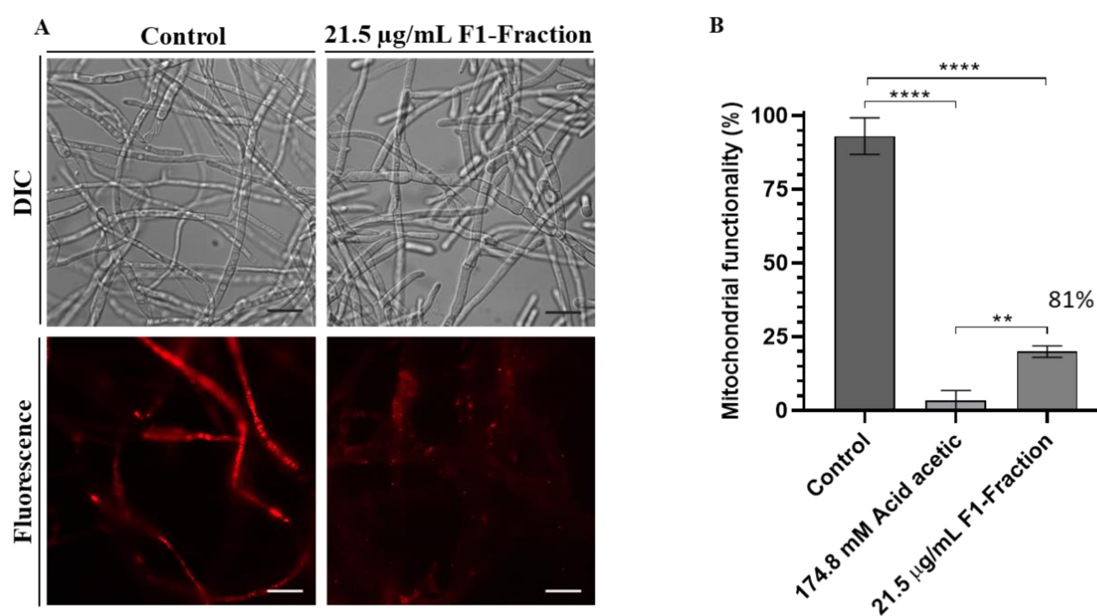


Figure 6: Mitochondrial functionality. (A) Fluorescence microscopy of *Colletotrichum scovillei* treated with 21.5 µg/mL F1-Fraction for 24 h and control (untreated) incubated with 50 nM Mitotracker Red. DIC: Differential interface contrast. Scale bars = 20 µm. (B) Effect of F1-Fraction on the reduction of tetrazolium salt (WST-1) to formazan by mitochondrial dehydrogenases. The cells were treated with 21.5 µg/mL F1-Fraction for 24 h. Then, 10 µL of WST-1 solution was added to the culture medium and incubated for 1 h at 37°C. Formazans were measured at 450 nm. The asterisk indicates a significant difference from the control (untreated hyphae) according to Tukey's test * $p < 0.1$, ** $p < 0.05$, *** $p < 0.01$, **** $p < 0.001$.

3.1.4.5. Changes in cell ultrastructure after treatment with F1-Fraction

Transmission electron microscopy was used to examine the condition of hyphae cultivated in the presence of F1-Fraction. Ultrastructural analysis of transverse sections revealed that the control hyphae (untreated) exhibited organelles arranged in a well-defined cytoplasm (Figure 7A), an intact cytoplasmic membrane, and an electrodense cell wall (Figure 7B). In the normal cell, most of the cytoplasmic organelles are enveloped in a double membrane, as electrodense particles of glycogen aggregated (GL), nucleus (N) and mitochondria (M) were visible and remained intact. In contrast, the hyphae treated with the F1-Fraction showed alterations, such as detachment of the cytoplasmic membrane from the cell wall with protuberant retraction of the cytoplasm (Figure 7C), rupture of the cytoplasmic membrane, and disintegration of cytoplasmic organelles (Figure 7D). Cell damage was more intense in some hyphae, with completely disorganized cytoplasm, intense vacuolization (V) and lysis of part of the fungal cells (Figure 7E).

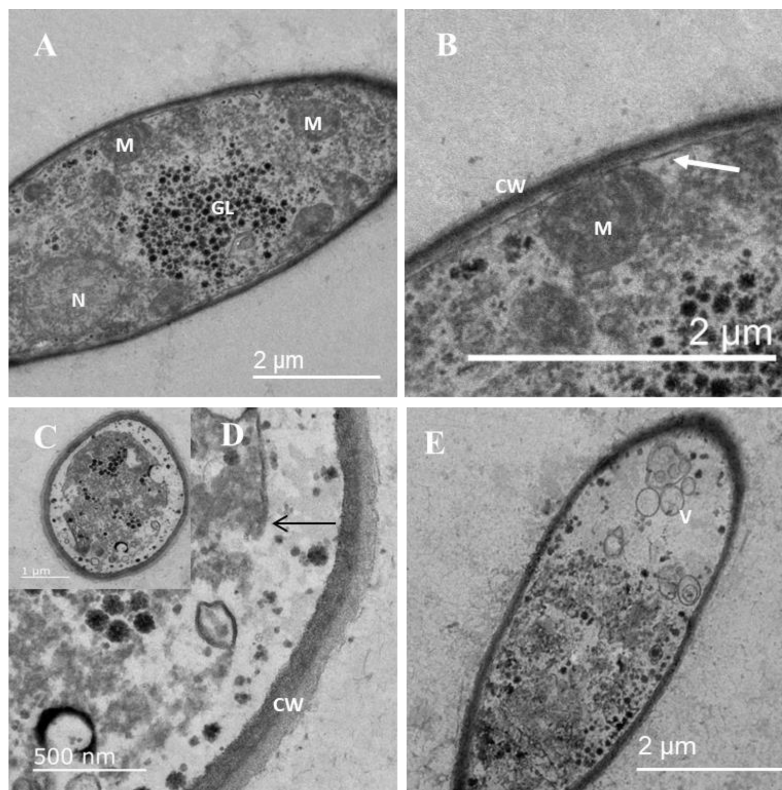


Figure 7: Changes in the ultrastructure of *C. scovillei* after treatment with F1-Fraction. (A-B) Control (untreated). M- mitochondria; GL- electrodense particles of aggregated glycogen; N-nucleus; CW- cell wall; White arrow- intact cytoplasmic membrane. (C-E) Cells treated with 21.5 µg/mL F1-Fraction for 24 h. Black arrow pointing to ruptured cytoplasmic membrane. V- autophagic vacuole.

3.1.4.6. Identification of peptides in the F1-Fraction

The peptide fragments obtained by MS were analyzed, and fragments with low scores and contamination were excluded. After analyzing the posttranslational protein modifications of the peptides, we obtained three peptide fragments corresponding to a first protein (I) and two peptide fragments corresponding to a second protein (II) (Figure 8). The peptide residues of Protein I *RGPCFTTGSCDDHCKN* and *VVQTEAKTCENLADTYR* in a BLASTp search of the NCBI database limited to finding homologous proteins in *C. chinense* showed 42% and 39% identity to the defensin peptides PHT96429.1 and PHU20899.1, respectively. The peptide fragment *NKEHLISGR* was not identical to any protein in this database. Protein II (GACTGDHNCALVCRNEGFSGGNCRG) obtained from the sequenced peptides *GACTGDHNCALVCR* and *NEGFSGGNCRG* was matched with PHU17080.1, PHU11333.1, and PHU11335.1 defensin peptides from *C. chinense* deposited in the NCBI database, with similarities of 80, 68, and 60%, respectively. By performing fragment alignment in Clustal W, we obtained 66% coverage of mature protein I and 52% coverage of mature protein II (Figure 8). Thus, as both identified proteins were similar to defensins, we named protein I CcDef1 and protein II CcDef2.

Identification	Mature peptide (positions 10, 20, 30, 40, 50)	P(%)	I(%)	E-value
CcDef1	KTCENLADTYRGPCFTTGSCDDHCKNKEHLISG-----------------			
PHT96429.1	KVCQRRSKTWPGPCINTGNCSRQCKNQEDGRFGACHRSGIGFACFCYFNC	57	42	1e-04
PHU20899.1	KVCQRRSKTWSGPCIHTDNCNRQCMNREDARSGACHKSGFGAACFCYFNC	57	39	0.002
CcDef2	-----------GACTGDHNCALVCRNEGFSGGNCRG------------			
PHU17080.1	RVCISQSHGFKGPCGHDHNCALVCRNEGFSGGDCIGVFIRKCYCTKNC	84	80	1e-12
PHU11333.1	RTCESQSHRFKGACASETNCASVCQTEGFSGGDCRG-FRRRCFCTRPC	80	68	6e-09
PHU11335.1	RHCESQSQRFKGPCVRRKNCAAVCETEGFPGGDCRG-FRRRCFCTRPC	64	60	6e-05

Figure 8: Multiple sequence alignment of amino acid residues from peptides of *Capsicum chinense* fruits from the F1-Fraction obtained by reversed-phase chromatography on an HPLC system with defensin-like proteins of *Capsicum chinense*. P (%) indicates the percentage of positive residues (that present the same physicochemical features). I (%) indicates the percentage of identical residues and are written in italics. The residues outside the cover in the alignment are shown in silver. Gaps (-) were introduced for better alignment.

3.1.4.7. Identification of defensins expressed in *C. chinense* pepper pericarp

Considering the similarities within the defensin sequences and the limited similarity to a particular isoform of defensin among the fragments identified through MS, particularly for protein 1, we sought to investigate the specific isoforms expressed within the pericarp of *C. chinense* peppers. RNA extracted from the pericarp of *C. chinense* fruits was utilized to synthesize cDNA. This cDNA synthesis aimed to identify the presence of five specific defensin sequences, those displaying the highest similarities in our MS analysis (PHT96429.1, PHU20899.1, PHU17080.1, PHU11333.1, and PHU11335.1). To achieve this, we designed primers tailored for each defensin sequence (Table 1), except for sequences PHT96429.1 and PHU20899.1, which share identical 5' and 3' ends; therefore, the same pair of primers would amplify both sequences. Following the PCR reactions employing the four pairs of primers listed in Table 1, we successfully observed amplification for two of them, those designed for Def-PHU11333.1 and Def-PHU11335.1. The amplicons were ligated into pGEMT-easy vector and subsequently sequenced. Analysis of the sequences of two colonies, named Primer2Colony1 (P2C1), Pimer2Colony2 (P2C2) obtained with the primers designed to PHU11333.1 resulted in a sequence 100% identical to the expected peptide (P2C2) and another sequence (RTCESQSQRFKGPCVRRKNCAAVCETEGFPGGDCRGFRRRCFCTRPC - P2C1) that exhibited 81% identity to PHU11333.1 and 97% identity to PHU11335.1. The only variation was found at the 5' end of the sequence, where a histidine from PHU11335.1 was replaced by a threonine in our sequence. Furthermore, we sequenced three colonies obtained from the PCR utilizing primer set four, targeting PHU11335.1, and these colonies were named Primer4Colony1 (P2C1), Primer4Colony2 (P4C2), and Primer4Colony3 (P4C3). The same sequence was obtained for the three colonies (named P4 only), corresponding to a protein 81% identical to PHU11335.1 and 97% identical to PHU11333.1. Given the similarities in the 5' end (only two residues different) and 3' end (identical) of both sequences, it is not surprising that the primer designed to amplify PHU11335.1 resulted in a sequence more similar to PHU11333.1. When we compare the colony P2C2 with P4, we observe that they are 97% similar, differing only in one amino acid at position

2 of the 5' end, where a histidine is substituted with a threonine. The sequences of the three defensin isoforms identified in our extract are shown in Figure 9.

```
P2C1          RTCESQSQRFKGPCVRRKNCAAVCETEGFPGGDCRGFRRRCFCTRPC
PHU11335.1    RHCESQSQRFKGPCVRRKNCAAVCETEGFPGGDCRGFRRRCFCTRPC
P2C2          RTCESQSHRFKGACASETNCASVCQTEGFSGGDCRGFRRRCFCTRPC
PHU11333.1    RTCESQSHRFKGACASETNCASVCQTEGFSGGDCRGFRRRCFCTRPC
P4            RHCESQSHRFKGACASETNCASVCQTEGFSGGDCRGFRRRCFCTRPC
              * *****:****.*.  .***:**:****.*****************
```

Figure 9: Multiple sequence alignment of the defensins identified in the pericarp *Capsicum chinense.* The most similar sequences found in the NCBI database (PHU11333.1and PHU11335.1).

3.1.4.8. Characterization of enzyme inhibitory activity

F1-Fraction showed a remarkable ability to inhibit *T. molitor* α-amylase enzyme activity, with 86% inhibition observed (Figure 10A). On the other hand, F1-Fraction had no effect on trypsin activity (Figure 10B). These results indicate that the components present in F1-Fraction have specific inhibitory properties directed to α-amylase.

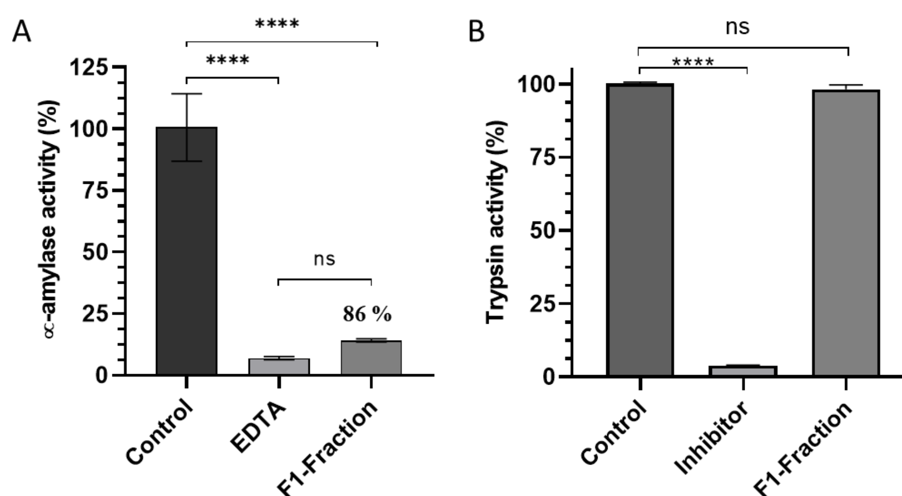


Figure 10: Inhibition of enzymatic activities. A- α-amylase inhibitory activity assay. B- Trypsin inhibitory activity assay. The asterisk indicates a significant difference from the control (untreated hyphae) according to Dunnett's test **** $p < 0.001$. ns- not significant difference. % inhibition percentage.

3.1.5. DISCUSSION

Colletotrichum fungi are among the most commonly reported pathogenic fungi, with a wide range of hosts and levels of aggression. As a result, they are considered one of the most hazardous to agriculture(Araújo et al., 2022). In Brazil, *C. scovillei* is the most common species that infects field peppers and sweet peppers, causing anthracnose. Despite technological developments in manufacturing, anthracnose can still cause major economic losses (Giacomin et al., 2021). Therefore, new control alternatives are needed. In this scenario, AMPs with promising antifungal potential emerge as a promising solution (Zhang et al., 2023b). They offer advantages such as fast and broad-spectrum inhibitory activity, minimal chance of resistance development, low toxicity for mammalian cells, and a wide range of potential applications (Luong et al., 2020). *Capsicum* peppers are a promising source of AMPs with antifungal activity. AMPs have already been found in the most diverse parts of the plant, such as leaves and roots (Pereira et al., 2018),seeds (Silva et al., 2021), flowers (Mulla et al., 2021), and fruits (Ribeiro et al., 2023). Furthermore, several AMPs in *Capsicum* AMPs have already been explored in heterologous (Liu et al., 2006; Kovaleva et al., 2011) or synthetic systems (Velázquez-Hernández et al., 2021; Taveira et al., 2022).

The G3 fraction was previously obtained by molecular exclusion chromatography as described by Resende et al. (2021). Subsequently, we performed reversed-phase chromatography of the G3 fraction and obtained 5 fractions, F1-F5. However, only the F1-Fraction showed growth inhibition potential (Figure 4). In support of our findings, Santos et al. (2020) reported the presence of a fraction rich in defensin-like peptides and lipid transfer proteins from other *C. chinense* fruits, which displayed inhibitory activity against *Fusarium solani* (39%) and *Fusarium oxysporum* (26%) at a concentration of 100 µg/mL. Notably, inhibition of *Colletotrichum scovillei* growth was observed only at a higher concentration of 200 µg/mL. Interestingly, our study demonstrated that F1-Fraction, even at the same concentrations, exhibited superior potency. This was substantiated by the determination of the MIC, which was defined as 200 µg/mL. Furthermore, we also observed that the F1-Fraction possessed α-amylase inhibitor activity, as illustrated in Figure 10. These findings provide credence to the notion that the peptides found in the F1-Fraction have antifungal and α-amylase inhibitory activities.

MS analysis confirmed the presence of defensins within the F1-Fraction, and we successfully identified the sequence PHU11333.1 in our *C. chinense* fruit extract. Additionally, we detected two defensin isoforms closely resembling PHU11335.1 (P2C1) and PHU11333.1 (P4) with only one residue variation compared to the sequences deposited in the NCBI database, as seen in Figure 6.

Defensins, LTPs, and protease inhibitors are examples of AMPs that have strong antifungal activity against fungi that infect plants, as well as other biological functions such as enzyme inhibition (Dias e Franco, 2015). The defensin PsDef2 from *Pinus sylvestris* presents antifungal activity against *Fusarium sporotrichiella* and *Saccharomyces cerevisiae*, oomycota against *Phytophthora gonapodyides* and antibacterial activity; in addition, it presents inhibition of different α-amylases of insects (Bukhteeva et al., 2022). BraDef (BraDef1 + BraDef2), another plant defensin, at 12.6 µg/mL, reduced the hyphal growth of *C. gloeosporioides* by approximately 56% and caused membrane disruption (Pacheco-Cano et al., 2020).

Understanding the mechanism of action requires an understanding of the processes that occur during growth suppression. This knowledge is vital for the potential future application of these substances as microbicidal agents. To understand the mechanism of action of the F1-Fraction, we used the concentration corresponding to the IC_{50}. We first observed an increase in ROS, which is one of the first signs that the cell is under stress. The endogenous rise in ROS can be regarded as a crucial indicator of cellular damage. Filamentous fungi have a sophisticated system of regulating and eliminating ROS produced at basal levels by cell physiology or excess stress. These systems can be divided into enzymatic (such as thioredoxin protein catalases, superoxide dismutases, and peroxidases) and non-enzymatic (such as glutathione and ascorbic acid) systems (Zhang et al., 2020b).

Disruptions in redox homeostasis can lead to oxidative stress, which disrupts the functioning of various cellular structures and organelles, ultimately diminishing fungal virulence or growth. Our results, as illustrated in Figure 5, indicate that hyphae treated with F1-Fraction have high levels of ROS, suggesting that they are in an oxidative burst. Other defensins, such as RsAFP2 (Aerts et al., 2007), NaD1 (van der Weerden et al., 2010), and HsAFP1 (Aerts et al., 2011), also induce oxidative stress by ROS. Similar results were reported by Ali et al. (2014) when treating hyphae of *Saprolegnia* spp. with exposure to boric acid (1 g/L) for 24 h. The

authors also observed that the hyphae that presented high levels of ROS also suffered a loss of mitochondrial functionality. This reduction in mycelial growth was verified by the authors. Gebara et al. (2020) obtained the F2 fraction from *C. annuum* fruit extract obtained by reversed-phase chromatography and composed of two defensins called *Ca*Def2.1 and *Ca*Def$_{2.2}$. This fraction, in addition to inhibiting the growth of *C. tropicalis* yeasts, also caused an increase in ROS in the cells.

Mitochondria are the main source of intracellular ROS production and are also one of the targets of these molecules. Consequently, additional generation of ROS occurs (Pan, 2011). Our results showed that in addition to the increase in ROS, the hyphae showed mitochondrial degeneration, as they lost mitochondrial membrane potential and dehydrogenase enzyme activity. Li et al. (2014) established a causal link between oxidative stress, mitochondrial dysfunction, and fungal cell degeneration, which corroborates our results. Mitochondrial membrane potential is essential for mitochondrial homeostasis, protein process regulation, including ATP synthesis, and control of ROS generation. A change in the potential of the mitochondrial membrane can reduce energy output. In this sense, due to intensive efforts in energy production to repair the cell and prevent the destruction of organelles, cells have less energy for development. As a result, we can deduce that these disturbances caused by toxic molecules cause damage to the physical structure. These mechanisms of action become permanent and fatal for the fungus.

We employed transmission electron microscopy to examine the treated hyphae and confirm any alterations in their morphology. When compared to the control, the hyphae showed substantial changes, as illustrated in Figure 7. These include cytoplasm retraction and disarray, cytoplasmic membrane breakdown, and the presence of putative autophagic vacuoles. Other natural antifungal agents have been shown to induce structural harm to fungi (Seyedjavadi et al., 2020; Hoyos et al., 2012) Ultrastructural damage similar to our data was observed by Mosquera-Sánchez et al. (2020) Decreased cytoplasmic space, vacuolation, and accumulation of electron-dense particles around the cell wall were also observed when *Colletotrichum* sp. isolated from coffee leaves were treated with zinc oxide nanoparticles that have an antifungal effect. In filamentous fungi, cells subjected to autophagy show an increase in the volume of vacuoles as a result of the degradation and recycling of molecules and organelles that are damaged under stress conditions (Shoji et al., 2010).

In view of the above, the F1-Fraction that presents antifungal activity against an important pathogen of peppers is composed of defensins. Three defenses were found to be expressed in the extract of the pericarp of *C. chinense* peppers, which may be the subject of studies in the control of plant pathogens. In addition, they can be studied in insect control, as they have α-amylase inhibitory activity.

The defensin peptides identified in our study may contribute to the observed growth inhibition of *C. scovillei*. Additionally, the potent inhibitory activity against α-amylase indicates the potential of the F1-Fraction in modulating carbohydrate metabolism. Collectively, our results are in line with previous studies and highlight the importance of defensins and related peptides as promising candidates for the development of antimicrobial agents and enzyme inhibitors in plant defense mechanisms.

3.1.6. CONCLUSIONS

The present study identified defensins not yet described in *Capsicum chinense*, with a structure strongly conserved with defensins from other species. These defensins exhibit potent antifungal and α-amylase inhibitory activities. The inhibition mechanisms of fungal growth involve chemical mechanisms, such as oxidative stress, and physical mechanisms, such as the destruction of structures and organelles. We observed an increase in ROS, which possibly led to oxidative burst, loss of mitochondrial functionality, and cytoplasm retraction, as well as an increase in vacuoles that were possibly autophagic. These data support the ability of plant defensins to exert potent antifungal action against phytopathogens. These molecules are strong candidates for novel biotechnological antifungal agents.

3.2. CONTROL OF PEPPER PLANT PATHOGENS, *IN VITRO* AND *IN VIVO*, BY A PEPTIDE BIOINSPIRED FROM *Capsicum annuum* DEFENSIN

3.2.1. INTRODUCTION

Traditional antibiotics and antifungals are used to control plant pathogenic diseases, but with their continued use and long-term testing for resistance selection, they also pose danger due to damage caused to humans and the environment. Consequently, there is an urgent need for the research and development of new compounds with different antimicrobial activities and mechanisms of action as alternatives to address serious problems and difficulties in controlling plant diseases (Lima et al., 2021).

The number of pathogens that affect pepper plants is very broad and includes fungi (e.g., the genus *Colletotrichum*) and bacteria (e.g., *Xanthomonas* spp.) (Parisi et al., 2020). For example, anthracnose is one of the main diseases affecting plants in the *Capsicum* genus and is characterized by rounded necrotic lesions of different diameters. The main causative agent of this disease is the fungus *Colletotrichum gloeosporioides*, and infections can also be caused by fungi from other species (Than et al., 2008). Bacteria of the genus *Xanthomonas*, in turn, are the causes of bacterial spots in plants of the Solanaceae family. This disease is characterized by the formation of necrotic or nonnecrotic spots distributed mainly on the edges of leaves, with brown lesions also occurring on the stem, petals, and fruits (Potnis et al., 2015)

Considering the growing need for sustainable agriculture, the search for new molecules that can assist in this control has been of fundamental importance. Given this background, AMPs have emerged as promising molecules for the development of new molecules with potential agrochemicals to control plant diseases. AMPs are important components of the plant defense response and share several common characteristics, such as small size, generally have a net positive charge at physiological pH and are amphipathic (Li et al., 2021).

However, the use of natural AMPs, which have biotechnological potential in disease control, faces several challenges, such as degradation by proteases, inactivity in saline concentrations, cytotoxicity, and expensive and laborious purification processes (Benfield e Henriques, 2020; Sarkar et al., 2021; Zhang et al., 2023b).

Despite these limitations, the structure of AMPs is considered an excellent model for rational design, generating increasing interest in the development of synthetic AMPs bioinspired from natural AMPs. These materials are designed to preserve or enhance antimicrobial potential, overcoming the shortcomings associated with natural analogues. Thus, strategies aimed at using natural AMPs in the design and description of more improved versions at controlling pathogens have attracted significant attention (Wang et al., 2018; Cardoso et al., 2020; Shwaiki et al., 2021; Zhang et al., 2023b).

Peppers belonging to the *Capsicum* genus are promising materials for the isolation and purification of AMPs with antimicrobial properties (Oliveira et al., 2022). A prominent example was the research carried out Taveira et al. (2022), in which the authors designed and synthesized a peptide bioinspired on a segment of a natural defensin called *Ca*Def2.1, which was isolated from *C. annuum* fruits for Gebara et al. (2020). As a result of these changes, researchers developed a synthetic peptide called *Ca*Def2.1$_{G27\text{-}K44}$, with a sequence of 18 amino acid residues: GLTRLRRILFRLLLWRTK. These peptides with substitutions in amino acid residues caused an increase in the net charge, greater hydrophobicity, and increased antimicrobial activity against yeasts of the genus *Candida* and strains of gram-positive bacteria, including *Mycobacterium tuberculosis*.

In this study, we investigated *Ca*Def2.1$_{G27\text{-}K44}$ to expand the spectrum of inhibitory activity against plant pathogens, especially pepper pathogens such as *X. euvesicatoria* and *C. scovillei*. In this work, we also investigated the ability of the *in*

vivo application and control of common bacterial blight on pepper plants, *C. annuum*.

3.2.2. LITERATURE REVIEW

3.2.2.1. Synthetic peptides bioinspired by plant AMPs

The extensive biodiversity of plant species provides a vast array of genetic resources for the exploration of new natural AMPs. However, this diversity also requires significant efforts to select and identify these molecules. The steps of isolating, purifying, and identifying AMPs involve a series of techniques and processes necessary for their extraction and purification (Tang et al., 2018).

Despite the promising advances in high-throughput screening techniques, the purification of natural AMPs remains laborious and complicates the scalable production of peptides with high yields. Additionally, natural AMPs are generally synthesized at low rates by their biological sources, are susceptible to degradation by proteases, or exhibit low bioavailability (Cunha et al., 2017).

The chemical synthesis of AMPs has been identified as an alternative to natural extraction due to its potential to reduce production time. Furthermore, rational modifications are designed to enhance the biological activities of these molecules, overcome resistance to pathogenic agents, and increase microbial killing, thereby providing potent activities with a lower propensity to select for resistance (Deslouches et al., 2013).

Rational synthesis is employed to increase stability and often improve the bioactivity of AMPs. Modifications may include the substitution of amino acid residues that are more susceptible to proteolytic cleavage, modifications of amino acids in the motif region of the molecule that interacts with the target of action, or an increase in net charge. In addition, chemical modifications may include N- or C-terminal acetylation or amidation, changes in post-translational glycosylation patterns of glycosylated peptides, chimerization by the addition of specific functional

groups, or peptide cyclization. These modifications have been employed with varying degrees of success to enhance the stability of AMPs (Cunha et al., 2017; Bednarska et al., 2017; Evans et al., 2020).

It is often the case that natural AMPs are susceptible to proteolysis, as they are composed of L-amino acids that are recognised by proteases. Therefore, rational design aimed at sequence modifications containing D-amino acid analogs substituted for L-amino acids can consistently increase the post-translational stability of the peptide without altering its biological function (Gan et al., 2021).

This information was confirmed by Fuente-Núñez et al. (2015). In a study on peptide design, the authors demonstrated that D-enantiomeric peptides were the most potent in inhibiting pre-formed biofilms of Gram-negative bacteria, including wild-type and multi-antibiotic-resistant strains. Additionally, these peptides exhibited strong synergy with conventional antibiotics, reducing the concentrations of antibiotics required for complete biofilm inhibition by up to 64 times.

Peptide cyclization is a strategy aimed at improving serum stability, which refers to the AMP's ability to remain active and intact in the bloodstream for an extended period without being rapidly degraded or eliminated. This technique involves linking the N and C terminal structures or forming internally cross-linked disulfide bridges, resulting in the cyclization of the AMP. This process conceals sites susceptible to proteolytic cleavage by aminopeptidases, contributing to the maintenance of the peptide's integrity (Harris et al., 2015).

The following sections will explore in more detail the physicochemical characteristics of AMPs that are subject to modifications capable of influencing their antimicrobial activity

3.2.2.2. Physicochemical characteristics of AMPs as targets for modifications

3.2.2.2.1. Size

The majority of AMPs are characterized by their small size, containing fewer than 100 amino acid residues. Comparative studies have been conducted to investigate the relationship between peptide size and activity. Smaller peptides tend

to have greater permeability and can penetrate microbial membranes more effectively, while larger peptides may have more complex structures that increase their stability and specificity against certain pathogens (Gan et al., 2021).

In a study conducted by Park et al. (2007), the synthetic peptide 15Mer HP-A3 (A3-NT), which is smaller than its original form, exhibited a 2- and 4-fold increase in antibacterial and antifungal activity, respectively, without causing hemolysis, compared to the original peptide from *Helicobacter pylori*.

In contrast, Lyu et al., (2016) emphasized the importance of maintaining size for antimicrobial efficacy. In their study, the AMP PMAP-36 demonstrated activity against bacteria and anti-Candida. In an attempt to optimize this activity, the researchers synthesized peptides derived from PMAP-36 with reduced sizes. After the experiments, they found no significant evidence of improved antimicrobial activity in the smaller peptides. However, a reduction in hemolysis was observed as the peptide size decreased.

These results suggest that smaller peptides have a higher safety margin compared to larger ones. It is important to note that the removal of key amino acids or functional regions can compromise antimicrobial activity.

3.2.2.2.2. Tertiary structure

The tertiary structure, especially the formation of the α-helix, plays a crucial role in the activity of AMPs. This is mainly due to the ability of the α-helix to facilitate the insertion of AMPs into the plasma membrane. The amphipathic α-helix structure is preferably rigid and elongated, with the polar groups of the amino acids positioned on one side of the helix, while the hydrophobic groups face inward, away from water. When an AMP with an α-helix structure approaches the membrane, the hydrophobic region of the helix tends to insert itself into the lipid region of the bilayer, while the polar region interacts with the hydrophilic groups present on the membrane surface. This arrangement allows AMP to efficiently cross the membrane and reach its intracellular target or exert its antimicrobial activity on the cell membrane (Malmsten, 2015).

In a study by Chen, Mant, Hodges (2002), L-amino acids were substituted for D-amino acid residues in the V13K peptide sequence. Although this did not affect charge and hydrophobicity, the substitutions did affect the ability of the peptides to correctly adopt the folded α-helix. Thus, they found that the V13K peptide composed of D-amino acids showed a reduction in antimicrobial activity compared to those composed of L-amino acids

Games et al., (2020) investigated the mechanism of action of two peptides (Mo-CBP3-PepI and Mo-CBP3-PepII) against *Candida albicans*. When in contact with micelles, Mo-CBP3-PepI changes its structure from a disordered form to an α-helix, increasing its interaction with the micelle and becoming 8 times more effective. On the other hand, Mo-CBP3-PepII does not change its structure. These results highlight the importance of the α-helix in antimicrobial interaction, symbolizing the interaction of peptides with the negatively charged membrane of microorganisms.

3.2.2.2.3. Positive net charge

Changes in the positive charge of AMPs play a critical role in their biological activity. In general, this charge is imparted by arginine or lysine residues. The presence of histidine in AMPs is less common due to the size of its imidazole group, which can interfere with the effective insertion of the peptide into the membrane. Jiang et al., (2008) promoted modifications in the V13K sequence to investigate the effect of fluid charge on antimicrobial and hemolytic activity. V13K variants with charges below +4 were found to be completely inactive. On the other hand, V13K variants with positive charges of +9 and +10 showed an increase in hemolytic activity, but without a correlation with an increase in antimicrobial activity. In contrast, V13K variants with charges from +4 to +8 showed high antimicrobial activity and low hemolytic activity. These results demonstrate the existence of a critical threshold for the net positive charge of AMPs, which is critical for the efficacy of their antimicrobial and hemolytic activities.

Mello et al., (2019) showed that increasing the net positive charge by replacing the Asp amino acids at positions 37 and 38 with two Arg residues in the

peptides γ_{31-45}*Pv*D_1^{++} e γ_{33-41}*Pv*D_1^{++}, guarantees them potent anti-inflammatory activity anti-*Candida* at low concentrations.

3.2.2.2.4. Hydrophobicity and amphipathicity

Amphipathicity refers to the property of a molecule to have regions with affinity for both polar and non-polar solvents, with a hydrophilic and a hydrophobic region. This is an important property for AMPs, which must interact with both water and the lipid membranes of microbial cells to exert their function. The presence of hydrophobic regions facilitates the insertion of peptides into lipid bilayers, disrupting their integrity and leading to cell disruption. Hydrophobicity also contributes to the selectivity of AMPs, making them selectively toxic to microorganisms. The stability of peptides is also affected by hydrophobicity, making them more resistant to enzymatic degradation. Finally, the ability to form secondary structures such as alpha helices is facilitated by hydrophobic regions in the peptide sequence, and these structures are often associated with antimicrobial activity (Kabelka e Vácha, 2018). In general, increasing the hydrophobicity on the surface of an α-helical amphipathic peptide improves antimicrobial activity (Wieprecht et al., 1997).

3.2.2.3. Examples of synthetic AMPs

Some examples of rationally designed AMPs bioinspired by plant AMPs have already been described in the literature. Sometimes these peptides show not only improved antimicrobial activities but also a lower propensity to select resistant microorganisms compared to native analogues. Some of these synthetic peptides are presented in Table 4 below.

Table 1: Synthetic peptides bioinspired by natural AMPs.

Natural peptide	Synthetic peptide	Microorganisms susceptible to synthetic peptide	Reference
Pg-AMP1	Guavanin 2	*Escherichia coli, Pseudomonas aeruginosa, Klebsiella pneumoniae, Acinetobacter baumannii, Staphylococcus aureus, Streptococcus pyogenes, Listeria ivanovii, Enterococcus faecalis, Candida albicans, Candida parapsilosis*	(Porto et al., 2018)
PaDBS1	PaDBS1R6	*Escherichia coli, Staphylococcus aureus, Enterococcus faecalis, Pseudomonas aeruginosa*	(Fensterseifer et al., 2019)
*Pv*D$_1$	$\gamma_{31\text{-}45}$*Pv*D$_1^{++}$	*Candida albicans Candida buinensis*	(Mello et al., 2019)
Rs-AFP2	Rs-AFP2(G9R) RsAFP2 (V39R)	*Fusarium culmorum*	(Samblanx et al., 1997)
Ib-AMP1 and IbAMP4	MCD26 MCD30 MCE01 MCE02	*N. crassa, B. cinerea, F. culmorum, S. cerevisiae, P. pastoris*	(Thevissen et al., 2005)
Pp-TH	Pp-TH (D32R)	*R. meliloti, X. campestris, C. Michiganensis, F. oxysporum, P. cucumerina, B. cinerea*	(Vila-Perelló et al., 2003)
*Ca*Def2.1	*Ca*Def2.1$_{G27\text{-}K44}$	*C. albicans*, *C. buinensis* , *C. parapsilosis* e *C. tropicalis*	(Taveira et al., 2022)

Table 1 – Cont.

Natural peptide	Synthetic peptide	Microorganisms susceptible to synthetic peptide	Reference
Mo-CBP3	Mo-CBP3-PepI Mo-CBP3-PepII Mo-CBP3-PepIII	*C. albicans,* C. tropicalis, *C. parapsilosis, C. krusei*	(Oliveira et al., 2019)
Rc -2S-Alb	Rc Alb-PepII	*Klebsiella pneumoniae* *Candida parapsilosis*	(Dias et al., 2020)
JcTI-I	Jc TI-PepI	*Candida krusei*	(Souza et al., 2022)

3.2.3. MATERIAL AND METHODS

3.2.3.1. Synthetic peptide synthesis

The peptides *Ca*Def2.1 (a fragment of the original *Ca*Def2.1 defensin without modifications) and *Ca*Def2.1$_{G27\text{-}K44}$ were designed and synthesized by AminoTech (São Paulo, Brazil), whose quality and purity were analyzed (≥ 95%) using high-performance liquid chromatography (RP-HPLC) and mass spectrometry, as described by Taveira et al. (2022). The bioinspired peptides were solubilized in DMSO (10%) and used in all the assays.

3.2.3.2. Microorganisms

The bacterium *X. euvesicatoria* ENA 4135, which causes common bacterial blight, and the fungus *C. scovillei* were obtained from the Laboratório de Melhoramento Genético Vegetal (LMGV), Centro de Ciências e Tecnologias Agropecuárias (CCTA), Universidade Estadual do Norte Fluminense Darcy Ribeiro (UENF), Campos dos Goytacazes, RJ, Brasil.

3.2.3.3. Pepper plant cultivation

The plants used in the inoculation trial with *X. euvesicatoria* were produced from seeds of the *C. annuum* genotype Ikeda and accession 1381 provided by LMGV. One seed per cell was sown in polystyrene trays filled with Vivatto® commercial substrate. The trays were kept in a greenhouse and watered once a day until the seeds germinated. The plants were subsequently transferred to a plastic pot containing washed sand and planting soil (1:1). After the development of two pairs of definitive leaves, 18 plants of each genotype were inoculated to evaluate the resistance reaction to the bacterial spot and the activity of the *Ca*Def2.1$_{G27\text{-}K44}$ peptide.

3.2.3.4. Preparation of the bacterial inoculum

X. euvesicatoria bacteria were subcultured in a Petri dish containing dextrose yeast glucose sucrose (DYGS) agar (HiMedia Laboratories LLC, Pennsylvania, USA) as a stock. After 24 h at 30°C, 2 or 3 colonies were transferred to 10 mL of DYGS liquid media (HiMedia Laboratories LLC, Pennsylvania, USA) and grown until they reached the log phase of growth. The bacterial inoculum was adjusted for turbidity to the McFarland 0.5 standard at 600 nm and contained 1.5×10^8 CFU/mL bacteria. The suspension was further diluted to produce 5×10^5 CFU/mL, which was considered the working solution containing bacterial cells.

3.2.3.5. Preparation of the fungal inoculum

To obtain *C. scovillei* conidia, the fungus was initially cultivated for 7 days at 30°C on 4% Sabouraud agar (Merck KGaA). Then, 10 mL of Sabouraud broth (Merck KGaA) was added to the fungus plate, and the conidia were released using a Drigalski loop. To remove mycelial fragments and hyphae, the liquid was filtered through sterile gauze, and the solution containing conidia was adjusted by dilution until a concentration of 10^4 conidia/mL was reached.

3.2.3.6. Plate microdilution inhibition assay

The inhibitory potential of the synthetic peptides *Ca*Def2.1 and *Ca*Def2.1$_{G27\text{-}K44}$ on microorganisms was tested using the broth microdilution method in sterile 96-well plates (polystyrene, U-bottom, Nunc, Thermo Scientific) according to the CLSI

M27-A (Clinical and Laboratory Standards Institute (CLSI), 2008; Balouiri et al., 2016)). The test was conducted in a final volume of 100 µL of the suspension containing microorganisms following the procedures mentioned in sections 3.2.3.4 and 3.2.3.5.

In the bacterial inhibition assay, 20 to 0.3 µM/mL concentrations of the synthetic peptides were applied, and absorbance readings were taken after 48 h of incubation at 30°C. The bacterial cell density (OD 595 nm) was measured using a plate reader (EZ Read 400, Biochrom Ltd., Cambridge, UK).

In the antifungal assay, we used concentrations of 200 to 3.2 µM/mL of synthetic peptide. Absorbance readings were taken after 24 h of incubation at 30°C, and cell density (OD 620 nm) was measured using the same plate reader.

Inhibition percentages were calculated using the formula [100(tABS$_{620}$ 100/cABS$_{620}$)], assuming that the control represented 100% growth, where tABS620 is the mean absorbance at 620 nm of the sample treated with synthetic peptides and cABS620 is the mean absorbance at 620 nm of the control sample.

The minimum inhibitory concentration (MIC) was determined as the lowest concentration of peptide that visibly inhibited microbial growth in the plate. The IC_{50} was determined by statistical analysis using a linear regression curve.

3.2.3.7. Viability of *X. euvesicatoria* treated with *Ca*Def2.1$_{G27\text{-}K44}$

After the inhibition assay described in section 3.2.3.6, viability was also evaluated after incubation with different concentrations of *Ca*Def2.1$_{G27\text{-}K44}$. After the growth absorbance was measured, each assay well was incubated with 5 µL of 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, a monosodium salt (WST-1) (Sigma–Aldrich Co.). Using a microplate reader (EZ Read 400), the absorbance of the formazan crystals in the mixture was measured at 450 nm. Then, the determination of viability was determined by the equation: % viability = [(450t/595t × 100)/(450c/595c)]. The assays were performed in three different experiments in triplicate. The minimum bactericidal concentration (MBC) was also determined as the lowest concentration at which there was no growth of CFU after 48 h in agar medium from the total volume of each well of the microdilution assay.

3.2.3.8. Determination of the time of death of *X. euvesicatoria* treated with ***Ca*Def2.1$_{G27\text{-}K44}$**

To determine the minimum period required to cause loss of viability and death of bacteria incubated with the *Ca*Def2.1$_{G27\text{-}K44}$ peptide at the MBC dose, the cells were prepared as described in subsection 3.2.3.4 (Preparation of bacterial inoculum). After the incubation time (0, ½, 1, 3 and 6 h) in the presence of the peptide, the cells were centrifuged, the supernatant was discarded, and 100 µL of PBS was added to wash the cells and spread them on the agar plate DYGS. Bacterial suspensions without *Ca*Def2.1$_{G27\text{-}K44}$ treatment were used as controls. The time 0 h refers to the time required to incubate the cells with peptide and immediately wash them in PBS, centrifuge them and plate them this time frame lasts approximately 5 min. After plating, the CFU were determined after 42 h of incubation at 30°C. Cell death was defined as loss of cell division capacity or loss of clonogenic capacity in culture medium in the absence of *Ca*Def2.1$_{G27\text{-}K44}$. Each experiment was repeated in triplicate and tested twice.

3.2.3.9. Control of common bacterial blight on pepper leaves by ***Ca*Def2.1$_{G27\text{-}K44}$**

Inoculation of *X. euvesicatoria* was carried out on leaves at the V3-V4 vegetative stage using the syringe infiltration method. The bacterial cell suspension was obtained by adding autoclaved deionized water to the DYGS medium plate and growing for 48 h to obtain the bacteria in solution. The bacterial inoculum was prepared at a concentration of 10^6 CFU/mL and immediately applied to the plant leaves in an approximate volume of 200 µL. Every 40 min, an application 300 µL of *Ca*Def2.1$_{G27\text{-}K44}$ (which corresponds to the volume necessary for the CMB to be applied) was applied by infiltration at concentrations adjusted to 1× MBC and 2× MBC. We used deionized water as a negative control. We applied a rating scale comparison to evaluate the severity of bacterial spot (*X. euvesicatoria*) on *C. annuum* leaves for comparison. The description of the symptoms in the Table 2 (Silva et al., 2017). The control was evaluated by the Dunnet test (P<0.001) using the GraphPad Prism program (version 8.0.2).

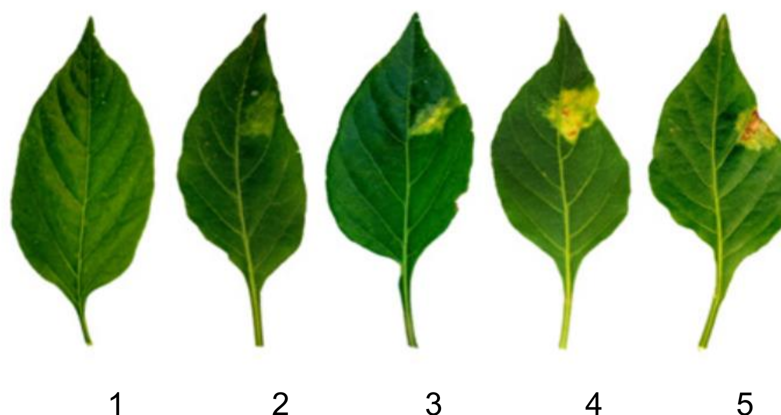


Figure 1: Rating scale for evaluating the severity of bacterial spot (*X. euvesicatoria*) on *C. annuum* leaves (Silva et al., 2017).

Table 2: Description of the rating scales used to determine the severity of common bacterial blight caused by *X. euvesicatoria* on pepper leaves inoculated by the infiltration method.

Leaf inoculation by infiltration method (10^6UFC/mL^{-1})	
Note – Phenotype Symptoms	**Symptoms**
1- Highly resistant	No apparent injury
2- Resistant	Presence of chlorotic lesion
3- Moderately susceptible	Presence of chlorotic lesion with necrotic spots
4- Susceptible	Necrotic lesion with presence of chlorotic halo
5- Highly susceptible	Coalescence of necrotic areas with the presence of a chlorotic halo

3.2.3.10. Morphological analysis of *C. scovillei* hyphae after inhibition

After the inhibition assay, the hyphae treated with the peptide at the concentration that had the greatest inhibitory effect on *C. scovillei* were subjected to phase differential contrast microscopy on an optical microscope model Axioplan A2 (Zeiss, Germany). Images of 10 random fields were collected, and hyphal diameter analysis was performed using measurements in the AxioVision LE program (length

measurement tool). Statistical analyses the analysis of variance of the means of the treated hyphae compared to the mean of the control hyphae were conducted using Prism software (version 8.0.2).

3.2.3.11. Conidia germination inhibition assay

Conidia were acquired as described in subsection 3.2.3.5. However, the conidia were dispersed in sterile water. The conidia concentration was adjusted to 2×10^4 conidia/mL. Conidia were treated with 300 or 400 μM/mL *Ca*Def2.1$_{G27\text{-}K44}$ peptide or without peptide (control). We used 70 mM sucrose as a carbon source to stimulate germination. The samples were incubated at 30°C and examined via optical microscopy every 3 h. From each treatment, fifty cells were randomly chosen. To maintain uniformity, the analyzed cells were in the same plane of focus. Using the AxionVision LE program (length measurement tool), the sizes of the conidia and hyphae were measured along the length of the longitudinal axis. Statistical analyses the analysis of variance of the means of the treated hyphae compared to the mean of the control hyphae were conducted using Prism software (version 8.0.2).

3.2.3.12. Cytoplasmic membrane permeabilization detection assay

The fungal conidia were prepared according to sections 3.2.3.5. The assays were carried out at a concentration of 400 μM/mL *Ca*Def2.1$_{G27\text{-}K44}$. At each 3 h period, the control and treatment groups were incubated for 15 min with 0.2 μM SYTOX™ Green (Invitrogen, Carlsbad, CA, USA) before being observed. Positive control cells were treated with 30 mM Triton X-100 (Sigma–Aldrich Co.) for 30 min after to adjust the fluorescence intensity and duration of exposure for capturing fluorescent signals, and these parameters were subsequently used for all additional treatments. An Axioplan A2 microscope (Zeis, Germany) equipped with fluorescent filters was used for fluorescein detection (excitation wavelengths 450–490 nm; emission wavelength 500 nm). The average of 40 cells in random fields was recorded via DIC and fluorescence for statistical evaluation. The percentage of fluorescent cells, an indicator of permeabilized cells, was calculated according to

the formula ([average number of fluorescent cells × 100]/average number of cells observed in DIC) for each sample (Thevissen et al., 1999).

The permeabilization of the bacterial membranes was verified using the MBC (5 μM/mL) of *Ca*Def2.1$_{G27\text{-}K44}$ and the SYTOX™ Green probe at a concentration of 0.2 μM. The inoculum was prepared according to section 3.2.3.4, with some modifications. In this test, the cells were resuspended in phosphate-buffered saline (PBS), pH 7.0. As a membrane permeabilization control, 16 mM Triton X-100 was used. The cells were incubated for 1 h, after which the cells were automatically read every 5 mins on a Chameleon V plate reader (USA & Canada IN/US Systems). The assay was performed in triplicate with two independent replicas.

3.2.3.13. Verification of the endogenous increase in reactive oxygen species

The increase in reactive oxygen species (ROS) was verified using the 2,7-dichlorofluorescein diacetate (H_2DCFDA) probe (Calbiochem — EMD, San Diego, CA, USA). The preparation of the cells was in accordance with section 3.2.3.5. At each 3 h interval, the control and treatment groups were incubated with 20 μM H_2DCFDA for 20 min. The positive control was incubated with 2.6 M hydrogen peroxide for 30 min before the probe was added. For visualization, an A2 microscope (Zeis, Germany) equipped with fluorescent filters was used for fluorescein detection (excitation wavelengths 450–490 nm; emission wavelength 500 nm). Positive control cells were used to adjustment of the fluorescence intensity and duration of exposure to capture fluorescent signals (Mello et al., 2011). The percentage of fluorescent cells, an indication of oxidative stress, was calculated according to the formula ([average number of fluorescent cells × 100]/average number of cells observed in DIC) for each sample.

3.2.3.14. Lipid peroxidation detection assay

Lipid peroxidation was checked by oxidizing BODIPY™ 581/591 C11 reagent (Thermo Fisher Scientific, Inc., Waltham, MA, USA). This reagent is found in living cell membranes and, when oxidized by lipid hydroperoxides, changes the

fluorescence emission peak from 590 nm to 510 nm. Cell preparation was carried out in the same way as in section 3.2.3.5. (Preparation of the fungal inoculum). Control and treatment cells (400 µM/mL *Ca*Def2.1$_{G27-K44}$ and positive control) were incubated with 20 µM probe for 40 min before completing a 3 h interval between observations. The positive control was incubated with 2.6 M hydrogen peroxide for 30 min before the probe was added. The positive control was used to adjust the intensity and duration of exposure when capturing fluorescence images. The cells were examined using an optical microscope equipped with fluorescent filters (Axioplan. A2, Zeis, Germany).

3.2.3.15. Assessment of the mitochondrial membrane potential

The membrane potential was assessed using a JC-1 probe (Thermo Fisher Scientific, Inc., Waltham, MA, USA). The preparation of conidia was carried out according to section 3.2.3.5. The control, cells treated with 400 µM/mL *Ca*Def2.1$_{G27-K44}$ and the positive control that was pre-incubated with 30 mM Triton X-100 for 40 min, were treated with 6 µM JC-1 (Thermo Fisher) for 40 min at 30°C. Cells were examined using a specific optical device equipped with fluorescent filters (Axioplan. A2, Zeis, Germany).

3.2.4. RESULTS

3.2.4.1. Characterization of the antimicrobial activity of synthetic peptides

The *Ca*Def2.1 peptide did not inhibit the growth *of C. scovillei* (Figure 2 A) or *X. euvesitoria* (Figure 2C). In contrast, the *Ca*Def2.1$_{G27-K44}$ peptide had an inhibitory effect on both microorganisms, as shown in Figure 2B and 2D. When the rate of inhibition was plotted in relation to the dosage administered, the curve resembled a nearly linear line that increased consistently with dose. As a result, it can be seen that the inhibitory effect of *Ca*Def2.1$_{G27-K44}$ on microorganisms was dose dependent. For *X. euvesicatoria*, 81 and 16% growth were inhibited in cells

treated with *Ca*Def2.1$_{G27-K44}$ at concentrations of 2.5 and 1.2 µM/mL, respectively. The MIC was determined to be 5 µM/mL, which is consistent with that of MBC. The IC_{50} was determined by linear regression to be 1.75 µM/mL. For *C. scovillei*, at concentrations of 100 and 200 µM/mL, the percentage of inhibition was 24 and 64%, respectively. At lower concentrations, no significant inhibition was observed.

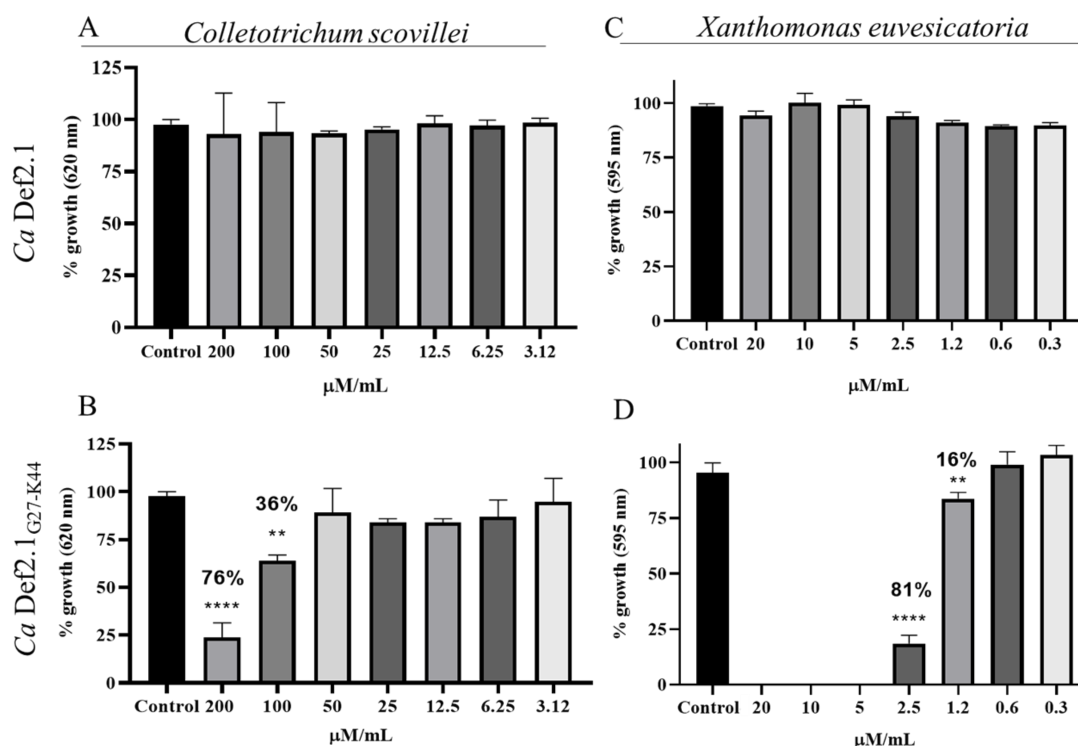


Figure 2: Inhibitory effect of *Ca*Def2.1 and *Ca*Def2.1$_{G27-K44}$ peptides on the growth of microorganisms. (A) Test of the *Ca*Def2.1 peptide with *C. scovillei.* (B) Test of the *Ca*Def2.1$_{G27-K44}$ peptide with *C. scovillei*. (C) Test of the CaDef2.1 peptide with *X. euvesicatoria.* (D) Test of the *Ca*Def2.1$_{G27-K44}$ peptide with *X. euvesicatoria.* Asterisks indicate significant differences in relation to the control, as assessed by the Dunnet test (** $p < 0.05$, **** $p < 0.001$). %, represents the percentage of inhibition.

3.2.4.2. Determination of the minimum bactericidal concentration (MBC) of *Ca*Def2.1$_{G27-K44}$ on *X. euvesicatoria*

After the inhibition assay, cells treated with 20, 10, or 5 µM/mL of *Ca*Def2.1$_{G27-K44}$ completely lost viability. This was verified by the use of WST-1 salt, which showed no conversion into formazan crystals. Furthermore, when these cells

were cultured in fresh media, no growth was observed, indicating that they lost their ability to proliferate and become clonogenic. However, at concentrations of 2.5 and 1.25 µM/mL, although growth was inhibited, the cells remained 100% viable and exhibited mitochondrial enzymatic activity, as shown in Figure 3A, and reproductive capacity, as shown in Figure 3B. Therefore, we set the MBC concentration at 5 µM/mL.

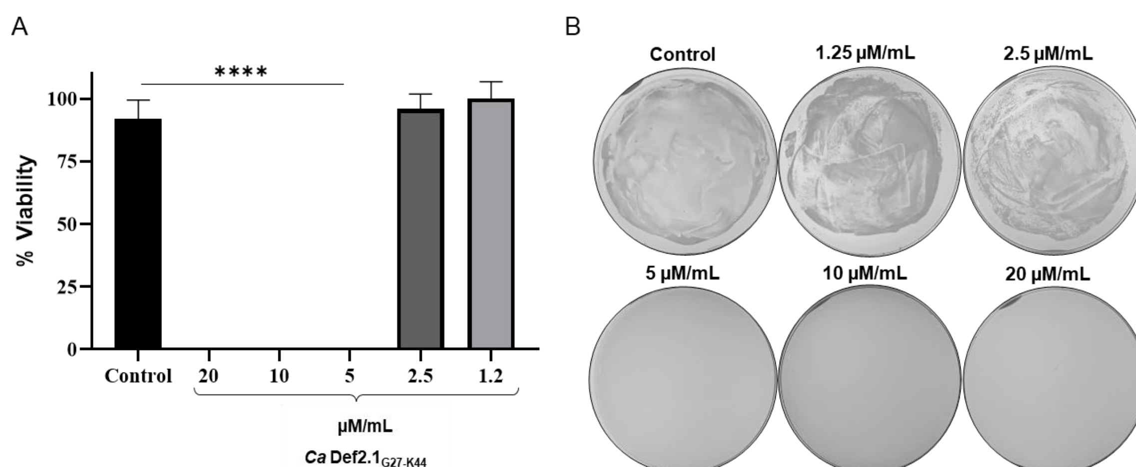


Figure 3: Viability of *X. euvesicatoria* after treatment with *Ca*Def2.1$_{G27\text{-}K44}$ in the microdilution assay. (A) Test with WST-1. (B) Cells were plated on DYGS agar medium and grown for 48 h. Asterisks indicate significant differences (P<0.001) between the control and treatment groups at their respective times.

3.2.4.3. Determination of time to death of *X. euvesicatoria* treated with the MIC of *Ca*Def2.1$_{G27\text{-}K44}$

The CaDef2.1$_{G27\text{-}K44}$ peptide causes the progressive death of *X. euvesicatoria*, as evidenced by the decreasing number of CFUs with increasing incubation time, as shown in Figure 4. The number of CFUs after ½ and 1 hour of incubation was significantly reduced, while the control had numerous CFUs, there were only 97 and 16 CFUs on the plates, respectively. As a result, at least 3 hours of incubation with the peptide are required for the complete killing of 5 × 10^5 CFU/m.

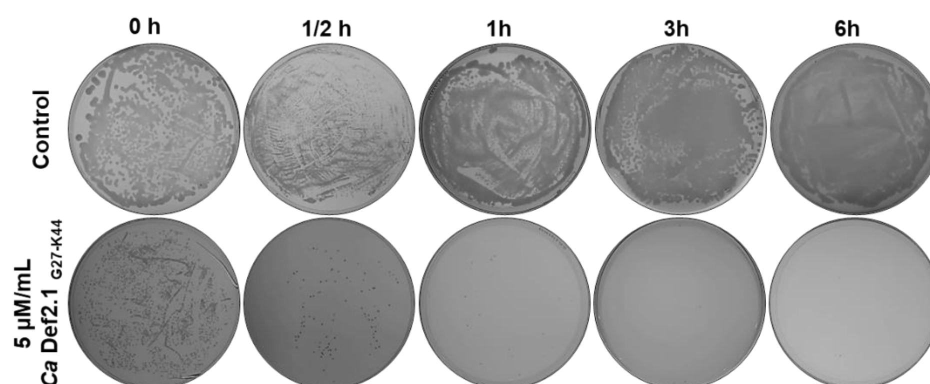


Figure 4: Time course of cell death in *X. euvesicatoria* triggered by MBC (5 µM/mL) *Ca*Def2.1$_{G27\text{-}K44}$. The number of UFCs was unknown at all times. At time 0, the number of CFU was unknown, and at time ½ and 1 h, the number of CFU was 97 and 16, respectively. After 3 h of incubation with the peptide, the cells lost all of their vitality. The plates were incubated for 48 h for CFU growth.

3.2.4.4. Permeabilization of *X. euvesicatoria* cytoplasmic membranes

X. euvesicatoria cells treated with MBC underwent membrane permeabilization, as shown in Figure 5, as indicated by a gradual increase in SYTOX™ Green fluorescence uptake within 60 min of observation. An increase in fluorescence uptake was also observed in cells treated with the ionic detergent Triton X-100. The control cells exhibited basal fluorescence.

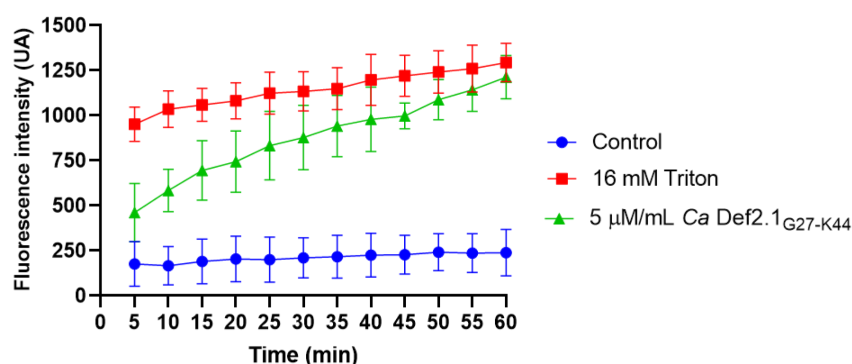


Figure 5: Permeabilization of the cytoplasmic membranes of *X. euvesicatoria* treated with 5µM/mL *Ca*Def2.1$_{G27\text{-}K44}$. Fluorescence intensity capture in arbitrary units (AUs) was performed every 5 min for 60 min. Control cells were incubated under the same conditions in the absence of peptide. A positive control was treated with Triton X-100.

3.12.4.5. Control test for *X. euvesicatoria* infection in pepper leaves by CaDef2.1$_{G27\text{-}K44}$

The experiment revealed that, compared to the control control group, the pepper leaves of the *C. annuum* Ikeda variety subjected to treatments with 1 or 2 times the minimum bactericidal concentration (MBC) presented a significantly reduced mean disease severity score. The MBC treatment and the 2X MBC treatment had average scores of 2.3 and 2, respectively, indicating a resistant phenotype but with the presence of chlorotic lesions, which was consistent with the expected results. In contrast, the control group had a score of 3, characterizing a moderately susceptible phenotype with the presence of chlorotic lesions and necrotic areas. However, when the test was carried out on pepper leaves of the *C. annuum* 1381 variety, no significant differences were observed between the treatments and the control. This lack of significance can be attributed to the high resistance of *C. annuum* 1381 to common bacterial blight, which prevents the development of the disease in the control group and, consequently, creates an inadequate contrast for evaluation (Figure 6).

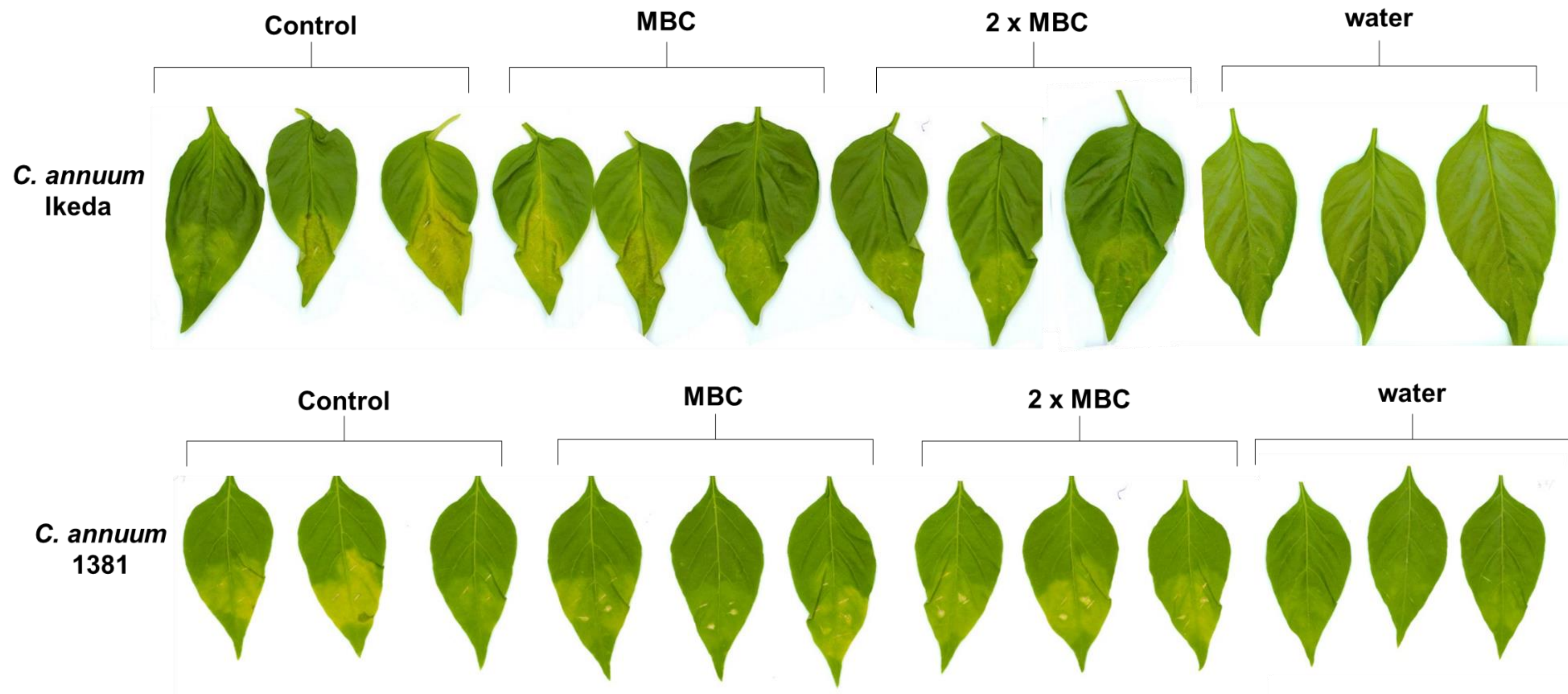


Figure 6: Symptoms of common bacterial blight caused by *X. euvesicatoria* on leaves of *C. annuum* peppers 8 days after inoculation. Control inoculation with deionized water. MBC- Minimum bactericidal concentration (5 µM/mL) of *Ca*Def2.1$_{G27\text{-}K44}$

.

3.2.4.5. Morphological changes in *C. scovillei* hyphae treated with *Ca*Def2.1$_{G27\text{-}K44}$

Optical microscopy of hyphae treated with 200 µM/mL *Ca*Def2.1$_{G27\text{-}K44}$ revealed morphological changes, such as poor development with the formation of globular structures, short secondary branches, and an increase in diameter. The control hyphae were long and had few short secondary branches (Figure 7A). The average diameter of the control hyphae was 7.8 ± 2.8 µm, whereas the hyphae treated with 200 µM/mL *Ca*Def2.1$_{G27\text{-}K44}$ had an average diameter of 15.6 ± 3.4 µm, indicating a significant increase in dilation compared to the untreated hyphae (Figure 7B).

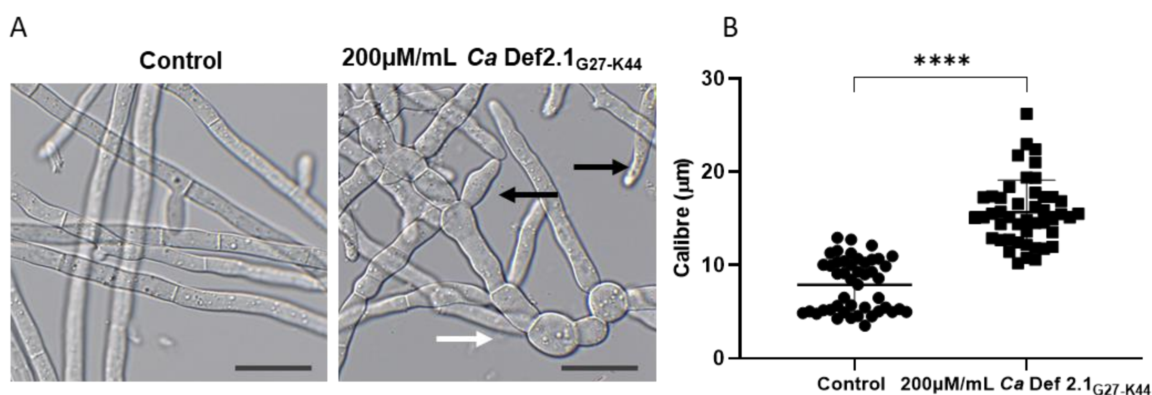


Figure 7: Visualization of *C. scovillei* hyphae treated with 200 µM/mL *Ca*Def2.1$_{G27\text{-}K44}$ for 24 h and control hyphae (untreated). A- Differential phase contrast microscopy of hyphae with and without peptide treatment. The white arrow indicates globular structures in the hyphae. The black arrow highlights the secondary branching of the hyphae. B- Comparison of the diameters of control hyphae and hyphae treated with 200 µM/mL *Ca*Def2.1$_{G27\text{-}K44}$. Bar = 20 µm. Asterisks indicate significant differences ($P<0.001$) between the control and treatment by T test.

3.2.4.6. Inhibition of *C. scovillei* conidia germination by *Ca*Def2.1$_{G27\text{-}K44}$

We investigated the ability of 300 and 400 µM/mL to prevent conidia germination. In the control group, conidia germination with germ tube emission occurred within 3 hours. A concentration of 300 µM/mL did not prevent germination, although it caused a delay in emission from the tube. In contrast, conidia treated

with 400 µM/mL did not germinate, indicating that this concentration is capable of preventing conidia germination. Additionally, some of the conidia in the 400 µM/mL treatment group had an irregular surface with a rough appearance (Figure 8).

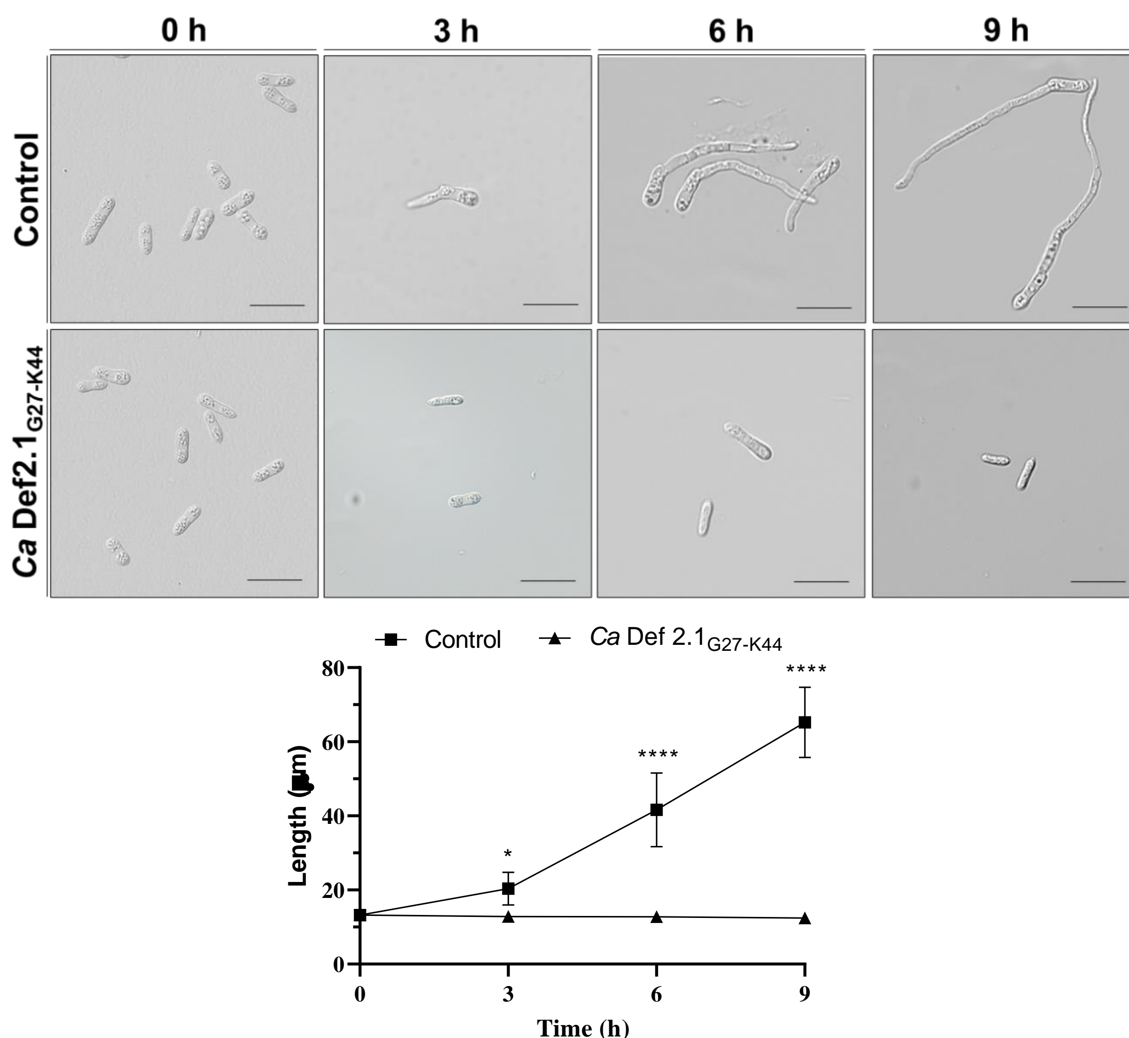
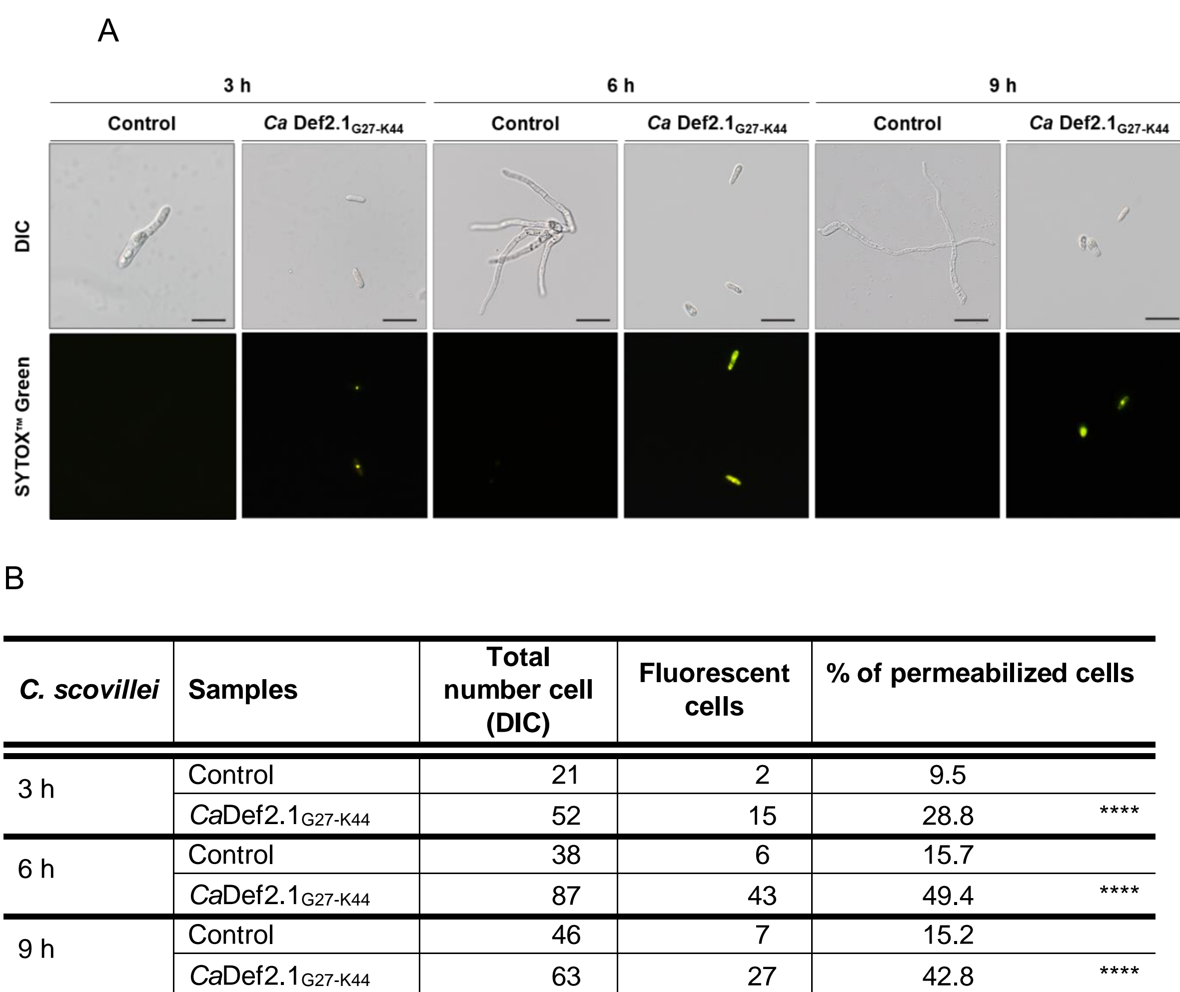


Figure 8: **Analysis of the inhibition of *C. scovillei* conidia germination by *Ca*Def2.1$_{G27-K44}$.** (A) Microscopy images taken every 3 h of incubation at 30°C with 400 µM/mL *Ca*Def2.1 $_{G27-K44}$. Bar = 20 µm. B) Evaluation of the sizes of germinated and nongerminated conidia. Asterisks indicate significant differences ($P<0.001$) between the control and treatment groups.

3.2.4.7. Cytoplasmic membrane permeabilization of *C. scovillei* treated with *Ca*Def2.1$_{G27-K44}$.

Fluorescence microscopy images revealed that conidia treated with 400 µM/mL *Ca*Def2.1$_{G27\text{-}K44}$ exhibited damage to the cytoplasmic membrane. As was evidenced by the entry of the SYTOX™ Green probe into the cells, which reacted with nucleic acids and produced a fluorescent signal, as shown in Figure 9A. In contrast, the control group cells showed some labeling, but there was no significant difference according to the Tukey test. Notably, the number of fluorescent conidia treated with the peptide increased by 70% within 3 to 6 hours, followed by a 13% reduction in fluorescent cells by 9 hours (Figure 9B).

A



B

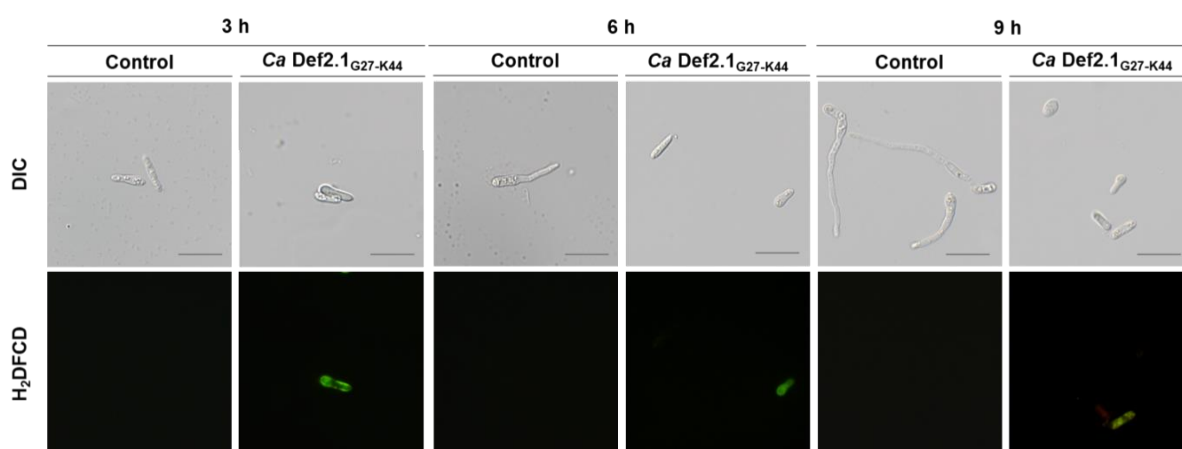
C. scovillei	Samples	Total number cell (DIC)	Fluorescent cells	% of permeabilized cells	
3 h	Control	21	2	9.5	
	*Ca*Def2.1$_{G27\text{-}K44}$	52	15	28.8	****
6 h	Control	38	6	15.7	
	*Ca*Def2.1$_{G27\text{-}K44}$	87	43	49.4	****
9 h	Control	46	7	15.2	
	*Ca*Def2.1$_{G27\text{-}K44}$	63	27	42.8	****

Figure 9: Permeabilization of *C. scovillei* plasma membranes treated with *Ca*Def2.1$_{G27\text{-}K44}$. (A) Optical microscopy images of DIC and fluorescence of *C. scovillei* incubated for 3, 6, and 9 h with 400 µM/mL *Ca*Def2.1$_{G27\text{-}K44}$. The control cells were incubated under the same temperature and humidity conditions as those used for the test in the absence of the peptide. Bar = 20 µm. (B) Cell count positive for SYTOX™ Green fluorescence. Asterisks indicate significant differences ($P<0.001$) between the control and treatment groups at the indicated times.

3.2.4.8. Detection of endogenous increase in ROS

C. scovillei conidia incubated with 400 µM/mL *Ca*Def2.1$_{G27-K44}$ for 3 hours produced 49% H_2DCFDA-positive cells, indicating an increase in ROS levels induced by the peptide. The control conidia did not show significant staining. After 6 hours, an increase to 57% of positive cells was observed, which slightly decreased to 55% after 9 hours. The fluorescence intensity in *C. scovillei* cells treated with *Ca*Def2.1$_{G27-K44}$ was highest during the first 3 h. However, the intensity in the positive cells was slightly reduced at 6 and 9 hours (Figure 10 A and B).

A



B

C. scovillei	Samples	Total number cells (DIC)	Fluorescent cells	% of permeabilized cells	
3 h	Control	30	0	0	
	*Ca*Def2.1$_{G27-K44}$	61	30	49.2	****
6 h	Control	52	8	15.3	
	*Ca*Def2.1$_{G27-K44}$	70	40	57.1	****
9 h	Control	21	4	19.4	
	*Ca*Def2.1$_{G27-K44}$	52	29	55.7	****

Figure 10: Endogenous increase in ROS in *C. scovillei* treated with *Ca*Def2.1$_{G27-K44}$. (A) Optical microscopy images of DIC and fluorescence images of *C. scovillei* incubated for 3, 6, and 9 h with 400 µM/mL *Ca*Def2.1$_{G27-K44}$. The control cells were incubated under the same temperature and humidity conditions as those used for the test in the absence of the peptide. Bar = 20 µm. (B) Count of cells positive for H_2DCFDA fluorescence. *Indicates a significant difference in relation to the control at the indicated time.

3.2.4.9. Lipid peroxidation in *C. scovillei* treated with *Ca*Def2.1$_{G27-K44}$.

C. scovillei conidia treated with 400 µM/mL *Ca*Def2.1$_{G27-K44}$ for 3, 6, or 9 hours exhibited lipid peroxidation similar to that in positive control cells induced by hydrogen peroxide, as shown in Figure 11. Fluorescent cells loaded with C11-BODIPY in red indicate unoxidized lipid molecules, while the oxidized form of C11-BODIPY appears in green. Notably, at all time points, the control cells exhibited red staining and no (or negligible signal at 9 h) green fluorescence, indicating the absence of lipid oxidative stress. The most intense green fluorescence was observed after 6 hours of treatment with the peptide, suggesting a peak of oxidative stress during this period

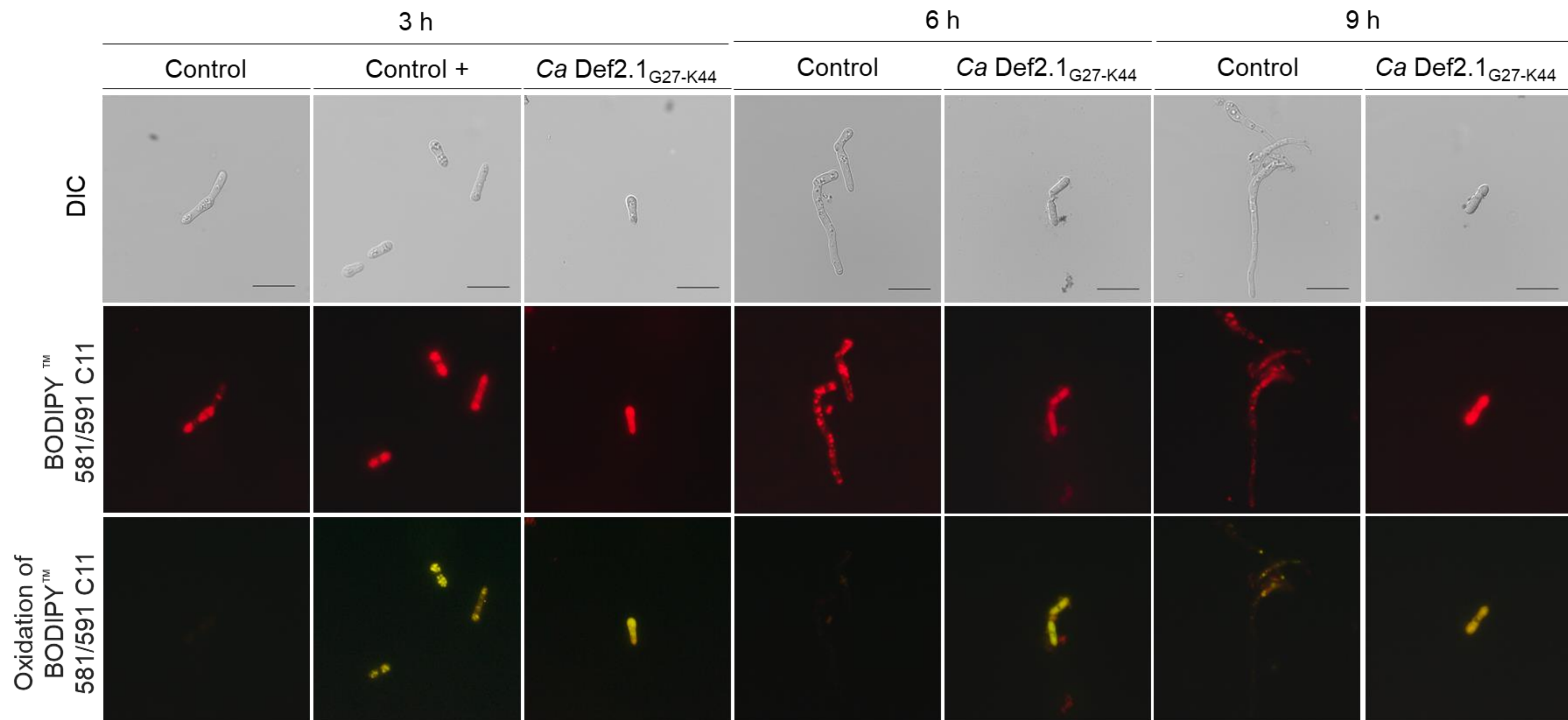


Figure 11: Assessment of lipid peroxidation in *C. scovillei* treated with 400 µM/mL *Ca*Def2.1$_{G27\text{-}K44}$. Peroxidation was visualized using the BODIPY™ 581/591 C11 sensor at observation intervals of 3, 6 and 9 h. In the reduced state, lipid labeling occurs in red, while in the oxidized state, lipid labeling occurs in green. As a positive control, 2.6 M hydrogen peroxide was used for 30 min before the probe was applied. Bar = 20 µm

3.2.4.10. Loss of mitochondrial functionality caused by *Ca*Def2.1$_{G27-K44}$ in *C. scovillei*

JC-1 dye demonstrated potential-dependent accumulation in mitochondria. At higher potentials, the JC-1 dye monomer forms red fluorescent "J aggregates", which accumulate inside the mitochondria, as shown in Figure 12, in control cells. Under conditions of low membrane potential, the JC-1 dye is in the form of green monomers, as evidenced by treating conidia with 30 mM Triton X-100 for 40 min. Analysis of cells treated with 400 µM/mL *Ca*Def2.1$_{G27-K44}$ in the first 3 h revealed a loss of mitochondrial functionality, as indicated by the accumulation of JC-1 monomers, although the red coloration still remained active. However, after 6 h, the cells lost their red fluorescence, while the JC-1 monomers continued to fluoresce. This phenomenon suggested the loss of mitochondrial viability over time, as indicated by treatment with the synthetic peptide.

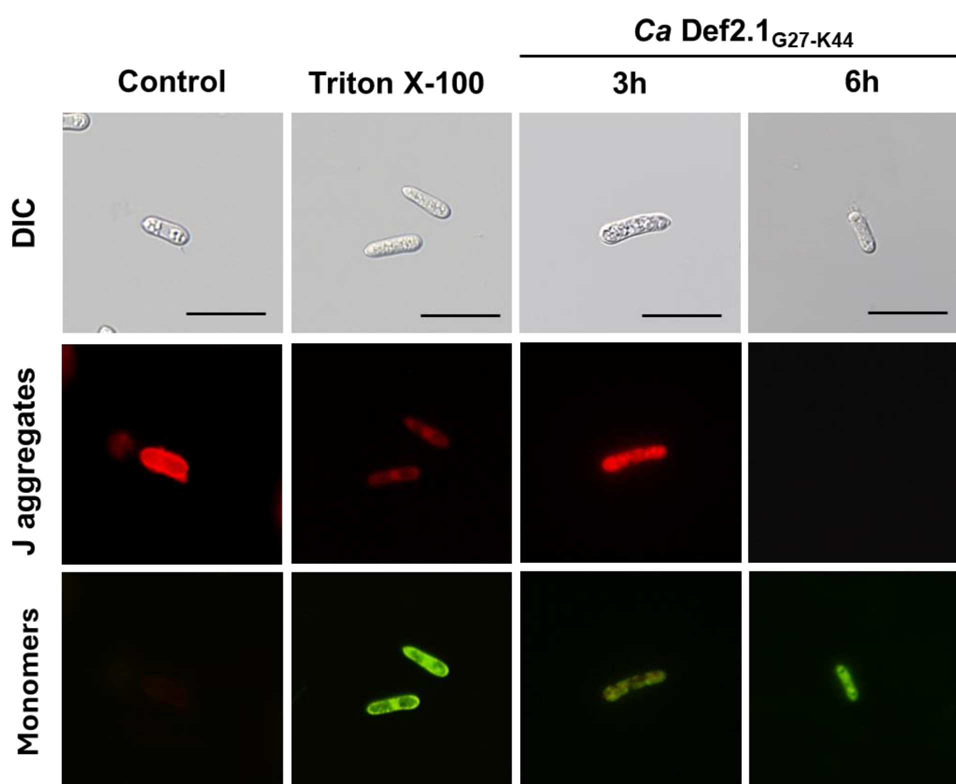


Figure 12: Loss of mitochondrial membrane potential in *C. scovillei* conidia treated with 400 µM/mL *Ca*Def2.1$_{G27-K44}$. At higher potentials, the JC-1 dye monomer forms red fluorescent "J aggregates." Under conditions of low membrane potential, the JC-1 dye is in the form of green monomers. Bar = 20 µm.

3.2.5. DISCUSSION

Advances in chemical synthesis have contributed significantly to reducing the cost of producing bioactive synthetic peptides. These advances have opened promising paths for obtaining new antimicrobial agents that are committed to safeguarding not only human health but also agriculture and livestock, facing the growing threat of antimicrobial resistance (Shwaiki et al., 2021).

Taveira et al.(2022) developed the AMP *Ca*Def2.1$_{G27\text{-}K44}$. This AMP was bioinspired by a specific segment of a defensin identified in *C. annuum*, from glycine 27 to lysine 44 of the primary structure of the original peptide. Modifications to the residues were implemented to increase the positive charge and hydrophobicity. The results showed that *Ca*Def2.1$_{G27\text{-}K44}$ has anti-*Candida* activity and antibacterial activity against *Mycobacterium tuberculosis*. Furthermore, it is known for its lack of toxicity in macrophage and monocyte cultures, highlighting its potential as a possible safe antimicrobial agent for therapeutic application.

In this study, the antimicrobial potential of the *Ca*Def2.1$_{G27\text{-}K44}$ peptide in controlling pepper pathogenic microorganisms was investigated. Anthracnose, caused by fungi of the genus *Colletotrichum*, represents a significant threat to several crops, with the species *C. scovillei* being the main pathogen responsible for pathogenicity in peppers. Common bacterial blight, caused by several species of *Xanthomonas*, is one of the most devastating diseases affecting sweet pepper and chili pepper plants (Russomanno e Kruppa, 2010; Parisi et al., 2020).

It was found that the modified synthetic peptide demonstrated effective antimicrobial activity against several pathogens affecting pepper plants, Figure 2. On the other hand, unmodified synthetic peptides do not exhibit antimicrobial activity. Other examples of synthetic AMPs whose antimicrobial activities are enhanced after rational amino acid modifications include AamAP1-lysine (Almaaytah et al., 2014), Guavanina (Cardoso et al., 2020), PaDBS1R6F10 (Fensterseifer et al., 2019) and Pp-TH(D32R) (Vila-Perelló et al., 2003). These results reinforce the importance of physicochemical characteristics, such as net positive charge and hydrophobicity as fundamental elements for successful antimicrobial activity.

*Ca*Def2.1$_{G27\text{-}K44}$ was more effective at reducing bacterial viability at lower concentrations (5 µM/mL) than at reducing antifungal activity, Figure 3. The

morphological changes in *C. scovillei* hyphae treated with 200 µM/mL led to the inhibition of development, the formation of uncharacteristic globular structures and increased caliber of these hyphae, Figure 7. These data suggest that the peptide directly affects cell wall integrity and cell morphology. At higher concentrations (400 µM/mL), *Ca*Def2.1$_{G27\text{-}K44}$ is capable of inhibiting conidia germination, Figure 8. We verified that the shared mechanism of action in both microorganisms was membrane permeabilization.

Membrane permeabilization as one of the most common mechanisms for pathogen death, in which electrostatic interactions between AMP (cationic) and phospholipids (negatively charged) can lead to the approach and, consequently, the insertion of AMP in the lipid bilayer. This process results in the formation of pores, which is widely discussed in the literature (Bin Hafeez et al., 2021). In the context of this study, it was observed that *Xanthomonas spp* cells exhibited membrane permeabilization within the first minutes of incubation (Figure 5). Bacterial cells suffer more quickly from the effects of *Ca*Def2.1$_{G27\text{-}K44}$. Similar mechanisms were observed for Zhang et al. (2023a), a catelecidin derived from green algae that inhibits bacterial growth by permeabilizing the bacterial cell membrane. Similarly, the membranes of the conidia of *C. scovillei* were also permeabilized in the first hour of incubation, after which the number of conidia increased until 6 hours and subsequently remained constant. However, as these cells have more complex walls, other targets of action were also investigated.

Soares et al. (2017) showed that dysfunctions in metabolism, with increased ROS and damage to cellular structures, can lead to delayed membrane permeabilization. This is because ROS play multifaceted roles by acting as cellular stress signals in some situations. ROS can trigger adaptive responses in pathogens, promoting the development of stress resistance pathways. However, in other circumstances, free radicals can be toxic to cells, resulting in the oxidation of essential components for cellular metabolism, such as proteins, lipids and nucleic acids (Pan, 2011). In the results, it was seen that conidia treated with *Ca*Def2.1$_{G27\text{-}K44}$ showed intense fluorescence in the first 3 hours of incubation, losing intensity over time (Figure 10), but not the number of fluorescent cells.

Subsequently, we examined lipid peroxidation and mitochondrial functionality and found that the cellular structures sensitive to increased ROS were compromised. Dysfunction of these components can impact the selective capacity of membranes and result in the loss of ionic homeostasis and energy, vital factors

for proper cell function (Gaschler e Stockwell, 2017). Barbosa et al.(2006) showed that the content of lipid bodies is associated with the germination process. Therefore, the loss of integrity of these structures reinforces the premise that this phenomenon causes growth inhibition. Huang et al. (2023) reported that Citral, a component of *Litsea cubeba* essential oil, could induce oxidative stress and subsequently lead to a rapid increase in the content of malondialdehyde, one of the most significant products of lipid peroxidation. Consequently, the authors saw that this irreversible damage led to the inhibition of pathogen growth.

Finally, the ability of *Ca*Def2.1$_{G27\text{-}K44}$ to inhibit the development of *X. euvesicatoria* in vivo was explored. It was observed that leaf treatment with the peptide commonly led to a reduction in the development of bacterial blight in *C. annuum* Ikeda, but the method used was not completely effective for all the pepper cultivars. However, the use of AMPs in agriculture is already a reality. To date, 18 peptides have been commercialized as green agents for plant protection (Zhang et al., 2023b). This finding shows the exploratory potential of these molecules. However, further tests and in-depth investigations into the application of AMPs in controlling these diseases still need to be conducted.

3.2.6. CONCLUSION

These results support the thesis that rational modifications to enhance the physicochemical characteristics, particularly the net charge and hydrophobicity, of bioinspired synthetic peptides are efficient strategies for developing new antimicrobial agents. Through this work, we also expanded the activity spectrum of *Ca*Def2.1$_{G27\text{-}K44}$, demonstrating its ability to inhibit phytopathogens at low concentrations. Membrane permeability was a common mechanism for both microorganisms. Additionally, we also observed an increase in ROS production and lipid peroxidation, a decrease in mitochondrial functionality, and morphological changes in *C. scovillei* treated with *Ca*Def2.1$_{G27\text{-}K44}$. Finally, when exploring *in vivo* application, a reduction in the development of bacterial blight in leaves treated with *Ca*Def2.1$_{G27\text{-}K44}$ was observed. The extensive use of AMPs in agriculture has demonstrated their exploratory potential, but further investigation is required to fully understand their applicability in disease control.

3.3. DIFFERENTIAL EXPRESSION OF ANTIMICROBIAL PEPTIDES IN *Capsicum annuum* FRUITS, RESISTANT AND SUSCEPTIBLE TO ANTHRACNOSE, IN RESPONSE TO INFECTION WITH *Colletotrichum scovillei*

3.3.1. INTRODUTION

Plants have developed a complex defense system to minimize the harmful effects of abiotic and biotic stresses. These mechanisms include effective structural and chemical barriers, which can be both pre-formed and inducible, playing a crucial role in mitigating these stresses (Pandey et al., 2017).

When plants detect the initial signs through plant cell surface-anchored pattern recognition receptors (PRRs), they recognize pathogen-derived molecules known as microbe-associated molecular patterns (MAMPs), pathogen-associated molecular patterns (PAMPs), or damage-associated molecular patterns (DAMPs). Upon recognition, PRRs recruit regulatory receptor kinases, forming complexes that trigger a series of responses known as MAMP-, PAMP-, or DAMP-triggered immunity (MTI, PTI, or DPI), which work to eliminate potential pathogenic infections (Nishad et al., 2020).

. However, plant pathogens have also developed strategies to overcome PTI, facilitating effector-triggered susceptibility (ETS). In response, plants have developed an additional defense system that recognizes pathogen effectors or host targets modified by effectors, leading to effector-triggered immunity (ETI). ETI

activation can result in the deposition of callose at the infection site, production of pathogenesis-related proteins (PR proteins) such as chitinases, β-1,3-glucanases, proteases that hydrolyze fungal cell wall components, protease inhibitors, and antimicrobial peptides (AMPs), among others (Ferreira et al., 2007; Sels et al., 2008).

AMPs are short cationic peptides, up to 100 amino acid residues long, characterized by a positive net charge at physiological pH and amphipathic properties (Savitskaya et al., 2023). They exhibit activity against various phytopathogens, including gram-positive and gram-negative bacteria, oomycetes, fungi (unicellular and filamentous), protozoa, and viruses (Zhang et al., 2023).

The main families of AMPs include thionins, hevein-like proteins, knottin-type peptides, α-hairpinins, snakins, cyclotides, lipid transfer proteins, defensins, and some protease inhibitors (Lima et al., 2022). The last three families have been the most characterized and are also described as being involved in plant defense responses.

AMPs emerge as promising molecules in the field of plant protection, benefiting from the diversity of structures and sequences available for manipulation, a broad spectrum of activities, and a low risk of environmental contamination. These peptides have the ability to induce components that promote plant development and trigger responses during the infection process.

Although several studies highlight peppers of the *Capsicum* genus as promising sources of AMPs with in vitro activity (Afroz et al., 2020; Oliveira et al., 2022), there is still a significant gap in the thematic approach needed for an in-depth understanding of the interaction between the pathogen and the host at the molecular level. This understanding is crucial for the effective introduction of AMPs with defensive characteristics, aiming to establish robust innate defense mechanisms for commercially important crops.

Fungi belonging to the *Colletotrichum* genus are among the ten most scientifically and economically significant fungal pathogens (Dean et al., 2012), causing considerable crop losses, especially in fruits, vegetables, and ornamental plants, both during production and post-harvest (Dean et al., 2012). Anthracnose stands out as the primary disease, responsible for reductions in pepper production, with post-harvest losses ranging from 10% to 80%, causing serious economic

damage globally, particularly affecting subsistence farmers in developing countries located in tropical and subtropical regions (Prajapati et al., 2020).

Given this context, the *Capsicum-Colletotrichum* pathosystem presents itself as an appropriate scenario for investigations. Thus, the objective of this study was to analyze the expression profile of three AMP families—a defensin, a lipid transfer protein (LTP), and a Kunitz-type protease inhibitor (KTI)—in response to infection by *Colletotrichum scovillei* in *Capsicum annuum* pepper fruits, resistant and susceptible to anthracnose.

3.3.2. LITERATURE REVIEW

In the previous chapters, we discussed the main general and physicochemical characteristics of plant AMPs. Next, we will discuss some families of antimicrobial peptides from plants identified in this work and reported in the literature to have exhibited activity against phytopathogens.

3.3.2.1. Defensins

Plant defensins belong to the cis-defensin superfamily, a classification based on the parallel positioning of a double disulfide bridge between the central β-sheet and the α-helix. The comparison between cis-defensins and trans-defensins is presented in Figures 2A and 2B, respectively (Shafee et al., 2016).

Defensins are one of the largest groups of plant AMPs studied to date and a major component of the plant innate immune system. They exhibit diversity in their amino acid residue sequences; however, some positions have highly conserved residues. For example, the eight cysteines (C1-C8/C2-C5/C3-C6/C4-C7) represented in Figure 1C, which form disulfide bonds. They also have two glycine residues (positions 12 and 32), an aromatic residue (position 10), and a glutamate (position 27), the position numbers refer to the NaD1 defensin from *Nicotiana alata* as a reference (PARISI et al., 2019).

The three-dimensional structure is highly conserved and is presented in Figure 1A. It consists of a triple chain of antiparallel β-sheets connected to an α-

helix by intramolecular disulfide bridges. This conformation is known as the cysteine-stabilized alpha-beta (CSαβ) motif. The diversity in the primary sequence structure causes slight variations in the spatial arrangement of the loops, which certainly contributes to the wide range of biological activities (Carvalho e Gomes, 2009; Shafee et al., 2016).

Another highly conserved region important for the biological activity of defensins is the γ-core, located between loops 4-7, composed of the residues $GXCX_{3-9}C$ (where X can represent any amino acid residue) (Yount e Yeaman, 2004). This region is described as being responsible for the antimicrobial activity in most defensins. In studies conducted by Sagaram et al. (2011) on the antifungal activity of MsDef1 (defensin from *Medicago sativa*) and MtDef4 (defensin from *Medicago truncatula*), it was found that the antimicrobial activity was related to the γ-core region. Thus, the close structural relationship between these elements is reflected in the broad spectrum of activity exhibited by this group of peptides (Samblanx et al., 1997; Sagaram et al., 2011).

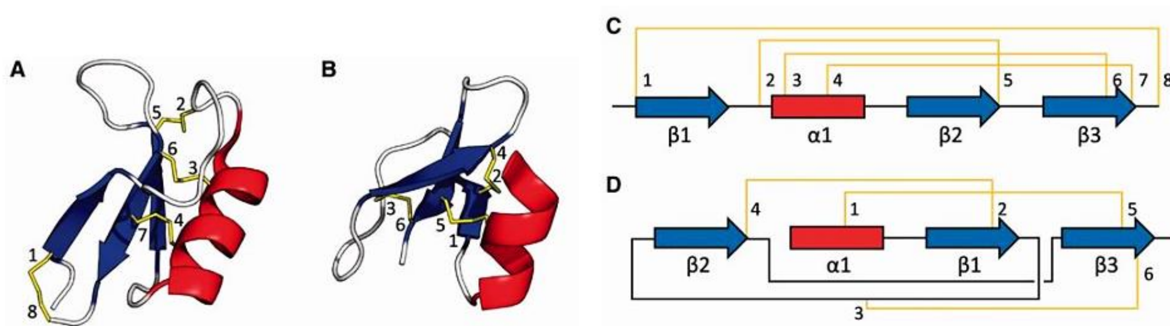


Figure 1: Schematic representation of different classes of defensins. (A) *Cis*-defensins, NaD1 from *Nicotiana alata* (PDB:1MR4). (B) *Trans*-defensins, human β-defensin (*Homo sapiens*) HBD1 (PDB:1JV). (C) Disulfide bond arrangement in "*Cis*" defensins and (D) Disulfide bond arrangement in "*Trans*" defensins. α-helices are indicated in red, β-sheets in blue, and disulfide bonds in yellow. The secondary structure and cysteines are numbered according to their sequence order (Shafee et al., 2016).

Plant defensins can be classified into two main categories: Class I and Class II. Class I defensins have an endoplasmic reticulum (ER) signal sequence followed by a mature defensin domain and are directed to the secretory pathway, lacking

signals for post-translational modifications or subcellular targeting. As a result, these defensins accumulate in cell walls and the extracellular space. On the other hand, Class II defensins are produced from larger precursors that include an ER signal sequence, a mature domain, and a C-terminal pro-peptide (CTPP) composed of 27–33 amino acids. These precursor defensins are directed to the vacuole via the CTPP, where they undergo proteolytic processing to release the mature defensin and are then stored. Most Class II defensins are produced by species of the Solanaceae family (Shafee et al., 2016; Costa et al., 2020).

The large variation in the amino acid sequences of defensins imparts different biological activities. These activities include regulation of development and sexual reproduction, antibacterial activity (Weerden e Anderson, 2013), inhibition of insect digestive enzymes such as trypsin and α-amylases (Pelegrini et al., 2008), influence on heavy metal tolerance (Mirouze et al., 2006), antitumor activity (Figueira et al., 2017), inhibition of ion channels in cells (Spelbrink et al., 2004), and inhibition of protozoa (Nascimento et al., 2015). Antifungal activity is the best-characterized function of defensins. Table 1 categorizes the antifungal activity described for plant defensins.

Table 1. Defensins with antifungal activity. Extracted from (Parisi et al., 2019).

Defensin name	Specie	Plant tissue	Activity on fungal pathogen
NaD1	*Nicotiana alata*	flowers	*Aspergillus nidulans, Botrytis cinerea, Candida albicans, Colletotrichum graminicola, Cryptococcus gattii, Cryptococcus neoformans, Fusarium graminearum, F. oxysporum, Puccinia coronate, P. sorghi, Saccharomyces cerevisiae, Thielaviopsis basicola, Verticillium dahliae*
RsAFP2	*Raphanus sativa*	seed	*Alternaria longipes, A. solani, A.brassicola, Ascochyta pisi, Aspergillus flavus, B. cinerea, C. albicans, C.krusei. C. glabrata, Cercospora beticola, Cladosporium sphaerosperm, Colletotrichum lindemuthianum, Fusarium culmorum F. graminearum, F. solani, F. oxysporum, Leptosphaeria maculans, Mycosphaerella fijiensis, Rhizoctonia solani, Sclerotinia sclerotiorum Septoria nodorum, Septoria tritici, Trichoderma hamatum, Tricho fiton mentagrophytes, Trichoderma viride, Verticilium alboatrum, V. dahlia, Venturia inaequalis*
Psd1	*Pisum sativum*	seed	*Aspergillus niger, Avicularia. versicolor, Fusarium solani, F. moniliformae, F. oxysporum, Neurospora crassa, S. cerevisiae, T. mentagrophytes*
HsAFP1	*Heuchera sanguinea*	seed	*A. flavus, B. cinerea, C. albicans, C. glabrata, Candida krusei, Cladsporium sphaerospermum, F. culmorum, F. solani, Leptosphaeria maculans, N. crassa, Penicllium digitatum, Septoria tritici, Verticillium albo-atrum, T. viride*
***Pv*D$_1$**	*Phaseolus vulgaris*	seed	*C. albicans, Candida tropicalis, F. oxysporum, F. solani, Fusarium laterithium, Kluyveromyces marxiannus, S. cerevisiae*
MsDef1	*Medicago sativa*	seed	*F. graminearum, N. crassa V. dália*
MtDef4	*Medicago trucatula*	seed	*F. graminearum, N. crassa, Puccinia tritici*

Table 1 – Cont.

Defensin name	Specie	Plant tissue	Activity on fungal pathogen
DmAMP1	*Dalia merkii*	seed	*Alternaria brassicicola, A. flavus, B. cinerea, C. albicans, C. glabrata, C. krusei, C. sphaerospermum, F. solani, F. culmorum, L. maculans, N. crassa, P. digitatum, S. cerevisiae, S. tritici, Trichoderma viride, V. albo-atrum*
NsD1, NsD2	*Nigella sativa*	seed	*A. niger, B. cinerea, Bipolaris sorokiniana, F. culmorum, F. graminearum, F. oxysporum*
PhD1, PhD2	*Petunia híbrida*	flowers	*B. cinerea, F. oxysporum*
Zm-ESR6	*Zea Mays*	seed	*B. cinerea, F. oxysporum, Plectosphaerella cucumerina*
PTH1	*Solanum tuberosum*	tuber	*Clavibacter michiganesis, F. solani, Pseudomonas solanacearum*
TvD1	*Tephrosia vilosa*	leaves	*Alternaria helianthi, B. cinerea, Curvularia sp, Fusarium moniliforme, F. oxysporum, Pheaoisariopsis personata*
Vv-AMP1	*Vigna unguiculata*	berry	*Alternaria longipes, B. cinerea, F. graminearum, F. oxysporum, F. solani, V. dahliae*

3.3.2.2. Lipid transfer proteins (LTPs)

Lipid transfer proteins (LTPs) are described as polypeptides capable of transporting a variety of hydrophobic molecules in vitro, including fatty acids, fatty acyl-CoA, phospholipids, glycolipids, and cutin monomers, in small amounts at a time. They use hydrophobic cavities in their tertiary structure to stabilize the membrane-free lipid molecules (Missaoui et al., 2022).

Most LTPs are cationic with an isoelectric point between 9 and 11 and have a tertiary structure composed of organized α-helices that form a hydrophobic cavity. This family of peptides consists of a structure with about 70 to 100 amino acid residues, including 8 to 10 cysteine residues that form 4 or 5 disulfide bonds. (Maximiano e Franco, 2021).

LTPs, like other AMPs, exhibit a wide variety of amino acid sequences and, for educational purposes, have been classified based on their characteristics. In the most recent system presented by Edstam et al., (2011), they were categorized based on the position of introns in the coding sequence, the presence of glycosylphosphatidylinositol modification sites, the spacing of eight cysteines, and sequence similarity. This system resulted in the identification of five main types of lipid transfer proteins (LTPs): LTP1, LTP2, LTPc, LTPd, and LTPg. Additionally, five minor types (LTPe, LTPf, LTPh, LTPj, and LTPk) were identified, which have fewer members (Amador et al., 2021).

LTP1 and LTP2 types are the most abundant in most species and therefore have been more extensively discussed in the literature. Type 1 LTPs (LTP1s), shown in Figure 2 A and B, have a molecular mass of approximately 9 kDa, four α-helices connected by loops, and a flexible C-terminal arm that completes the hydrophobic cavity. Type 2 LTPs (LTP2s), shown in Figure 2 C and D, have a molecular mass of around 7 kDa, a high isoelectric point, three α-helices, and two simple loops in the C-terminal region. The hydrophobic cavity formed in the tertiary structure is more compact compared to the same region in LTP1s(Missaoui et al., 2022; Tam et al., 2015; Edstam et al., 2011).

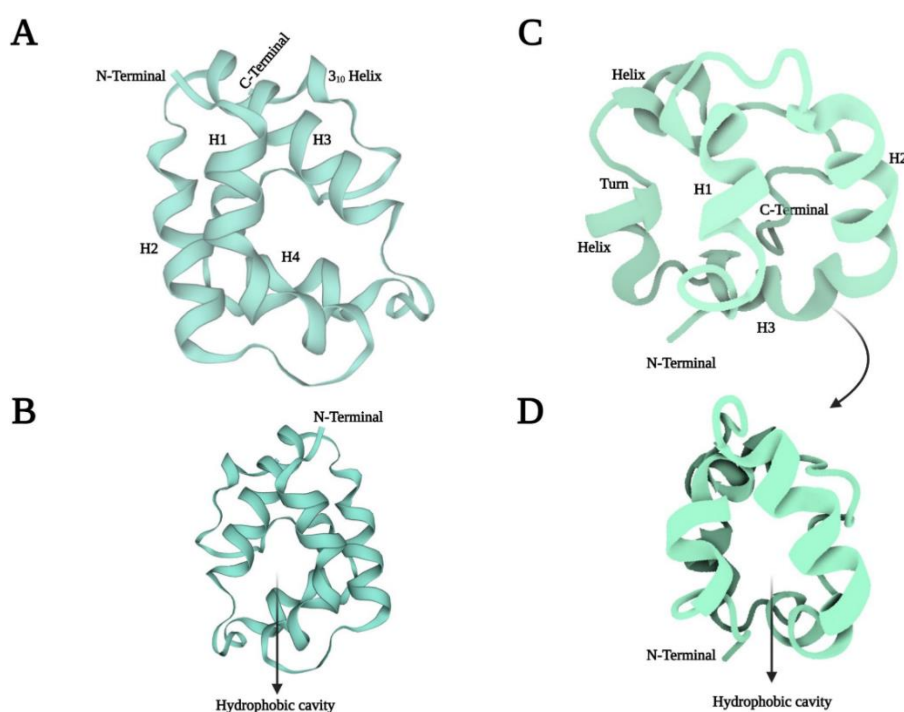


Figure 2: Three-dimensional structure of ns-LTPs. A and B - LTP1 (PDB ID: 3GSH). C and D - LTP2 (PDB ID: 1L6H).

The ns-LTPs perform a variety of physiological functions, including the transport of intracellular lipids, the deposition of wax or cutin on cell walls, the response to abiotic stress, and others (Yeats e Rose, 2008; Odintsova et al., 2019; Maximiano e Franco, 2021). With regard to their antimicrobial activity, it has been proposed that they interact with the phospholipids of the cytoplasmic membrane, causing permeabilization (Carvalho e Gomes, 2007; Bard et al., 2016).

As molecules involved in the plant defense mechanism, LTPs bind to lipid molecules secreted by plants or pathogenic microorganisms and interact with receptors. This interaction triggers signal transduction mediated by secondary messenger molecules, resulting in a cascade of mitogen-activated protein kinase (MAPK) activation. This process induces transcription factors, protective factors, pathogenesis-related proteins (PR proteins), and other antimicrobial peptides (AMPs), ultimately leading to systemic acquired resistance (SAR) (Finkina et al., 2016).

Table 2 illustrates the antifungal activity of ns-LTPs against various fungal pathogens.

Table 2:List of LTPs with antifungal activity, extracted from Maximiano; Franco (2021).

LTP name	Species	Tissue	Activity on fungal pathogen
Radish ns-LTP	*Raphanus sativus*	Seeds	*Alternaria brassicola* *Ascockyta pisi* *Botrytis cinera* *Colletotrichum lindemuthianum* *Fusarium culmorum* *Fusarium oxysporum fsp. Pisi* *Fusarium oxysporum fsp. Licopersici* *Nectria hematococa* *Phoma betae* *Pyricularia oryzae* *Trichoderma hamatum* *Verticillium dáliae*
Cw_{18}, Cw_{20}, Cw_{21}	*Hordeum vulgare*	Leaves	*Fusarium solani* *Alternaria brassicola*

Table 2 – Cont.

LTP name	Species	Tissue	Activity on fungal pathogen
Ace-AMP1	*Allium cepa*	Seeds	*Ascockyta pisi* *Botrytis cinera* *Colletotrichum lindemuthianum* *Fusarium culmorum* *Fusarium oxysporum fsp. Pisi* *Fusarium oxysporum fsp. Licopersici* *Nectria hematococa* *Phoma betae* *Pyrenopkora tritici-repentis* *Pyricularia oryzae* *Verticillium dáliae*
LTP1.10	*Oryza sativa*	Leaves	*Piricularia oryza*
Ha-AP10	*Helianthus annuus*	Seeds	*Fusarium solani f. sp. Eumártio* *Alternaria brassicae*
***Bc*-nsLTP**	*Brassica campestris*	Seeds	*Fusarium oxysporum* *Mycosphaerella arachidicola*
LJ-AMP$_2$	*Leonurus japonicus*	Seeds	*Fusarium solani f. sp. eumártio* *Alternaria brassicae* *Botrytis maydis* *Rhizoctonia cerealis* *Aspergillus niger* *Fusarium oxysporum* *Penicillium digitatum* *Saccharomyces cerevisiae*

3.3.2.3. Protease inhibitors

Protease inhibitors (PIs) are proteins that play a significant role in various physiological activities in plants. They regulate the activity of endogenous proteases and participate in biotic and abiotic defense mechanisms, exhibiting both antimicrobial and insecticidal activity (Rodríguez-Sifuentes et al., 2020; Moloi e Ngara, 2023).

Plants produce inhibitory polypeptides that can suppress enzymatic activities in response to the attack of proteinases produced by phytopathogenic microorganisms. This phenomenon was first observed in tomatoes infected with *Phytophthora infestans*, where elevated levels of trypsin and chymotrypsin

inhibitors were correlated with the plants' resistance to the pathogen (Woloshuk et al., 1991).

Pis are classified according to the catalytic site of the target enzyme into serine protease inhibitors, cysteine protease inhibitors, aspartic protease inhibitors, and metalloproteases protease inhibitors (Cotabarren et al., 2020). The majority of PIs with antimicrobial activity are serine protease inhibitors, which can be classified into Kunitz-type inhibitors (18–24 kDa), Bowman-Birk inhibitors (4-8 kDa), potato type I and II inhibitors, cereal trypsin/α-amylase inhibitors, among others. They are classified based on the degree of homology in amino acid residue sequences, the position of cysteine residues, the location of the reactive site, and the molecular mass (Christeller e Laing, 2005).

Table 3 provides examples of inhibitors with antimicrobial activity.

Table 3: Presents a list of plant proteases that have antimicrobial properties.

Name	Specie	Classification	Size (kDa)	Microrganismo	Reference
PG-2	*Solanum tuberosum* L. cv. Golden Valley	Kunitz	3.2	*Candida albicans* *Clavibacter michiganensis* *Staphylococcus aureus* *Rhizoctonia solani*	(Kim et al., 2013)
TcTI	*Theobroma cacao*	Kunitz	23	*Moniliophythora perniciosa*	(Amaral et al., 2022)
PDI	*Conyza dioscoridis*	Kunitz	25	*Enterococcus faecalis* *Salmonella entérica* *Klebsiella pneumoniae* *Bacillus cereus* *Aspergillus niger* *Botrytis cinérea* *Fusarium solani* *Penicillium digitatum*	(Karray et al., 2020)
WTI	*Triticum aestivum*	Bowman-Birk	8.5	*Botrytis cinerea* *Colletotrichum acutatum* *Didymella bryoniae* *Fusarium culmorum* *Fusarium graminearum* *Septoria tritici* *Thielaviopsis basicola* *Verticillium dahliae*	(Chilosi et al., 2000)

Table 3 – Cont.

Name	Specie	Classification	Size (kDa)	Microrganismo	Reference
Unnamed	*Dolichos biflorus*	Bowman-Birk	16	*Alternaria alternata,* *Fusarium oxysporum* *Aspergillus niger*	(Kuhar et al., 2013)
ClCPI	*Cassia leiandra*	inibidores tripsina/α-amilase	16.6	*Candida tropicalis*	(Melo et al., 2019)
***Ca*CPin-II**	*Capsicum annuum* L. var. annuum	Tipo II de batata	~ 6	*Candida albicans* *Candida buinensis*	(Cherene et al., 2023)
PT-1	*Solanum tuberosum* L cv. Gogu	Tipo II de batata	5,6	*Candida albicans*, *Rhizoctonia solani* *Clavibacter michiganense*	(Kim et al., 2005)
ApTIA, **ApTIB** and **ApTIC**	*Acacia plumosa*	Kunitz	~20	*Aspergillus niger* *Thielaviopsis paradoxa* *Colletotrichum* sp	(Lopes et al., 2009)
JcTI-I	*Jatropha curcas*	Inibidor de tripsina	~10	*Salmonella enterica* *Staphylococcus aureus*	(Costa et al., 2014)

The mechanism by which microorganisms are inhibited may be dependent on the inhibition of the activity of endogenous or exogenous proteases, which results in a reduction in the availability of nutrients for development or the blockage of important metabolic pathways. For example, the JcTI-I inhibitor, extracted from the *Jatropha curcas* seed, exhibits potent inhibitory activity. At a concentration of 5µg/mL, the inhibitor was found to be capable of inhibiting 100% of the endogenous proteases extracted from *Salmonella enterica* and 84.6% of those extracted from *S. aureus* (Brogden, 2005; Costa et al., 2014).

Blocking crucial metabolic pathways can impact not only nutritional pathways, but also physiological pathways, such as the capacity to suppress the activity of proteases that are essential for development. Chitin is a crucial component of the fungal cell wall and is synthesized from the substrate uridine diphosphate N-acetylglucosamine (UDP-GlcNAc) by chitin synthase catalysis (Machida e Saito, 1993). Inhibiting this process can result in the development of aberrant fungal cell morphology, alterations in the permeability of the cell wall, and ultimately, the demise of the fungus. Consequently, the inhibition of chitin synthase may be a crucial strategy for the control of pathogens and the regulation of its activity represents a promising target for the development of antifungal drugs (Liu et al., 2023b).

Additionally, PIs can act on the integrity of the cytoplasmic membrane, a mechanism that is independent of the active site for binding to proteases. In this case, the antimicrobial potential is due to the characteristics of net charge and hydrophobicity that act on the membranes (Clemente et al., 2019). The ApTI peptide, derived from *Adenanthera pavonina*, has been demonstrated to compromise membrane integrity, resulting in cell lysis in *Escherichia coli* and the release of nucleic acids into the medium (Almeida et al., 2020).

Due to their prevalence and characteristics, such as notable resistance to high temperatures and stability against changes in pH, denaturing agents, ionic strength, and proteolysis, inhibitors have attracted interest from researchers in various fields as promising molecules for biotechnological, biomedical, and agronomic applications. These include the control of herbivores and phytopathogens (Cotabarren et al., 2020).

3.3.3. MATERIAL AND METHODS

3.3.3.1. Biological materials

The pepper species *Capsicum annuum* GBUEL103 (susceptible to bacterial spot, pepper yellow mosaic virus, and anthracnose) and GBUEL104 (resistant to bacterial spot, pepper yellow mosaic virus, and anthracnose) (Bento et al., 2017). The fungal isolate *Colletotrichum scovillei* (GenBank MN121780, MN121791, MN121802, MN121811 and MN121822) was cultivated on potato-dextrose-agar (PDA) medium at pH 7.0 and incubated in the dark at 25°C for seven days. Both strains were kindly provided by the State University of Londrina (UEL).

3.3.3.2. Pepper cultivation

The pepper seeds were planted in an organic substrate designed for vegetable cultivation. Once the second pair of definitive leaves had emerged, the seedlings were transferred to 5-liter plastic pots containing a mixture of sand and substrate in a 2:1 (weight:weight) ratio. The plants were cultivated in a greenhouse in accordance with the recommended practices for growing peppers. The fruits were harvested 35 days after anthesis (DAA), at which stage they were green in color. The fruits were carefully removed from the plant and subjected to a 5-minute disinfection process in a 1% sodium hypochlorite solution. Subsequently, the fruits were subjected to three consecutive washes with distilled water, each lasting one minute. In this manner, the fruits were prepared for the infiltration of *C. scovillei* conidia.

.

3.3.3.3. Inoculation of the fungus *C. scovillei* in pepper fruits

An aqueous solution containing 10^6 conidia/ mL of C. *scovillei* suspension was prepared by scraping the spores cultivated in PDA medium into a Petri dish. Inoculation was carried out under laboratory conditions using the infiltration method in the central region of the fruit, with a Micro Syringe Model 1705 TLL (Hamilton, Switzerland). To ensure consistent inoculum volume and uniformity in lesion size,

the depth of infiltration was set at 1 mm. Control fruits underwent a similar treatment, using distilled water.

Pepper fruits were then incubated in the dark for 24 hours at 25°C, followed by 12-hour light/dark exposure cycles in a humid chamber. Samples were collected at intervals of 24 and 48 hours post-inoculation (HPI). Three biological replicates were conducted, resulting in a total of 24 samples, as detailed in Table 4. All samples were immediately frozen in liquid nitrogen and stored at -80 °C until RNA extraction.

Table 4: Identification of *Capsicum annuum* samples.

Genotype	Characteristic	Sample	Hours	Sample ID
GBUEL103	Susceptible to anthracnose, pepper yellow mosaic virus and bacterial spot	Mock	24	SM24
			48	SM48
		Inoculated	24	SI24
			48	SI48
GBUEL104	Resistant to anthracnose and bacterial spot	Mock	24	RM24
			48	RM48
		Inoculated	24	RI24
			48	RI48

3.3.3.4. RNA extraction from fruits

Total RNA from the samples was extracted using the SV Total RNA Isolation System kit (Promega), following the instructions provided by the manufacturer. The evaluation of the quantity, purity and integrity of the RNA was carried out by spectrophotometry, using the NanoDrop ND-1000 equipment (Thermo Fisher Scientific, Waltham, MA, USA), in addition to integrity analysis by electrophoresis in 1% agarose gel.

3.3.3.5. cDNA synthesis

Complementary DNAs (cDNAs) were synthesized for all samples using the GoScript Reverse Transcription System Kit (Promega, Madison, Wisconsin, USA), according to the manufacturer's instructions. For this process, 1 µg of total RNA was used in a reaction with a final volume of 20 µL.

3.3.3.6. Identification of genes related to pathogen-responsive antimicrobial proteins and peptides

The cDNA sequences of genes for antimicrobial peptides and pathogen-responsive proteins, respectively, the genes for a defensin, a nonspecific lipid transfer protein, and a Kunitz-type protease inhibitor (NCBI accession numbers: The previously described query sequences AF442388.1, NM_001288696.1, and XM_047402942.1, which have been shown to be responsive to biotic stress in plants, were used in the transcriptome dataset of C. annuum inoculated with C. scovillei (Baba, 2018). Antimicrobial peptide gene primers were designed using the CLC Genomics Workbench v.9.5.3 program (http://www.qiagenbioinformatics.com/) to amplify nucleotide sequences ranging from 100 to 130 base pairs with an annealing temperature of 55 °C ± 2 °C.

3.3.3.7. Expression analysis of candidate genes by RT qPCR

The transcriptional profiles of the genes were analyzed using the 7500 Fast Real-Time PCR System equipment (Thermo Fisher Scientific, Waltham, MA, USA). Each reaction consisted of a total volume of 10 µL, comprising 5 µL of GoTaq qPCR Master Mix (Promega, Madison, Wisconsin, USA), 1 µL of cDNA (25 ng.µL-1), 0.2-0.4 µL of each forward and reverse primer (10 µM), and the final volume adjusted with nuclease-free water. The amplification conditions were as follows: The thermal cycling program consisted of an initial denaturation step at 94 °C for 5 minutes, followed by 40 cycles of denaturation at 94 °C for 30 seconds, annealing at 55 °C for 45 seconds, and extension at 72 °C for 30 seconds. The dissociation curve was analyzed to ensure the specificity of the amplification. To ensure the reliability and transparency of the results, RT-qPCR validations were carried out in accordance

with the Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines. The genes whose expressions and characteristics were evaluated are described in Table 5. As an endogenous control, the *CaEF1α* gene was used, as established by Bin et al., (2012). All reactions were performed with three biological replicates to strengthen the robustness of the data obtained. The results were analyzed using 2 x 2 factorial statistics (factor 1: cultivar, factor 2: with or without inoculation).

Table 5: Primers for RT-qPCR expression analysis of genes coding for pepper antimicrobial peptides, identified in the *Capsicum annuum* transcriptome.

Gene ID	Feature	Nucleotide sequence	Amplicon size (bp)	Average efficience (%)
CA05g17340	Kunitz-type protease inhibitor KPI-D2.2	F: TTCCCTTGCAATAATCCTTCC R: AGGGGTTTGCCATTAGTATC	108	100,11%
CA07g03740	Defensin CADEF1 in response to pathogen infection, abiotic elicitors and environmental stresses	F: CACTGAGATGGGACCAATGA R: GGAGGCACAATTCGTCTCAC	100	89,84%
CA03g33250	Non-specific lipid-transfer protein AKCS9-like	F: GCCTTGCCTCTGCAATTATC R: GGAGGCACAATTCGTCTCAC	104	100,07%
CA06g07620	Elongation factor 1-alpha	F - TGAAGAATGGTGATGCTGG R - GACAACACCAACAGCAACA	132	100,00%

3.3.4. RESULTS

3.3.4.1. Gene identification

The reference gene sequences of antimicrobial proteins and peptides KTI, defensin and LTP were identified in the transcriptome of *C. annum* peppers inoculated with C. scovillei as *CA05g17340*, *CA07g03740* and *CA03g33250*, respectively.

3.3.4.2. Expression profiles of genes encoding AMPs in response to interaction with C. scovillei

The gene *CA03g33250* showed higher basal expression levels (non-inoculated treatments with *C. scovillei*) in the resistant cultivar compared to the susceptible cultivar, 24 h after inoculation (HAI) (Figure 3A). The presence of the pathogen did not regulate gene expression in the resistant cultivar, but it induced the transcriptional levels of *CA03g33250* in the susceptible cultivar (GBUEL103), reaching levels similar to those

Similarly, at 24 HAI, the basal transcript levels of *CA03g33250* are higher in the resistant cultivar. However, upon contact with the pathogen, its expression is induced in both cultivars. Interestingly, GBUEL104 shows significantly higher transcript levels of *CA03g33250* when in contact with *C. scovillei* compared to the susceptible cultivar.

For the gene *CA05g17340*, both the basal expression (in the absence of the pathogen) and the expression after contact with *C. scovillei* were higher in the resistant cultivar at both time points (Figure 3B). The presence of the fungus induced the expression of *CA05g17340* in GBUEL104 at both evaluation periods, but in GBUEL103 the induction occurred only at 48 HAI, indicating that the susceptible cultivar takes longer to respond in the presence of the pathogen.

Similar to what was observed for *CA03g33250*, the basal expression levels of *CA07g03740* are higher in the resistant cultivar at both evaluation periods (Figure 3C). The expression of *CA07g03740* is repressed in both cultivars at 24 HAI, but the transcript level is higher in the resistant cultivar than in the susceptible cultivar. Interestingly, at 48 HAI, induction of CA07g03740 is observed in both cultivars, with higher transcript levels observed in GBUEL104.

A – Gene *CA03g33250* (LTP) B– Gene *CA05g17340* (KTI) C– Gene *CA07g03740* (Defensina)

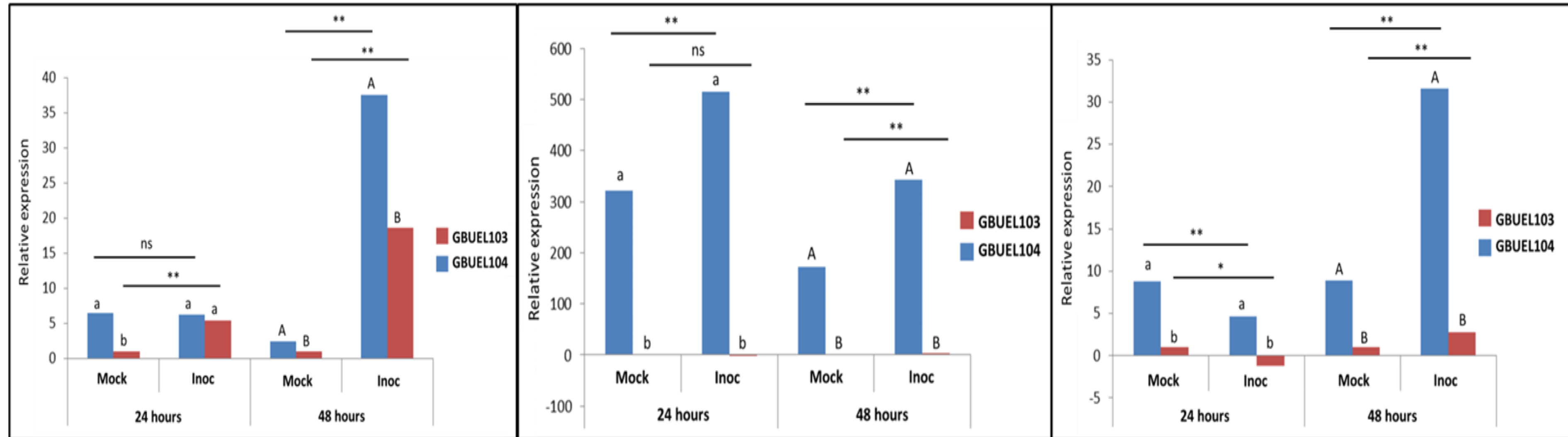


Figure 3: Analysis of the expression of three genes encoding antimicrobial peptides in response to *Colletotrichum scovillei* infection in two *Capsicum annuum* cultivars (susceptible to anthracnose - UEL103 and resistant to anthracnose - UEL104) at two inoculation times (24 and 48 hours). The samples were subjected to RT-qPCR using the comparative CT method ($2^{-\Delta\Delta CT}$). **A**- gene *CA03g33250* (LTP), B- gene *CA05g17340* (KTI), C- gene *CA07g03740* (defensin). The elongation factor α-1 gene was used as an endogenous control to normalize the level of target gene detection. Significant differences in the qRT-PCR results. Means followed by different lowercase letters differ from each other within the same inoculation treatment, in the evaluation after 24 hours of exposure, by Tukey's test at 5% significance. Means followed by different uppercase letters differ from each other within the same inoculation treatment, in the evaluation after 48 hours of exposure, by Tukey's test at 5% significance. * Means differ from each other within the same cultivar and evaluation period by Student's t-test at 5% significance. ** Means differ from each other within the same cultivar and evaluation period according to Student's t-test at 1% significance. ns: not significant.

3.3.5. DISCUSSION

To protect themselves against pathogen attacks and maintain viability, plants use natural components such as primary and secondary metabolites to defend against aggressors. Among these molecules antimicrobial peptides (AMPs) are included. In some cases, these resources are expressed constitutively, acting as a primary component in resistance. In other cases, they can be induced in response to infection (Zaynab et al., 2019). In recent years, several antimicrobial proteins and peptides have been described in peppers of the genus *Capsicum* (Oliveira, et al., 2022). Despite the challenging categorization due to their wide diversity, the potential for controlling pathogen growth is already established for many of these compounds (Oliveira et al., 2022).

In a study conducted by Maracahipes et al. (2019), the antimicrobial proteins and peptides present in two genotypes of *C. annuum* fruits, one susceptible and one resistant, were identified following infection with *C. gloeosporioides*. The protein profile of polyacrylamide gel extracts revealed the presence of several low-molecular-weight proteins in all treated samples. Additionally, antimicrobial peptides, including defensin, lipid transfer protein (LTP), and protease inhibitor, were identified through mass spectrometry. Furthermore, it was demonstrated that green fruits are more susceptible to infection, exhibiting the production of antimicrobial peptides in response to injury and fungal inoculation. However, aspects such as gene expression in specific tissues or under different types of stress, which can result in increased or decreased expression, are still poorly understood.

Thus, this study aimed to investigate the gene expression of some AMPs in *C. annuum* plants, specifically in the cultivars GBUEL103 (susceptible to bacterial spot, pepper yellow mosaic virus, and anthracnose) and GBUEL104 (resistant to bacterial spot, pepper yellow mosaic virus, and anthracnose). The experiment involved the inoculation of *C. scovillei* UEL8.1 and the analysis of fruits at different post-inoculation stages (24 and 48 hours). The main goal was to identify AMP genes responsive to fungal interaction and understand the differential responses between cultivars. The first step involved investigating AMP genes already described in the literature and expressed in the transcriptome of *C. annuum* fruits infected with *C. scovillei*. Thus, the expressed genes *CA03g33250, CA05g17340*, and *CA07g03740* were identified in the dataset. Analyzing the results of the differential expression, the

gene *CA03g33250* showed positive expression in all treatments, regardless of the cultivar, time, and type of inoculation. Remarkably, the resistant cultivar showed higher levels of expression, indicating a more effective response to fungal interaction. Regarding the gene *CA05g17340*, positive expression was observed in all treatments of the resistant cultivar, while in the susceptible cultivar, it was evident only in the 48-hour inoculation. There was a significant increase in expression levels in the inoculated treatments of both times. The gene *CA07g03740* exhibited positive expression in most treatments, except in SI24. The resistant cultivar consistently showed higher expression levels compared to the susceptible one. The repression of this gene in SI24 is noteworthy.

The gene responsible for encoding the defensin *CADEF1* shows reports of constitutive expression and induced transcription, which is notably intensified in the face of infections by *Xanthomonas campestris* pv. *vesicatoria*, abiotic stimuli, and various environmental stresses in pepper plants (Mee Do et al., 2004). Huai-Xia et al. (2020), for example, demonstrated a significant increase in *CADEF1* expression in peppers as part of a defense response against *Phytophthora capsici* infection. Other research, such as that conducted by Hussain et al. (2021), studied the influence of the transcriptional activator *CabHLH113* on the immunity of *C. annuum*, inducing an increase in transcriptional expression levels of the *CADEF1* gene in peppers resistant to *Ralstonia solanacearum*. The findings corroborate this trend, revealing that the gene *Ca07g03740*, which encodes the defensin CADEF1 in *C. annuum*, is regulated in plants resistant to infection by *C. scovillei.*

The role of the LTP AKCS9 is poorly addressed regarding its response to pathogens in plants. In a study conducted by Campos-Bermudez et al. (2013), grains of resistant hybrid maize to *Fusarium verticillioides* (L4637) infection showed positive expression of the AKCS9 protein, in contrast to the non-inoculated. Additionally, Xie et al. (2022) also briefly addressed the regulation of the nonspecific lipid transfer protein AKCS9 in banana peels after inoculation with *F. proliferatum*. The negative regulation of these proteins may favor *F. proliferatum* infection in later stages of the pathogenic process.

In the broader context of LTPs, studies indicate that the overexpression of genes activated by *NbLTP1*, an LTP from *Nicotiana benthamiana*, positively regulates plant immunity against viral infection. The results demonstrate that the gene *CA03g33250*, which encodes an LTP, exhibits positive expression at basal

levels, even before inoculation in both cultivars. The observed induction following inoculation with *C. scovillei* suggests the potential involvement of this protein in constitutive defense mechanisms against pathogens. This could include the synthesis of cutin and waxes as a defensive response to pathogen invasion.

Protease inhibitors (KTI) constitute an extensive protein group that plays a significant role in plant defense against pathogens. Their action involves the inhibition of pathogenic enzymes, which can result in the reduction of essential nutrient availability and interfere with their colonization capacity (Zhu-Salzman e Zeng, 2015). Other anti-virulence mechanisms include cell wall disruption and the formation of pores in the membrane. These actions aim to disrupt ion flow and, in some cases, promote membrane rupture, leading to the leakage of internal cellular components and consequently leading to cell death (Gutierrez-Gongora e Geddes-McAlister, 2021). Ribeiro et al. (2022) demonstrated that the coordinated presence of *NBS-LRR-WRKY* genes and protease inhibitors is a crucial element for effective resistance of cowpea against root-knot nematodes. In a comparative gene expression analysis study conducted by Gesteira et al. (2007) in *Theobroma cacao* (cacao) meristems infected by *Moniliophthora perniciosa*, sequences of KTIs inhibitors were exclusively identified in the resistant variety of cocoa. These findings corroborate with the results obtained in the research, where the expression of gene *CA05g17340* was observed exclusively in the resistant genotype (UEL103). The selective expression of these inhibitors in the resistant genotype indicates that they play a pivotal role in the defense response against the pathogen.

Although the studies were exclusively focused on three groups of AMPs (defensins, LTPs, and protease inhibitors) due to their recurrent description in *Capsicum*, it is relevant to note that there are other AMPs that may respond to pathogen attacks, such as snakins (Maróti et al., 2011). Consequently, future investigations may be expanded to incorporate additional AMP families in order to obtain a more comprehensive and detailed understanding of defense mechanisms in peppers of the genus *Capsicum*.

The identification and analysis of gene expression encoding AMPs in *Capsicum* peppers in response to *C. scovillei* infection offers valuable insights into the molecular mechanisms involved in defense against fungal infections. The resistant cultivar exhibited a more robust response, with greater regulation, in

comparison to the susceptible cultivar, which suggests that these genes may play a role in plant defense against the pathogen.

3.3.6. CONCLUSION

This study represents the first approach to the analysis of AMP expression belonging to the families of defensins, LTPs, and inhibitors in *C. annuum* in response to *C. scovillei* infection in fruits. The distinct transcriptional profiles observed among the three evaluated genes suggest that each plays a specific role in pepper fruit defense. *CA03g33250* gene exhibited late regulation, *CA05g17340* gene responded within the first 24 hours after inoculation, and CA07g03740 gene showed initial repression followed by induction. Notably, gene *CA05g17340* exhibited exclusive expression in the resistant cultivar, particularly after inoculation. This finding underscores the potential of this gene as a molecular marker of resistance against the pathogen. The results obtained reinforce the participation of inhibitors particularly in plant defense responses, providing advances in the understanding of this specific field. Additionally, the practical implications of this study are significant for understanding the molecular behavior of AMPs in resistant cultivars.

.

4. FINAL CONSIDERATIONS

This research aimed to investigate the potential of antimicrobial peptides (AMPs) found in plants of the genus *Capsicum* as control agents for phytopathogens in peppers. The search for defense molecules in plants that are responsive to infection and have the potential to inhibit pathogens is crucial for the development of sustainable strategies for controlling plant diseases. These molecules offer alternatives to chemical pesticides, contributing to sustainable agriculture and food security. Furthermore, by understanding plant defense mechanisms, it is possible to identify genes and proteins that confer resistance to pathogens, allowing the development of more resistant and productive cultivars. This contributes to environmental sustainability by reducing pollution caused by excessive use of pesticides.

To achieve this objective, in the first chapter, methods for purifying natural AMPs were used. In this case, a fraction rich in plant defensins was purified from pepper fruits. The second chapter concentrated on the antimicrobial activity of synthetic peptides based on *C. annuum* defensins. Finally, the third chapter analyzed the expression profile of AMP genes in pepper fruits infected with the phytopathogen *C. scovillei*. These methods not only identified new candidates for antimicrobial agents but also elucidated the role of AMPs in plant defense against pathogens.

Two species of peppers were utilized in this study*: C. chinense* and *C. annuum*. *C. chinense* was selected due to the paucity of reports on its identification in the literature, which renders it a highly exploitable source for the identification of

novel AMPs. However, the limitations of the genome of this species, such as scaffold assembly, restrict its application in molecular biology work. Scaffold assembly refers to the incomplete assembly of the genome into individual chromosomes, which can result in gaps and poorly represented regions, making it challenging to accurately identify genes and regulatory regions. In contrast, *C. annuum*, a species that has been extensively studied, has a well-annotated genome, making it suitable for different stages of work.

It is important to note that *C. chinense* and *C. annuum* belong to the same gene complex, indicating a close evolutionary relationship and the sharing of a relatively recent common ancestor. Despite sharing similar genomes and biological characteristics, there may be differences that distinguish them as separate species. The possibility that they share many similar genes and metabolic pathways suggests the future ability to expand knowledge about *C. annuum* to *C. chinense*.

Furthermore, this work reinforces the efficacy of rational modifications to improve the physicochemical characteristics of synthetic peptides as a strategy for proposing new agents with broad-spectrum antimicrobial activities. The application of AMPs in agriculture to control pests and diseases is already a reality, as is the case with Sero-X®, the first bioinsecticide inspired by a plant cyclopeptide. Therefore, the development of improved molecules that overcome the limitations that natural AMPs present is an interesting proposal. In this context, bioinspired synthetic peptides are a promising area of research. In the present study, the *Ca*Def2.1$_{G27\text{-}K44}$ peptide demonstrated activity against pepper pathogens, indicating its potential for further investigation.

Another perspective addressed in this study is the role of AMPs in plant defense against pathogen infection. The results obtained demonstrate that each AMP plays a specific role in the defense of pepper fruits. Late regulation was observed for protein LTP, while the protease inhibitor responded in the first 24 hours after inoculation, with priority expression in the resistant cultivar. In contrast, defensin showed initial repression, followed by induction. This difference in transcriptional behavior between genes suggests specific roles in plant defense. Nevertheless, further investigation of these genes is necessary to fully comprehend their role in plant defense. Collectively, the findings of these studies contribute significantly to the existing knowledge about AMPs in plants of the *Capsicum* genus and highlight their potential as alternatives for controlling diseases in agriculture.

REFERENCES

Aerts, A.M., Bammens, L., Govaert, G., Carmona-Gutierrez, D., Madeo, F., Cammue, B.P.A., Thevissen, K. (2011) The Antifungal Plant Defensin HsAFP1 from *Heuchera sanguinea* Induces Apoptosis in *Candida albicans*. Front Microbiol. doi: 10.3389/fmicb.2011.00047

Aerts, A.M., François, I.E.J.A., Meert, E.M.K., Li, Q.-T., Cammue, B.P.A., Thevissen, K. (2007) The Antifungal Activity of RsAFP2, a Plant Defensin from *Raphanus sativus*, Involves the Induction of Reactive Oxygen Species in *Candida albicans*. *Microb Physiol* 13: 243–247.

Afroz, M., Akter, S., Ahmed, A., Rouf, R., Shilpi, J.A., Tiralongo, E., Sarker, S.D., Göransson, U., Uddin, S.J. (2020) Ethnobotany and Antimicrobial Peptides From Plants of the Solanaceae Family: An Update and Future Prospects. Front Pharmacol. doi: 10.3389/fphar.2020.00565

Aguieiras, M.C.L., Resende, L.M., Souza, T.A.M., Nagano, C.S., Chaves, R.P., Taveira, G.B., Carvalho, A.O., Rodrigues, R., Gomes, V.M., Mello, É.O. (2021) Potent Anti-Candida Fraction Isolated from *Capsicum chinense* Fruits Contains an Antimicrobial Peptide That is Similar to Plant Defensin and is Able to Inhibit the Activity of Different α-Amylase Enzymes. *Probiotics Antimicrob Proteins* 13: 862–872.

Ali, A., Bordoh, P.K., Singh, A., Siddiqui, Y., Droby, S. (2016) Post-harvest development of anthracnose in pepper (*Capsicum* spp): Etiology and management strategies. *Crop Prot* 90: 132–141.

Ali, S.E., Thoen, E., Evensen, Ø., Wiik-Nielsen, J., Gamil, A.A.A., Skaar, I. (2014) Mitochondrial Dysfunction Is Involved in the Toxic Activity of Boric Acid against *Saprolegnia*. *PLoS One* 9: e110343.

Allende, D., Simon, S.A., McIntosh, T.J. (2005) Melittin-Induced Bilayer Leakage Depends on Lipid Material Properties: Evidence for Toroidal Pores. *Biophys J* 88: 1828–1837.

Almaaytah, A., Tarazi, S., Abu-Alhaijaa, A., Altall, Y., Alshar'i, N., Bodoor, K., Al-Balas, Q. (2014) Enhanced Antimicrobial Activity of AamAP1-Lysine, a Novel Synthetic Peptide Analog Derived from the Scorpion Venom Peptide AamAP1. *Pharmaceuticals* 7: 502–516.

Almeida, L.H. de O., Oliveira, C.F.R. de., Rodrigues, M. de S., Neto, S.M., Boleti, A.P. de A., Taveira, G.B., Mello, É. de O., Gomes, V.M., Santos, E.L. dos., Crusca, E., Franco, O.L., Cardoso, M.H. e S., Macedo, M.L.R. (2020) Adepamycin: design, synthesis and biological properties of a new peptide with antimicrobial properties. *Arch Biochem Biophys* 691: 108487.

Altschul, S.F., Gish, W., Miller, W., Myers, E.W., Lipman, D.J. (1990) Basic local alignment search tool. *J Mol Biol* 215: 403–410.

Amador, V.C., Santos-Silva, C.A., Vilela, L.M., Oliveira-Lima, M., de Santana Rêgo, M., Roldan-Filho, R.S., Oliveira-Silva, R.L., Lemos, A.B., de Oliveira, W.D., Ferreira-Neto, J.R., Crovella, S., Benko-Iseppon, A.M. (2021) Lipid Transfer Proteins (LTPs)—Structure, Diversity and Roles beyond Antimicrobial Activity.

Anaya-López, J.L., López-Meza, J.E., Baizabal-Aguirre, V.M., Cano-Camacho, H., Ochoa-Zarzosa, A. (2006) Fungicidal and cytotoxic activity of a *Capsicum chinense* defensin expressed by endothelial cells. Biotechnol Lett. doi: 10.1007/s10529-006-9060-4

Araújo, M. do S.B. de., Sudré, C.P., Graça, G.A. da., da Silva Alencar, A.A., da Costa Geronimo, I.G., Rodrigues, R. (2022) A new approach to quantify

anthracnose symptoms in inoculated *Capsicum* spp. fruits. *Trop Plant Pathol* 47: 386–401.

Avery, S. V., Singleton, I., Magan, N., Goldman, G.H. (2019) The fungal threat to global food security. *Fungal Biol* 123: 555–557.

Baba, V.Y. (2018) ANÁLISE DE TRANSCRIPTOMA E DE METABÓLITOS SECUNDÁRIOS EM FRUTOS DE *Capsicum annuum* NA INTERAÇÃO COM Colletotrichum gloeosporioides. Universidade Estadual de Londrina 91p.

Baba, V.Y., Powell, A.F., Ivamoto-Suzuki, S.T., Pereira, L.F.P., Vanzela, A.L.L., Giacomin, R.M., Strickler, S.R., Mueller, L.A., Rodrigues, R., Gonçalves, L.S.A. (2020) Capsidiol-related genes are highly expressed in response to *Colletotrichum scovillei* during *Capsicum annuum* fruit development stages. *Sci Rep* 10: 12048.

Babu, B.S.., Pandravada, S.R., Prasada Rao, R.D.V.J., Anitha, K., Chakrabarty, S.K., Varaprasad, K.S. (2011) Global sources of pepper genetic resources against arthropods, nematodes and pathogens. *Crop Prot* 30: 389–400.

Bakare, O.O., Gokul, A., Fadaka, A.O., Wu, R., Niekerk, L.-A., Barker, A.M., Keyster, M., Klein, A. (2022) Plant Antimicrobial Peptides (PAMPs): Features, Applications, Production, Expression, and Challenges. *Molecules* 27: 3703.

Balouiri, M., Sadiki, M., Ibnsouda, S.K. (2016) Methods for in vitro evaluating antimicrobial activity: A review. *J Pharm Anal* 6: 71–79.

Barbosa, A.C., Carmo, A.E. do., Graf, L., Tomaz, R., Souza, C.F. de., Mendes, J., Randi, M.A.F., Buchi, D., Schadeck, R.J.G. (2006) Morphology and lipid body and vacuole dynamics during secondary conidia formation in *Colletotrichum acutatum* : laser scanning confocal analysis. *Can J Microbiol* 52: 117–124.

Bard, G.C.V., Zottich, U., Souza, T.A.M., Ribeiro, S.F.F., Dias, G.B., Pireda, S., Da Cunha, M., Rodrigues, R., Pereira, L.S., Machado, O.L.T., Carvalho, A.O., Gomes, V.M. (2016) Purification, biochemical characterization, and antimicrobial activity of a new lipid transfer protein from *Coffea canephora* seeds. Genet Mol Res. doi: 10.4238/gmr15048859

Bastos, R.W., Rossato, L., Goldman, G.H., Santos, D.A. (2021) Fungicide effects on human fungal pathogens: Cross-resistance to medical drugs and beyond. *PLOS Pathog* 17: e1010073.

Bednarska, N.G., Wren, B.W., Willcocks, S.J. (2017) The importance of the glycosylation of antimicrobial peptides: natural and synthetic approaches. *Drug Discov Today* 22: 919–926.

Benfield, A.H., Henriques, S.T. (2020) Mode-of-Action of Antimicrobial Peptides: Membrane Disruption vs. Intracellular Mechanisms. Front Med Technol. doi: 10.3389/fmedt.2020.610997

Bento, C.S., de Souza, A.G., Sudré, C.P., Pimenta, S., Rodrigues, R. (2017) Multiple genetic resistances in *Capsicum* spp. Genet Mol Res. doi: 10.4238/gmr16039789

Bin Hafeez, A., Jiang, X., Bergen, P.J., Zhu, Y. (2021) Antimicrobial Peptides: An Update on Classifications and Databases. *Int J Mol Sci* 22: 11691.

Bin, W.S., Wei, L.K., Ping, D.W., Li, Z., Wei, G., Bing, L.J., Gui, P.B., Jian, W.H., Feng, C.J. (2012) Evaluation of appropriate reference genes for gene expression studies in pepper by quantitative real-time PCR. Mol Breed. doi: 10.1007/s11032-012-9726-7

Boto, A., De La Lastra, J.M.P., González, C.C. (2018) The road from host-defense peptides to a new generation of antimicrobial drugs.

Broekaert, W. (1990) An automated quantitative assay for fungal growth inhibition. *FEMS Microbiol Lett* 69: 55–59.

Brogden, K.A. (2005) Antimicrobial peptides: pore formers or metabolic inhibitors in bacteria?. *Nat Rev Microbiol* 3: 238–250.

Bukhteeva, I., Hrunyk, N.I., Yusypovych, Y.M., Shalovylo, Y.I., Kovaleva, V., Nesmelova, I. V. (2022) Structure, dynamics, and function of PsDef2 defensin from *Pinus sylvestris. Structure* 30: 753-762.e5.

Campos-Bermudez, V.A., Fauguel, C.M., Tronconi, M.A., Casati, P., Presello, D.A., Andreo, C.S. (2013) Transcriptional and Metabolic Changes Associated to the

Infection by *Fusarium verticillioides* in Maize Inbreds with Contrasting Ear Rot Resistance. *PLoS One* 8: e61580.

Cardoso, M.H., Orozco, R.Q., Rezende, S.B., Rodrigues, G., Oshiro, K.G.N., Cândido, E.S., Franco, O.L. (2020) Computer-Aided Design of Antimicrobial Peptides: Are We Generating Effective Drug Candidates?. Front Microbiol. doi: 10.3389/fmicb.2019.03097

Carvalho, A. de O., Gomes, V.M. (2009) Plant defensins-Prospects for the biological functions and biotechnological properties. *Peptides* 30: 1007–1020.

Carvalho, A. de O., Gomes, V.M. (2007) Role of plant lipid transfer proteins in plant cell physiology-A concise review. *Peptides* 28: 1144–1153.

Chen, Y., Mant, C.T., Hodges, R.S. (2002) Determination of stereochemistry stability coefficients of amino acid side-chains in an amphipathic α-helix. *J Pept Res* 59: 18–33.

Cherene, M.B., Gomes, V.M., Taveira, G.B., Rodrigues, R. (2023a) Antifungal activities of leaf extracts from the *Capsicum* genus. *DELOS Desarro LOCAL Sosten* 16: 3002–3018.

Cherene, M.B., Taveira, G.B., Almeida-Silva, F., da Silva, M.S., Cavaco, M.C., da Silva-Ferreira, A.T., Perales, J.E.A., de Oliveira Carvalho, A., Venâncio, T.M., da Motta, O.V., Rodrigues, R., Castanho, M.A.R.B., Gomes, V.M. (2023b) Structural and Biochemical Characterization of Three Antimicrobial Peptides from *Capsicum annuum* L. var. annuum Leaves for Anti-Candida Use. Probiotics Antimicrob Proteins. doi: 10.1007/s12602-023-10112-3

Chilosi, G., Caruso, C., Caporale, C., Leonardi, L., Bertini, L., Buzi, A., Nobile, M., Magro, P., Buonocore, V. (2000) Antifungal Activity of a Bowman–Birk-type Trypsin Inhibitor from *Wheat kernel*. *J Phytopathol* 148: 477–481.

Christeller, J., Laing, W. (2005) Plant Serine Proteinase Inhibitors. *Protein Pept Lett* 12: 439–447.

Clemente, M., Corigliano, M., Pariani, S., Sánchez-López, E., Sander, V., Ramos-Duarte, V. (2019) Plant Serine Protease Inhibitors: Biotechnology Application in Agriculture and Molecular Farming. *Int J Mol Sci* 20: 1345.

Clinical and Laboratory Standards Institute (CLSI). (2008) Reference method for broth dilution. *Ref method broth dilution Antifung susceptibility Test yeasts Approv Stand 3th ed* 28: 0–13.

Corrêa, J.A.F., Evangelista, A.G., Nazareth, T. de M., Luciano, F.B. (2019) Fundamentals on the molecular mechanism of action of antimicrobial peptides. *Materialia* 8: 100494.

Costa, H.P.S., Oliveira, J.T.A., Sousa, D.O.B., Morais, J.K.S., Moreno, F.B., Monteiro-Moreira, A.C.O., Viegas, R.A., Vasconcelos, I.M. (2014) JcTI-I: a novel trypsin inhibitor from *Jatropha curcas* seed cake with potential for bacterial infection treatment. Front Microbiol. doi: 10.3389/fmicb.2014.00005

Costa, L.S.M., Pires, Á.S., Damaceno, N.B., Rigueiras, P.O., Maximiano, M.R., Franco, O.L., Porto, W.F. (2020) In silico characterization of class II plant defensins from *Arabidopsis thaliana*. *Phytochemistry* 179: 112511.

Cotabarren, J., Lufrano, D., Parisi, M.G., Obregón, W.D. (2020) Biotechnological, biomedical, and agronomical applications of plant protease inhibitors with high stability: A systematic review. *Plant Sci* 292: 110398.

da Cunha, N.B., Cobacho, N.B., Viana, J.F.C., Lima, L.A., Sampaio, K.B.O., Dohms, S.S.M., Ferreira, A.C.R., de la Fuente-Núñez, C., Costa, F.F., Franco, O.L., Dias, S.C. (2017) The next generation of antimicrobial peptides (AMPs) as molecular therapeutic tools for the treatment of diseases with social and economic impacts.

da Silva, M.S., Gomes, V.M., Taveira, G.B., de Azevedo dos Santos, L., Maracahipes, Á.C., Rodrigues, R., de Oliveira Carvalho, A., Fernandes, K.V.S., Oliveira, A.E.A. (2021) Bifunctional Inhibitors from *Capsicum chinense* Seeds with Antimicrobial Activity and Specific Mechanism of Action Against Phytopathogenic Fungi. *Protein Pept Lett* 28: 149–163.

da Silva Pereira, L., do Nascimento, V.V., de Fátima Ferreira Ribeiro, S., Rodrigues, R., Fernandes, K.V.S., de Oliveira Carvalho, A., Vasconcelos, I.M., dos Santos Bento, C., Sudré, C.P., Zottich, U., Gomes, V.M. (2018) Characterization of *Capsicum annuum* L. leaf and root antimicrobial peptides: antimicrobial activity against phytopathogenic microorganisms. *Acta Physiol Plant* 40: 107.

de la Fuente-Núñez, C., Reffuveille, F., Mansour, S.C., Reckseidler-Zenteno, S.L., Hernández, D., Brackman, G., Coenye, T., Hancock, R.E.W. (2015) D-Enantiomeric Peptides that Eradicate Wild-Type and Multidrug-Resistant Biofilms and Protect against Lethal *Pseudomonas aeruginosa* Infections. *Chem Biol* 22: 196–205.

DEAN, R., VAN KAN, J.A.L., PRETORIUS, Z.A., HAMMOND-KOSACK, K.E., DI PIETRO, A., SPANU, P.D., RUDD, J.J., DICKMAN, M., KAHMANN, R., ELLIS, J., FOSTER, G.D. (2012) The Top 10 fungal pathogens in molecular plant pathology. *Mol Plant Pathol* 13: 414–430.

Deslouches, B., Steckbeck, J.D., Craigo, J.K., Doi, Y., Mietzner, T.A., Montelaro, R.C. (2013) Rational Design of Engineered Cationic Antimicrobial Peptides Consisting Exclusively of Arginine and Tryptophan, and Their Activity against Multidrug-Resistant Pathogens. *Antimicrob Agents Chemother* 57: 2511–2521.

Dias, L.P., Souza, P.F.N., Oliveira, J.T.A., Vasconcelos, I.M., Araújo, N.M.S., Tilburg, M.F.V., Guedes, M.I.F., Carneiro, R.F., Lopes, J.L.S., Sousa, D.O.B. (2020) RcAlb-PepII, a synthetic small peptide bioinspired in the 2S albumin from the seed cake of *Ricinus communis*, is a potent antimicrobial agent against *Klebsiella pneumoniae* and *Candida parapsilosis*. *Biochim Biophys Acta - Biomembr* 1862: 183092.

Dias, R. de O., Franco, O.L. (2015) Cysteine-stabilized αβ defensins: From a common fold to antibacterial activity. *Peptides* 72: 64–72.

Diz, M.S.S., Carvalho, A.O., Gomes, V.M. (2003) Purification and molecular mass determination of a lipid transfer protein exuded from *Vigna unguiculata* seeds. *Brazilian J Plant Physiol* 15: 171–175.

do Amaral, M., Freitas, A.C.O., Santos, A.S., dos Santos, E.C., Ferreira, M.M., da Silva Gesteira, A., Gramacho, K.P., Marinho-Prado, J.S., Pirovani, C.P. (2022) TcTI, a Kunitz-type trypsin inhibitor from cocoa associated with defense against pathogens. *Sci Rep* 12: 698.

Do Nascimento, V. V., Mello, É.D.O., Carvalho, L.P., De Melo, E.J.T., Carvalho, A.D.O., Fernandes, K.V.S., Gomes, V.M. (2015) PvD1 defensin, a plant antimicrobial peptide with inhibitory activity against *Leishmania amazonensis. Biosci Rep* 35: 1–7.

Edstam, M.M., Viitanen, L., Salminen, T.A., Edqvist, J. (2011) Evolutionary History of the Non-Specific Lipid Transfer Proteins. *Mol Plant* 4: 947–964.

Evans, B.J., King, A.T., Katsifis, A., Matesic, L., Jamie, J.F. (2020) Methods to Enhance the Metabolic Stability of Peptide-Based PET Radiopharmaceuticals. *Molecules* 25: 2314.

Fensterseifer, I.C.M., Felício, M.R., Alves, E.S.F., Cardoso, M.H., Torres, M.D.T., Matos, C.O., Silva, O.N., Lu, T.K., Freire, M. V., Neves, N.C., Gonçalves, S., Lião, L.M., Santos, N.C., Porto, W.F., de la Fuente-Nunez, C., Franco, O.L. (2019) Selective antibacterial activity of the cationic peptide PaDBS1R6 against Gram-negative bacteria. *Biochim Biophys Acta - Biomembr* 1861: 1375–1387.

FERREIRA, R.B., MONTEIRO, S., FREITAS, R., SANTOS, C.N., CHEN, Z., BATISTA, L.M., DUARTE, J., BORGES, A., TEIXEIRA, A.R. (2007) The role of plant defence proteins in fungal pathogenesis. *Mol Plant Pathol* 8: 677–700.

Figueira, T.N., Oliveira, F.D., Almeida, I., Mello, É.O., Gomes, V.M., Castanho, M.A.R.B., Gaspar, D. (2017) Challenging metastatic breast cancer with the natural defensin PvD $_1$. *Nanoscale* 9: 16887–16899.

Finkina, E.I., Melnikova, D.N., Bogdanov, I. V., Ovchinnikova, T. V. (2016) Lipid Transfer Proteins As Components of the Plant Innate Immune System : Structure , Functions , and Applications. 8: 47–61.

Fones, H.N., Bebber, D.P., Chaloner, T.M., Kay, W.T., Steinberg, G., Gurr, S.J. (2020) Author Correction: Threats to global food security from emerging fungal and oomycete crop pathogens. *Nat Food* 1: 455–456.

Franco, O.L., Rigden, D.J., R. Melo, F., Bloch, C., Silva, C.P., Grossi de Sá, M.F. (2000) Activity of wheat α-amylase inhibitors towards bruchid α-amylases and structural explanation of observed specificities. *Eur J Biochem* 267: 2166–2173.

Games, P.D., daSilva, E.Q.G., Barbosa, M. de O., Almeida-Souza, H.O., Fontes, P.P., deMagalhães-Jr, M.J., Pereira, P.R.G., Prates, M.V., Franco, G.R., Faria-Campos, A., Campos, S.V.A., Baracat-Pereira, M.C. (2016) Computer aided identification of a Hevein-like antimicrobial peptide of bell pepper leaves for biotechnological use. BMC Genomics. doi: 10.1186/s12864-016-3332-8

Gan, B.H., Gaynord, J., Rowe, S.M., Deingruber, T., Spring, D.R. (2021) The multifaceted nature of antimicrobial peptides: current synthetic chemistry approaches and future directions. *Chem Soc Rev* 50: 7820–7880.

Gaschler, M.M., Stockwell, B.R. (2017) Lipid peroxidation in cell death. *Biochem Biophys Res Commun* 482: 419–425.

Gebara, R. da S., Taveira, G.B., Santos, L. de A. dos., Calixto, S.D., Simão, T.L.B.V., Lassounskaia, E., Muzitano, M.F., Teixeira-Ferreira, A., Perales, J., Rodrigues, R., Carvalho, A. de O., Gomes, V.M. (2020) Identification and Characterization of Two Defensins from *Capsicum annuum* Fruits that Exhibit Antimicrobial Activity. *Probiotics Antimicrob Proteins* 12: 1253–1265.

Gesteira, A.S., Micheli, F., Carels, N., Da Silva, A.C., Gramacho, K.P., Schuster, I., Macêdo, J.N., Pereira, G.A.G., Cascardo, J.C.M. (2007) Comparative Analysis of Expressed Genes from Cacao Meristems Infected by *Moniliophthora perniciosa*. *Ann Bot* 100: 129–140.

Ghiasi, Z., Esmaeli, F., Aghajani, M., Ghazi-Khansari, M., Faramarzi, M.A., Amani, A. (2019) Enhancing analgesic and anti-inflammatory effects of capsaicin when loaded into olive oil nanoemulsion: An in vivo study. *Int J Pharm* 559: 341–347.

Giacomin, R.M., Ruas, C. de F., Baba, V.Y., De Godoy, S.M., Sudré, C.P., Bento, C. dos S., Da Cunha, M., Da Costa Geronimo, I.G., Rodrigues, R., Gonçalves, L.S. (2021) Phenotypic, molecular and pathogenic characterization of

Colletotrichum scovillei infecting *Capsicum* species in Rio de Janeiro, Brazil. *PeerJ* 9: e10782.

Gupta, P., Mahajan, A. (2015) Green chemistry approaches as sustainable alternatives to conventional strategies in the pharmaceutical industry. *RSC Adv* 5: 26686–26705.

Gutierrez-Gongora, D., Geddes-McAlister, J. (2021) From Naturally-Sourced Protease Inhibitors to New Treatments for Fungal Infections. *J Fungi* 7: 1016.

Harris, K.S., Durek, T., Kaas, Q., Poth, A.G., Gilding, E.K., Conlan, B.F., Saska, I., Daly, N.L., van der Weerden, N.L., Craik, D.J., Anderson, M.A. (2015) Efficient backbone cyclization of linear peptides by a recombinant asparaginyl endopeptidase. *Nat Commun* 6: 10199.

Hein, M.J.A., Kvansakul, M., Lay, F.T., Phan, T.K., Hulett, M.D. (2022) Defensin–lipid interactions in membrane targeting: mechanisms of action and opportunities for the development of antimicrobial and anticancer therapeutics. *Biochem Soc Trans* 50: 423–437.

Hernández-Huerta, J., Tamez-Guerra, P., Gomez-Flores, R., Delgado-Gardea, M.C.E., García-Madrid, M.S., Robles-Hernández, L., Infante-Ramirez, R. (2021) Prevalence of *Xanthomonas euvesicatoria* (formally X. perforans) associated with bacterial spot severity in *Capsicum annuum* crops in South Central Chihuahua, Mexico. *PeerJ* 9: e10913.

Hoyos, J.M.Á., Alves, E., Rozwalka, L.C., Souza, E.A. de., Zeviani, W.M. (2012) Antifungal activity and ultrastructural alterations in *Pseudocercospora griseola* treated with essential oils. *Ciência e Agrotecnologia* 36: 270–284.

Huang, J., Liu, S., Liu, R., Yi, Y., Li, C., Xiao, Z., Tu, J., Xiao, J. (2023) Mechanisms of Litsea cubeba essential oil in the control of *Colletotrichum scovillei* in pepper (*Capsicum annuum* L.): Cell membrane/wall perspective. *Physiol Mol Plant Pathol* 127: 102103.

Hussain, A., Noman, A., Arif, M., Farooq, S., Khan, M.I., Cheng, P., Qari, S.H., Anwar, M., Hashem, M., Ashraf, M.F., Alamri, S., Adnan, M., Khalofah, A., Al-zoubi, O.M., Ansari, M.J., Khan, K.A., Sun, Y. (2021) A basic helix-loop-helix

transcription factor *CabHLH113* positively regulate pepper immunity against *Ralstonia solanacearum*. *Microb Pathog* 156: 104909.

Jiang, Z., Vasil, A.I., Hale, J.D., Hancock, R.E.W., Vasil, M.L., Hodges, R.S. (2008) Effects of net charge and the number of positively charged residues on the biological activity of amphipathic α-helical cationic antimicrobial peptides. *Pept Sci* 90: 369–383.

Kabelka, I., Vácha, R. (2018) Optimal Hydrophobicity and Reorientation of Amphiphilic Peptides Translocating through Membrane. *Biophys J* 115: 1045–1054.

Karray, A., Alonazi, M., Smaoui, S., Michaud, P., Soliman, D., Ben Bacha, A. (2020) Purification and Biochemical Characterization of a New Protease Inhibitor from *Conyza dioscoridis* with Antimicrobial, Antifungal and Cytotoxic Effects. *Molecules* 25: 5452.

Kępińska-Pacelik, J., Biel, W. (2021) Alimentary Risk of Mycotoxins for Humans and Animals. *Toxins (Basel)* 13: 822.

Khani, S., Seyedjavadi, S.S., Hosseini, H.M., Goudarzi, M., Valadbeigi, S., Khatami, S., Ajdary, S., Eslamifar, A., Amani, J., Imani Fooladi, A.A., Razzaghi-Abyaneh, M. (2020) Effects of the antifungal peptide Skh-AMP1 derived from *Satureja khuzistanica* on cell membrane permeability, ROS production, and cell morphology of conidia and hyphae of Aspergillus fumigatus. *Peptides* 123: 170195.

Kim, J.-Y., Gopal, R., Kim, S., Seo, C., Lee, H., Cheong, H., Park, Y. (2013) PG-2, a Potent AMP against Pathogenic Microbial Strains, from Potato (*Solanum tuberosum* L cv. Gogu Valley) Tubers Not Cytotoxic against Human Cells. *Int J Mol Sci* 14: 4349–4360.

Kim, J.-Y., Park, S.-C., Kim, M.-H., Lim, H.-T., Park, Y., Hahm, K.-S. (2005) Antimicrobial activity studies on a trypsin–chymotrypsin protease inhibitor obtained from potato. *Biochem Biophys Res Commun* 330: 921–927.

Kim, S., Park, M., Yeom, S.-I., Kim, Y.-M., Lee, J.M., Lee, H.-A., Choi, D., et al. (2014) Genome sequence of the hot pepper provides insights into the evolution of pungency in *Capsicum* species. *Nat Genet* 46: 270–278.

Kovaleva, V., Krynytskyy, H., Gout, I., Gout, R. (2011) Recombinant expression, affinity purification and functional characterization of *Scots pine* defensin 1. *Appl Microbiol Biotechnol* 89: 1093–1101.

Kuhar, K., Kansal, R., Subrahmanyam, B., Koundal, K.R., Miglani, K., Gupta, V.K. (2013) A Bowman–Birk protease inhibitor with antifeedant and antifungal activity from *Dolichos biflorus*. *Acta Physiol Plant* 35: 1887–1903.

Larkin, M.A., Blackshields, G., Brown, N.P., Chenna, R., McGettigan, P.A., McWilliam, H., Valentin, F., Wallace, I.M., Wilm, A., Lopez, R., Thompson, J.D., Gibson, T.J., Higgins, D.G. (2007) Clustal W and Clustal X version 2.0. *Bioinformatics* 23: 2947–2948.

Lee, J.-H., Venkatesh, J., Jo, J., Jang, S., Kim, G.W., Kim, J.-M., Han, K., Ro, N., Lee, H.-Y., Kwon, J.-K., Kim, Y.-M., Lee, T.-H., Choi, D., Van Deynze, A., Hill, T., Kfir, N., Freiman, A., Davila Olivas, N.H., Elkind, Y., Paran, I., Kang, B.-C. (2022) High-quality chromosome-scale genomes facilitate effective identification of large structural variations in hot and sweet peppers. Hortic Res. doi: 10.1093/hr/uhac210

León, I.R., Neves-Ferreira, A.G.C., Valente, R.H., Mota, E.M., Lenzi, H.L., Perales, J. (2007) Improved protein identification efficiency by mass spectrometry usingN-terminal chemical derivatization of peptides fromAngiostrongylus costaricensis, a nematode with unknown genome. *J Mass Spectrom* 42: 781–792.

Li, J., Hu, S., Jian, W., Xie, C., Yang, X. (2021) Plant antimicrobial peptides: structures, functions, and applications. *Bot Stud* 62: 5.

Li, L., Hu, X., Xia, Y., Xiao, G., Zheng, P., Wang, C. (2014) Linkage of Oxidative Stress and Mitochondrial Dysfunctions to Spontaneous Culture Degeneration in *Aspergillus nidulans*. *Mol Cell Proteomics* 13: 449–461.

Li, Y., Rebuffat, S. (2020) The manifold roles of microbial ribosomal peptide–based natural products in physiology and ecology. *J Biol Chem* 295: 34–54.

Lima, A.M., Azevedo, M.I.G., Sousa, L.M., Oliveira, N.S., Andrade, C.R., Freitas, C.D.T., Souza, P.F.N. (2022) Plant antimicrobial peptides: An overview about classification, toxicity and clinical applications. *Int J Biol Macromol* 214: 10–21.

Lima, M.F., Ragassi, C.F., Bianchetti, L.B., Faleiro, F.G., Cerrados, E. (2017) Characterization of a pepper collection (*Capsicum frutescens* L .) from Brazil. 16: 1–18.

Lima, P.G., Oliveira, J.T.A., Amaral, J.L., Freitas, C.D.T., Souza, P.F.N. (2021) Synthetic antimicrobial peptides: Characteristics, design, and potential as alternative molecules to overcome microbial resistance. *Life Sci* 278: 119647.

Lima, P.G., Souza, P.F.N., Freitas, C.D.T., Oliveira, J.T.A., Dias, L.P., Neto, J.X.S., Vasconcelos, I.M., Lopes, J.L.S., Sousa, D.O.B. (2020) Anticandidal activity of synthetic peptides: Mechanism of action revealed by scanning electron and fluorescence microscopies and synergism effect with nystatin. J Pept Sci. doi: 10.1002/psc.3249

Liu, F., Zhao, J., Sun, H., Xiong, C., Sun, X., Wang, X., Wang, Z., Jarret, R., Wang, J., Tang, B., Xu, H., Hu, B., Suo, H., Yang, B., Ou, L., Li, X., Zhou, S., Yang, S., Liu, Z., Yuan, F., Pei, Z., Ma, Y., Dai, X., Wu, S., Fei, Z., Zou, X. (2023a) Genomes of cultivated and wild *Capsicum* species provide insights into pepper domestication and population differentiation. *Nat Commun* 14: 5487.

Liu, K., Jiang, H., Moore, S.L., Watkins, C.B., Jahn, M.M. (2006) Isolation and characterization of a lipid transfer protein expressed in ripening fruit of *Capsicum chinense*. *Planta* 223: 672–683.

Liu, L., Wu, H., Long, Y., Yang, X., Du, C., Xu, Y., Ji, Q. (2023b) Novel spiro[pyrrolidine-2,3′-quinoline]-2′-one derivatives containing piperazine fragment as potential chitin synthase inhibitors and antifungal agents: Design, synthesis and biological evaluation. *Eur J Med Chem* 260: 115777.

Lopes, J.L.S., Valadares, N.F., Moraes, D.I., Rosa, J.C., Araújo, H.S.S., Beltramini, L.M. (2009) Physico-chemical and antifungal properties of protease inhibitors from Acacia plumosa. *Phytochemistry* 70: 871–879.

Luong, H.X., Thanh, T.T., Tran, T.H. (2020) Antimicrobial peptides – Advances in development of therapeutic applications. *Life Sci* 260: 118407.

Lyu, Y., Yang, Y., Lyu, X., Dong, N., Shan, A. (2016) Antimicrobial activity, improved cell selectivity and mode of action of short PMAP-36-derived peptides against bacteria and *Candida*. *Sci Rep* 6: 27258.

Machida, S., Saito, M. (1993) Purification and characterization of membrane-bound chitin synthase. *J Biol Chem* 268: 1702–1707.

Malmsten, M. (2015) Interactions of Antimicrobial Peptides with Bacterial Membranes and Membrane Components. *Curr Top Med Chem* 16: 16–24.

Maracahipes, Á.C., Taveira, G.B., Mello, E.O., Carvalho, A.O., Rodrigues, R., Perales, J., Teixeira-Ferreira, A., Silva, M.S., Rocha, G.L., Fernandes, K.V.S., Gomes, V.M. (2019a) Biochemical analysis of antimicrobial peptides in two different *Capsicum* genotypes after fruit infection by *Colletotrichum gloeosporioides*. Biosci Rep. doi: 10.1042/BSR20181889

Maracahipes, Á.C., Taveira, G.B., Sousa-Machado, L.Y., Machado, O.L.T., Rodrigues, R., Carvalho, A.O., Gomes, V.M. (2019b) Characterization and antifungal activity of a plant peptide expressed in the interaction between *Capsicum annuum* fruits and the anthracnose fungus. Biosci Rep. doi: 10.1042/BSR20192803

Maróti, G., Kereszt, A., Kondorosi, É., Mergaert, P. (2011) Natural roles of antimicrobial peptides in microbes, plants and animals. *Res Microbiol* 162: 363–374.

Maximiano, M.R., Franco, O.L. (2021) Biotechnological applications of versatile plant lipid transfer proteins (LTPs). *Peptides* 140: 170531.

Mee Do, H., Chul Lee, S., Won Jung, H., Hoon Sohn, K., Kook Hwang, B. (2004) Differential expression and in situ localization of a pepper defensin (*CADEF1*)

gene in response to pathogen infection, abiotic elicitors and environmental stresses in *Capsicum annuum*. *Plant Sci* 166: 1297–1305.

Mello, É. de O., Taveira, G.B., Carvalho, A. de O., Gomes, V.M. (2019) Improved smallest peptides based on positive charge increase of the γ-core motif from PvD1 and their mechanism of action against *Candida* species. *Int J Nanomedicine* Volume 14: 407–420.

Mello, E.O., Ribeiro, S.F.F., Carvalho, A.O., Santos, I.S., Da Cunha, M., Santa-Catarina, C., Gomes, V.M. (2011) Antifungal Activity of PvD1 Defensin Involves Plasma Membrane Permeabilization, Inhibition of Medium Acidification, and Induction of ROS in Fungi Cells. *Curr Microbiol* 62: 1209–1217.

Melo, I.R.S., Dias, L.P., Araújo, N.M.S., Vasconcelos, I.M., Martins, T.F., de Morais, G.A., Gonçalves, J.F.C., Nagano, C.S., Carneiro, R.F., Oliveira, J.T.A. (2019) ClCPI, a cysteine protease inhibitor purified from *Cassia leiandra* seeds has antifungal activity against Candida tropicalis by inducing disruption of the cell surface. *Int J Biol Macromol* 133: 1115–1124.

Mendelson, M., Laxminarayan, R., Limmathurotsakul, D., Kariuki, S., Gyansa-Lutterodt, M., Charani, E., Singh, S., Walia, K., Gales, A.C., Mpundu, M. (2024) Antimicrobial resistance and the great divide: inequity in priorities and agendas between the Global North and the Global South threatens global mitigation of antimicrobial resistance. Lancet Glob Heal. doi: 10.1016/S2214-109X(23)00554-5

Mirouze, M., Sels, J., Richard, O., Czernic, P., Loubet, S., Jacquier, A., François, I.E.J.A., Cammue, B.P.A., Lebrun, M., Berthomieu, P., Marquès, L. (2006) A putative novel role for plant defensins: a defensin from the zinc hyper-accumulating plant, Arabidopsis halleri , confers zinc tolerance. *Plant J* 47: 329–342.

Missaoui, K., Gonzalez-Klein, Z., Pazos-Castro, D., Hernandez-Ramirez, G., Garrido-Arandia, M., Brini, F., Diaz-Perales, A., Tome-Amat, J. (2022) Plant non-specific lipid transfer proteins: An overview. *Plant Physiol Biochem* 171: 115–127.

Moloi, S.J., Ngara, R. (2023) The roles of plant proteases and protease inhibitors in drought response: a review. *Front Plant Sci* 14: 1165845.

Moscone, E.A., Scaldaferro, M.A., Grabiele, M., Cecchini, N.M., Sánchez García, Y., Jarret, R., Daviña, J.R., Ducasse, D.A., Barboza, G.E., Ehrendorfer, F. (2007) THE EVOLUTION OF CHILI PEPPERS (*CAPSICUM* - SOLANACEAE): A CYTOGENETIC PERSPECTIVE. *Acta Hortic* 137–170.

Mosquera-Sánchez, L.P., Arciniegas-Grijalba, P.A., Patiño-Portela, M.C., Guerra–Sierra, B.E., Muñoz-Florez, J.E., Rodríguez-Páez, J.E. (2020) Antifungal effect of zinc oxide nanoparticles (ZnO-NPs) on *Colletotrichum* sp., causal agent of anthracnose in coffee crops. *Biocatal Agric Biotechnol* 25: 101579.

Moura, D.S., Ryan, C.A. (2001) Wound-inducible proteinase inhibitors in pepper. Differential regulation upon wounding, systemin, and methyl jasmonate. *Plant Physiol* 126: 289–298.

Mulla, J.A., Kibe, A.N., Deore, D.D., Jadhav, A.R., Tamhane, V.A. (2021) Molecular characterization of diverse defensins (γ-thionins) from *Capsicum annuum* flowers and their effects on the insect pest *Helicoverpa armigera*. *Plant Gene* 26: 100284.

Nascimento, V. V., Mello, É. de O., Carvalho, L.P., de Melo, E.J.T., Carvalho, A. de O., Fernandes, K.V.S., Gomes, V.M. (2015) PvD1 defensin, a plant antimicrobial peptide with inhibitory activity against *Leishmania amazonensis*. Biosci Rep. doi: 10.1042/BSR20150060

Neitzke, R.S., Vasconcelos, C.S., Barbieri, R.L., Vizzotto, M., Fetter, M.R., Corbelini, D.D. (2015) Variabilidade genética para compostos antioxidantes em variedades crioulas de pimentas (*Capsicum baccatum*). *Hortic Bras* 33: 415–421.

Nishad, R., Ahmed, T., Rahman, V.J., Kareem, A. (2020) Modulation of Plant Defense System in Response to Microbial Interactions. Front Microbiol. doi: 10.3389/fmicb.2020.01298

Odintsova., Slezina., Istomina., Korostyleva., Kovtun., Kasianov., Shcherbakova., Kudryavtsev. (2019) Non-Specific Lipid Transfer Proteins in *Triticum kiharae*

Dorof. et Migush.: Identification, Characterization and Expression Profiling in Response to Pathogens and Resistance Inducers. *Pathogens* 8: 221.

Oliveira, A.P.B.F., Resende, L.M., Rodrigues, R., de Oliveira Mello, É., Taveira, G.B., de Oliveira Carvalho, A., Gomes, V.M. (2022) Antimicrobial peptides of the genus *Capsicum*: a mini review. *Hortic Environ Biotechnol* 63: 453–466.

Oliveira, J.T.A., Souza, P.F.N., Vasconcelos, I.M., Dias, L.P., Martins, T.F., Van Tilburg, M.F., Guedes, M.I.F., Sousa, D.O.B. (2019) Mo-CBP3-PepI, Mo-CBP3-PepII, and Mo-CBP3-PepIII are synthetic antimicrobial peptides active against human pathogens by stimulating ROS generation and increasing plasma membrane permeability. *Biochimie* 157: 10–21.

ONUS, A.N., PICKERSGILL, B. (2004) Unilateral Incompatibility in Capsicum (Solanaceae): Occurrence and Taxonomic Distribution. *Ann Bot* 94: 289–295.

Pacheco-Cano, R.D., Salcedo-Hernández, R., Casados-Vázquez, L.E., Wrobel, K., Bideshi, D.K., Barboza-Corona, J.E. (2020) Class I defensins (BraDef) from broccoli (*Brassica oleracea* var. italica) seeds and their antimicrobial activity. *World J Microbiol Biotechnol* 36: 30.

Pan, Y. (2011) Mitochondria, reactive oxygen species, and chronological aging: A message from yeast. *Exp Gerontol* 46: 847–852.

Pandey, P., Irulappan, V., Bagavathiannan, M. V., Senthil-Kumar, M. (2017) Impact of Combined Abiotic and Biotic Stresses on Plant Growth and Avenues for Crop Improvement by Exploiting Physio-morphological Traits. Front Plant Sci. doi: 10.3389/fpls.2017.00537

Parisi, K., Shafee, T.M.A., Quimbar, P., van der Weerden, N.L., Bleackley, M.R., Anderson, M.A. (2019) The evolution, function and mechanisms of action for plant defensins. *Semin Cell Dev Biol* 88: 107–118.

Parisi, M., Alioto, D., Tripodi, P. (2020) Overview of Biotic Stresses in Pepper (Capsicum spp.): Sources of Genetic Resistance, Molecular Breeding and Genomics. *Int J Mol Sci* 21: 2587.

Park, Y., Park, S., Park, H., Shin, S.Y., Kim, Y., Hahm, K. (2007) Structure-activity relationship of HP (2–20) analog peptide: Enhanced antimicrobial activity by N-terminal random coil region deletion. *Pept Sci* 88: 199–207.

Parthasarathy, A., Borrego, E.J., Savka, M.A., Dobson, R.C.J., Hudson, A.O. (2021) Amino acid–derived defense metabolites from plants: A potential source to facilitate novel antimicrobial development. *J Biol Chem* 296: 100438.

Pelegrini, P.B., Lay, F.T., Murad, A.M., Anderson, M.A., Franco, O.L. (2008) Novel insights on the mechanism of action of α-amylase inhibitors from the plant defensin family. *Proteins Struct Funct Bioinforma* 73: 719–729.

Pereira, L. da S., do Nascimento, V.V., Ribeiro, S. de F.F., Rodrigues, R., Fernandes, K.V.S., Carvalho, A. de O., Vasconcelos, I.M., Bento, C. dos S., Sudré, C.P., Zottich, U., Gomes, V.M. (2018) Characterization of *Capsicum annuum* L. leaf and root antimicrobial peptides: antimicrobial activity against phytopathogenic microorganisms. *Acta Physiol Plant* 40: 107.

Perry, L., Dickau, R., Zarrillo, S., Holst, I., Pearsall, D.M., Piperno, D.R., Berman, M.J., Cooke, R.G., Rademaker, K., Ranere, A.J., Raymond, J.S., Sandweiss, D.H., Scaramelli, F., Tarble, K., Zeidler, J.A. (2007) Starch fossils and the domestication and dispersal of chili peppers (*Capsicum* spp. L.) in the Americas. Science (80-). doi: 10.1126/science.1136914

Porto, W.F., Irazazabal, L., Alves, E.S.F., Ribeiro, S.M., Matos, C.O., Pires, Á.S., Fensterseifer, I.C.M., Miranda, V.J., Haney, E.F., Humblot, V., Torres, M.D.T., Hancock, R.E.W., Liao, L.M., Ladram, A., Lu, T.K., de la Fuente-Nunez, C., Franco, O.L. (2018) In silico optimization of a guava antimicrobial peptide enables combinatorial exploration for peptide design. *Nat Commun* 9: 1490.

Potnis, N., Timilsina, S., Strayer, A., Shantharaj, D., Barak, J.D., Paret, M.L., Vallad, G.E., Jones, J.B. (2015) Bacterial spot of tomato and pepper: diverse X anthomonas species with a wide variety of virulence factors posing a worldwide challenge. *Mol Plant Pathol* 16: 907–920.

Pouny, Y., Rapaport, D., Mor, A., Nicolas, P., Shai, Y. (1992) Interaction of antimicrobial dermaseptin and its fluorescently labeled analogs with phospholipid membranes. *Biochemistry* 31: 12416–12423.

Prajapati, M.K., Rawat, S., Singh, P., Shankar, K. (2020) Cultural and morphological characterization of *Colletotrichum capsici* causing anthracnose of chilli (*Capsicum anum* L.). *J Pharmacogn Phytochem* 9: 1985–1989.

Răileanu, M., Borlan, R., Campu, A., Janosi, L., Turcu, I., Focsan, M., Bacalum, M. (2023) No country for old antibiotics! Antimicrobial peptides (AMPs) as next-generation treatment for skin and soft tissue infection. *Int J Pharm* 642: 123169.

Resende, L.M., de Oliveira Mello, É., de Lima Aguieiras, M.C., Nagano, C.S., Chaves, R.P., Taveira, G.B., da Silva, M.S., de Oliveira Carvalho, A., Rodrigues, R., Gomes, V.M. (2021) Inhibition of Serine Protease, α-Amylase and Growth of Phytopathogenic Fungi by Antimicrobial Peptides from *Capsicum chinense* Fruits. *Probiotics Antimicrob Proteins* 15: 502–515.

RIBEIRO, C.S. da C., LOPES, C.A., CARVALHO, S.I.C. de., HENZ, G.P., REIFSCHNEIDER, F.J.B. (2008) *Pimentas Capsicum*. Brasília, DF 200p.

Ribeiro, D.G., Mota, A.P.Z., Santos, I.R., Arraes, F.B.M., Grynberg, P., Fontes, W., Castro, M. de S., de Sousa, M.V., Lisei-de-Sá, M.E., Grossi-de-Sá, M.F., Franco, O.L., Mehta, A. (2022) NBS-LRR-WRKY genes and protease inhibitors (PIs) seem essential for cowpea resistance to root-knot nematode. *J Proteomics* 261: 104575.

Ribeiro, M.C., Gebara, R.S., Taveira, G.B., de O. Carvalho, A., Rodrigues, R., Mello, E.O., Nagano, C.S., Chaves, R.P., Gomes, V.M. (2023) Anti-Candida Potential of Peptides from Immature and Ripe Fruits of *Capsicum chinense* Jacq. *Probiotics Antimicrob Proteins* 15: 1124–1136.

Ribeiro, S.F.F., Carvalho, A.O., Da Cunha, M., Rodrigues, R., Cruz, L.P., Melo, V.M.M., Vasconcelos, I.M., Melo, E.J.T., Gomes, V.M. (2007) Isolation and characterization of novel peptides from chilli pepper seeds: Antimicrobial activities against pathogenic yeasts. *Toxicon* 50: 600–611.

Ribeiro, S.F.F., Fernandes, K.V.S., Santos, I.S., Taveira, G.B., Carvalho, A.O., Lopes, J.L.S., Beltramini, L.M., Rodrigues, R., Vasconcelos, I.M., Da Cunha, M., Souza-Filho, G.A., Gomes, V.M. (2013) New small proteinase inhibitors from *Capsicum annuum* seeds: Characterization, stability, spectroscopic analysis and a cDNA cloning. *Biopolymers* 100: 132–140.

Roach, R., Mann, R., Gambley, C.G., Shivas, R.G., Rodoni, B. (2018) Identification of *Xanthomonas* species associated with bacterial leaf spot of tomato, capsicum and chilli crops in eastern Australia. *Eur J Plant Pathol* 150: 595–608.

Rodrigues, G., Maximiano, M.R., Franco, O.L. (2021) Antimicrobial peptides used as growth promoters in livestock production. *Appl Microbiol Biotechnol* 105: 7115–7121.

Rodríguez-Sifuentes, L., Marszalek, J.E., Chuck-Hernández, C., Serna-Saldívar, S.O. (2020) Legumes Protease Inhibitors as Biopesticides and Their Defense Mechanisms against Biotic Factors. *Int J Mol Sci* 21: 3322.

Russomanno, O.M.R., Kruppa, P.C. (2010) DOENÇAS FÚNGICAS DAS PLANTAS MEDICINAIS, AROMÁTICAS E CONDIMENTARES – PARTE AÉREA. *Biologico* 72: 31–37.

Sagaram, U.S., Pandurangi, R., Kaur, J., Smith, T.J., Shah, D.M. (2011) Structure-Activity Determinants in Antifungal Plant Defensins MsDef1 and MtDef4 with Different Modes of Action against *Fusarium graminearum*. *PLoS One* 6: e18550.

Samblanx, G.W. de., Goderis, I.J., Thevissen, K., Raemaekers, R., Fant, F., Borremans, F., Acland, D.P., Osborn, R.W., Patel, S., Broekaert, W.F. (1997) Mutational Analysis of a Plant Defensin from Radish (*Raphanus sativus* L.) Reveals Two Adjacent Sites Important for Antifungal Activity. *J Biol Chem* 272: 1171–1179.

Santos, L. de A. dos., Taveira, G.B., Silva, M.S. da., Gebara, R. da S., Pereira, L. da S., Perales, J., Teixeira-Ferreira, A., Mello, É. de O., Carvalho, A. de O., Rodrigues, R., Gomes, V.M. (2020) Antimicrobial peptides from *Capsicum*

chinense fruits: agronomic alternatives against phytopathogenic fungi. Biosci Rep. doi: 10.1042/BSR20200950

Sarkar, T., Chetia, M., Chatterjee, S. (2021) Antimicrobial Peptides and Proteins: From Nature's Reservoir to the Laboratory and Beyond. Front Chem. doi: 10.3389/fchem.2021.691532

Savitskaya, A., Masso-Silva, J., Haddaoui, I., Enany, S. (2023) Exploring the arsenal of antimicrobial peptides: Mechanisms, diversity, and applications. *Biochimie* 214: 216–227.

Saxena, A., Raghuwanshi, R., Gupta, V.K., Singh, H.B. (2016) Chilli Anthracnose: The Epidemiology and Management. Front Microbiol. doi: 10.3389/fmicb.2016.01527

Sels, J., Mathys, J., De Coninck, B.M.A., Cammue, B.P.A., De Bolle, M.F.C. (2008) Plant pathogenesis-related (PR) proteins: A focus on PR peptides. *Plant Physiol Biochem* 46: 941–950.

Sengupta, D., Leontiadou, H., Mark, A.E., Marrink, S.-J. (2008) Toroidal pores formed by antimicrobial peptides show significant disorder. *Biochim Biophys Acta - Biomembr* 1778: 2308–2317.

Seyedjavadi, S.S., Khani, S., Eslamifar, A., Ajdary, S., Goudarzi, M., Halabian, R., Akbari, R., Zare-Zardini, H., Imani Fooladi, A.A., Amani, J., Razzaghi-Abyaneh, M. (2020) The Antifungal Peptide MCh-AMP1 Derived From *Matricaria chamomilla* Inhibits *Candida albicans* Growth via Inducing ROS Generation and Altering Fungal Cell Membrane Permeability. Front Microbiol. doi: 10.3389/fmicb.2019.03150

Shafee, T.M.A., Lay, F.T., Hulett, M.D., Anderson, M.A. (2016) The Defensins Consist of Two Independent, Convergent Protein Superfamilies. *Mol Biol Evol* 33: 2345–2356.

Shenkarev, Z.O., Balandin, S. V., Trunov, K.I., Paramonov, A.S., Sukhanov, S. V., Barsukov, L.I., Arseniev, A.S., Ovchinnikova, T. V. (2011) Molecular Mechanism of Action of β-Hairpin Antimicrobial Peptide Arenicin: Oligomeric

Structure in Dodecylphosphocholine Micelles and Pore Formation in Planar Lipid Bilayers. *Biochemistry* 50: 6255–6265.

Shoji, J., Kikuma, T., Arioka, M., Kitamoto, K. (2010) Macroautophagy-Mediated Degradation of Whole Nuclei in the Filamentous Fungus *Aspergillus oryzae*. *PLoS One* 5: e15650.

Shwaiki, L.N., Lynch, K.M., Arendt, E.K. (2021) Future of antimicrobial peptides derived from plants in food application – A focus on synthetic peptides. *Trends Food Sci Technol* 112: 312–324.

Silva, L.R.A., Rodrigues, R., Pimenta, S., Correa, J.W.S., Araújo, M.S.B., Bento, C.S., Sudré, C.P. (2017) Inheritance of bacterial spot resistance in *Capsicum annuum* var. annuum. Genet Mol Res. doi: 10.4238/gmr16029631

Slavokhotova, A.A., Shelenkov, A.A., Andreev, Y.A., Odintsova, T.I. (2017) Hevein-like antimicrobial peptides of plants. *Biochem* 82: 1659–1674.

Smith, P.K., Krohn, R.I., Hermanson, G.T., Mallia, A.K., Gartner, F.H., Provenzano, M.D., Fujimoto, E.K., Goeke, N.M., Olson, B.J., Klenk, D.C. (1985) Measurement of protein using bicinchoninic acid. *Anal Biochem* 150: 76–85.

Soares, J.R., Melo, E.J.T. de., Cunha, M. da., Fernandes, K.V.S., Taveira, G.B., Pereira, L. da S., Pimenta, S., Trindade, F.G., Regente, M., Pinedo, M., Canal, L. de la., Gomes, V.M., Carvalho, A. de O. (2017) Interaction between the plant ApDef1 defensin and *Saccharomyces cerevisiae* results in yeast death through a cell cycle- and caspase-dependent process occurring via uncontrolled oxidative stress. *Biochim Biophys Acta - Gen Subj* 1861: 3429–3443.

Souza, L.A.L., Dias, L.P., Araújo, N.M.S., Carneiro, R.F., Nagano, C.S., Teixeira, C.S., Silva, R.G.G., Oliveira, J.T.A., Sousa, D.O.B. (2022) JcTI-PepI, a synthetic peptide bioinspired in the trypsin inhibitor from *Jatropha curcas*, presents potent inhibitory activity against *C. krusei*, a neglected pathogen. *Biochimie* 200: 107–118.

Spelbrink, R.G., Dilmac, N., Allen, A., Smith, T.J., Shah, D.M., Hockerman, G.H. (2004) Differential Antifungal and Calcium Channel-Blocking Activity among Structurally Related Plant Defensins. *Plant Physiol* 135: 2055–2067.

Sudré, C.P., Gonalves, L.S.A., Rodrigues, R., do Amaral Jnior, A.T., Riva-Souza, E.M., dos S. Bento, C. (2010) Genetic variability in domesticated *Capsicum* spp as assessed by morphological and agronomic data in mixed statistical analysis. *Genet Mol Res* 9: 283–294.

Tam, J., Wang, S., Wong, K., Tan, W. (2015) Antimicrobial Peptides from Plants. *Pharmaceuticals* 8: 711–757.

Tang, S.-S., Prodhan, Z.H., Biswas, S.K., Le, C.-F., Sekaran, S.D. (2018) Antimicrobial peptides from different plant sources: Isolation, characterisation, and purification. *Phytochemistry* 154: 94–105.

Taveira, G.B., de Oliveira Mello, É., Simão, T.L.B.V., Cherene, M.B., de Oliveira Carvalho, A., Muzitano, M.F., Lassounskaia, E., Pireda, S., de Castro Miguel, E., Basso, L.G.M., Da Cunha, M., da Motta, O.V., Gomes, V.M. (2022) A new bioinspired peptide on defensin from *C. annuum* fruits: Antimicrobial activity, mechanisms of action and therapeutical potential. *Biochim Biophys Acta - Gen Subj* 1866: 130218.

Taveira, G.B., Mathias, L.S., da Motta, O. V., Machado, O.L.T., Rodrigues, R., Carvalho, A.O., Teixeira-Ferreira, A., Perales, J., Vasconcelos, I.M., Gomes, V.M. (2014) Thionin-like peptides from *Capsicum annuum* fruits with high activity against human pathogenic bacteria and yeasts. *Biopolymers* 102: 30–39.

Than, P.P., Prihastuti, H., Phoulivong, S., Taylor, P.W.J., Hyde, K.D. (2008) Chilli anthracnose disease caused by *Colletotrichum* species. *J Zhejiang Univ Sci B* 9: 764–778.

Thevissen, K., François, I.E.J.A., Sijtsma, L., Amerongen, A. van., Schaaper, W.M.M., Meloen, R., Posthuma-Trumpie, T., Broekaert, W.F., Cammue, B.P.A. (2005) Antifungal activity of synthetic peptides derived from *Impatiens balsamina* antimicrobial peptides Ib-AMP1 and Ib-AMP4. *Peptides* 26: 1113–1119.

Thevissen, K., Terras, F.R.G., Broekaert, W.F. (1999) Permeabilization of fungal membranes by plant defensins inhibits fungal growth. *Appl Environ Microbiol* 65: 5451–5458.

TSUKATANI, T., OBA, T., UKEDA, H., MATSUMOTO, K. (2003) Spectrophotometric Assay of Yeast Vitality Using 2,3,5,6-Tetramethyl-1,4-benzoquinone and Tetrazolium Salts. *Anal Sci* 19: 659–664.

van der Weerden, N.L., Hancock, R.E.W., Anderson, M.A. (2010) Permeabilization of Fungal Hyphae by the Plant Defensin NaD1 Occurs through a Cell Wall-dependent Process. *J Biol Chem* 285: 37513–37520.

Velázquez-Hernández, M.E., Ochoa-Zarzosa, A., López-Meza, J.E. (2021) Defensin γ-thionin from *Capsicum chinense* improves butyrate cytotoxicity on human colon adenocarcinoma cell line Caco-2. *Electron J Biotechnol* 52: 76–84.

Vieira Bard, G.C. (2014) Vicilin-like peptides from *Capsicum baccatum* L. seeds are α-amylase inhibitors and exhibit antifungal activity against important yeasts in medical mycology. Biopolymers. doi: 10.1002/bip

Vila-Perelló, M., Sánchez-Vallet, A., García-Olmedo, F., Molina, A., Andreu, D. (2003) Synthetic and structural studies on *Pyrularia pubera* thionin: a single-residue mutation enhances activity against Gram-negative bacteria. *FEBS Lett* 536: 215–219.

Von Borowski, R.G., Zimmer, K.R., Leonardi, B.F., Trentin, D.S., Silva, R.C., de Barros, M.P., Macedo, A.J., Gnoatto, S.C.B., Gosmann, G., Zimmer, A.R. (2019) Red pepper *Capsicum baccatum*: source of antiadhesive and antibiofilm compounds against nosocomial bacteria. *Ind Crops Prod* 127: 148–157.

Walsh, B.M., Hoot, S.B. (2001) Phylogenetic Relationships of *Capsicum* (Solanaceae) Using DNA Sequences from Two Noncoding Regions: The Chloroplast atpB - rbcL Spacer Region and Nuclear waxy Introns. *Int J Plant Sci* 162: 1409–1418.

Wang, Y., Cui, P., Zhang, Y., Yang, Q., Zhang, S. (2018) Augmentation of the antibacterial activities of Pt5-derived antimicrobial peptides (AMPs) by amino

acid substitutions: Design of novel AMPs against MDR bacteria. *Fish Shellfish Immunol* 77: 100–111.

Weerden, N.L. van der., Anderson, M.A. (2013) Plant defensins: Common fold, multiple functions. *Fungal Biol Rev* 26: 121–131.

Wieprecht, T., Dathe, M., Beyermann, M., Krause, E., Maloy, W.L., MacDonald, D.L., Bienert, M. (1997) Peptide Hydrophobicity Controls the Activity and Selectivity of Magainin 2 Amide in Interaction with Membranes. *Biochemistry* 36: 6124–6132.

Woloshuk, C.P., Meulenhoff, J.S., Sela-Buurlage, M., van den Elzen, P.J., Cornelissen, B.J. (1991) Pathogen-induced proteins with inhibitory activity toward *Phytophthora infestans*. *Plant Cell* 3: 619–628.

Xie, L., Wu, Y., Duan, X., Li, T., Jiang, Y. (2022) Proteomic and physiological analysis provides an elucidation of *Fusarium proliferatum* infection causing crown rot on banana fruit. *Microbiol Res* 256: 126952.

Yeats, T.H., Rose, J.K.C. (2008) The biochemistry and biology of extracellular plant lipid-transfer proteins (LTPs). *Protein Sci* 17: 191–198.

Yount, N.Y., Yeaman, M.R. (2004) Multidimensional signatures in antimicrobial peptides. *Proc Natl Acad Sci* 101: 7363–7368.

Yuan, J., Wang, J., Li, X., Zhang, Y., Xian, J., Wang, C., Zhang, J., Wu, C. (2023) Amphiphilic small molecule antimicrobials: From cationic antimicrobial peptides (CAMPs) to mechanism-related, structurally-diverse antimicrobials. *Eur J Med Chem* 262: 115896.

Zaynab, M., Fatima, M., Abbas, S., Sharif, Y., Umair, M., Zafar, M.H., Bahadar, K. (2018) Role of secondary metabolites in plant defense against pathogens. *Microb Pathog* 124: 198–202.

Zaynab, M., Fatima, M., Sharif, Y., Zafar, M.H., Ali, H., Khan, K.A. (2019) Role of primary metabolites in plant defense against pathogens. *Microb Pathog* 137: 103728.

Zhang, C., Zhang, R.-K., Feng, Y., Sun, S.-N., Fan, Z.-C. (2023a) Green algae-derived triple CATH_BRALE multimer protein potently inhibits bacterial growth by permeabilizing the bacterial cell membrane. *Process Biochem* 130: 555–565.

Zhang, H.-X., Feng, X.-H., Ali, M., Jin, J.-H., Wei, A.-M., Khattak, A.M., Gong, Z.-H. (2020a) Identification of Pepper CaSBP08 Gene in Defense Response Against *Phytophthora capsici* Infection. Front Plant Sci. doi: 10.3389/fpls.2020.00183

Zhang, Q.-Y., Yan, Z.-B., Meng, Y.-M., Hong, X.-Y., Shao, G., Ma, J.-J., Cheng, X.-R., Liu, J., Kang, J., Fu, C.-Y. (2021) Antimicrobial peptides: mechanism of action, activity and clinical potential. *Mil Med Res* 8: 48.

Zhang, R., Xu, L., Dong, C. (2022) Antimicrobial Peptides: An Overview of their Structure, Function and Mechanism of Action. *Protein Pept Lett* 29: 641–650.

Zhang, Y.-M., Ye, D.-X., Liu, Y., Zhang, X.-Y., Zhou, Y.-L., Zhang, L., Yang, X.-L. (2023b) Peptides, new tools for plant protection in eco-agriculture. *Adv Agrochem* 2: 58–78.

Zhang, Z., Chen, Y., Li, B., Chen, T., Tian, S. (2020b) Reactive oxygen species: A generalist in regulating development and pathogenicity of phytopathogenic fungi. *Comput Struct Biotechnol J* 18: 3344–3349.

Zheng, J., Zhou, Y., Li, Y., Xu, D.-P., Li, S., Li, H.-B. (2016) Spices for Prevention and Treatment of Cancers. *Nutrients* 8: 495.

Zhu-Salzman, K., Zeng, R. (2015) Insect Response to Plant Defensive Protease Inhibitors. *Annu Rev Entomol* 60: 233–252.

Zottich, U., Da, M., Carvalho, A.O., Dias, G.B., Silva, N.C.M., Santos, I.S., Viviane, V., Miguel, E.C., Machado, O.L.T., Gomes, V.M. (2011) Biochimica et Biophysica Acta Puri fi cation , biochemical characterization and antifungal activity of a new lipid transfer protein (LTP) from *Coffea canephora* seeds with α -amylase inhibitor properties. *BBA - Gen Subj* 1810: 375–383.