ANTIMICROBIAL PEPTIDES OF *Capsicum annuum* FRUITS IN RESPONSE TO FUNGUS *Colletotrichum gloeosporioides*

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DEDICATED to God, for all the blessings bestowed upon my life; to my mother Maria Aparecida, who spared no efforts to help me get here; and to my wife Rozane Franci, for always being on my side.

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SUMMARY

RESUMO

Maracahipes, Álan Chrisleyr; DS.c.; Universidade Estadual do Norte Fluminense Darcy Ribeiro; Março de 2019; Peptídeos antimicrobianos de frutos de *Capsicum annuum* em resposta ao fungo *Colletotrichum gloeosporioides*; Orientadora: Valdirene Moreira Gomes: Conselheiros: André de Oliveira Carvalho e Rosana Rodrigues.

Existem vários problemas fitossanitários que vêm causando sérios danos às culturas de *Capsicum*, incluindo a antracnose. Após o ataque de patógenos, várias moléculas relacionadas a defesa são produzidas, entre elas estão as proteínas relacionadas à patogênese (proteínas PR). Nestas proteínas estão incluídas alguns peptídeos antimicrobianos (AMPs), como inibidores de tripsina, defensinas de plantas, tioninas e proteínas transferidoras de lipídios (LTPs). As defensinas de plantas são peptídeos básicos de baixa massa molecular entre 5 e 6 kDa, com a capacidade de inibir vários patógenos, especialmente fungos. O objetivo deste trabalho foi investigar, identificar e caracterizar as diferenças na expressão de proteínas e peptídeos antimicrobianos em frutos imaturos e maduros de *Capsicum annuum*, de dois diferentes tratamentos (Ikeda e UENF1381), em resposta à infecção por *C. gloeosporioides*. O fungo foi inoculado em frutos de *Capsicum* pela deposição de uma suspensão de esporos (10^6 conídios/mL), e após 24 e 48 horas os frutos foram retirados da câmara úmida e submetidos a um processo de extração de proteínas. A análise proteica e a caracterização dos extratos foram realizadas por eletroforese em gel de tricina, *Western blotting*, sequenciamento por espectrometria de massa. Ensaios de

inibição de tripsina, detecção de inibidores de protease por zimografia reversa e ensaios de atividade de β-1,3-glucanase também foram realizados. Os extratos também foram testados quanto à sua capacidade de inibir o crescimento de fungos de *C. gloeosporioides* "*in vitro*". O extrato IIF48 (Inoculated immature fruit 48HAI – fruto imaturo inoculado, 48 após a inoculação), o qual apresentou alta atividade antimicrobiana, foi submetido à purificação das proteínas por sistema de HPLC e, em seguida, foi realizada a eletroforese em gel de tricina para obtenção do perfil proteico das frações obtidas. Ensaios de inibição do crescimento foram realizados com o fungo *C. gloeosporioides* com todas as frações derivadas do HPLC. A fração F7, a qual apresentou o maior resultado de inibição, foi e submetida ao sequenciamento N-terminal. Com a fração F7 foram realizados ensaios de permeabilização da membrana plasmática, indução endógena de espécies reativas de oxigênio (ROS) e de funcionalidade mitocondrial. Os resultados mostraram a presença de várias proteínas de baixa massa molecular em todos os extratos obtidos, e em alguns tratamentos, peptídeos antimicrobianos como defensinas, proteínas transferidoras de lipídios (LTP) e inibidores de tripsina. Foi demonstrado também que frutos verdes são mais responsivos à infecção, no qual a presença de peptídeos antimicrobianos em resposta à injúria e inoculação do fungo foi maior quando comparada em frutos maduros em qualquer tratamento. O extrato IIF48 apresentou o maior percentual de inibição contra o fungo *C. gloeosporioides*, causando a permeabilização da membrana, induzindo ROS endógeno e interferindo na funcionalidade mitocondrial. A sequência obtida da banda de 5 kDa da fração F7 mostrou similaridade à defensina de planta e foi denominada *Def-IFCa*, este peptídeo purificado também mostrou alta atividade antimicrobiana contra o fungo *C. gloeosporioides.* Concluímos que existem peptídeos antimicrobianos nos frutos imaturos de *C. annuum* com capacidade de inibição do fungo *C*. *gloeosporioides.*

ABSTRACT

Maracahipes, Álan Chrisleyr; DS.c.; Universidade Estadual do Norte Fluminense Darcy Ribeiro; Março de 2019; Antimicrobial peptides of *Capsicum annuum* fruits in response to fungus *Colletotrichum gloeosporioides*; Advisor: Valdirene Moreira Gomes: Committee members: André de Oliveira Carvalho e Rosana Rodrigues.

Several phytosanitary problems have been causing serious damage to *Capsicum* crops, anthracnose among them. After pathogen attack, many defense molecules are produced, among which are pathogenesis-related (PR) proteins. These proteins include some antimicrobial peptides (AMPs) such as trypsin inhibitors, plant defensins, thionins, and lipid transfer proteins (LTPs). Plant defensins are basic low molecular weight peptides with the ability to inhibit various pathogens, especially fungi. This study investigates, identifies, and characterizes differences in the expression of antimicrobial proteins and peptides in immature and mature *Capsicum annuum* fruits from two different treatments (Ikeda and UENF1381) in response to *C. gloeosporioides* infection. The fungus was inoculated in *Capsicum* fruits by deposition of 20 µl of the spore suspension (10^6 conidia/mL), and the fruits stored in a humid chamber. After 24 and 48 hours, the fruits were removed from the humid chamber and subjected to a protein extraction process. Extracts were characterized by tricine gel electrophoresis, Western blotting, mass spectrometry sequencing, trypsin inhibition assays, protease inhibitor detection by reverse zymography, and β-1,3-glucanase activity assays. Extracts were also tested for their ability to inhibit the *in vitro* growth of fungus *C. gloeosporioides*.

Extract IIF48 (Inoculated immature fruit 48HAI – 48 h after inoculation), which had high antimicrobial activity, was subjected to protein purification by HPLC. Subsequently, electrophoresis was performed in tricine gel, in addition to the assay of growth inhibition of *C. gloeosporioides*. Fraction F7, which presented the highest inhibition result, was subjected to N-terminal sequencing, plasma membrane permeabilization assay, endogenous induction of reactive oxygen species (ROS), and mitochondrial functionality assay. The results showed the presence of several low molecular weight proteins in all the extracts obtained. Some treatments showed antimicrobial peptides like defensins, lipid transfer proteins (LTP), and trypsin inhibitors. The sequence obtained from the 5 kDa band of the F7 fraction showed similarity to plant defensin and was called Def-IFCa. This purified peptide showed high antimicrobial activity against the fungus *C. gloeosporioides*. We conclude that there are antimicrobial peptides in immature fruits of *C. annuum* which can inhibit *C. gloeosporioides* fungi.

1. INTRODUCTION

1.1. Pathosystem *Capsicum-Colletotrichum*

1.1.1. Pathogen: *Colletotrichum gloeosporioides*

Anthracnose is a disease of complex etiology caused by different species of the genus *Colletotrichum*, such as *Colletotrichum acutatum*, *C. gloeosporioides*, *C. capsici*, *C. coccodes*, *C. dematium*, *C. musae*, *C. truncatum*, among several other described species. This disease has been causing serious problems for the cultivation of species of the family Solanaceae, including tomato, chilli and sweet pepper, eggplant, gilo, among others. In Brazil, species *C. acutatum* and *C. gloeosporioides* are the most frequent. These species preferentially infect leaves, stems, and fruits at any stage of maturity: immature, maturing, and mature. Fungicide application along with integrated management has been used in the cultivation of *Capsicum* aiming to control/combat the disease, but little or no success has been obtained, besides increasing the costs of production and the price of the final product (Sun et al., 2015).

Ramdial and Rampersaid (2015) argue that anthracnose can affect *Capsicum* fruits both in the pre- and postharvest phases, causing serious losses (ranging from 50% to 100%) to the producer, with a 30% yield loss in the first harvest. Tozze Jr. et al. (2006) point out that due to the more variable latent period of some species of the genus *Colletotrichum*, fruits may present no symptoms during cultivation, and lesions begin to appear during harvesting, postharvest

handling, and in the transport or commercialization of fruits, generating losses for both the commerce and the consumer.

In *Capsicum*, anthracnose occurs mainly in hot and rainy periods, with ideal temperature above 27º C, and relative humidity of 80%. Conidia are dispersed through raindrops or through the contact of biotic agents. The symptoms of the disease in *Capsicum* fruits are characterized by lesions consisting of circular, dark-colored depressions that may be of different diameters, where a mass of orange to pink conidia appears (Figure 1) (Maracahipes et al., 2017; Oo and Oh, 2016). In leaves and stems, lesions are less apparent, with gray and/or brown stains (Ramdial and Rampersaid, 2015). Under ideal conditions of temperature and humidity, the conidia germinate forming the germ tube with a circular structure at the end, called appressorium. The appressorium is used to force the host tissue wall both chemically and physically (Maracahipes, 2014).

In the course of the infection process, the appressorium adheres to the surface of the plant tissue through a thick layer of adhesive material called hemicellulose mucilage. Shortly after, a penetration peg with the ability to break the cuticle and enter the tissue host is produced. The appressorium also has the ability to germinate and produce other appressoria in chain, or in the germination of a germ tube, with the potential to produce phialidic conidia (conidia produced from terminal cells) at its end (Menezes, 2013; Than et al., 2008).

The cycle of pathogen infection in the host includes tissue colonization by an infection that can be intracellular hemibiotrophic (colonizing the host, causing necrosis, and continuing to grow and reproduce in the plant's dead tissue), subcuticular intramural (under the cuticle), or established intracellularly. Fungi of the genus are considered as hemibiotrophic or facultative biotrophic, since they initially show a biotrophic infection strategy where there is colonization of the membrane and cell wall, with hyphae colonizing one or two cells and producing secondary necrotrophic hyphae (Than et al., 2008).

Figure 1. Lesion caused by infection of *Colletotrichum gloeosporioides* in immature and mature fruits of *Capsicum* spp., seven days after inoculation of 20 µl of the spore suspension at 10^6 conidia/mL.

1.1.2. Host: *Capsicum* sp.

In contrast to the effects of the pathogen on the host, studies have been developed aiming to find solutions to combat anthracnose. For that purpose, one of the most efficient ways has been discovering genotypes resistant to the development of the disease. Maracahipes (2014) identified and selected genotypes as sources of resistance to *C. gloeosporioides* among 88 genotypes of the Germplasm Active Bank (GAB) of UNEMAT. The following species are highlighted as anthracnose resistant genotypes: *C. baccatum* var. *pendulum*, *C. chinense*, and *C. frutescens*. The fungus developed with lower aggressiveness in the fruits during the dry period, with higher temperatures and lower relative humidity, characteristics contrary to those required by the fungus for its development. The maturation stage was also assessed. Immature fruits were more resistant to disease than mature fruits, showing no anthracnose symptoms; in some fruits the lesion was present, but without conidia development.

There are reports of difference in the resistance pattern of *Capsicum* fruits regarding the maturation stage of fruits. Immature fruits are more resistant to the

pathogen than mature fruits. Some studies showed that resistance to *Colletotrichum* was controlled by two dominant genes in green fruits, and two susceptible genes in immature fruits. In *C. baccatum*, resistance against *C. acutatum* was monogenic recessive in immature fruits, and monogenic dominant in mature fruits. For *C. capsicum* in *C. baccatum*, resistance was controlled by a single recessive gene at all stages of maturation (Lin et al., 2007; Kim et al., 2008; Mahasuk et al., 2009; Pakdeevaraporn et al., 2005).

Figure 2. Differences in the resistance response between immature and mature fruits of the *Capsicum annuum* accession UENF1381, two days after inoculation of 20 µl of the suspension (10^6 conidia/mL) of spores of the fungus *Colletotrichum gloeosporioides*.

When collecting *Colletotrichum* isolates from the field in Trinidad, Ramdial and Rampersaid (2014) noticed that red ripe fruits were the ones that presented most symptoms of the disease, whereas in immature fruits symptoms were less common. Of the 129 isolates collected, 72% were *C. truncatum* and 28% *C. gloeosporioides*, confirming that the latter fungus species has a strong economic

importance for the cultivation of *Capsicum*, as it is found in chilli and sweet pepper crops in several countries of different continents.

Due to differences in the resistance of *Capsicum* fruits to different *Colletotrichum* species, studies on the genes that control this resistance and how their inheritance behaves throughout the generations have been of fundamental importance for the release of anthracnose-resistant peppers. Kim et al. (2008) discusses the inheritance of resistance to *C. capsici* in the progenies of the cross between varieties “Daepoongcho” (resistant) and “Yeoju” (susceptible). The authors concluded that a single recessive gene controls such resistance, and that throughout the generations other smaller genes may influence a greater or lesser resistance. Moreover, they state that disease control depends on the stage of infection. Mahasuk et al. (2009) inoculated 13 isolates of *Colletotrichum* in the parents of the cross between *C. chinense* ‘PBC932’ and *C. baccatum* ‘PBC80’ and in their progenies, evaluating immature and mature fruits. The frequency distribution of disease evaluation scores in populations F2 and RC1 (backcrossing 1) indicated a recessive gene controlling resistance at the immature stage of green fruits, and a dominant gene at the mature stage.

Sun et al. (2015) mapped quantitative trait loci (QTLs) for resistance of an interspecific cross between accessions of *C. chinense* and *C. annuum* against *C. acutatum*. The results showed that resistance is dominant in both maturation stages (immature and mature), depending on the same genetic control. The authors also concluded that resistance genes for both maturation stages were not inherited independently. Both progenies showed resistance at the immature stage and susceptibility at the mature stage, a pattern different from that of the parents, suggesting that resistance to *C. acutatum* may be recombined in some individuals. Voorrips et al. (2004) identified a major QTL for *C. capsici* and three QTLs for *C. gloeosporioides* in a cross between *C. annuum* and *C. chinense*, conferring resistance in some progenies.

Studies that prioritize research on physical and chemical barriers, constitutive and/or induced by the plant, conferring resistance to pest and diseases have been gaining prominence as a possible and effective tool in the control of diseases such as anthracnose. The elucidation of how these compounds, such as secondary metabolites, pathogenesis-related proteins, antimicrobial peptides, among others, are produced and how they act, conferring

resistance to the host against the attack of pathogens, has been of fundamental importance in the improvement of plant resistance. Nineteen secondary metabolites from immature and mature fruits of *Capsicum* were characterized by Baba et al. (2019) in response to anthracnose caused by *C. gloeosporioides*. Immature fruits of the resistant genotypes showed high concentrations of ferulic acid, gallic acid, caffeic acid, chlorogenic acid, trigonelline, and paraxanthine between the 1st and the 8th day after inoculation. The amount of these metabolites was higher in resistant compared to susceptible genotypes inoculated with the pathogen.

It has been suggested that the induction of local resistance mediated by salicylic acid (AS) could protect immature pepper fruits against infection by the fungus *C. gloeosporioides*, as it inhibits appressorium development. The AS-mediated transcriptional regulation of genes plays a key role in the expressed resistance against pathogen infection in mature pepper fruits (Do et al., 2004).

Among the chemical defenses produced by the plant to restrain the attack of pathogens and pests is the production of pathogenesis-related proteins or PR proteins. These proteins are part of the group of late-formed proteins, since they are absent or at very low levels constitutively in the plant, being produced in greater quantity after some biotic or abiotic stress. Pathogenesis-related (PR) proteins are known to act directly against the aggressor, causing losses to its metabolism. Some proteins act directly on the plasma membrane of the pathogen, causing membrane permeabilization and consequently the loss of ions and even death. Moreover, some PR proteins act in the inhibition of spore germination and in hyphae formation, while others act in the oxidation of cell wall components, and others are involved in the transport of lipids from the membrane, destabilizing it and inhibiting proteases and α-amylases excreted by insects (Gorjanović, 2009; Stangarlin et al., 2011).

2. OBJECTIVES

2.1. General objective

Investigating the differences in the expression of antimicrobial proteins and peptides in immature and mature fruits of *C. annuum* in response to *C. gloeosporioides* infection, in addition to identifying and characterizing antimicrobial peptides present in this response.

2.2. Specific objectives

1. Investigating the differences in protein profiles between susceptible (Ikeda) and resistant (UENF1381) treatments of *C. annuum* in response to *C. gloeosporioides*;
2. Investigating the differences in protein profiles between immature and mature fruits of *C. annuum* in response to *C. gloeosporioides*;
3. Purifying antimicrobial peptides present in *C. annuum* fruits in response to *C. gloeosporioides*;
4. Analyzing the inhibition ability of *C. annuum* samples under the growth of the filamentous fungus *C. gloeosporioides*;
5. Analyzing the effect of antimicrobial peptide fractions of *C. annuum* fruits on membrane permeabilization of *C. gloeosporioides*;

6. Investigating the induction of endogenous ROS in *C. gloeosporioides* cells in the presence of antimicrobial peptides from *C. annuum*;
7. Analyzing the mitochondrial functionality of *C. gloeosporioides* cells in the presence of antimicrobial peptides from C. annuum.

3. CHAPTERS

3.1. BIOCHEMICAL ANALYSIS OF ANTIMICROBIAL PEPTIDES IN TWO DIFFERENT *Capsicum* GENOTYPES AFTER FRUIT INFECTION BY *Colletotrichum gloeosporioides*

3.1.1. INTRODUCTION

Sweet and chili peppers belong to the Solanaceae family and to the *Capsicum* genus, which encompasses more than 31 described species, with only five being domesticated (*Capsicum annuum* var. *annuum*; *C. baccatum* var. *pendulum* and *umbilicatum*; *C. chinense*; *C. frutescens*; and *C. pubescens*) (Maracahipes et al., 2016). During the cultivation of *Capsicum*, the plants may be vulnerable to several pests and diseases, what may lead to severe losses for farmers. Among these diseases is the anthracnose.

Anthracnose is a disease of complex etiology, caused by distinct isolates of different species of *Colletotrichum* (Pereira et al., 2011). It is one of the most destructive diseases of sweet and chili peppers in tropical and subtropical regions, and its spread occurs mainly in crops which are grown outdoors during periods of high temperature and high humidity. The fungus produces conidia (spores) as

reproductive structures, which are released and disseminated by splashes of rain, wind, and insects, among other vectors (Santos et al., 2015).

In a favorable environment for their development, the conidia germinate, producing the appressorium at the end of the germ tube or at the end of the hyphae of the mycelium, which are differentiated structures used to force entry into the surface of the plant. Thus, the fungus penetration into the host tissue, what follows with tissue colonization with rapid hyphae growth (Araújo e Stadnik, 2013). The disease mainly affects the fruits, causing necrotic lesions of circular shape, with dark coloration and variable diameters, from which a mucilaginous mass of orange color evolves, i.e., the conidia (Pereira et al., 2011).

Several plant proteins are produced upon pathogens attack, some of which have low molecular masses, below 10 kDa, and a broad spectrum of action (Stangarlin et al., 2011). These molecules are known as proteins related to pathogenesis or PR proteins, which may be particularly enriched in certain families, such as protease inhibitors (PR-6), β-1,3-glucanase (PR-2), defensins (PR-12), thionins (PR-13), and lipid transfer proteins (LTPs) (PR-14). Among these families are also the antimicrobial peptides (AMPs), which have been especially prominent in recent years, gaining attention in researches related to plant defenses (Moguel-Salazar et al., 2011). Several of these peptide families may also be present at constitutive levels in various plant tissues, especially in seeds, thus contributing to the resistance of healthy plants against various pathogens. These AMPs can be expressed in high concentrations upon the aggression of some pathogen or upon exposure to biotic or abiotic stresses. Effectively, antimicrobial peptides tend to act directly in the region of injury against the aggressor and can be found in several plant organs, such as seeds, reproductive organs, tubers, fruits and flowers, and in almost all plant species (Silva et al., 2017).

AMPs may have activity against various bacteria, fungi, viruses and parasites and represent rapid and effective defense establishment capability. The storage of AMPs in plants can play a particularly essential role in protecting young plants that are vulnerable during the early stages of their life cycle (Santos et al., 2017). It is evident that antimicrobial peptides are an important tool in the defense of plants against several injuries and that they present great potential for use in the launching of resistant cultivars, but more studies are necessary for a better comprehension of their mechanism of action, to facilitate their *in vivo* use in

agronomic applications. Thus, the objective of this work was to identify proteins and antimicrobial peptides in fruits of two different genotypes of *Capsicum annuum* infected with *Colletotrichum gloeosporioides* fungus.

3.1.2. REVIEW

3.1.2.1. Defense of plants

Plants suffer attacks from various pathogens throughout their life cycle and, unlike animals, there is no complex defense system that can specialize with each attack. One of the first lines of plant defense is summarized in receptors present in the outer layers of plants, which recognize when plants are under pathogen attack, activating a signaling cascade to restrain it (Wit, 2007). The defenses present in the plants are divided into two groups - constitutive defense and induced defense. Constitutive or preformed defenses are those that are already present in the plant even before any biotic or abiotic stress, and are generally part of the compounds formed during plant development. These defenses can be chemical or physical, the latter including the cuticle layer and the cell wall, which act as a physical barrier to prevent pathogen penetration (Ferreira et al., 2007).

Induced defenses, in turn, are those that appear after the attack by some pathogen in the host, and count on a series of short and medium term responses, such as hypersensitivity reaction, tissue lignification, activation of several chemical barriers at the site of lesion, concomitant generation of reactive oxygen species (ROS), and increased concentration of pathogenesis-related proteins (Heil, 2010).

3.1.2.2. Pathogenesis-related (PR) proteins

When under pathogen attack, several protein molecules are produced for defense, differing in molecule size, location (intra- or extracellular), and in the mechanisms of action and phytopathogenic targets. Initially, PRs were discovered and described as abundant proteins produced and accumulated in the extracellular space of *N. tabacum* plants under the attack of the tobacco mosaic

virus (TMV). Currently, such molecules are known as pathogenesis-related proteins or PR proteins, distributed in 17 protein families (Table 1). Among these families, the following are highlighted: proteinase inhibitors (PR-6), defensins (PR-12), thionins (PR-13), and lipid transfer proteins (LTPs) (PR-14) (Fernandes et al., 2009; Van Loon and Van Strien, 1999; Barros et al., 2010).

The production of this group of proteins in the plant occurs mainly through the presence of signaling molecules known as elicitors, which may have endogenous or exogenous origin. It is also known that PR proteins are regulated by abiotic agents such as ethylene, jasmonic acid, and salicylic acid, which tend to regulate signaling cascades for their production. Several PRs are involved in the degradation of structural polysaccharides present in the cell wall of microorganisms, altering their architecture and hindering infection, development, and proliferation (Fernandes et al., 2009; Balasubramanian et al., 2012).

Some families of PR proteins are represented by antimicrobial peptides (AMPs), which, especially in recent years, have been gaining space in research related to plant defenses (Sels et al., 2008; Martins, 2010).

Table 1. Families of pathogenesis-related proteins (PR Proteins).

Family	First Described Member	Activity
PR-1	Tobacco PR-1	Antifungal, anti-oomycetes
PR-2	Tobacco PR-2	β-*1,3*-glucanase
PR-3	Tobacco P, Q	Chitinase
PR-4	Tobacco R	Chitinase
PR-5	Tobacco S	Thaumatin-like
PR-6	Tomato Inhibitor I	Protease Inhibitor
PR-7	Tomato P69	Endoproteinase
PR-8	Cucumber Chitinase	Chitinase
PR-9	Tobacco Peroxidase	Peroxidase
PR-10	Parsley PR-1	Ribonuclease-like
PR-11	Tobacco Class V Chitinase	Chitinase
PR-12	Defensins	Antifungal
PR-13	Thionins	Antifungal
PR-14	Barley LTP4	Lipid Transfer Protein
PR-15	Barley OxOa	Oxalate oxidase
PR-16	Barley OxOLP	Oxalate oxidase-like
PR-17	Tobacco PRp27	Unknown

3.1.2.3. Antimicrobial Peptides (AMPs)

Antimicrobial peptides (AMPs) generally have a molecular mass up to approximately 10 kDa, with positive overall charge and the presence of disulfide bridges linking cysteine (Cys) residues, which are responsible for the greater stability of the molecule. These peptides can act against various species of bacteria, fungi, viruses, and parasites, and have the ability to establish rapid and effective defense. The storage of AMPs in plants can play an essential role in protection, mainly for young plants, which are vulnerable in the early stages of their life cycle (Guaní-Guerra et al., 2010). Antimicrobial peptides tend to act efficiently directly in the region of injury against the aggressor, and can be found in several plant organs such as seeds, reproductive organs, tubers, fruits, and flowers, and in almost all plant species (Peters et al., 2010).

Considering their structural characteristics, AMPs can be grouped into 13 distinct families, as shown in Table 2, where we highlight: lipid transport proteins (LTPs) (which have biological functions in processes such as cutin synthesis, β-oxidation, and defense against pathogens), snakins, plant defensins, thionins (which were the first proteins in which *in vitro* activity against plant pathogens was detected), hevein-like peptides, knotin-type peptides, sheferdins (the only plant AMPs described as having no disulfide bridges and represented by glycine- and histidine-rich linear polypeptide chains), MBP-1 peptides, macrocyclic peptides, and small peptides called Ib-AMPs (isolated from *Impatiens balsamin* seeds) (Garcia-Olmedo et al., 2001; Benko-Iseppon et al., 2010).

Antimicrobial peptides (AMPs) can be categorized according to their interaction with a given pathogen. For example, antiviral peptides may cause membrane instability (and consequently make the virus unable to infect host cells) only by aggregating into the viral envelope or membrane of the host cell. Viral receptor blockade may occur in this process, as AMPs prevent the passage of viral particles and even cause rupture of the viral envelope. Both enveloped RNA and DNA viruses may be targets of these AMPs. Furthermore, there are antiviral AMPs that have the ability to cross the cell membrane and lodge in the cytoplasm and/or organelles, altering the gene expression of the host cells and corroborating with the host defense system against the virus (Bahar and Ren, 2013; Horne et al., 2005; Robinson et al., 1998; Tamamura et al.,1996).

There are antibacterial AMPs, amphipathic molecules with a hydrophobic domain and a hydrophilic domain, which aim to destabilize the lipid bilayer of the bacterial plasma membrane, although some research has already shown that these AMPs can cause bacterial death without damaging the plasma membrane through inhibition of important metabolic processes such as protein synthesis (Brogden, 2005). Park et al. (1998) showed that the AMP Buforin II can cross the plasma membrane of bacterial cells without destabilizing them, and associate with DNA.

Antifungal AMPs, also amphipathic, have the fungal cell wall as target, and can cause the death of the pathogen through damage such as pore formation and permeabilization of the fungal membrane. Some AMPs require some wall component to bind and permeate, as is the case of plant defensin NaD1, which has the ability to permeabilize the *F. oxysporum* membrane through binding to

glycosylated cell wall proteins. Several antifungal AMPs have the ability to induce the production of endogenous ROS and even apoptosis. Plant defensin PvD1 can kill *C. albicans* and *F. oxysporum* cells by membrane permeabilization and oxidative stress caused initially by the production of ROS and nitric oxide (Costa, 2014; Bahar and Ren, 2013). Finally, antiparasitic AMPs present a mechanism of action similar to the other AMPs, with the ability to kill parasites through the formation of pores in the cell membrane (Bahar and Ren, 2013).

Table 2. Families of antimicrobial peptides and their classification, structural characteristics, database (PDB - Protein database), and number of disulfide bridges (DB). Table adapted from Benko-Iseppon et al., 2010.

Family	Name	Protein Structure	PDB entry	DB	Cys Arrangement*
Plant Defensin	Rs-AFP2		1AYJ	4	3-C-10-C-5-C-3-C-9-C-8-C-1-C-3-C
α- and β-Thionin (8-Cystein-type)	Alpha-1-purothionin		1BHP	4	2-CC-7-C-3-C-8-C-3-C-1-C-8-C-6
Thionin (6-cystein-type)	Crambin		1AB1	3	2-CC-11-C-9-C-5-C-7-C-6
non-specific Lipid transfer Protein	Ace-AMP1		1T12	4	3-C-9-C-12-CC-18-C-1-C-23-C-15-C-4
Hevein-like protein	Ace-AMP2		1HEV	4	3-C-4-C-4-CC-5-C-6-C-2
Knotin	Mj-AMP1		1DKC	3	1-C-6-C-8-CC-3-C-10-C-3
Macadamia	MiAMP1		1C01	3	10-C-9-C-1-C-25-C-14-C-11-C
Maize-AMP	MBP-1	-	-	-	6-C-3-C-13-C-3-C-4
Puroindolines	Puroindoline A	-	-	-	10-C-8-C-7-C-9-WR[W]$_2$K[W]$_2$K-C-6-C-9-CRC-35-C-5-C-7

Table 2. Cont.

Family	Name	Protein Structure	PDB entry	DB	Cys Arrangement*
Snakin	SN1	-	-	-	$[X]_n$-C-3-C-2-RC-8-C-3-C-2-CC-2-C-1-CVP-1-G-2-GN-3-C-1-CY-10-KCP
Family	**Name**	**Protein Structure**	**PDB entry**	**DB**	**Cys Arrangement***
Cyclotide	Kalata B1		1BH4	3	1-C-3-C-4-C-4-C-1-C-4-C-6

The mechanisms of action of AMPs on the membrane of the pathogen can be divided basically into three models: transmembrane or barrel-stave channel, toroidal pore, and carpet model (Table 3). In the transmembrane or barrel-stave model, monomers are inserted into the membrane forming a barrel-like pore, where the hydrophobic side of the peptide lies outside, contacting the membrane lipids, and the hydrophilic side is in the inside of the pore. As other monomers add to the barrel-like structure, the pore becomes larger and the cytoplasmic contents of the cell can be extravasated (Costa, 2014).

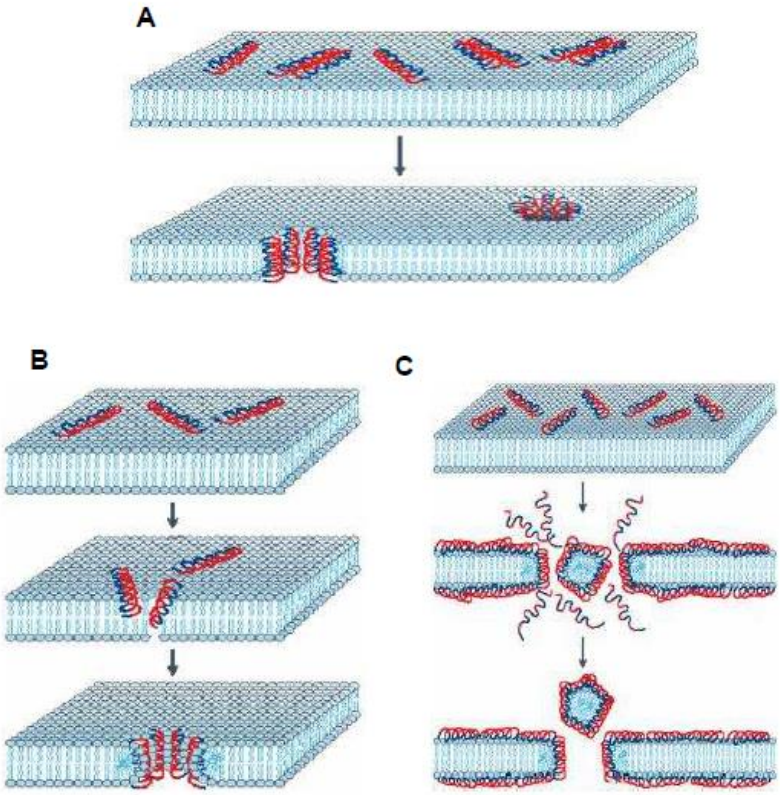

Figure 1. Scheme of the mechanism of action of antimicrobial peptides (AMPs) in interaction with membranes of microorganisms. Hydrophobic regions of the peptide (dark blue) align with the central lipid bilayer region, and hydrophilic regions of the peptide (red) form the inner region of the pore. (A) Transmembrane or barrel-stave channel; (B) Toroidal pore; and (C) Carpet model.

In the toroidal pore model, peptides are inserted into the membrane, forming a lipid monolayer with a high capacity to fold through the pore. The pore wall is formed with part of the inserted peptide and part of the polar heads of the lipid membrane. In the carpet model, AMPs are attracted to the membrane electrostatically, and accumulate on the surface forming a carpet. In this model, when there is a high accumulation of these AMPs in the membrane, disintegration and collapse of the cell membrane occurs (Costa, 2014; Taveira, 2016).

These AMPs may be of great importance in plant breeding, since they constitute a possible tool to be used against the attack of several pathogens throughout the plant cycle, aiming to generate materials resistant to pests and diseases. Studies that aim to understand MPAs in plants, and how they can be used in a beneficial way in plant breeding, are necessary and may contribute to the generation of resistant cultivars.

3.1.3. MATERIALS AND METHODS

3.1.3.1 Plants and fruit picking

UENF 1381 and 'Ikeda' had been evaluated for disease resistance, including bacterial spot (*Xanthomonas* spp.) and anthracnose (*Colletotrichum gloeosporioides*). UENF 1381 is a genebank chili pepper accession identified as resistant to bacterial spot (Silva et al., 2017) and anthracnose. 'Ikeda' is a sweet pepper variety susceptible to bacterial spot and also to anthracnose (Bento et al., 2017).

The experiment was carried out from June 2015 to December 2016 at the *Universidade Estadual do Norte Fluminense Darcy Ribeiro* (UENF), located in the Campos dos Goytacazes municipality, Rio de Janeiro, Brazil. Seeds of *C. annuum* from the UENF1381 accession and the cultivar Ikeda, from the Genebank of the *Laboratório de Melhoramento Genético Vegetal (LMGV),* were seeded in polystyrene foam of 128 cells containing commercial substrate and maintained in a growth chamber with a controlled temperature of 28 °C and a photoperiod of 12 h. After the seedlings exceeded 10 cm in height, they were transplanted into 5 L pots and placed in a greenhouse.

The flowers were marked during the anthesis, after a period of 30 days for immature fruits and 45 days for ripe fruits, the marked fruits were collected and taken to the *Laboratório de Fisiologia e Bioquímica de Microrganismos (LFBM)* for disinfestation, inoculation and extraction of the proteins from the fruits. UENF1381 access was selected as a resistant genotype, and Ikeda cultivar was selected as susceptible genotype (Table 3).

Table 3. Samples derived from control (not inoculated) or inoculated fruits with the fungus *Colletotrichum gloeosporioides*, at intervals of 24 and 48 h after inoculation (HAI).

Genotypes	Feature	Samples	Identification
UENF1381	Resistant to anthracnose and bacterial spot	Inoculated immature fruit (24 HAI)	IIF24
		Control immature fruit (24)	CIF24
		Inoculated immature fruit (48 HAI)	IIF48
		Control immature fruit (48)	CIF48
		Inoculated ripe fruit (24 HAI)	IRF24
		Control ripe fruit (24)	CRF24
		Inoculated ripe fruit (48 HAI)	IRF48
		Control ripe fruit (48)	CRF48
IKEDA	Susceptible to anthracnose, *Pepper yellow mosaic virus*, and bacterial spot	Inoculated immature fruit (24 HAI)	IIF24
		Control immature fruit (24)	CIF24
		Inoculated immature fruit (48 HAI)	IIF48
		Control immature fruit (48)	CIF48
		Inoculated ripe fruit (24 HAI)	IRF24
		Control ripe fruit (24)	CRF24
		Inoculated ripe fruit (48 HAI)	IRF48
		Control ripe fruit (48)	CRF48

3.1.3.2. Obtaining conidia solution of *Colletotrichum gloeosporioides*

The isolate of *C. gloeosporioides* was given in by LMGV-UENF, cultured in Petri dishes containing Potato Dextrose Agar (PDA), and maintained in an incubator at a temperature of 28 ± 2°C for 7 days at 28°C with a 12 hour photoperiod. For inoculation of the fungus into *C. annuum* fruits, a spore solution was made and adjusted using a Neubauer chamber at a concentration of 10^6 conidia/mL (Amorim, 1995).

3.1.3.3. Inoculation of *Colletotrichum gloeosporioides* into fruits.

Immature and ripe fruits were disinfected in 70% alcohol, 0.5% sodium hypochlorite and ultrapure water, for 1 minute at each stage. An injury was created in the middle region of each fruit with a sterilized needle and this point was used for the inoculation with 20 µL of the *C. gloeosporioides* fungus spore solution. Control (not inoculated) fruits were established, in which a drop of ultrapure water was deposited after injury. The trays with the fruits were packed in a transparent

plastic box with a lid containing cotton wads wetted with water to form a humid chamber (Maracahipes et al., 2016).

3.1.3.4. Extraction fruit proteins

Protein extraction was performed according to the methodology proposed by Taveira et al. (2014), in which immature and ripe fruits were removed from the humid chamber at two different intervals: 24 h and 48 h after inoculation (Table 1). The peduncle and seeds of all fruits were removed and discarded.

Forty grams of inoculated fruits and 40 g of not inoculated fruits (controls) from each interval were used for protein extraction The fruit powders were processed in 200 mL of extraction buffer (10 mM Na2HPO4, 15 mM NaH2PO4, 100 mM KCl, 1.5% EDTA, pH 5.4) (Sigma) for 15 min with a multiprocessor, and the mixture was shaked for 2 h, at 4 ºC. The suspension was centrifuged at 15,400 x g for 45 min at 4°C and then the supernatant was filtered with a filter paper; the pellet was discarded. Ammonium sulfate at 70 % saturation was added to the solution, which was shaken for 40 min and kept at 4 ºC, overnight. The next day, the solution was centrifuged at 15,400 x g for 45 min at 4 °C and the supernatant was discarded. The precipitated material was resuspended and placed in a water bath for 15 min at 80 °C before centrifuged at 15,400 x g for 30 min. The final precipitate was discarded and the supernatant was dialyzed using semipermeable membranes (exclusion cutoff 1,000 Da) at 4 ºC for a period of three days using distilled water, with three exchanges of water daily. After this period, the obtained samples were lyophilized, resuspended in water, conditioned in microcentrifuge tubes and stored at -18 ºC. Quantitative protein determinations were done by the Bradford method (1976) with bovine serum albumin as the standard.

3.1.3.5. Tricine gel electrophoresis

The one-dimensional gel was assembled according to the methodology proposed by Schagger and Von Jagon (1987), where 20 µg/ml of sample from each treatment were prepared with 5% sample buffer and β-mercaptoethanol (1%), totaling 20 µL. 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 applied to each well of the gel. The run was performed under an amperage of 400 A and voltage between 22 and 26

V, overnight. At the end of the run, the gel was removed from the glass plates and immersed in Coomassie R dye solution, then in a distain solution; the gel image was recorded with the aid of a gel documentation system.

3.1.3.6. Western Blotting

After the tricine gel run, the gels were withdrawn from the plates and immersed in transfer buffer (182 mM glycine, 25 mM Tris and 20% methanol) for 20 min. Similarly, nitrocellulose membranes (cut to the same size as the gels) were immersed in transfer buffer for 20 min. Proteins from the gels were electroblotted onto nitrocellulose membranes using a commercial (semi-dry) transfer cell. A "sandwich" was mounted consisting of the filter paper, membrane and tricine gel in the transfer tank, in the following order: 5 filter papers, membrane, gel, 5 filter papers. The transfer run lasted for 2 h at maximum voltage and 56 volt amperage.

After transfer, the “sandwiches” were carefully disassembled, and the membranes were reversibly stained with Ponceau S (0.1%) to determine the success of the transfer. The membranes were then subjected to the following treatments: immersion in blocking buffer (10 mM NaH_2PO_4 (PBS pH 7.4) + 0.15 M NaCl + 2% skim powdered milk) at room temperature for a period of 1 hour, with the aim of blocking nonspecific sites on the membrane; incubation in blocking buffer containing the primary antibody (anti-LTP at a titer of 1:1000) for a period of 16 h at 4°C; washing in PBS pH 7.4 at room temperature (five washes of 10 min each); incubation in blocking buffer, this time containing the secondary antibody (rabbit anti-IgG conjugated to peroxide, 1:1000), for a period of 2 h at room temperature; and washing in PBS pH 7.4 at room temperature (five washes of 10 min each). The immune reaction on the membrane were developed by immersing the membranes in a 10 mg/mL solution of 3,3'-diaminobenzidine (DAB) with 10 µL of hydrogen peroxide (Towbin et al., 1979).

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

The copper reagent for the determination of reducing sugars was prepared according to a method described by Somogy (1952), and the arsenomolybdate reagent was prepared according to a method described by Nelson (1944). The

determination of β-1,3-glucanase activity in the samples was done according to a method described by Fink et al. (1988). The reagents were added in test tubes containing 20 µg/ml of proteins, 125 µL of laminarin (2 mg/mL in 50 mM sodium acetate buffer, pH 5.0) and adjusted to a final volume of 500 µL with 50 mM sodium acetate buffer, pH 5.0. The mixture was incubated at 37 °C in an incubator for 12 h. After the incubation period, 500 mL of the copper reagent was added, and the mixture was boiled for 10 min, then placed at room temperature, and 1000 µL of the arsenomolybdate reagent was added. For a reaction control, the protein sample was replaced by assay buffer. A unit of glucanase activity was defined as the concentration of the enzyme that yields an absorbance of 0.001 when read at 500 nm.

3.1.3.8. Trypsin inhibition assay

The trypsin inhibitory activity of the UENF1381 and IKEDA samples (Table 1) was measured based on the hydrolytic activity of bovine commercial trypsin over BAPNA substrate after incubation with the extracts. Tris-HCl buffer (50 mM, pH 8.0), 25 µL of BAPNA substrate (5 mM), 20 µg of samples and 10 µL of trypsin (1µg/ml) were placed in microcentrifuge tubes. There were alternative blank treatments for all samples tested, without the addition of either the substrate or the enzyme. The tubes were incubated in a water bath at 37°C for 30 min. To stop the reaction, 100 µL of acetic acid was added. Afterward, photometric reading of the treatments was performed on the basis of released p-nitroanilide at wavelength of 405 nm (Ribeiro et al., 2013).

3.1.3.9. Reverse zymographic detection of protease inhibition

Inhibition tests of trypsin on gel were performed using a methodology of Felicioli et al. (1997), where the samples were separated on polyacrylamide gel (12% SDS-PAGE) co-polymerized with 0.1% gelatin, under semi-denaturing conditions (sample buffer did not contain SDS, urea or β-mercaptoethanol). After an electrophoretic run, the gels were placed in wash buffer (0.1 M Tris/HCl pH 8.0 containing 2.5% Triton X-100) twice for 60 min to remove SDS. They were immersed in incubation buffer (50 mM Tris-HCl pH 8.0 containing 20 mM CaCl2 and 50 µg/ml trypsin) at 37°C for 1 h. The gels were then rinsed with distilled water

for removal of the trypsin excess. The non-digested proteins were stained by a solution of 0.2% Coomassie Brilliant Blue G 250, 45% methanol and 10% acetic acid for 30 min, and destained thereafter. The dark blue bands on the gel show the potential presence of protease inhibitors, indicating the inability of trypsin to digest gelatin in those regions.

3.1.3.10. Effect of the extracts on fungal growth

The fungus *C. gloeosporioides* was transferred from stock to a Petri dish containing PDA medium and grown for approximately seven days at 28°C. After this period, 10 mL of PDA medium were poured over the plate containing the fungus, and the conidia were released with the aid of a Drigalski spatula. This suspension was filtered through gauze to prevent the passage of mycelial debris. These conidia were quantified in a Neubauer chamber (Laboroptik) under an optical microscope.

A quantitative assay for fungal growth inhibition was performed following the protocol developed by Broekaert *et al*. (1997) with modifications, as follows. To verify the effect of protein extractions on *C. gloeosporioides* growth, $1x10^4$ conidia mL^{-1} in 200 µL of PDA medium were incubated at 25 ^oC in 96-well microplates (Nunc) in the presence of protein extracts at a concentration of 50, 100 and 200 µg mL^{-1}. Optical readings at 620 nm were collected at the start and at every 6 h, for 60 h. Fungal growth in medium without the addition of protein extracts was determined as control. Graphics of absorbance versus time were plotted. The experiments were done in triplicate and repeated three times, and the inhibition percentage was calculated by the formula according to Vieira *et al*. (2015) with the following modifications: the inhibition percentages were assessed against a control representing 100% growth based on the formula [100 – (ABS620 × 100/ cABS620)], where ABS620 was the average absorbance reading at 620 nm of protein extractions-treated cells at 60 h and cABS620 was the average absorbance reading at 620 nm of the control cells at 60 h.

3.1.3.11. Amino acid sequencing by mass spectrometry analysis

For internal sequencing of the *Capsicum* fruits peptides, after electrophoresis in tricine gel, the gel was removed and subjected to staining and

destaining steps. Protein bands of interest were extracted and digest from the gel and subjected to a mass spectrometry evaluation (Léon et al., 2007). In brief, the peptides digest was first co-crystalized with a large molar excess of the α-cyano-4-hydroxycinnamic acid matrix before being analyzed by matrix-assisted laser desorption time-of-flight mass spectrometry (MALDI-TOF-MS), using an AB SCIEX TOF/TOFTM 5800 System spectrometer (AB SCIEX) in the reflectron mode. Up to ten of the most intense ion signals with a signal to noise ratio above 20 were selected as the precursors for MS/MS. External calibration in MS mode was performed using a mixture of four peptides: des-Arg1-Bradykinin (m/z = 904.47), angiotensin I (m/z = 1,296.69), Glu1-fibrinopeptide B (m/z = 1,570.68) and ACTH (18–39) (m/z = 2,465.20). MS/MS spectra were externally calibrated using known fragment ion masses observed in the MS/MS spectrum of Glu1-fibrinopeptide B. MS/MS database searching was performed against the NCBIprot databases using the Mascot software (www.matrixscience.com). The search parameters included two missed tryptic cleavages allowed and non-fixed modifications of methionine (oxidation) and fixed cysteine (carbamidomethylation). Searches for sequence homology were performed with the BLAST program (Altschul et al., 1997).´

3.1.3.12. Statistical analysis

Data from trypsin assays, glucanase assays and fungal growth inhibition were evaluated using one-way ANOVA. Mean differences at $p < 0.05$ were considered to be significant. All statistical analyses were performed using the Graph Pad Prism software (version 5.0 for Windows). The IC50 was calculated based on a linear regression curve and it was defined as the protein extracts concentration required to inhibit 50% of microorganism growth in the conditions tested.

3.1.4. RESULTS AND DISCUSSION

The fungus *C. gloeosporioides* is the main responsible for anthracnose in fruits of *Capsicum* in Brazil, and can appear in any part of the plant, from its

development in the field to even in fruits stored in the post-harvest phase (França et al., 2015). *Colletotrichum* species can infect pepper fruits at any stage of maturation. There are studies showing a strong incidence of *C. acutatum* and *C. gloeosporioides* in immature fruits (Ramdial e Rampersad, 2015; Harp et al., 2013). Kim et al. (2010) found several accessions of *Capsicum* resistant to *C. acutatum*, but only in immature stage of the fruits. In order to detect different proteins and peptides, which could be differentially expressed in ripe and immature fruits, two distinct genotypes of *Capsicum* with different resistance levels towards the fungus were chosen.

The pattern of the low molecular weight proteins of the ripe and immature fruits of *C. annuum* inoculated with *C. gloeosporioides*, at 24 and 48 h after inoculation, was analyzed by tricine gel electrophoresis (Figure 2). In the fruits of UENF1381, all the treatments showed a similar protein profile, with at least five bands ranging from 6 to 12 kDa, except for the IIF48 treatment, which presented an additional band of 5 kDa. This band was subjected to mass spectrometry sequencing. For the Ikeda genotype, the immature fruits showed a different protein profile compared to the ripe fruits, where the immature fruits under different treatments showed at least five bands of 5 to 12 kDa, while the ripe fruits presented bands of 5 to 10 kDa. The IIF48 treatment showed a band at approximately 7 kDa, which was also subjected to mass spectrometry sequencing.

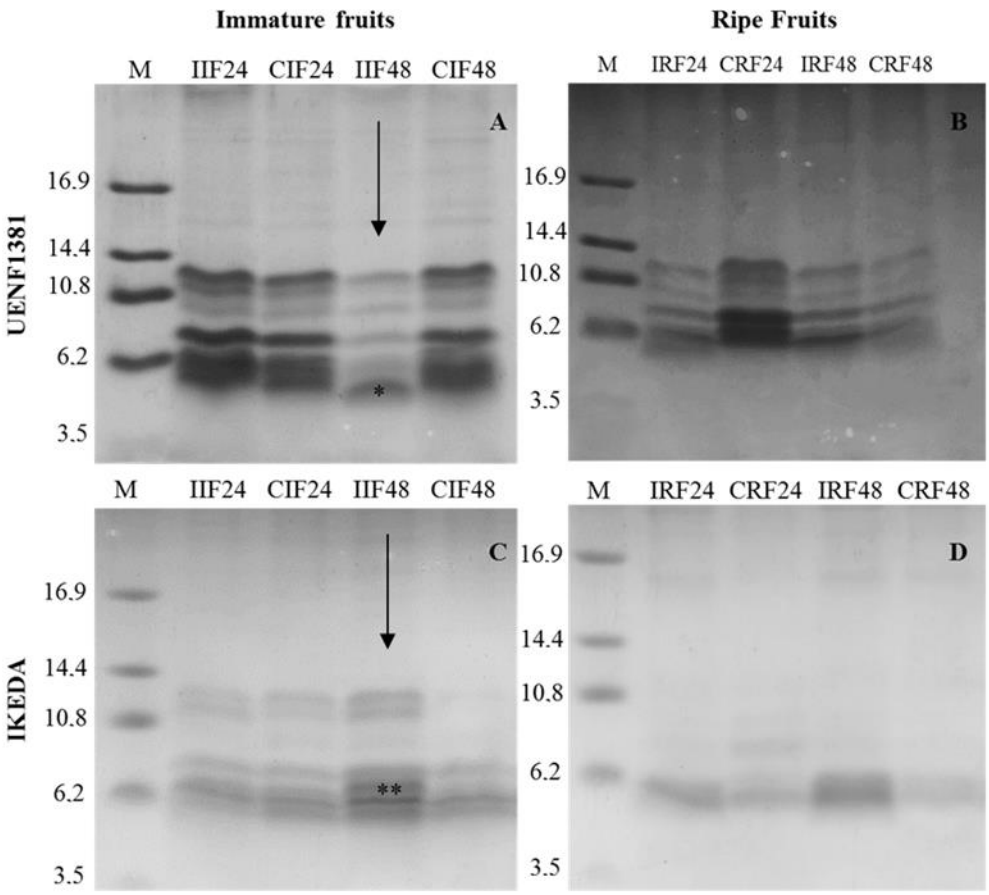

Figure 2. Tricine gel electrophoresis showing low molecular weight protein pattern of *Capsicum annuum* extracts from UENF1381 and the IKEDA cultivars in response to the fungus *Colletotrichum gloeosporioides*. M- Low molecular weight marker; I- Inoculated; C- Control (Not inoculated); 24 - 24 h after inoculation; 48 - 48 h after inoculation; * Defensin; ** Protease inhibitor.

Capsicum extracts have been shown to inhibit the growth and hyphae formation of various phytopathogenic fungi. In the inhibition test of the filamentous fungus *C. gloeosporioides* (Figure 3), different extracts, IIF48 (A) and CIF48 (B) of UENF1381, and IIF48 (C) and CIF48 (D) of Ikeda were tested at different concentrations (50, 100 and 200 µg/mL). The fungal growth was inhibited by all extracts and at all concentrations tested. From 50 µg/mL, *C. gloeosporioides* growth inhibition was observed in all the extracts tested, with values ranging from 75.64% (IIF48) to 100% inhibition when the concentration used was 200 µg/mL. The fractions IIF48 and CIF48 from Ikeda showed a higher percentage of inhibition at all tested concentrations when compared to fractions IIF48 and CIF48 from UENF1381. The fungal growth inhibition tests were corroborated by the images obtained by optical microscopy (Figure 3a, b, c and d), in which fewer *C.*

gloeosporioides hyphae were observed. At 200 µg/mL of extracts, total absence of hyphae was perceived. Taveira et al. (2017) isolated an antimicrobial peptide of *Capsicum annuum*, belonging to the Thionin family of peptides, called *CaThi*, which inhibited 83% of the growth of *Fusarium solani* at the concentration of 50 µg/mL^{-1}. Additionally, the authors showed *CaThi* prevents hyphae formation and permeates membranes of the pathogen. The results about antimicrobial activity reaffirm the richness of antimicrobial peptides in *Capsicum* extracts, and their potential for use against various phytopathogenic fungi of agronomic interest.

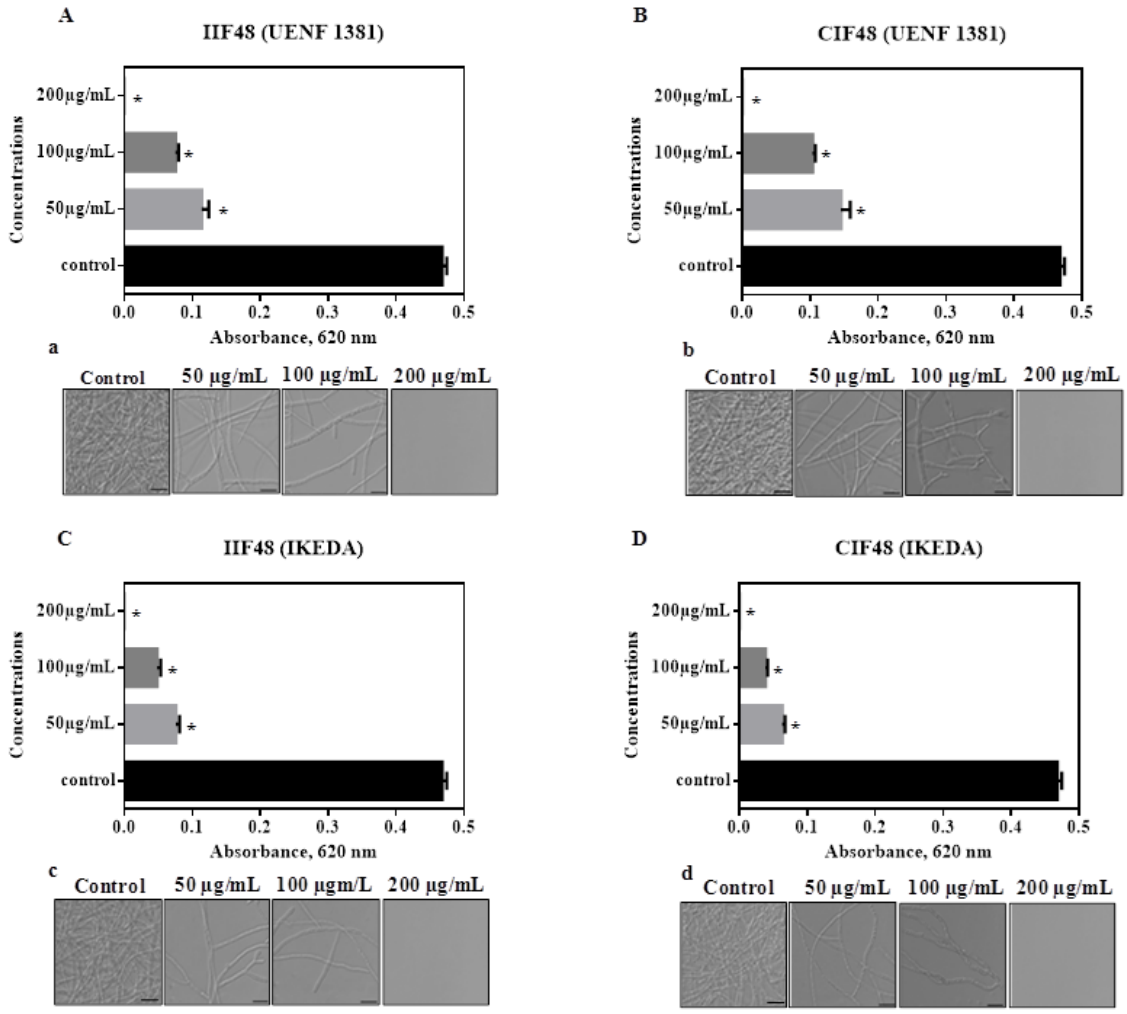

Figure 3. The effect of protein extracts IIF48 (A) and CIF48 (B) of UENF1381 and IIF48 (C) and CIF48 (D) of IKEDA on the growth of the plant pathogen *Colletotrichum gloeosporioides*, at concentrations of 50µg/ml, 100 µg/ml and 200µg/ml. (*) indicates significant differences by the One-way Tukey test ($P < 0.05$). Images of *C. gloeosporioides* cells by light microscopy after different incubation times with IIF48 (a) and CIF48 (b) of UENF1381 and IIF48 (c) and CIF48 (d) of IKEDA. Control cells without protein extractions. Bars = 20 µm, 63x objective. Experiments were performed in triplicate.

The sequencing of the 5 kDa band in the IIF48 treatment (highlighted by * in Figure 2) from fruits of UENF1381 was performed by mass spectrometry, and a sequence fragment with 40 amino acid residues was obtained, later named *Ca*Def1. Alignment of the fragment called *Ca*Def1 (Figure 4) showed that it had 90 to 100% similarity to the above-described defensin sequences. Similar antimicrobial peptides by alignment were flower-specific defensin-like *Capsicum annuum* (GenBank XP_016579688.1), defensin-like protein *C. chinense* (NCBI Reference Sequence: XP_016579688.1), Defensin-like protein *C. baccatum* (NCBI Reference Sequence: PHT42128.1), and flower-specific defensin-like *C. baccatum* (NCBI Reference Sequence: XP_016579687.1.). It can be seen that all six cysteine residues (Cys) are well conserved in all aligned sequences. Van der Weerden and Anderson (2013) performed the alignment of 139 plant defensins, separated into 17 groups, in which it was possible to visualize the conserved amino acid regions inside and outside the groups, mainly the positions of the eight Cys residues of all the peptides, which were completely conserved. In a study done by Rogozhin et al. (2011), where the objective was to isolate and characterize seed defensins from the Ranunculaceae family, two sequences with 30 amino acids were obtained, containing eight residues of Cys. On the basis of the alignment, it was concluded that these sequences were defensins, named Ns-D1 and Ns-D2. An inhibition assay was performed, and Ns-D1 and Ns-D2 showed high inhibition activity against the hyphae of *Bipolaris sorokiniana*, *Fusarium solani* and *Botrytis cinerea*, as well as cause rupture in spores of *B. sorokiniana* which had already germinated, after 48 h of incubation.

Identification	Sequences	% I
	* * * * *	
*Ca*Def1	SKYFTGLCWTDSSCRKVCIEKDKFQDGHCSKIQRNCLCTK	-
Flower-specific defensin-like *C. annuum*	SKYFTGLCWTDSSCRKVCIEKDKFQDGHCSKIQRNCLCTK	100%
Defensin-like *C. chinense*	SKYFTGLCWTDSSCRKVCIEKDKFQDGHCSKIQRNCLCTK	98%
Defensin-like *C. baccatum*	SKYFTGLCWADSSCRKVCIEKDKFQDGHCSKLQRNCLCTK	95%
Flower-specific defensin-like *C. baccatum*	SKYFQGLCWIDSSCRKVCIEKDNFQDGHCSKLQRNCLCTK	90%

Figure 4. Alignment of 40 amino acid residues from the 5 kDa peptide of immature fruits of the UENF1381 cultivar of *Capsicum annuum*, 48 h after inoculation with the fungus *Colletotrichum gloeosporioides*. The Cys residues are conserved in all aligned sequences, being highlighted by *. The different amino acid residues between aligned sequences are underlined. The sequences were obtained from SWISS-PROT and aligned by Clustal Omega. The 5 kDa peptide was named *Ca*Def1 (*Capsicum annuum* Defensin 1) and showed homology with the sequences: Flower-specific defensin-like *Capsicum annuum*, GenBank XP_016579688.1; Defensin-like protein *C. chinense*, NCBI Reference Sequence: XP_016579688.1; Defensin-like protein *C. baccatum*, NCBI Reference Sequence: PHT42128.1; Flower-specific defensin-like *C. baccatum,* NCBI Reference Sequence: XP_016579687.1.

Carvalho and Gomes (2009) showed that defensins are common in the plant kingdom and are involved in several *in vitro* activities, acting as microbial inhibitors, protease inhibitors, α-amylase inhibitors, translation process inhibitors, zinc tolerance mediators, enzymes and even as ion channel blockers. This studies show the great potential of defensins as defense proteins in plants against several pathogens, stressing their importance and the need for studies for its further application in agronomic scenarios.

A ±7 kDa band of the IKEDA fruit treatment IIF48 (highlighted by ** in Figure 2) was also subjected to mass spectrometry sequencing, and a 16 amino acid residue fragment was obtained, which, after alignment, showed similarity to serine protease inhibitors (Figure 5). This peptide was named *Ca*TI2 because of its homology to a pepper seed peptide previously isolated and determined to be a trypsin inhibitor, called *CaTI* (Ribeiro et al., 2007). The fragment was aligned with the following proteins: protease inhibitor IB-like of *C. annuum* (NCBI reference sequence: XM_016687591); trypsin inhibitor 1-like of *C. annuum* (NCBI reference

sequence: XM_016689257.1); trypsin inhibitor 1-like of *Nicotiana sylvestris* (NCBI reference sequence: XM_009776647.1); and trypsin inhibitor 1-like of *Solanum tuberosum* (NCBI reference sequence: XM_006363611.1). In a work done by Mishra et al. (2012), *C. annuum* leaves were subjected to various treatments, such as aphid infestation or mechanical injury with added oral secretion (OS) of *Helicoverpa armigera* or water. Silva et al. (2017) showed that a trypsin inhibitor isolated from *C. annuum* seeds showed inhibitory activity against phytopathogenic fungi such as *F. oxysporum*, *C. gloeosporioides* and *C. lindemuthianum*, hindering their growth and causing morphological changes in the hyphae, reaffirming the important role of protease inhibitors in plant defense and as a possible tool in plant breeding for pest and disease resistance.

Identification	Sequences	% I
CaIkdTi	KDWWPELLGVPAGLAR	-
Proteinase inhibitor I-B-like	KDKWPELLGVPAGLAR	94%
Trypsin inhibitor 1-like Ca	KDTWPELLGVPAKLAR	94%
Trypsin inhibitor 1-like Ns	KDTWPELLGVPAKLAR	88%
Trypsin inhibitor 1-like St	KSSWPELLGVPAKLAR	81%

Figure 5. Alignment of 16 amino acid residues from the 7 kDa peptide of immature fruits of the IKEDA cultivar of *Capsicum annuum*, 48 h after inoculation with the fungus *Colletotrichum gloeosporioides*. The sequences were obtained from SWISS-PROT and aligned by Clustal Omega. The 7 kDa peptide was named *CaIkdTi* (*Capsicum annuum* IKEDA trypsin inhibitor). The different amino acid residues between aligned sequences are underlined. The I-B-like protease inhibitor of *Capsicum annuum*, NCBI Reference Sequence: XM_016687591.1; trypsin inhibitor 1-like Ca of *Capsicum annuum*; NCBI Reference Sequence: XM_016689257.1; Trypsin inhibitor 1-like Ns of *Nicotiana sylvestris*, NCBI Reference Sequence: XM_009776647.1; Trypsin inhibitor 1-like St of *Solanum tuberosum*, NCBI Reference Sequence: XM_006363611.1.

Analyzing Western blotting using anti-LTP antibody and a pure 9 kDa LTP of *Capsicum* seeds as control, it was possible to visualize two bands of the same size as the control in the treatments CIF24 and CIF48 (Figure 6). Our results show that these bands reacted positively with the anti-LTP antibody, suggesting that the protein in the band is immunorelated to LTPs. It is believed that in these immature

fruits, inoculation with the fungus *C. gloeosporioides* is associated with a decrease in the expression of this protein. In the fruits of the Ikeda, there was a poor positive reaction in treatments CIF24, IIF48 and CIF48, and there were no positive reactions with the anti-LTP antibody in the ripe fruit treatments. Diz et al. (2011) identified an LTP in *C. annuum* seeds called *Ca-LTP1,* which has inhibitory activity and can cause several morphological changes in the fungus *C. lindemunthianum*, an anthracnose fungus found in several cultivated species, for example, in *Phaseolus vulgaris* L., and may cause up to 100% losses in the production and in the vegetative parts of the plant (Cruz et al., 2014).

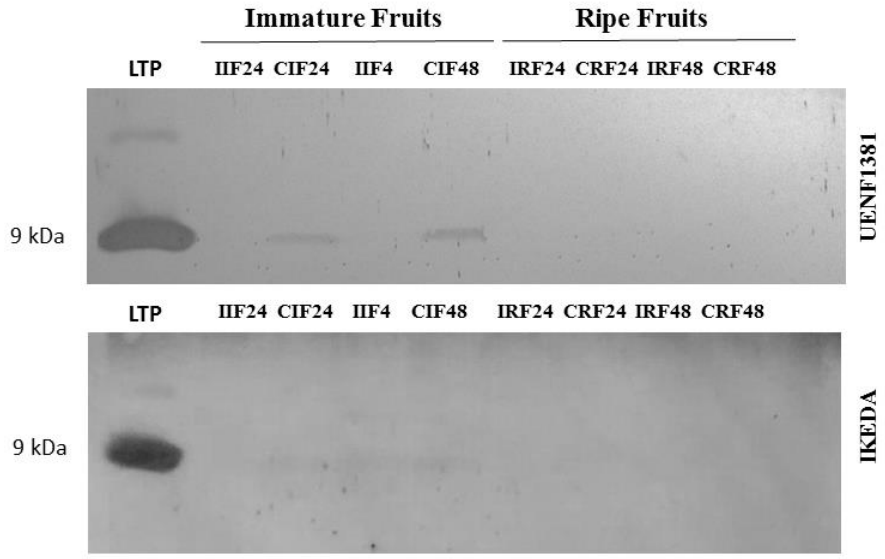

Figure 6. Western blotting for the detection of LTPs in immature and ripe fruits of *Capsicum annuum* (UENF1381) from the UENF BAG, inoculated with fungus *Colletotrichum gloeosporioides*, at 24 and 48 h after inoculation. Membrane revealed with DAB. LTP - Control with purified LTP of seeds of *Capsicum annuum*; I-inoculated; C-Control (not inoculated).

According to Carvalho and Gomes (2007), there are two families of LTPs: LTP1, which has a molecular mass close to 10 kDa, a basic pH and an isoelectric point between 9 and 10, and LTP2, which has a molecular mass of approximately 7 kDa and a high isoelectric point. Both families of LTPs have *in vitro* inhibition activity against fungi and bacteria, which may have great value in programs for the improvement of *Capsicum* resistance to diseases, since the inhibitory action of

LTP can be used *in vivo* to combat several pathogens, including *C. gloeosporioides,* with the goal of releasing an anthracnose-resistant cultivar. Some studies, such as that of Wang et al. (2004), have shown antimicrobial activity of the mung bean LTP against other agronomically important pathogens such as *Fusarium solani*, *F. oxysporum*, *Pythium aphanidermatum* and *Sclerotium rolfsii*.

From the trypsin inhibition assay, it was possible to verify that there is inhibition of trypsin in all tested treatments (Figure 7A), with significant differences in all the treatments in respect to the blank. However, only the samples IIF48 and IRF24 from UENF1381 presented significant differences in respect to their individual controls, CIF48 and CRF24, respectively. It was observed for all treatments of ripe fruits, except for CRF24 and IRF24 de UENF1381, that the percentage of inhibition decreased in relation to immature fruits, without significant differences by Tukey's test. These data are in agreement with the mass spectrometry sequencing, where there was the identification of trypsin inhibition in the immature fruits of IKEDA (Figure 5). Pearce et al. (1988) showed that some protease inhibitors of wild tomato species were present in a large quantity in immature fruits, acting as defensive products against herbivory, and during maturation of the fruits the levels of inhibitors decreased turning them edible and facilitating seed dispersal.

Protease inhibitors were also detected by zymography (Figure 7B), and the results corroborate those from the trypsin inhibition assays (Figure 7A). Ribeiro et al. (2013) identified and purified a protein with trypsin inhibitory activity, called *CaTI,* from the *C. annuum* seed. This protein had the ability to permeabilize the plasma membrane of *Saccharomyces cerevisia*e and *Candida albicans* cells after 24 h of growth. Silva et al. (2017) reported growth inhibition of the fungi *C. gloeosporioides* and *C. lindemuthianum* by the *CaTI* inhibitor, in addition to showing the ability of the protein to permeabilize the membrane of all the tested fungi, what indicates the importance of protease inhibitors in the defense of plants against several pathogens. The protease inhibitors are constitutively present in all plant kingdoms, being mainly located in the reserve tissues, in addition to being produced in response to injuries caused by insects or even by fungi and bacteria (Macedo et al., 2016; Ryan, 1990; Sumikawa et al., 2010). Alternatives aiming at the reduction of pest and disease damages to current crops are of great interest

as a whole, mainly for plant breeding, and the use of PR proteins have great potential for such purposes.

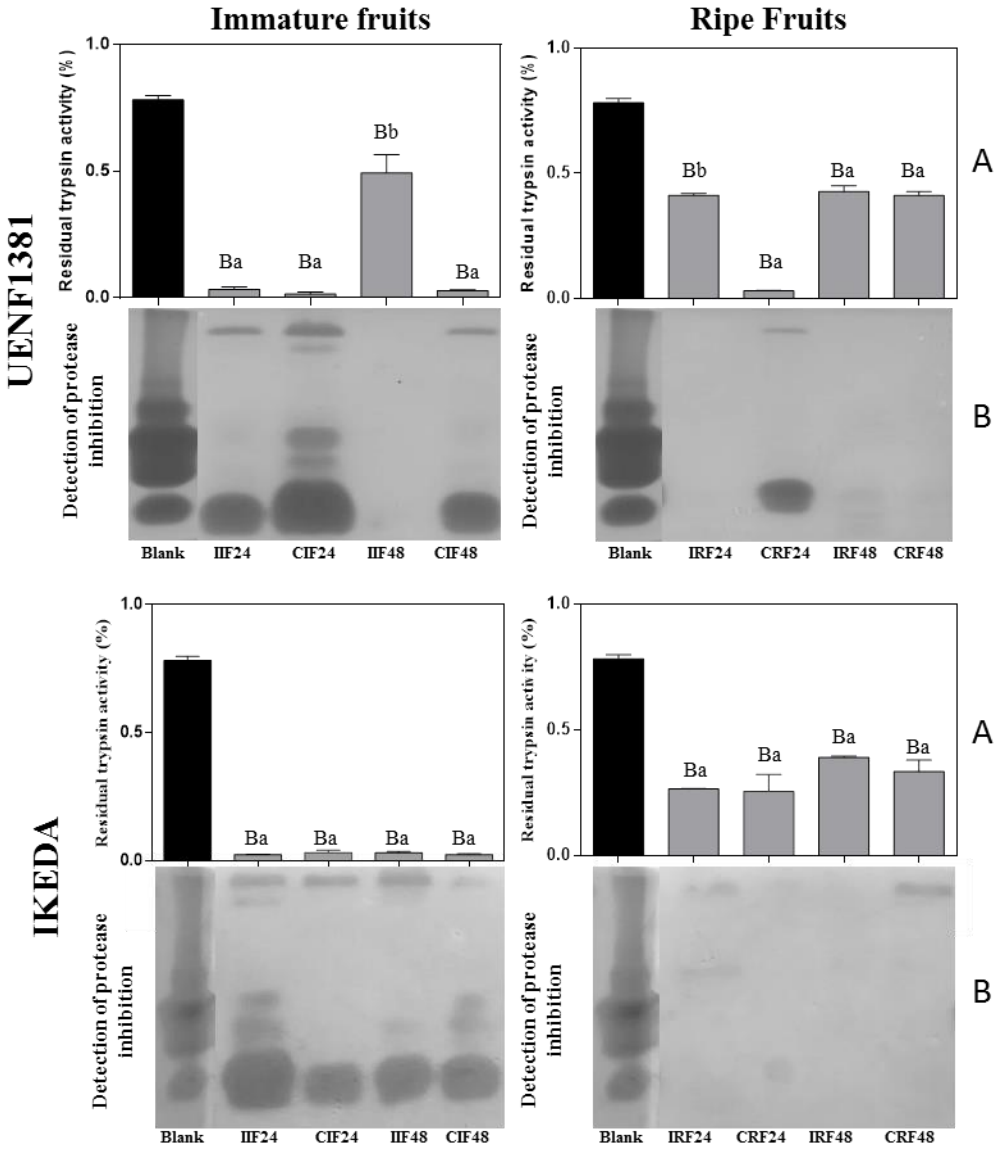

Figure 7. Inhibitory activity assay of trypsin (A) and reverse zymographic detection of protease inhibition (B) on immature and ripe fruits samples of *Capsicum annuum,* from UENF1381 and IKEDA, 24 h and 48 h after inoculation with the fungus *Colletotrichum gloeosporioides*. I- inoculated; C- Control (not inoculated); Trypsin – Control assay (commercial trypsin). Means followed by the same capital letter does not differ in relation to the control of the test, by Tukey test at 5% probability. Means followed by the same lowercase letter do not differ from the uninoculated control by the Tukey test at 5% probability.

The activity of β-1,3-glucanase was detected in all analyzed treatments (24 h and 48 h, immature and ripe, UENF1381 and Ikeda) (Figure 8), but with higher activity in ripe fruits of the Ikeda. In the fruits of UENF1381, β-glucanase activity was low and similar at all-time intervals and did not differ significantly between immature and ripe fruits or between treated immature or ripe fruits and their respective controls. In Ikeda fruits, the β-glucanase activity was higher, being predominant in ripe fruits, showing activity well above the control, with significant differences at 0.05% probability for treatments IRF24, IRF48 and CRF48 between each other and in relation to the general control. It should be noted that the β-1,3-glucanase activity in the Ikeda ripe fruits inoculated for 24 h (IRF24) was much higher than in the fruits subjected to the other treatments, differing significantly from all the other treatments analyzed, including the uninoculated control (CRF24). In ripe 48 h inoculated fruits of the Ikeda (IRF48), the difference with respect to the control (CRF48) decreases, but the value remains significantly different from all tested treatments. According to Balasubramanian & Vashisht (2012), β-1,3-glucanases play an important role in plant defense by means of hydrolytic degradation of β-1,3/1,6-glucans present in the cell wall of pathogenic fungi, and are used as elicitors that activate a signaling cascade, resulting in the production of other proteins used in plant defense.

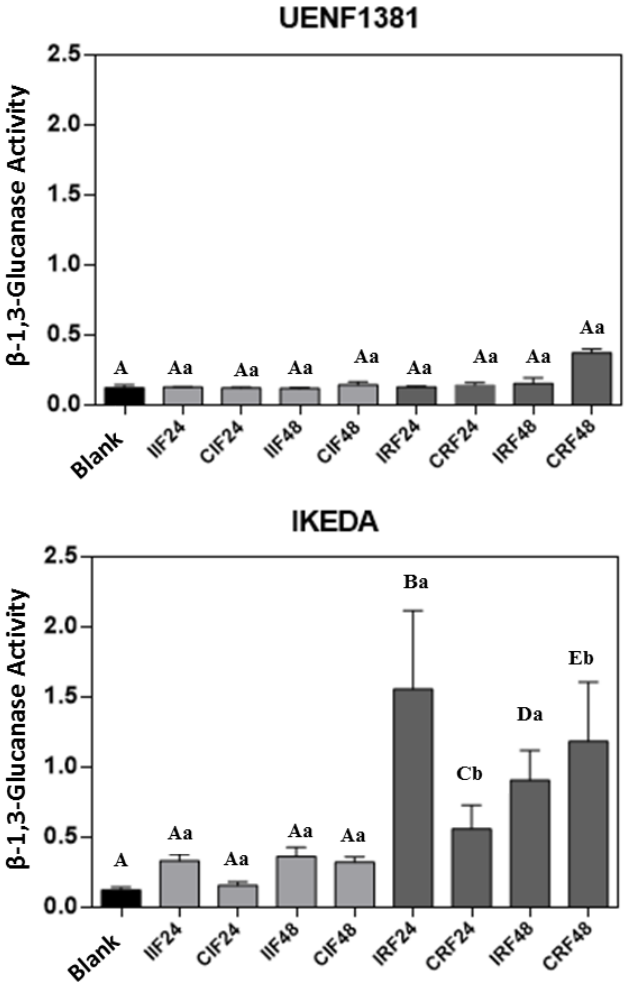

Figure 8. β-1,3-Glucanase activity of immature and ripe fruits of UENF1381 and IKEDA *Capsicum annuum*, 24 h and 48 h after inoculation with the fungus *Colletotrichum gloeosporioides*. I- inoculated; C- Control (not inoculated); Means followed by the same capital letter does not differ in relation to the control of the test, by Tukey test at 5% probability. Means followed by the same lowercase letter do not differ from the uninoculated control by the Tukey test at 5% probability.

Aggarwal et al. (2011) quantified the levels of glucanase expression over time in leaves of 12 wheat genotypes inoculated with *Bipolaris sorokiniana* and found that some genotypes were resistant to the fungus; seven genes were identified in the response to the fungus, among them the *Glucanase I* and *Glucanase II* genes. The *Glucanase II* gene was significantly upregulated in resistant genotypes 16 and 24 h after inoculation; its expression levels were increased up to seven fold compared to the control. It was also observed that the *Glucanase I* and *Glucanase II* genes were not found in the susceptible treatment.

These results are consistent with the results obtained in the present work since the highest levels of β-1,3-glucanase activity occurred in ripe fruits of the Ikeda. However, the Ikeda in this experiment are considered susceptible to treatment.

3.1.5. CONCLUSIONS

In conclusion, it is shown that *C. annuum* fruits present several low molecular weight proteins in the diverse analyzed treatments, particularly antimicrobial peptides such as defensin, lipid transfer protein (LTP) and protease inhibitor. These proteins inhibit the early growth of *C. gloeosporioides*, causing hyphal morphological alterations. The findings reported in this paper on the presence of different defense proteins and antimicrobial peptides in distinct genotypes of *Capsicum*, which are differentially expressed in ripe and immature fruits, suggest an important role for these proteins, especially in constitutive host defense mechanisms against microbial pathogens. This may contribute for the development of biological control of fungal pathogens typical of *Capsicum.*

3.2. ISOLATION AND CHARACTERIZATION OF A *Capsicum annuum* IMMATURE FRUIT DEFENSIN THAT EXHIBIT HIGH ANTIMICROBIAL ACTIVITY AGAINST *Colletotrichum gloeosporioides*

3.2.1. INTRODUCTION

Plant defensins are basic low-molecular-weight peptides ranging from 5 to 6 kDa, have 45 to 55 amino acid residues, with eight conserved cysteine residues linked by four disulfide bonds that ensure increased strength and structural stability. Studies have shown that plant defensins have inhibitory activity against fungal species of the genera *Colletotrichum*, *Fusarium*, *Aspergillus*, *Alternaria*, *Nectria*, and *Candida*, among others (Carvalho & Gomes, 2009). Defensins may interact with some components of the plasma membrane and have the ability to internalize in the cell through receptors. Consequently, the production of reactive oxygen species (ROS) caused by defensin can lead to pathogen cell death. The defensins present toxicity to some microbial cells and are therefore of interest in the production of new antimicrobial agents for both medicinal and biotechnological purposes (Vriens et al., 2014).

Seo et al. (2014) and Meyer et al. (1996) identified defensins in various *Capsicum* tissues, including the floral parts and fruits at different stages of maturation. The appearance of defensin in pepper fruits was related to fruit ripening or induced by wounds and/or pathogen attacks. The promoters of genes encoding defensins had in their sequence transcription factors binding sites

related to the signaling of jasmonic acid and ethylene, and overexpression of the gene occurred mainly in immature fruits of *Capsicum* through the exogenous application of methyl jasmonate.

In a previous work, we observed differences in the protein profile of the crude extract of immature fruits of the *C. annuum* accession uenf1381 48 h after inoculation with the fungus *C. gloeosporioides*, named IIF48, when compared to protein profiles at 24 , 72 and 96 h after inoculation. IIF48 was able to inhibit 100% of the growth of the fungus *C. gloeosporioides* at 200 μg $mL^{-1.}$, and by subjecting the 5 kDa sample to sequencing by mass spectrometry, the sequence obtained showed similarity to a defensin (Maracahipes et al., 2018). Based on the above pieces of information, the objective of this work was to purify the peptides and study the mechanisms of action of the antimicrobial peptides present in IIF48 (immature fruits of *Capsicum annuum* inoculated with *Colletotrichum gloeosporioides*).

3.2.2. REVIEW

3.2.2.1. Plant defensins

Under the attack of plant pathogens, several defense molecules with broad-spectrum activity are produced to prevent the pathogen from colonizing and even killing the host. Among these molecules produced are the antimicrobial peptides, which have a strong inhibitory activity against several microorganisms. These AMPs have plant defensins, which are small molecules with about 45 to 55 amino acid residues. These molecules are basic, with eight conserved cysteine (Cys) residues that confer stability to the molecule through four disulfide bridges, and have a three-dimensional structure with αβ motif consisting of an α-helix and three β-sheets (Thevissen et al., 2003).

Defensins are part of a large superfamily called cis-defensins, whose members share a structural similarity (Figure 4), although they have acquired a different mode of action throughout evolution, resulting in defensins with multiple

functions. The structure of plant defensins can be divided into two parts. The first is formed by seven loops, with the eight cysteine residues conserved in almost all plant species; the second part is formed by sequences with high variability, determining the different biological functions and the different mode of action of each defensin within the family (Parisi et al., 2018).

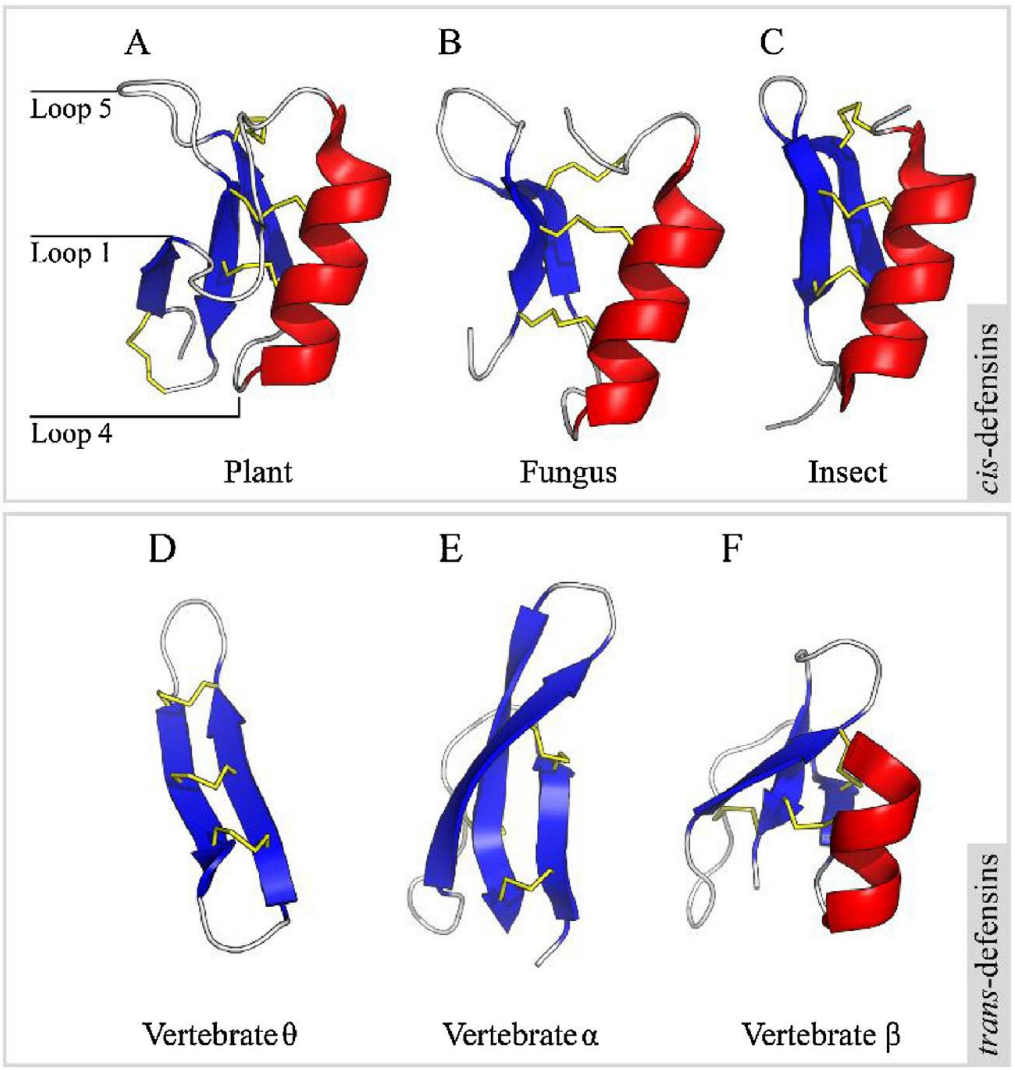

Figure 1. Structure of defensins from different organisms (plants, fungi, insects, and vertebrates), with common structures such as an α-helix and two to three β-sheets in each molecule (Figure adapted from Parisi et al., 2018).

Initially, defensins were classified as γ-thionins due to similar characteristics such as small molecule (around 5 kDa) and the same number of disulfide bridges

as α- and β-thionins. Later, more in-depth studies revealed that the three-dimensional and functional structure of plant defensins closely resembled the defensins of mammals and insects, being reclassified. Initially, defensins were isolated from radish, tobacco flowers, potato flowers, and petunia pistils. It is now known that these proteins are present throughout the plant kingdom. Several defensins have already been purified from different plant tissues such as roots, stems, leaves, seeds, and floral organs, and have already been detected more accurately in the cell wall, in the extracellular space of seeds, in the epidermis, in vascular bundles, in the stomata, in parenchyma cells, and in other peripheral regions of plant tissues. Plant defensins are not toxic to plant and mammalian cells, but showed *in vitro* biological activities against several pathogens. These activities include amylase inhibition, ion channel blockade, antibacterial activity, and inhibition of microbial growth (Meyer et al., 1996; Mello et al., 2014; De Coninck et al., 2010; Carvalho and Gomes, 2011).

It is believed that the various locations (mainly extracellular) of defensins are given as a function of the N-terminal signal peptide, which may be present or absent next to the primary peptide sequence that leads to the location in which defensin will be secreted. Some defensins are directed to the apoplast, as is the case of defensins NaD1 isolated from tobacco flowers, which are deposited in the vacuole due to a C-terminal domain in addition to the amino acid sequence; others are retained in the intracellular space, such as AhPDF1.1 from *Arabidopsis halleri*, which are directed to the vacuolar space but retained in the intracellular space. The extracellular location of defensins may be advantageous, since they are released soon after the pathogen's attack, being characterized as a chemical defense (De Coninck et al., 2013).

Due to the four disulfide bridges, defensins present a globular structure that confers greater resistance and stability against several changes in the environment such as pH variations and temperature variations (from 0º to 90º C), and resistance to digestion of proteolytic enzymes (Carvalho and Gomes, 2011). A common feature of defensins is the binding (affinity) to some lipids, usually occurring in the loop 5 region, where its amino acid sequence can be used to predict in which lipids a given defensin binds to the membrane. The loop 5 region is also responsible for antifungal activity. Defensins of the family Solanaceae have

a crack coated with cationic amino acids in this region, with affinity at pH 4.5 (Parisi et al., 2018).

Studies have shown that some plant defensins may not directly affect the plasma membrane of the pathogen, but may lead to increased potassium efflux and increased calcium uptake, thereby causing a homeostatic imbalance and consequently membrane permeabilization. It is believed that the interaction between defensins and plasma membrane occurs through complex lipids such as sphingolipids, and sterols such as glucosylceramide (GlcCer) (Thevissen et al., 2003).

Variability in the sequence of nonconserved regions provides defensins with different functions, such as inhibition of protein synthesis in cells, inhibiting translation and elongation of the polypeptide chain; inhibition of trypsins and α-amylases, hindering nutrient uptake and starch digestion in the insect gut; and antibacterial activity, as is the case of fabatins from *Vicia faba*, which are active against the bacteria *Pseudomonas aeruginosa*, *Escherichia coli*, and *Enterococcus hirae* (it is noteworthy that antibacterial activity is more common in the defensins of vertebrates). Moreover, other functions include tolerance to heavy metals, as is the case of defensins from the zinc hyperaccumulator species *Arabidopsis thaliana*, which make the plant tolerant to the metal; blockade of sodium (Na^+), potassium (K^+), and calcium (Ca^{2+}) ion channels, as is the case of defensins y1- and y2-zeathionin from *Zea mays*, which inhibit Na^+ channels; antiproliferative activity of cancer cells through binding of defensins to a phospholipid and disruption of the plasma membrane, causing cell death; and inhibition of root growth and plant development, which can be considered as a negative effect on plants but that can also be understood as a protection against other parasitic organisms (Parisi et al., 2018).

Among all the *in vitro* activities described for plant defensins, the best known and studied is the ability to inhibit the growth of filamentous fungi and yeasts. The first plant defensins described with antimicrobial activity were reported by Terras et al. (1992), which were two defensins isolated from *R. sativus* seeds: Rs-AFP1 and Rs-AFP2. After research and tests with several phytopathogens, it was found that these two defensins were able to impair the development and to inhibit the growth of species such as *Alternaria brassicola*, *A. helianthi*, *A. solani*, *Aspergillus versicolor*, *Bipolaris maydis*, *B. sorokiniana*, *Botrytis cinerea*,

Cladosporium colocasiae, *C. sphaerospermum*, *Colletotrichum lindemuthianum*, *Curvularia* sp., *Diploidia maydis*, *Fusarium avenaceum*, *F. culmorum*, *F. decemcellulare*, *F. graminearum*, *F. laterithium*, *F. moliniforme*, *F. oxysporum*, *F. oxysporum* var. *cubense*, *F. verticillioides*, *F. solani*, *Heterobasidion annosum*, *Kluyveromyces marxiannus*, *Mycosphaerella arachidicola*, *M. fijinesis*, *Nectria haematococca*, *Penicillium digitatum*, *P. expansum*, *Pericularia oryzae*, *Phaeoisariopsis personata*, *Phoma batae*, *Physalospora piricola*, *Pichia membranifacies*, *Plectosphaerella cucumerina*, *Rhizoctonia solani*, *Septoria tritici*, *Trichoderma hamatum*, *T. rubrum*, *T. viride*, *Verticilium albo-atrum* and *V. dahlae,* oomycetes *Phythophtora infestans*, *P. parasitica*, *P. parasitica* var. *nicotianae*, *P. palmivora*, *Perenospora hyoscyani, tabaciana* e *Pythium debaryanum*, *A. niger*, *Neurospora crassa* e *Saccharomyces cerevisiae, Candida albicans*, *C. crusei*, *C. dubliniensis*, *C. guilliermondii*, *C. parapsilosis,* and *C. tropicalis* (Carvalho and Gomes, 2011).

Other defensins from different plant species have also been tested in microbial growth inhibition assays, including pathogens of agronomic interest, as is the case of defensin Ah-AMP1 from *Aesculus hippocastanum* (horse chestnut), which was able to inhibit the growth of the fungus *Fusarium culmorum*. Defensins Rs-AFP1, Rs-AFP2, Br-AFP1, and Br-AFP2, isolated from turnip (*Brassica rapa*), were able to inhibit the growth of *Verticillium dahliae*. Defensin Dm-AMP1, isolated from *Dhalia merckii*, inhibited by 50% the growth of the fungus *Botrytis cinerea* (Mello, 2015; Terras et al., 1993). Furthermore, Sathoff et al. (2018) demonstrated that the y-core region of peptides MsDef1, MtDef4, MtDef5, RsAFP-2, and So-D2 showed antifungal activity against *P. medicaginis* and *F. solani* at low concentrations, besides inhibiting spore germination, mycelial growth, and causing elongation of the germ tube. The y-core region also showed antibacterial action against *E. coli, Pseudomonas syringae* pv*. syringae, Sinorhizobium meliloti* and *X. alfalfae* subsp*. alfalfae*.

Since the 1990s, the first defensins have been isolated and characterized from different plant species. Studies have elucidated their mechanism of action and associated different functions to the protein, in addition to the primordial function of plant defense. Table A1 (Annex 1) shows a summary of almost all the plant defensins described, elucidating from which tissue and from which species the peptide was isolated, and which biological functions are performed in that

species, ranging from the autoincompatibility of *Brassica oleracea* pollen to the sweet flavor of the fruit pulp of *Pentadiplandra brazzeana*, but highlighting the antibacterial functions that plant defensins play in several pathosystems.

Regarding the in vivo action of defensins, studies such as Terras et al. (1995) demonstrate that RsAFP1 and RsAFP2 are secreted by radish seeds soon after germination, forming a safer environment against phytopathogenic fungi. Defensins ZmESR6a and ZmESR6b from maize are produced shortly after pollination, protecting the developing embryo (Balandin et al., 2005). Some studies point to the overexpression of defensins, as is the case of DEF2, which increased the resistance of tomato to *B. cinerea*, and of AtPDF1.1 in *A. thaliana* against the nonhost pathogen *Cercospora beticola*.

Studying *Capsicum* fruits, Seo et al. (2014) evaluated the biotechnological potential of transgenic peppers, inserting defensin J1-1 from immature fruits aiming to confer resistance to mature fruits against the fungus *C. gloeosporioides*. After genetic transformation with *Agrobacterium*, the authors observed an increase in J1-1 accumulation, conferring some resistance to anthracnose in mature fruits, as the lesions decreased considerably in size.

Carvalho and Gomes (2011) discuss some biotechnological applications of plant defensins, such as in transgenic plants resistant to a wide range of pathogens, since plant defensins have antimicrobial action against several microorganisms, thus reducing the use of chemical pesticides. These applications are considered to be practical in phytoremediation, since they can provide plants with resistance to heavy metals such as zinc, allowing the cultivation of plants in environments contaminated by industrial activities, mining, among others. Finally, antimicrobial peptides (AMPs) also have potential in the creation of new therapies, since these peptides are nontoxic to mammalian cells and are effective against several diseases, including fungal diseases caused by different pathogens such as *Candida* species, which can cause various infections in animals.

3.2.3. MATERIAL AND METHODS

3.2.3.1 Location of the experiment

The study was conducted at Universidade Estadual Norte Fluminense Darcy Ribeiro, Campos dos Goytacazes, Rio de Janeiro, Brazil. Immature fruits of *C. annuum* of the access UENF1381, resistant to fungus *C. gloeosporioides*, were harvested 30 days after the anthesis, and taken to Laboratório de Fisiologia e Bioquímica de Micro-organismos (LFBM) where they were disinfestated by sequentially immersion in alcohol 70%, sodium hypochlorite 0.5 % and ultrapure water. After disinfestation, an injury was made in the middle region of each fruit using a sterilized needle, where a drop of 20 µl of *C. gloeosporioides* spore solution (10^6 conidia/mL) was inoculated. The fruits were packed in a humid chamber and after 48 h, the peduncle and the seeds were removed, and the fruits were submitted to protein extraction.

3.2.3.2. Obtaining the IIF48 sample

Protein extraction was performed according to Taveira et al., (2013). Briefly, the fruits (without seeds) were processed into 200 mL of phosphate buffer for 15 min with a multiprocessor and the extraction was placed at 4 °C for 2 h with shaking. The extraction was centrifuged for 45 min at a temperature of 4 °C and a rotation of 15.400 x *g*, and then it was filtered with a paper filter and the insoluble sediment was discarded. The ammonium sulfate at 70% saturation was added to the crude extract and stirred for 40 min at 4 °C overnight. The next day, the solution was centrifuged for 45 min at 4 °C and 15.400 x *g*, and the supernatant was discarded. The precipitate was resuspended and placed in a water bath for 15 min at 80 °C and then centrifuged for 30 min at 15.400 x *g*. The precipitate was discarded and the supernatant stored.

The samples were dialyzed with semipermeable membranes (exclusion cutoff 1.000 Da) at 4 °C for a period of three days with distilled water, with three daily water exchanges. After this period, the samples were lyophilized, resuspended in water, conditioned in microcentrifuge tubes and stored at -18 °C. Quantitative determinations of protein were done by the Bradford method (1976) with bovine serum albumin as standard.

3.2.3.3. HPLC Chromatography

The IIF48 fraction was subjected to reverse phase chromatography using the C18 column (Pre-Packed Column RT 250-4) (MERK) for protein separation from the crude extract. The run had duration of 100 min, with flow of 0.4 mL/min, being injected 0.5 mL of filtered sample at the beginning of the run. The column was initially equilibrated only with solvent A (H_2O with 0.1% TFA) (Sigma-Aldrich) for the first two min. From two to 90 min there was a gradient of solvent B (100% propanol (Sigma-Aldrich) in 0.1% TFA) at a concentration of 1% to 40%, between 90 and 94 min the concentration of solvent B inreased to 60%. From 95 min onwards the concentration of solvent B was reduced to 0%. After the chromatography, the fractions were diluted in water, lyophilized and had their protein content quantified.

3.2.3.4. Electrophoresis in Tricine Gel

The one-dimensional electrophoresis was performed according to Schagger and Von Jagon (1987), where 20 µg of sample was prepared with 5% sample buffer and β-mercaptoethanol (1%), totaling 20 µL. 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 applied to each well of the one-dimensional gel. The run was performed with amperage of 400 A, and tension between 22 and 26 V overnight. At the end of the run, the gel was removed from the glass plates and immersed in the Coomassie R dye solution, then in a distain solution; the gel image was recorded with the aid of a gel documentation system.

3.2.3.5. Analysis of inhibition of fungal spores growth

The fungus *C. gloeosporioides* was transferred from the stock and placed to grow on Petri dishes containing Sabouraud agar for approximately fifteen days at 30 °C. After growth, 10 mL of Sabouraud broth were poured onto the plate containing the fungi and the spores were liberated with the aid of a Drigalski loop. This suspension was filtered in gauze to prevent the passage of mycelial debris that could be in solution together with the spores. These spores were then

quantified in the Neubauer chamber in the presence of an optical microscope (Axiovison A2, Zeiss).

Initially, fungal spores (1 x 10^4 cells/mL) were incubated in Sabouraud broth containing different concentrations of the crude extract and / or the peptides containing fractions obtained from *C. annuum* fruits, and the assay final volume adjusted to 200 µL. The assay was performed on cell culture microplates (96 wells) at 30 °C for a period of 24 h. The cell growth was determined by optical density, monitored every 6 h, in a microplate reader at a wave-length of 620 nm. Every test was done in triplicate. The entire procedure was performed under aseptic conditions, in a laminar flow hood, according to a methodology adapted from Broekaert *et al.* (1997).

The statistical analyses were performed using the Graph Pad Prism software (version 5.0 for Windows). The IC50 was calculated based on a linear regression curve and it was defined as the protein extracts concentration required to inhibit 50% of microorganism growth in the conditions tested.

3.2.3.6. N-terminal sequencing by Edman degradation

To obtain the primary structure of peptide of the F7 fraction, 50 µg of protein were lyophilized in a microfuge tube and subjected to Edman degradation sequencing in a Shimadzu PPSQ-23A automatic protein sequencer. The F7 fraction (50 µg) was loaded onto a trifluoroacetic acid (TFA), treated glass fiber membrane and its N-terminal end was sequenced by coupling the peptide with the phenylisothiocyanate (PITC) reagent which reacted with the N-amino terminal residue to form N-phenylthiocarbamoyl (PTC). Then a molecular rearrangement occurs with the cleavage of the first peptide bond, forming the anilinotiazolinone (ATZ) and the remainder of the peptide without the first amino acid. Still in diluted acid medium, there is the conversion of ATZ to phenylthiohydantoin (PTH) which are referred to high pressure reverse phase liquid chromatography. Quantification and identification of amino acids are performed by comparison with a PTH amino acids standard. Sequence alignment was performed in the Blast program, the percentage of sequence identity obtained with the aligned sequences was performed on the Needleman-Wunsch Global Align Protein Sequences, and the

signal peptide of the aligned sequences was determined by the SignalP 4.1 tool. server.

3.2.3.7. Effect of fractions on membrane permeabilization

The membrane permeabilization of fungal cells treated with the different peptide fractions present in *C. annuum* fruits was evaluated using the SYTOX Green fluorescent dye, according to the methodology described by Thevissen et al. (1999), with some modifications. SYTOX Green is a dye that has high affinity for nucleic acids and penetrates cells only when their membrane is compromised. Immediately after the fungal growth inhibition assay, aliquots of fungal growth were incubated under constant shaking for 15 min at 30 ºC with the SYTOX Green fluorescent dye at a final concentration of 0.2 µM according to instructions provided by the manufacturer. The cells were analyzed under an optical microscope (Axiovison A2, Zeiss) equipped with a set of fluorescent filters (excitation wavelengths, 450 to 490 nm; emission wavelength, 500 nm).

3.2.3.8. Intracellular ROS induction assay

To evaluate if the mechanism of action of fractions involves the induction of oxidative stress, the fluorescent probe 2', 7'-dichlorofluorescein diacetate (H_2DCFDA) was used to measure intracellular ROS, according to methodology described by Mello et al. (2011). After growth inhibition against *C. gloeosporioides* fungus, 50 µl of these cells grown in the absence and presence of 100 µg mL^{-1} of the peptide fractions were incubated with H_2DCFDA, which is capable of diffusing through plasma membrane of the cells and be hydrolyzed by intracellular esterases to form a non-fluorescent molecule. With increased ROS production, this molecule will react with intracellular ROS forming a fluorescent molecule. After 30 min incubation at room temperature with constant shaking the cells were analyzed under an optical microscope (Axiovison 4, Zeiss) equipped with a set of fluorescent filters (excitation wavelength between 450-490 nm and 500 nm emission). The results represent triplicate experiments.

3.2.3.9. Mitochondrial Functional Determination Assay

Mitochondrial functionality was assessed by fluorescent dye Rhodamine 123 (Sigma-Aldrich). Rhodamine 123 is a cationic fluorescent dye that has high affinity to the membrane electrical potential s, thus, it marks active mitochondria in living cells. After the growth inhibition assay, the fungal cells were resuspended and incubated with 10 $\mu g.mL^{-1}$ of Rhodamine 123 under constant shaking at 500 rpm and protected from light for 2 h, and then analyzed by DIC on the optical microscope equipped with a fluorescence filter (excitation wavelength of 506 nm, emission wavelength of 530 nm). The control cells had the same treatment of treated cells with the exclusion of the peptide fractions (Taveira et al., 2018).

3.2.4. RESULTS AND DISCUSSION

3.2.4.1. Purification of IIF48 extract

The IIF48 sample presented low-molecular-weight bands ranging from 5 to 11 kDa and presented a band of 5 kDa that did not appear in the control treatment (without inoculation) (Figure 2, topic 3.1.4.). The sample was subjected to reversed-phase chromatography, and after fractionation using a C18 column, 13 fractions were obtained (F1 to F12 fractions and the unretained fraction (NR)) (Figure 2). These fractions were submitted to tricine gel electrophoresis (Figure 3), where several bands with molecular weights between 4 and 10 kDa were labeled in almost all fractions except NR and F1. There was a single band marking for fractions F2, F4, F5 and F12 and a major band for the F7 fraction. From the 52 min to the 100 min run, all material was collected together as fraction 12 (F12).

Similar to the present work, Santos et al. (2017) purified and characterized peptides extracted from seeds of *Capsicum annuum*, *C. baccatum* and *C. chinense* and obtained a chromatogram with 8 fractions and peptides with low molecular weights for the three species studied. Vieira-Bard et al. (2014) isolated antimicrobial peptides from *C. baccatum* seeds; after HPLC chromatography, 7 different fractions were obtained, and one (H4) fraction showed similarity with vicillins. Dias et al. (2013) performed reversed-phase chromatography of *C. chinense* seeds and obtained three fractions (PEF1, PEF2 and PEF3) showing

peptides with molecular weights between 5 and 8.5 kDa and inhibitory activity against yeast growth.

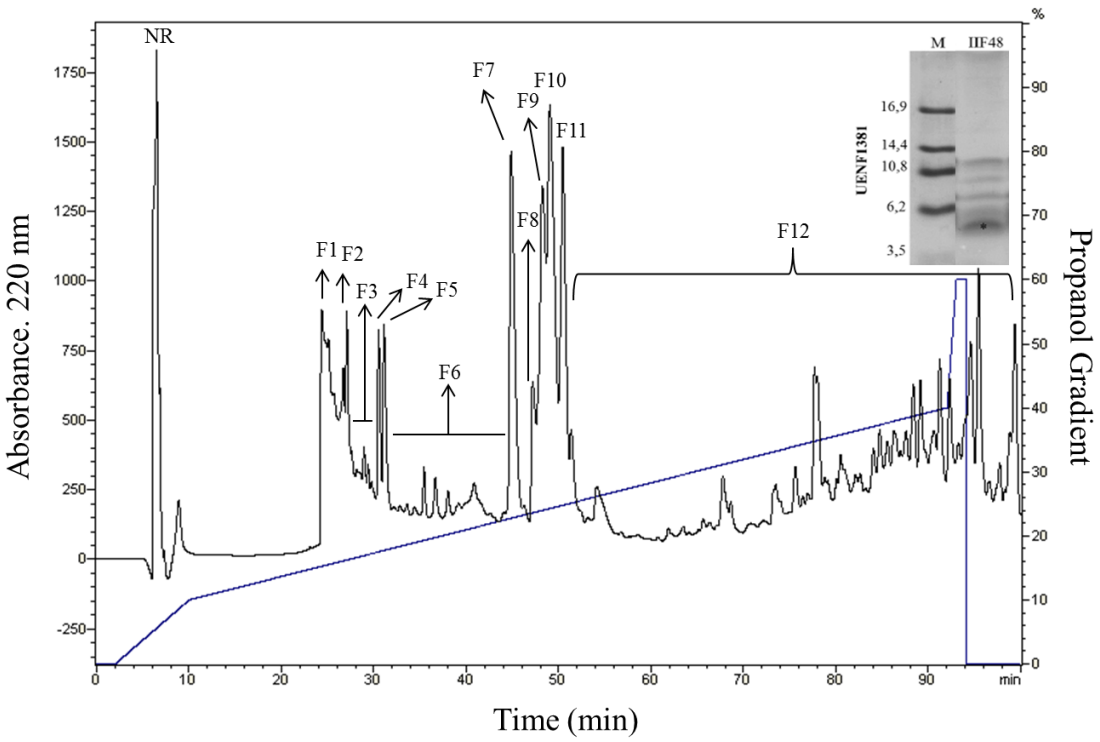

Figure 2. Chromatogram of the IIF48 fraction from fruits of *C. annuum* in reversed-phase C18 column. The sample IIF48 was separated into 12 fractions, named F1 to F12, and one non-retained fraction (NR). Insert: Image of the IIF48 gel electrophoresis showing its protein profile. (M) Molecular mass marker in kiloDaltons. The column was equilibrated and run with 0.1% TFA (Solvent A) and eluted using a gradient (oblique line) of 100% propanol in 0.1% TFA (Solvent B). The flow used was 0.5 mL min^{-1}.

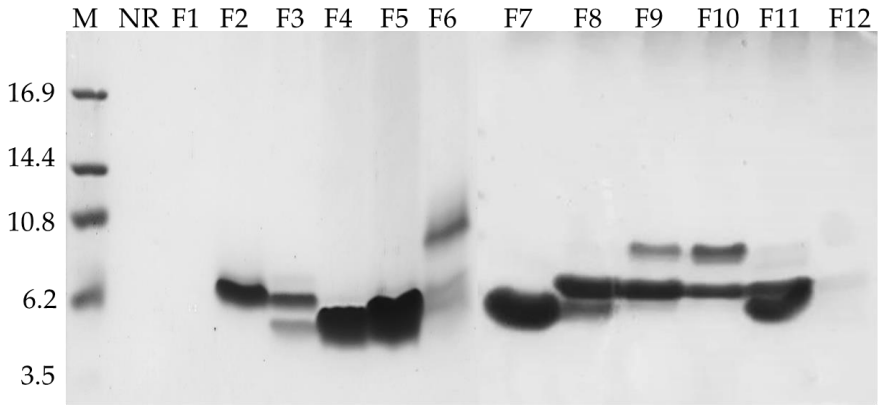

Figure 3. Electrophoretic visualization in Tricine-SDS-PAGE of the 13 fractions obtained by the fractionation of the IIF48 fraction of *C. annuum* fruit (NR and F1 to F12) by reversed-phase C18 column in HPLC. (NR) non-retained fraction. (M) refers to molecular mass markers in kiloDaltons.

3.2.4.2. Growth inhibition assay of the fungus *C. gloeosporioides in vitro*

A growth inhibition assay against *C. gloeosporioides* with the 13 fractions obtained in HPLC was performed (data not shown), but only four fractions (F3, F4, F5 and F7) showed significant differences in relation to the control (in the absence of peptide fractions) (Figure 4). The F3 fraction showed 12% growth inhibition against *C. gloeosporioides*, the F4 fraction 18.38%, F5 displayed 15.30% growth inhibition, and the most active fraction, F7, inhibited 73.94% of the *C. gloeosporioides* growth at a concentration of 200 µg mL^{-1}. Optical microscopy results (Figure 4b) corroborated the results of the inhibition assay, where we clearly observed the greatest reduction in *C. gloeosporioides* hyphae in the presence of the F7 fraction.

Taveira et al. (2018) tested the ability of *CaThi*, a thionin-like peptide purified from mature fruits of *C. annuum*, to inhibit the growth of the filamentous fungus *Fusarium solani* and found that the protein inhibited 21, 50 and 83% of fungal growth at concentrations of 12.5, 25 and 50 µg mL^{-1}, respectively. They also observed that there was a strong reduction in the number of colony forming units after 48 h of incubation using 50 µg mL^{-1} of *CaThi*. Santos et al. (2017) tested the fractions obtained from seeds of *Capsicum* spp. against various filamentous fungi and found that all eight fractions were able to inhibit the growth of *Fusarium lateritium*, *Fusarium solani* and *Fusarium oxysporum* but were not able to inhibit the growth of *C. gloeosporioides*.

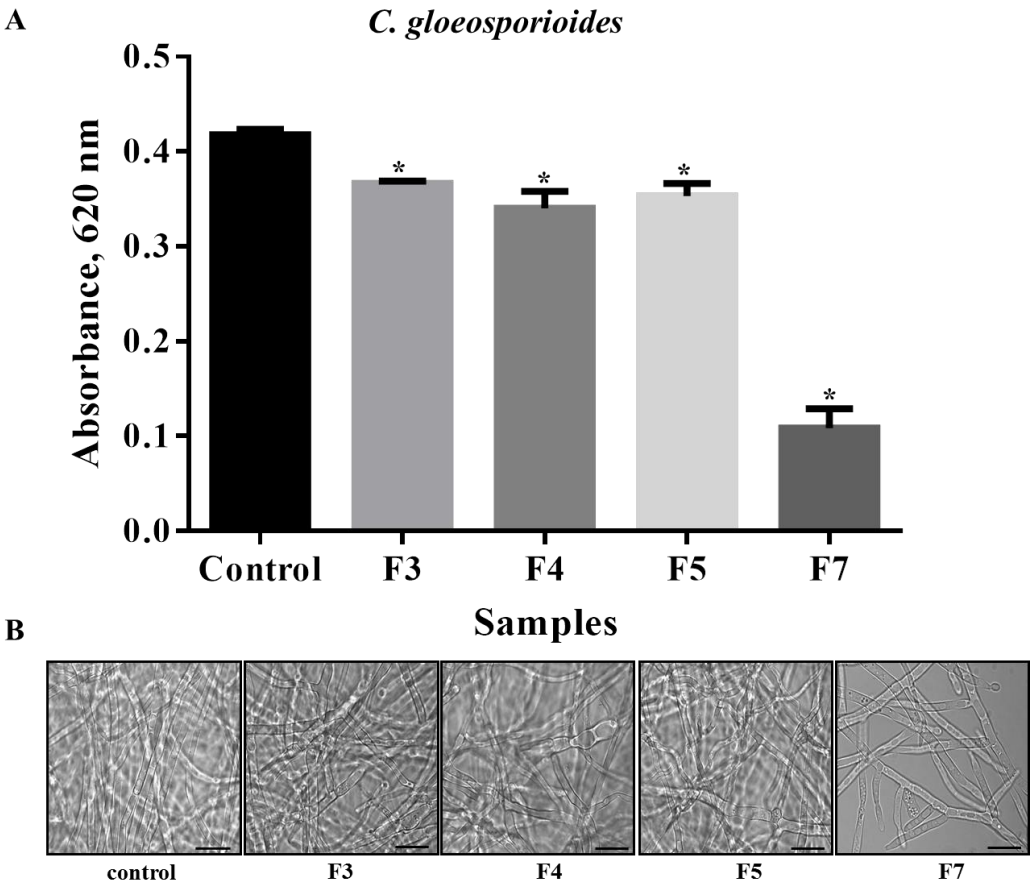

Figure 4 (A) The effect of F3, F4, F5 and F7 fractions on the growth of the phytopathogen *Colletotrichum gloeosporioides*. Control (absence of fractions) and 200 µg mL^{-1} of each fraction. (*) Indicates significance by the one-way ANOVA test ($P < 0.05$). **(B)** Images of *C. gloeosporioides* cells by light microscopy after 24 h of incubation. Control cells without fractions. Bars = 20 µm. Experiments were performed in triplicate.

Silva et al. (2017) tested *CaTI*, a protease inhibitor obtained from *C. annuum* seeds, on the growth of fungi *Colletotrichum lindemuthianum*, *C. gloeosporioides*, *F. solani* and *F. oxysporum* and found inhibition against fungi of the genus *Colletotrichum*, but not for those of the genus *Fusarium*. In microscopic analysis, it was concluded that *CaTI* reduced the growth of the mycelium of *C. gloeosporioides*. For *C. lindemuthianum* and *F. oxysporum*, abnormal growth of the hyphae was observed, and for *F. solani,* a cluster of cells was observed in the control treatment.

The J1-1/GST defensin of *Capsicum* showed inhibitory activity towards the growth and development of anthracnose fungi, maintaining antifungal activity and interrupting the process of fungal infection of plant tissues Seo et al. (2014).

These results confirm the presence of several antimicrobial peptides in *Capsicum*, capable of inhibiting several phytopathogenic fungi of agronomic interest *in vitro*, and the need for further study of these molecules for use in plant breeding for disease resistance. The results obtained in the present work show a fraction with the ability to inhibit the growth of a fungal species that causes anthracnose, one of the most destructive diseases of peppers.

3.2.4.3. Protein sequencing by Edman degradation

Because of its strongest inhibitory activity, the protein band of the F7 fraction (6 kDa band) was sequenced via Edman degradation. From this analysis a fragment with 18 amino acid residues was obtained (Figure 5), including the N-terminal region, with three cysteine residues and one locus where two amino acids were identified: a glutamine and a leucine. The sequence obtained was called *Def*-IF*Ca* (immature fruit *Capsicum annuum* defensin). In the alignment, the sequence obtained showed a similarity of 94% with sequences already deposited in the NCBI for two proteins induced by stress in *C. annuum*: Stress-induced protein 18 (Sequence ID: AHI85723.1); Stress-induced protein 19 (Sequence ID: AHI85724.1): a protein described as flower specific *defensin-like* from *C. annuum* (Sequence ID: XP_016579689.1); and a protein described as thionin-like of *C. annuum* with Sequence ID: ABY66953.1. For the alignment, only the mature protein was used, and the percentage of positive amino acids found in the sequences was 94%. Cysteines are conserved in all sequences.

Identification	Sequences		I%	P%
Def-IFCa (Q)	1 EICCQVPTTPFLCTNDPQ----------	18	-	-
Def-IFCa (L)	1 EICCLVPTTPFLCTNDPQ----------	18	-	-
AHI85723.1	18 EICCKVPTTPFLCTNDPQCKALCSKVNYEDGHCFDILSKCVCMNRCVQDAKTLAAELLEEEFLKQ	82	94	94
AHI85724.1	27 EICCKVPTTPFLCTNDPQCKALCSKVNYEDGHCFDILSKCVCMNRCVQDAKTLAAELLEEEFLKQ	91	94	94
XP_016579689.1	27 EICCKVPTTPFLCTNDPQCKTLCSKVNYEDGHCFDILSKCVCMNRCVQDAKTLAAELIEEEFLKQ	91	94	94
ABY66953.1	27 EICCKVPTTPFLCTNDPQCKTLCSKVNYEDGHCFDILSKCVCMNRCVQDAKTLAAELIEEEFLKQ	91	94	94

Figure 5. Alignment of the 18 amino acid residues of F7 fraction of 6 kDa obtained by Edman degradation sequencing. Alignment was performed by Clustal Omega, and the sequence obtained was designated *Def*-IF*Ca*. The sequence obtained showed 94% similarity to the sequences of *Capsicum annuum*: Sequence ID: AHI85723.1 Stress-induced protein 18; Sequence ID: AHI85724.1 Stress-induced protein 19; Sequence ID: XP_016579689.1 flower defensin-like from *C. annuum*; and Sequence ID: ABY66953.1 Thionin-Like. The percentage of identity between the sequences was determined with Needleman-Wunsch Global Align Protein Sequences methods, and the signal peptides were omitted. I% - percentage of identity. P% - percentage of positive residues.

Plant defensins are present throughout the plant kingdom and can trigger several defense-related functions, such as inhibitors of α-amylase enzyme, present mainly in the insect chewing process, and antimicrobial activity against various pathogens (Carvalho & Gomes, 2009). Carvalho and Gomes (2011) presented some new characteristics that transgenic plants possess after a superexpression of defensins. Among these characteristics are resistance to heavy metals in *Arabidopsis thaliana*, resistance to fruit and root rot of *Carica papaya*, resistance to *Botrytis cinerea* in *Solanum lycopersicon,* and resistance to early dying disease in *Solanum tuberosum* caused by *Verticillium dahlia*, among other characteristics in different cultures such as sweet taste in *Zea mays*. Van del Weerden & Anderson (2013) compared the sequences of more than 139 plant defensins and related the biological functions that each performs. They concluded that although they exhibit a common fold with a β-sheet connected to an α-helix with three sulfide bridges, the overall level of identity between the plant defensins was less than 35% and defensins differed in the activity performed, concluding that their sequences are divergent, which helps to diversify the activities performed by them as antifungal and antibacterial activities, enzymatic inhibitory activities, and important roles in heavy metal tolerance.

3.2.4.4. Plasma membrane permeabilization

Due to the strong inhibitory activity of the F7 fraction, tests for membrane permeation, endogenous increases in ROS and mitochondrial functionality were performed for this fraction only.

The membrane permeabilization assay (Figure 6) showed that the F7 fraction was able to cause structural alterations in the plasma membrane of *C. gloeosporioides*, resulting in the permeabilization of fungal cells as indicated by the green fluorescence. It was possible to visualize a few hyphae developing in the presence of the F7 fraction but deformations were present, such as swollen hyphae and disordered growth. The absence of spores in the controls was also observed, indicating that the spores developed into hyphae; however, it was possible to observe spores in the tests with the F7 fraction, indicating that the fraction is able to delay the germination process. However, none of the spores observed in the tests showed fluorescence.

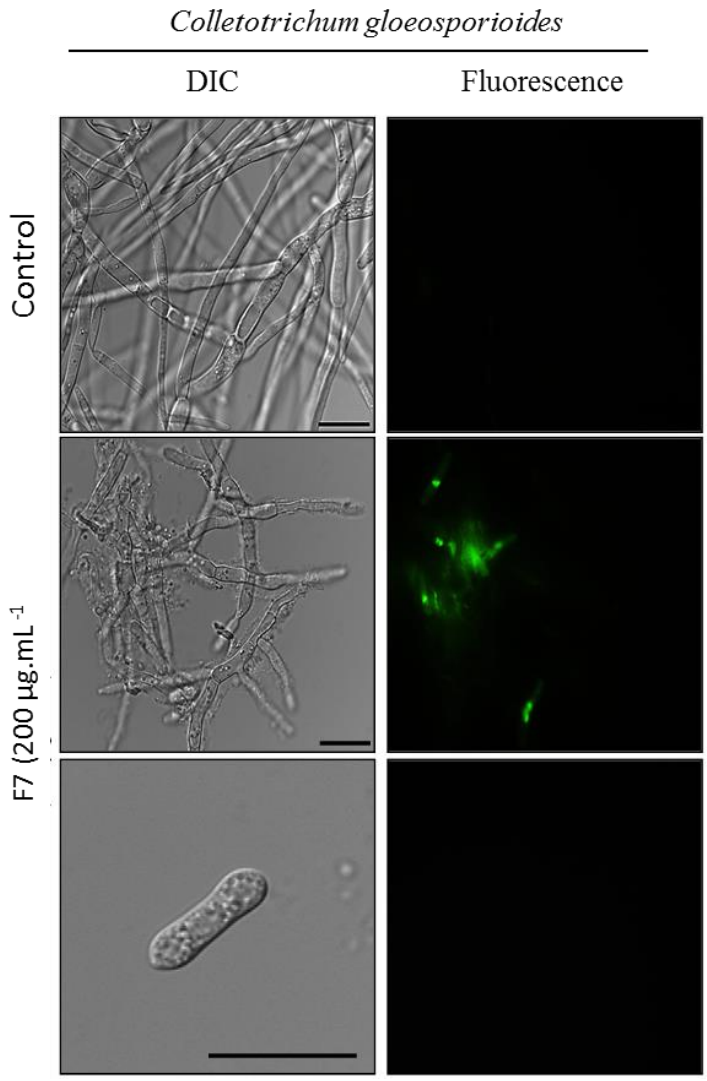

Figure 6. Membrane permeabilization assay of cells and spore of *C. gloeosporioides* by fluorescence microscopy using the fluorescent probe Sytox Green. Cells were treated with 200 µg mL^{-1} of fraction F7 and then assayed for membrane permeabilization. Control cells were treated only with the Sytox Green probe. Bars = 20 µm.

Taveira et al. (2017) observed plasma membrane permeabilization of *F. solani* cells after 12 h of incubation with 50 µg mL^{-1} of *CaThi*. The presence of propidium iodide after 24 h of incubation was observed, indicating necrotic cell death of some cells. The appearance of fluorescent spots was also observed in the cell nuclei after 48 h of incubation with *CaThi*. That work also showed that after 60 h of incubation, the *CaThi-FITC* peptide was inside the fungal cells. Taveira et al. (2018) tested *CaThi* on *Saccharomyces cerevisiae* and *Candida tropicalis* cells

and concluded that the protein can induce apoptosis in *S. cerevisiae* cells. Santos et al. (2017) showed that the Fa5 fraction of *Capsicum annuum* seeds was able to permeabilize the membrane of the fungi *Fusarium lateritium*, *F. solani* and *F. oxysporum* after 24 h of incubation with 200 µg mL^{-1} of the fraction. In a study conducted by Seo et al. (2014), a large amount of the J1-1 peptide (defensin) was located in the cells invaded by the pathogen and excreted onto the surface of the cell and around the fungus, which shows a broad mechanism of action of defensin in its utilization by the plant in defense against pathogens.

3.2.4.5. ROS induction assay

An assay was performed to verify whether the F7 fraction caused the increase in the endogenous production of ROS, where the fungus was incubated along with the fraction for a period of 24 h. In figure 6, it is possible to verify the fluorescent labeling in both the hyphae and the spores of *C. gloeosporioides*, indicating the increase in the endogenous production of ROS in these structures in the presence of the fraction, which does not happen in the control (without the F7 fraction) (Figure 7). It is suggested that the increase in oxidative stress induced by the F7 fraction may be the basis of the inhibitory effect against this fungus. Santos et al. (2017) showed that 200 µg mL^{-1} of Fa5 was able to induce ROS in several *Fusarium* species but was not able to induce ROS in *C. gloeosporioides*. Silva et al. (2017) found that *CaTI* was able to induce endogenous ROS in all fungi tested at a concentration of 64 µg mL^{-1}. According to Camejo et al. (2016), ROS act as antimicrobial molecules present in the cell wall that serve to prevent the entry of pathogens.

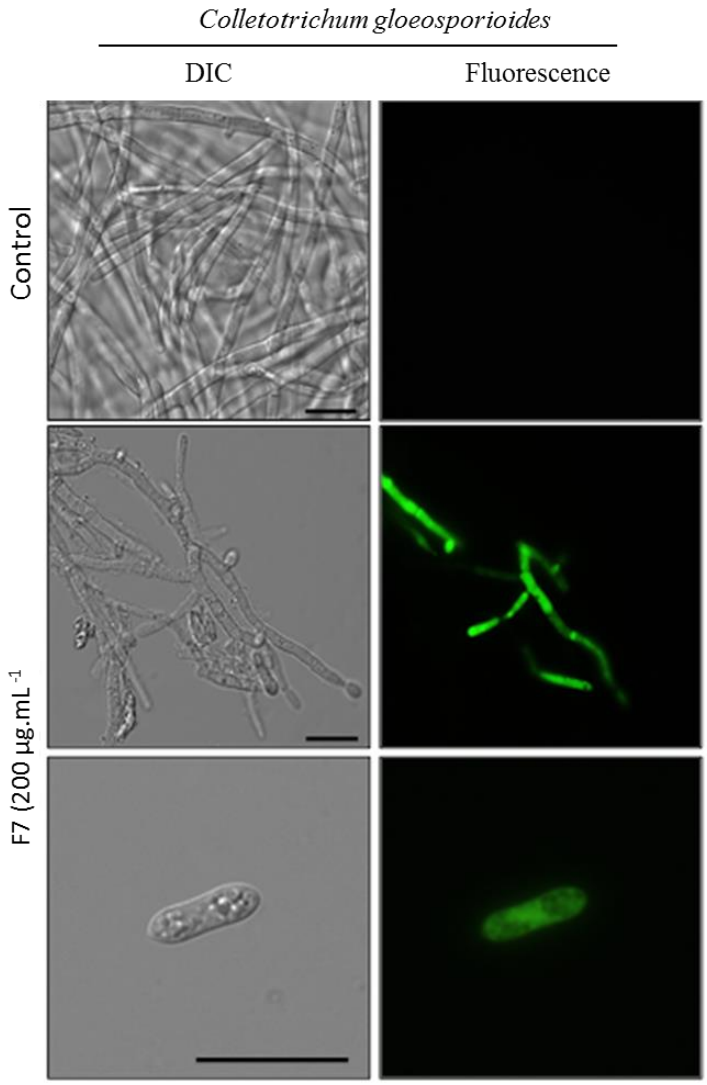

Figure 7. Cells and spore of *C. gloeosporioides* after ROS induction assay by light microscopy using the fluorescent probe 2,7-dichlorofluorescein diacetate. Cells were treated with 200 µg mL^{-1} of fraction F7 for 24 h and then assayed for oxidative stress. Control cells were treated only with 2,7-dichlorofluorescein diacetate probe. Bars = 20 µm.

3.2.4.6. Analysis of mitochondrial functionality

Figure 8 shows the assay to verify the mitochondrial functionality of fungal cells. After 24 h of incubation of *C. gloeosporioides* cells with 200 µg mL^{-1} of fraction F7, the cells had decreased mitochondrial activity compared to that of the control cells (without fraction), which can be observed by the low fluorescence signal of Rhodamine 123 dye in both treated hyphae and spores. For control cells

that have functional mitochondria, a strong fluorescence signal of Rhodamine 123 is observed.

These data show that the F7 fraction, where the *Def*-IF*Ca* protein has been identified, is toxic to *C. gloeosporioides*, causing strong inhibition of cell growth, in addition to causing damage to the few hyphae that have developed and to the spores. It is suggested that this identified fraction and the associated protein may contribute to anthracnose resistance in the immature stage of the fruits of *C. annuum* and are thus important tools to be examined in the improvement of *Capsicum* breeding for resistance to pests and diseases.

Taveira et al. (2018) presented the results of the action of *CaThi* in cells of *C. tropicalis*, where after 24 h of treatment with 10 µg mL^{-1} of the protein, there was a decrease in mitochondrial signals. Aerts et al. (2011) evaluated the effects of HsAFP1 antifungal defensin on mitochondrial functionality and ROS production because mitochondria are the main source of endogenous ROS in *Candida albicans* cells. Vieira et al. (2015) evaluated the mitochondrial functionality of *C. albicans* in the presence of the *Lp-Def 1* defensin of *Lecythis pisonis* and concluded that there is loss of mitochondrial functionality after 36 h of incubation.

All these results corroborate each other, stating the toxicity that *Def*-IF*Ca* defensin of immature fruits of *C. annuum* presents in the hyphae and conidia of the filamentous fungus *C. gloeosporioides*. The data continue to affirm the presence of antimicrobial peptides in fruits of *Capsicum* and can be an effective tool in the control of anthracnose, thus reducing crop losses caused by the disease.

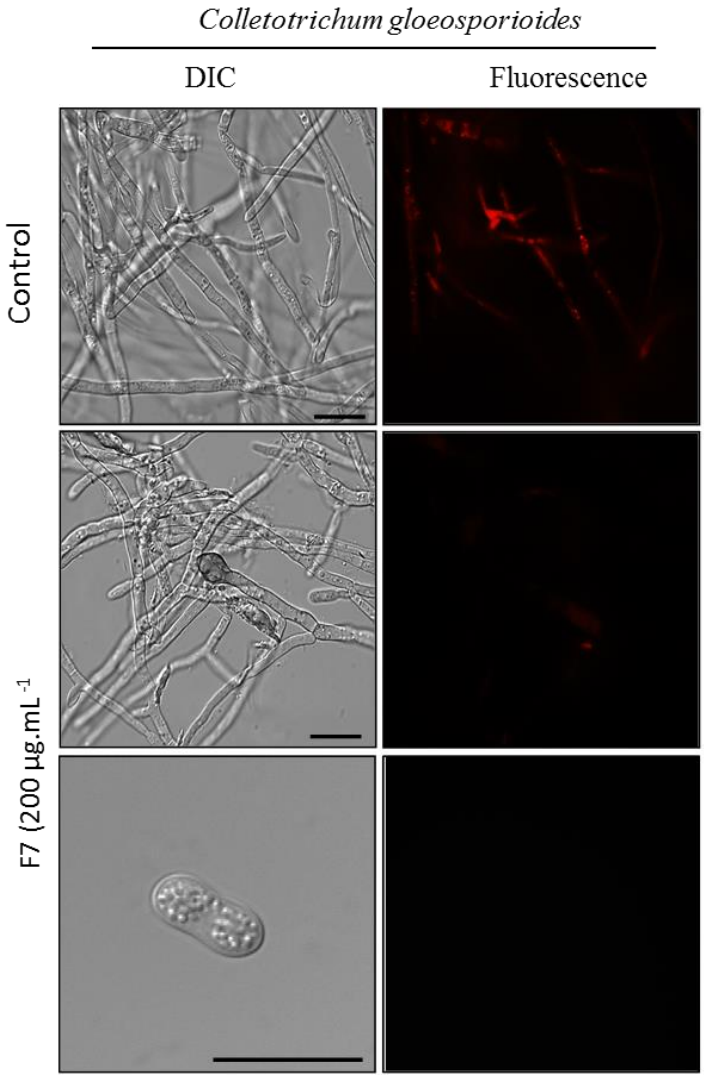

Figure 8. Cells and spore of *C. gloeosporioides* after mitochondrial functionality assay, visualized by fluorescence microscopy using Rhodamine 123 fluorescent probe. Cells were treated with 10 µg mL^{-1} of CaThi for 24 h and then analyzed for mitochondrial functionality. Control cells were treated only with the Rhodamine 123 probe. Bars = 20 µm.

3.2.5. CONCLUSION

Fraction F7, which possess the antimicrobial peptide *Def*-IF*Ca*, presented toxicity against the fungus *C. gloeosporioides*, causing inhibition of growth, membrane permeabilization, induction of endogenous ROS and decreased mitochondrial functionality of the pathogen.

4. GENERAL CONCLUSIONS

1. There are differences in the electrophoretic profile between the 24-h and 48-h samples of immature *C. annuum* fruits from treatments Ikeda (susceptible) and UENF1381 (resistant) in response to the inoculation of the fungus *C. gloeosporioides*;
2. The IIF48 samples of the susceptible and resistant treatments showed growth-inhibitory capacity of up to 100% against *C. gloeosporioides*;
3. Defensin CaDef1 was identified and characterized in the resistant IIF48 sample, and a protease inhibitor was identified in the crude extract of IIF48 from the susceptible treatment;
4. Lipid transfer proteins (LTPs) were immunodetected in uninoculated samples of immature fruits of UNEF1381;
5. Protease inhibitors were detected in the samples of immature fruits from treatments Ikeda and UENF1381;
6. The highest activity of β-1,3-glucanase was detected in the samples of mature fruits from the susceptible treatment (Ikeda);
7. Fraction F7 showed the greatest inhibitory capacity against the growth of *C. gloeosporioides*;
8. Fraction F7 was able to permeabilize the plasma membrane, increase the production of endogenous ROS, and prevent the mitochondrial activity of the fungus *C. gloeosporioides*;
9. The plant defensin of fraction F7 was named Def-IFCa.

REFERENCES

Aerts, A.M., Bammens, L., Govaert, G., Carmona-Gutierrez, D., Madeo, F., Cammue, B., Thevissen, K. (2011) The antifungal plant defensin HsAFP1 from *Heuchera sanguinea* induces apoptosis in *Candida albicans*. *Front in microbial*, 2:47-56.

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(1):39-47.

Altschul, S.F., Madden, T.L., Schaffer, A.A., Zhang, J., Zhang, Z., Miller, W., Lipman, D.J. (1997) Gapped blast and psi-blast: a new generation of protein database search programs. *Nucl Acids Res*, 25:3389-402.

Amorim, L. (1995) Avaliação de doenças. *In:* Bergamin Filho, A., Kimati, H., Amorim, L, *Manual de Fitopatologia*; 3 ed. São Paulo: Ceres, p. 647-671.

Araújo, L., Stadnik, M.J. (2013) Multiple appressoria and conidial anastomosis tubes in the infection process of *Colletotrichum gloeosporioides* on apple. *Bragantia*, 72(2):180-183.

Baba, V.Y., Constantino, L.V., Ivamoto, S.T., Moreira, A.F.P., Madeira, T.B., Nixdorf, S.L., Rodrigues, R., Gonçalves, L.S.A. (2019) *Capsicum-Colletotrichum* interaction: Identification of resistance sources and

quantification of secondary metabolites in unripe and ripe fruits in response to anthracnose infection. *Sci Hortic*, 246:469-477.

Bahar, A.A., Ren, D. (2013) Antimicrobial peptides. *Pharmaceuticals*, 6(12):1543-1575.

Balandín, M., Royo, J., Gómez, E., Muniz, L.M., Molina, A., Hueros, G. (2005) A protective role for the embryo surrounding region of the maize endosperm, as evidenced by the characterization of *ZmESR-6*, a defensin gene specifically expressed in this region. *Plant mol biol*, 58(2):269-282.

Balasubramanian, V., Vashisht, D., Cletus, J., Sakthivel, N. (2012) Plant β-1, 3-glucanases: their biological functions and transgenic expression against phytopathogenic fungi. *Biotechnol Lett*, 34(11):1983-1990.

Barros, F.C., Sagata, É., de Castro Ferreira, L.C., Juliatti, F.C. (2010) Indução de resistência em plantas à fitopatógenos. *Biosci J*, 26(2):231-239.

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. *Genet Mol Res*, 16(3):1-13.

Bradford, M.M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analyt Biochem*, 72(1-2):248-254.

Broekaert, W.F., Cammue, B.P., De Bolle, M.F., Thevissen, K., De Samblanx, G.W., Osborn, R.W., Nielson, K. (1997) Antimicrobial peptides from plants. *Crit Rev Plant Sci*, 16(3):297-323.

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

Camejo, D., Guzmán-Cedeņo, Á., Moreno, A. (2016) Reactive oxygen species, essential molecules, during plant–pathogen interactions. *Plant Physiol Biochem*, 103:10-23.

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

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

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

Costa, F.G. (2014) Caracterização do efeito antifúngico de peptídeos presentes na peçonha de escorpiões em linhagens de *Cryptococcus neoformans*. Dissertação (Mestrado em Biologia Molecular) – Brasília – DF, Universidade de Brasília – UNB, 67p.

Cruz, M.F.A., Araujo, L., Polanco, L.R., Rodrigues, F.A. (2014) Aspectos microscópicos da interação feijoeiro-*Colletotrichum lindemuthianum* mediados pelo silício. *Bragantia*, 73(3):284-291.

De Coninck, B.M., Sels, J., Venmans, E., Thys, W., Goderis, I.J., Carron, D., Delaure´, S.L., Cammue, B.P.A., De Bolle, M.F.C., Mathys, J. (2010) *Arabidopsis thaliana* plant defensin AtPDF1. 1 is involved in the plant response to biotic stress. *New Phytol*, 187(4):1075-1088.

De Coninck, B., Cammue, B.P., Thevissen, K. (2013) Modes of antifungal action and in planta functions of plant defensins and defensin-like peptides. *Fungal Biol Rev*, *26*(4):109-120.

Dias, G.B., Gomes, V.M., Pereira, U.Z., Ribeiro, S.F.F., Carvalho, A.O., Rodrigues, R., Machado, O.L.T., Fernandes, K.V.S., Ferreira, A.T.S., Da Cunha, M. (2013) Isolation, characterization and antifungal activity of proteinase inhibitors from *Capsicum chinense* Jacq. seeds. *The prot J,* 32(1):15-26.

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

Do, H.M., Lee, S.C., Jung, H.W., Sohn, K.H., Hwang, B.K. (2004) Differential expression and in situ localization of a pepper defensin (*CADEF1*) gene in response to pathogen infection, abiotic elicitors and environmental stresses in *Capsicum annuum*. *Plant Sci*, 166(5):1297-1305.

Felicioli, R., Garzelli, B., Vaccari, L., Melfi, D., Balestreri, E. (1997) Activity staining of protein inhibitors of proteases on gelatin containing polyacrylamide gel electrophoresis. *Analyt Biochem*, 244:176-178.

Fernandes, C.D.F., Vieira Júnior, J.R., da SILVA, D.S.G., Reis, N.D., Antunes Júnior, H. (2009) Mecanismos de defesa de plantas contra o ataque de agentes fitopatogênicos. *Embrapa Rondônia-Documentos*, (INFOTECA-E).

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(2):270-275.

Ferreira, R.B., Monteiro, S., Freitas, R., Santos, C.N., Chen, Z., Batista, L.M., Duarte, J., Borges, A., Teixeira A.R. (2007) The role of plant defence proteins in fungal pathogenesis. *Mol Plant Pathol,* 8:677–700.

França, G.S., Costa-Carvalho, R.R., Neves, R.P., Araujo, E.R., Laranjeira, D. (2015) Controle pós-colheita da antracnose do pimentão pela *levedura Rhodotorula glutinis Biosci J,* 31(2):451-459.

García-Olmedo, F., Rodriguez-Palenzuela, P., Molina, A., Alamillo, J.M., Lopez-Solanilla, E., Berrocal-Lobo, M., Poza-Carrion, C. (2001) Antibiotic activities of peptides, hydrogen peroxide and peroxynitrite in plant defense. *FEBS Lett*, 498:219–222.

Guaní-Guerra, E., Santos-Mendoza, T., Lugo-Reyes, S.O., Terán, L.M. (2010) Antimicrobial peptides: general overview and clinical implications in human health and disease. *Clin Immunol*, 135:1-11.

GORJANOVIĆ, S. (2009) A review: biological and technological functions of barley seed pathogenesis - related proteins (PRS). *J I Brewing*, 115(4): 334–360.

Harp, T.L., Kuhn, P., Roberts, P.D., Pernezny, K.L. (2013) Management and cross-infectivity potential of *Colletotrichum acutatum* causing anthracnose on bell pepper in Florida. *Phytoparasitica*, 42:31–39.

Heil, M. (2010) Plastic defence expression in plants. *Evol Ecol*, 24:555-569.

Horne, W.S., Wiethoff, C.M., Cui, C., Wilcoxen, K.M., Amorin, M., Ghadiri, M.R., Nemerow, G. R. (2005) Antiviral cyclic d, l-α-peptides: Targeting a general biochemical pathway in virus infections. *Bioorg Med Chem*, 13(17):5145-5153.

Kim, S.H., Yoon, J.B., Do, J.W., Park, H.G. (2008) A major recessive gene associated with anthracnose resistance to *Colletotrichum capsici* in chili pepper (*Capsicum annuum* L.). *Breed Sci*, 58(2):137-141.

Kim, J.S., Jee, H.J., Gwag, J.G., Kim, C.K., Shim, C.K. (2010) Evaluation on red pepper germplasm lines (*Capsicum* spp.) for resistance to anthracnose caused by *Colletotrichum acutaum*. *Plant Pathol J*, 26(3):273-279.

León, I.R., Neves-Ferreira, A.G., Valente, R.H., Mota, E.M., Lenzi, H.L., Perales, J. (2007) Improved protein identification efficiency by mass spectrometry using N-terminal chemical derivatization of peptides from *Angiostrongylus costaricensis*, a nematode with unknown genome. *J. Mass Spectrom*, 42:781-92.

Lin, S.W., Gniffke, P.A., Wang, T.C. (2007) Inheritance of resistance to pepper anthracnose caused by *Colletotrichum acutatum*. *Acta hort*, 760:329-334.

Macedo, M.L.R., Ribeiro, S.F., Taveira, G.B., Gomes, V.M., de Barros, K.M., Maria-Neto, S. (2016) Antimicrobial Activity of ILTI, a Kunitz-Type Trypsin Inhibitor from Inga laurina (SW.) Wild. *Curr Microbiol*, 72(5):538-544.

Mahasuk, P., Taylor, P.W.J., Mongkolporn, O. (2009) Identification of two new genes conferring resistance to *Colletotrichum acutatum* in *Capsicum baccatum*. *Phytopathology*, 99(9):1100-1104.

Maracahipes, A.C. (2014) Reação de acessos de *Capsicum* spp. a *Colletotrichum gloeosporioides*. Dissertação (Mestrado em Genética e Melhoramento de Plantas) – Cáceres – MT, Universidade do Estado de Mato Gross – UNEMAT, 65p.

Maracahipes, A.C., Viscovini, K.K.C.G., Silva Annunciatto, E., Neves, L.G., da Luz, P.B., Araujo, K.L. (2016) Genetic diversity of the germplasm active bank of' *Capsicum* of UNEMAT based on components resistant to the fungus *Colletotrichum gloeosporioides*. *Aust J Crop Sci*, 10(7):940-948.

Maracahipes, A.C., Correa, J.W.S., Teodoro, P.E., Barelli, M.A.A., Neves, L.G. (2017) Correlation study of resistance components in the selection of *Capsicum* genotypes resistant to the fungus *Colletotrichum gloeosporioides*. *Genet Mol Res*, 16(3):gmr16039720.

Martins, M.L.B. (2010) Proteínas e peptídeos relacionados à defesa de plantas e análise proteômica de folhas de tomate inoculadas com *Xanthomonas campestris* pv. *Vesicatoria.* Dissertação (Mestrado em Bioquímica Agrícola) – Viçosa – MG, Universidade federal de Viçosa – UFV, 109p.

Menezes, M. (2013) Aspectos biológicos e taxonômicos de espécies do gênero *Colletotrichum*. *An Acad Pernamb Ciênc Agron*, 3:170-179.

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.

Mello, É.O. (2014) Defensinas de sementes de *Phaseolus vulgaris* L.: superexpressão e estudo da atividade antimicrobiana e do mecanismo de ação. Tese (Doutorado em Biocências) – Campos dos Goytacazes – RJ, Universidade Estadual do Norte Fluminense Darcy Ribeiro – UENF, 130p.

Meyer, B., Houlne, G., 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(2):615-622.

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(1):217-231.

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

Nelson, N. (1944) A photometric adaptation of the Somogyi method for the determination of glucose. *J Biol Chem*, 153(2):375-380.

Oo, M.M., Oh, S.K. (2016) Chilli anthracnose (*Colletotrichum* spp.) disease and its management approach. *Korean J of Agr Sci*, 43:153-162.

Pakdeevaraporn, P., Wasee, S., Taylor, P.W.J., & Mongkolporn, O. (2005) Inheritance of resistance to anthracnose caused by *Colletotrichum capsici* in *Capsicum*. *Plant Breed*, 124(2):206-208.

Parisi, K., Shafee, T.M., Quimbar, P., van der Weerden, N.L., Bleackley, M.R., Anderson, M.A. (2018) The evolution, function and mechanisms of action for plant defensins. *Semin Cell Dev Biol*, 17:30469-X.

Park, C.B., Kim, H.S., Kim, S.C. (1998) Mechanism of action of the antimicrobial peptide buforin II: buforin II kills microorganisms by penetrating the cell membrane and inhibiting cellular functions. *Biochem Biophys Res Commun*, 244(1):253-257.

Pearce, G., Ryan, C.A., Liljegren, D. (1988) Proteinase inhibitors I and II in fruit of wild tomato species: transient components of a mechanism for defense and seed dispersal. *Planta*, 175(4):527-531.

Pereira, M.J.Z., Massola-Junior, N.S., Sussel, A.A.B., Sala, F.C., Costa, C.P., Boiteux, L.S. (2011) Reação de acessos de *Capsicum* e de progênies de

cruzamentos interespecíficos a isolados de *Colletotrichum acutatum*. *Hortic Bras,* 29:569-576.

Peters, B.M., Shirtliff, M.E., Jabra-Rizk, M.A. (2010) Antimicrobial Peptides: Primeval molecules or future drugs. *PLoS Pathog*, 3:6-10.

Ramdial, H., Rampersad, S.N. (2015) Characterization of *Colletotrichum* spp. causing anthracnose of bell pepper (*Capsicum annuum* L.) in Trinidad. *Phytoparasitica*, 43(1):37-49.

Ribeiro, S.F., Carvalho, A.O., Da Cunha, M., Rodrigues, R., Cruz, L.P., Melo, V.M., Vasconcelos, I.M., Melo, E.J., Gomes, V.M. (2007) Isolation and characterization of novel peptides from chilli pepper seeds: antimicrobial activities against pathogenic yeasts. *Toxicon*, 50(5):600-611.

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., Souza-Filho 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.

Robinson Jr, W.E., McDougall, B., Tran, D., Selsted, M.E. (1998) Anti-HIV-1 activity of indolicidin, an antimicrobial peptide from neutrophils. *J Leukoc Biol*, 63(1):94-100.

Rogozhin, E.A., Oshchepkova, Y.I., Odintsova, T.I., Khadeeva, N.V., Veshkurova, O.N., Egorov, T.A., Grishin, E.V., Salikhov, S.I. (2011) Novel antifungal defensins from *Nigella sativa* L. seeds. *Plant Physiol Biochem*, 49(2):131-137.

Ryan, C.A. (1990) Protease inhibitors in plants: genes for improving defenses against insects and pathogens. *Rev Anual Fitopatol*, 28(1):425-449.

Santos, P.C.M., Lima, W.G., Bezerra, C.S., Michereff, S.J., Câmara, M.P.S. (2015) Diversidade genotípica e patogênica de *Colletotrichum musae* no estado de pernambuco. *Rev Bras Frutic,* 37(2):355-366.

Santos, L.A.D., Taveira, G.B., Ribeiro, S.F.F., Pereira, L.S., Carvalho, A.O., Rodrigues, R., Oliveira, A.E.A., Machado, O.L.T., Araujo, J.S.,

Vaconcelos, 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.

Sathoff, A.E., Velivelli, S., Shah, D., Samac, D.A. (2018) Plant defensin peptides have antifungal and antibacterial activity against human and plant pathogens. *Phytopathology*, (ja).

Schägger H., Von-Jagow G. (1987) Tricine-sodium dodecyl sulfate polyacrylamide gel electrophoresis for the separation of proteins in the range from to 100 kDa. *Analyt Biochem*, 166:368-79.

Seo, H.H., Park, S., Park, S., Oh, B.J., Back, K., Han, O., Kim, J., Kim, Y.S. (2014) Overexpression of a Defensin Enhances Resistance to a Fruit-Specific Anthracnose Fungus in Pepper. *PLoS one*, 9(5):e97936.

Sels, J., Mathys, J., De Coninck, B.M., Cammue, B.P., De Bolle, M. F. (2008) Plant pathogenesis-related (PR) proteins: a focus on PR peptides. *Plant Physiol Biochem*, *46*(11):941-950.

Silva, M.S., Ribeiro, S.F.F., Taveira, G.B., Rodrigues, R., Fernandes, K.V.S., Vaconcelos, I.M., Carvalho, A.O., 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*, 1:1-1.

Stangarlin, J.R., Kuhn, O.J., Toledo, M.V., Portz, R.L., Schwan-estrada, K.R.F., Pascholati, S.F. (2011) A defesa vegetal contra fitopatógenos. *Sci Agr paran*, 10:18-46.

Somogyi, M. (1952) Notes on sugar determination. *J of Biolog Chem*, 195:19-23.

Sumikawa, J.T., de Brito, M.V., Macedo, M.L.R., Uchoa, A.F., Miranda, A., Araujo, A.P.U., Silva-Lucca, R.A., Sampaio M.U., Oliva, M.L.V. (2010) The defensive functions of plant inhibitors are not restricted to insect enzyme inhibition. *Phytochemistry*, 71(2-3):214-220.

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.

Tamamura, H., Ishihara, T., Otaka, A., Murakami, T., Ibuka, T., Waki, M., Matsumoto, A., Yamamoto, N., Fujii, N. (1996) Analysis of the interaction of an anti-HIV peptide, T22 ([$Tyr^{5,12}$, Lys^{7}]-polyphemusin II), with gp 120 and CD4 by surface plasmon resonance. *Biochim Biophys Acta*, 1298(1):37-44.

Taveira, G.B., Mathias, L.S., Motta, O.V., Machado, O.L.T., Rodrigues, R., Carvalho, A.O., Teixeira-Ferreira, A., Perales, J.P., Vasconcelos, I.M., Gomes, V.M. (2014) Peptides From *Capsicum annuum* Fruits With High Activity Against Human Pathogenic Bacteria and 8 Yeasts. *Pept Sci,* 102:30-39.

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(1):12-26.

Taveira, G.B., Mello, É.O., Carvalho, A.O., Regente, M., Pinedo, M., De La Canal, L., Rodrigues, R., and 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*. *Pept Sci*, 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:BSR20180119.

Terras, F., Schoofs, H., De Bolle, M., Van Leuven, F., Rees, S., Vanderleyden, J., Cammue, B. Broekaert, W. (1992) Analysis of two novel classes of plant antifungal proteins from radish (*Raphanus sativus* L.) seeds. *J Biol Chem*, 267:15301-15309.

Terras, F.R.G., Torrekens, S., Van Leuven, F., Osborn, R.W., Vanderleyden, J., Cammue, B.P., Broekaert, W.F. (1993) A new family of basic cysteine-rich plant antifungal proteins from *Brassicaceae* species. *FEBS Lett*, 316:233-240.

Terras, F.R.G., Ergermont, K., Kovaleva, V., Raikhel, N.V., Osbourn, R.W., Kester, A., Rees, S.B., Torrekens, S., Van Leuden, F., Vanderleyden, J., Cammue, B.P.A. Broekaert, W.F. (1995) Small cystein-rich antifungal proteins from radish: their role in host defense. *Plant Cell*, 7:573-588.

Than, P.P., Prihastuti, H., Phoulivong, S., Taylor, P.W., Hyde, K.D. (2008) Chilli anthracnose disease caused by *Colletotrichum* species. *J. Zhejiang Univ Sci B*, 9(10):764-778.

Thevissen, K., Terras, F.R., Broekaert, W.F. (1999) Permeabilization of fungal membranes by plant defensins inhibits fungal growth. *Appl Environ Microbiol*, 65(12):5451-5458.

Thevissen, K., Ferket, K.K., François, I.E., Cammue, B.P. (2003) Interactions of antifungal plant defensins with fungal membrane components. *Peptides*, 24(11):1705-1712.

Towbin H., Staehelin T., Gordon J. (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc Natl Acad Sci*, 76:4350-4354.

Tozze-Júnior, H.J., Mello, M.B., Júnior, N.S.M. (2006) Caracterização morfológica e fisiológica de isolados de *Colletotrichum* sp. causadores de antracnose em solanáceas. *Crops*, 32(1):71-79.

Van der Weerden, N.L., Anderson, M.A. (2013) Plant defensins: common fold, multiple functions. *Fungal Biol Rev*, 26(4):121-131.

Van Loon, L.C., Van Strien, E.A. (1999) The families of pathogenesis-related proteins, their activities, and comparative analysis of PR-1 type proteins. *Physiol Mol Plant Pathol,* 55(2):85-97.

Vieira-Bard, G.C., Nascimento, V.V., Oliveira, A.E.A., Rodrigues, R., Da Cunha, M., Dias, G.B., Vasconcelos, I.M., Carvalho, A.O., Gomes, V.M. (2014)

Vicilin-like peptides from *Capsicum baccatum* L. seeds are α-amylase inhibitors and exhibit antifungal activity against important yeasts in medical mycology. *Pept Sci*, 102(4):335-343.

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 (Shanghai)*, 47(9):716-729.

Voorrips, R.E., Finkers, R., Sanjaya, L., Groenwold, R. (2004) QTL mapping of anthracnose (*Colletotrichum* spp.) resistance in a cross between *Capsicum annuum* and *C. chinense*. *Theor Appl Genet,* 109(6):1275-1282.

Vriens, K., Cammue, B., Thevissen, K. (2014) Antifungal plant defensins: mechanisms of action and production. *Molecules*, 19(8):12280-12303.

Wang, S.Y., Wu, J.H., Ng T.B., Ye, X.Y., Rao, P.F. (2004) A non-specific lipid transfer protein with antifungal and antibacterial activities from the mung bean. *Peptides*, 25:1235–42.

Wit, P.J.G.M. (2007) How plants recognize pathogens and defend themselves. *Cell Mol Life Sci*, 64:2726-2732.

APPENDIX

Table A1. Inventory of some plant defensins described.

Plant species	Defensin name	Family	Tissue	Biological activity
Adzuckia angularia	RBAFP	Fabaceae	seed	inhibitory activity against protein translation synthesis, antifungal, mitogenic activity, inhibitory effect toward HIV reverse transcriptase
Aesculus hippocastanum	*Ah*-AMP$_1$	Hippocastanaceae	seed	antifungal, antibacterial
Arabidopsis halleri	*Ah*-PDF1.1	Brassicaceae	shoot	heavy metal tolerance; antifungal
Arabidopsis thaliana	PDF1.1	Brassicaceae	seed, infected leaf	antifungal
Beta vulgaris	AX$_1$, AX$_2$	Chenopodiaceae	leaf	antifungal
Brassica campestris	*BSD1*	Brassicaceae	stamen	antifungal
Brassica napus	*Bn*-AMP$_1$, *Bn*-AMP$_2$	Brassicaceae	seed	antifungal
Brassica rapa	*Br*-AMP$_1$ *Br*-AMP$_2$	Brassicaceae	seed	antifungal antifungal, antibacterial
Brassica oleracea	PCP-A$_1$	Brassicaceae	pollen coating	self-incompatibility
Cassia fistula	5459 5144	Fabaceae	seed	inhibitory activity against trypsin without inhibitory activity against trypsin
Capsicum annuum	CADEF$_1$ J1	Solanaceae	leaf (cloned) red fruit	antifungal

Table A1. Cont.

Plant species	Defensin name	Family	Tissue	Biological activity
Capsicum chinense	γ-thionin	Solanaceae	cloned	antifungal, inhibitory activity toward cancer cell
Clitoria ternatae	*Ct*-AMP$_1$	Fabaceae	seed	antifungal, antibacterial
Dhalia merckii	*Dm*-AMP$_1$	Asteraceae	seed	antifungal, antibacterial
	Dm-AMP$_2$			antifungal
Elaeis guineensis	EGAD$_1$	Arecaceae	tissue culture	expression associated with epigenetic somaclonal variation
Echinochloa crusgalli	*Ec*-AMP-D$_1$	Poaceae	seed	antifungal
	Ec-AMP-D$_2$			
Ginkgo biloba	*Gbd*	Ginkgoaceae	leaf	not determined
Gymnocladus chinensis	gymnin	Fabaceae	seed	antifungal, mitogenic, inhibitory activity toward cancer cells, inhibitory effect toward HIV reverse transcriptase
Helianthus annuus	*SD*2	Asteraceae	flower (cloned)	not determined
	Ha-DEF$_1$		root (cloned)	antifungal, inhibitory activity against parasitic plant
Heuchera sanguinea	*Hs*-AMP$_1$	Saxifragaceae	seed	antifungal
Hordeum vulgare	γ-hordothionin	Poaceae	endosperm	inhibitory activity against protein translation synthesis, inhibitory activity against insect α-amylase
	ω-hordothionin			inhibitory activity against protein translation synthesis

Table A1. Cont.

Plant species	Defensin name	Family	Tissue	Biological activity
Ipomoea batatas	SPD_1	Convolvulaceae	tuber	dehydro- and monodehydoascorbate reductase activity, antifungal, antibacterial, inhibitory activity against trypsin
Lens culinaris	*Lc*-def	Fabaceae	germinated seed	antifungal
Lepidium meyenii	*Lm*-def	Asteraceae	leaf (cloned)	inhibitory activity against oomycete
Medicago sativa	$MsDEF_1$	Fabaceae	seed	antifungal, blocks mammalian L-type Ca^{+2} channels, inhibitory activity against plant root growth
Mendicago trucatula	$MtDEF_2$	Fabaceae	seed	antifungal, inhibitory activity against plant root growth
Nicotiana alata	*Na*D_1	Solanaceae	pistil	antifungal
Nicotiana megalosephon	*Nm*Def02	Solanaceae	inoculated leaves	antifungal
Nigella sativa	*Ns*-D_1, *Ns*-D_2	Ranunculaceae	seed	antifungal, antibacterial
Pachyrrhizus erosus	SPE10	Fabaceae	seed	antifungal
Pentadiplandra brazzeana	brazzein	Pentradiplandraceae	fruit pulp	sweet taste
Petunia hybrida	PhD_1, PhD_2	Solanaceae	petal	antifungal
Phaseolus limensis	BLBAMP	Fabaceae	seed	antifungal, inhibitory effect toward HIV reverse transcriptase
	limyin			antifungal, inhibitory activity toward cancer cells

Table A1. Cont.

Plant species	Defensin name	Family	Tissue	Biological activity
Phaselus vulgaris	PBAFP	Fabaceae	seed	inhibitory activity against protein translation synthesis; antifungal; mitogenic activity; inhibitory effect toward HIV reverse transcriptase
	PvD_1			antifungal; inhibit glucose stimulated acidification of the medium
	WCBAFP			antifungal, antibacterial, activity against protein translation synthesis, inhibitory activity toward cancer cells, mitogenic, inhibitory effect toward HIV reverse transcriptase
	vulgarinin			antifungal, antibacterial, activity against protein translation synthesis, inhibitory activity toward cancer cells, inhibitory effect toward HIV reverse transcriptase
Pinus sylvestris	*Ps*Def1	Pinaceae	seedlings	antifungal
Pisum sativum	*Ps*D_1, *Ps*D_2	Fabaceae	seed	antifungal
Prunus persica	*Pp*-Def_1	Rosaceae	bark (cloned)	antifungal
Raphanus sativus	*Rs*-AFP_1	Brassicaceae	seed	antifungal
	Rs-AFP_2			antifungal, weak antibacterial, inhibitory activity against plant root growth
Saccharum officinarum	Sd1, Sd3, Sd5	Poaceae	SUCEST plasmids	antifungal
Sinapis alba	*Sa*-AFP_1, *As*-AFP_2	Asteraceae	seed	antifungal
Solanum lycopersicon	DEF2	Solanaceae	clone	antifungal, probable developmental signal for male reprodutive development
Solanum tuberosum	PTH_1	Solanaceae	tuber	antifungal, antibacterial, weak activity against *Leishmania*

Table A1. Cont.

Plant species	Defensin name	Family	Tissue	Biological activity
Sorghum bicolor	*Sl*α$_1$, *Sl*α$_2$, *Sl*α$_3$	Poaceae	seed	inhibitory activity against insect α-amylase
Spinacea oleracea	*So*D$_1$	Poaceae	leaves	antibacterial
	*So*D$_{2-7}$			antifungal, antibacterial
Stellaria media	Sm-AMP-D1, Sm-AMP-D2	Caryophyllaceae	seed	antifungal
Tephrosia villosa	*Tv*D$_1$	Fabaceae	leaf	anifungal; inhibitory activity against plant root growth
Trichosanthes kirilowii	TDEF1	Curcubitaceae	leaf (cloned)	antifungal
Trigonella foenum-graecum	Tfgd1	Fabaceae	leaf (cloned)	antifungal
Triticum aestivum	TAD1	Poaceae	crown tissue	antibacterial, cold induction
Triticum turgidum	γ$_1$-purothionin γ$_2$-purothionin	Poaceae	endosperm	not determined
Vigna angularis	*Va*D1	Fabaceae	seed	antifungal, antibacterial, weak inhibitory activity against insect, inhibitory activity against protein translation synthesis
Vigna radiata	*Vr*CRP	Fabaceae	developing seed	antifungal, inhibitory activity against insect, inhibitory activity against insect cell culture,
	*Vr*D$_1$			inhibitory activity against protein translation synthesis
Vigna sesquipedalis	sesquin	Fabaceae	seed	antifungal, antibacterial, inhibitory activity toward cancer cells, inhibitory effect toward HIV reverse transcriptase

Table A1. Cont.

Plant species	Defensin name	Family	Tissue	Biological activity
Vigna unguiculata	VUDEF	Fabaceae	seed	antifungal, inhibitory activity against insect α-amylase
	Cp-thionin			inhibitory activity against trypsin
	Cp-thionin II			antibacterial
Vitis vinifera	*Vv*-AMP$_1$	Vitaceae	berry	antifungal
Wasabia japonica	WT$_1$	Brassicaceae	leaf	antifungal, weak antibacterial
Zea mays	γ$_1$-zeathionin	Poaceae	seed	sodium channel blocker
	γ$_2$-zeathionin (PDC-1)			sodium channel blocker, antifungal
	Zm-ESR6			antifungal, antibacterial