Capsicum annuum L. LEAF AND ROOT ANTIMICROBIAL PEPTIDES: CHARACTERIZATION AND MECHANISM OF ACTION ON THE INHIBITION OFTHE PHYTOPATHOGENIC MICROORGANISMS

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

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(Advisor)

DEDICATION

DEDICATED to God, the one who is with me at all times; my family for vibrating with each of my achievements; to the friends who welcomed and cared for me.

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To God, who made me dream and fly so high, who helped me and gave me the strength to complete this work. Words can not describe how good and patient You are with me. Thank You for having always helped me, for which I am very happy and grateful;

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SUMMARY

ABSTRACT

Lídia da Silva Pereira; DS.c.; Universidade Estadual do Norte Fluminense Darcy Ribeiro; April 2021; Identification and characterization of antimicrobial peptides from *Capsicum annuum* L. and mechanism of action on the development of phytopathogenic microorganisms; Advisor: Valdirene Moreira Gomes; Committee members: André de Oliveira Carvalho e Rosana Rodrigues.

Diseases caused by phytopathogenic microorganisms are difficult to control and can affect plants at different stages of development. Many resistance genes and antimicrobial peptides (AMPs) have been identified and related to the resistance process in plants of the genus *Capsicum.* In recent years, studies have shown that peppers, especially the UENF1381 cultivar are resistant to phytopathogenic microorganisms. This paper has aimed to identify, purify, and characterize AMPs from leaves and roots of *Capsicum annuum* L. (UENF1381) and to investigate the differences in protein expression of leaves and roots in response to infection by *Xanthomonas euvesicatoria*. Furthermore, it has aimed to evaluate the activities of the extracts on different enzyme families and the inhibitory activity on *Xanthomonas euvesicatoria* and *Colletotrichum scovill*ei. Mechanisms of action of the extracts and purified fractions on *C. scovill*ei were also determined. Initially, self-fertilized pepper seeds were cultivated for 45 days in glass jars containing ½-MS medium. Then, leaves were inoculated with *Xanthomonas euvesicatoria* (10^8 CFU/mL) or water (control). Leaf and root samples were collected at 12, 24, and 48 h after inoculation for extraction. The activity of the extracts on different enzyme

families was analyzed by incubating them with the trypsin, α-amylase and β-1,3-glucanase enzymes. The effect of the extract and purified fractions on fungi and bacteria was also tested at different concentration levels. The mechanism of action on fungi was studied by the plasma membrane permeabilization, induction of reactive oxygen species (ROS), mitochondrial functionality, and caspase activation.

it was identified that leaf protein and root extracts from *C. annuum* showed most bands with low molecular mass (6 to14 kDa), and that an isolated leaf peptide called L1 and an isolated root peptide called R1 had their expression differentiated in response to infection. Both peptides showed similarity to lipid transfer proteins and serine proteinase inhibitors, respectively. It had been found that every leaf and root extracts has inhibited trypsin and α-amylase activity and is able to inhibit significantly the *C. scovillei* growth. For *X. euvesicatoria* inhibitory assay, only two extracts named LC_{48} (leaf extract inoculated with water after 48 h) and IL_{48} (leaf extract inoculated with *X. euvesicatoria* after 48 h) were able to inhibit *X. euvesicatoria*. On the basis of the results of differentiated expression of protein in response to *X*. *euvesicatoria* and characterization by mass spectrometry, the LC_{48} and RC_{48} (root extract inoculated with water after 48 h) extracts were selected for peptide purification in HPLC system. It was obtained twenty-three fractions from LC_{48} extract, named L1' to L23, and it was obtained twenty-seven fractions from RC_{48} extract, named R1' to R27. The effect of purified fractions on *C*. *scovillei* growth was tested with 10 µg/mL of fractions. Only the L1' fraction was able to inhibit the fungus growth (88.4%) significantly being able to permeabilize the membrane, lead to endogenous ROS induction and decrease mitochondrial activity. Results from this study can contribute to use peptides as potential molecules in microbial control and in the development of new *Capsicum* cultivars resistant to microorganisms.

Keywords: Pepper; Plant defense; Phytopathogens

RESUMO

Lídia da Silva Pereira; DS.c.; Universidade Estadual do Norte Fluminense Darcy Ribeiro; Abril de 2021; Identificação e caracterização de peptídeos antimicrobianos de *Capsicum annuum* L. e mecanismo de ação sobre o desenvolvimento de microrganismos fitopatogênicos; Orientadora: Valdirene Moreira Gomes; Conselheiros: André de Oliveira Carvalho e Rosana Rodrigues.

Doenças causadas por microrganismos fitopatogênicos são de difícil controle e podem afetar as plantas em diferentes estágios de seu desenvolvimento. Vários genes de resistência e peptídeos antimicrobianos (AMPs, do Inglês antimicrobial peptides) foram identificados e relacionados ao processo de resistência de plantas do gênero *Capsicum*. Nos últimos anos, estudos têm mostrado que plantas de pimentas, principalmente o acesso UENF1381, apresentam resistência a microrganismos fitopatogênicos. Este trabalho teve como objetivo identificar, purificar e caracterizar AMPs de folhas e raízes de *C. annuum* L. UENF 1381 e investigar as diferenças na expressão protéica das folhas e raízes em resposta à infecção por *Xanthomonas euvesicatoria*. Além disso, objetivou avaliar as atividades dos extratos em diferentes famílias de enzimas e avaliar a atividade inibitória sobre *Xanthomonas euvesicatoria* e *Colletotrichum scovillei*. Mecanismos de ação dos extratos e frações purificadas sobre *C. scovillei* também foram determinados. Inicialmente, sementes autofecundadas de pimenta foram cultivadas por 45 dias em frascos de vidro contendo meio ½-MS. Em seguida, as folhas foram inoculadas com *Xanthomonas euvesicatoria* (10^8 UFC/mL) ou água

(controle). Amostras de folhas e raízes foram coletadas em 12, 24 e 48 h após a inoculação para extração. A atividade dos extratos em diferentes famílias de enzimas foi analisada pela incubação das enzimas tripsina, α-amilase e β-1,3-glucanase. O efeito de extratos e frações purificadas, em diferentes concentrações, sobre o fungo e a bactéria também foi testado. O mecanismo de ação sobre o fungo foi estudado através de ensaios de permeabilização da membrana plasmática, indução de espécies reativas de oxigênio (ROS, do inglês reactive oxigen species), funcionalidade mitocondrial e ativação de caspase. Identificou-se que proteínas de extratos de folhas e raízes de *C. annuum* apresentaram maioria de bandas com baixa massa molecular (6 a 14 kDa) e que um peptídeo isolado de folha denominado L1 e um peptídeo isolado de raiz denominado R1 tiveram suas expressões diferenciados em resposta à infecção. Ambos os peptídeos apresentaram similaridade com proteínas transportadoras de lipídeos e inibidores de serina proteinase, respectivamente. Observamos que todos os extratos de folhas e raízes inibiram significativamente a atividade da tripsina e α-amilase e foram capazes de inibir significativamente o crescimento de *C. scovillei*. Para o ensaio de inibição da *X. euvesicatoria*, apenas dois extratos denominados LC_{48} (extrato de folha inoculado com água após 48 h) e IL_{48} (extrato de folha inoculado com *X. euvesicatoria* após 48 h) foram capazes de inibir bactéria. Com base nos resultados de expressão diferenciada de proteínas em resposta a *X. euvesicatoria* e caracterização por espectrometria de massa, os extratos LC_{48} e RC_{48} (extrato de raiz inoculada com água após 48 h) foram selecionados para purificação de peptídeos em sistema HPLC. A partir do extrato LC_{48}, foram obtidas 23 frações, denominadas L1' a L23 e do extrato RC_{48}, 27 frações, denominadas R1' a R27. O efeito das frações purificadas no crescimento de *C. scovillei* foi testado usando 10 µg/mL de frações. Apenas a fração L1' foi capaz de inibir significativamente o crescimento do fungo (88,4%), sendo capaz de permeabilizar a membrana, levar à indução endógena de ROS and diminuir a atividade mitocondrial. Os resultados deste estudo podem contribuir para o uso de peptídeos como moléculas potenciais no controle microbiano e no desenvolvimento de novas cultivares de *Capsicum* resistentes a microrganismos.

Palavras-chave: Pimenta; Defesa de planta; Fitopatógenos

1. INTODUCTION

1.1. The Solanaceae family and *Capsicum* genus

The Solanaceae family includes a wide group of vascular plants of great diversity and number of species. There are between 3000-4000 species distributed among 150 genera (Knapp et al., 2004; Wu and Tanksley, 2010; Gebhardt, 2016). A large geographic distribution of plants of the Solanaceae family is found in Central and South America, regions believed to be the center of origin of these plants due to the wide variety of species and genera (Astley, 2007; Hunziker, 1979; McLeod et al., 1982).

In Brazil, there are about 34 plant genera of the Solanaceae family, approximately 449 species, of which 215 are endemic to the country. Most of the plants in this family found in Brazil are endemic to the Atlantic Rainforest (Mata Atlântica), a South American ecosystem hosting a great variety of animal and plant species (Stehmann et al., 2020).

Plants belonging to the Solanaceae family are economically important and include the potato, eggplant, tomato, and *Capsicum*. Prominent among these are the peppers of the *Capsicum* genus, whose demand is growing. *Capsicum*, commonly called pepper, is currently cultivated worldwide and is the leading vegetable and spice crop that originated in America. It is distributed and consumed in both fresh and dry forms. The main cultivated species are *C*. *annuum*, *C*.

pubescens, *C*. *chinense*, *C*. *baccatum*, and *C*. *frutescens* (Tripodi and Kumar, 2019).

Capsicum is native to tropical and subtropical America and distributed over a wide region of Mexico and northern Central America, the Caribbean, the Bolivian lowlands, the northern lowland of the Amazon plain, and the mid-elevation southern Andes. Archaeological evidence suggests *Capsicum* has been used in these regions since 6000 BC (Basu and De, 2003; Davenport, 1970; Perry et al., 2007). These vegetables are used widely around the world and are not only known for their economic, but also for their nutritional value (Morales-Soto et al., 2013; Salehia et al., 2018; Tripodi and Kumar, 2019).

Capsicum plants are used as a highly nutritious food. The peppers are a source of vitamins C and E, provitamin A, carotenoids, flavonoids, mineral elements, and phenolic compounds. These substances have antioxidant activity and their bioactive properties have been reported in the literature (Morales-Soto et al., 2013). Table 1 shows highlights the benefits of *Capsicum ssp.* for health based on reports by Salehi et al. (2018).

Most *Capsicum* plant fruits contain a group of pungent components called capsaicin (methyl vanillyl nonenamide), a lipophilic chemical compound with a range of physiological and pharmacological properties that can produce a strong to moderate burning sensation in the mouth, eyes, and skin.. Capsaicin also works to protect against consumption by mammals and insects. It is more highly concentrated in certain structures, such as the placental and internal tissues, with lower concentrations present in other parts of the fruit, such as seeds. The presence of capsaicin in *Capsicum* is dependent upon genetic factors and the Scoville Scale is used to measure the degree of spiciness or pungency of fruits. (Guillen et al., 2018).

Table 1. Example of health benefits of the consumption of *Capsicum* spp. Salehi et al. (2018).

PAIN RELIEF - Topical pain relief - Neuropathic pain - Post herpetic neuralgic pain - Morton's neuroma - Arthritis
GASTROINTESTINAL HEALTH BENEFITS - Digestion stimulant - Increase in fat digestion - Increase in gastrointestinal motility - Amelioration of gastroesophageal reflux - Protection of gastric mucosa
METABOLIC SYNDROME - Anti-hypercholeremic effect - Anti-lithogenic effect - Hypo-glycemic effect - Increase in thermogenesis - Decrease in respiratory quotient
CANCER - Apoptosis in cell lines - Related to some carcinogenic process at high concentrations - Protective effect at low concentrations
OTHERS - Improves physical activity - Better iron status - Decrease in plasma cholesterol and modulation of plasma lipids - Beneficial vascular activity

Capsicum fruits are found in a variety of sizes, shapes, colors, flavors, and also pungency levels. Their different colors attract birds that disperse the seeds (Fig. 1) (Guzman and Bosland, 2017). *Capsicum* is a diploid species with 12 chromosomes (2n = 2x = 24). There are also species with 13 chromosomes (2n = 2x = 26) as well as one tetraploid species (2n = 4x = 48), which is *C*. *annuum* var. *glabriusculum*, the wild form of pepper (Carrizo García et al., 2016).

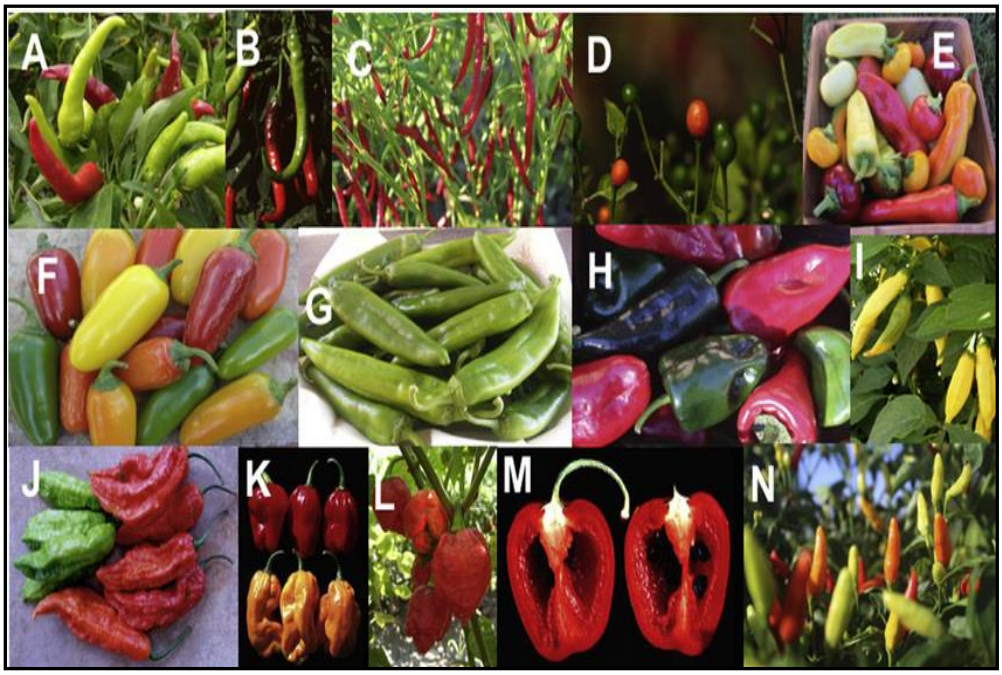


Figure 1. Images of *Capsicum* species variety and pod types. *Capsicum annuum* pod types are A) Asian, B) Cayenne, C) Chile de Arbol, D) Chiltepin, E) Hungarian Paprika, F) Jalapeño, G) New Mexican, and H) Poblano. A *Capsicum baccatum* pod type is I) Aji. *Capsicum chinense* pod types are J) Bhut Jolokia, K) Habanero, and L) Scorpion. A *Capsicum pubescens* pod type is M) Rocoto and a *Capsicum frutescens* pod type is N) Tabasco (Guzman and Bosland, 2017).

Between 1994 and 2019, about 1.8 to 4.2 million tons of dried *Capsicum* and 12 to 38 million tons of fresh ones were produced globally. China is the largest producer, with12 million tons of fresh, followed by Mexico, which produced about 2 million tons and Turkey which 1.8 million tons (FAOSTAT, 2021). The production share of dry and fresh pepper by region is shown in Figure 2.

Despite the increasing in production of *Capsicum* plants, these crops are facing several problems, including diseases caused by phytopathogenic microorganisms (Savary et al., 2012; Carrizo García et al., 2016). Species of fungus *Colletotrichum* and bacterium *Xanthomonas* are important plant pathogens that cause disease and large losses to the production of *Capsicum* and other food crops (Schwartz et al., 2015; Suwannarat et al., 2017). One strategy used in *Capsicum* breeding programs has involved the identification of peppers with certain genetic traits, such as those related to high productivity, resistance to diseases, and the production of bioactive compounds (Padilha and Barbieri, 2016).

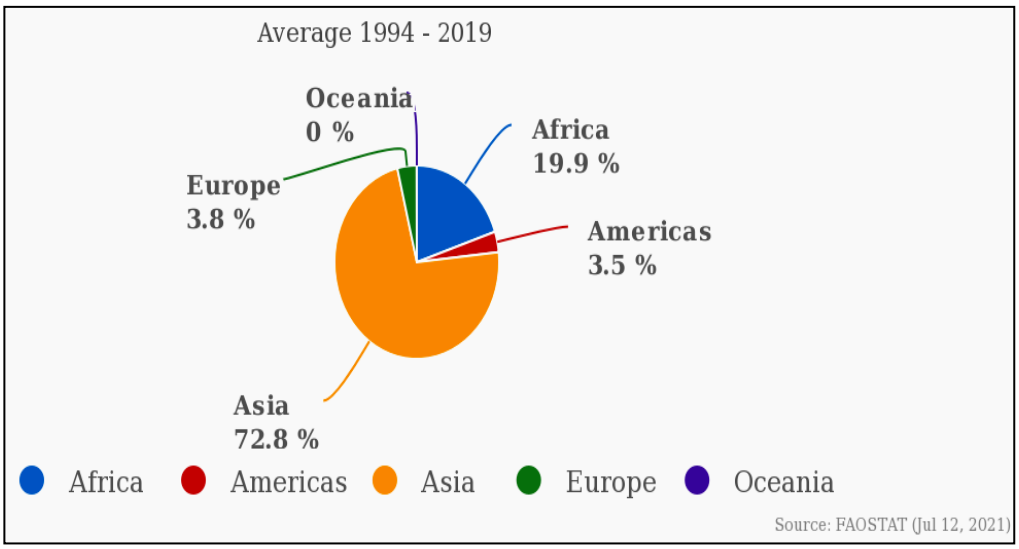


Figure 2. Production share of Chillies and peppers, dry by region (FAOSTAT, 2021).

C. *annuum* UENF1381 accession chili pepper is accession present in the Universidade Estadual do Norte Fluminense Darcy Ribeiro (UENF) genebank described as resistant to bacterial spot, anthracnose and PepYMV (Pepper yellow mosaic virus) (Riva et al., 2004; Bento et al., 2017) and also in this accession, in recent years, a group of molecules called antimicrobial peptides (AMPs) has been identified mainly in fruits and seeds of UENF1381 (Silva et al., 2017; Taveira et al., 2018; Maracahipes et al., 2019). AMPs participate in the innate defense response of organisms, these molecules have been described as potential candidates for design of medicines and for the control of diseases of plants and animals (Huan et al., 2020).

Based on knowledge aimed at new biotechnological strategies will be new forms of control and prevention of diseases caused by phytopathogenic microorganisms, consequently promoting diminishing of the use of pesticides and reducing their impact on the environment (Ilyas et al., 2017). The aforementioned potential of peptides from *C. annuum* in the control of microorganisms that cause disease in plants, this work aims to identify, characterize and advance the knowledge of the mechanisms of action of *C. annuum* UENF1381 leaf and root peptides with antimicrobial potential, since there is little information about defenses in these organs.

2. OJECTIVES

2.1. General objectives

The objectives of this work were the identification, purification, and characterization of proteins and antimicrobial peptides of *Capsicum annuum* L. UENF1381 and investigations into differences in the protein expression of leaves and roots in response to *Xanthomonas euvesicatoria* infection as well as to evaluate the activities of the extracts on different families of enzymes and inhibitory activity and possible mechanisms of action of the extracts and fractions on the development of phytopathogenic microorganisms.

2.2. Specific objectives

1. Investigate the differences in protein profiles between extracts (leaf and root) inoculated and not inoculated with *X. euvesicatoria*;
2. Identify proteins and antimicrobial peptides in the leaves and roots of *C. annuum* that are responsive to *X. euvesicatoria* infection;
3. Purify proteins and antimicrobial peptides of *C. annuum* and characterize their molecular masses and primary structures;
4. Determine the inhibitory activity of the leaf and root extracts from *C. annuum* on the following enzymes: trypsin, α-amylase and detection of the β-1,3-glucanase;

5. Analyze the inhibitory effects *in vitro* of leaf and root extracts and fractions from *C. annuum* on the development of the bacterium *X*. *euvesicatoria* and fungus *Colletotrichum scovillei*;
6. Investigate the mechanisms of action of leaf and root extracts and fractions from *C. annuum* on the development of the *C. scovillei.*

3. REVIEW

3.1. *Capsicum* diseases

The main diseases that affect the plant genus *Capsicum* are the bacterial spot, caused by bacteria of the genus *Xanthomonas* and the anthracnose caused by the fungi *Colletotrichum* spp. (Obradovic *et al*., 2004; Potnis et al., 2015; Sun et al., 2015).

Bacteria of the genus *Xanthomonas* are the cause of spot bacterial in Solanaceae family plants. The characteristics of this genus are part of a phytopathogens group that threaten approximately 400 species of the Solanaceae family, mainly tomatoes and peppers. The disease is characterized by the creation of necrotic spots or not, distributed mainly on the edges of the leave, also occurring brown spots on the stem, petals, and fruit. In fruit, this disease causes deformations and whitish to browning stains, hindering its commercialization (Jones et al., 2002; Potnis et al., 2015). On the other hand, fungi of the genus *Colletotrichum* are the cause of anthracnose, one of the most notable diseases that occur in plants in different cultures, including the plant genus *Capsicum*. In Brazil, the *Colletotrichum gloeosporioides* fungus is the main agent that causes this disease (Mendes et al., 1998; Azevedo et al., 2006; Oliveira et al., 2017). Anthracnose is the main disease in *Capsicum annuum*, characterized by rounded necrotic lesions in different diameters, whose center consists of an orange color conidia, and highest occurrence in rain seasons. The fungus infection occurs

under temperatures from 20 ºC to 24 ºC and high relative humidity, and the water being an essential factor for germination and dispersion of the fungal spores in the crop (Serra and Silva, 2005; Azevedo et al., 2006;). Pathogens of this genus are responsible for large losses in agriculture because they can infect plants in different stages of development. The losses occur mainly in the fruit post-harvest. In Brazil, losses are estimated around 30% in commercialized fruit (Serra and Silva, 2005).

Diseases caused by phytopathogenic microorganisms affect the marketing of important crops (Dias, 2010; Buttimer et al., 2017), for example, sweet peppers are considered one of the ten most important species economically in the Brazilian market. With the increase in demand for this crop, planting has been done mainly in greenhouses, supporting at most two or more consecutive harvests, considering the soil contamination with phytopathogenic microorganisms from previous harvests (Charlo et al., 2009).

According to the National Health Surveillance Agency (ANVISA, Brazilian acronym, 2018), pesticides applied in 1/3 of the vegetables consumed in Brazil is higher than the acceptable level, becoming them unsuitable for consumption. Bell pepper leads contamination lists with 91.8% in relation to other crops. It is fundamental to find new control strategies for *C*. *annuum* phatogens.

3.2. Control of diseases in *Capsicum*

Diseases caused by phytopathogenic microorganisms are a serious problem for agriculture (Dias, 2010; Buttimer et al., 2017). The methods adopted traditionally to minimize diseases are largely conditioned by using chemical pesticides in the soil, seeds, and during plant growth. However, *C. annuum* cultivated areas have been contaminated by salt due to the frequent use of mandatory pesticides and fertilizers (Hahm et al., 2017). Brazil is one the largest consumer of chemical pesticides in the world. (Pignati et al., 2017). Identification and selection of strains resistant to microorganisms are also used, but they are restricted to a few cultures and microorganisms (Lapin et al., 2013). For fungal diseases, different *Capsicum* species, mainly *Capsicum annuum*, have been reported as highly resistant to different fungi types (Mishra; Guangshun, 2018; Parisi et al., 2020); for bacterial spot, for example, some resistance genes have

been identified in peppers, including the Bs1, Bs3, Bs3, bs5, and bs6 genes among others (Jones et al., 2002; Stall et al., 2009).

These resistance genes, when interacting with the respective bacterial genes (virulence genes) lead to an incompatibility and a hypersensitivity response, protecting the plant against the occurrence of the disease. Despite the development of resistant cultivars to bacterial spot, the great genetic diversity of bacterial strains hampers the development of cultivars with durable resistance (Minsavage et al., 1990; Vallejos et al., 2010).

Currently, research has focused on identifying secondary plant metabolites, possible tools to control pest microorganisms, for example, alkaloids, glycoalkaloids, terpenoids, organic acids, alcohols, and antimicrobial peptides (AMPs). The family Solanaceae contains many species that are producer of toxic substances for insects and microorganisms (Chowanski et al., 2016). Increased restricted use of pesticides and research into new disease control strategies in plants, AMPs have shown that is a promising strategy for this purpose (Moguel-Salazar et al., 2011). In recent years, reports about the occurrence of AMPS from diverse species of plants, including *Capsicum,* have been mentioned more frequently (Silva et al., 2017; Taveira et al., 2017; Pereira et al., 2018;).

Studies have investigated the use of plant AMPs as promising molecules in microbial control. Many AMPs have been used as a model for development of synthetic AMPS, and also the resistance process of transgenic plants that express these molecules has been studied (Montesinos, 2007; Montesinos and Bardaj, 2008). The growth of plants that express antimicrobial proteins/peptides has proved to be a compelling strategy to control microorganisms that cause diseases in plants.

Proteins and peptides with antimicrobial activity have a great wide of action against different microorganisms, and genetic modifications to increase the expression of molecules already existent in plants have shown a promising approach for increasing species productivity. The success of this approach would guarantee that the external dependence on imported pesticides would be minimized, besides creating alternatives to control diseases caused by microorganisms based on natural compounds, to which these organisms have not evolved yet and, therefore, are vulnerable (Ilyas et al., 2019).

3.3. Antimicrobial peptides (AMPs)

Plants are constantly exposed to a wide number of pathogenic organisms such as fungi, viruses, and bacteria. In this way, plants have developed many defense mechanisms in their evolution process, among them, the synthesis of components with high antimicrobial activity (Meneguetti et al., 2017). Among the compounds produced by plants, the antimicrobial peptides (AMPs) stand out with a comprehensive inhibitory activity against many microorganisms (Benko-Iseppon et al., 2010). AMPs are parts existent in plants or are induced after being attacked by fungi, bacteria, viruses, or parasites, and can be isolated from different parts of the plant tissue or organs, mainly seeds and fruit, but they also occur in flowers, leaves, and roots (Egorov et al., 2005; Campos et al., 2018; Tang et al., 2018).

AMPs are characterized by their small size (ten to one hundred amino acid residues), most of them showing α-amphipathic helix conformation, which are cationic with two or more positive charges and have a large percentage of hydrophobic amino acids (40% to 60%) (Strempel et al., 2014). It has been found that the presence of positively charged amino acids (lysine and arginine) and hydrophobic are essential characteristics for antimicrobial activity (Jiang et al., 2008; Lee et al., 2016; Oñate-Garzón et al., 2017).

AMPs are divided into families (Fig. 3), according to amino acid spacing among cysteine, position, length, and molecular weight. The families that are well-known are thionin, defensins, lipid transfer proteins (LTP), and proteinase inhibitors (Nawrot et al., 2014; Tam et al., 2015).

Thionins are cysteine-rich peptides about forty-five to forty-seven amino acid residues, with a molecular weight around 5 kDa. The thionin family is separated into two subgroups; the first subgroup is composed of eight cysteine residues linked by four bonds, and the second subgroup is composed of six cysteine residues linked by three disulfide bonds (Stec, 2006; Odintsova et al., 2018). Thionins are believed to be involved in protection against plant pathogens, including bacteria and fungi, acting directly on the photogene membrane (Azmi and Hussain, 2021).

Plant defensins, for example, are small AMPs that have a molecular weight between 5 and 8 kDa and are basic. They are part of the innate immune system of plants and in general are widely distributed in the kingdom plantae.

They are consisted of forty-five to fifty-four amino acid residues, among which there are eight conserved cysteines in the primary structure binding in pairs, composing four disulfide bridges. These bridges stabilize the three-dimensional structure, and two of them compose the motif stabilized by cysteines, characteristic of peptides with antimicrobial activity (Carvalho and Gomes, 2011; Guillén-Chable et al., 2017).

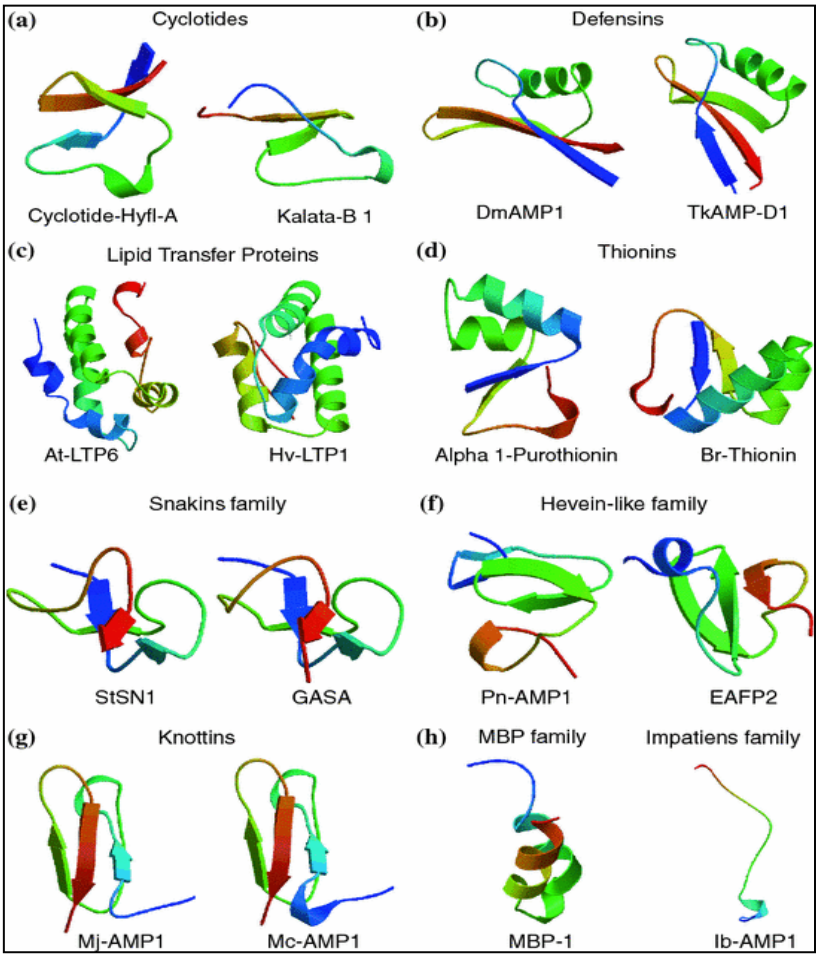


Figure 3. 3D ribbon structures of different family members of plant AMPs. The structures were computed using SWISS-MODEL. Cyclotide Hyfl-A: *Hybanthus floribundus* (P84647), Kalata-B1: *Oldenlandia affinis* (P56254), DmAMP1: *Dahlia merckii* (P0C8Y4), Tk-AMP-D1: *Triticum kiharae* (P84963), At-LTP6: *Arabidopsis thaliana* (Q9LDB4), Hv-LTP1: *Hordeum vulgare* (A8YPK3), Alpha-1-Purothionin: *Triticum aestivum* (P01543), Br-Thionin: *Brassica rapa* subsp. pekinensis (Q9SBK8), StSN1: *Solanum tuberosum* (Q948Z4), GASA: *Fagus sylvatica* (Q0VYL5), Pn-AMP1: *Ipomoea nil* (P81591), EAFP2: *Eucommia ulmoides* (P83596), Mj-AMP1: *Mirabilis jalapa* (P25403), Mc-AMP1: *Mesembryanthemum crystallinum* (O81338), MBP-1: *Zea mays* L. (P28794), Ib-AMP1: *Impatiens balsamina* (O24006). AMP name: plant name (GenBank or UniProt ID). By Goyal and Mattoo, (2016).

The lipid transfer protein (LTP) family are divided into two groups - LTP1 and LTP2, 9 and 7 kDa, respectively. LTP1 is composed approximately of ninety-ninety-three amino acid residues and LTP2 approximately seventy amino acid residues (Diz et al., 2011; Yamuna et al., 2019). The antimicrobial activity of LTPs had been discovered by scanning protein extracts of plants that were able to inhibit phytopathogens *in vitro* (Molina et al., 1993). Bacteria and fungi are among the inhibited phytopathogens, but the activity is more remarkable against fungi (Kader, 1996; Carvalho and Gomes, 2007). Molina et al. (1993) isolated four LTPs from barley leaves and one from corn leaves and found that these peptides have biological activity against *Clavibacter michiganensis* subsp. *sepedonicus, Pseudomonas solanacearum* bacteria, and *Fusarium solani* fungus. Since the discovery of LTPs as peptides able to inhibit phytopathogens, it has been speculated that this effect may be due to the interaction between LTPs and membranes, which probably leads to a permeabilization effect (Yeats and Rose, 2008; Wimley et al., 2019).

Studies have identified genes involved in the expression of LTPs in the plant genus *Capsicum*. For example, Jung et al. (2003) isolated three genes from plants of the genus *Capsicum*, named CALTPI, CALTPII, and CALTPIII, being encoded to LTP. It has been found that the gene CALTPIII was only transcribed in leaves, roots, flowers, and fruit tissues when infected by *Xanthomonas campestris* pv. *vesicatoria*, *Phytophthora capsici,* and *Colletotrichum gloeosporioides*. The gene CALTPIII was also expressed in the upper leaves after the lower leaves having been immunized by pathogenic or non-pathogenic bacteria (Jung et al., 2006).

Serine proteinase inhibitors (PIs) have been described in many species of plants (Volpicella et al., 2011; Rustgi et al., 2018). In plants, PIs can be found as constitutive components present in the reserve tissues (tubers and seeds) or as a response to the perception of attack by insects and pathogens, inhibiting the action of digestive proteases present mainly in mammals and insects, as well as enzymes present in bacteria and fungi (Kidrič et al., 2014; Clemente et al., 2019). Most of these inhibitors are molecules from 3 to 24 kDa, which can inhibit both trypsin and chymotrypsin, and some of them are able to inhibit both enzymes (Richardson, 1977). Most of the PIs studied belonging to three main plant families, Leguminosae (Fabaceae), Solanaceae, and Gramineae (Poaceae) (Brzin;

Kidric, 1995; Katoch et al., 2014), can be divided into different classes, including metallo, cysteine , and aspartyl-PIs, depending on the mechanistic types of proteases with which they interact (Rawlings et al., 2004, 2010).

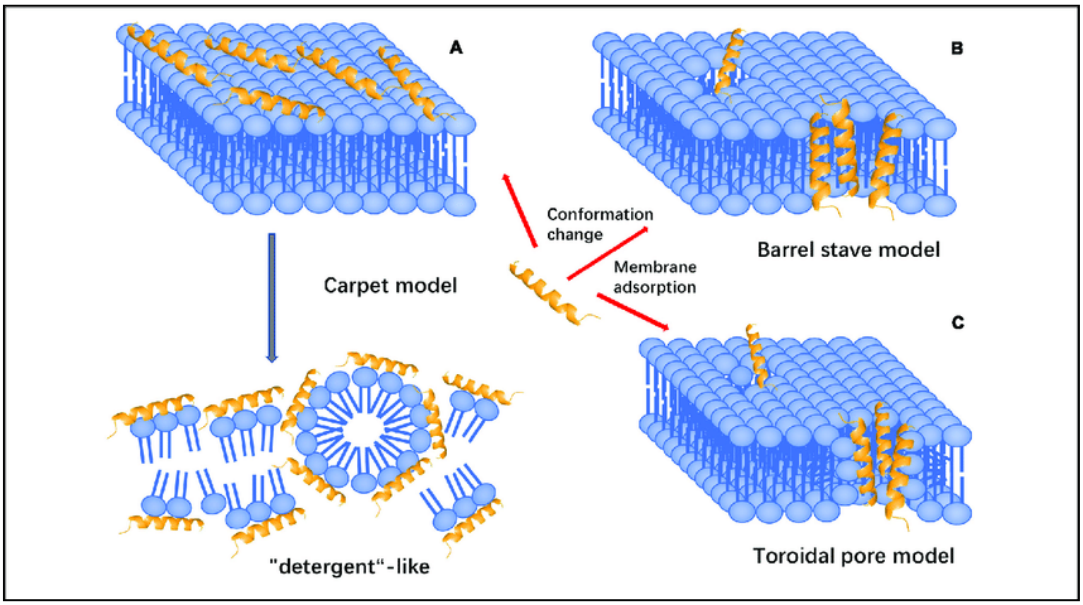
The interest in peptides, especially those isolated from plants with double ability to inhibit the specific proteases activity and microbial growth, has attracted the attention of many researchers; many studies have shown those abilities (Paiva et al., 2013; Ribeiro et al., 2013; Silva et al., 2020).

Thus, AMPs are remarkable molecules to control microorganism growth due to their chemical nature, that is, they are cationic (most of them), binding to the microorganism membranes (charged negatively), causing damage to these structures or are internalized, interacting with intracellular targets, preventing the synthesis of proteins important for the microorganism reproduction (Pardoux et al., 2020). AMPs had been called antimicrobials due to the ability to interact with membranes from different microorganisms (charged negatively) and lead these microorganisms to the death through complex mechanisms (Bechinger et al., 2017).

3.4. Mechanism of action of AMPs

The action mechanism of every AMP families is unclear; many studies indicate the membrane permeabilization as one of the models by which peptides act, although these models is not able to show in detail how AMPs kill microorganisms (Ma et al., 2020). It is speculated that the biological activity of AMPs is the result of interaction with phospholipids in cell membranes, and intracellular targets are also linked to the action mechanism of these molecules (Zasloff, 2002; Matsuzaki, 2009; Mishra and Guangshun, 2012). On the basis of the effect on target cell involvement, AMPs can be divided into two groups. The first and most well-known group is composed of AMPs that act through the permeabilization and rupture of the membrane, leading the microorganism to death (Toke, 2005). A more detailed representation of the different mechanisms of AMPs interaction with the microorganism membranes is presented by Huanet et al. (2020) (Scheme 1). In general, this process occurs because it allows interaction and insertion of peptides (charged positively) in the microorganism membranes

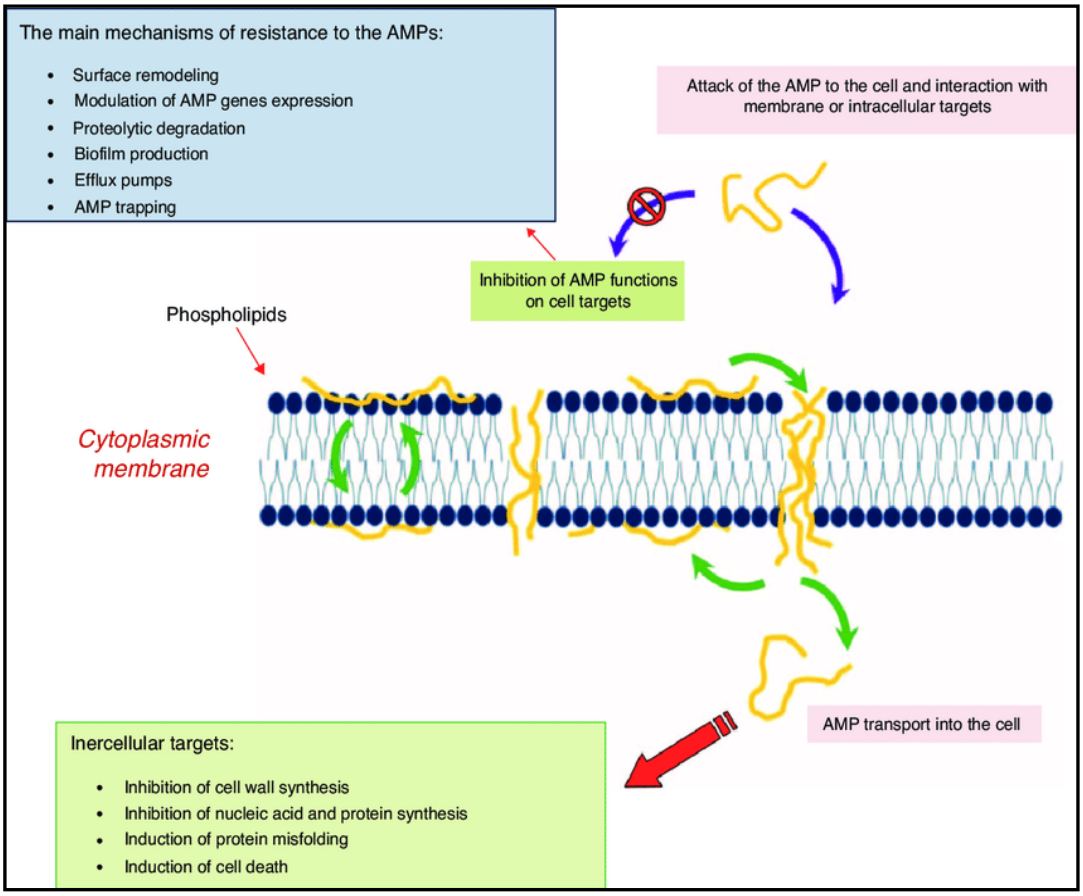
charged (negatively), leading to the formation of pores and loss of cellular homeostasis (Kumar et al., 2018).



Scheme 1. Representation models of action for extracellular AMP activity. (A) Carpet model: accumulation of AMPs on the surface and then destruction of the cell membrane in the manner of “detergent”. (B) Barrel stave model: AMPs aggregate with each other and are inserted into the bilayer of the cell membrane in the form of multimers and arrange parallel to the phospholipids, then form a channel. (C) Toroidal pore model: accumulation of AMPs vertically embed in the cell membrane, and then, bend to form a ring hole Huan et al. (2020).

The second group is the AMPs that need to reach intracellular targets to exert their activity. This action can occur independently or synergistically with membrane permeabilization (Yeaman and Yount, 2003). However, AMPs penetrate the microorganism membranes in both cases. Thus, the AMPs that pass through the membrane are also able to involve intracellular targets and act in low concentrations (Le et al., 2017; Kumar et al., 2018). There are two ways for AMPs to enter the microorganism's cell, through direct penetration or through endocytosis. After entering the cytoplasm, AMPs will be directed to act on the target and, depending on the destination, AMPs can be divided into the following categories: (a) inhibition of protein biosynthesis; (b) inhibition of nucleic acid biosynthesis; (c) inhibition of protease activity; and (d) inhibition of cell division,

among others (Huan et al., 2020). A scheme of the AMP intracellular targets is shown below.



Scheme 2. The main mechanisms of cell killing by antimicrobial peptides (AMPs) through intracellular targets (Hancock and Sahl, 2006; Jenssen et al., 2006; Nicolas, 2009).

The mechanisms of AMPs' action reported about Solanaceae were similar to those mentioned above. Changes in cell membrane potential and permeability, membrane pore induction, and cell aggregation were the possible mechanisms reported for bacteria. On the other hand, the antifungal activity of AMPs was due to the induction of cell membrane permeabilization, inhibition of germination, and alteration of hyphae growth (Afroz et al., 2020). Solanaceae, including the genus *Capsicum,* contains a variety of AMPs with promising antimicrobial activity that could be a potential source for the development of innovative approaches to plant protection in agriculture. The resistance acquired by diseases by AMPs can

reduce losses in the yield, quality, and safety for agricultural products (Moguel-Salazar et al., 2011).

3.5. AMPs of *Capsicum annuum* L.

Most studies about *Capsicum* are carried in *C*. *annuum*. In addition to being the most consumed pepper species, *C*. *annuum* has a wide variety of bioactive compounds that act against different microorganisms, mainly fungi and bacteria (Batiha et al., 2020). For example, bell pepper extracts great activity potential against the gram negative bacteria: *Salmonella typhimurium, Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa,* and *Escherichia coli* (Shayan et al., 2013).

Several resistance genes identified in *C*. *annuum* are linked to the induction of defense proteins/peptides. In particular, antimicrobial peptides (AMPs), which are small groups of proteins that widely exist in nature. AMPs have been studied for the past 25 years, due to their great ability to inhibit microorganisms (Mejía-Argueta et al., 2020).

Meyer et al. (1996) identified that green fruits of *C. annuum* when injured activated the *JI, jI*-1 and *jI*-2 genes, which encoded a group of AMPs called defensin, which are related to the plant defense response against invading microorganisms. Other defensin-encoding genes were also identified: Houlné et al. (1998) found the *j1* ± 1 and *j1* ± 2 genes and Oh et al. (1999) kept record of the gene named *j1*-1, activated in response to infection by the fungus *Colletotrichum gloesosporioides.*

From another research, when studying *C*. *annuum* fruits, Seo et al. (2014) observed a peptide, called *J1*-1, which the suggested role is protection of the reproductive organs. *J1*-1 was undetectable in the green stage, so it was isolated from ripe pepper fruit and thus able to inhibit the growth of fungi *Fusarium oxysporum* and *Botrytis cinerea*. Guillén-Chable et al. (2017) cloned the *J1*-1 gene and, by affinity chromatography, obtained a defensin which showed antibacterial activity against *Pseudomonas aeruginosa*.

Oh et al. (1999) characterized a gene denominated PepThi, which product is similar to an AMP family, the thionins. It was verified that after interaction with *Colletotrichum gloeosporioides*, PepThi was inducible in green fruits. In addition,

PepThi was also induced by salicylic acid. The conclusion of that work showed that PepThi is highly induced through the interaction with the fungus and with different signal transductions during the ripening of the fruit as a way to protect the reproductive organs. Thionin encoded genes have also been found by other authors. Lee et al. (2000) identified a gene called CATHION1 in *C*. *annuum* leaves infected by the bacterium *Xanthomomas campestre* and oomycete *Phytophthora capsici*. This suggests that CATHION1 expression is activated in response to disease leading to hypersensitivity reactions (HR) wich result in plant protection. In *C*. *annuum* plants infected with *X*. *campistris,* a gene called *CADEF1*, encode a defensin, was also isolated (Do et al., 2004).

Another plant AMP family has also been identified in *C*. *annuum*, the lipid transfer proteins (LTPs). Three cDNAs corresponding to the CALTPI, CALTPII and CALTPIII genes of proteins from the *C*. *annuum* LTP family were characterized by Jung et al. (2003). These genes accumulated in the leaf, stem, and fruit tissues when infected with the bacterium *X*. *campestris*, the oomycete *Phytophthora capsici* and the fungus *C*. *gloesoporoides*. Chen et al. (2011) also identified an LTP called CaMF2 isolated from anther of *C*. *annuum*. CaMF2 is related to three specific LTPs already identified in *C*. *annuum*. Park et al. (2002) cloned a LTP (CaLTP1) that was isolated of *C*. *annuum* leaf when inoculated with the tobacco mosaic virus (TMV). The amino acid sequence of CaLTP1 is highly similar to that of other plant LTPs. The analysis showed that a small family of genes from LTP-related sequences was present in the pepper genome. Transcripts homologous to CaLTP1, accumulated abundantly in the old leaves and flowers. CaLTP1 expression was induced in the ncompatible interaction i with the virus. CaLTP1 transcripts began to accumulate 24 hours after virus inoculation, reaching a maximum level at 48 hours.

Protease inhibitors (PIs), another AMP family, are the focus of interest for many researchers in *C. annuum*, because of their structure and great functional diversity and relevance in plant defense. The first study on the efficacy of PIs was performed by Tamhane et al. (2005), who purified from leaf extract two PIs called CapA1 and CapA2. In the analysis of tests with the insect *Helicoverpa armigera*, it was found that the PIs of *C*. *annuum* inhibited more than 60% of the total proteolytic activity of the larvae. Subsequently, Mishra et al. (2013) reported the presence of two *C*. *annuum* genes (CanPI-15 and -7) that encoded PIs. The

activity and structural stability of these CanPIs were revealed under various pH, temperature and denaturing conditions. In *Capsicum annuum* L. (UENF1381), several PIs have been identified as active againstphytopathogenic microorganisms (Moulin et al., 2014; Bard et al., 2015).

3.6. AMPs of *Capsicum annuum* L. UENF1381

Capsicum annuum L. (UENF1381) is an accession present in the UENF gemoplasma bank and is described as resistant to for multiple disease resistance (Riva et al., 2004; Bento et al., 2017).

In *C. annuum* UENF1381 the presence of several AMPs has been reported, such as thionins, defensins, LTPs and proteinase inhibitors mainly in seeds and fruits, which are active against phytopathogenic and of medical importance microorganisms (Diz et al., 2011; Santos et al., 2017; Taveira et al., 2018; Maracahipes et al., 2019).

Diz et al. (2006; 2011) identified in a peptide fraction of *C*. *annuum* UENF138 seeds the presence of an lipid transfer proteins (LTP). This fraction showed high antimicrobial activity against *Candida albicans*, *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*. In addition, of human salivary α-amylase inhibition was also observed. Cruz et al. (2010) also identified an LTP in a fraction isolated of *C. annuum* UENF1381 seeds.

Other AMPs were identified by Ribeiro et al. (2007; 2013) in *C*. *annuum* UENF138 seeds: the proteinase inhibitors called CaTI1, CaTI2 and CaTI3, each with approximately 6 kDa. Those AMPs inhibited the activity of the trypsin and chymotrypsin enzymes Silva et al. (2017) found that CaTI, a trypsin inhibitor from *C. annuum* seeds also turn permeable the membrane of phytopathogenic fungi, such as *C*. *gloeosporioides* and *C*. *lindemuthianum*. Bard et al. (2015) in turn identified a serine protease inhibitor called HyPep in hybrid seeds (Ikeda x UENF 1381). HyPep was present in F3 fraction. HyPep showed inhibitory activity against yeasts, such as *S*. *cerevisiae*, *C*. *albicans*, *C*. *tropicalis* and *Kluyveromyces marxianus*. In addition, HyPep was also able to inhibit insect and human salivary α-amylase activities.

In *C*. *annuum* UENF1381 fruits, Taveira et al. (2014) characterized a thionin (CaThi). A enriched fraction of fruit extract called F1 was able to inhibit the growth

of yeasts such as *S. cerevisiae*, *C. albicans*, *C. tropicalis,* and bacteria such as *Escherichia coli* and *Pseudomonas aeruginosa*. Both fractions had a 6 kDa peptide a peptide similar to plant thionin. Another fraction called F3 also showed inhibitory activity against *C*. *albicans* and *C*. *tropicalis*. For the mechanism of action, it was demonstrated that membrane permeabilization was correlated to the yeast growth inhibition process.

Continuing his studies, Taveira et al. (2016) tested whether CaThi in combination with the drug fluconazole was able to inhibit yeasts of the *Candida* genus. It was observed the combination of both molecules caused strong inhibitory activity against *C*. *albicans*, *C*. *tropicalis*, *C*. *parapsilosis*, *C*. *pelliculosa*, *C*. *buinensis,* and *C*. *mogii*. It is important to highlight alone at the same concentration and in combinations no inhibitory activity was observed. Similar to the previous results, the mechanism of action characterized was membrane permeabilization.

In order to study other mechanisms of action used by CaThi in combination with fluconazole, Taveira et al. (2017) identified that CaThi is capable of inhibiting the germination of *Fusarium solani* conidia, in addition to permeabilizing the plasma membrane, inducing an increase in H_2O_2 levels and activating caspases. Other AMPs such as defensins and LTPs have also been identified in fruits and showed activity mainly against phytogenic fungi (Maracahipes et al., 2019).

In leaves and roots from *C. annuum* UENF1381, Pereira et al. (2018) standardized two methodologies for the extraction of AMPs: acid extraction and extraction in 50% ethanol. The extracts obtained by both methods were subject to the High Performance Liquid Chromatography (HPLC) and the protein profile accompanied by uni and bidimensional gel electrophoresis. The results showed the presence of low molecular weight proteins. The alcoholic and acid extracts of leaf and root were able to inhibit the growth of *C. lindemuthianum* and only the alcoholic root extract was able to inhibit *C. gloeosporioides*. The antibiogram with *X. euvesicatoria* using the alcoholic and acid extracts in different concentrations were also performed, where it has been identified that these extracts are able to inhibit the growth of this bacterium. In addition, the L1′ band originating from the leafy acid extraction was sequenced. The N-terminal sequence of L1′ revealed that this protein shows 85% similarity to a small portion of U-Lim protein, previously identified in *Capsicum* and associated with a defensive function. This was the first

work to characterize AMPs in leaf and root from *C*. *annuum* UENF1381. Thus, the continuation of the identification of defense proteins/peptides in leaf and root of *C*. *annuum* UENF1381 is proposed in this work, since there is little information about defenses in these organs.

4. MATERIALS AND METHODS

4.1. Plant material

Seeds from *Capsicum annuum* L. (UENF1381 accession chili pepper) (Fig. 4) were provided by the Laboratório de Melhoramento Genético Vegetal (LMGV), Centro de Ciências e Tecnologias Agropecuárias (CCTA), this accession present in the Universidade Estadual do Norte Fluminense Darcy Ribeiro (UENF) genebank Rio de Janeiro (RJ), Brazil, and is described as resistant to bacterial spot, anthracnose and PepYMV (Pepper yellow mosaic virus) (Riva et al., 2004; Bento et al., 2017) and also to several AMPs has been identified in this accession (Santos et al., 2017; Pereira et al., 2018; Taveira et al., 2018; Maracahipes et al., 2019).

Figure 4. *Capsicum annuum* L. (UENF1381).

4.2. Microorganisms

The microorJganisms used in this research were supplied by the LMGV, UENF, RJ, Brazil. *Xanthomonas* euvesicatoria, isolate 4135, was cultured in DYGS medium (2 g/L glucose, 1.5 g/L bacteriological peptone, 1.5 g/L yeast extract, 0.5 g/L K_2HPO, 1.5 g/L glutamic acid, 0.5 g/L $MgSO_4$ and 18 g/L agar, pH adjusted to 7) at 28 °C. The phytopathogenic fungus *Colletotrichum scovillei* isolate 8.1 was cultured in Potato Dextrose Agar (PDA), at 30 °C. These cultures were kept in the Laboratório de Fisiologia e Bioquímica de Microrganismos (LFBM), Centro de Biociências e Biotecnologia (CBB), UENF, RJ, Brazil.

4.3. Insect larvae

Larvae of the *Tenebrio molitor* was obtained from a colony maintained in the Laboratório de Química e Função de Proteínas e Peptídeos (LQFPP), CBB, UENF, RJ, Brazil. The insects were reared on wheat germ diet at natural photoperiod, 28 °C, and 70% relative humidity. Larvae weighing ≥120 mg were

used in the bioassays and for dissection to isolate the peritrophic membranes for analyses.

4.4. Plant cultivation

Capsicum annuum L. UENF1381 seeds were planted in a greenhouse, and during the flowering periods the flowers were protected with paper bags. Due the possibility of cross-fertilization in plants of *Capsicum* self-fertilized seeds were used in this work. *In vitro* cultivation was used in this work to evaluate plants inoculated with *X*. euvesicatoria under controlled conditions and free from pathogen attack, unlike conditions observed under greenhouse conditions.

The seeds obtained from self-fertilization were initially washed in commercial liquid-detergent and rinsed in running water. After the seeds were immersed in 70% alcohol for 1 min and in 0.7% NaClO with three drops of Tween 20 added in an 80 mL volume for 15 min in an aseptic environment. Subsequently, seeds were rinsed for three times in deionized and autoclaved water.

Seeds germination rate (Fig. 5) was assessed in the dark for 15 cultivation days in Petri dishes (90 x 15 mm) containing medium composed of half of the MS mineral (½ MS), White vitamin complex (Murashige and Skoog, 1962), and 100 mg/L myo-inositol, without sucrose, with pH adjusted to 5.7 ± 0.1 and solidified in 2 g/L Phytagel (Sigma).

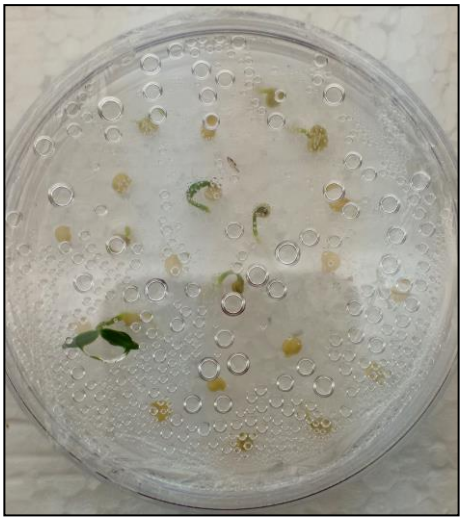


Figure 5. Seeds germinated in Petri dishes containing MS medium.

Then plants were transplanted into 350 mL flasks (Fig. 6) (125 mm x 60 mm) with the same medium with the addition of 20 g/L sucrose and maintained for approximately 30 days in a cultivation room in Laboratório de Fitotecnia, Setor de Horticultura, UENF, RJ, Brazil, at 27 ± 2 °C for 30 days under a 16 h light: dark photoperiod with irradiance of 50 µmol m^{-2} s^{-1} provided by OSRAM® day-light fluorescence lamps (Walter et al., 2018).

Figure 6. Plants grown in flasks containing MS medium.

4.4.1. Plant infection

X. *euvesicatoria* was grown in DYGS (Dextrose Yeast Glutamato) culture medium for 36 h at 28 °C, and bacterial colonies were suspended in sterile water and cellular density adjusted to a of 10^8 colony forming unit (CFU/mL) via a spectrophotometer using a wavelength of 600 nm. The bacterial inoculum was tested on a susceptible cultivar (Ikeda) (Fig. 7). Inoculation was performed after 45 days, using the method of suspended bacterial infiltration at the mesophyll (Fig. 8). (Bongiolo Neto et al., 1986; Costa et al., 2002; Riva et al., 2004). Uninoculated and mock inoculated plant with water were used as controls.

The plants were collected at 12, 24 and 48 h after infection, were dissected into leaves and roots and were separately stored in aluminium foil bags,

immersed in liquid nitrogen and immediately transferred to an ultrafreezer (− 70 °C) until subsequent use for protein extraction.

Figure 7. Ikeda inoculated with *X. euvesicatoria*

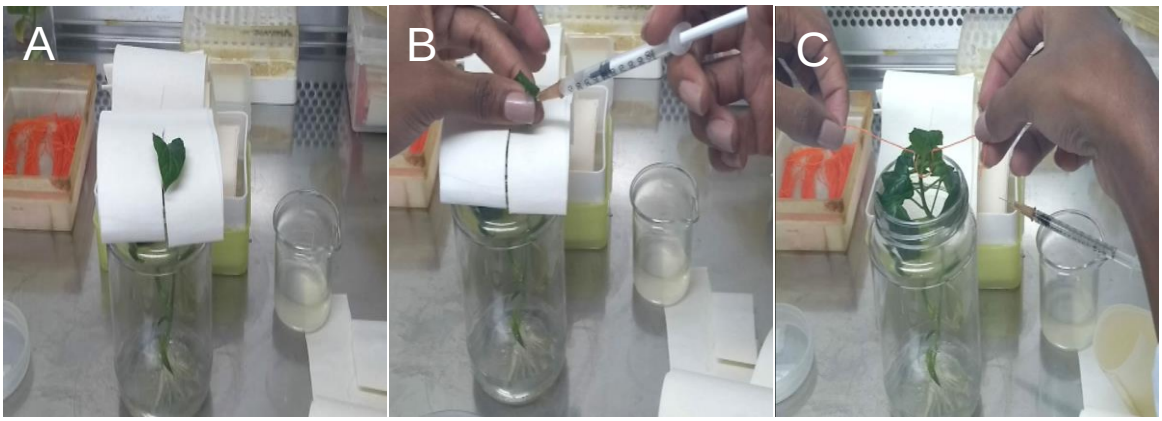
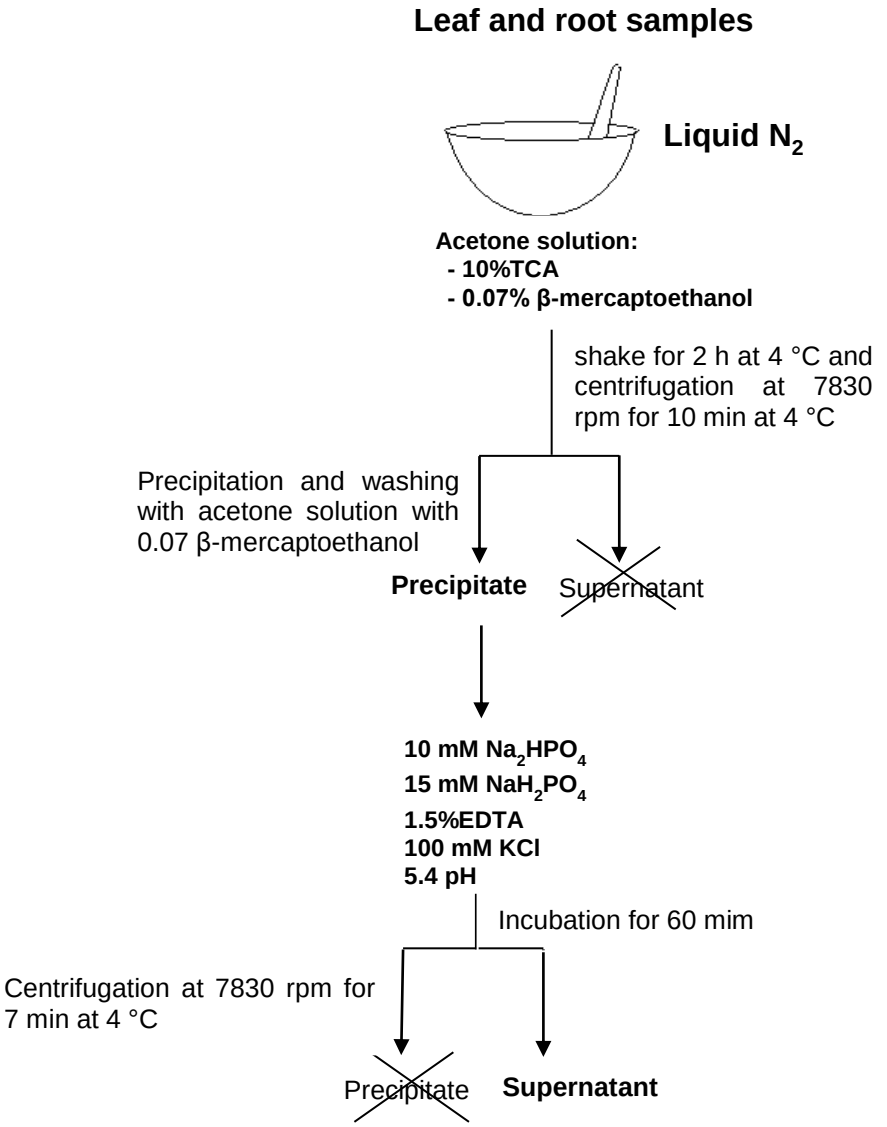


Figure 8. Plant infection. A- protection of the leaf to be infection with filter paper; B- leaf infection; C- marking with infection leaf line.

4.5. Extraction, purification and characterization of the peptides of leaf and root from *Capsicum annuum*

4.5.1. Extraction of leaves and roots from *Capsicum annuum*

Plant tissue extractions (leaves and roots) were performed according to Granier (1998) and Egorov (2005) (Scheme 3). The samples were named as described in Table 2.



Scheme 3. Extraction of leaves and roots according to Granier, (1988) and Egorov (2005).

Table 2. Samples identification of *Capsicum annuum* UENF1381, Resistant to bacterial spot, anthracnose and pepper yellow mosaic virus (PepYMV). NC= not inoculated; L= leaf; R= root; C= control (mock inoculated); I= inoculated 12, 24 and 48h after inoculation.

Samples	Identification
Leaf not inoculated	L_{NC}
Leaf mock inoculated (12)	LC_{12}
Leaf mock inoculated(24)	LC_{24}
Leaf mock inoculated (48)	LC_{48}
Inoculated leaf (12)	IL_{12}
Inoculated leaf (24)	IL_{24}
Inoculated leaf (48)	IL_{48}
Root not inoculated	R_{NC}
Root mock inoculated (12)	RC_{12}
Root mock inoculated (24)	RC_{24}
Root mock inoculated (48)	RC_{48}
Inoculated root (12)	IR_{12}
Inoculated root (24)	IR_{24}
Inoculated root (48)	IR_{48}

4.5.2. Purification of peptides of leaf and root from *Capsicum annuum*

Based on the results of differentiated expression of proteins in response to *X*. *euvesicatoria* and mass spectrometry characterization LC_{48} and RC_{48} extracts were selected for purification. The extracts were subjected to reversed phase chromatography on HPLC system (Shimadzu) using a reversed-phase column C18 with a C8 guard column. The column was equilibrated with solution A (2% acetonitrile containing 0.1% TFA), at a flow rate of 0.5 mL/min, for 95 min and at 33 °C. Proteins were eluted with a gradient of solution B (80% acetonitrile in 0.1% TFA). The column was further washed with the solution A for 10 min; next, a gradient was applied by adding Solution B for approximately 85 min. The elution profile was monitored using a diode array detector. After being collected, the leaf and root fractions were lyophilized dry for further analysis.

4.5.3. Protein quantification

Quantitative protein determinations were made as method described by Smith et al. (1985) with modifications. In brief total soluble protein content was determined by Bicinchoninic (BCA) method and bovine serum albumin (BSA) was used as a protein standard.

4.5.4. Gel electrophoresis

Sodium dodecyl sulfate (SDS)-tricine gel electrophoresis was performed according to Schägger and Von Jagow (1987), where different leaf and root extracts (30 µg/mL) and fractions (10 µg/mL) were prepared with 5% sample buffer and β-mercaptoethanol (1%). The samples were placed in a water bath at 80 °C for 5 min, centrifuged at 15,000 x *g* for 3 min and then load to each well of the gel. The electrophoresis was performed at 400 A and 22 V, overnight. An ultra-low ranger molecular weight marker was used (Sigma-Aldrich). After running the gels were stained with Coomassie R or stained with silver nitrate (Morrissey, 1981).

4.5.5. Peptide identification by mass spectrometry analysis

The peptide identification was performed by Laboratório de Bioquímica Marinha (BioMar-Lab), Departamento de Engenharia de Pesca, Universidade Federal do Ceará (UFC), Ceará, Brazil. After separation by gel electrophoresis, the protein bands of interest the peptides L1 and R1 present in the LC_{48} and RC_{48} extracts, respectively, were extracted from the gel.

Then, the selected peptides were digested by trypsin according Shevchenko et al. (2006) and subjected to mass spectrometry evaluation. The instrument used was a hybrid mass spectrometer (ESI-Q-ToF) (Synapt HDMS, Waters Corp, MA, USA). The machine parameters were adjusted as described by Carneiro et al. (2013). For sequencing of the L1' fraction obtained on the HPLC system, after electrophoresis in tricine gel, the gel was subjected to staining and destaining. Protein band of interest were extracted and subjected to a mass spectrometry evaluation. The sequences of the peptides were compared

with the sequences reported in NCBI-BLAST amino acid databases and were presented for automatic alignment using the Clustal Omega program online (Sievers and Higgins, 2018).

4.6. Inhibition of trypsin activity

The inhibitory activity of the peptides was determined by measuring the activity residual hydrolytic content of trypsin through the use of N-benzoyl-DL-arginyl-p-nitroanilide (BApNA) substrate (stock 5 mM), after pre-incubation with with 30 µg/mL of leaf and root extracts from *C*. *annuum* UENF1381 (Table 2). The proteolytic activity was measured using synthetic peptide derivates of *p*-nitroanilide (0.0625 mM each) in 50 mM Tris-HCl buffer, pH 8.0 at 37 °C, in a final volume of 200 µL. The reaction was interrupted by adding 100 µL of 30% acetic acid (v/v) and the hydrolysis of the substrate was accompanied photometrically by the measurement of absorbance of p-nitroanilide released at 405 nm using a spectrophotometer (EZ Read 400) (Macedo et al., 2007).

4.7. Inhibition of α-amylase activity

The intestinal α-amylase activity of *Tenebrio molitor* larvae was performed as previously described (Bernfeld et al. 1986). Initially, the reaction mixture for determination α-amylase unit of activity was assayed in water at 37 °C for 30 min using 1% starch (Sigma-Aldrich Co.) as the substrate and intestinal tract α-amylase (2 µL). The 3,5-dinitrosalicilic acid (DNS) reagent solution was prepared by combining 30 ml of solution 1 (4.5% NaOH) + 88 ml of solution 2 (1% DNS + 25.5% potassium/sodium tartrate) + 10 ml of solution 3 (2.2 ml of 10% NaOH + 1 g of phenol + water q.s.p. 10 mL). After the addition of DNS, the reaction was stopped by heating to 100 °C for 5 min, 400 µL of water was added, and the absorbance at 540 nm was monitored (UVVIS-1203, Shimadzu). One unit of α-amylase activity was defined as the quantity of enzyme that increased the absorbance at 540 nm by 0.1 absorbance units over 30 min. α-amylase inhibitory assays with the leaf and root extracts were carried out as described for the determination of α-amylase activity in units, with leaf and root extracts (30 µg/mL). EDTA was used with positive control.

4.8. Determination of β-1,3-glucanase activity

The determination of β-1,3-glucanase activity of leaf and root extracts was performed according to a method described by Fink et al. (1988). The experiment was carried out in test tubes containing 30 µg/mL of leaf and root extracts from *C*. *annuum* UENF1381 125 µL of laminarin (2 mg/L in 50 mM sodium acetate buffer, pH 5.0) and the final volume adjusted to 500 µL with 50 mM sodium acetate buffer, pH 5.0. The mixture was incubated at 37 °C for 12 h (Biochemistry Oxygen Demand (BOD) incubato). After the incubation period, 500 µL of the copper reagent (Somogyi, 1952) was added; the mixture was boiled for 10 min and then allowed at room temperature, and then 1000 µL of the arsenomolybdate reagent (Nelson, 1944) was added. The β-1,3-glucanase activity was defined as the concentration of the enzyme that yielded an absorbance of 0.001 when read at 500 nm. For control reaction the extracts were replaced by assay buffer.

4.9. Antibiogram

To evaluate the effects of the leaf and root extracts from *C*. *annuum* on the growth of *X*. *euvesicatoria*, the method previously described by Filho and Romeiro (2009) was used. To assess the formation of inhibition halo, bacteria were grown on 30 mL of DYGS broth and kept in an orbital shaker (Tecnal TE420) for 24 h at 28 °C and 100 rpm. After this period, 100 µL of the bacteria-enriched aliquot was transferred to an Erlenmeyer flask containing 30 mL of 0.8% semi-solid at the temperature of 40 °C. The semi solid DYGS broth containing the *X*. *euvesicatoria* was poured into Petri dishes. After medium solidification, paper discs of approximately 6 mm in diameter were placed over medium, and to each disc was added 5 µL leaf and root extracts (100 µg/ mL). In one of the discs, 5 µL of water was added to establish a control. The halos were evaluated (measuring from the edge of the paper discs to the end of the halo) at 24 h periods with a hardened stainless steel digital caliper (Stainless Hardened).

4.10. Effect of total extracts and fractions on fungal growth

To assay the effect of extracts and fractions (leaf and root fractions from the HPLC system) on *C. scovillei* growth, the conidia (20.000 cells/mL in 1 mL PDA medium) were incubated at 30 °C in microplates in the presence of the different extracts (100 and 200 µg/mL) and fractions (10 µg/mL). Optical readings at 620 nm were taken (Plate chameleon™ V, Mikrowin program) at 48 h to evaluate fungal growth. Cell growth without the addition of extracts was also determined as control. The entire assay was performed under aseptic conditions in a laminar flow hood (Trox technic®) (Broekaert et al., 1990).

4.10.1. Effect of extracts and L1' fraction on membrane permeabilization

Plasma membrane permeabilization assay was accessed by *Sytox Green* fluorescent probe (Thevissen et al., 1999) with some modifications. One hundred microliter aliquots of the *C. scovilei* suspension that had been grown in the presence of leaf and root extracts (100 µg/mL) and L1' fraction (10 µg/mL) for 24 h, period for fungus growth, were incubated with 0.2 µM *Sytox Green* in 96-well microplates for 30 min at 25°C under periodic agitation and then were observed using a DIC microscope (Axio Imager A2, *Zeiss*) equipped with a fluorescence filter (excitation wavelengths, 450 to 490 nm; emission wavelength, 500 nm). Negative (no protein extract added) controls were also run to evaluate the baseline membrane permeability. The images were viewed through Axiovision version 4.0 (Zeiss). All fluorescence images were taken with the same exposure time (excitation wavelengths, 450 to 490 nm; emission wavelength, 500 nm).

4.10.2. Intracellular ROS induction assay

After fungal growth inhibition assays, 50 µL of these cells grown in the absence and presence of the leaf and root extracts (100 µg/mL) and L1' fraction (10 µg/mL) for 24 h were incubated with 200 µM of the fluorescent probe 2',7'-dichlorofluorescein diacetate (H_2DCFDA). After 30 min incubation at room temperature with constant shaking, the cells were analysed according to the section 4.10.1. (Mello et al., 2011).

4.10.3. Mitochondrial functionality determination assay

Following the growth inhibition assay in presence of the leaf and root extracts (100 µg/mL) and L1' fraction (10 µg/mL) for 24 h, mitochondrial functionality was assessed by the fluorescent probe Rhodamine 123 (Sigma-Aldrich). The fungal cells were resuspended and incubated with 10 µg/mL Rhodamine 123 after 15 min of incubation at room temperature with constant shaking, and then cells were analysed according to the section 4.10.1. with modifications in the fluorescence filter (excitation wavelength, 506 nm; emission wavelength, 530 nm) (Taveira et al., 2018).

4.10.4. Caspase activity detection

Detection of active caspase was performed using the CaspACE FITC-VAD-FMK *in Situ* Marker (Promega), as described in the manual. For this test only the L1' fraction was used. Following the growth inhibition assay in presence of the L1' fraction (10 µg/mL) for 24 h. One hundred microliter aliquots of the suspensions of *C*. *scovilei* were incubated with 50 µM FITC-VAD-FMK. After incubation for 20 min at 30 °C the cells were observed by fluorescence microscopy as describe in section 4.10.1.

4.11. Statistical analysis

All data were obtained from experiments performed in triplicate and evaluated using a one-way ANOVA. Mean differences at $*p < 0.05$ were considered to be significant. All statistical analyses were performed using GraphPad Prism software (version 5.0 for Windows).

5. RESULTS AND DISCUSSION

5.1. Characterization of enzymatic and antimicrobial activities of leaf and root extracts from *Capsicum annuum* against phytopathogenic microorganisms

5.1.1. SDS-tricine-PAGE of leaf and root extracts from *Capsicum annuum*

In this work, we studied the induction of proteins and peptides from *C. annuum* inoculated with the bacterium *Xanthomonas euvesicatoria*. The protein profile of the leaf and root extracts from *C. annuum* was determined by SDS-tricine gel electrophoresis. Electrophoresis revealed that leaf and root extracts were rich in low molecular weight proteins. Both extracts presented major proteins above 6-14 kDa and the presence of proteins above 17 kDa was also observed (Fig. 9).

It was observed in the extracts the diferential expression of low molecular weight bands. In leaf exctracts, a band named L1 of approximately 6 kDa was induced in control plants (mock inoculated) and was repressed in plants inoculated with *Xanthomonas euvesicatoria* .

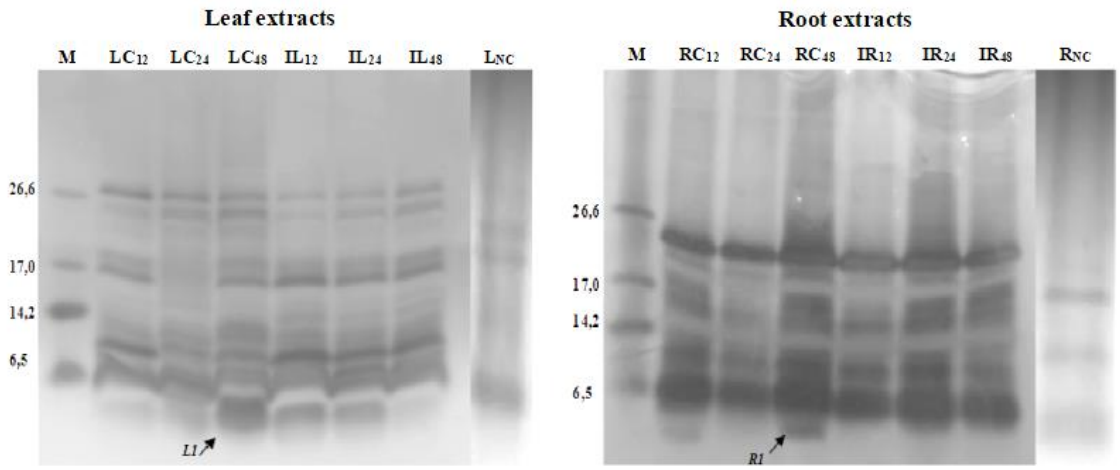


Figure 9. Electrophoretic visualization of proteins of leaf and root extracts from *Capsicum annuum* L. UENF1381 (30 µg/mL) subjected to C18 reversed-phase column in HPLC chromatography by SDS-tricine-PAGE. **(A)** L_{NC} - Leaf not inoculated; LC_{12} - Leaf mock inoculated (12); LC_{24} - Leaf mock inoculated (24); LC_{48} - Leaf mock inoculated (48); IL_{12} - Inoculated leaf (12); IL_{24} - Inoculated leaf (24); IL_{48} - Inoculated leaf (48); **(B)** R_{NC} - Root not inoculated; RC_{12} - Root mock inoculated (12); RC_{24} - Root mock inoculated (24); RC_{48} - Root mock inoculated (48); IR_{12} - Inoculated root (12); IR_{24} - Inoculated root (24); IR_{48} - Inoculated root (48). L1- non-specific-transfer protein; R1- ethylene-responsive proteinase inhibitor; M-low molecular mass markers (kDa). The protein bands were stained with silver nitrate.

Although protein is present in all leaf extracts only in the LC_{48} and IL_{12} extracts, protein induction occur. In the leaf samples inoculated with the bacteria, a decrease in protein concentration was observed (Fig. 9). In root extracts, a band named R1 of approximately 5,6 kDa was present only in the RC_{12} and RC_{48} extracts (Fig. 9). Pathogens express efector proteins to infect plants. These molecules are of fundamental importance for pathogen development in plant tissues. This fact suggests that protein suppression is important for pathogen development, allowing recognition of pathogen virulence genes by plant resistance genes to establish a defence response (Kushalappa et al., 2016; Villamil et al., 2019).

5.1.2. Mass spectrometry analysis

L1 and R1 bands (selected from the LC_{48} and RC_{48} extracts, respectively) had their expression suppressed in plants inoculated with *X*. *euvesicatoria*. The bands were submitted to mass spectrometry for amino acid residues identification.

The L1 sequencing resulted in two fragment of 13 and 11 amino acid residues (Fig. 10a), and the R1 sequence resulted in a fragment of 42 amino acid residues (Fig. 10b). The L1 and R1 sequences were compared with the sequences reported in NCBI-BLAST amino acid databases, and the sequences were subjected to automatic alignment using the Clustal Omega program. For the alignment, only the mature proteins were used, and the amino acid identity and positivity percentages were calculated.

Band L1 showed similarity sequences with non-specifc transfer proteins: 79% identity and 87% positivity with a sequence of non-specifc lipid-transfer protein (nsLTP) from *C. baccatum* plant (Sequence ID: PHT38497.1); 75% identity and 83% positivity with a sequence of nsLTP from *C. chinense* plant (Sequence ID: PHU22162.1); 75% identity and 83% positivity with a sequence of nsLTP1—like from *C. chinense* plant (Sequence ID: XP_016565683.1); and 75% identity and 83% positivity with a sequence of nsLTP from *C. annuum* plant (Sequence ID: PHT86247.1) (Fig. 10a).

Several plant defense genes encoding for LTPs have already been identifed in *C. annuum* (Do et al., 2004), for example, the CALTPI and CALTPIII genes, which show differences in structure and sequence, are transcriptionally activated in *C. annuum* tissues by pathogen infection, abiotic and environmental stresses. In seeds and fruits of *Capsicum* species, peptides similar to LTPs were characterized, however there are no reports of the presence of these peptides in leaves of that plant (Cruz et al., 2010; Diz et al., 2011; Maracahipes et al., 2019; Santos et al., 2020).

Band R1 showed similarity with the sequence of ethylene-responsive proteinase inhibitor 1: 90% identity and 88% positivity with the predicted sequence of ethylene-responsive proteinase inhibitor 1-like from *C. annuum* (Sequence ID: XP_016567356.1); 90% identity and 88% positivity with the sequence of ethylene-responsive proteinase inhibitor 1 from *C. baccatum* (Sequence ID: PHT57973.1); 83% identity and 90% positivity with the predicted sequence of trypsin inhibitor 1 from *Nicotiana attenuata* (Sequence ID: XP_019254743.1); and 83% identity and 90% positivity with the predicted sequence of trypsin inhibitor 1 from *Nicotiana sylvestris* (Sequence ID: XP_009774947.1) **(**Fig. 10b**)**.

The presence of protease inhibitors in different *Capsicum* species was reported by Silva et al. (2017). The seeds of *Capsicum annuum* (UENF1381

access), *C*. *baccatum* (UENF 1732 access), *C*. *baccatum* (UENF 1496 access), *C*. *chinense* (UENF 1498 access) and *C*. *frutescens* have shown the ability to inhibit trypsin activity.

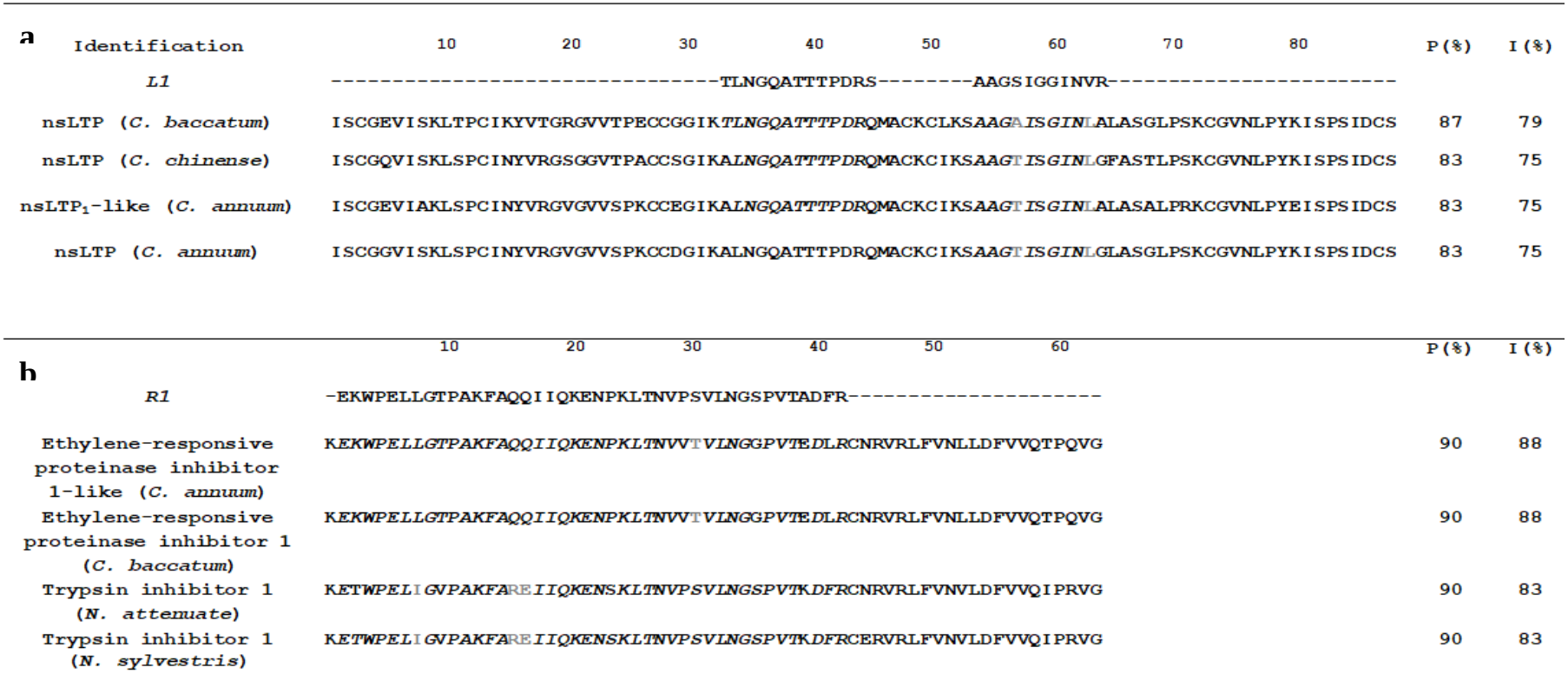


Figure 10. Alignment of amino acid residues from peptides of leaves (L1) and roots (R1) of *Capsicum annuum* (UENF1381) from LC_{48} and RC_{48} samples, respectively. The sequences showen were obtained from Blast and aligned by Clustal Omega. **(a)** The leaf peptide named L1 with approximately 6 kDa showed similarity with the following sequences: Non-specific lipid-transfer protein (nsLTP) from *Capsicum baccatum* (Sequence ID: PHT38497.1); nsLTP from *Capsicum chinense* (Sequence ID: PHU22162.1); $nsLTP_1$ - like from *C. chinense* plant (Sequence ID: XP_016565683.1); and nsLTP from *C. annuum* plant (Sequence ID: PHT86247.1). **(b)** The root peptide named R1 with approximately 5.6 kDa showed similarity with the following sequences: predicted sequence of ethylene-responsive proteinase inhibitor 1-like from *C. annuum* (Sequence ID: XP_016567356.1); ethylene-responsive proteinase inhibitor 1 from *C. baccatum* (Sequence ID: PHT57973.1); predicted sequence of trypsin inhibitor 1 from *Nicotiana attenuata* (Sequence ID: XP_019254743.1); and predicted sequence of trypsin inhibitor 1 from *Nicotiana sylvestris* (Sequence ID: XP_009774947.1). P% indicates the percentage of positive residues (that present the same physic-biochemical features) and are written in gray. I% indicates the percentage of identical residues and are written in italics. Gaps (-) were introduced for better alignment.

5.1.3. Inhibitory activity of extracts on trypsin

The inhibitory potential of leaf and root extracts from *C*. *annuum* on trypsin enzyme activity was evaluated. The leaf and root extracts were able for inhibiting trypsin activity at a concentration of 30 µg/mL (Fig. 11). The leaf extracts showed an inhibition of over 90% of the enzyme activity (Fig. 11), and the root extracts showed an inhibitory range of 56.62 to 94.6% (Fig. 11).

Different studies discuss that *Capsicum* plants respond to herbivores by accumulating metabolites and defensive proteinase inhibitors in their tissues (Mishra et al., 2012). Moulin et al. (2014) also identifed in leaves from *C. baccatum* var. pendulum the presence of trypsin inhibitor. Bard et al. (2015) identifed a 6 kDa peptide in hybrid seed extract (Ikeda×UENF 1381), identifed as a serine protease inhibitor that is capable of inhibiting human salivary and the insect *Callosobruchus maculatus* α-amylases.

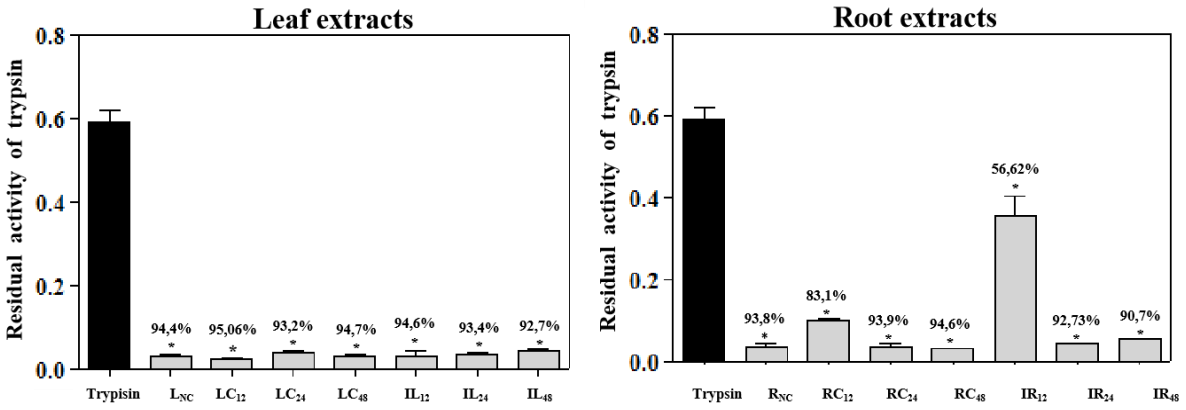


Figure 11. Inhibitory activity of trypsin in the presence of 30 µg/mL of leaf and root extracts from *C. annuum*. The assays were performed in water and at 37 °C. The values are the means (±SD) of triplicate experiments. Asterisks indicate significant differences ($p < 0.05$) between each experimental treatment and the control and above the bars are show the inhibition percentages.

5.1.4. Inhibitory activity of extracts on α- amylase

The α-amylase inhibitory activity of leaf and root extracts was measured using starch as a substrate on the insect (*Tenebrio molitor*) (Fig. 12). The leaf extract, at the tested concentration of 30 µg/mL, was able to inhibit over 90% of

the enzyme activity **(**Fig. 12**)**. The root extracts showed an inhibitory range of 84.3 to 96.7% (Fig. 12).

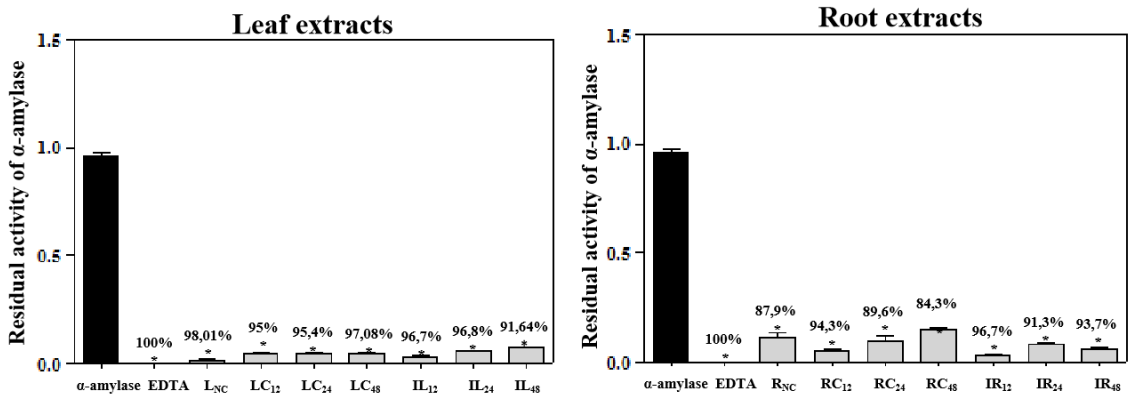


Figure 12 . Inhibitory activity of α-amylase (*Tenebrio molitor*) in the presence of 30 µg/mL of leaf and root extracts from *C. annuum*. The assays were performed in water and at 37 °C. The values are the means (±SD) of triplicate experiments. Asterisks indicate significant differences ($p < 0.05$) between each experimental treatment and the control and above the bars are show the inhibition percentages.

Silva et al. (2018) characterized an antimicrobial peptide from *Vigna unguiculata* called *Vu-LTP*, which is capable of inhibiting the activity of human salivary α-amylase and intestinal α-amylases from *C. maculatus.* Gadge et al. (2015) identifed a α-amylase/trypsin inhibitor in *Cajanus cajan* seeds. It has been observed that some α-amylases present in plants are bifunctional molecules because they have the ability to inhibit the activities of α-amylases and proteases. In *Capsicum* plants, several proteins with bifunctional activity have already been identifed. Pereira et al. (2018) found that leaf and root extracts from *C*. *annuum* have the ability to inhibit both α-amylase and trypsin activities and Santos et al. (2017) found this double inhibitory activity to a peptide from *C*. *annuum* fruits.

5.1.5. Determination of β-1,3-glucanase activity

The activity of β-1,3-glucanase was detected in leaf and root extracts (Fig. 13). The enzyme activity was low, although all the inhibitory activity of extracts were signifcant when compared to the control. Better results were observed with

R_{NC} and IR_{48}. In these extracts, the β-1,3-glucanase activity was higher than that of the control (Fig. 13). Similar results were reported by Maracahipes et al. (2019) in fruit extracts from *C*. *annuum*. β-1,3-glucanases are enzymes present in various plant species and exhibit physiological functions primarily related to defense. Aggarwal et al. (2011) monitored the presence of β-1,3-glucanase in 12 wheat genotypes inoculated with *Bipolaris sorokiniana*. It was observed that 7 genotypes were resistant to the fungus, that genes related to the resistance process were present only in resistant plants and also that the enzyme accumulates in response to the pathogen. It was observed the induction of proteins with β-1,3-glucanase activities in the leaves of *C*. *annuum*, during infection caused by phytopathogenic microorganisms (Egea-Gilabert et al., 1996; Wang et al., 2013). As β-1,3-glucanase protects against phytopathogenic fungi invasion of plants it can be used as a biological control in agricultural applications (Bertoldo and Mazaro 2018; Castoria et al.,1997).

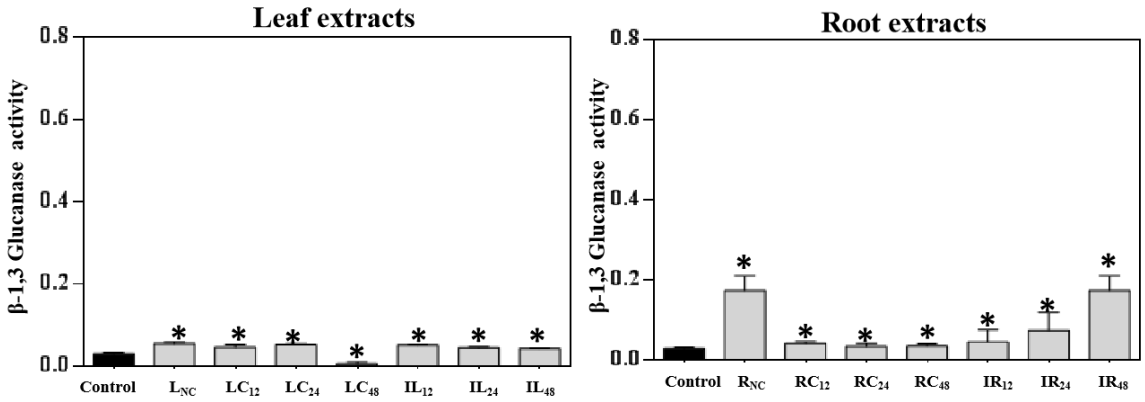


Figure 13. Determination of β-1,3-glucanase activity in the presence of 30 µg/mL of leaf and root extracts from *C. annuum.* The values are the means (±SD) of triplicate experiments. Asterisks indicate significant differences ($p < 0.05$) between each experimental treatment and the control.

5.1.6. Efect of extracts on bacterial growth

We evaluated the effect of leaf and root extracts on the growth of *X*. *euvesicatoria*. The inhibition halo was measured at 24 and 48 h. The extracts

L_{NC}, LC_{12}, LC_{24}, IL_{12}, IL_{24}, R_{NC}, RC_{12}, RC_{24}, RC_{48}, IR_{12}, IR_{24}, IR_{48} and control were not able to inhibit bacterial growth (data not shown). Only the extracts LC_{48} and IL_{48} were able to promote the formation of a significant inhibition halo at a concentration of 100 µg/mL. After 48 h, with LC_{48}, a halo of 0.47 mm in diameter was observed, and with IL_{48}, a halo with a diameter of 1.92 mm was observed (Figure 14). These results differ from Pereira et al. (2018), where root extracts were more effective against this bacterium.

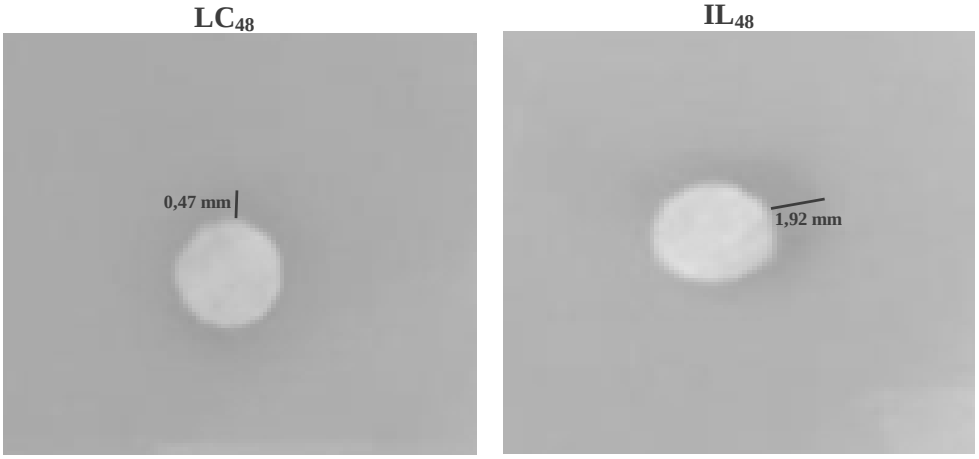


Figure 14. *Xanthomonas euvesicatoria* antibiogram in the presence of 100 µg/mL of leaf extract from *Capsicum annuum* for 24 h at 28 °C. LC_{48}-water-inoculated leaf extract after 48 h of inoculation and IL_{48}-*X. euvesicatoria*-inoculated leaf extract after 48 h of inoculation. Inhibition halo in mm.

5.1.7. Efect of extracts on fungal growth

To evaluate the efect of leaf and root extracts from *C. annuum* on *C. scovillei* growth, the fungus was first grown for a period of 48 h in the presence of 200 µg/mL of leaf and root extracts (Fig. 15). The leaf extracts L_{NC}, LC_{12}, LC_{24}, LC_{48}, IL_{12} and IL_{48} were able to inhibit 100% of the fungal growth, and the IL_{24} extract inhibited approximately 96.7%. Only the root extracts R_{NC} and RC_{48} were able to inhibit 100% of the fungal growth. Moreover, the RC_{12} extract inhibited approximately 9.6% of the fungal growth, the RC_{24} extract inhibited approximately

61.09%, the IR_{12} extract inhibited approximately 79.5%, the IR24 extract inhibited approximately 40.08% and the IR48 extract inhibited approximately 48.77%.

In the next experiment, a lower concentration (100 µg/mL) of all extracts was used. The leaf extracts L_{NC}, LC_{12}, LC_{24}, LC_{48}, IL_{12}, IL_{24} and IL_{48} were able to inhibit from 51.3 to 64.3% of the fungal growth. The root extracts RC_{12}, RC_{24}, RC_{48}, IR_{12}, IR_{24} and IR_{48} were able to inhibit from 35.48 to 52.48% of the fungal growth, and the R_{NC} extract was able to inhibit 98.14%.

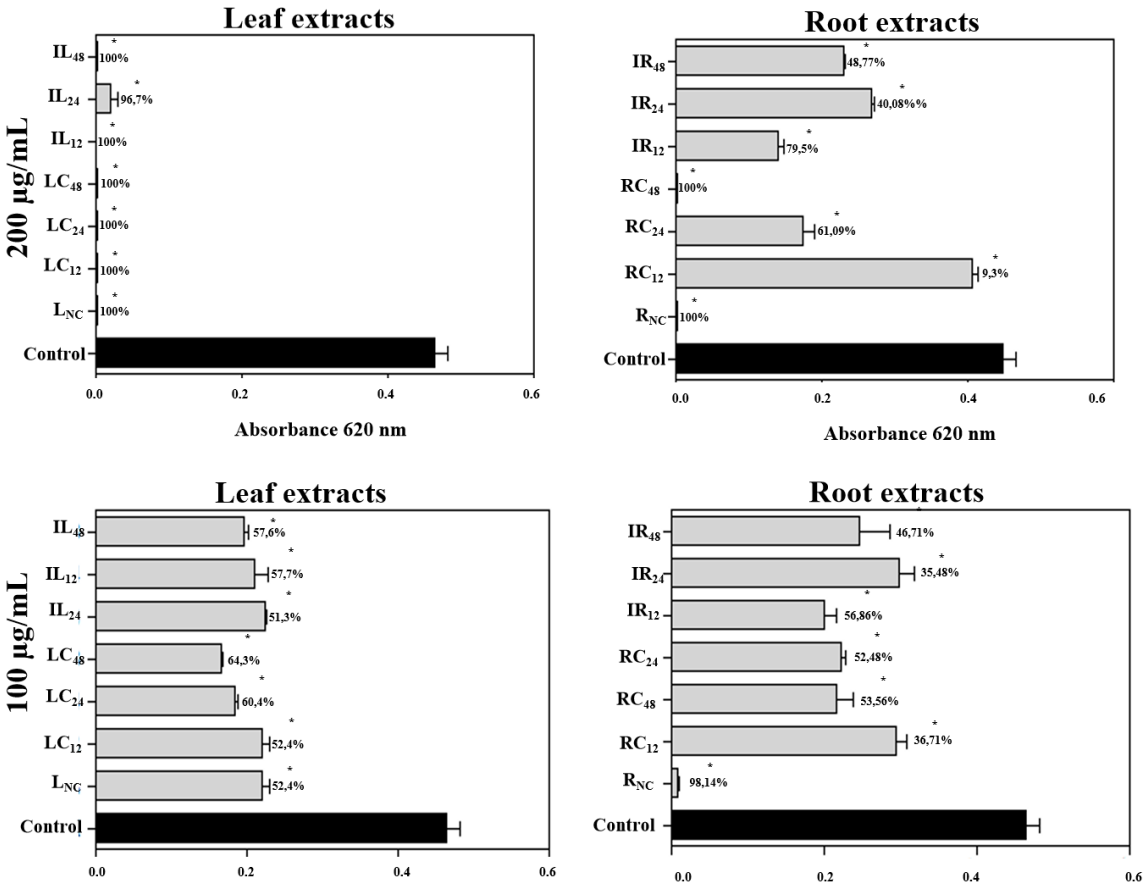


Figure 15. Growth inhibition assay of *C. scovillei* in the presence of 200 amd 100 µg/mL of leaf and root extracts. The growth was observed until 48 h. The values are the means (±SD) of triplicate experiments. Asterisks indicate significant differences ($p < 0.05$) between the experimental treatment and control at 48 h of the experiment and the control and above the bars are shown the inhibition percentages.

The Figure 16 shows some morphological changes, were observed in leaf extracts LC_{12}, LC_{24}, LC_{48}, IL_{12}, IL_{24} and IL_{48} and the root extracts R_{NC}, RC_{24}, RC_{48}, IR_{12} and IR_{48}, such as the formation of vacuoles and an increase in hyphal ramifications when compared with the control. The presence of spores without germinating can also be observed. The changes were observed 24 h after incubation of fungual cells with 100 µg/mL leaf and root extracts.

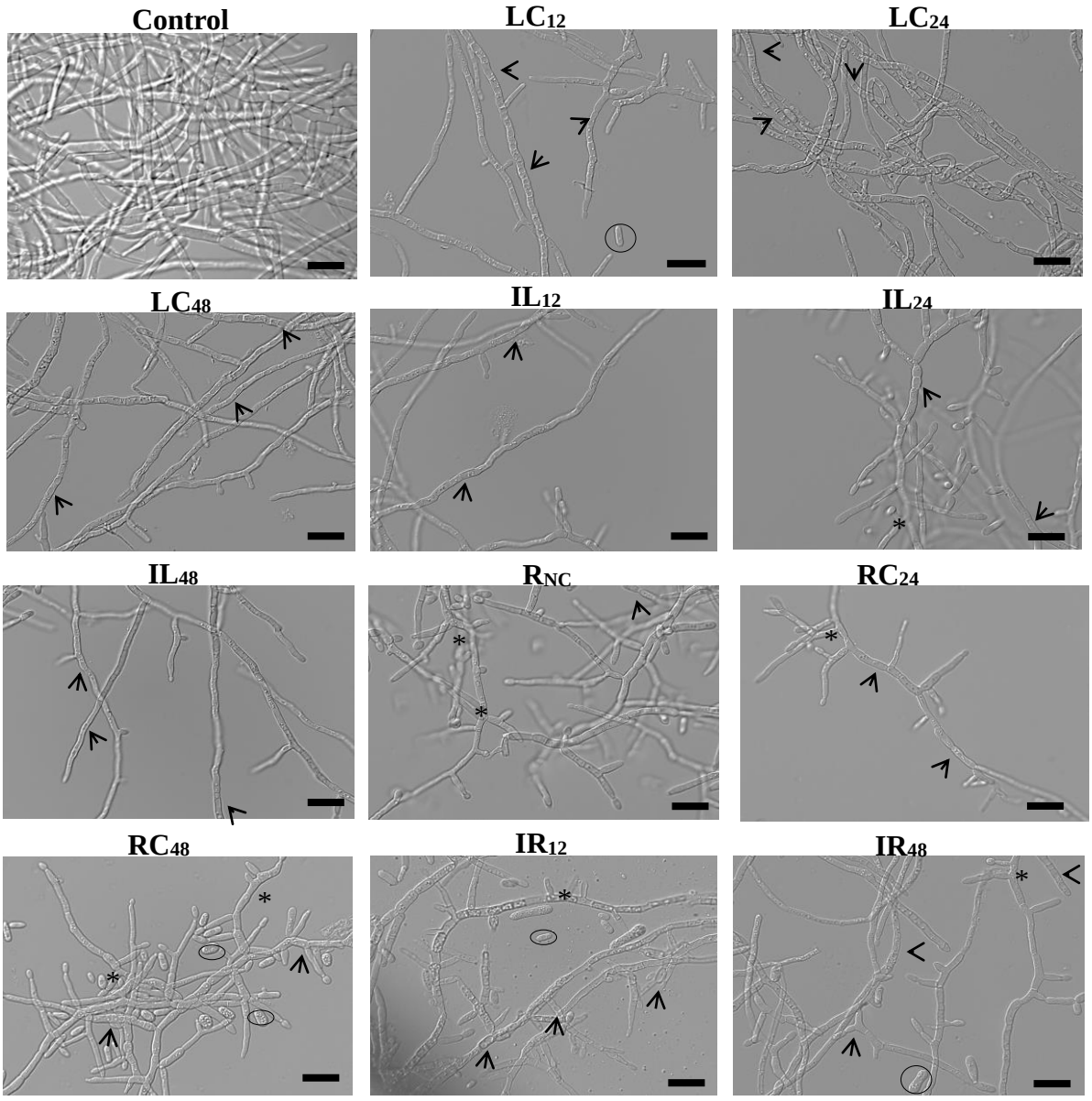


Figure 16. Morphological changes of *C. scovillei* fungus after treatment with 100 µg/mL of leaf and root extracts from *C*. *annuum*. Morphological changes were observed 24 h after exposure, and control cells were not treated with the extracts. Bars = 20 µm.The arrows indicate vacuolization, Asterisks indicate hyper-branching and circles indicate spores without germinating.

Capsicum plants have several biological activities, among them, antifungal activity. *Capsicum* extracts have been able to inhibit fungal growth in low concentrations. Soumya and Bindu (2012) demonstrated the antifungal efcacy of leaf and fruit extracts from *C*. *frutescens* L. against the fungi *Aspergillus favus*, *A*. *niger*, *Penicillium* sp. and *Rhizopus* sp. At 10 mg/mL the leaf extract inhibited 88.06% of *A. favus* growth, while the fruit extract inhibited 88.33% of the *A. niger*. Maracahipes et al. (2019) identifed diferent AMPs in *Capsicum annuum* fruits such as defensin, LTP and protease inhibitor.

In their studies, it was found that *C. annuum* fruit extracts are potent inhibitors of the fungus *C. gloeosporioides* growth*.* At 200 µg/mL total inhibition of the pathogen was verifed. Pereira et al. (2018) identifed that leaf and root extracts from *C*. *annuum* inhibit the growth of the fungus *C. lindemuthianum.* Root extracts were also able to inhibit the total growth of the fungus *Colletotrichum gloeosporioides* at 1000 µg/mL. The mechanism of action used by these molecules present in the extracts was suggested as membrane permeabilization.

5.1.8. Plasma membrane permeabilization

To evaluate whether the leaf and root extracts were able to permeate the membranes of *C*. *scovillei* and thus begin to understand the mechanism of action of these peptides, we incubated the fungus, treated with extracts at 100 µg/mL after 24 h, with the fluorescent probe *Sytox Green* and observed the fuorescence by microscopy. Figure 17 shows *C*. *scovillei* control hyphal with normal morphology. The LC_{12} and RC_{24} extracts were able to permeabilize the membranes of *C*. *scovillei.* The leaf extracts L_{NC}, LC_{24}, LC_{48}, IL_{12}, IL_{24}, IL_{48} and the root extracts R_{NC}, RC_{12}, RC_{48}, IR_{12}, IR_{24} and IR_{48} were not able to permeabilize the membrane because they did not exhibit fuorescence (data not shown).

Most AMPs are cationic and amphipathic, this tast characteristic allows for stronger interaction with negative charges present the membrane of microorganism. Thus, conformational chage, which can act by several mechanisms. The mechanism of action of AMPs are highly infuenced by the structure of the peptide and by the characteristic of the lipid membrane of the microorganism (Bechinger and Gorr, 2017).

The membrane permeabilization is one of the most described mechanisms of action for AMPs. Several studies show that *Capsicum* peptides are able to permeate the membrane of microorganisms. For example, fractions (F4 and F5) of fruit extracts of *C. chinense* are able to permeate the membrane of the fungi *Fusarium solani* and *F*. *oxysporum*, and furthermore the fractions were able to induce the increase of reactive oxygen species in *F*. *Solani* cells. Some authors have described membrane permeation and endogenous ROS increase as correlated mechanisms (Santos et al., 2020).

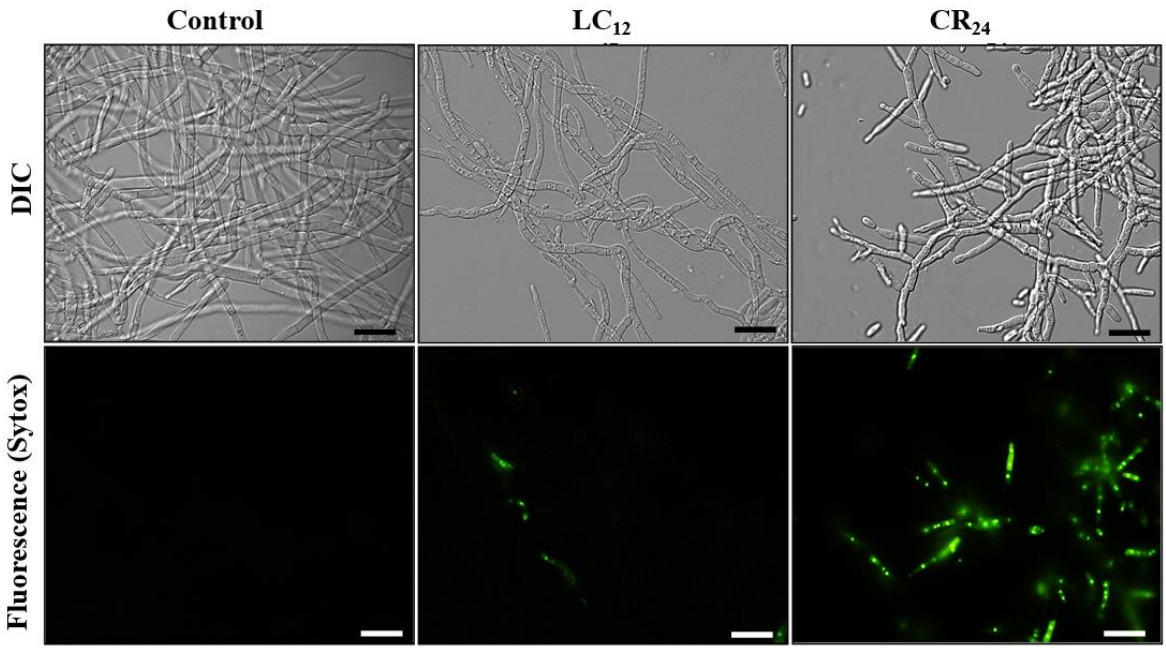


Figure 17. Membrane permeabilization assay of *C*. *scovillei* cells by fluorescence microscopy using the fluorescent probe *Sytox Green*. Cells were treated with 100 µg/mL of extracts and then assayed for membrane permeabilization. Control cells were treated only with the *Sytox Green* probe. Bars = 20 µm.

5.1.9. Intracellular ROS induction

Evaluation of the leaf and root extracts showed that they could induce increase in reactive oxygen species (ROS) levels. *C*. *scovillei* was treated with 100 µg/ mL of extracts and then incubated for 30 mim with the probe 2′,7′-dichlorofuorescein diacetate (H_2DCFDA) and observed by fuorescence

microscopy. In Figure 18, the fungus in the control is observed the normal morphology.

The LC_{24} and IR_{12} extracts caused changes in fungal morphology and an increase in ROS levels, as observed by an increase in fuorescence. The leaf extracts L_{NC}, LC_{12}, LC_{48}, IL_{12}, IL_{24}, IL_{48} and the root extracts R_{NC}, RC_{12}, RC_{48}, IR24 and IR_{48} did not induce increase reactive oxygen species levels (data not shown).

Studies suggest that membrane permeability is the main mechanism that would lead to the death of microorganisms (Lei et al., 2019). Others studies have suggested membrane permeabilization as an event linked to production of ROS (Koprivnjak and Peschel, 2011; Malanovic and Lohner, 2016). Gebara et al. (2020) shows some fractions of *C*. *annuum* fruit extract induced changes in the membrane of some strains of yeasts of the *Candida* species leading to permeabilization and observed that production of ROS was induced by fractions in some yeast strains. In *C*. *annuum* fruits, Maracahipes et al. (2019) noted that 200 µg/mL of a fraction called F7 was able to permeate the membrane of *C*. *gloeosporioides* and induce an increase in ROS. In addition, loss of mitochondrial viability was observed.

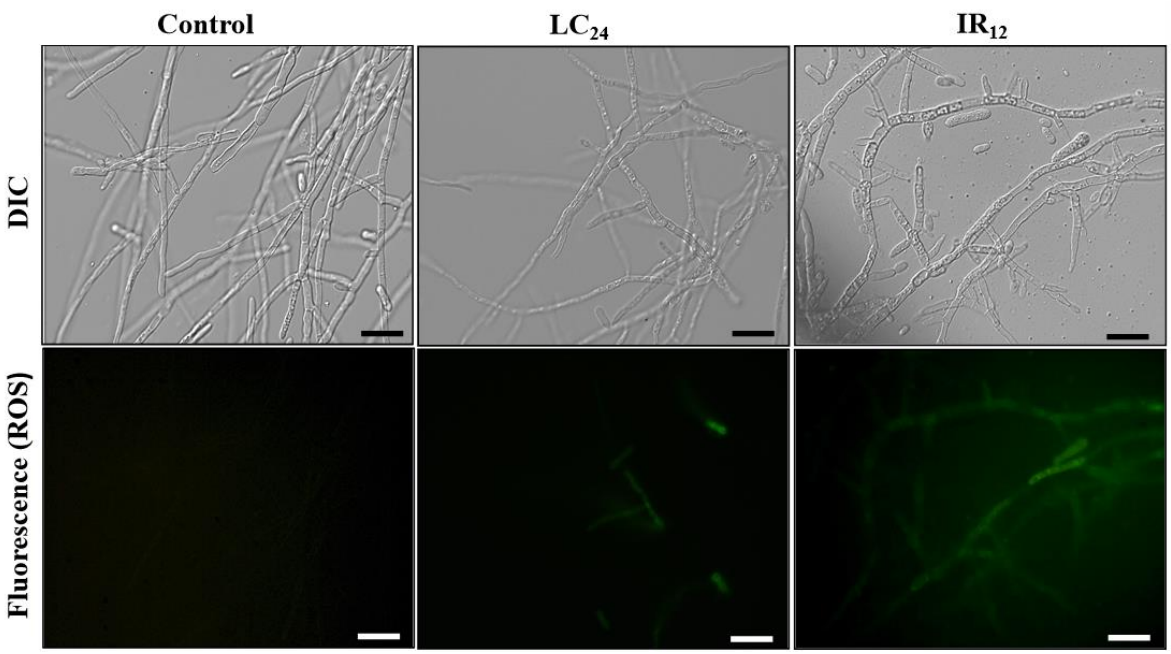


Figure 18. Cells of *C*. *scovillei* after ROS induction assay by light microscopy using the H2DCFDA probe. Cells were treated with 100 µg/mL of extracts for 24 h and then assayed for oxidative stress. Control cells were treated only with H2DCFDA. Bars = 20 µm.

5.1.10. Mitochondrial functionality assay

Figure 19 shows the results to the mitochondrial functionality of *C*. *scovillei* after 24 h of incubation with 100 µg/mL of diferent extracts. In leaf extracts L_{NC}, LC_{12}, LC_{24}, LC_{48} and the root extracts R_{NC}, RC_{12}, RC_{24}, IR_{12} and IR_{24}, the cells had decreased mitochondrial activity compared to control cells (without extract treatments), as observed by the low fuorescence signal of Rhodamine 123 probe.

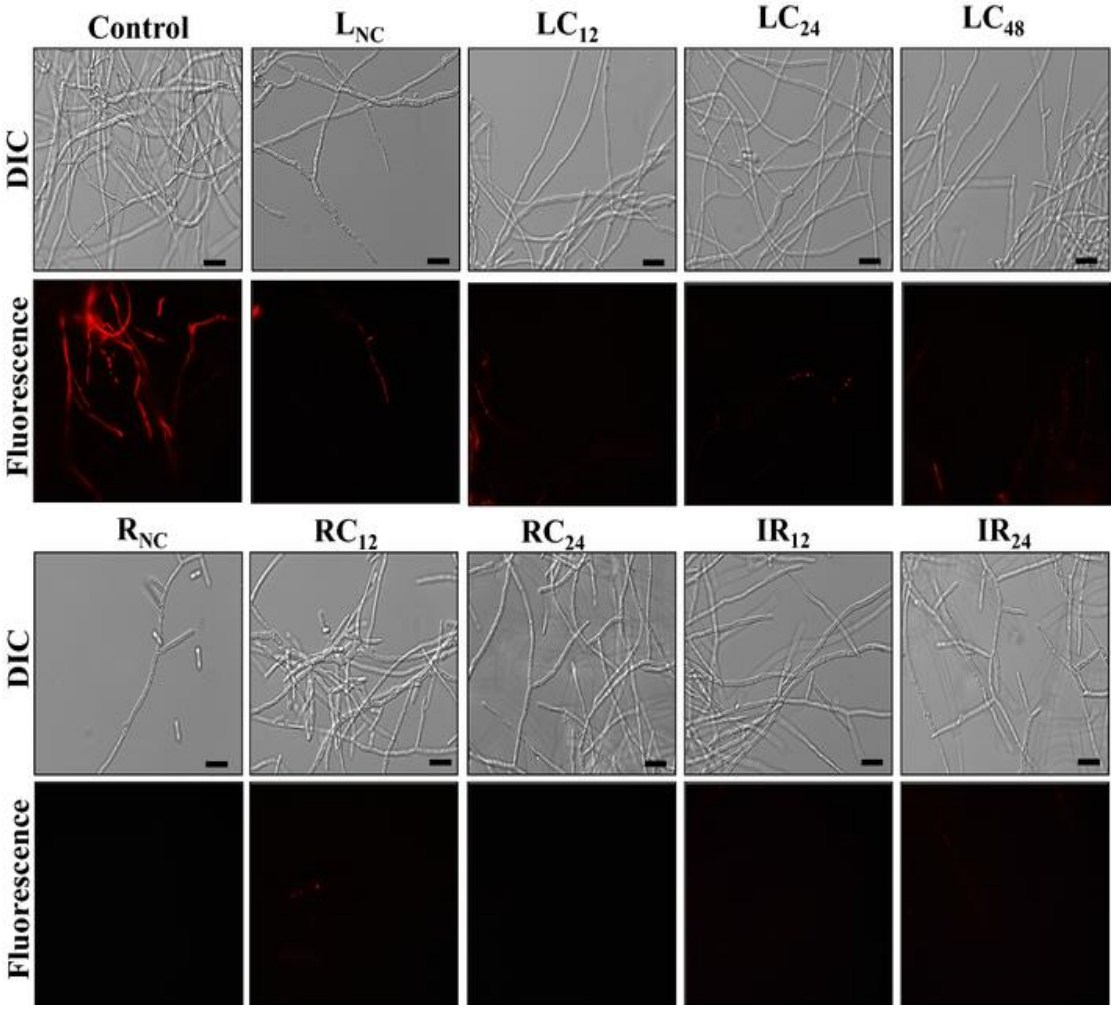


Figure 19. Cells of *C. scovillei* after the mitochondrial functionality assay, visualized by fluorescence microscopy using the Rhodamine 123 fluorescent probe. Cells were treated with 100 µg/mL of leaf and root extracts for 24 h and then analysed for mitochondrial functionality. Control cells were treated only with the Rhodamine 123 probe. Bars = 20 µm.

It is reported that some of the cationic AMPs interact with fungal organelles, such as mitochondria, leading to fulgal death. It is observed that cationic AMPs that are rich in amino acids such as histidine have high activity against fungal cells. These peptides can interact with intracellular mitochondria, lead to the efux of ATP without cell lysis and block mitochondrial respiration, which can lead to damage to the mitochondrial membrane and the plasma membrane, and trigger ion efuxes and cell death (Li et al., 2018; Taniguchi et al., 2019).

5.2. Purification and characterization of *Capsicum annuum* leaf and root antimicrobial peptides: antimicrobial activity against *Colletotrichum scovillei*

In the first part of this paper, it was showed that there is inhibitory activity against phytopathogenic microorganisms and inhibitory activity in different enzyme families in leaf and root extracts from *C. annuum* (UENF 1381). It had been identified that proteins from leaf and root extracts from *C. annuum* (UENF1381) showed that most bands had a low molecular mass (6 to 14 kDa) and that every leaf and root extracts inhibited trypsin and α-amylase activity significantly, and that are able to inhibit *C. scovillei* growth meaningly.

After finding these results with the extracts, partial purification of the AMPs of the LC_{48} and RC_{48} extracts was done by HPLC chromatography. These extracts were chosen for partial purification because two different families of antimicrobial peptides had been identified in these fractions. The sequences of L1 (present in LC_{48}) and R1 (present in RC_{48}) were compared to the sequences reported in NCBI-BLAST protein database. For the alignment, L1 band showed similarity to the sequences of non-specific transfer protein, and R1 band showed similarity to the sequence of proteinase inhibitor, as well as they were suppressed in plants inoculated with *X*. *euvesicatoria*.

5.2.1. Purification and characterization of LC_{48} and RC_{48} extracts

The profile of the LC_{48} extract is shown in Figure 20A. Peaks were named L1', L2, L3, L4, L5, L6, L7, L8, L9, L10, L11, L12, L13, L14, L15, L16, L17, L18, L19, L20, L21, L22, and L23 and retention times ranging from 7 to 66 min. The HPLC chromatographic profile of the RC_{48} extract is shown in Figure 20B, in

which, peaks were named R1', R2, R3, R4, R5, R6, R7, R8, R9, R10, R11, R12, R13, R14, R15, R16, R17, R18, R19, R20, R21, R22, R23, R24, R25, R26, and R27 and retention times from 7 to 64 min.

Many parts of plants are sources of biologically active compounds such as AMPs. AMPs had shown one of the main barriers to plant immunity to biotic and abiotic factors. Most methods for extracting and isolating AMPs or for obtaining specific AMPs are based on the plant's organs. These processes do not comprise the molecular diversity of all AMPs' family existent in the plant (Barashkova et al., 2020).

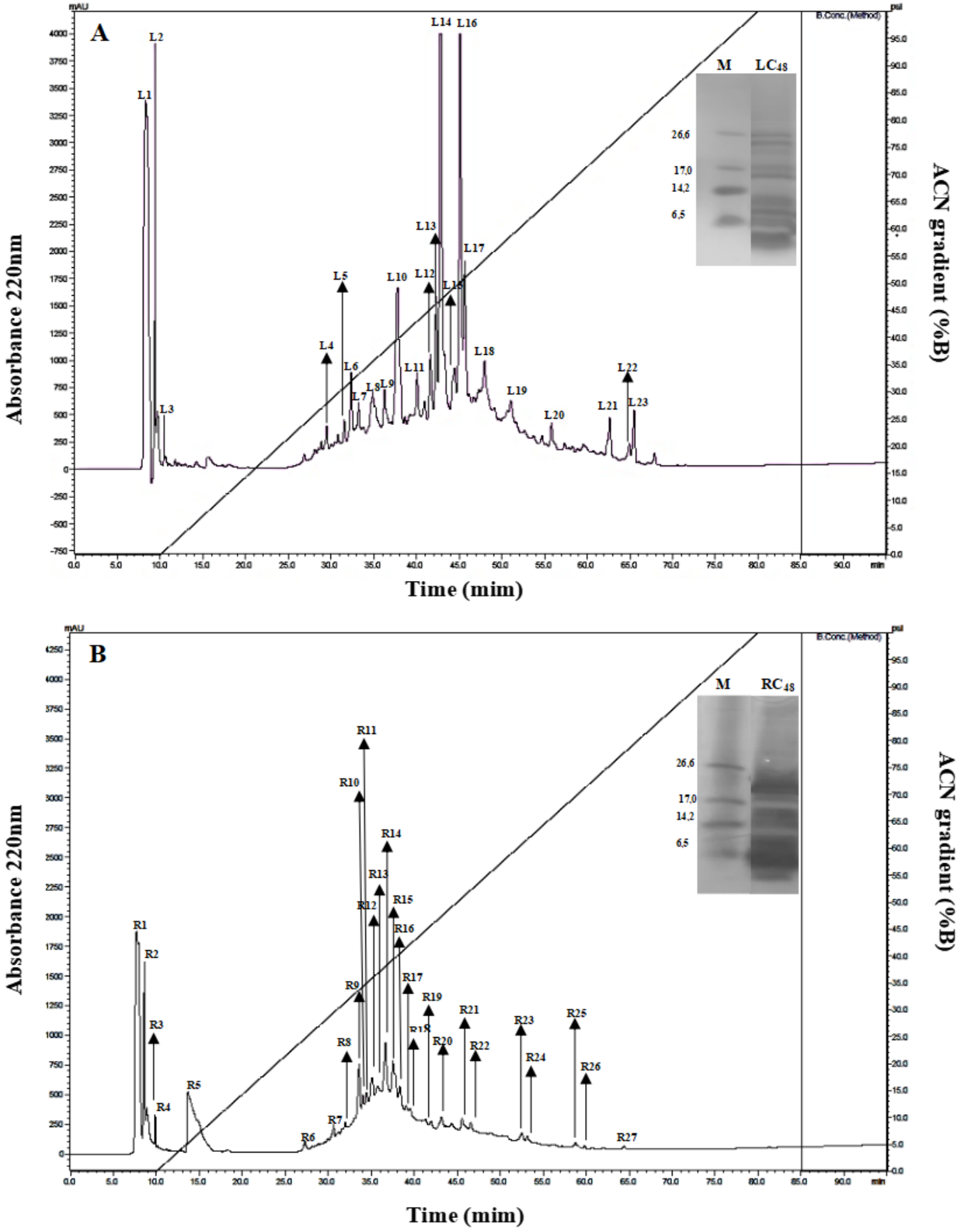


Figure 20. Chromatogram of LC_{48} **(A)** and RC_{48} **(B)** extracts in reverse-phase C2/C18 with guard column C8 chromatogram . Peptides were eluted with solvents A (0.1% TFA and 0.2% ACN v/v) and B (0.1% TFA and 80% ACN v/v) and monitored at 220 nm with a discontinuous gradient of increasing ACN. The lines represent the ACN gradient at a flow rate of 0.5 ml/mim.

The protein profiles of chromatographic fractions were analyzed by gel electrophoresis (Fig. 21). Most fractions showed protein bands of approximately 6.5 kDa, but proteins greater than 14 kDa were also present. Fractions L2, L3, L4, L5, R1', R2, R3, R4, R5, and R7 had no protein bands; fractions R9 and R15 were not applied to the gel because their protein content was not enough. It is important to note that many studies involve AMPs isolated from seeds and fruit, and this paper is one of the main studies showing the abundance and diversity of peptides in leaf and root of plants of the genus *Capsicum* (Pereira et al., 2021). Furthermore, it is clearly visible that the fractions obtained from leaves have a greater diversity of proteins than the fractions obtained from roots, which validates the data already described for other plant genera (Aleinein et al., 2015; Pereira et al., 2018; Tang et al., 2018; Teixeira et al., 2006).

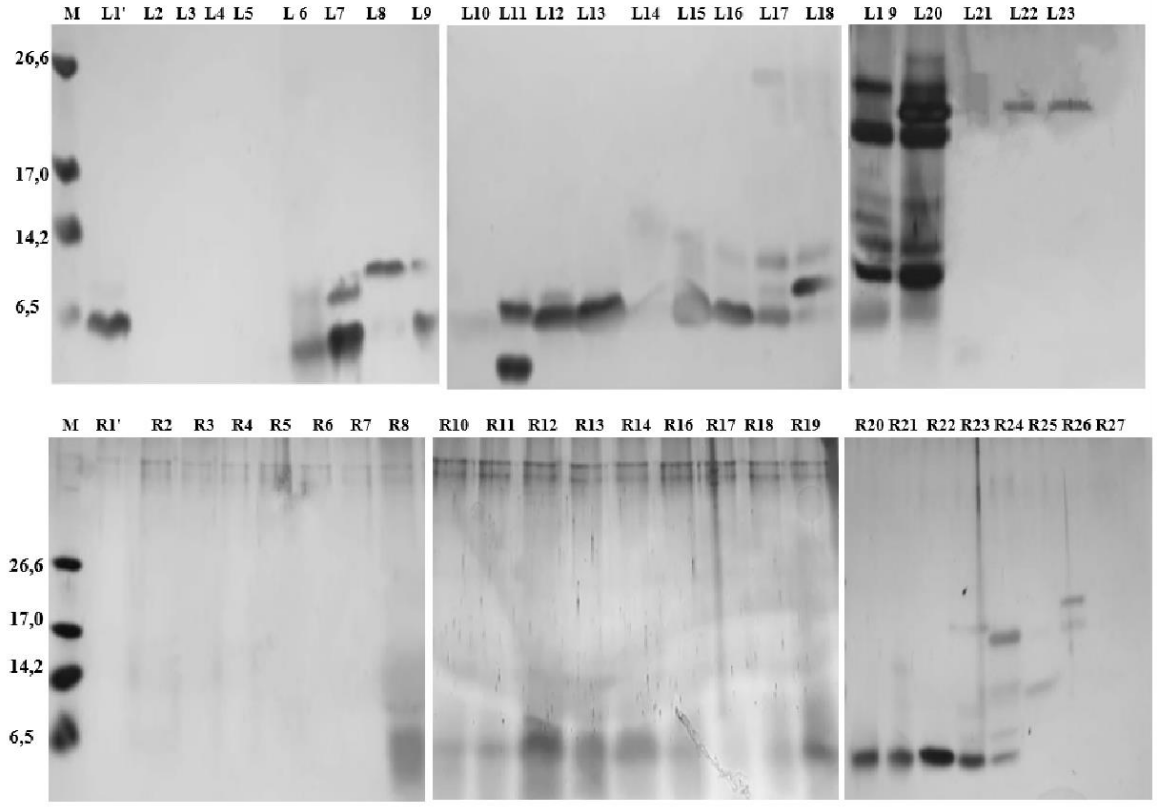


Figure 21. Electrophoretic visualization of proteins from extracts of *Capsicum annuum* L. UENF1381 leaves (LC_{48}) and roots (RC_{48}) (10 µg/mL), subjected to reverse-phase chromatography, by SDS-tricine-PAGE. The protein bands were stained with silver nitrate.

5.2.2. Efect of HPLC fractions on fungal growth

Figure 22 shows the growth of *C.scovillei* in the presence of 10 µg/mL leaf fractions obtained from C18 column. Whereas the L1', L2, L4, L6, L7, L8, L9, L10, L11, L12, L13, L14, L15, L16, L17, L18, L19, L20, L22, and L23 fractions were tested, L3 and L5 had no sufficient protein content and were not tested.

Fungus growth is inhibited by L1' (about 88.4%), L2 (32.52%), L8 (13.4%), L9 (34.52%), L10 (15.58%), L14 (7.47%), and L19 (12.13%). L4, L6, L7, L11, L12, L13, L15, L16, L17, L18, L20, L22, and L23 fractions had not inhibited the growth of *C.scovillei* at the concentration tested (10 µg/mL) (Fig. 22).

Figure 22 also shows the growth curve of *C. scovillei* in the presence of 10 µg/mL of the L1' fraction until 48 h. In addition to the decrease in growth of the test in relation to the control, it was found that the control growth had started before the test.

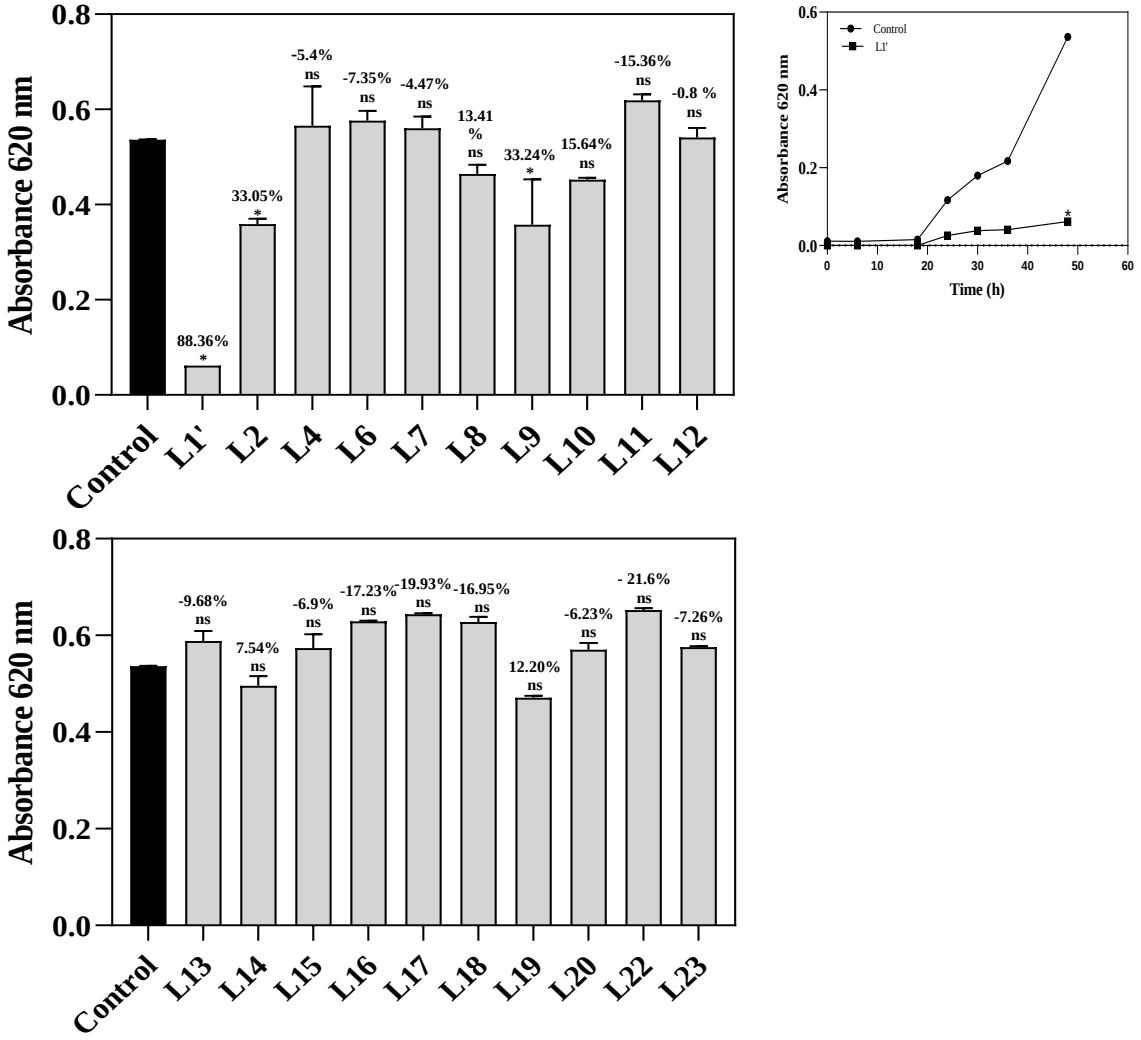


Figura 22. Growth inhibition assay of *C. scovillei* in the presence of 10 µg/mL of leaf fractions from *C*. *annuum*. The growth was observed until 48 h. (B) Insertion: Growth curve of the L1' fraction until 48 h. The values are the means (±SD) of triplicate experiments. Asterisks indicate significant differences ($p < 0.05$) between the experimental treatment and control at 48h.

Figure 23 shows the growth of *C. scovillei* in the presence of 10 µg/mL root fractions obtained from C18 column. Only R12, R13, R14, R19, R20, R22, and R26 fractions were used in this experiment, as the other fractions had not enough protein content for the test. It was found that R14 and R19 have inhibited the fungal growth from 3% to 4%. R22 and R26 have inhibited the fungal growth in approximately 11.76% and 6.53%, respectively. R12, R13, R20, and R21 fractions were not able to significantly inhibit the growth of *C. scovillei* at the concentration of the tested samples (Fig. 23).

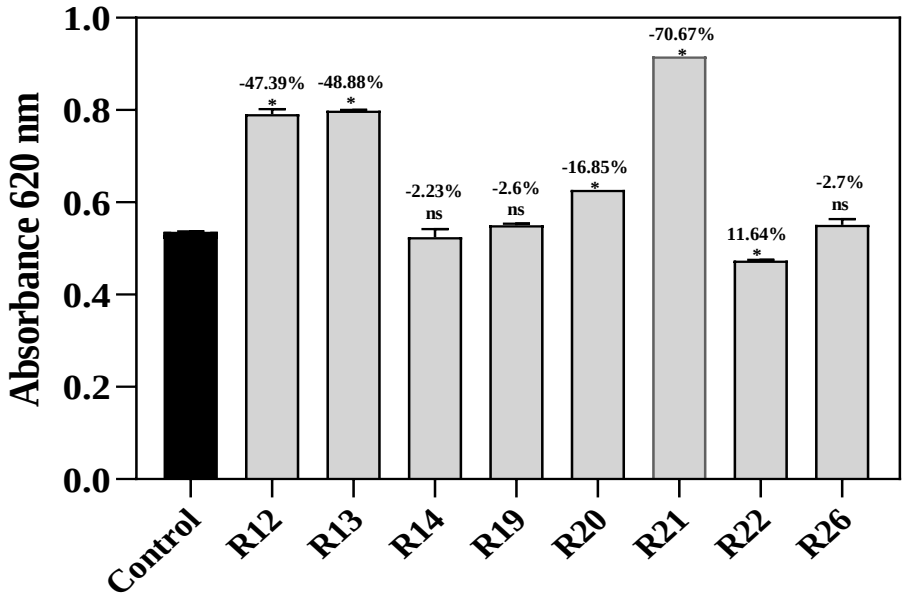


Figure 23. Growth inhibition assay of *C. scovillei* in the presence of 10 µg/mL of root fractions from *C*. *annuum*. The growth was observed until 48 h. The values are the means (±SD) of triplicate experiments. Asterisks indicate significant differences ($p < 0.05$) between the experimental treatment and control at 48 h.

The genera *Capsicum* and *Solanum* are the most usual (from 35% to 38%) on literature about identification and characterization of AMPs carried out on plants of the family Solanaceae (Afroz et al., 2020). Brito-Argáez *et al*. (2016) reported antifungal activity on a ~7.57 kDa peptide from *C*. *chinense* seeds (MIC 3–15 µg/mL). Taveira et al. (2016) identified a thionin called CaThi in *C. annuum* fruit that showed strong antimicrobial activity against six *Candida* species with IC_{50} ranging from 10 to 40 µg/mL. CaThi was able to permeabilize the membrane of all six yeasts.

The mechanisms of AMPs action identified in Solanaceae are similar to those of other AMPs reported, including changing in potential and permeability of membranes, membrane pore formation, and cell aggregation. Induction of germination and growth alterations have been reported as mechanisms of antifungal activity (Afroz et al., 2020).

5.2.3. Plasma membrane permeabilization

Analyses of leaf and root fractions effects on the *C*. *scovillei* growth revealed that fraction L1' had the highest percentage of inhibition (88.4%). Thus, L1' was chosen to continue to study the mechanism of action. So far, the results of the L1' sequence have not been obtained yet.

After the growth inhibition test for 24 h in the presence of L1', the membrane permeabilization was tested with *Sytox Green* probe (Fig. 24). Sytox Green probe passes through and marks the cell nucleus, although it does not have the ability to pass through the membrane if the cell membrane is compromised. It was found that L1' at 10 µg/mL was able to permeabilize the membrane of *C*. *scovillei*, allowing the probe to pass through and marking. Permeabilization was verified by comparing the punctual labeling among cells cultivated in the presence of L1' to the control cells (absence of L1'), in which markings were not found (Fig. 24).

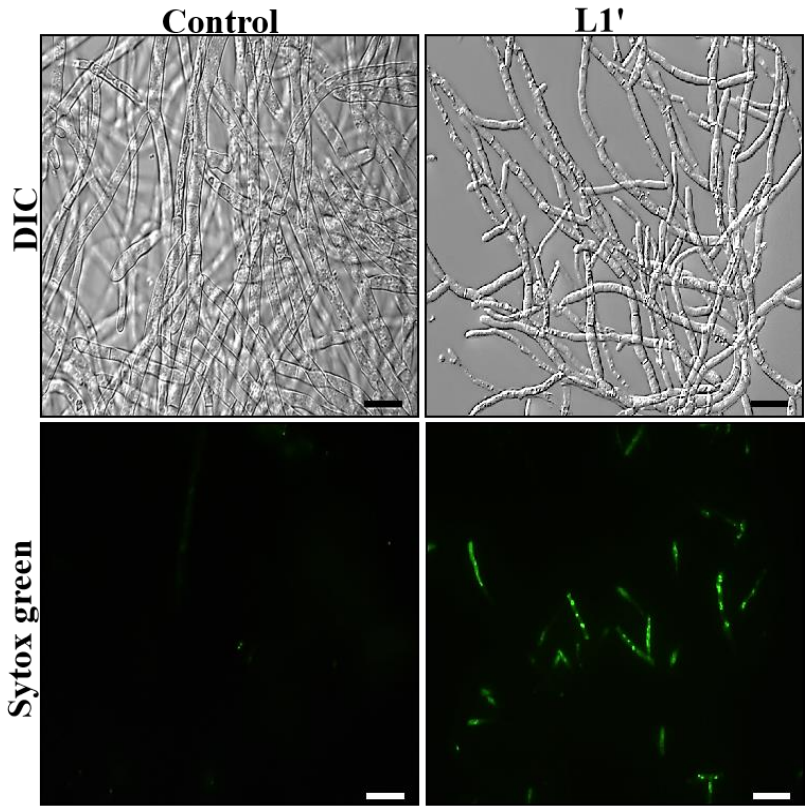


Figure 24. Images of *C*. *scovillei* hyphal by fluorescence microscopy using the fluorescent probe *Sytox Green*. Hyphal were treated with 10 µg/mL of L1' for 24 h and then assayed for membrane permeabilization. Control hyphal were treated only with the *Sytox Green* probe. Bars = 20 µm.

Damage to cell membranes can cause imbalance in osmotic pressure and, therefore, inhibit the biosynthesis of some components from the fungal plasma membrane. For example, ergosterol, an essential component of the fungal cell membrane structure ensures cell viability, retaining the integrity of the plasma membrane and maintaining the passage of cell substances (Ma et al., 2020). The cell membrane is the second barrier of fungal cells, and most AMPs have antifungal activity by membrane permeabilization (Rautenbach et al., 2016; Khan *et al*., 2019). This event is due to the positive net charge of AMPs, provided mainly by the lysine and arginine residues (Rios et al., 2016).

For example, PvD1, an isolated defensin from *Phaseolus vulgaris* seeds (Games et al., 2008), has antifungal activity against *Fusarium* species (*F. oxysporum*, *F. solani,* and *F. lateritium*) and *Candida* species (*C. albicans*, *C. tropicalis*, *C. parapsilosis,* and *C.* guilliermondii) by this mechanism (Mello et al., 2011).

5.2.4. Intracellular ROS induction

Following the study of the action mechanism, hyphal was incubated with probe dichlorodihydrofluorescein diacetate (H_2DCFDA) to investigate whether the L1' fraction was able to induce an increase in the production of ROS after the fungal growth inhibition test (presence of L1') and control (absence of L1').

Probe H_2DCFDA is able to diffuse through the cell membrane and the molecule reacts to the intracellular ROS, creating a fluorescent molecule, when hydrolyzed by esterases that catalyze the hydrolysis reaction, forming non-fluorescent molecules as the ROS production increases (Delattin et al., 2014).

In addition to permeabilizing the membrane, it was found that the L1' fraction was also able to lead to an increase in ROS in the same concentration tested (10 µg/mL) (Fig. 25).

Seyedjavadi et al. (2020) found that the MCh-AMP1 (at 32 and 64 µg/mL), an AMP isolated from *Matricaria chamomilla* L., showed fungicidal activity against *Candida albicans*. MCh-AMP1 increased the permeability of the yeast cell membrane and induced the production of ROS. Those authors found that MCh-AMP1 used these two mechanisms to cause the death of the cell of *C. albicans*. In another study, Khani et al. (2020) isolated a peptide called Skh-AMP1 from

Satureja khuzistanica. At lower concentration levels (40 and 80 µM), Skh-AMP1 showed strong antifungal activity against *Aspergillus* and *Candida* species, resulting in permeabilization of plasma membrane, production of ROS, and loss of mitochondrial functionality.

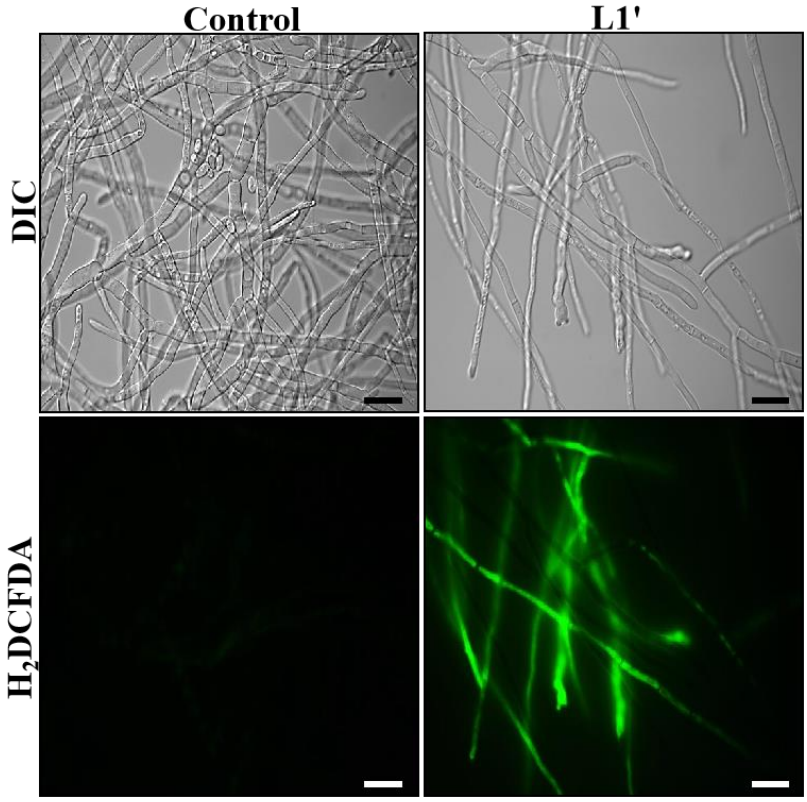


Figure 25. Images of of *C. scovillei* hyphal after ROS induction assay by fluorescence microscopy using the H_2DCFDA probe. Hyphal were treated with 10 µg/mL of L1' for 24 h and then assayed for oxidative stress. Control hyphay were treated only with H_2DCFDA. Bars = 20 µm.

5.2.5. Mitochondrial functionality determination assay

To assess the presence of intracellular targets, mitochondrial functionality was also assessed in this paper with rhodamine 123 probe (Fig. 26). Rhodamine 123 is a cationic probe at physiological pH that bears high affinity with the electrical potential of membranes and is able to mark active mitochondria. This probe is, thus, an indicator of cell viability. Therefore, after the fungal growth inhibition test, rhodamine 123 probe was incubated with test (in the presence of

L1') and control (absence of L1'). Results showed high labelling in the control, indicating the viability of the control hyphal. The test had shown loss in fluorescence intensity when compared to the control, indicating that L1' tends to decrease mitochondrial functionality at the concentration tested (Fig. 26).

At the same concentration of 10 µg/mL, Vieira et al. (2015) found that a defensin called Lp-Def1 was able to inhibit *C. albicans* growth by increased ROS induction and a significant loss of mitochondrial function. Sadat et al. (2020) identified a peptide isolated from *Matricaria chamomilla* L., called MCh-AMP1, at concentration higher than 32 and 64 µg/mL with the same action mechanism against *Candida albicans*. Maracahipes et al. (2019) found that 200 µg/mL of fruit fraction from *C*. *annuum* was also able to decrease mitochondrial functionality of *C*. *gloeosporioides*.

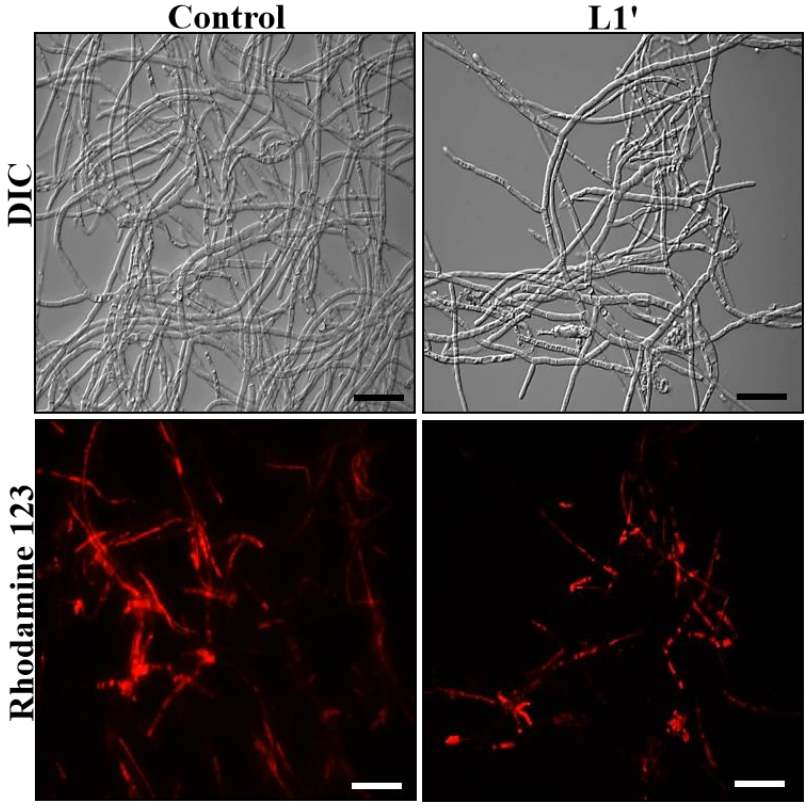


Figure 26. Images of *C*. *scovillei* hyphal after mitochondrial functionality assay by fluorescence microscopy using the fluorescent probe Rhodamine 123.Hyphal were treated with 10 µg/mL of L1' for 24 h and then analysed for mitochondrial functionality. Control hyphay were treated only with Rhodamine 123 probe. Bars = 20 µm.

5.2.6. Caspase activity detection

Caspase activity was detected by the probe CaspACE FITC-VAD-FMK, which is able to penetrate the cell and bind to it to active caspases irreversibly. No labelling was found in the test sample (presence of L1') or in the control sample (absence of L1') (Fig. 27), indicating that L1' is not able to activate the capase pathway at the concentration tested (10 µg/mL). However, Soares et al. (2017) isolated a defensin from *Adenanthera pavonina,* called ApDef1, that was able to cause death to *Saccharomyces cerevisiae* cells (LD of 7.8 µM). Among many action mechanisms used, ApDef1 was able to pass through the membrane, increase the production of H_2O_2, condense the chromatin, and activate caspases.

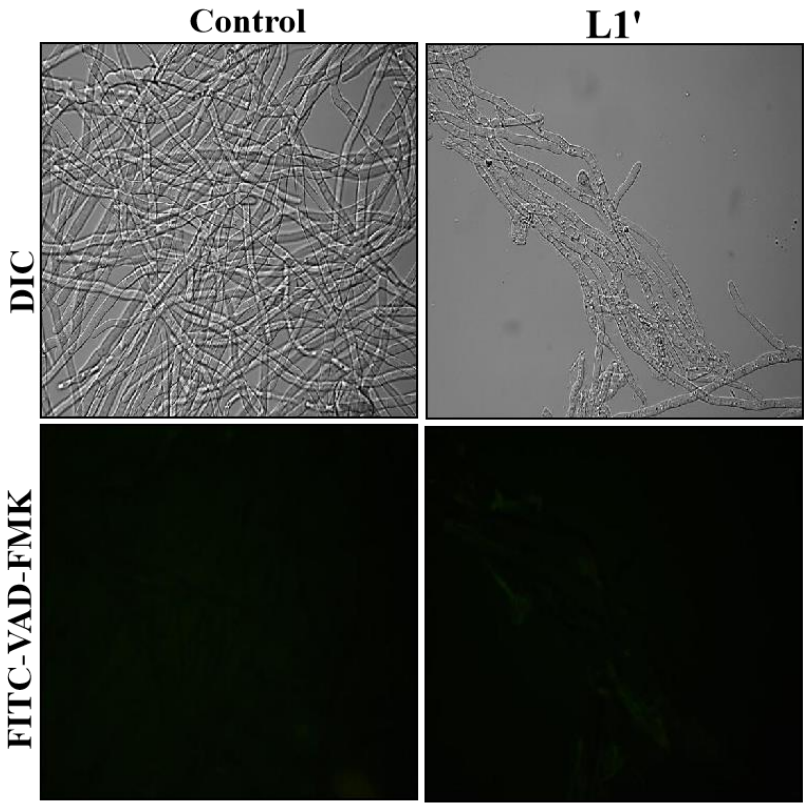


Figure 27. Images of *C. scovillei* hyphal after detection of metacaspase activity assay by fluorescence microscopy using the fluorescent probe CaspACE FITC-VAD-FMK probe. Hyphal were treated with 10 µg/mL of L1' for 24 h and then analysed for metacaspase activity. Control hyphal were treated only with CaspACE FITC-VAD-FMK probe. Bars = 20 µm.

Phytogenic microorganisms have caused significant damage to agricultural production, thus, threatening agriculture and food security (Nazarov et al., 2020). Many strategies used to control microorganisms require the use of chemicals; however, some studies have shown the harmful effect of these chemicals on human, animals, and their toxic effects on the environment (Nicolopoulou-Stamati et al., 2016; Bertero et al., 2020; Hassaan and El Nemr, 2020). It has also been shown that the indiscriminate use of these products can create resistance in phypathogenic microorganisms (Rangasamy et al., 2017). Thus, the investigation of new strategies that can be used to control phytopathogenic diseases is highly recommended.

Many phytosanitary issues have been caused serious damage to *Capsicum* crops and anthracnose being among them (Diao et al., 2017). After pathogen attack, many defense molecules are produced; these proteins include some AMPs, such as lipid transfer proteins (LTPs) and proteinase inhibitors (Iqbal et al., 2019). In the last years, our group has been identified AMPs in different parts of the plant genus *Capsicum*. The data continue to show the occurrence of AMPs in leaves and roots of *Capsicum* and can be an effective tool to control *C. scovillei,* thus, reducing crop losses caused by the disease (Diz et al., 2011; Maracahipes et al., 2019; Pereira et al., 2021).

In recent years, studies have investigated the use of antimicrobial peptides (AMPs) from plant as promising molecules in microbial control. AMPs from plants have attractive resources for pharmaceutical product and agricultural pesticides development (Das et al., 2019; Tam et al., 2015).

This paper shows an overview of the extract and fraction potential from *C. annuum*; their interaction with microorganisms can provide a roadmap for the creation of pepper resistant cultivars and biotechnological application of AMPs to control microorganisms that cause diseases in plants.

6. GENERAL CONCLUSIONS

- Leaf and root extracts from *C. annuum* presented in Tricin-SDS-PAGE a majority of proteins bands with a low molecular mass (6 to 14 kDa);
- L1 and R1 bands (selected from the LC_{48} and RC_{48} extracts, respectively) demonstrated suppressed expression in plants inoculated with *X*. *euvesicat*oria;
- The identity of L1 by mass similarity to the non-specific transfer protein and the identity of R1 was similar to proteinase inhibitor, proteins already identified in plants of the genus *Capsicum;*
- Leaf and root extracts were able to significantly inhibit the activity of trypsin and α-amylase enzymes and β-1,3-glucanase activity were detected with R_{NC} and IR_{48} extracts;
- Only the LC_{48} and IL_{48} extracts at 100 μg/mLexhibited the ability to inhibit the growth of *X*. *euvesicatoria* bacteria.
- All extracts were able to significantly inhibit the growth of *C*. *scovillei* and it was identified that most extracts showed the ability to decrease the pathogen's mitochondrial functionality;
- The L1' fraction was able to significantly inhibit *C*. *scovillei* growth through membrane permeabilization, induction of endogenous ROS and a decrease in mitochondrial functionality was observed.

REFERENCES

Afroz, J., Jubyda, F.T., Sharmin, S., Faruq, M., Dey, A.K., Ibrahim, M., Farzana, T., Sarkar, M. (2020) Multidrug resistant clinical strains isolated from tracheal aspirates of patients in Dhaka, Bangladesh. *J Res Med Sci*, 8(3):1094.

Aggarwal, R., Purwar, S., Kharbikar, L., Gupta, S. (2011) Induction of a wheat β-1,3-glucanase gene during the defense response to *Bipolaris sorokiniana*. *Acta Phytopathol Entomol Hung*, 46:39–47.

Aguilar-Toal, J.E., Deering, A. J., Liceaga, A.M. (2020). New insights into the antimicrobial properties of hydrolysates and peptide fractions derived from chia seed (*Salvia hispanica* L.). *Probiotics Antimicrob*, 12(4):1571–1581.

Aleinein, R.A., Schäfer, H., Wink, M. (2015) Rhizosecretion of the recombinant antimicrobial peptide ranalexin from the transgenic Tobacco hairy roots. *Bot Sci*, 1:45–55.

ANVISA, UFPR. Seminário de mercado de agrotóxico e regulação. Brasilia: *ANVISA*, Acesso em: 11 de abril de 2018.

Astley, D. (2007) Genetic resources, chromosome engineering and crop improvement: vegetable crops*. Exp Agr*, 3:530*.*

Azevedo, C.P., Filho, A.C., Henz, P.G., Reis, A. (2006) Recomendações de manejo da antracnose do pimentão e das pimentas. *EMBRAPA*, 1414–9850.

Azmi, S., Hussain, M.K. (2021) Analysis of structures, functions, and transgenicity of phytopeptides defensin and thionin: a review. *Beni-Suef Univ J Basic Appl Sci*, 10:5.

Barashkova, A.S., Rogozhin, E.A. (2020) Isolation of antimicrobial peptides from different plant sources: Does a general extraction method exist?. *Plant Methods*, 16:143.

Bard, G.C.V., Nascimento, V.V., Ribeiro, S.F.F., Rodrigues, R., Pereles, J., Teixeira-Ferreira, A., Carvalho, A.O., Fernandes, K.V., Gomes, V.M. (2015) Characterization of peptides from *Capsicum annuum* hybrid seeds with inhibitory activity against α-amylase, serine proteinases and fungi. *Protein J*, 34:122–129.

Basu, S.K., De, A.K. (2003) *Capsicum*: historical and botanical perspectives. In: De, A.K. (ed) *Capsicum*: the genus *Capsicum*. Taylor and Francis, London. 1–15.

Batiha, G.E., Alqahtani, A., Ojo, O.A., Shaheen, H.M., Wasef, L., Elzeiny, M., Ismail, M., Shalaby, M., Murata, T., Zaragoza-Bastida, A., Rivero-Perez, N., Magdy Beshbishy, A., Kasozi, K.I., Jeandet, P., Hetta, H.F. (2020) Biological properties, bioactive constituents, and pharmacokinetics of some *Capsicum* spp. and capsaicinoids. *Int J Mol Sci*, *21*(15):5179.

Bechinger, B., Gorr, S-U. (2017) Antimicrobial peptides mechanisms of action and resistance. *J Dent Res*, 96:254–260.

Benko-Iseppon, A.M., Lins Galdino, S., Calsa Jr, T., Akio Kido, E., Tossi, A., Carlos Belarmino, L., Crovella, S. (2010) Overview on plant antimicrobial peptides. *Curr Protein Pept Sci*, 11(3):181–188.

Bento, C.S., De Souza, A.G., Sudré, C.P., Pimenta, S., Rodrigues, R. (2017) Multiple genetic resistances in *Capsicum* spp. *Int Genet Mol Biol*, 16(3):2–13.

Bernfeld, P. (1995) Amylase α e β. *Methods Enzymol*, 1:149–158.

Bertero, A., Chiari, M., Vitale, N., Zanoni, M., Faggionato, F., Biancardi, E., Biancardi, A., Caloni, F. (2020) Types of pesticides involved in domestic and wild animal poisoning in Italy. *Sci Total Environ*, 707, 136129.

Bertoldo, E., Mazaro, S.M. (2018) β-1,3 glucanases: uma revisão sob a ótica da defesa vegetal. *Sci Agrar Parana*, 17:1–13.

Bongiolo Neto, A., Reifschneider, F.J.B., Takatsu, A. (1986) Levantamento de grupos de *Xanthomonas campestris* pv. vesicatoria no Brasil. *Fitopatol Bras*, 10:325.

Brito-Argáez, L., Tamayo-Sansores, J.A., Madera-Piña, D., Garcıa-Villalobos, F.J.,´ Moo-Puc, R.E., Kú-González, Á., Villanieva, M.A., Islas-Flores, I. (2016) Biochemical characterization and immunolocalization studies of a *Capsicum chinense* Jacq. protein fraction containing DING proteins and anti-microbial activity. *Plant Physiol Biochem*, 109:502–514.

Broekaert, W.F., Terras, F.R.G., Cammue, B.P., Vanderleyden, J. (1990) An automated quantitative assay for fungal growth inhibition. *FEMS Microbial Lett*, 69:55–59.

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

Brzin , J., Kidric, M. (1995) Proteinases and their inhibitors in plants: role in normal growth and in response to various stress conditions. *Biotechnol Genet Eng Rev*, 13:421–467.

Buttimer, C., McAuliffe, O., Ross, R.P., Hill, C.O., Mahony, J., Coffey, C. (2017) Bacteriophages and bacterial plant diseases. *Front Microbiol*, 8(34):1–5.

Campos, M.L., Lião, L.M., Alves, E.S.F., Simoni, L.M., Dias, C., Franco, O.L. (2018) A structural perspective of plant antimicrobial peptides. *Biochem Journal*, 45(21):3359–3375.

Carneiro, R.F., Melo, A.A., Almeida, A.S., Moura, R.M., Chaves, R.P., Sousa, B.L., Nascimento, K.S., Sampaio, S.S., Lima, J.P.M.S., Cavada, B.S., Nagano, C.S., Sampaio, A.H. (2013) H-3, a new lectin from the marine sponge *Haliclona caerulea*: Purification and mass spectrometric characterization. *Int J Biochem Cell B*, 45:2864–2873.

Carrizo García, C., Barfuss, M., Sehr, E., Barboza, G., Samuel, R., Moscone, E., Ehrendorfer, F. (2016) Phylogenetic relationships, diversification and expansion of chili peppers (*Capsicum*, Solanaceae). *Annals of Botany*, 18(1):35–51.

Carvalho, A., Gomes, V.M. (2011) Plant defensins and defensin-like peptides-biological activities and biotechnological applications. *Curr Pharm Des*, 17(38):4270–4293.

Carvalho, A.O., Gomes, V.M. (2007) Role of plant lipid transfer proteins in plant cell physiologya concise review. *Peptides*, 28(5):1144–1153.

Castoria, R., De Curtis, F., Lima, G., De Cicco, V. (1997) β-1, 3-glucanase activity of two saprophytic yeasts and possible mode of action as biocontrol agents against postharvest diseases. *Postharvest Biol Technol*, 12:293–300.

Castro, M.S., Fontes, W. (2005). Plant defense and antimicrobial peptides. *Protein Pept Lett*, 12(1):13–18.

Charlo, H.C.O., Castoldi, R., Fernandes, C., Vargas, P.F., Braz, L.T. (2009) Cultivo de híbridos de pimentão amarelo em fibra da casca de coco. *Hortic Bras*, 27:155−159.

Chen, C., Chen, G., Hao, X., Cao, B., Chen, Q., Liu, S., Lei, J. (2011) Plant Science CaMF2, an anther-specific lipid transfer protein (LTP) gene, affects pollen development in *Capsicum annuum* L. *Plant Sci*, 81(4):439−448.

Chowanski, S., Adamski, Z., Marciniak, P., Rosinski, G., Büyükgüzel, E., Büyükgüzel, K., Falabella, P., Scrano, L., Ventrella, E., Lelario, F., Bufo, S.A. (2016) A review of bioinsecticidal activity of *Solanaceae* alkaloids. *Toxins*, 8(60):2–28.

Clemente, M., Corigliano, M.G., Pariani, S.A., Sánchez-López, E.F., Sander, V.A., Ramos-Duarte, V.A. (2019) Plant serine protease inhibitors: biotechnology application in agriculture and molecular farming. *Int J Mol Sci*, 20(6):1345.

Costa, R.A., Rodrigues, R., Sudré, C.P. (2002) Resistência à mancha-bacteriana em genótipos de pimentão. *Hortic Bras*, 20:86–89.

Cruz, L., Ribeiro, S., Carvalho, O.A., Vasconcelos, I., Rodrigues, R., Cunha, M., Gomes, V. (2010) Isolation and partial characterization of a novel lipid transfer protein (LTP) and antifungal activity of peptides from chilli pepper seeds. *Protein Pept Lett*, 17:311–318.

Das, K., Datta, K., Karmakar, S., Datta, S.K. (2019) Antimicrobial Peptides - Small but Mighty Weapons for Plants to Fight Phytopathogens. *Protein Pept Lett,* 26(10): 720–742.

Davenport, W.A. (1970) Progress report on the domestication of *Capsicum* (chili peppers). *Proc Assoc Am Geogr*, 2:46–47.

Delattin, N., Cammue, B.P., Thevissen, K. (2014) Reactive oxygen species-inducing antifungal agents and their activity against fungal biofilms. *Future Med. Chem.* 6:77–90.

Diao, Y.Z., Zhang, C., Liu, F., Wang, W.Z., Liu, L., Cai, L., Liu, X.L. (2017) *Colletotrichum* species causing anthracnose disease of chili in China. *Persoonia*. 38: 20-37

Dias, J. (2010) Impact of improved vegetable cultivars in overcoming food insecurity. *Euphytica*, 176:125–136.

Diz, M.S., Carvalho, A.O., Rodrigues, R., Neves-Ferreira, A.G., Da Cunha, M., Alves, E.W., Okorokova-Façanha, A.L., Oliveira, M.A., Perales, J., Machado, O.L., Gomes, V.M. (2006) Antimicrobial peptides from chili pepper seeds causes yeast plasma membrane permeabilization and inhibits the acidification of the medium by yeast cells. *Biochim Biophys Acta*, 1760(9):1323–32.

Diz, M.S.S., Carvalho, A.O., Ribeiro, S.F.F., Da Cunha, M., Beltramini, R., Nascimento, V.V., Machado, O.L.T., Gomes, V.M. (2011) Characterization, immunolocalization and antifungal activity of lipid transfer protein from chilii pepper (*Capsicum annuum*) seeds with novel α- amylase inhibitory properties. *Physiol Plant*, 142(3):233–246.

Do, H.M, Lee, S.C., Jung, H.W., Sohn, K.H., Hwang, B.K. (2004) Diferential 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.

Egea-Gilabert, C., Alcázar, M.D., Candela, M. (1996) β-1,3-glucanase and chitinase as pathogenesis-related proteins in the defense reaction of two *Capsicum annuum* cultivars infected with cucumber mosaic virus. *Biol Plant*, 38:437–443.

Egorov, T.A., Odintsova, I.T., Pukhalsky, A.V., Grishin, V.E. (2005) Diversity of wheat anti-microbial peptides. *Peptides*, 26(11):2064–2073.

FAOSTAT., 2019. http://www.fao.org/faostat/en/#data/QC/visualize (accessed on 12 July 2021).

Filho, R.L., Romeiro, R.S. (2009) Sensibilidade de *Xanthomonas vesicatoria* a antibióticos para desenvolvimento de um meio semi-seletivo. *Rev Tróp Ciênc Agric Biol*, 3:28–39.

Fink, W., Liefland, M., Mendgen, K. (1988) Chitinases and β-1,3-glucanases in the apoplastic compartment of oat leaves (*Avena sativa* L.). *Plant Physiol*, 88:270–275.

Gadge, P.P., Wagh, S.K., Shaikh, F.K., Tak, R.D., Padul, M.V., Kachole, M.S. (2015) A bifunctional α-amylase/trypsin inhibitor from pigeonpea seeds: Purifcation, biochemical characterization and its bioefcacy against *Helicoverpa armigera*. *Pestic Biochem Physiol*, 125:17–25.

Games, P.G., Dos Santos, I.S., Mello, E.O., Diz, M.S.S., Carvalho, A.O., de Souza-Filho, G.A., Da Cunha, M., Vasconcelos, I.M., Ferreira, B.S., Gomes, V.M. (2008) Isolation, characterization and cloning of a cDNA encoding a new antifungal defensin from *Phaseolus vulgaris* L. seeds. *Peptides*, 29:2090–2100.

Gebara, R.S., Taveira, G.B., Santos, L.A., Calixto, S.D., Simão, T.L.B.V., Lassounskaia, E., Muzitano, M.F., Teixeira-Ferreira, A., Perales, J., Rodrigues, R., Carvalho, A.O., Gomes, V.M. (2020) Identifcation and characterization of two defensins from *Capsicum annuum* fruits that exhibit antimicrobial activity. *Probiotics Antimicrob Proteins*, 12:1253–1265.

Gebhardt, C. (2016) The historical role of species from the Solanaceae plant family in genetic research. *Theor Appl Genet*, 129:2281–2294.

Goyal, R., Mattoo, A. (2016) Plant Antimicrobial Peptides. Host defense peptides and their potential as therapeutic agents, *Springer Cham*, 111–136.

Granier, F. (1988) Extraction of plant proteins for two-dimensional electrophoresis. *Electrophoresis*, 9:712–718.

Guillen, N.G., Tito, R., Mendoza, N.G. (2018) Capsaicinoids and pungency in *Capsicum chinense* and *Capsicum baccatum* fruits. *Pesq Agropec Trop*, 48(3):237–244.

Guillén-Chable, F., Arenas-Sosa, I., Islas-Flores, I., Corzo, G.; Martinez-Liu, C., Estrada, G. (2017) Antibacterial activity and phospholipid recognition of the recombinant defensin *J1*-1 from *Capsicum* genus. *Protein Expr Purif*, 136:45–51.

Guzman, I., Bosland, P. (2017) Sensory properties of chile pepper heat – and its importance to food quality and cultural preference. *Appetite*, 117:186–190.

Hahm, M.S., Son, J.S., Hwang, Y.J., Kwon, D. K., Ghim, S.Y.J. (2017) Alleviation of salt stress in pepper (*Capsicum annum* L.) Plants by plant growth-promoting Rhizobacteria. Microbiol Biotechnol, 27(10):1790–1797. https://doi.org/10.1007/s11274-011-0979-9.

Hancock, R.E., Sahl, H-G. (2006) Antimicrobial and host-defense peptides as new antiinfective therapeutic strategies. *Nat Biotechnol*, 24:1551–1557.

Hassaan, M.A., El Nemr, A. (2020) Pesticides pollution: Classifications, human health impact, extraction and treatment techniques. *Egypt J Aquat Res*, 46(3), 207–220.

Houlné, G., Meyer, B., Schantz, R. (1998) Alteration of the expression of a plant defensin gene by exon shuffling in bell pepper (*Capsicum annuum* L.). *Mol Gen Genet*, 259:504–510.

Huan, Y., Kong, Q., Mou, H., Yi, H. (2020) Antimicrobial peptides: classification, design, application and research progress in multiple. *Front Microbiol*, 11:1–21.

Hunziker, A.T. (1979) South american Solanaceae: a synoptic survey. In: the biology and taxonomy of the Solanaceae. *Academic Press*, 49–85.

Ilyas H, H., Datta, A., Bhunia, A. (2017). An approach towards structure based antimicrobial peptide design for use in development of transgenic plants: a strategy for plant disease management. *Curr Med Chem*, 24(13): 1350–1364.

Ilyas, H., Kim, J., Lee, D., Malmsten, M., Bhunia, A.J. (2019) Structural insights into the combinatorial effects of antimicrobial peptides reveal a role of aromatic-aromatic interactions in antibacterial synergism. *Biol Chem*, 294(40):14615–14633.

Iqbal, A., Khan, R., Shehryar, K., Imran, A., Ali, F., Attia, S., Shah, S., Mii, M. (2019) Antimicrobial peptides as effective tools for enhanced disease resistance in plants. *Plant Cell*, 139(38):1–15.

Jenssen, H., Hamill, P., Hancock, R.E. (2006) Peptide antimicrobial agents. *Clin Microbiol Rev*, 19:491–511.

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

Jones, J.B., Minnsavage, G.V., Roberts, P.D., Johnson, R.R., Kousik, C.S., Subramanian, S., Stall, R.E. (2002) A non-hypersensitive resistance in pepper to the bacterial spot pathogen is associated with two recessive genes. *Phytopathology*, 92:273–277.

Jung, H.W., Kim, W. Hawang, B.K. (2003) Three pathogen-inducible genes encoding lipid transfer protein from peper are differentially activated by pathogens, abiotic, and environmental stresses. *Plant Cell Environ*, 26(6):915–928.

Jung, H.W., Lim, C.W., Hwang, B.K. (2006) Isolation and functional analysis of pepper lipid transfer protein III (CALTP III) gene prometer during signaling to paphogen, abiotic and environmental stresses. *Plant Science*, 170:258–266.

Kader, J-C. (1996) Lipid-transfer protein in plants. *Annu Rev Plant Physiol Plant Mol Biol*, 47:627–654.

Katoch, M., Rani, S., Kumar, S., Chahota, R.K. (2014) Plant protection with the use of protease inhibitors- a scientific review. *Life Sci Leafl*, 53:1–21.

Khan, R.S., Iqbal, A., Malak, R., Shehrya, K., Attia, S., Ahmed, T., Khan, M.A., Arif, M., Mii, M. (2019) Plant defensins: types, mechanism of action and prospects of genetic engineering for enhanced disease resistance in plants. *3 Biotech*, 9(5):192.

Khani, S., Seyedjavadi, S.S., Hosseini, H.M., Goudarzi, M., Valadbeigi, S., Khatami, S., Adjdary, S., Eslamifar, A., Amani, J., Fooladi, A.A.I.M., Razzagli-Abjaneh, 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.

Kidrič, M., Kos, J., Sabotič, J. (2014) Proteases and their endogenous inhibitors in the plant response to abiotic stress. *Bot Serbica*, 38:139–158.

Knapp, S., Bohs, L., Nee, M., Spooner, D.M. (2004) Solanaceae - a model for linking genomics with biodiversity. *Comp Funct Genom*, 5:285−291.

Koprivnjak, T., Peschel, A. (2011) Bacterial resistance mechanisms against host defense peptides. *Cell Mol Life Sci,* 68:2243–2254.

Kumar, P., Kizhakkedathu, J., Straus, S. (2018) Antimicrobial peptides: diversity, mechanism of action and strategies to improve the activity and biocompatibility *in vivo*. *Biomolecules*, 8(4):doi:10.3390/biom8010004.

Kushalappa, A.C., Yogendra, K.N., Karre, S. (2016) Plant innate immune response: Qualitative and quantitative resistance. *Crit Rev Plant Sci*, 35:38–55.

Lapin, D., Van den Ackerveken, G. (2013) Susceptibility to plant disease: more than a failure of host immunity. *Trends Plant Sci*, 18(10):546–54.

Le, C.F., Fang, C.M., Sekaran, S.D. (2017) Intracellular targeting mechanisms by antimicrobial peptides. *Antimicrob Agents Chemother*, 61(4):e02340–16.

Lee, S.C., Lee, Y.K., Kim, K.D., Hwang, B.K. (2000) In situ hybridization study of organ-and pathogen-dependent expression of a novel thionin gene in pepper (*Capsicum annuum*). *Physiol Plant*, 110(3):384-392.

Lee, T.H., Hall, K.N., Aguilar, M.I. (2016) Antimicrobial peptide structure and mechanism of action: a focus on the role of membrane structure. *Curr Top Med Chem*, 16:25–39.

Lei, J., Sun, L., Huang, S., Zhu, C., Li, P., He, J., Mackey, V., Coy, D.H., He, Q. (2019) The antimicrobial peptides and their potential clinical applications. *Am J Transl Res*, 11(7), 3919–3931.

Li, C., Liu, H., Yang, Y., Xu, X., Lv, T., Zhang, H., Liu, K., Zhang, S., Chen, Y. (2018) N-myristoylation of antimicrobial peptide *CM4* enhances its anticancer activity by interacting with cell membrane and targeting mitochondria in breast cancer cells. *Front Pharmacol*, 9:1297.

Ma, H., Zhao, X., Yang, L., Su, P., Fu, P., Peng, J., Yang, N., Guo, G. (2020) Antimicrobial Peptide AMP-17 affects *Candida albicans* by disrupting its cell wall and cell membrane integrity. *Infect Drug Resist*, 13:2509–2520.

Macedo, M.L., Garcia, V.A., Freire, M., Richardson, M. (2007) Characterization of a Kunitz trypsin inhibitor with a single disulfide bridge from seeds of *Inga laurina* (SW.) *Willd Phytochemistry*, 68:1104–1111.

Malanovic, N., Lohner, K. (2016) Gram-positive bacterial cell envelopes: The impact on the activity of antimicrobial peptides. *Biochim Biophys Acta*, 1858:936–946.

Maracahipes, A.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. (2019) Biochemical analysis of antimicrobial peptides in two different *Capsicum* genotypes after fruit infection by *Colletotrichum gloeosporioides*. *Biosci Rep*, 39(4):1–14.

Matsuzaki, K. (2009) Control of cell selectivity of antimicrobial peptides. *Biochim Biophys Acta*, 1788(8):1687−1692.

McLeod, M.J., Guttman, S.I., Eshbaugh, W.H. (1982) Early evolution of chili peppers (*Capsicum*). *Econ Bot*, 36:361−368.

Mejía-Argueta, E.L., Santillán-Benítez, J.G., Ortiz-Reynoso, M. (2020) Antimicrobial peptides, an alternative to combat bacterial resistance. *Acta Biol Colomb*, 25(2):294–302.

Mello, E.O., Ribeiro, S.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.

Mendes, M.A.S., Silva, V.L., Dianese, J.C., Ferreira, M.A.S.V., Santos, C.E.N., Gomes Neto, E., Urben, A.F., Castro, C. (1998) Fungos em plantas no Brasil. Brasil, SPL, 555.

Meneguetti, B.T., Machado, L.D., Oshiro, K.G., Nogueira, M.L., Carvalho, C.M., Franco, O.L., (2017) Antimicrobial Peptides from Fruits and Their Potential Use as Biotechnological Tools-A Review and Outlook. *Front Microbiol*, 7, 2136.

Meyer, B., Houlné, C., Pozueta-Romero, J., Schantz, M-L., Schantz, R. (1996) Fruit-specific expression of a defensin-type gene family in bell pepper: upregulation during ripening and upon wounding. *Plant Physiol*, 112:615–622.

Minsavage, G.V., Dahlbeck, D., Whalen, M.C., Kearney, B., Bonas, U., Staskawicz, B., Stall, R.E. (1990) Gene-for-gene relationships specifying disease resistance in *Xanthomonas campestris* pv. vesicatoria: pepper interactions. *Mol Plant Microbe Interact*, 3(1):41–47.

Mishra, B., Guangshun, W. (2012) The importance of amino acid composition in natural amps: an evolutional, structural, and functional perspective. *Front Immunol*, 3:221.

Mishra, M., Joshi, R.S., Gaikwad, S., Gupta, V.S., Giri, A.P. (2013) Structural-functional insights of single and multi-domain *Capsicum annuum* protease inhibitors. *Biochem Biophys Res Commun,* 430(3):1060–1065.

Mishra, M., Mahajan, N., Tamhane, V.A., Kulkarni, M.J., Baldwin, I.T., Gupta, V.S., Giri, A.P. (2012) Stress inducible proteinase inhibitor diversity in *Capsicum annuum*. *BMC Plant Biol*, 12:2–14.

Mishra, R., Rout, E., Joshi, R.K. (2018) Identification of Resistant Sources Against Anthracnose Disease Caused by *Colletotrichum truncatum* and *Colletotrichum gloeosporioides* in *Capsicum annuum* L. *Proc Natl Acad Sci India Sect B Biol Sci*, 89:517–524

Moguel-Salazar, F., Brito-Argáez, L., Díaz, M.B., Islas, I.F. (2011) A review of a promising therapeutic and agronomical alternative: antimicrobial peptides from *Capsicum* sp. *Afr J Biotechnol*, 10(86):19918–19928.

Molina, A., Segura, A., Garcia-Olmedo, F. (1993) Lipid transfer proteins (nsLTP) from barley and maize leaves are potent inhibitors of bacterial and fungal plant pathogens. *FEBS Lett*, 2:119–122.

Montesinos, E. (2007) Antimicrobial peptides and plant disease control. *FEMS Microbiol Lett*, 270(1):1–11.

Montesinos, E., Bardaj, E. (2008) Synthetic antimicrobial peptides as agricultural pesticides for plant- disease control. *Chem Biodivers*, 5(7):1225–1237.

Morales-Soto, A., Gómez-Caravaca, A.M., García-Salas, P., Segura-Carretero, A., Fernández-Gutiérrez, A. (2013) High-performance liquid chromatography coupled to diode array and electrospray time-of-flight mass spectrometry detectors for a comprehensive characterization of phenolic and other polar compounds in three pepper (*Capsicum annuum* L) samples. *Food Res Int*, 51:977−984.

Morrissey, J.H. (1981) Silver stain for proteins in polyacrylamide gels: a modified procedure with enhanced uniform sensitivity. *Anal Biochem*, 117:307–310.

Moulin, M.M., Rodrigues, R., Ribeiro, S.F., Gonçalves, L.S., Bento, C.S., Sudré, C.P., Vasconcelos, I.M., Gomes, V.M. (2014) Trypsin inhibitors from *Capsicum baccatum* var. pendulum leaves involved in pepper yellow mosaic virus resistance. *Genet Mol Res*, 13:9229–9243.

Murashige, T., Skoog, F. (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol Plant*, 15:473–497.

Nawrot, R., Barylski, J., Nowicki, G., Broniarczyk, J., Buchwald, W., Goź dzicka-Józefiak, A. (2014) Plant antimicrobial peptides. *Folia Microbiol*, 59(3):181–196.

Nazarov, P.A., Baleev, D.N., Ivanova, M.I., Sokolova, L.M., & Karakozova, M.V. 2020. Infectious Plant Diseases: Etiology, Current Status, Problems and Prospects in *Plant Protection. Acta Naturae,* 12(3), 46–59.

Nelson, N. (1944) A photometric adaptation of the Somogyi method for the determination of glucose. *J Biol Chem*, 153:375–380.

Nicolas, P. (2009) Multifunctional host defense peptides: intracellular-targeting antimicrobial peptides. *FEBS J*, 276:6483–6496.

Nicolopoulou-Stamati, P., Maipas, S., Kotampasi, C., Stamatis, P., Hens, L. (2016) Chemical pesticides and human health: the urgent need for a new concept in agriculture. *Front Public Health*. 4, 148.

Obradovic, A., Mavridis, A., Rudolph, K., Janse, J.D., Arsenijevic, M., Jones, J.B., Minsavage, G.V., Wang, J-F. (2004) Characterization and PCR-based typing of *Xanthomonas campestris* pv. *vesicatoria* from peppers and tomatoes in Serbia. *Eur J Plant Pathol*, 110:285–292.

Odintsova, T., Slezina, M., Istomina, E. (2018) Plant thionins: structure, biological functions and potential use in biotechnology. *Vavilov Journal of Genetics and Breeding*, 22(6):667–675.

Oh, B.J., Ko, M.K., Kostenyuk, I., Shin, B., Kim, K.S. (1999) Coexpression of a defensin gene and a thionin-like gene via different signal transduction pathways in pepper and *Colletotrichum gloeosporioides* interactions. *Plant Mol Biol*, 41:313–319.

Oliveira, C.V.S., Matos, K.S., de Albuquerque, D.M.C. (2017) Identification of *Colletotricum* isolates from *Capsicum chinense* in Amazon. *Genet Mol Res*, 29(2): gmr16029601.

Oñate-Garzón, J., Ausili, A., Manrique-Moreno, M., Torrecillas, A., Aranda, F.J., Patiño, E., Gomez-Fernández, J.C. (2017) The increase in positively charged residues in cecropin D-like *Galleria mellonella* favors its interaction with membrane models that imitate bacterial membranes. *Arch Biochem Biophys*, 629:54–62.

Padilha, H.K.M., Barbieri, R.L. (2016) Plant breeding of chili peppers (*Capsicum,* Solanaceae) - A review. *Aust J Basic & Appl Sci*, 10(15):148–154.

Paiva, P.M.G., Pontual, E.V., Coelho, L.C.B.B., Napoleão, T.H. (2013) Protease inhibitors from plants: Biotechnological insights with emphasis on their effects on microbial pathogens. *Formatex Research Center,* 641–649.

Pardoux, É., Boturyn, D., Roupioz, Y. (2020) Antimicrobial peptides as probes in biosensors detecting whole bacteria: a review. *Molecules*, 25(8):1998.

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*(7):2587.

Park, C-J., Shin, R., Park, J.M., Lee, G-J., You, J-S., Paek, K-H. (2002) Induction of pepper cDNA encoding a lipid transfer protein during the resistance response to tobacco mosaic virus. *Plant Mol Biol*, 48:243–254.

Pereira, L.P., Souza, T.A.M., Walter, R., Sudré, C.P., Dos Santos, L.A., Maracahipes, A.C., Taveira, G.B., Carvalho, V.S., Nagano, C.S., Chaves, R., Rodrigues, R., Carvalho, A.O., Gomes, V.M.G. (2021) Identifcation of enzyme inhibitors and antimicrobial activities from *Capsicum annuum* L. protein extracts against *Colletotrichum scovillei. Hortic Environ Biotechnol*, doi.org/10.1007/s13580-020-00323-w.

Pereira, L.S., Nascimento, V.V., Rodrigues, R., Fernandes, K., Valevski S., Sudré, C., Ribeiro, S.F.F., Vasconcelos, I.M., Zottich, U., Bento, C., Gomes, V.M. (2018) Characterization of *Capsicum annuum* L. leaf and root antimicrobial peptides: antimicrobial activity against phytopathogenic microorganisms. *Acta Physiol Plant*, 40:1–10.

Perry, L., Dickau, R., Zarrillo, S., Hoist, I., Pearsall, D.M., Piperno, D.R., Berman, M.J., Cooke, R.G., Rademaker, K., Ranere, A., Raymond, 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*, 315:986–988.

Pignati, W.A., Lima, F.A.N.S., Lara, S.S., Correia, M.L.M., Barbosa, J.R., Leão, L.H.C., Pignati, M.G. (2017) Spatial distribution of pesticide use in Brazil: a tool for health surveillance. *Ciênc Saúde Coletiva*, 22 (10):3281–3293.

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 *Xanthomonas* species with a wide variety of virulence factors posing a worldwide challenge. *Mol Plant Pathol*, 16(9):907–920.

Rangasamy, K., Murugan, A., Devarajan, N., Samykannu, G.,Parray, J., Nagarajan, A., Shameem, N., Alqarawi, A., Hashem, A., Abd-Allah, E. (2017) Pesticide degrading natural multidrug resistance bacterial flora. *Microb Pathog*, 114, 304–310.

Rautenbach, M., Troskie, A.M., Vosloo, J.A. (2016) Antifungal peptides: to be or not to be membrane active. *Biochimie*, 130:132–145.

Rawlings, N.D., Barrett, A.J., Bateman, A. (2010) MEROPS: the peptidase database. *Nucleic Acid Res*, 38:227–233.

Rawlings, N.D., Tolle, D.P., Barrett, A.J. (2004) Evolutionary families of peptidase inhibitors. *Biochem J*, 378:705–716.

Ribeiro S.F., Fernandes K.V., Santos I.S., Taveira G.B., Carvalho A.O., Lopes J.L., Beltramini L.M., Rodrigues R., Vasconcelos I.M., Da Cunha M., SouzaFilho G.A., Gomes V.M. (2013) New small proteinase inhibitors from *Capsicum annuum* seeds: characterization, stability, spectroscopic analysis and a cDNA cloning. *Pept Sci*, 100(2):132–140.

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

Richardson, M. (1991) Seed storage proteins: The enzyme inhibitors. *Protein Expr Purif*, 136:45–51.

Rios, A.C., Moutinho, C.G., Pinto, F.C., Del Fiol, F.S., Jozala, A., Chaud, M.V, M.M.D.C., Teixeira, J.A., Balcão, V.M. (2016) Alternatives to overcoming bacterial resistances: state-of-the-art. *Microbiol Res*, 191:51–80.

Riva, E.M., Rodrigues, R., Pereira, M.G., Sudré, C.P., Karasawa, M., Amaral Júnior, A.T. (2004) Inheritance of bacterial spot disease in *Capsicum annuum* L. *Crop Breed Appl Biot*, 4(4):490−494.

Rustgi, S., Boex-Fontvieille, E., Reinbothe, C., von Wettstein, D., Reinbothe, S. (2018) The complex world of plant protease inhibitors: Insights into a Kunitz-type cysteine protease inhibitor of *Arabidopsis thaliana*. *Commun Integr Biol*, 11:e1368599.

Sadat, S.S., Soghra, K., Ali, E., Soheila, A., Mehdi, G., Raheleh, H., Reza, A., Hadi, Z-Z., Ali, I.F.A., Jafar, A., Mehdi, R-A. (2020) The antifungal peptide MCh-AMP1 derived from *Matricaria chamomilla* inhibits *Candida albicans g*rowth via inducing ROS generation and altering fungal cell membrane permeability. *Front Microbiol*, 10:3150

Salehia, B., Hernández-Álvarezc, A.J., Contreras, M.D.M., Martorelle, M., Ramírez-Alarcóne, K., Melgar-Lalannef, G., Matthewsg, K.R., Sharifi-Radh, M., Setzeri, Q.N., Nadeemj, M., Yousafk, Z., Sharifi-Radl, J. (2018) This is a repository copy of potential phytopharmacy and food applications of *Capsicum* spp.: A comprehensive review. *Nat Prod Commun,* 13(11):1543−1556.

Santos, L.A., Taveira, G.B., Ribeiro, S.F.F., Pereira, L.S., Rodrigues, R., Oliveira, A.E.A., Machado, O.L.T., Araujo, J.S., Vasconcelos, I.M., Gomes, V.M. (2017) Purification and characterization of peptides from *Capsicum annuum* fruits which are α-amylase inhibitors and exhibit high antimicrobial activity against fungi of agronomic importance. *Protein Expr Purif*, 132:97−107.

Santos, L.A., Taveira, G.B., Silva, M.S., Gebara, R., Pereira, L.S., Perales, J., Teixeira-Ferreira, A., Mello, E.O., Carvalho, A.O., Rofrigues, R., Gomes, V.M.

(2020) Antimicrobial peptides from *Capsicum chinense* fruits: Agronomic alternatives against phytopathogenic fungi. *Biosci Rep*, 40:1–5.

Savary, S., Ficke, A., Jean-Noël, A., Hollier, C. (2012) Crop losses due to diseases and their implications for global food production losses and food security. *Food Secur*, 4:519−537.

Schägger, H., von Jagow, G. (1987) Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 100 kDa. *Anal Biochem,* 166:368–379.

Schwartz, A.R., Potnis, N., Timilsina, S., Wilson, M., Patané, J., Martins JrJ., Minsavage, G.V., Dahlbeck, D., Akhunova, A., Almeida, N., Vallad, G.E., Barak, J.D., White, F.F., Miller, S.A., Ritchine, D., Goss, R., Bart, R.S., Setubal, J.C., Jones, J.B., Staskawicz, B.J. (2015) Phylogenomics of *Xanthomonas* field strains infecting pepper and tomato reveals diversity in effector repertoires and identifies determinants of host specificity. *Front Microbiol*, 6:1–17.

Seo, H.H., Park, S., Park, S., Oh, B.J., Back, K., Han, O., Kim, J.I., Kim, Y. S. (2014) Overexpression of a defensin enhances resistance to a fruit-specific anthracnose fungus in pepper. *PloS One*, 9(5): e97936.

Serra, I.M.R.S., Silva, G.S. (2005) Caracterização morfofisiológica de isolados de *Colletotrichum gloeosporioides* agentes de antracnose em frutíferas. *EMBRAPA*, 6:1679–8869.

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*, 10:3150.

Shayan, S., Saeidi, S. (2013) Antibacterial and antibiofilm activities of extract *Capsicum annuum* L on the growth and biofilm formation of common pathogenic strains. Intl. *Res J Appl Basic Sci*, 5:5(4):513-518.

Shevchenko, A., Tomas, H., Havlis, J., Olsen, J.V., Mann, M. (2006) In-gel digestion for mass spectrometric characterization of proteins and proteomes. *Nat Protoc*, 1:2856–2861.

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.

Sievers, F., Higgins, D.G. (2018) Clustal Omega for making accurate alignments of many protein sequences. *Protein Sci*, *27*(1), 135–145.

Silva, F.C.V., Nascimento, V.V., Machado, O.L.T., Pereira, L.S., Gomes, V.M., Carvalho, A.O. (2018) Insight of the α-amylase inhibitory activity of plant lipid transfer proteins. *J Chem Inf Model*, 8:2284–2304.

Silva, M.S., Ribeiro, S.F., Taveira, G.B., Rodrigues, R., Fernandes, K.V., Carvalho, A.O., Vasconcelos, I.M., Mello, E.O., Gomes, V.M. (2017) Application and bioactive properties of *CaTI*, a trypsin inhibitor from *Capsicum annuum* seeds: membrane permeabilization, oxidative stress and intracellular target in phytopathogenic fungi cells. *J Sci Food Agric*, 97(11):3790–3801.

Silva, M.S., Gomes, V.M., Taveira, G.B., Santos, L.A., Maracahipes, A.C., Rodrigues, R., Carvalho, A.O., Fernandes, K.V.S., Oliveira, A.E.A. (2020) Bifunctional inhibitors from *Capsicum chinense* seeds with antimicrobial activity and specific mechanism of action against phytopathogenic fungi. *Protein Pept Lett*, 27:1–1.

Smith, P.K., Krohn, R.I., Hermanson, G.T., Maallia, A.K., Gartner, F.H., Provenzano, M.D., Fujimoto, E.K., Goeke, N.M., Oslon, B.J., Klenk, D.C.

(1985) Measurement of protein using bicinchoninic acid. *Anal Biochem*, 150:76–85.

Soares, J.R., Melo, E.J.T., Cunha, M., Fernandes, K.V.S., Taveira, G.B., Pereira, L.S., Trindade, F.G., Regente, M., Pinedo, M., Canal, L.L., Gomes, V.M., Carvalho, A.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.

Somogyi, M. (1952) Notes on sugar determination. *J Biol Chem*, 195:19–23.

Soumya, S.L., Bindu, N. (2012) Antifungal efcacy of *Capsicum frutescens* extracts against one prevalent fungal strains associated with groundnut storage. *J Agric Technol*, 8:739–750.

Stall, R.E., Jones, J.B, Minsavage, G.V. (2009) Durability of resistance in tomato and pepper to Xanthomonads causing bacterial spot. *Annu Rev Phytopathol*, 47:265–284.

Stec, B. (2006) Plant thionins – the structural perspective. *Cell Mol Life Sci*, 63:1370–1385.

Stehmann, J.R., Mentz, L.A., Agra, M.F., Vignoli-Silva, M., Giacomin, L.L., Rodrigues, I.M.C. Jardim Botânico do Rio de Janeiro. Available from: http://floradobrasil.jbrj.gov.br/jabot/floradobrasil/FB14716 (accessed Mar 2020).

Strempel, N., Strehmel, J., Overhage, J. (2014) Potential application of antimicrobial peptides in the treatment of bacterial biofilm infections. *Curr Pharm Des*, 21:67–84.

Sun, C., Mao, S.L., Zhang, Z.H., Palloix, A., Wang, L.H., Zhang, B.X. (2015) Resistances to anthracnose (*Colletotrichum acutatum*) of *Capsicum* mature

green and ripe fruit are controlled by a major dominant cluster of QTLs on chromosome P5. *Sci Horti*, 181:81–88.

Suwannarat, S., Steinkellner, S., Ongkumarn, P., Sangchote, S. (2017) Diversity of *Colletotrichum* spp. isolated from chili pepper fruit exhibiting symptoms of anthracnose in Thailand. *Mycol Prog*, 16(3):677–686.

Tam, J.P., Wang, S., Wong, K.H., Tan, W.L. (2015) Antimicrobial peptides from plants. *PHRM*, 8(4):711–757.

Tamhane, V.A., Chougule, N.P., Giri, A.P., Dixit, A.R., Sainani, M.N., Gupta, V.S. (2005) In vivo and in vitro effect of *Capsicum annum* proteinase inhibitors on Helicoverpa armigera gut proteinases. *Biochim Biophys Acta*, 1722(2):156–767.

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.

Taniguchi, M., Saito, K., Aida, R., Ochiai, A., Saitoh, E., Tanaka, T. (2019) Wound healing activity and mechanism of action of antimicrobial and lipopolysaccharide-neutralizing peptides from enzymatic hydrolysates of rice bran proteins. *J Biosci Bioeng*, 128:142–148.

Taveira, G.B., Carvalho, A.O., Rodrigues, R., Trindade, F.G., Da Cunha, M., Gomes, V.M. (2016) Thionin-like peptide from *Capsicum annuum* fruits: mechanism of action and synergism with fluconazole against *Candida* species. *BMC Microbiol*, 16:12.

Taveira, G.B., Mathias, L.S., da Motta, O.V., Machado, O.L., 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(1):30–9.

Taveira, G.B., Mellho, E.O., Carvalho, A.O., Regente, M., Pinedo, M., de La Canal, L., Rodrigues, R., Gomes. V. M. (2017) Antimicrobial activity and mechanism of action of a thionin-like peptide from *Capsicum annuum* fruits and combinatorial treatment with fluconazole against *Fusarium solani*. *Biopolymers*, 108(3): e23008.

Taveira, G.B., Mello, É.O., Souza, S.B., Monteiro, R.M., Ramos, A.C., Carvalho, A.O., Rodrigues, R., Okorokov, L.A., Gomes, V.M. (2018) Programmed cell death in yeast by thionin-like peptide from *Capsicum annuum* fruits involving activation of capases and extracelullar H^+ flux. *Biosci Rep*, 38:1–12.

Teixeira, F.R., Lima, M.C.O.P., Almeida, H.O., Romeiro, R.S., Silva, D.J.H., Pereira, P.R.G., Fontes, E.P.B., Baracat-Pereira, M.C. (2006) Bioprospection of cationic and anionic antimicrobial peptides from bell pepper leaves for inhibition of *Ralstonia solanacearum* and *Clavibacter michiganensis* ssp. michiganensis growth. *J Phytopathol*, 54:418–21.

Thery, T., Lynch, K.M., Arendt, E.K. (2019) Natural antifungal peptides/proteins as model for novel food preservatives, In Comprehensive reviews in food science and food safety, 18(5): 1327–1360.

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.

Toke, O. (2005) An interesting review that discusses the therapeutic potential of AMPs. Antimicrobial peptides: new candidates in the fight against bacterial infections. *Biopolymers*, 80(6):717–735.

Tripodi, P., Kumar, S. (2019) The *Capsicum* crop: an introduction. *Springer Cham*, 1−8.

Vallejos, C.E., Jones, V., Stall, R.E., Jones, J.B., Minsavage, G.V., Schultz, D.C., Rodrigues, R., Olsen, L.E., Mazourek, M. (2010) Characterization of two

recessive genes controlling resistance to all races of bacterial spot in peppers. *Theor Appl Genet*, 121: 37–46.

Vieira, M.E.B., Vasconcelos, I.M., Machado, O.L.T., Gomes, V.M., Carvalho, A.O. (2015) Isolation, characterization and mechanism of action of an antimicrobial peptide from *Lecythis pisonis* seeds with inhibitory activity against *Candida albicans*, Acta Biochim Biophys Sin, 47(9):716–729.

Villamil, J.C.M., Mueller, A.N., Demir, F., Meyer, U., Ökmen, B., Hüynck, J.S., Breuer, M., Dauben, H., Win, P., Huesgen, P.F., Doehlemann, G. (2019) A fungal substrate mimicking molecule suppresses plant immunity via an inter-kingdom conserved motif. *Nat Commun*, 1576:1–15.

Volpicella, M., Leoni, C., Costanza, A., De Leo, F., Gallerani, R., Ceci, L.R. (2011) Cystatins, serpins and other families of protease inhibitors in plants. *Curr Protein Pept Sci*, 12:386–398.

Walter, R., Carvalho, V.S., Generoso, A.L., Rodrigues, R., Gravina, G.A. (2018) Cultivation of immature *Capsicum* spp. embryos for incompatible-crossing embryo rescue. *Acta Sci Agron*, 40:2–8.

Wang, J., Li, D., Zhang, Y., Zhao, Q., He, Y., Gong, Z. (2013) Defence responses of pepper (*Capsicum annuum* L.) infected with incompatible and compatible strains of *Phytophthora capsici*. *Eur J Plant Pathol*, 136:625–638.

Wimley, W. C., & Hristova, K. (2019) The mechanism of membrane permeabilization by peptides: still an enigma. *Aust J Chem*, *73*(3):96–103.

Wu, F., Tanksley, S.D. (2010) Chromosomal evolution in the plant family Solanaceae. *BMC Genomics*, 11(1):182.

Yamuna, S., Nithesh, K.V., Bala, S.D., Kumaravel, K. (2019) Antimicrobial peptides from plants and their mode of action. *AIJRSTEM*, 265–269.

Yeaman, M., Yount, N. (2003) Mechanisms of antimicrobial peptide action and resistance. *Pharmacol Rev*, 55(1):27–55.

Yeats, T.H., Rose, J.K.C. (2008) The biochemistr y and biology of extracellular plant lipid-transfer proteins (LTPs). *Protein Sci*, 17:191-8.

Zasloff, M. (2002) Antimicrobial peptides of multicellular organisms. *Nature*, 415:389-395.