POTENTIAL OF ANTIMICROBIAL PEPTIDE OF SEEDS OF *Coffea canephora* cv ROBUSTA TROPICAL IN THE CONTROL OF PHYTOPATHOGENIC MICRORGANISMS

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Orientadora: Profª. Drª Valdirene Moreira Gomes

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SUMMARY

RESUMO

BARD, Gabriela Costa Vieira Universidade Estadual do Norte Fluminense Darcy Ribeiro; Outubro de 2016 “POTENCIAL DE PEPTÍDEOS ANTIMICROBIANOS DE SEMENTES DE *Coffea canephora* cv ROBUSTA TROPICAL NO CONTROLE DE MICRORGANISMOS FITOPATOGÊNICOS”; Orientadora: Drª Valdirene Moreira Gomes; Conselheiros: Drª Rosana Rodrigues e Dr André de Oliveira Carvalho.

Peptídeos antimicrobianos, os quais desempenham um papel importante na defesa das plantas, têm sido identificados particularmente de sementes. Neste trabalho nós isolamos e imunolocalizamos uma proteína transportadora de lipídeos (LTP), denominada *Cc*-LTP2, de sementes de *Coffea canephora.* Também descrevemos sua atividade antimicrobiana contra vários fungos fitopatogênicos de importância econômica e contra a bactéria *Xanthomonas euvesicatoria*. Os peptídeos de sementes de *C. canephora* foram inicialmente extraídos em tampão ácido e submetidos à cromatografia de troca iônica e de fase reversa. Um peptídeo purificado de aproximadamente 9 kDa foi então submetido ao seqüenciamento de aminoácidos. As análises mostraram que o peptídeo era semelhante a LTPs isoladas de várias plantas e foi denominado de *Cc*-LTP2. A localização tecidual de LTPs de *Coffea canephora* indicou localização tanto nas paredes celulares do parênquima paliçádico como localização intracelular no em grandes vacúolos. Os resultados de imunohistoqúimica e histoquímica de tecidos de sementes de *C. canephora* sobrepostos mostrou que LTPs e corpos lipídicos estão presentes em organelas, apoiando a hipótese de que as LTPs de sementes estão envolvidas na mobilização de lipídeos durante a germinação. *Cc*-LTP2 não inibiu o desenvolvimento dos fungos fitopatogênicos,

mas inibiu o crescimento de *X. euvesicatoria.Cc*-LTP2 promoveu a permeabilização das membranas e induziu a produção endógena de espécies reativas de oxigênio em todos os fungos testados. Outro objetivo do presente estudo foi avaliar a atividade antimicrobiana dos peptídeos *Cc*-LTP2 e *Cc*-GRP isolados de sementes de *C. canephora* e sua possível atividade sinérgica com a droga azólica Fluconazol. *Cc*-LTP2 e *Cc*-GRP separadamente e em combinação com 20 µg/mL de Fluconazol foram avaliados quanto à atividade antimicrobiana sobre o fungo *Fusarium solani*, e os efeitos destes peptídeos sozinhos e em conjunto com o Fluconazol sobre a permeabilização de membranas e a indução de estresse oxidativo foram determinados. Os nossos resultados mostraram que estes peptídeos, em uma concentração de 400 µg/mL combinados com 20 µg/mL de Fluconazol foram capazes de inibir o crescimento do fungo testado, promover alterações no seu padrão de crescimento, permeabilizar sua membrana e induzir espécies reativas de oxigênio (ROS). Alguns desses resultados não foram observados com os peptídeos sozinhos ou com o Fluconazol sozinho, sugerindo que os peptídeos e o Fluconazol atuem sinergicamente promovendo uma potencialização da atividade antimicrobiana. Por fim os peptídeos *Cc*-LTP e *Cc*-GRP foram submetidos à clonagem, porém com a estratégia empregada não foi possível a obtenção da sequencia dos fragmentos clonados.

Palavras chaves: Peptídeos; *Coffea canephora*; Atividade antimicrobiana

ABSTRACT

BARD, Gabriela Costa Vieira; Universidade Estadual do Norte Fluminense Darcy Ribeiro; October, 2016; POTENTIAL OF ANTIMICROBIAL PEPTIDE OF SEEDS OF *Coffea canephora* cv ROBUSTA TROPICAL IN THE CONTROL OF PHYTOPATHOGENIC MICRORGANISMS Advisor Drª Valdirene Moreira Gomes; Consultants: Drª Rosana Rodrigues e Dr André de Oliveira Carvalho.

Antimicrobial peptides, which play a role in plant defense, have been identified and isolated particularly from seeds. In this work we isolated and immunolocalized a new lipid transfer protein (LTP), named *Cc*-LTP2, from *Coffea canephora* seeds. We report its antimicrobial activity against various phytopathogenic fungi of economic importance, and against the bacterium *Xanthomonas euvesicatoria*. Peptides from *C. canephora* seeds were initially extracted using acid buffer and subjected to ion-exchange and reverse-phase chromatographies. A purified peptide of approximately 9 kDa was then subjected to amino acid sequencing. The analyses showed that it was similar to LTPs isolated from various plants and was named *Cc*-LTP2. The tissue and subcellular localization of *C. canephora* LTPs indicated that they were located in cell walls and intracellular palisade parenchyma, mainly in large vacuoles. The results of immunohistochemistry and histochemistry superposed from *C. canephora* seed tissues showed that LTPs and lipid bodies are present in organelles, supporting the hypothesis that LTPs from seeds are involved in lipid mobilization during germination. *Cc*-LTP2 did not inhibit the development of the phytopathogenic fungi but did inhibit *X. euvesicatoria. Cc*-LTP2 also increased membrane permeability and induced endogenous production

of reactive oxygen species in all the fungi tested. Another objective of the present study was to evaluate the antimicrobial activity of the *Cc*-LTP2 and *Cc*-GRP peptides isolated from *Coffea canephora* seeds and their possible and synergistic activity with the azole drug Fluconazole. *Cc*-LTP2 and *Cc*-GRP alone or in combination with 20 µg/mL fluconazole were evaluated for their antimicrobial activity on the fungus *Fusarium solani*, and the effects of these peptides alone or in combination with the drug on the permeability of membranes and the induction of oxidative stress were determined. Our results show that these peptides at a concentration of 400 µg/mL combined with 20 µg/mL of fluconazole were able to inhibit the growth of the tested fungi, promote changes in their growth pattern, permeabilize the membrane, and induce reactive oxygen species (ROS). Some of these results were observed with the peptides alone or with fluconazole alone, suggesting that the peptides act synergistically, promoting the potentiation of antimicrobial action. Finally *Cc*-LTP and *Cc*-GHRP peptides were subjected to cloning, but with the strategy employed were not possible to obtain the sequence of the cloned fragment.

Keywords: Peptides; *Coffea canephora*; Antimicrobial activity

1 – INTRODUCTION

Plant diseases caused by viruses, bacteria and fungi affect crops and are responsible for significant losses or reductions in the quality of agricultural products (Agrios, 2005). Phytopathogenic fungi are the main organisms causing diseases in plants, acting as obligate or facultative parasites (Kalpana et al., 2005; Di Maro et al., 2010). The genus *Fusarium* harbors some of the main pathogens of agronomic importance; it is responsible for losses of billions of dollars. Most of their representatives are saprophytic and live on soil, plants, and other organic substrates, especially in regions of tropical and temperate climate. Fungi of this genus produce mycotoxins that not only affect harvest and productivity but are also harmful to animals and humans (Kimura et al., 2007). Additionally, some species of the genus *Fusarium*, such as *Fusarium solani*, *F. oxysporum*, *F. incarnatum-equiseti*, *F. fujikuroi*, *F. chlamydosporum*, *F. dimerum* and *F. sporotrichioides*, have emerged as important opportunistic human pathogens that can cause systemic infections (Dignani and Anaissie 2004; Zhang et al., 2006). This is because these species have been shown to be more insensitive to the classically used antifungal agents, especially those derived from azole, e.g., fluconazole (O'Donnell et al., 2008; van Diepeningen and Hoog, 2016).

Today, the control of fungal diseases is achieved mainly via pesticides based on chemical fungicides, but the use of these products can have a negative impact on the environment and on human health. Moreover, their long-term use may be beneficial in the selection of resistant phytopathogenic fungi, reducing the

efficiency of the method in the long run and making conventional pesticides less efficient (Gurgel et al., 2005; Komárek et al., 2010)

To combat the increasingly resistant phytopathogenic fungi and at the same time reduce the negative impacts on human health and the environment caused by the classic pesticides, numerous strategies have been employed such as the use of artificially selected resistant genotypes, production of resistant transgenic varieties and biological control through microorganisms, among others. Therefore, the discovery of novel antifungal agents, particularly those produced by the plants themselves, has been the objective of many studies focusing on the analysis of plant-isolated antimicrobial proteins and peptides involved in defense mechanisms. This raises the possibility of using the biotechnology of these molecules for the development of new strategies to control diseases caused by pathogenic fungi (Xuan et al., 2003; Mahlo et al., 2010; Pane et al., 2013).

In this scenario, plant antimicrobial peptides, which are low-molecular-mass molecules that are part of the innate immunity of many plants as their first line of defense, have been shown to be a sustainable and effective alternative in the combat against phytopathogenic microorganisms (Komárek et al., 2010; Pane et al., 2013). In the impossibility of escape of an environment, especially in the face of different biotic and/or abiotic stresses that pose risks to survival, plants had to develop biochemical or morphological defense mechanisms (Pichersky and Lewinsohn, 2011). These mechanisms are basically divided into constituents when they are part of the normal development plan of the plant and are induced when they are directly involved in the response to some stress factor, which includes several physical, molecular, biochemical and morphological changes. An example of such alterations may be the release of oxidative compounds, expression of defense-related genes, production of antimicrobial compounds and/or programmed cell death (Leitner et al., 2005; Mithofer and Boland, 2012). In the last decade, several studies have focused on the analysis of antimicrobial proteins and peptides isolated from cultivated or wild plants that are involved in the defense mechanisms of the plant, raising the possibility of the biotechnological use of these molecules for the development of new strategies for the control of diseases caused by pathogenic fungi. This will be an important step both for the development of strategies that reduce agricultural losses and for the development of new antifungal drugs.

The genus *Coffea*, belonging to the family Rubiaceae, comprises approximately 100 species (Matielo et al., 2005). This genus is of fundamental importance for the Brazilian economy, since Brazil is the largest producer and the second largest consumer of coffee in the world (FAO, 2010). However, despite this great annual production, the coffee crop suffers many losses due to numerous diseases that affect both the field phase and the storage phase. This leads to excessive use of agrochemicals, which culminates in environmental damage of different sorts. To contain this excessive use of such toxic compounds, much research has been carried out on coffee and several other plants aiming to isolate proteins and peptides with antimicrobial activity and possibly related to the defenses of such plants.

In recent years, our group has been isolating and characterizing different antimicrobial proteins and peptides present in seeds which are involved in plant defense mechanisms. Our group recently isolated two peptides from *Coffea canephora* coffee seed that, after sequencing, exhibited homology with lipid carrier proteins (LTPs) and with plant glycine-rich proteins and were named *Cc*-LTP2 and *Cc*-GRP. These peptides showed inhibitory activity on the growth of fungi, especially yeasts. In this way, with this project, we aim to continue the study of these peptides through their cloning and overexpression as well as to deepen the knowledge about their antimicrobial activity and mechanism of action. With the obtained results, we intend to contribute to the understanding of the action of these peptides in the control of fungal diseases of agronomic importance. The approach used in this project will be of great importance not only for fundamental aspects, but also for the possible applied aspects.

This thesis reports studies to continue the characterization and antimicrobial activity of *C. canephora* seed peptides on the growth of fungi of agronomic importance as well as to provide better understanding of the mechanisms of action of these peptides on filamentous fungi and to use them as biotechnological tools through their cloning for subsequent gene overexpression and production of recombinant peptides.

2 – OBJECTIVES

2.1 – General objective

To isolate, characterize and clone the anti-microbial peptides *Cc*-LTP2 and *Cc*-GRP from *C. canephora* seeds and evaluate their antimicrobial activities and mechanisms of action on the growth of phytopathogenic microorganisms separately and in combination with fluconazole.

2.2 – Specific objectives

- To purify *Cc*-LTP2 and *Cc*-GRP from *C. canephora* seeds;
- To determine the location of the *Cc*-LTP2 peptide in the mature seed;
- To evaluate the antimicrobial activity of *Cc*-LTP2 on phytopathogenic fungi and on the phytopathogenic bacterium *Xanthomonas euvesicatoria*;
- To evaluate the effect of *Cc*-LTP2 on the permeabilization of membranes of phytopathogenic fungi;
- To evaluate the induction of oxidative stress in cells of phytopathogenic fungi treated with *Cc*-LTP2;
- To evaluate the antimicrobial activity of the peptides *Cc*-LTP2 and *Cc*-GRP, in combination with fluconazole, on the growth of the fungus *F. solani*;
- To evaluate the effect of *Cc*-LTP2 and *Cc*-GRP, in combination with fluconazole, on membrane permeabilization of the fungus *F. solani*;

- To evaluate the induction of oxidative stress in cells of *F. solani* treated with *Cc*-LTP2 and *Cc*-GRP in combination with fluconazole;
- To evaluate the flow of H^+ in cells of *F. solani* in the presence of *Cc*-LTP2 and in combination with fluconazole;
- To clone the antimicrobial peptides *Cc*-LTP2 and *Cc*-GRP heterologously.

3 – CHAPTERS

3.1 – PURIFICATION, BIOCHEMICAL CHARACTERIZATION, AND ANTIMICROBIAL ACTIVITY OF A NEW LIPID TRANSFER PROTEIN FROM *Coffea canephora* SEEDS

3.1.1 – INTRODUCTION

The genus *Coffea* belongs to the Rubiaceae family and encompasses about 100 species, of which only five are grown commercially; the two most widely marketed species are *Coffea arabica* L. (Arabica coffee) and *Coffea canephora* Pierre (cultivars robust and conilon) (Matiello, 2005). Coffee culture is extremely important to the Brazilian economy because Brazil is the largest producer and the second largest consumer of coffee in the world. However, this productivity is constantly threatened by diseases that attack the coffee both in the field and during storage, causing losses that can even prevent the production of the plant. This leads to an overuse of pesticides, which cause numerous environmental problems. In Brazil, the productivity of many economically important crops is reduced by diseases caused by phytopathogenic fungi. Hundreds of species promote disease and rot during grain production and storage, and several cause major damage to crops; examples include *Rhizoctonia solani*, *Colletotrichum*

lindemuthianum, *C. gloeosporioides*, *C. truncatum*, *C. gossypii* var. cephalosporioides, frogeye leaf spot *Cercospora kikuchii* (and others of the same genera), *Fusarium solani* (and others of the same genera), *Phakopsora pachyrhizi*, *Ramularia areola*, and *Hemileia vastatrix* (Tamm et al., 2011).

Research into the mechanisms by which plants resist phytopathogens has revealed many types of antimicrobial peptides (AMPs), which are rich in cysteine residues (Gonçalves et al., 2013; Moulin et al., 2014). The AMPs produced by plants include lipid transfer proteins (LTPs), which can be divided into two families based mainly on their molecular weight: LTP1 proteins are approximately 9 kDa and contain 90–95 amino acid residues; and LTP2 proteins are approximately 7 kDa and contain 70 amino acids. Both families share some features such as an abundance of cysteine residues (eight in total), four intramolecular disulfide bridges, and a net positive charge at physiological pH. They also share structural characteristics: they form into four α-helices and a long C-terminal tail that is devoid of secondary structure with the exception of a 3_{10}-type helix in LTP1 proteins, and three α-helices and two single-turn helices in LTP2 proteins. The main structural characteristic of both families is a hydrophobic cavity; in LTP1 proteins, it is a tunnel-like structure and in LTP2 proteins, it is a triangular box. These cavities allow both LTP families to bind and transport lipid molecules (Carvalho and Gomes, 2007).

LTPs were named according to their ability to transfer between lipid membranes *in vitro* (Kader, 1975). The transferred lipids include phosphatidylinositol, phosphatidylcholine, and galactolipids (Carvalho and Gomes, 2007). Lipid transfer capability has led to the idea that LTPs are involved in a cytoplasmic function that regulates membrane biogenesis and intracellular fatty acids(Tsuboi et al., 1992), but more in-depth studies have proven the existence of an extracellular addressing signal peptide (Carvalho and Gomes, 2007). Many LTPs are located in the cell wall (Tsuboi et al., 1992; Carvalho et al., 2004), but there are exceptions: LTPs are also localized in other cellular organelles such as glyoxysomes (Tsuboi et al., 1992) and vacuoles (Carvalho et al., 2004).

Further studies are necessary to explain the role of LTPs in intracellular lipid transport, but numerous biological functions have been proposed for this class of proteins, e.g., monomer transfer for the synthesis of cutin (Domínguez et al., 2015); β-oxidation (Tsuboi et al., 1992), defense signaling (Maldonado et al.,

2002), and protection of plants against fungi, bacteria, and viruses (Carvalho and Gomes, 2007). Although the toxicity mechanism of LTPs has not yet been fully elucidated, it is thought to be related to the ability of these proteins to interact with cell membranes and promote their permeability through the formation of pores, leading to the efflux of intracellular ions and cell death (Carvalho and Gomes, 2007).

Consequently, the exploration of novel natural antimicrobial agents such as proteins and substances involved in secondary metabolism has increased. Several compounds with toxic activity against insects, such as legumins and cyclotides (Huang et al., 2009), and against fungi, such as LTP and glycine-rich protein (GRP) (Zottich et al., 2011; Zottich et al., 2013), have been isolated from coffee. In the present paper, we report the characterization, immunolocalization, and antimicrobial activity of a new LTP called *Cc*-LTP2, which can be isolated from *C. canephora* seeds.

3.1.2 – REVIEW

3.1.2.1 – Coffee

Over the last 50 years, coffee consumption has increased considerably. Today, more than 70 countries produce coffee, and more than 50% of this production originate from only three countries: Brazil, Vietnam and Indonesia (FAO, 2015).

Coffee production in Brazil accounts for one-third of the world production, making Brazil the largest coffee producer in the world, a position it has held for more than 150 years, and also the world's leading exporter of coffee, followed by Vietnam and Colombia (FAO, 2015). Brazil also ranks second among beverage-consuming countries. According to ABIC (Brazilian Association of the Coffee Industry), coffee has been the foodstuff most consumed daily by 78% of the Brazilian population over 10 years, which represents 79.7 L/inhabitant/year. For this reason, coffee cultivation is one of the most important agricultural activities in Brazil, especially for the generation of jobs and income in inland municipalities

(Rubim, 2009). In the national scenario, the largest producing states of this grain are Minas Gerais, Espírito Santo, São Paulo, Bahia, Paraná and Rondônia, which, together, correspond to more than 98% of the national production (CONAB, 2016).

The genus *Coffea* belongs to the family Rubiaceae, the fourth largest family of angiosperms, with more than 13,000 species distributed in about 650 genera that occupy tropical regions for the most part. The family Rubiaceae belongs to the superclass Asteridae, which diverged from the clade Rosidae between 114 and 125 million years ago (Wikström et al., 2001), which comprises two thirds of the species of angiosperms. Because of its economic importance, *Coffea* is the most studied genus of the family Rubiaceae. This genus comprises 124 species, 66 of which are native to the Indian Ocean Islands and only two are native to the African continent and are the most widely cultivated and commercialized species in the world: *Coffea arabica* and *Coffea canephora* (Hamon et al., 2016). These two species are cultivated In Brazil, where *C. arabica* accounts for 83.2% of the national coffee production and is cultivated in the states of Minas Gerais (largest producer), São Paulo, Paraná, Bahia, Espírito Santo, Goiás and Rio de Janeiro. *Coffea canephora* corresponds to 22.2% of the national productivity, and the main producing states are Espírito Santo (largest producer), Rondônia and Bahia (CONAB, 2016).

The species *C. arabica* and *C. canephora* differ in several respects. The species *C. canephora* (Figure 5), also known as Robusta coffee, is diploid (2n = 22) and cross-fertilized, thus having a high rate of genetic variability. Its seedlings are clonal varieties of several individuals selected for superiority in some agronomic traits such as resistance to pathogens like *Hemileia vastatrix* Berk & Broome, which causes the coffee rust; *Colletotrichum kahawae* Waller & Bridge, which causes the Coffee Berry Disease; and nematodes of the genus *Meloidogyne Goeldi*, 1887 (Lashermes et al., 2008). In spite of this, *C. canephora* is less appreciated in relation to *C. arabica*, mainly because of its high content of caffeine and soluble solids, which gives it a very strong coffee flavor, causing it to be more frequently used in soluble form and in blends with *C. arabica* (Leroy et al., 2006; Chalfoun and Reis, 2010). Unlike *C. canephora*, *C. arabica* is an allotetraploid species (2n = 4x = 44) and reproduces by self-fertilization, which considerably decreases its genetic variability, although it is more susceptible to pests and diseases than *C. canephora*. It is reproduced by seeds and presents a

certain homogeneity, which may be important for the maintenance of genetic characteristics of economic interest (Ferrão et al., 2010).

Figure 1 – Flowering and fruiting of *C. canephora* (Fonte – http://www.cafepoint.com.br/comunidade/fique-atento/ufv-e-iac-debatem-viabilidade-do-robusta-em-minas-e-sao-paulo-48703n.aspx).

3.1.2.2 – Lipid transport proteins (LTPs)

Lipid transport proteins (LTPs) were discovered approximately 40 years ago and named after their ability to transfer lipids between a donor membrane and an acceptor membrane *in vitro* (Kader, 1996; Carvalho and Gomes, 2007). They are a homogeneous class of basic proteins with a molecular mass between 7 kDa and 10 kDa found in different plant species, in different organs from seeds to flowers, fruits and leaves, in addition to being rich in cysteine (Botton et al., 2002; Liu et al., 2011). Lipid transport proteins have different expression patterns at different stages of development and different tissues under various levels of physiological stress (Liu et al., 2011). They can also be found in organisms such as fungi, bacteria and some animals (Park et al., 2002).

Lipid transport proteins are classified into two large multigenic families called LTP1 and LTP2, which share common characteristics such as the presence of eight cysteine residues forming four disulfide bonds and a high isoelectric point (between eight and nine). Both families also have a hydrophobic cavity that accommodates the lipids to be transported. However, the two families have little similarity to each other in amino acid sequence and different molecular masses, with representatives of the LTP1 family having between 8 kDa and 10 kDa, while

the representatives of the LTP2 family are smaller, having around 7 kDa (Carvalho and Gomes, 2007).

Representatives of the LTP1 family have about 90-95 amino acid residues of which eight are conserved cysteines at similar positions throughout their primary structure forming four disulfide bonds. The secondary structure of the LTP1 family is composed of four α-helices and a c-terminal loop that forms a tunnel-shaped cavity that crosses both sides of the molecule (Carvalho and Gomes, 2007) (Figure 2).

In addition to the cysteine residues, other residues are also conserved in the LTPs1 structure, namely Arg 44, Tyr 79 and Ile 81, which are directly related to lipid binding. Cheng et al. (2004) showed that in rice LTP1, these residues are responsible for significant structural differences in the LTP structure between the form bound to the fatty acid and the unbound form. In the present study, we found that glycerolipids (Lerche et al., 1997; Guerbette et al., 1999; Douliez et al., 2000), fatty acids (Tsuboi et al., 1992; Lerche et al., 1997) and their hydrophobic cavities can vary in size according to the lipids they accommodate, and they can also accommodate more than one lipid at a time (Lerche et al., 1998; Charvolin et al., 1999).

Compared with the LTP1 family, LTP2 is considerably less studied. Their representatives have around 70 amino acid residues; they also have four α-helices stabilized by four disulfide bridges that form a triangular hollow cavity (Figure 2), which are more flexible despite being smaller than the cavity of LTPS1, providing these proteins with a five times greater capacity of lipid transfer when compared with LTP1 representatives, including sterols (Samuel et al., 2002, Pons et al., 2003; Cheng et al., 2004).

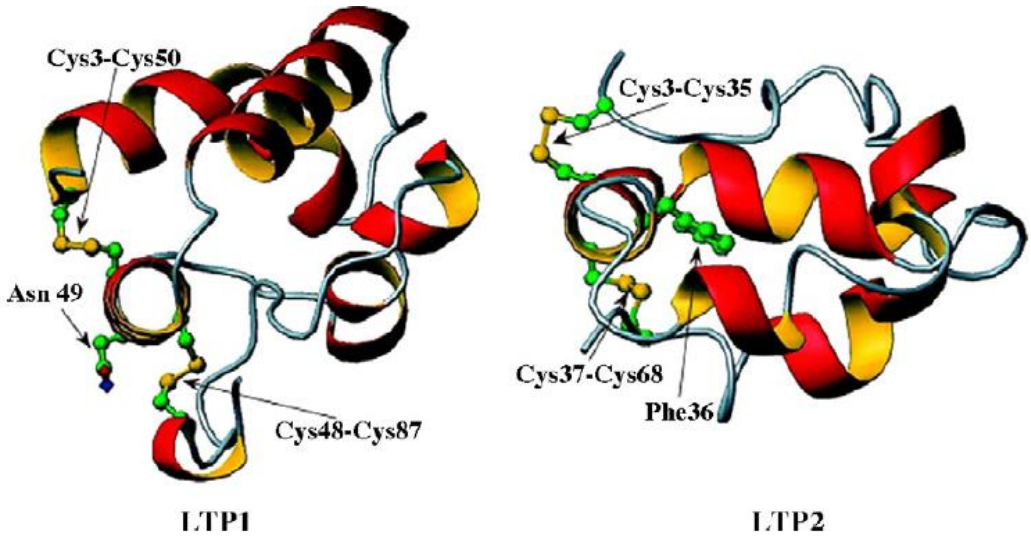

Figure 2 - Representation of the three-dimensional structure of two lipid transport proteins (LTP1 and LTP2) representing families 1 and 2, respectively. Disulfide bridges in yellow. Source: Carvalho and Gomes (2007).

3.1.2.3 – Biological functions of LTPs

The biological function of LTPs in plants is not yet clear; however, studies have shown that these proteins are related to different functions such as cutin synthesis (Dominguez et al., 2015); abiotic stresses like drought (Jang et al., 2004), salinity (Jang et al., 2004), high temperature (Jang et al., 2004) and injury (Choi et al., 2008); and biotic stresses (Jung et al., 2003).

Many LTPs were located in the cell walls of the epidermis and this location may be related to a role in the formation and deposition of the cell wall or cuticular material (Sterk et al., 1991; Liu et al., 2015). In tobacco, TobLTP2 facilitates the loosening of the cell wall and extension through the attachment of the cavity with hydrophobic molecules of the cell wall/xyloglucan cell wall, followed by secretion of TobLTP2. Loosening of the cell wall by LTPs may be critical in cell expansion or in directional growth sites leading to cell specialization (Nieuwland et al., 2005).

The study of HaAP10, an LTP isolated from sunflower seeds, showed the relationship between seed germination and defense against the pathogen. HaAP10 has antifungal activity and the ability to permeabilize the membrane of fungi (Regente et al., 2005). This peptide has an extracellular localization in dry seeds, but when these seeds are soaked, it is rapidly translocated to structures related to intracellular oil mobilization (Pagnussat et al., 2012), indicating a change

in the HaAP10 function from seed protection to mobilization of lipids stored in the seeds to support the growth of seedlings during germination.

The study of the antimicrobial activity of LTPs started with in vitro assays with radish (*Raphanus sativus*) protein extracts (Terras et al., 1992), barley (*Hordeum vulgare*) (Molina et al., 1993), *Arabidopsis thaliana* (Almeida et al., 1993), onion (*Alium cepa*) and other seeds (Cammue et al., 1995). Each of these isoforms inhibited the growth of pathogens (bacteria and fungi) to varying degrees depending on the isoform and the pathogen.

Many LTPs have been isolated from seeds of different species and characterized as antimicrobial peptides due to their strong antifungal and/or antibacterial activity in vitro, including corn (Sossountzov et al., 1991), sunflower (Regente De La Canal, 2000), Mung bean (Wang et al., 2004), wheat (Boutrot et al., 2005), *Brassica campestris* (Lin et al., 2007), pepper (Diz et al., 2011) (Zatane et al., 2011), coffee (Zottich et al., 2011) and lentils (Gizatullina et al., 2013), participating in the defense of seeds against microorganisms.

In recent studies, our group isolated two LTPs from pepper seeds (*Capsicum annum*), *Ca*-LTP, which showed antimicrobial activity against the phytopathogenic fungus *Colletotrichum lindemunthianum* and yeast *Candida tropicalis* (Diz et al., 2011) and another from coffee seeds (*Coffea canephora*), *Cc*-LTP, which was active against different yeasts of the genus *Candida* (Zottich et al., 2011). Both were able to promote permeabilization of the membranes of the microorganisms. *Ca*-LTP and *Cc*-LTP were also able to inhibit the activity of the human salivary ɣ-amylase enzyme *in vitro*, an ability first described for LTPs.

3.1.3 – MATERIAL AND METHODS

3.1.3.1 – Plant material

C. canephora (p. ex Fr.) seeds of cultivar Robusta Tropical (INCAPER 8151) were collected at the pinhead stage from coffee trees on the INCAPER experimental farm in Linhares, ES, Brazil.

3.1.3.2 – Microorganisms

Pathogenic fungi *Colletotrichum lindemuthianum*, *C. gloeosporioides*, *Fusarium solani*, and *F. lateritium* were cultured in Sabouraud agar and preserved in the Laboratório de Fisiologia e Bioquímica de Microrganismo (LFBM), at the Universidade Estadual do Norte Fluminense – Darcy Ribeiro (UENF), in the Campos dos Goytacazes, RJ, Brazil. Pathogenic fungi were maintained in Sabouraud 2% glucose agar (Merck, USA). *Xanthomonas euvesicatoria* and *Colletotrichum* spp. (53/1) isolates were supplied by the Laboratório de Melhoramento Genético Vegetal, (LMGV), at UENF in the Campos dos Goytacazes Rio de Janeiro, Brazil.

3.1.3.3 – Purification of *Cc*-LTP2

The peptides from coffee seeds were extracted using the method described by Egorov et al. (2005). The crude extract was centrifuged at 15,000 × *g* (at 4°C) for 10 min, and the supernatant was dialyzed extensively, recovered by freeze-drying, resuspended in 50 mM Tris-HCl, pH 8.0, and subjected to chromatographic methods. A diethylaminoethanol (DEAE)-Sepharose anion-exchange column was used for further separation of proteins. This column was equilibrated and initially eluted with 50 mM Tris-HCl, pH 8.0, followed by elution with the same buffer containing 1 M NaCl. The flow rate was 50 mL/h, and 4-mL fractions were collected. The D1 fraction was dialyzed against distilled water, recovered by freeze-drying (Freezone 4.5, Labconco), diluted with 0.1% (v/v) trifluoroacetic acid (TFA; Fluka), and injected into a high performance liquid chromatography (HPLC) (Prominence, Shimadzu) C18 reverse-phase column (Shim-pack VP-ODA 250L × 4.6, Shimadzu) (attached to a C8 pre-column (20 × 4.6 mm, Pelliguard, Sigma-Aldrich). The solvent flow rate was 0.5 mL/min and the solvent progressed from 100% solvent A (0.1% TFA in water) for 10 min, 0 to 50% solvent B (100% 2-propanol (Merck) containing 0.1% TFA) for 50 min, 50% solvent B for 5 min, and finally returned to 0% solvent B for 10 min. Elution of proteins was monitored by on-line measurement of the absorbance at 220 and 280 nm. Eleven fractions were obtained, and the H11 fraction was subjected to a second HPLC (Prominence, Shimadzu) run in the same C18 reverse-phase

column (attached to a C8 pre-column; 20 × 4.6 mm, Pelliguard, Sigma-Aldrich). The solvent flow rate was 0.2 mL/min and the solvent progressed from 100% solvent A (0.1% TFA in water) for 10 min, 0 to 50% solvent B (100% 2-propanol (Merck) containing 0.1% TFA) for 100 min, 50% solvent B for 5 min, and finally returned to 0% solvent B for 10 min. Elution of proteins was monitored by on-line measurement of the absorbance at 220 and 280 nm of the three fractions obtained. Quantitative determinations of protein levels were made using the bicinchoninic acid method described by Smith et al. (1985) with modifications. Ovalbumin was used as standard protein.

3.1.3.4 – Tricine gel electrophoresis

Tricine–sodium dodecyl sulfate-polyacrylamide gel (16.4%) electrophoresis (tricine–SDS-PAGE) was carried out according to the method described by Schägger and von Jagow (1987).

3.1.3.5 – Aminoacid sequence analysis

For the amino acid sequence analysis, the 9-kDa band was subjected to tricine–SDS-PAGE, transferred to a polyvinylidene difluoride (PVDF, Millipore) membrane, and stained with Ponceau S (0.1%). The 9-kDa band was excised from the membrane and briefly washed in different substances as follows: 100 µL water, 400 µL methanol with vortexing, and 100 µL chloroform with vortexing. Finally, the last wash was removed and the membrane was air-dried. The N-terminal amino acid sequence of the peptide blotted onto PVDF was determined by Edman degradation carried out in a Shimadzu PSQ-23 protein sequencer. PTH-amino acids were detected at 269 nm after separation on a reverse-phase C18 column (250 × 4.6 mm) under isocratic conditions, according to the manufacturer instructions. Searches for sequence similarity were carried out using the BLASTp program.

3.1.3.6 – Histochemical analysis

To evaluate the presence of lipids and protein, microchemical tests were carried out on sections of fresh seed endosperm without prior treatment according to standard plant morphology techniques (Jensen, 1962). The slides were then examined and documented using a camera (PowerShot A640; Canon, New York, USA) attached to a light microscope (Axioplan, Zeiss, Oberkochen, Germany).

3.1.3.7 – Immunohistochemistry assay

Antisera anti-LTP proteins from *C*. *canephora* seeds were prepared by immunization of white New Zealand rabbits with the 9-kDa band that showed sequence similarity to LTP. Purified antibodies were obtained by affinity chromatography of the crude immune serum in a column of Protein A covalently bound to Sepharose CL-4B. Briefly, crude sera were chromatographed in a column (0.5 × 4 cm) prepared with that adsorbent, equilibrated with 0.1 M phosphate buffer, pH 7.6, and eluted with 1 M acetic acid. The IgG fractions were recovered by dialysis in 0.15 M NaCl and freeze-dried. Pre-immune sera were collected before immunization (Zottich et al., 2011).

For tissue localization of LTPs, seed and root fragments were fixed in a solution of 0.01% glutaraldehyde and 4% formaldehyde in a 0.2 M sodium cacodylate buffer at pH 7.4 for 2 h at room temperature. The samples were then dehydrated in an ascending series of methanol solutions and embedded in LR Gold resin. Sections (0.5-µm thick) were obtained using an ultramicrotome (Reichert Ultracut S), mounted on slides, and submitted to the tissue localization assay. Slides were immunolabeled using a silver intensification kit (Inten SE silver enhancement kit, Amersham, RPN 491, Buckinghamshire, UK) following the manufacturer instructions. Anti-LTP serum (primary antibody) was used at a 1:300 dilution. Secondary antibody was used at a 1:200 dilution. The reaction was visualized by light microscopy via the deposition of a precipitate on gold colloidal particles coupled to a secondary antibody (Silva dos Santos et al., 2004). In the control, the primary anti-serum from root and seed sections was replaced by a pre-immune serum. The slides were then investigated and documented using a camera (PowerShot A640, Canon, New York) attached to a light microscope (Axioplan, Zeiss, Oberkochen).

3.1.3.8 – Effect of proteins on fungal growth

To prepare conidia of *F. solani*, *F. lateritium*, *C. lindemuthianum*, *C. gloeosporioides*, and other *Colletotrichum* spp., fungal extracts were cultured for 12 days at 30°C on petri dishes containing Sabouraud agar;Sabouraud culture medium (10 mL) was then added to the dishes, which were gently agitated for 1 min to liberate spores with the help of a Drigalski spatula. The conidia were quantified in a Neubauer chamber (Laboroptik) for appropriate dilutions. A quantitative assay of fungal growth inhibition was carried out by following the protocol developed by Broekaert et al. (1990), with some modifications.

To assay the effect of *Cc*-LTP2 on fungi growth, conidia (2000 conidia/mL) were incubated at 30°C on 100-µL microplates in the presence of the *Cc*-LTP2 (200 and 400 µg/mL). Optical readings at 620 nm were taken at zero time and every 6 h for the following 48 h. Cell growth control without addition of peptides was determined.

3.1.3.9 – SYTOX Green uptake assay

Fungal plasma membrane permeabilization was measured by the SYTOX Green uptake assay, as described previously by Thevissen et al. (1999) with some modifications. SYTOX Green dye only penetrates cells when the plasma membrane has been structurally compromised. Once inside the fungal cytoplasm, it binds to nucleic acids and forms a fluorescent complex; it can therefore be used to visualize the permeabilization of the fungal plasma membrane. Aliquots (100 µL) of the suspensions that had been grown in the presence of *Cc*-LTP2 (200 µg/mL) were incubated with 0.2 µM SYTOX Green in 1.5-mL microcentrifuge tubes for 30 min at 25°C with periodic agitation. Cells were observed under an Axiophot differential interference contrast microscope (Zeiss) equipped with a fluorescence filter set for fluorescein detection (excitation wavelength 450–490 nm; emission wavelength 500 nm). Negative (no *Cc*-LTP2 added) controls were also run to evaluate the baseline membrane permeability.

3.1.3.10 – Reactive oxygen species (ROS) induction assay

A dye that can be used to indicate the presence of reactive species was used to determine whether the mechanism of action of *Cc*-LTP2 involves the induction of oxidative stress. Induction of the endogenous production of ROS in various fungi treated with 200 µg/mL *Cc*-LTP2 after the growth inhibition assay was evaluated using the fluorescent dye 2',7'-dichlorofluorescein diacetate (Calbiochem; EMD), as described by Aerts et al. (2007) with some modifications. Incubation was performed as described in the SYTOX Green uptake assay section. The incubation time was 24 h of growth in the presence or absence of *Cc*-LTP2. An aliquot was incubated with constant agitation for 2 h with fluorescent dye to a final concentration of 20 µM, according to the manufacturer instructions. These cells were then transferred to slides, covered with coverslips, and examined using an Axiophot fluorescence microscope (Zeiss) equipped with a fluorescence filter set for fluorescein detection (excitation wavelength 450–490 nm; emission wavelength 500 nm).

3.1.3.11 – Effect of proteins on bacterial growth

The method described by Filho and Romero (2009) with modifications, was employed to evaluate the effects of *Cc*-LTP2 on the growth of the bacterium *X. euvesicatoria*. To evaluate the formation of inhibition zones at different times and concentrations, bacteria were grown in liquid DYGS medium (30 mL) and kept in a shaker (Tecnal TE420) under agitation for 24 h, at 28°C and 100 rpm. A bacteria-enriched aliquot (100 µL) was then transferred to an Erlenmeyer flask containing 0.8% semi-solid DYGS (30 mL), and heated to 40°C. The DYGS was poured into petri dishes. After medium solidification, two paper disks (approximately 6 mm in diameter) were loaded, one with 5 µL (75 µg/mL) *Cc*-LTP2 and the other with 5 µL water (the control). The plates were evaluated for 48 h using a Stainless Hardened® digital caliper.

3.1.4 – RESULTS

3.1.4.1 – LTP purification and characterization

Initially, proteins from *C. canephora* seeds were subjected to acidic extraction, as previously described (Egorov et al., 2005). The final extract was then subjected to anion-exchange chromatography on DEAE-Sepharose resin, from which was obtained two fractions termed D1 and D2 (Figure 3A). The D1 fraction was eluted from the column equilibration buffer and represented the fraction of basic proteins, whereas the D2 fraction, which was retained on the column, was eluted with 1 M NaCl and contained acidic proteins. Thus, fraction D1 was lyophilized and subjected to a second purification step through the reverse-phase C18 column of the HPLC system. To better maintain the biological activity of the LTPs, the purification was carried out in 2-propanol solvent (Zottich et al., 2011), where 11 peaks were obtained (H1–H11, indicated only to H11; Figure 3B). As the subsequent purification process, the H11 peak was subjected to a second round of reverse-phase chromatography on the HPLC system, also in a C18 column, and three peaks were obtained (Figure 3C). The major peak was analyzed by denaturing tricine–SDS-PAGE and showed only one band (molecular approximately 9 kDa; Figure 3C).

The N-terminal amino acid sequence of the 9-kDa peptide from the major peak showed similarity to the first 20 amino acids of LTPs isolated from various plants (Figure 4); in particular, there was 67% identity with *Musa acuminata* (XP009396871), 70% identity with*Solanum lycopersicum* (XP009798115), and 75% identity with *Nicotiana sylvestris* (XP009796551).

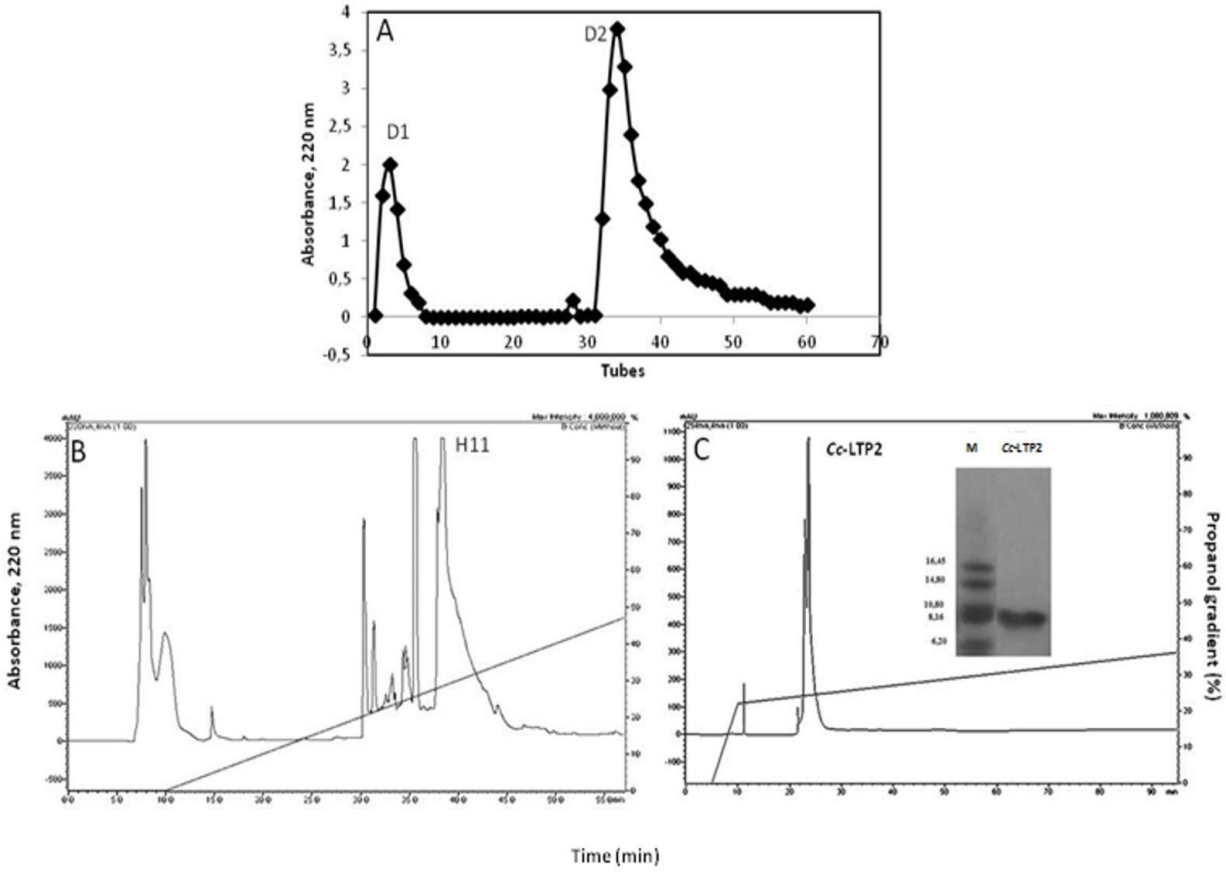

Figure 3 – Purification of *Cc*-LTP2 from *Coffea canephora* seeds. (A) Chromatogram of the protein extract of *C. canephora* in diethylaminoethanol (DEAE)-Sepharose anion-exchange chromatography. D1, non-retained fraction; D2, retained fractions eluted in 1 M NaCl.Sample elution profile was monitored at 220 nm. (B) Chromatogram of the D1 fraction of *C. canephora* in a C18 reversed-phase column. The 2-propanol gradient is represented by the oblique line. H11 retained fraction and eluted in the 2-propanol gradient. The sample elution profile was monitored at 220 nm. (C) Chromatogram of the H11 fraction of *C. canephora* in a C18 reversed-phase column. The 2-propanol gradient is represented by the oblique line. H11 retained fraction and eluted in the 2-propanol gradient, which contain the 9-kDa protein band. The sample elution profile was monitored at 220 nm. In detail, 16.4% tricine-SDS-PAGE of the 9-kDa peptide from *C. canephora* seeds obtained after reverse-phase chromatography. (M) Refers to the molecular weight marker (Da).

Coffea canephora(Cc-LTP2)	1	I	T	C	Q	Q	V	T	X	E	L	E	P	C	V	P	Y	L	T	-	Q	G	21	I		P
Coffea canephora(Cc-LTP1)	1	I	T	C	Q	Q	V	T	S	E	L	G	P	C	V	P	Y	L	T	G	Q	G	21			
Musa acuminata	26	I	T	C	G	Q	V	T	S	D	L	S	P	C	I	P	Y	A	T	G	K	G	46	67		77
Solanum lycopersicum	25	I	T	C	G	Q	V	D	A	N	L	A	P	C	V	P	F	L	T	-	Q	G	44	70		75
Nicotiana sylvestris	23	I	T	C	G	Q	V	D	A	A	L	A	P	C	V	P	Y	L	T	-	Q	G	42	75		75

Figure 4 – Comparison of N-terminal sequence of the purified 9-kDa peptide from *Coffea canephora* seeds with other similar peptides described. The sequences of the following proteins are presented: *Coffea canephora* (Zottich et al., 2011); *Musa acuminata* (XP009396871); *Solanum lycopersicum* (XP009798115); *Nicotiana sylvestris* (XP009796551). (I) represents the percentage of identical amino acids, (P) represents the percentage of positive amino acids, and gaps (-) were included to improve alignment. Numbers flanking the amino acid sequences indicate the positions of amino acids in the peptide sequence. The 9-kDa peptide was designated *Cc*-LTP2.

3.1.4.2 – Immunolocalization of *Cc*-LTP2

The analysis of immunohistochemistry sections of tissue (hypocotyl and seed) from *C. canephora* treated with the anti-LTP antibodies revealed immunolabeling of *Cc*-LTPs in the cell walls and intracellular palisade parenchyma of the seed (Figure 5A) as compared with the control (Figure 5B). It is noteworthy that the presence of LTPs from *C. canephora* was additionally detected in the vacuoles (Figure 5C) but not in the control of the root radicle sections (Figure 5D), and was apparent in the vascular bundle of the hypocotyl (Figure 5E) but not in the control root hair zone (Figure 5F).

Proteins with a positive reaction (blue) occurred in all tissues with intracellular localization, and were more evident in tests on fibrous tissue (Figure 6A and B), which are known to be rich in oblique scores. The lipid substances present in all tissues analyzed by histochemistry are evident in the integument within the parenchyma cells of the seeds; the red-orange shade resulted from Sudan IV reagent in the form of droplets. Oil droplets are abundant and are found in all parts of the seed, including the epidermis and seed tegument (Figure 6C and D).

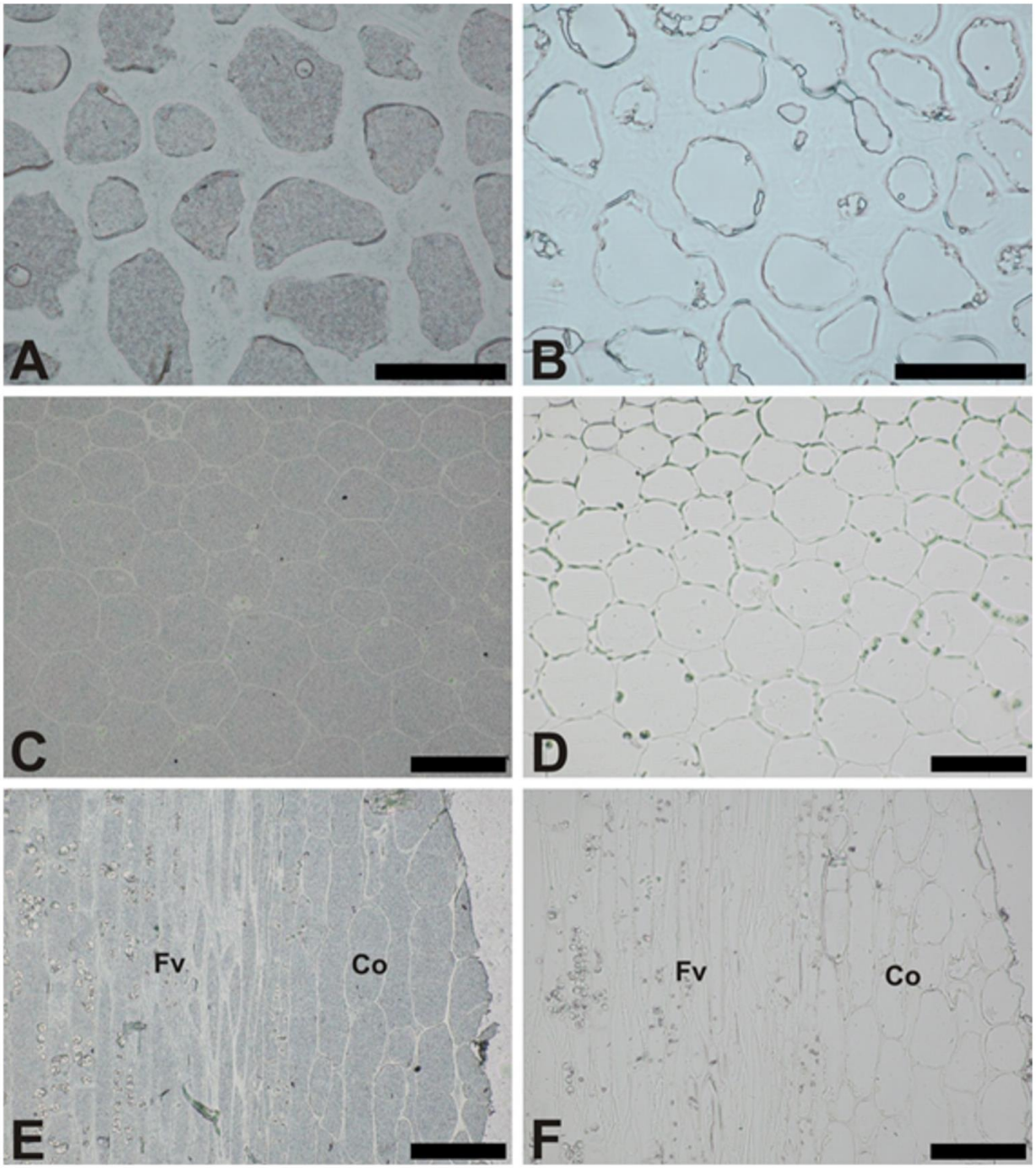

Figure 5 – Immunolocalization of lipid transfer proteins (LTPs) in seedling roots and seed sections of *Coffea canephora*by light microscopy using an anti-LTP serum followed by treatment with 10 nm colloidal gold-conjugated secondary antibodies, and visualized using a silver intensification kit. (A) Immunolabeling of LTPs in the seed endosperm. Note that the immunolabeling can be observed in the cell vacuole; (B) Control seed endosperm; (C) Immunolabelling of LTPs of transversal sections of radicle. Note that the immunolabeling can be observed in the cell vacuole; (D) Control of root radicle sections in which the primary anti-serum was replaced by pre-immune serum; (E) Immunolabelling of LTPs of the longitudinal sections of the root hair zone. Note that the immunolabeling can be observed in the cortex cells (Co) and vascular bundles (Fv); (F) Control of root hair zone in which the primary anti-serum was replaced by pre-immune serum. Bars: A, B, E, and F: 20 µm; C and D 50 µm. The results of immunolocalization are representative of one triplicate experiment.

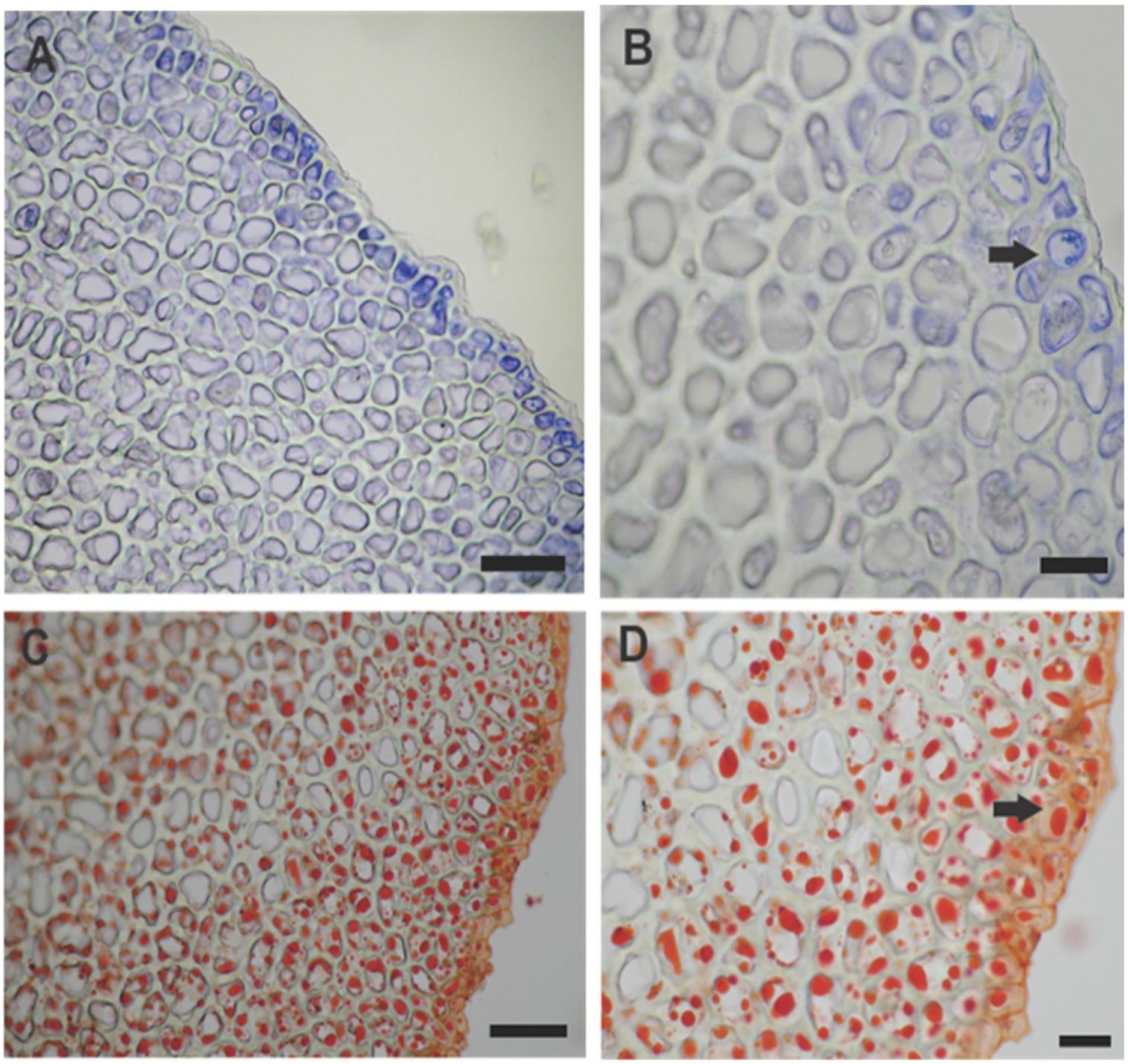

Figure 6 – Histochemical characterization of the seed endosperm in *Coffee canephora* treated with Coomassie Blue (A-B) and Sudan IV (C-D). (A) Cellular endosperm revealed with a positive blue reaction to Coomassie Blue; (B) Note detail for intracellular localization of protein bodies (arrow); (C) Lipids body and lipid portion of the outer periclinal cell wall revealed with a positive red-orange reaction to Sudan IV; (D) Note detail of the lipids body in the cellular endosperm (arrow). Bars: A, B, 10 µm C, D, 20 µm. The results of histochemistry are representative of one triplicate experiment

3.1.4.3 – Antimicrobial activity against phytopathogenic fungi

To analyze a possible antimicrobial role of *Cc*-LTP2, isolated in this work, antifungal assays were carried out with the pathogenic fungi *C. lindemuthianum*, *C. gloeosporioides*, *Colletotrichum* spp., *F. solani*, and *F. lateritium*, which are

pathogens of several very important economic crops. The fungi were grown for 48 h in the absence (control) and presence of 200 µg/mL and 400 µg/mL *Cc*-LTP2, with their growth monitored every 6 h. As can be seen in Figure 7, the two concentrations of *Cc*-LTP2 tested were not able to inhibit the growth of fungi when compared with the control curves.

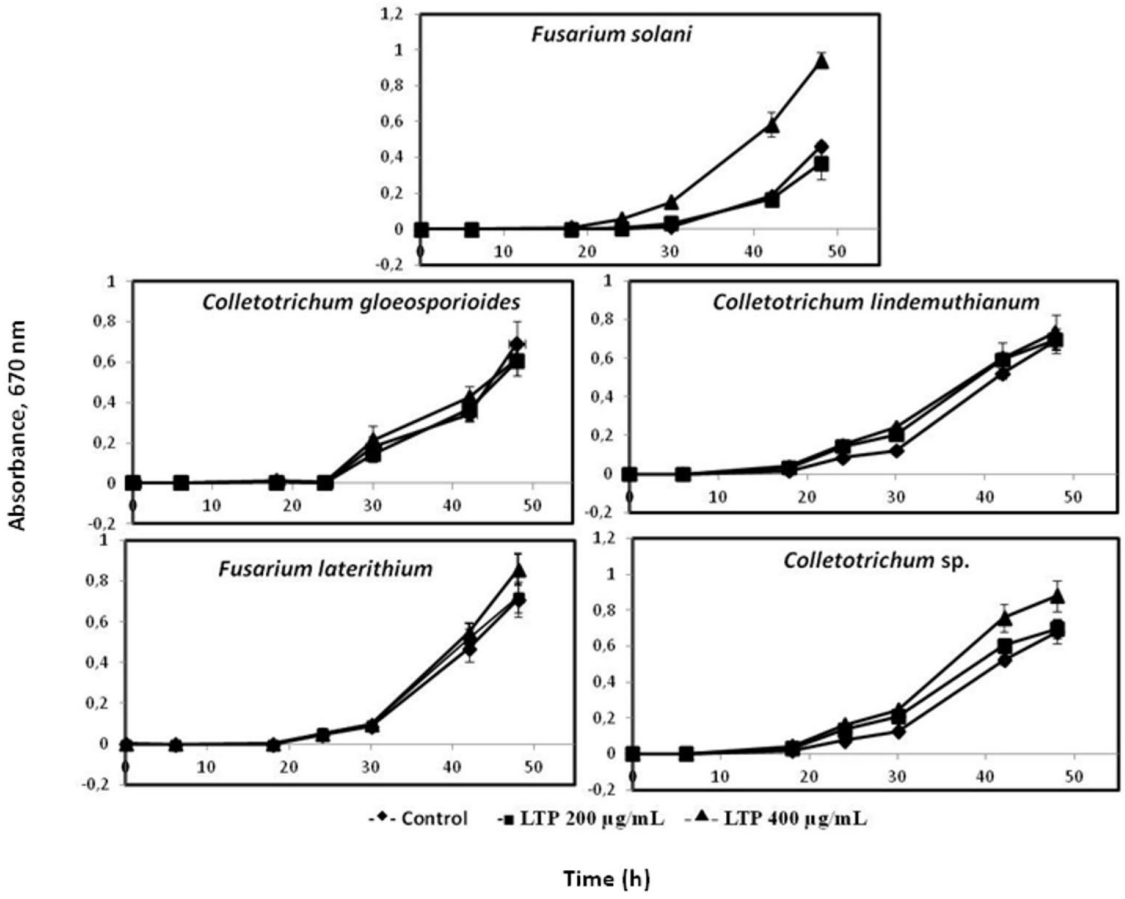

Figure 7 – Growth inhibition assay of *Fusarium solani*, *Fusarium lateritium*, *Colletotrichum* spp *Colletotrichum gloeosporioides*, and *Colletotrichumlindemuthianum* in the presence of *Cc*-LTP2. The growth was observed for 48 h. (-♦-) Control (-■-) 200 µg/mL (-▼-) 400 µg/mL. Values are means (±SD) of triplicates. The results of growth inhibition assays are representative of one triplicate experiment.

3.1.4.4– Membrane permeabilization and ROS induction

In a study with an LTP isolated from *Helianthus annuus*, Regente et al. (2005) showed for the first time the ability of these proteins to promote the membrane permeabilization of *F. solani* fungus spores. To determine whether the

Cc-LTP2 isolated in this study also had that ability, we conducted tests on membrane permeabilization in filamentous fungi using a growth inhibition assay. After 24 h, the fungus grown in the absence (control) and presence of 200 µg/mL *Cc*-LTP2 was incubated with the fluorescent dye SYTOX Green, then immediately investigated by optical fluorescence microscopy. This particular dye can only penetrate cells if they have a damaged or compromised membrane. As can be seen in Figure 8, *Cc*-LTP2 was able to perturb the membrane of all the tested fungi, damaging their structure.

Another property that has been described for some antimicrobial peptides is their ability to induce endogenous production of ROS. To determine whether the *Cc*-LTP2 isolated in this work was able to induce endogenous production of ROS, after 24 h, the fungus grown in the absence (control) and presence of 200 µg/mL *Cc*-LTP2 was incubated for 2 h with 2',7'-dichlorofluorescein diacetate dye and then immediately examined using an optical fluorescence microscope. In investigating the effects of *Cc*-LTP2, little or no fluorescence was observed in the control, indicating no production of ROS. In cells treated with 200 µg/mL *Cc*-LTP2, however, intense staining by 2',7'-dichlorofluorescein diacetate was observed, indicating a large increase in ROS after treatment with *Cc*-LTP2 (Figure 9).

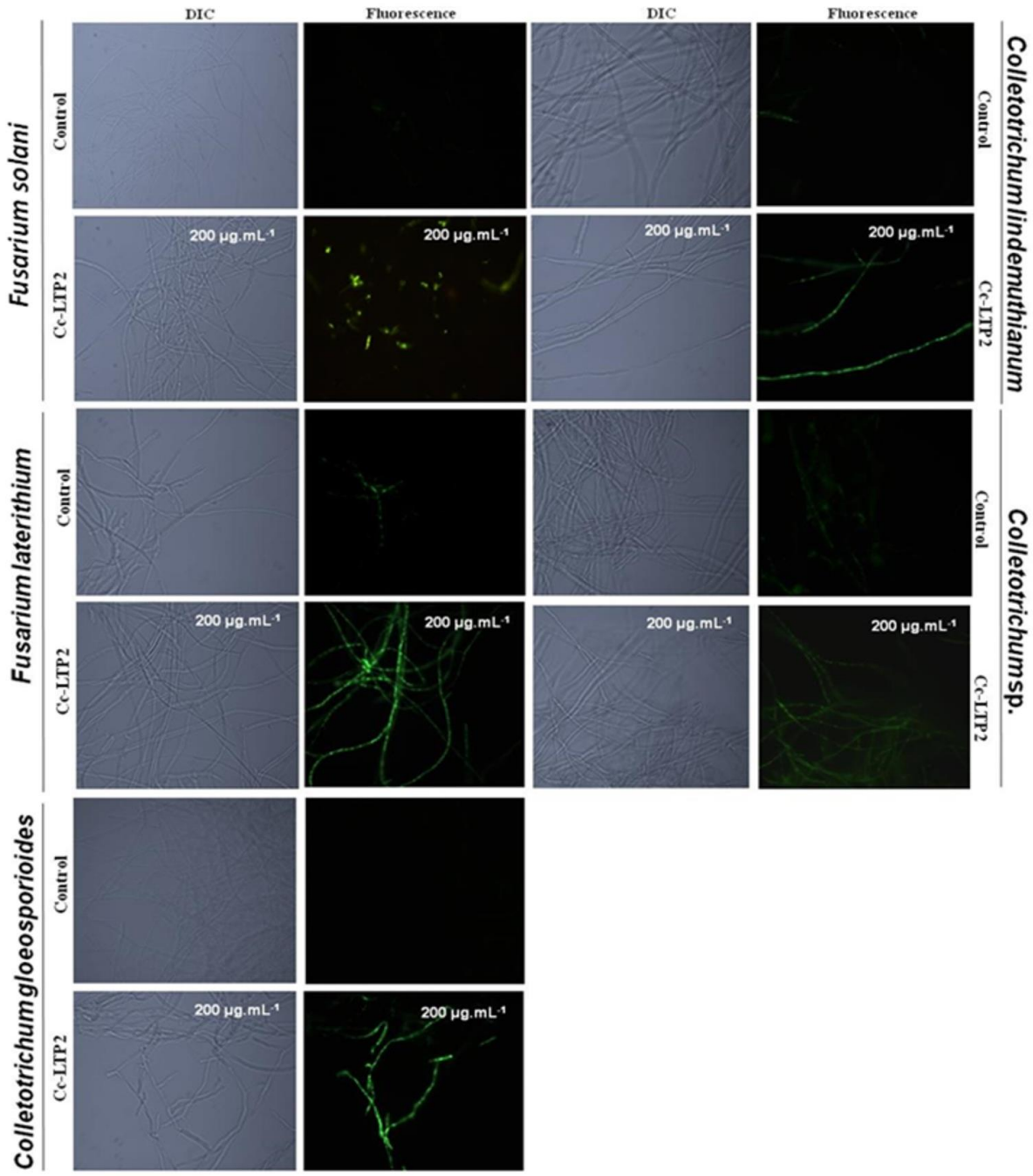

Figure 8 – Membrane permeabilization assays of cells of different filamentous fungi previously incubated with *Cc*-LTP2 at 200 µg/mL for 24 h. Cells were treated with SYTOX green for 30 min to evaluate membrane permeabilization. 400X magnification. The results of membrane permeabilization are representative of one triplicate experiment

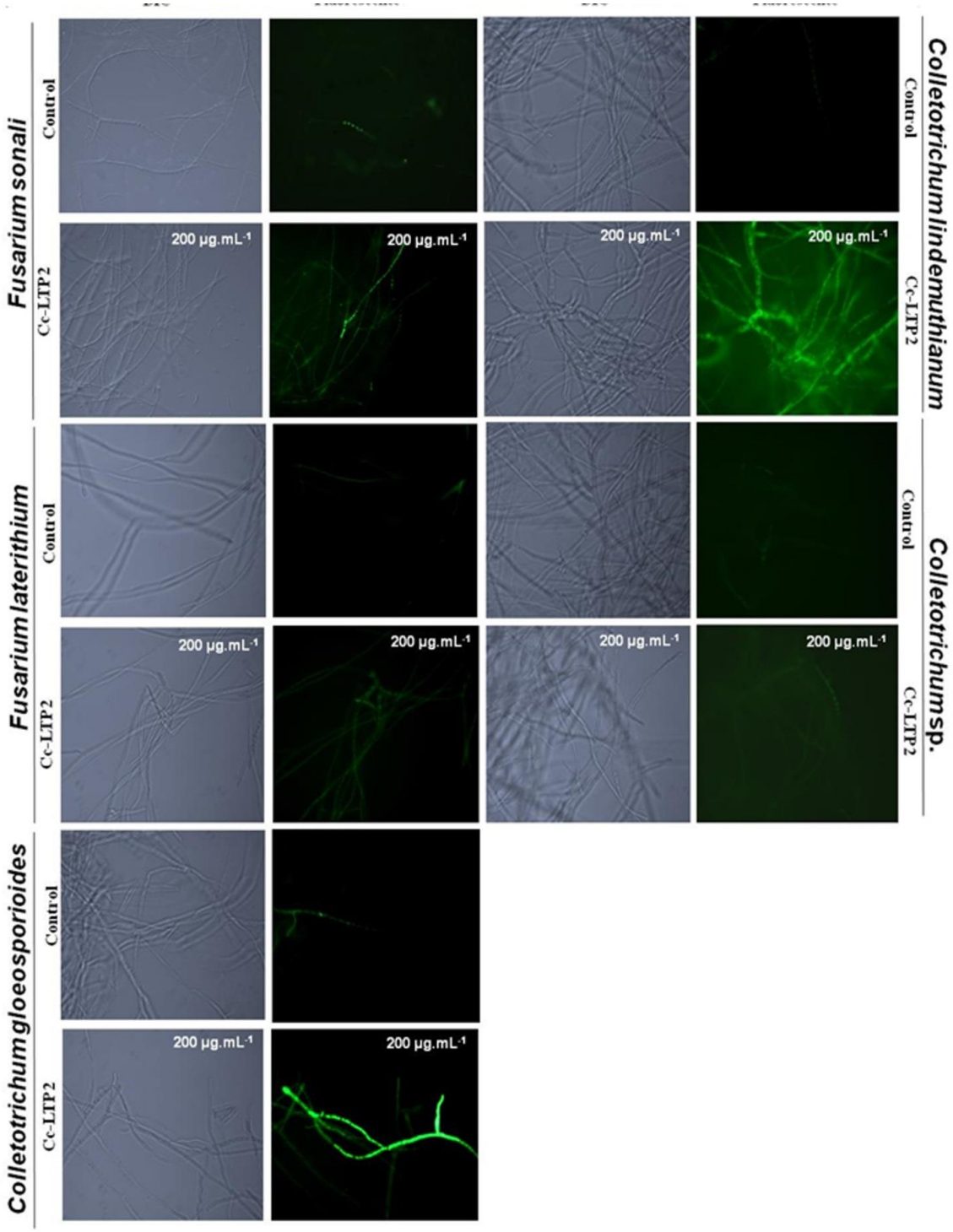

Figure 9 – Oxidative stress assays of cells of different filamentous fungi previously incubated with *Cc*-LTP2 at 200 µg/mL for 24 h. Cells were treated with 2',7'-dichlorofluorescein diacetate for 2 h to evaluate ROS production. 400X magnification. The results of oxidative stress are representative of one triplicate experiment.

3.1.4.5 – Effect of proteins on bacterial growth

To test the bactericidal activity of *Cc*-LTP2, we evaluated its effect on the growth of the gram-negative bacteria *X. euvesicatoria*, measuring the formation of inhibition zones over a 48-h period. In the presence of *Cc*-LTP2 at a concentration of 75 µg/mL, an inhibition zone of 0.25 mm diameter was formed, which was not observed in the control treatment (Figure 10).

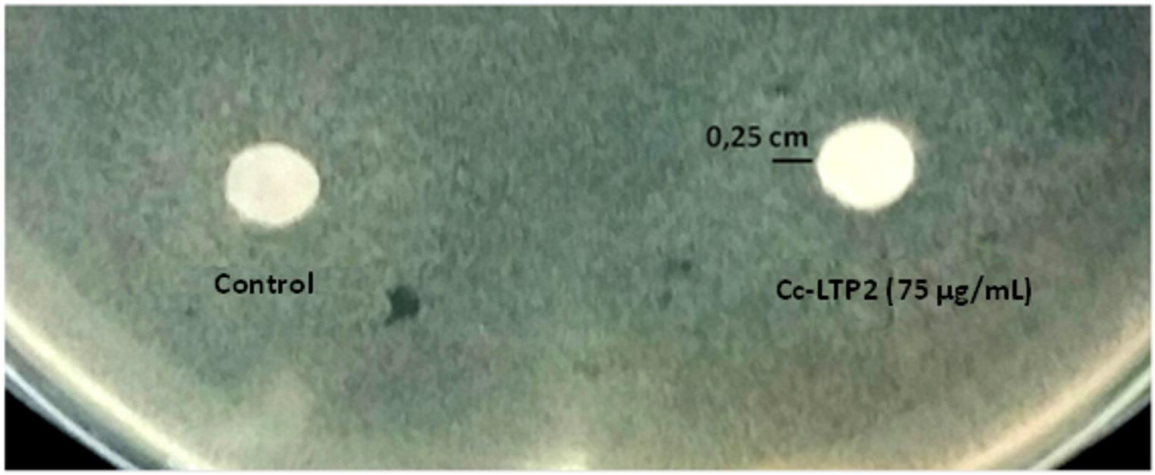

Figure 10 – Antibiogram of *Xanthomonas euvesicatoria*in the presence of 75 µg/mL C*c*-LTP2 after 48 h of growth. Bacterial growth inhibition is visualized as a clear zone around the paper disc containing the *Cc*-LTP2. The results of the antibiogram are representative of one triplicate experiment

3.1.5 – DISCUSSION

A key feature of plant antimicrobial peptides is that they are generally basic and have a net positive charge (Benko-Iseppon et al., 2010). Zottich et al. (2011) isolated a coffee LTP, which they named *Cc*-LTP1, and in the same study, they proved the existence of two isoforms of this protein. Therefore, we suggest the LTP isolated in this study is an isoform of *Cc*-LTP1 and thus we have named it *Cc*-LTP2.

LTPs are in general coded by several genes that belong to a multigene family, as demonstrated in *Arabidopsis* and rice (Liu et al., 2015). The results of immunohistochemistry in seed tissue compared with histochemistry showed that LTPs and lipids were present in the same tissues. There are few studies showing the localization of LTPs in intracellular spaces; the majority of plant LTPs are extracellular, and only two instances of LTP1 have been reported intracellularly (Tsuboi et al., 1992). Based on observations of lipid transport, LTPs have been implicated in the formation of wax and cutin (Cameron et al., 2006). It is also suggested that these proteins are involved in lipid mobilization during sunflower germination (Pagnussat et al., 2009). Accordingly, these results reinforce an important physiological role of seed LTPs in the mobilization of lipids during germination.

Among the numerous biological functions proposed for the LTPs is their ability to protect the plant from abiotic stresses such as low temperature, high salinity, and wounds (Carvalho and Gomes, 2007). Some are also involved in physiological processes such as pollen–stigma interaction (Huang et al., 2013; Tian et al., 2013) and organogenesis of nodules (Lei et al., 2014). Working on SAR (Systemic Acquired Resistance), Maldonado et al. (2002) showed that LTPs can also be directly related to defense signaling. Plant LTPs also show antimicrobial activity against a number of microorganisms, especially fungi (Diz et al., 2011; Zottich et al., 2011).

Although LTPs are known for their antimicrobial activity, there are literature reports of LTPs that showed little or no antimicrobial activity, such as Ns-LTP1 isolated from *Triticum aestivum* (Dubreil et al., 1998). The antimicrobial activity of LTPs was first described by Terras et al. (1992). Since then, several LTPs with antimicrobial activity against fungi and bacteria have been isolated from various plants. *Ca*-LTP, isolated from the seeds of *Capsicum annum* was able to inhibit the growth of the yeast *Candida tropicalis* by more than 70% (Diz et al., 2011). Zottich et al. (2011) isolated an LTP called *Cc*-LTP from coffee beans, *C. canephora*, which was able to inhibit the growth of the yeast *C. albicans* and promote morphological changes such as the formation of pseudohyphae in *C. tropicalis*. In the present study, the isolated LTP isoform *Cc*-LTP2 showed no activity against the tested fungi. Dubreil et al. (1998) detected the main nonspecific LTP, ns-LTP1e1, in wheat endosperm. In mature wheat seeds, this LTP was

specifically located within the aleurone cells but not in the cell walls, in marked contrast to most other plant LTP1 proteins. ns-LTP1e1 is not capable of inhibiting the growth of fungi, and a rather weak synergy was observed between ns-LTP1e1 and α-purothionins.

The complete mechanism of action of microorganism growth inhibition by the LTPs has not been fully determined. Nevertheless, because of the affinity of LTPs for lipids and their ability to inhibit the development of many phytopathogens, it has been postulated that the microbial inhibition is caused by the interaction between this peptide and cell membranes. This interaction may culminate in permeabilization of the cell membrane and a subsequent loss of cell viability (Carvalho and Gomes, 2007). The results observed in this study, in which peptides modulate the influx of ions through the membranes by their permeation, have been observed for several proteins and peptides isolated from various plants, including coffee (Zottich et al., 2011).

The *Cc*-LTP1 isolated from *C. canephora* was able to decrease cell viability and promote permeation of the membranes of the yeasts *C. albicans*, *C. tropicalis,* and *S. cerevisiae* (Zottich et al., 2011). Another LTP called *Ca*-LTP1, isolated from the seeds of *Capsicum annum*, showed the ability to permeabilize the membrane of *C. tropicalis*. Besides LTPs, other AMPs are able to increase membrane permeability in various microorganisms. Such AMPs include defensins such as PvD1 isolated from *Phaseolus vulgaris* (Mello et al., 2011), thionin (Taveira et al., 2014), and trypsin-chymotrypsin inhibitors (Ribeiro et al., 2012) isolated from *Capsicum annuum*. It is important to note that membrane permeation and growth inhibition are not necessarily related. Although this happens in many cases, there may be growth inhibition with no membrane permeabilization, and vice versa (Teixeira et al., 2012), as observed in this study with *Cc*-LTP2. Muñoz et al. (2012) elucidated the mechanism of action of cell-penetrating antifungal peptides using a hexapeptide called PAF26 as a model. Their results showed that this peptide has a dynamic antifungal mechanism of action that involves at least three stages: peptide interaction with the fungal cell wall and/or plasma membrane; its internalization; and a number of complex and specific intracellular effects whose relationship with the cell death of the target fungus is still unclear. This review was very important for the better characterization and study of cell-penetrating antifungal peptides, their permeabilization, and cell death. Interestingly, *Cc*-LTP1

coupled to fluorescein isothiocyanate, and its subsequent treatment with 4′,6-diamidino-2-phenylindole revealed the presence of the peptide in the nucleus of *S. cerevisiae*, and showed for the first time the intracellular localization of Cc-LTP (Zottich, 2012).

Interestingly, Aerts et al. (2007) showed that a defensin called RsAFP2, isolated from radishes, was able to inhibit the growth and induce endogenous production of ROS in *C. albicans* cells. In the presence of ascorbic acid as an antioxidant, these activities were inhibited, suggesting a connection between the antimicrobial activity of the defensin in question and ROS production mediated by it. Recently, another defensin, PvD1, was able to induce endogenous production of ROS in *C. albicans* cells (Mello et al., 2011).

In addition to antifungal activity, bactericidal activity has been described in many AMPs, including numerous LTPs. An LTP isolated from *Hordeum vulgare* demonstrated antimicrobial activity against the gram-negative bacteria *Pseudomonas solanacearum* (Tian et al., 2013).Another LTP from mung beans inhibited the growth of the gram-positive bacteria *Staphylococcus aureus* (Wang et al., 2004).

3.1.6 – CONCLUSIONS

Coffee is a very important crop for the Brazilian economy, but diseases caused by bacteria and fungi are responsible for significant losses or decline in the quality of the final product. A major problem is the indiscriminate use of pesticides and synthetic fungicides to control pests and diseases; they have numerous negative effects on the environment, and substantially increase production costs, making the product less competitive. There has also been an increase in plant diseases caused by fungi, which are becoming increasingly resistant to currently available fungicides, probably because of indiscriminate use. The *Cc*-LTP2 protein isolated in this study from *C. canephora* showed the ability to permeabilize the membrane and induce the production of ROS in various phytopathogenic fungi. It also inhibited the growth of the phytopathogenic bacteria *X. euvesicatoria. Cc*-

LTP2 is therefore fundamental for the sustainable development of new strategies to combat plant diseases.

3.2 – ANTIMICROBIAL ACTIVITY AND MECHANISMS OF ACTION OF THE ANTIMICROBIAL PEPTIDES *CC*-LTP2 AND *CC*-GRP ISOLATED FROM *Coffea canephora* SEEDS ON THE GROWTH OF PHYTOPATHOGENIC FUNGI IN COMBINATION WITH FLUCONAZOLE.

3.2.1 – INTRODUCTION

Plant diseases caused by viruses, bacteria and fungi affect crops and are responsible for significant losses or decreased quality of agricultural products (Agrios 2005). Plant pathogenic fungi are the main organisms responsible for diseases in plants and act as mandatory or facultative parasites (Kalpana et al., 2005; Di Maro et al., 2010), representing approximately 70% of these infections. The genus *Fusarium* harbours some of the major pathogens of agronomic importance that are responsible for losses that reach billions of dollars (van Diepeningen e Hoog 2016). Most representatives are saprophytes and live in soil, plants, and other organic substrates and are especially common in tropical and temperate regions. The fungi of this genus are responsible for producing mycotoxins that not only affect harvests and productivity but also are harmful to animals and humans (Kimura et al., 2007). Some species of *Fusarium*, such as *Fusarium solani*, *Fusarium oxysporum*, *F. incarnatum-equiseti*, *F. fujikuroi*, *F. chlamydosporum*, *F. dimerum* and *F. sporotrichioides* have emerged as important opportunistic human pathogens that are able to cause systemic infections (Dignani and Anaissie 2004; Zhang et al., 2006). Furthermore, these species have become

increasingly insensitive to classic antifungal derivatives of azoles, such as fluconazole (FLC) (O'Donnell et al. 2008, 2009; Van Diepeningen and Hoog 2016).

Today, the control of fungal diseases is mostly achieved using pesticides based on chemical fungicides. However, the use of such products may result in a negative impact on the environment and human health. In addition, their prolonged use may result in the selection of resistant phytopathogenic fungi, thus reducing the long-term efficiency of these fungicides, making conventional pesticides increasingly less effective (Gurgel et al., 2005; Komárek et al., 2010).

To combat the increasing resistance of phytopathogenic fungi and reduce the negative impacts on human health and the environment caused by classical pesticides, numerous strategies have been employed, such as the use of artificially selected resistant plant genotypes, production of resistant transgenic varieties, and biological control using other microorganisms. Therefore, the discovery of new antifungal agents, particularly those produced by the plants themselves, e.g., proteins and peptides, have also been the focus of many studies in cultivated plants. Antimicrobial proteins and peptides isolated from cultivated or wild plants that are involved in plant defence mechanisms provide the possibility of using these molecules to development new strategies to control diseases caused by pathogenic fungi (Xuan et al., 2003; Mahlo et al., 2010; Pane et al., 2013).

Antimicrobial peptides (AMPs) are evolutionarily ancient molecules considered part of the innate immune system of many species and are described both as components of constitutive defence, as well as components of induced defence (Castro and Fontes 2005; Sels et al., 2008; Peters et al., 2010). Plant AMPs are small molecules with molecular weights less than10 kDa that are rich in cysteine and are amphipathic, giving them an ability to interact with the membranes of target microorganisms. Among the AMPs in plants are the Lipid Transport Proteins (LTPs), a group composed of two superfamilies, LTP-1 and LTP-2, with molecular weights of approximately 9 kDa and 7 kDa, respectively (Carvalho and Gomes 2007). Some LTPs have been reported to inhibit the growth of bacteria (Molina et al. 1993) phytopathogenic fungi and yeasts (Diz et al., 2011; Zotich et al., 2011.).

Another important group of plant-derived proteins and peptides is the Glycine Rich Proteins (GRPs), which include sequences rich in repetitive glycine domains. More recently, some representatives of this class have demonstrated

antimicrobial activity and are capable of inhibiting the growth of phytopathogenic fungi, yeasts (Wang et al., 2003; Egorov et al., 2005; Zottich et al., 2013) and Gram-negative bacteria (Pelegrini et al., 2008).

In a previous report, we isolated two antifungal compounds from coffee seeds: a GRP named*Cc*-GRP that showed activity against phytopathogenic fungi and yeast in the Candida genus, and an LTP named*Cc*-LTP2 that, similar to *Cc*-GRP, promoted the permeability of the fungal membrane and induced the production of Reactive Oxygen Species (ROS) (Zottich et al., 2013; Bard et al., 2016). FLC, in combination with AMPs, has shown promising antifungal activity against important fungal pathogens such as yeasts of the *Candida* genus and *Cryptococcus neoformans* (Barbosa et al., 2007, Rossi et al., 2012; Taveira et al. (2016). Therefore, in this study, we investigated whether the *Cc*-GRP peptides and *Cc*-LTP2 could act synergistically with a commercial drug such as FLC to enhance its effects. The results reported here contribute to the future development of these AMPs as new alternative therapies for fungal pathogen infections of medical and agronomic importance.

3.2.2 – REVIEW

3.2.2.1 – Factors limiting the production and defense of the genus Coffea

Maintaining the quality and viability of coffee seeds during the storage period is a recurring concern among producers, since the constituents of seeds such as carbohydrates, lipids and proteins are a food source for a multitude of organisms, including insects and microorganisms that, when feeding on these seeds, make them unfit for consumption (Xavier-Filho, 1993).

Thus, one of the major problems faced by coffee farmers is the susceptibility of coffee to various diseases that can occur in both the field phase and in the storage phase. An example is coffee rust caused by the fungus *H. vastatrix*, which causes necrotic lesions in the form of leaf spots, leading to tissue death. Another disease that causes many losses is cercosporiosis, also caused by a fungus, *Cercospora coffeicola* (Carvalho et al., 2012). In addition to fungi and

some bacteria, insects are responsible for the destruction of coffee beans and crops around the world, e.g., the coffee borer, caused by the beetle *Hypothenemus hampei* (Tozani and Oliveira, 2006).

To contain these losses, coffee growers have made use of fertilizers and agrochemicals intensively, which leads to a large increase in the cost of production and often without the expected results. This exacerbated use of agrochemicals in coffee production has generated several environmental and food problems with negative repercussions on the production and export costs of coffee, which contributes to making the activity unfeasible (Carvalho et al., 2012). Therefore, the development of new control techniques that are more efficient, economical and non-polluting are of fundamental importance (Shewry and Lucas, 1997).

In coffee, cyclotides and legumins, purified seed proteins may be toxic to insects (Huang et al., 2009) and also present antimicrobial activity (Mazzafera and Robison, 2000). A chitinase called CacIXIP, a xylanase inhibitor, was isolated from arabica coffee leaves and showed the ability to inhibit the growth of fungal spores (Vasconcelos et al., 2011). Chandra et al. (2012) showed an anti-inflammatory activity of the aqueous extract of *C. arabica* that was able to prevent protein denaturation. Zottich et al. (2011) isolated a lipid carrier protein (LTP) from *C. canephora* seeds called *Cc*-LTP, which showed the ability to inhibit growth and cause damage to the membrane of the yeast *C. albicans*. This same protein was also able to inhibit human salivary α-amylase activity *in vitro*.

Runte et al. (2006) recently showed that the normal and decaffeinated extract of *C. arabica* was able to inhibit the growth of different bacteria such as *Staphylococcus epidermidis* and *Enterococcus faecali* among others, although these results were similar for both caffeinated and decaffeinated extracts. These results make it clear that the antimicrobial activity is not related to caffeine and that more research must be carried out to characterize the different components in coffee with some antimicrobial activity.

In recent studies by our research group on *C. canephora*, two peptides with antimicrobial activity were isolated. In these works, the sequences of the N-terminal regions demonstrated similarity with lipid carrier proteins (LTPs) and glycine-rich proteins (GRPs) of plants. The purified peptide of approximately 9 kDa was named *Cc*-LTP1; in two-dimensional electrophoresis, it showed presence of two isoforms with pIs between 8.0 and 10. This peptide presented antifungal

activity against *Candida albicans*, also promoting morphological changes in *C. tropicalis*. *Cc*-LPT1 stimulated acidification of the medium with glucose and permeabilized yeast plasma membranes. It was also shown that *Cc*-LPT1 was able to inhibit the activity of mammalian salivary amylase *in vitro*. Antifungal assays with *Cc*-LTP1 coupled to FITC and subsequent treatment with DAPI demonstrated the internalization of this peptide its presence d in the nucleus of *Sacharomices cerevisiae* (Zottich et al., 2011). The purified peptide of approximately 7 kDa (*Cc*-GRP) was efficient in inhibiting the growth of yeasts and filamentous fungi. The interaction with *Cc*-GRP culminated in morphological changes and permeabilization of membranes. *Cc*-GRP also inhibited the formation of the yeast colonies *C. albicans* and *C. tropicalis* (Zottich et al., 2013).

3.2.2.2 – Glycine-rich proteins (GRPs)

In plants, glycine-rich proteins (GRPs) are characterized by the presence of semi-repetitive conserved motifs rich in glycine. These proteins, in general, are involved in regulating plant development and usually exhibit a tissue-specific expression pattern (Mangeon et al., 2010). This expression can also be modulated by biotic and abiotic stresses (Sachetto-Martins et al., 2000).

Glycine-rich proteins are classified based on their general structure, considering the glycine repetition patterns and the presence of conserved motifs. Initially, four classes (I, II, III and IV) were described and, recently, a fifth class was proposed as class V. Class I GRPs have a signal peptide followed by a region with high-glycine (GGX) repeats. Class II GRPs may also contain a signal peptide and have a cysteine-rich C-terminal region. Class III GRPs may contain a signal peptide, but have a lower glycine content compared with the other classes. Class IV GRPs are also known as glycine-rich proteins with RNA binding domains (GR-RBPs), since, in addition to the glycine-rich domain, they have an RNA binding domain (RRM) and a cold shock domain (CSD) and can present zinc fingers in their structure. Because of this large diversity of domains present, class IV GRPs are subdivided into four sub-classes (a, b, c and d). The last class of GRPs described was class V, which is composed of proteins that have a high glycine content, but with a mixed repetition pattern (Mangeon et al., 2010) (Figure 1).

The great diversity of structure, variation in expression pattern and subcellular localization of plant GRPs suggest that they are involved in a multitude of processes (Mangeon et al., 2010). Some GRPs play a structural role in cell wall composition, such as PvGRP1.8, an GRP isolated bean that is located in the primary cell wall and is closely related to the development of the protoxilema (Ringli et al., 2001). Some class IV GRPs that exhibit a cold shock domain are related to acclimatization of plants at low temperatures (Kim et al., 2007).

Some GRPs may be involved in plant defense and, in some cases, this function is also related to the structural function of this protein. This was demonstrated by Ueki (2002), in which tobacco plants infected with TMV and exposed to low cadmium levels showed increased expression of the gene encoding NtCIG1, which is a GRP induced by low levels of cadmium. In parallel to this increased expression, the spread of the virus was contained due to an increase in callose deposition provided by GRP NtCIG1.

A number of factors can induce the expression of GRPs as plant hormones, e.g., abscisic acid (de Oliveira et al., 1990); methyl jasmonate (Molina et al., 1997); ethylene (Memelink et al., 1990); attack of pathogens such as viruses, fungi and nematodes; osmotic stresses (Xu et al., 1995); low temperatures (Carpenter et al., 1994); wounds (Showalter et al., 1991); and light (Kaldenhoff and Richter, 1989).

The GRPs Armadilidine H and Armadilidine Q, isolated from the terrestrial crustacean *Armadillidium vulgare* at very low concentrations, were able to inhibit the growth of countless gram-positive and gram-negative bacteria and were also active against the phytopathogenic fungus *Alternaria brassicicola*. Several morphological alterations were observed in cells of microorganisms grown in the presence of the two peptides. Armadilidine H and Armadilidine Q were further able to promote the membrane plating of different bacteria and of the fungus *Alternaria brassicicola* (Verdon et al., 2016).

The recombinant GRP Pg-AMP1 expressed in *Escherichia coli*, previously isolated from *Psidium guajava* seeds, was active against several gram-positive and gram-negative bacteria and was shown to be a promising peptide to be used as a tool for the biotechnological control of human infectious diseases (Tavares et al., 2012).

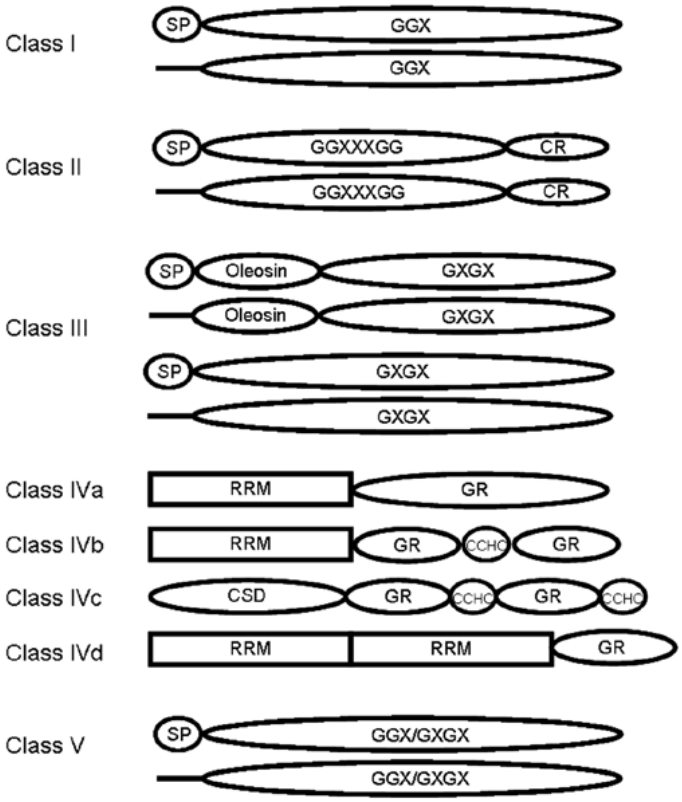

Figure 1 - Schematic representation of the classification of glycine-rich proteins of plants. SP - signal peptide; CR - cysteine-rich domain; Oleosin - conserved oleosin domain; RRM - RNA binding region; GR - glycine-rich domain; CCHC - zinc fingers; CSD - cold shock domain. Glycine-rich repeats are indicated in GGX, GGXXXGG, GXGX and GGX/GXGX, where G represents glycine and X is any amino acid. Source: Mangeonet al. (2010).

Recently, our group isolated a GRP from seeds of *Coffea canephora* that was named *Cc*-GRP and showed activity against different yeasts of the genus *Candida* and against the phytopathogenic fungi *Colletotrichum lindemuthianum* and *Fusarium oxysporum* (Zottich et al., 2013).

3.2.2.3 – Azole Drugs

Fluconazole belongs to the class of azole derivatives, more specifically to triazoles. It is a synthetic compound easily soluble in water that provides one of the most important characteristics of the drug: its high penetration into the biological fluids of all organisms (Casalinuovo et al., 2004). Azole has been one of the most commonly used antifungal drug classes for decades against various fungi, targeting the fungus' ergosterol biosynthesis pathway (Cowen 2008).

The mechanism of action of azole derivatives is by blocking the incorporation of the ergosterol acetate by inhibiting lanosterol 14-α-sterol demethylase or ERG11p, by an interference with cytochrome P-450 and leading to accumulation of an intermediate sterol in the biosynthesis pathway of ergosterol, 14-α-methyl-3,6-diol, which is toxic to the fungus membrane (Figure 2). This metabolite does not have the same form and physical properties as ergosterol, and its accumulation in the plasma membrane of the fungus leads to the formation of the membrane with altered properties, impairing the basic functions necessary for its development (Lupetti et al., 2002; Cowen 2008).

Among the azole derivatives, fluconazole is the most widely used and effective for the treatment of various fungal diseases. However, a number of early-treatment accounts are appearing in the literature because of the appearance of azole-resistant strains that overexpress multidrug efflux transporters including the ABC (ATP-binding cassette transporters) or MFS (*major facilitator superfamily*) (Cannon et al., 2009, Iwazaki et al., 2010).

There are at least three main mechanisms described for the acquisition of resistance to azoles. The first mechanism associated with azole resistance is overexpression of multidrug efflux transporters, resulting in decreased drug concentration in the fungal cell target enzyme, lanosterol 14-α-sterol demethylase. Another common mechanism of azole resistance involves the alteration or stimulation of the expression of the target enzyme to lanosterol 14-α-sterol demethylase, which is encoded by the ERG11 gene. Mutations in this gene prevent azoles from binding to the enzyme site. In *C. krusei*, the intrinsic resistance to fluconazole has been attributed to the reduced affinity of ERG11p to fluconazole. The third mechanism of resistance to azole drugs involves the development of alternative pathways. Mutations in the ERG3 gene prevent the formation of 14-α-methyl-3,6-diol, which is toxic to the fungus membrane, turning it into non-toxic 14-α-methylfecosterol, thereby maintaining the functionality of the plasma membrane and overriding the deleterious effects of azole on fungal membranes (Lupetti et al., 2002).

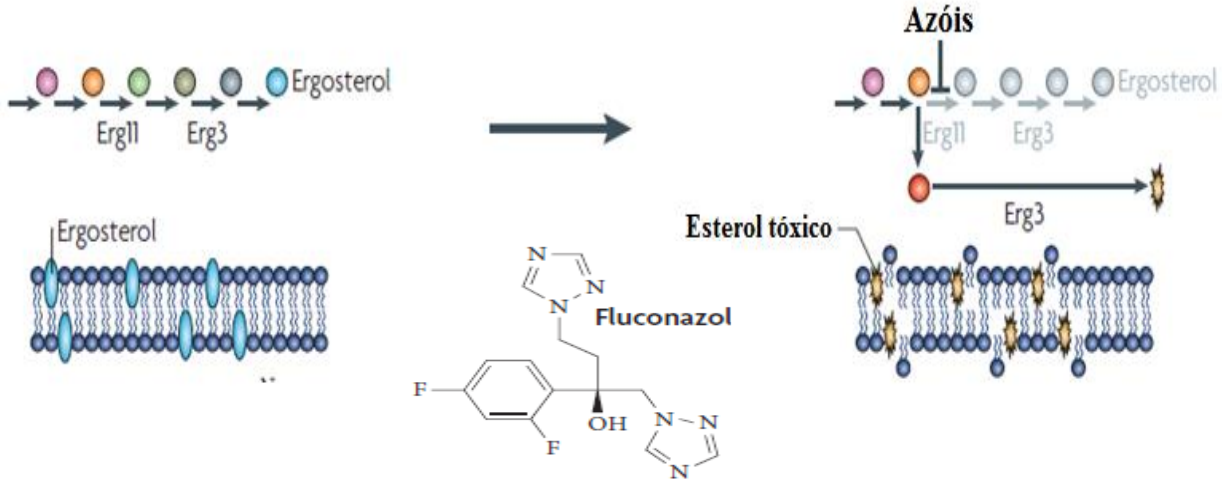

Figure 2: Schematic representation of the mode of action of azoles. Azole, e.g., fluconazole, inhibits ERG11p, causing blockade of ergosterol production and leading to accumulation of an intermediate sterol toxic to fungal cells (adapted from Cowen, 2008).

3.2.3 – MATERIALS AND METHODS

3.2.3.1 – Microorganisms

The pathogenic fungus *Fusarium solani* was cultured in Sabouraud agar and preserved in the Laboratório de Fisiologia e Bioquímica de Microrganismo (LFBM), at Universidade Estadual do Norte Fluminense - Darcy Ribeiro (UENF), in Campos dos Goytacazes, RJ, Brazil.

3.2.3.2 – *Cc-GRP* and *Cc*-LTP-2

Extraction and purification of the *Cc*-GRP from *Coffea canephora seeds* by chromatographic methods were performed as described by Zottich et al. (2013) and extraction and purification of the *Cc*-LTP2 from *Coffea canephora seeds* by chromatographic methods were performed as described by Bard et al. (2016).

3.2.3.3 – Synergism assay and determination of dry weight

To determine the synergistic activities, we combined FLC with *Cc*-GRP and/or*Cc*-LTP2. Initially, fungal spores (1 x 10^4 spore/mL) were incubated in Sabouraud broth containing 400 µg/mL of *Cc*-GRP and *Cc*-LTP2, but this did not inhibit the growth of the fungus tested, and 20 µg/mL of FLC was added, and the final volume was adjusted to 200 µL in vitro. The assay was performed in 96-well microplates (Nunc) at 30 °C for 48 h. Optical readings at 620 nm were taken at zero h and every 6 h thereafter for the following 48 h. Control cells were: 1) grown in the absence of *Cc*-GRP, *Cc*-LTP2 or FLC; 2) grown in the presence of FLC; or 3) grown in the presence of *Cc*-GRP or *Cc*-LTP2. Synergistic activity was deduced by comparing the dry weight of fungi from each control and combined drug treatment (FLC plus *Cc*-GRP) and (FLC plus *Cc*-LTP2). We define synergism as the action of the AMP combined with other substance that causes an enhanced decrease in the growth of the microorganism compared with the growth inhibition by a single substance. To test for synergism, assay cells (controls and tests) were analysed by DIC optical microscope (Axiovison 4, Zeiss). The data were obtained from triplicate experiments. The data were evaluated using a one-way ANOVA. Mean differences at $p < 0.05$ were considered to be significant. All statistical analyses were performed using GraphPad Prism software (version 5.0 for Windows).

3.2.3.4 – Plasma membrane permeabilization assay

Permeabilization of the fungal plasma membrane was measured by Sytox Green uptake according to the methodology described by Thevissen et al. (1999), with some modifications. Briefly, the pathogenic fungi *Fusarium solani* was incubated with 400 µg/mL of *Cc*-GRP or *Cc*-LTP2 separately and in combination with 20 µg/mL of FLC for 24 h. After this time, a 100 µL aliquot of cell suspension was incubated with 0.2 µM of Sytox Green in a 1.5 mL microcentrifuge tube for 30 min at 25 °C with constant agitation. Cells were analysed with a DIC optical microscope (Axiovison 4, Zeiss) equipped with a fluorescent filter set to detect fluorescein (excitation wavelength, 450–490 nm, emission wavelength 500 nm).

3.2.3.5 – Determining the induction of intracellular ROS in fungal cells

To evaluate whether the mechanism of action of *Cc*-GRP and *Cc*-LTP2 involves the induction of oxidative stress, the fluorescent probe 2,7-dichlorofluoresceindiacetate (H2DCFDA) was used to measure intracellular ROS following the methodology described by Mello et al. (2011). The pathogenic fungus *F. solani* was incubated with 400 µg/mL of *Cc*-GRP or*Cc*-LTP2 separately and in combination with 20 µg/mL of FLC for 24 hat 30 °C, and after this incubation, an aliquot of 50 µL for each combination was incubated with 200 µM of H2DCFDA in 1.5 mL microcentrifuge tubes for 1 h at 25 °C with constant agitation at 500 rpm. Cells were analysed with aDIC optical microscope (Axiovison 4, Zeiss) equipped with a fluorescent filter set to detect fluorescein (excitation wavelength, 450–490 nm, emission wavelength 500 nm). The experiments were performed in triplicate.

3.2.3.6 – Measurements of H^+ flux and current using anion-selective vibrating probe system

Extracellular voltage differences, proton flux and surface pH were measured in *Fusarium solani* fungi using an H^+ selective vibrating probe. Initially, the fungus *F. solani*was transferred from stock and placed to grow in 40 mL of Sabouraud broth for 48 h at 30 °C with constant agitation at 0.75 rpm. After 48 h, the suspension was filtered through gauze to prevent the passage of mycelial debris that might be in solution together with the conidia. A total of 1 µL of conidia solution was removed and added into the centre of a petri dish (40 x 10 mm) containing 1 mL of Sabouraud agar, which was grown in 24 h at 30 °C. After 24 h of growth, 2 mL of Sabouraud broth was gently poured into the Petri dish, and measurements of the H^+ flux were performed.

A detailed description of the experiment with the ion-selective vibrating probe technique used in this study is described in previous works (Feijó et al.,1999; Ramos et al., 2008, 2009).

Ion-specific vibrating microelectrodes were produced as described by Feijó et al. (1999) and Ramos et al. (2008). Micropipettes were pulled from 1.5 mm borosilicate glass capillaries and treated with dimethyl dichlorosilane (Sigma-Aldrich, UK). After silanization, they were backfilled with a 15 to 20 mm column of

electrolytes (15 mMKCl and 40 mM KH_2PO_4, pH 6.0 for H^+) and then front-loaded with a 20 to 25 µm column of the respective ion-selective liquid exchange cocktail(Fluka). An Ag/AgCl wire electrode holder (World Precision Instruments) was inserted into the back of the microelectrode and electrical contact with the bathing solution was established. The ground electrode was a dry reference (DRIREF-2, World Precision Instruments) that was inserted into the sample bath. To obtain a calibration curve, the microelectrodes were calibrated at the beginning and end of each experiment using standard solutions covering the experimental range of each ion.. Both the slope and intercept of the calibration line were used to calculate the respective ion concentration from the mV values measured during the experiments.

The *Cc*-LTP_2 (400 µg/mL) and *Cc*-LTP_2 (400 µg/mL) + FLC (20 µg/mL) treatments were performed in *F. Solani* cells after determination of each H^+ flux at the colony (n=6). Data acquisition was stopped and the respective treatments were added in the Petri-dishes and the H^+ flux was measured for a minimum of 5 min or until reach the steady state. After that, a background reference was taken and H^+ flux was recorded again.

3.2.4 – RESULTS

3.2.4.1 – Effect of the combination of *Cc*-LTP2 and *Cc*-GRP with fluconazole on the growth of *F. Solani*

Figure. 3 shows the growth curves of the fungus *F. solani* in the presence of *Cc*-LTP2 and *Cc*-GRP separately and in combination with FLC. For *Cc*-LTP2 it was found that neither of the concentrations tested separately (FLC (20 µg/mL) and *Cc*-LTP2 (400 µg/mL)) or in combination (FLC (20 µg/mL) + *Cc*-LTP2 (400 µg/mL)) could significantly inhibit fungal growth (Figure 3A). The same was not observed for *Cc*-GRP; the concentrations used individually for FLC (20 µg/mL) and *Cc*-GRP (400 µg/mL) were unable to inhibit the growth of the fungus. On the other hand, when the substances were combined with FLC (20 µg/mL) + *Cc*-GRP (400 µg/mL), *F. solani* growth was inhibited by 30% (Figure 3B).

The effect of *Cc*-LTP2 and *Cc*-GRP on the growth of *F. solani* was also evaluated by determining the dry weight of the fungus grown in the absence (control) and in the presence of *Cc*-LTP2 and *Cc*-GRP separately, and in combination FLC (Figure 4). Unlike the observation in the growth curve, analysis of the dry weight showed that *Cc*-LTP2 (400 µg/mL), when combined with FLC (20 µg/mL), induced a significant reduction in the fungal dry weight compared the control. However, the same was not observed for the *Cc*-LTP2 and FLC when tested separately. The significant reduction in the growth and the change in fungal growth pattern were also observed in the photos of the fungus (Figure 4A).

Cc-GRP (400 µg/mL), in combination with FLC (20 µg/mL),caused a significant reduction in the dry weight of the fungus compared to controls, indicating the inhibition of F*. solani* fungus growth similar to what was shown in the curve growth. *Cc*-GRP alone (400 µg/mL) also caused a significant reduction in the dry weight of the fungus, although the growth curve analysis had shown no growth inhibition. Observing *F. solani* growth directly on the plate did not show significant changes in its growth pattern, both in the absence (control) and in the presence of *Cc*-GRP (400 µg/mL) and FLC (20 µg/mL), separately or in combination (Figure 4B).

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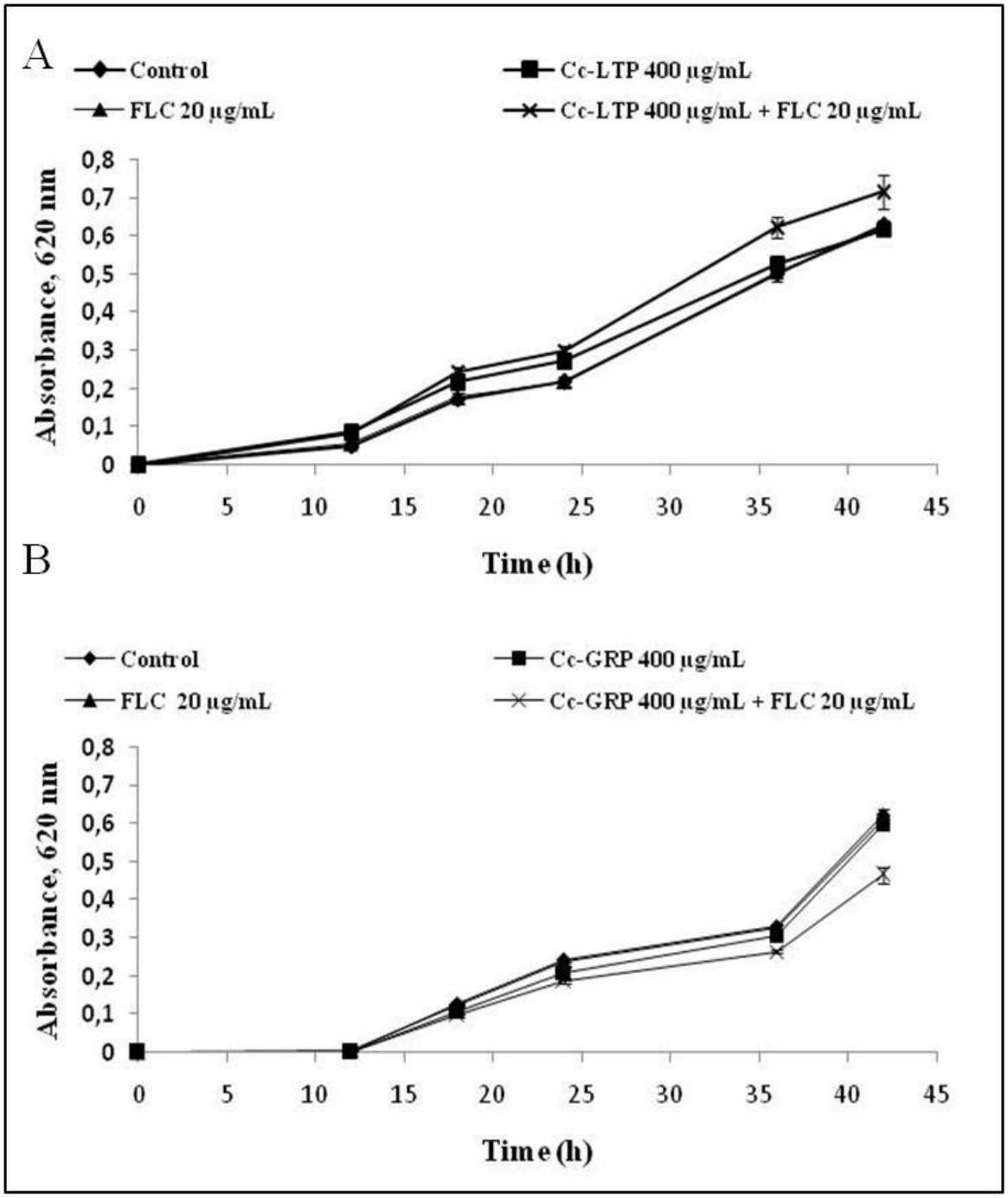


Figure 3 – (A) Effect of *Cc*-LTP2, FLC and *Cc*-LTP2 plus FLC on *F. solani* growth (B) Effect of *Cc*-GRP, FLC and *Cc*-GRP plus FLC on *F. solani.* Experiments were performed in triplicate.

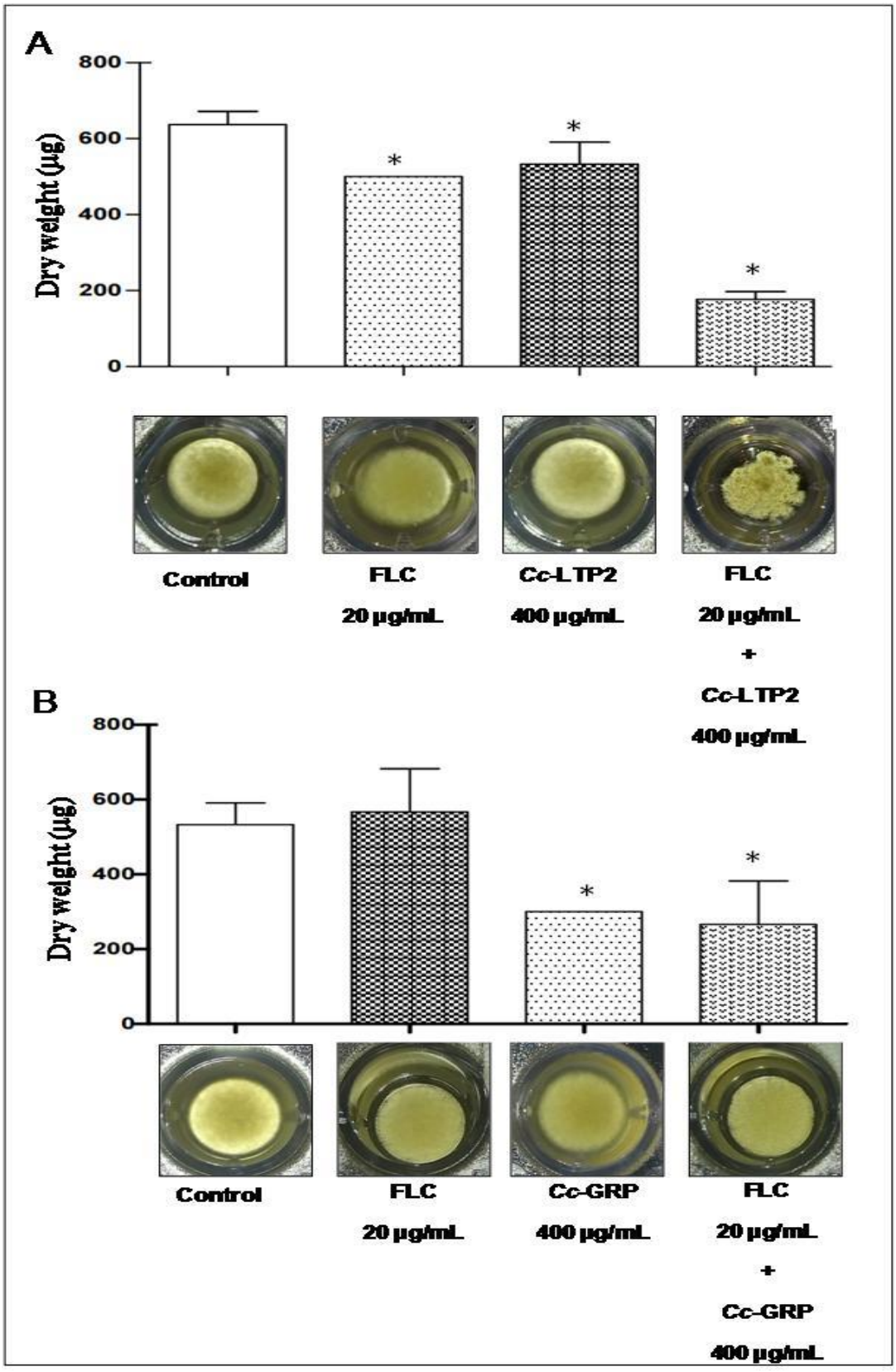


Figure 4 – Dry weight of the fungus *F. solani* and photographs of growth on microplates in the presence of (A) *Cc*-LTP2, FLC or *Cc*-LTP2 plus FLC and (B) *Cc*-GRP, FLC or *Cc*-GRP plus FLC.Asterisks indicate significant differences ($p < 0.05$) between the experimental treatment and the control after 48 h

Figure 5 shows the effect of FLC (20 µg/mL) on the growth of *F. solani*, both separately and in combination as follows: *Cc*-LTP2 (400 µg/mL) + FLC (20 µg/mL) and *Cc*-GRP (400 µg/mL) + FLC (20 µg/mL). The cells grown in the presence of FLC separately showed no change in their number or morphology and were similar to control cells. Cells treated with the two combinations showed a reduction in the amount of hyphae, as well as collapsed and disorganized cytoplasm compared to the control

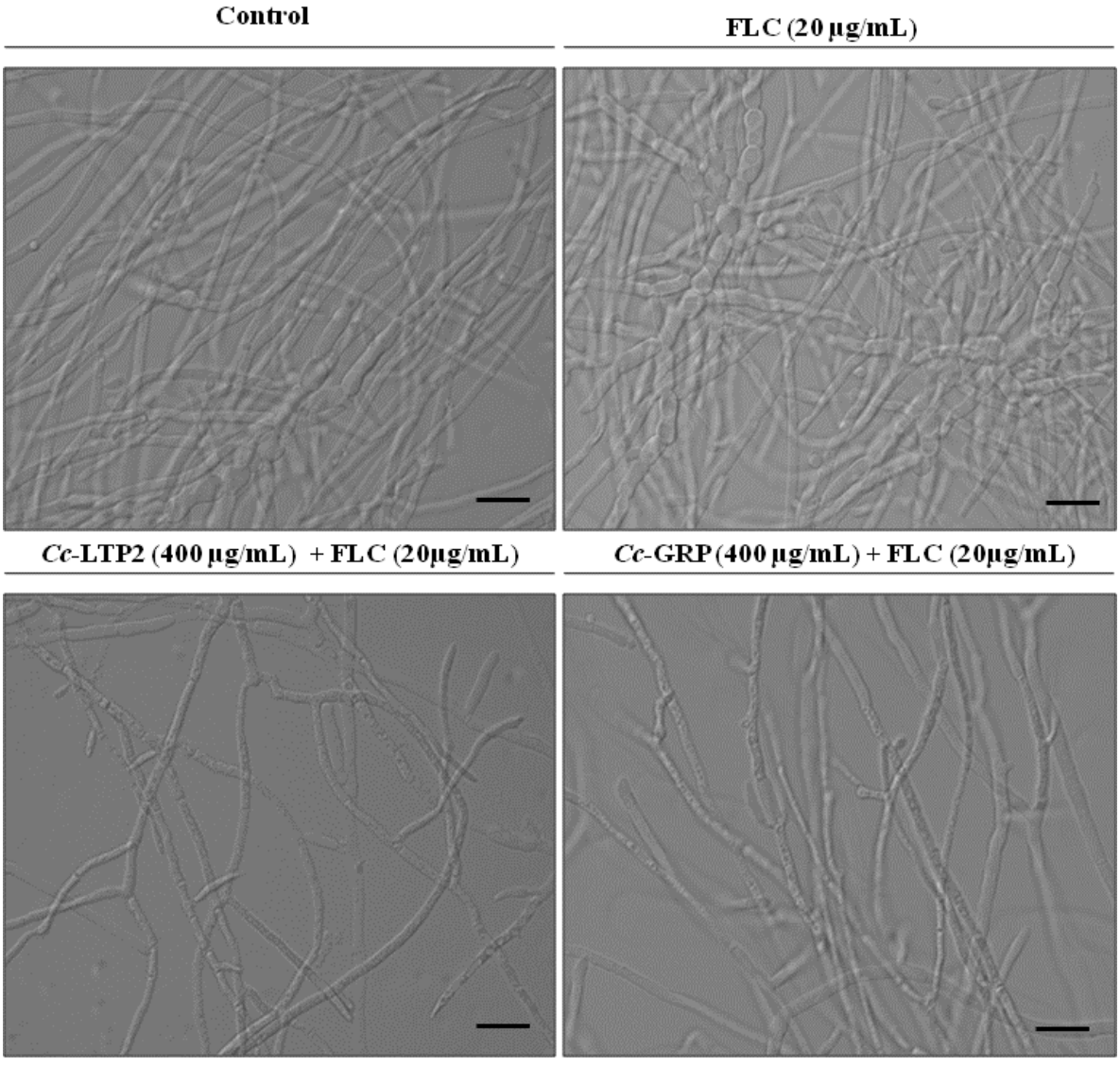


Figure 5 – Optical microscopy of the fungus *F. solani* growing in the presence of FLC, *Cc*-LTP2 plus FLC or*Cc*-GRP plus FLC. (Bars: 20 µm).

These results suggest that the *Cc*-LTP2 and *Cc*-GRP peptides acted in synergy with FLC to inhibit the growth of the fungus *F. solani* and promoted morphological changes in the fungal cells.

3.2.4.2 – Permeabilization of *Fusarium solani* Plasma Membrane

The peptides *Cc*-LTP2 and *Cc*-GRP, separately and in combination with FLC, were tested for the ability to permeabilize the membrane of the fungi *F. solani*. In Figure 6, it can be seen that fungi grown in the presence of FLC (20 µg/mL) and *Cc*-LTP2 (400 µg/mL) separately, as well as the control, showed no labelling for the dye Sytox Green, that is, they were not able to permeabilize the membrane of the fungus. However, the fungus grown in the presence of the combination *Cc*-LTP2 (400 µg/mL) + FLC (20 µg/mL) presented markings, showing that the joint action of these substances was able to promote structural changes in the plasma membrane of these fungi, promoting its permeabilization. The *Cc*-GRP peptide (400 µg/mL), both separately and in combination with FLC (20 µg/mL), induced permeabilization of the cells of the fungus *F. solani.*

3.2.4.3 – Oxidative Stress Assay

To assess whether *Cc*-LTP2 and *Cc*-GRP separately and in combination with FLC were able to induce the endogenous production of ROS, after 24 h, the fungus grown in the absence (control) or presence *Cc*-LTP2 and *Cc*-GRP separately and in combination with FLC was incubated for 15 min with the dye 2, 7 dichlorofluoresceindiacetate and then immediately analysed with a fluorescence optical microscope. As seen in Figure 7, the fungus grown in the presence of *Cc*-LTP2 and *Cc*-GRP separately as well as the control showed no fluorescence, but when *Cc*-LTP2 (400 µg/mL) was combined with FLC (20 µg/mL), the combination was able to induce endogenous production of reactive oxygen species (ROS). For *Cc*-GRP, both separately and in combination with FLC (20 µg/mL) was thus able to induce the endogenous production of ROS.

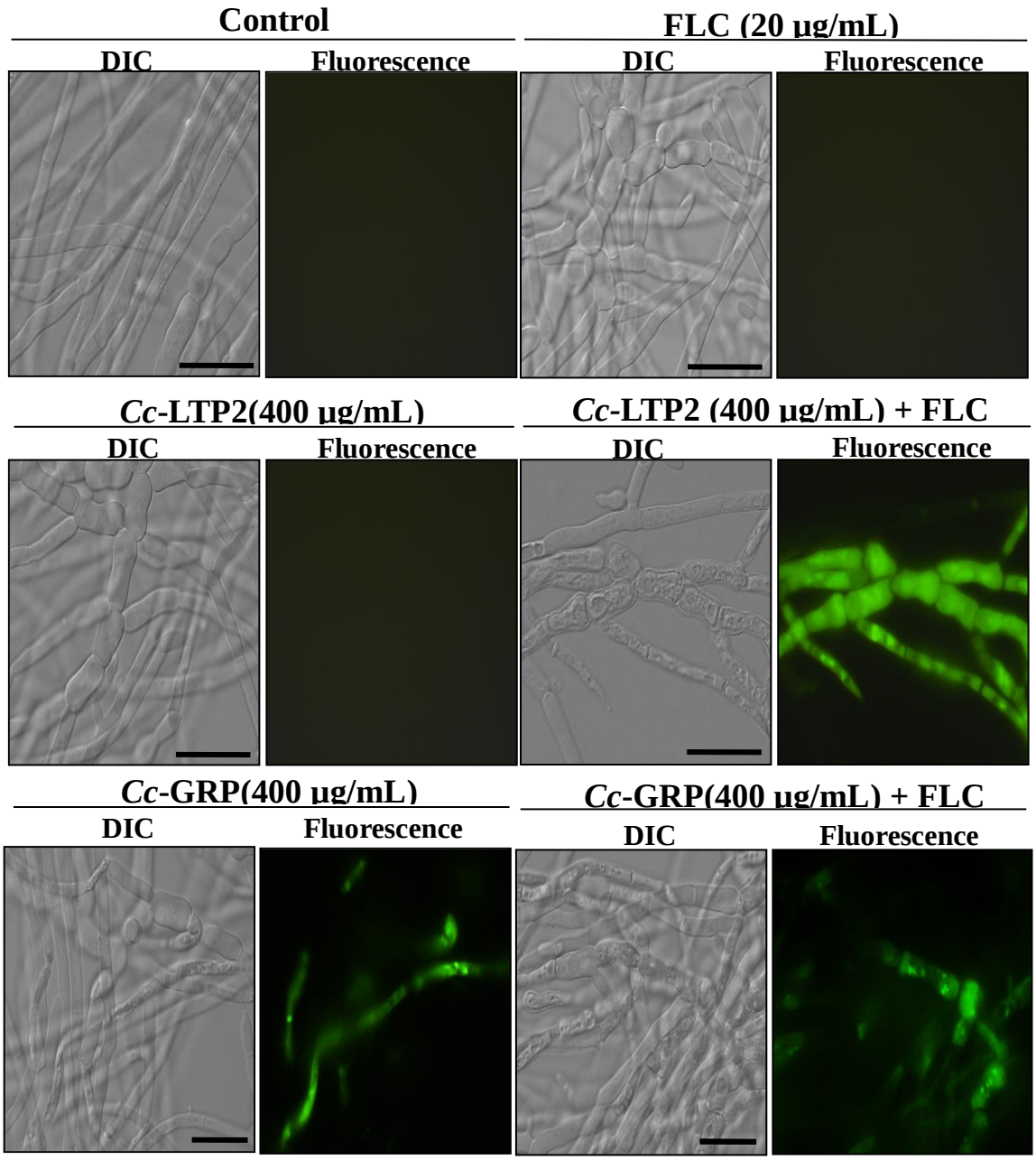


Figure 6 – Membrane permeabilization assay. Photomicrography of *F. solani* cells after membrane permeabilization assay by fluorescence microscopy using the fluorescent probe Sytox Green. Cells were treated with FLC, *Cc*-LTP2, *Cc*-LTP2 plus FLC, *Cc*-GRP or *Cc*-GRP plus FLC for 24 h and then assayed for membrane permeabilization. Control cells were treated only with Sytox Green. (Bars: 20 µm).

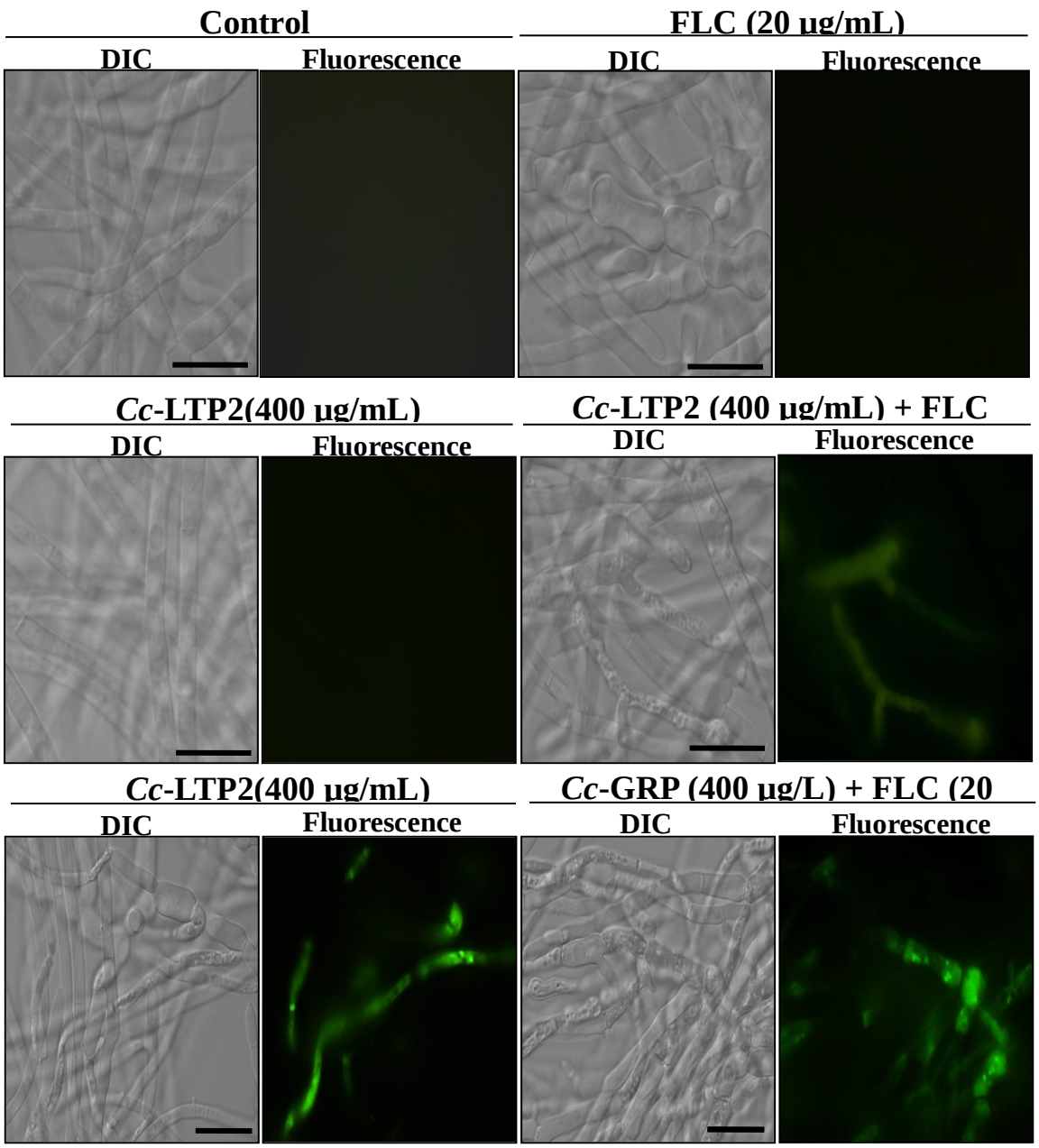


Figure 7 –Membrane oxidative stress assays. Photomicrographs of *F. solani* cells after membrane oxidative stress assays by fluorescence microscopy using the probe 2,7 -dichlorofluoresceindiacetate (H2DCFDA). Cells were treated with FLC, *Cc*-LTP2, *Cc*-LTP2 plus FLC, *Cc*-GRP or *Cc*-GRP plus FLC for 24 h and then assayed for membrane permeabilization. Control cells were treated only with Sytox Green. (Bars: 20 µm).

3.2.4.4 – Analysis of H+ Flow Using an Ion-selective Vibrating Electrode System

Extracellular voltage difference, proton fluxes and surface pH were measured in *F. solani* fungal using an H^+-selective vibrating probe. A stable H^+ voltage difference was recorded in the presence or absence of *Cc*-LTP2 peptide and *Cc*-LTP2 + FLC (Figure 8A). Before the exposure to 400 µg/mL *Cc*-LTP2, the fungal cells showed a steady-state extracellular H^+ efflux activity of 6.09 ± 0.71 pmol cm^{-2} min^{-1}, while after treatment with the peptide, the H^+ efflux showed an inhibition of approximately 67% ($p \leq 0.0001$, Tukey test; Figure 8B). After the addition of FLC (20 µg/mL), fungal cells had effluxes of approximately 2.66 ± 0.93, showing that inhibition was not enhanced by the combination of (*Cc*-LTP2 + FLC) (p=0,1198, Tukey test; Figure 8B). Consequently, the colony surface pH increased significantly when the fungal cells were exposed to *Cc*-LTP2 peptide (Figure 8C). However, 10 min after the removal of *Cc*-LTP2 peptide from the medium, the basal H^+ efflux and root surface pH returned to normal, suggesting that the inhibition is *Cc*-LTP2 peptide-dependent (data not shown).

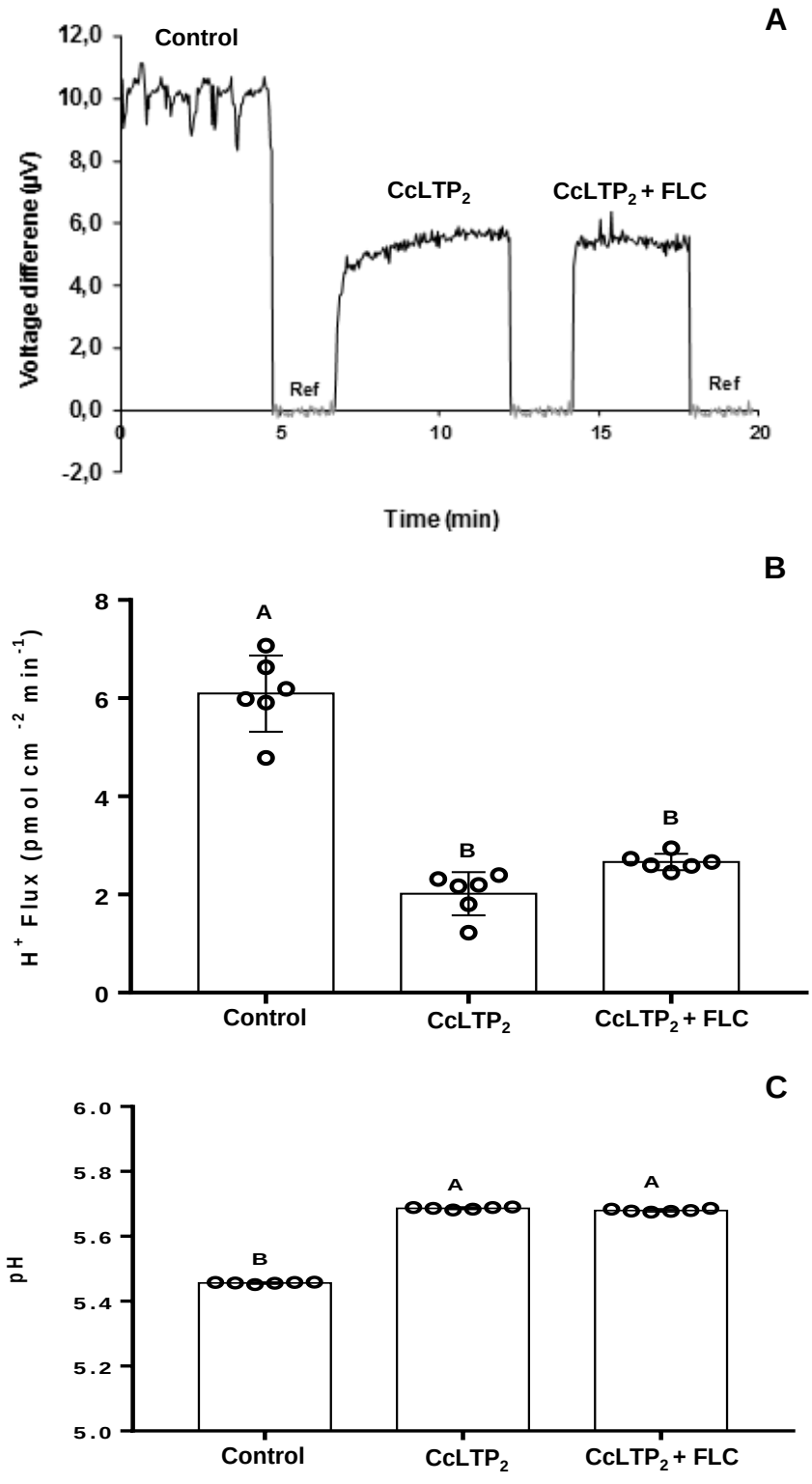


Figure 8 – Voltage difference (A), H^+ efflux rate (B) and root surface pH (C) in *F. solani* cells treated or not (control) with $CcLTP_2$ (400 µg/mL) or $CcLTP_2$ (400 µg/mL) + FLC (20 µg/mL). for the H^+ efflux and pH data, the means are significantly different by Student's*t*-test at p≤0.01. Ref represents the background reference.

3.2.5 – DISCUSSION

Diseases and pests that attack crops are responsible for large annual losses from the planting stage to storage, indicating a major threat to global food security. Pathogenic microorganisms such as viruses, fungi and bacteria are responsible for a drop of more than 10% in global food production (Strange et al., 2005). AMPs are emerging as a possible alternative to help combat microorganisms that have become increasingly resistant to drugs used commercially. These molecules have been isolated from different species from bacteria to vertebrates and are usually part of the innate immune systems of these organisms, where they play an important role in host defence against infection (Li et al., 2012; Goyal et al., 2014).

LTPs are among the many classes of peptides with antimicrobial activity that have been isolated from plants. They exhibit antimicrobial activity against various microorganisms, including viruses (Huang et al., 2013), bacteria (Molina et al., 1993) and fungi (Diz et al., 2011; Zottich et al., 2011). GRP has been described to be active against bacteria (Pellegrini et al., 2008), filamentous fungi (Wang et al., 2003; Egorov et al., 2005) and yeast (Zottich et al., 2013). The present work was designed to study the antimicrobial activity and the mechanism of action of these two peptides that represent the following classes: LTP (*Cc*-LTP2) and GRP (*Cc*-GRP). One of the major problems in combating fungal infections is the large number of pathogens that are becoming increasingly resistant to classical antibiotics such as FLC, which is used commercially to combat fungal infection (Liu et al., 2014). An alternative that is increasingly used is the combination of antimicrobial molecules such as AMPs with classic drugs such as FLC.

Therefore, in this study, to enhance the effects of *Cc*-LTP2 and *Cc*-GRP peptides through possible synergistic activity, they were combined with non-toxic concentrations of FLC. Analysis of the growth curves of the fungus *F. solani* in the presence of the combinations (*Cc*-LTP2 + FLC and *Cc*-GRP + FLC) showed that the combination of FLC with *Cc*-GRP promoted the inhibition of the fungus by approximately 30%, which was not observed for the combination with *Cc*-LTP2 using analysis of optical density (Figure 5). However, when we analysed the

growth of the fungus directly, we observed a significant reduction in fungal growth and changes in growth pattern (Figure 6A). Therefore, we employed dry weigh analysis of the fungus grown in the absence and presence of the combinations (*Cc*-LTP2 + FLC and *Cc*-GRP + FLC), and we observed a significant reduction in dry weight of fungi that were grown in the presence of the peptides in combination with a nontoxic concentration of the FLC. This showed that these peptides can act in synergy with FLC (Figure 4A and 4B).

Similar results were reported by Taveira et al. (2016), who showed synergistic effects between FLC and the peptide *Ca*Thi against six yeasts of medical importance, and this combination caused drastic morphological changes in the cells of these yeasts. The combinations (*Cc*-LTP2 + FLC and *Cc*-GRP + FLC) also promoted various morphological changes in *F. solani* cells, such as a reduction in the number of hyphae and disorganized and retracted cytoplasm (Figure 5). In the literature, different compounds have been described to act synergistically with FLC. Silva et al. (2014) demonstrated the synergistic action of a flavonoid in combination with FLC on the growth of the yeast *Candida tropicalis*. Zhai et al. (2010) showed that the combination of a classical antibiotic, polymyxin B, with FLC can be an alternative to combat systemic cryptococcosis.

Many AMPs have amphipathic characteristics, which confer them with the ability to interact with biological membranes. This interaction may lead to disorganization of the membrane and increased permeabilization, including the formation of pores, which may or may not be related to the antimicrobial activity of the peptide and the death of the microorganism (Jenssen et al., 2006). To assess whether the *Cc*-LTP2 and *Cc*-GRP peptides, separately and in combination with FLC could permeabilize the membrane of the fungus *F. solani*, the dye Sytox Green, which penetrates only cells that have a compromised membrane, was used. *Cc*-GRP applied separately or in combination with FLC was able to permeabilize the membrane of *F. Solani.* However, *F. solani* cells grown in the presence of *Cc*-LTP2 (400 µg /mL) and FLC (20 µg /mL) separately, as well as in the control medium, showed no fluorescence except when the two compounds were combined. Thus, the two compounds work synergistically to permeabilize the membrane of the fungus (Figure 6).

The results observed in this study show that peptides can modulate the influx of ions through the membrane by modifying the membrane permeability.

This phenomenon has been observed for several proteins and peptides isolated from different plants, including from coffee. Zottich et al. (2011) isolated a peptide they named *Cc*-LTP from *Coffea canephora* seeds, and it was able to permeabilize the membranes of different yeasts such as *C. albicans*, *C. Tropicalis* and *Saccharomyces cerevisiae.* The *Cc*-GRP evaluated in this study was previously isolated by Zottich et al. (2013) and was shown to inhibit the growth of *C. albicans* and *C. tropicalis* as well as to increase their membrane permeability .A combination of other compounds with FLC may also cause changes in the membranes of microorganisms. An example is glabridin, a secondary metabolite that acts synergistically with FLC to cause damage to the cell envelope of *C. albicans* and *C. tropicalis*, thereby potentiating the antifungal effects of FLC (Liu et al., 2014).

The action of AMPs on the membrane of microorganisms can change the flow of protons, causing an imbalance in homeostasis and may lead the microorganism's death Thevissen et al. (1999). The H^+ flux across the plasma membrane plays an essential role in the physiology of the fungal cell. The H^+ flux is usually mediated by the H^+ATPase pump, and interference with this stream can lead to cell death. These effects were monitored by measuring the flow of protons into the *F. solani* cells. The results presented herein show that after treatment with *Cc*-LTP2 separately and in combination with FLC, an efflux of H^+ resulted in a significant inhibition of growth by approximately 67%, demonstrating that this peptide can cause an imbalance in the homeostasis of H^+ (Figure 8). Similar results were reported by Diz et al. (2006) and Ribeiro et al. (2007), in which peptides present in a fraction that was obtained from *Capsicum annuum* seeds completely inhibited the efflux of H^+ in *S. cerevisiae* (100% inhibition).

Some studies have shown that increase in ROS production is a mechanism employed by many AMPs (Aerts et al., 2007). In fungi, ROS are reactive molecules generated as metabolic products of endogenous or exogenous sources. These molecules from oxygen intracellular metabolism can act on the activation of transcription factors through signal transduction (Waris and Ahsan 2007).Within cells, ROS molecules are normally in equilibrium with antioxidants; however, when this critical balance is interrupted, there is excessive production of ROS, resulting in significant cellular damage due to oxidative stress (Scandalios 2005). When examining whether *Cc*-LTP2 and *Cc*-GRP separately or in combination with FLC

were able to induce the endogenous production of ROS, it was found that the *Cc*-LTP2 and FLC alone did not induce the production of ROS; this was observed only when these two compounds were combined. *Cc*-GRP both separately and in combination with FLC was able to increase the endogenous production of ROS (Figure 7). Silva et al. (2014) showed that a flavonoid, when combined with FLC induced the production of phosphatidylserine, an important marker of apoptosis and induced endogenous production of ROS. Similar results were also observed when the compounds were used separately.

Many studies have attempted to combine classical antifungals such as FLC and amphotericin B with different compounds to combat resistant organisms. However, such studies have been mainly developed in the area of human pathogens. There were no studies similar to the one conducted in this work to attempt to combat pathogenic fungi that have become increasingly resistant, mainly representatives of the genus *Fusarium* which are the main pathogens of agronomic importance.

3.2.6 – CONCLUSION

The genus *Fusarium* is a group of filamentous fungi most studied, and contains some of the most important species of plant pathogens and economically affecting agricultural and horticultural. Another concern is that a large number of Fusarium species are not only able to cause infections in plants, but also infections in animals and humans. Therefore, the discovery of new antifungal agents is of paramount importance. Here, we demonstrated that *Cc*-LTP2 and *Cc*-GRP combined with FLC has antifungal effect against *F. solani*, and it works by permeabilizing the membrane and inducing oxidative stress response in this fungal. Our results show the combined treatment of *Cc*-LTP2 and *Cc*-GRP with FLC is a strong candidate for studies aimed at improving ways of combating F. solani, and this strategy is even more interesting because it can minimize selection of resistant microorganisms.

3.3 – CLONING OF THE ANTIMICROBIAL PEPTIDES OF *Coffea canephora* SEEDS *CC*-LTP AND *CC*-GRP

3.3.1 – INTRODUCTION

Antimicrobial peptides (AMPs) have attracted the attention of researchers in recent years because of their efficiency in inhibiting the growth of different pathogens. These peptides are found in nature and have been isolated from a wide variety of organisms (Keymanesh et al., 2009).

For a better characterization of the mechanisms of action of these AMPs, a high amount of these purified active molecules is required, and obtaining these peptides naturally can sometimes be a slow and low-yielding process. An alternative is the chemical synthesis of the peptides; however, this process is costly, especially for the synthesis of peptides that have disulfide bonds and many posttranslational modifications, rendering this technique inefficient for large-scale production (Li et al., 2011). Advances in recombinant DNA technology have provided an opportunity to produce AMPs in large quantities. This technology allows the cloning of genes into vectors specific for expression in prokaryotic and/or eukaryotic host cells. This has been considered the most effective method in terms of time and production costs (Xu et al., 2007; Parachin et al., 2012).

Many expression systems can be used to produce heterologous peptides with different conformations, sizes and complexity. Studies have shown that the main hosts for the production of AMPs are bacteria and yeasts, representing

97.4% of the expressed heterologous AMPs (Li et al., 2008). The bacterium *E. coli* is still the most widely used microorganism for the heterologous production of AMPs because of its rapid growth, high availability of commercial expression vectors and extensive available information about its genetics, biochemistry and physiology (Sorensen et al., 2005).

3.3.2 – REVIEW

3.3.2.1 – Plant antimicrobial peptides

Antimicrobial peptides (AMPs) are low-molecular-mass molecules important in the self-defense against infections by different pathogens, potentially representing a new class of antifungal drugs (Zasloff, 2002; Hancock and Sahl, 2006).

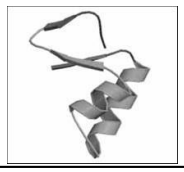
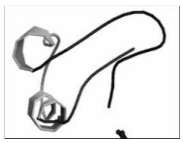
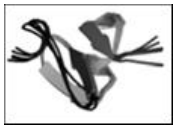
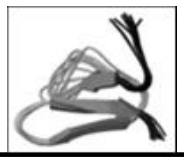
These molecules are considered part of the innate immune system of many species (Peters et al., 2010). Antimicrobial peptides have been described both as components of constitutive defense and as components of induced defense (Castro and Fontes, 2005; Sels et al., 2008). They belong to a diverse and abundant group of molecules and are produced in various tissues and cell types in plants, invertebrates and vertebrates (Berends, 2010; Mihajlovic and Lazaridis, 2010).

Plant AMPs have characteristics in common, including the presence of cysteine residues that form disulfide bonds (2, 3 or 4), providing stability to these molecules, and are mostly basic, presenting a positive net charge at physiological pH and molecular mass ranging from 2 kDa to 10 kDa. In addition, they have amphipathic properties that impart these molecules with the ability to interact with biological membranes, which may or may not be related to antimicrobial activity (Benko-Iseppon et al., 2010; Tam et al., 2015).

Plant antimicrobial peptides can be divided considering mainly their structural characteristics. They may be classified into families based on their sequence similarity, cysteine residues and disulfide bonding patterns, which in turn determine their lipid-transport proteins (LTPs), initially thought to be involved in the

transport of lipids between organelles, as follows: snakins, initially isolated from potatoes (*Solanum tuberosum*); plant defensins, initially isolated from barley seeds (*Hordeum vulgare*); thionines, wherein purothionin is isolated from wheat (*Triticum aestivum*), the first protein whose activity against plant pathogens was detected *in vitro*; hevein-type peptides, initially described as the most abundant rubber latex peptides; knotine-type peptides, initially isolated from marigold seeds (*Mirabilis jalapa*); MBP-1 peptides, isolated from maize (*Zea mays*); small peptides named Ib-AMPs, isolated from balsamine seeds (*Impatiens balsamina*); and proteinase inhibitors isolated from pepper seeds (*Capsicum annuum* L.) (adapted from Benko-Iseppon et al., 2010). In the present study, proteinase inhibition was observed in *Capsicum annuum* L. genotypes (Benko-Iseppon et al., 2010).

Quadro 1 - Families of plant antimicrobial peptides including structural characteristics, database and number of disulfide bridges.

FAMÍLIA	NOME	ESTRUTURA PROTÉICA	PDB[1]	DB[2]	REFERÊNCIA
Defensina de planta	Rs-AFP2		1AYJ	4	- Terras *et al*., 1992 - Aerts *et al*., 2007 - Tavares *et al.,* 2008
α- e β-tioninas	Alpha-1 purotionina		1BHP	4	- Ohtani *et al*., 1977
Tioninas	Crambina		1AB1	3	- Schrader-Fischer e Apel, 1994
Proteína transportadora de lipídeos	Ace-AMP1		1T12	4	-Cammue *et al.,* 1995 - Carvalho *et al.,* 2001 - Diz *et al*., 2006 - Zottich *et al., 2011*
Proteína do tipo heveína	Ace-AMP2		1HEV	4	-Broekaert *et al*., 1992 - Lipkin *et al*., 2005
Knotina	Mj-AMP1		1DKC	3	-Cammue *et al*., 1992 - García-Olmedo *et al.,* 2001

Continuação ...

Macadamia	MiAMP1		1C01	3	- Marcus *et al*., 1997 -McManus *et al*., 1999 - Stephens *et al*., 2005
Impatiens	ib-AMP1	-	-	-	- Tailor *et al*., 1997 - Patel *et al*., 1998 -Thevissen *et al 2005* - Wang *et al*., 2009
Milho-AMP	MBP-1	-	-	-	- Duvick *et al*., 1992
Puroindolinas	Puroindolina A	-	-	-	- Blochet *et al*., 1993 -Gautier *et al*., 1994 - Dhatwalia *et al*., 2009
Snakinas	SN1	-	-	-	- Segura *et al*., 1999 -Berrocal-Lobo *et al*., 2002
Ciclotídeos	Kalata B1		1JJZ	3	- Jennings *et al*., 2001 -Kamimori *et al*., 2005
Inibidores de proteinase	*CaTI*	-	-	-	- Ribeiro et al., 2007b

[1]1PDB refers to the ProteinDatabase and, [2]DB to the number of disulfide bridges

Although most AMPs are stabilized by disulfide bridges, there are still other plant-isolated peptides with antimicrobial activity, but these are not considered classical AMPs, since they are not rich in cysteine and may be totally free of cysteine or have only one residue, which gives such peptides great structural flexibility. This is also the case of glycine-rich proteins (GRPs) like *Cc*-GRP, a peptide of approximately 7k of the coffee isolate (*Coffea canephora*) that shows activity against phytopathogenic fungi and yeasts of the genus *Candida* (Zottich et al., 2013). Another example is Cn-AMP1, a cysteine-free peptide which has nine

amino acid residues, isolated from green coconut (*Cocos nucifera*), with antibacterial and antifungal activity (Silva et al., 2012). Sepherins I and II are AMPs isolated from roots of the shepherd's purse (*Capsellabursa pastoris*) plant that are rich in glycine and histidine and exhibit activity against gram-negative bacteria and fungi (Park et al., 2000).

3.3.2.2 – Biological activity and mechanisms of action of antimicrobial peptides

Plant AMPs have extensive antimicrobial activity on viruses, filamentous fungi, insects, yeasts, protozoa and bacteria, and some also have the ability to inhibit α-amylase from insects and mammals (Tam et al., 2015).

It is believed that the antibacterial activity of AMPs is related to their amphiphilic character and the presence of many conserved positively charged residues, which facilitates attachment and insertion into the bacterial membrane through the permeabilization of their membranes and pore formation (Hancock et al., Boman, 2000; Powers et al., 2004).

The antiviral activity of AMPs is attributed to membrane interaction in electrostatic association with negative charges of glycosaminoglycans facilitating the binding of AMPs and competing with the virus (Mettenleiter, 2002). One example is lactoferrin, a mammalian cationic peptide that prevents the binding of the herpes simplex virus (HSV) to heparan by blocking the cell-virus interaction (WuDunn and Spear, 1989; Laquerre et al., 1998). Some defensins have the ability to bind to glycoproteins, and these bonds prevent HSV from binding to the surface of host cells (Fernandez-de Caleya et al., 1972; Bruix et al., 1993).

The activity of AMPs against fungi was initially attributed to lysis of fungal cells or interference with cell wall synthesis, but there are no conserved domains clearly associated with antifungal activity (Laguerre et al., 1998; Jenssen et al., 2006). Chitin, for example, has been the target of several bioactive peptides. Our group has published many papers reporting the ability of MPAs to permeabilize the fungi membrane. In the present study, two LTPs, *Ca*-LTP1 and *Cc*-LTP strains isolated from pepper (Diz et al., 2011) and coffee (Zottich et al., 2011), were able to permeabilize the membrane of different yeasts of the genus *Candida*. A thionin

called CaThi isolated from pepper seeds (*Capsicum annum*) was also able to permeabilize the membrane of numerous yeasts (Taveira et al., 2016).

Several mechanisms of action have been proposed for MPAs and include mainly membrane permeabilization through the formation of stable pores (barrel or toroidal pore model) and membrane changes (molecular electroporation or balsa model) or micellization (carpet model). Soon after the entry of the peptides, there is an extrusion of ions and metabolites, a decrease in intracellular components, dissipation of the electrochemical potential and finally death of the pathogen cell, demonstrating the lytic potential of the AMPs (Teixeira et al., 2012).

In the barrel model, the peptide spans the membrane and forms a pore with the hydrophilic portion facing the inside of the pore. This type of transmembrane pore is induced by the very hydrophobic peptides, in which the hydrophobic domains interact with the interior of the membrane, whereas the hydrophilic face forms the porous coating (Figure 1A). In the carpet model, the peptides bind in parallel to the membrane. This model explains the activity of antimicrobial peptides such as ovispirin (Yamaguchi et al., 2001), dermaseptin, cecropin (Shai, 1999) and some magainins (Papo and Shai, 2002). The peptides bind parallel to the membrane, forming a kind of carpet, and prevent its rearrangement. After the binding, the peptides form transient holes in the membrane that disintegrate as micelles, and the peptides thus enter the membrane (Figure 1B) (Yeaman and Yount, 2003; Teixeira et al., 2012). In the toroidal pore model, the peptide binds to the membrane and inserts itself, and, at the moment of insertion, the peptide forces a curving of the membrane and the polar faces of the peptides associate with the polar heads of the lipids, thus forming a toroidal pore. This type of transmembrane pore is induced by some magainins, protegrins and melitin (Yeaman and Yount, 2003; Teixeira et al., 2012). Another proposed mechanism for the action of some AMPs is molecular electroporation (Figure 1D). In this mechanism, pore formation in the membrane occurs under the influence of an external electric field (Miteva et al., 1999). Molecular electroporation occurs only when the peptides have a charge density sufficient to generate an electric field. This model has been proposed to explain the formation of membrane pores by annexin V (Karshikov et al., 1992). Pore sizes 2-4 nm in diameter were reported by conventional electroporation and by at least two other cationic peptides, polymyxin B and melittin. This mechanism is particularly important for describing the action of

peptides that exhibit antimicrobial activity without apparent formation of transmembrane pores, providing a novel insight into the means by which peptides increase membrane permeability without necessarily causing its rupture (Teixeira et al., 2012). Lastly, another mechanism of action that has been proposed for AMPs is the sinking raft model. This model proposes that when the peptide binds to a lipid domain, it produces a disproportionate mass locally that directs the translocation of the peptide by increasing the curvature of the local membrane (Figure 1E). In this model, the formation of transient pores occurs after the dissipation of the membrane-induced mass imbalance in the peptides (Pokorny et al., 2002; Teixeira et al., 2012).

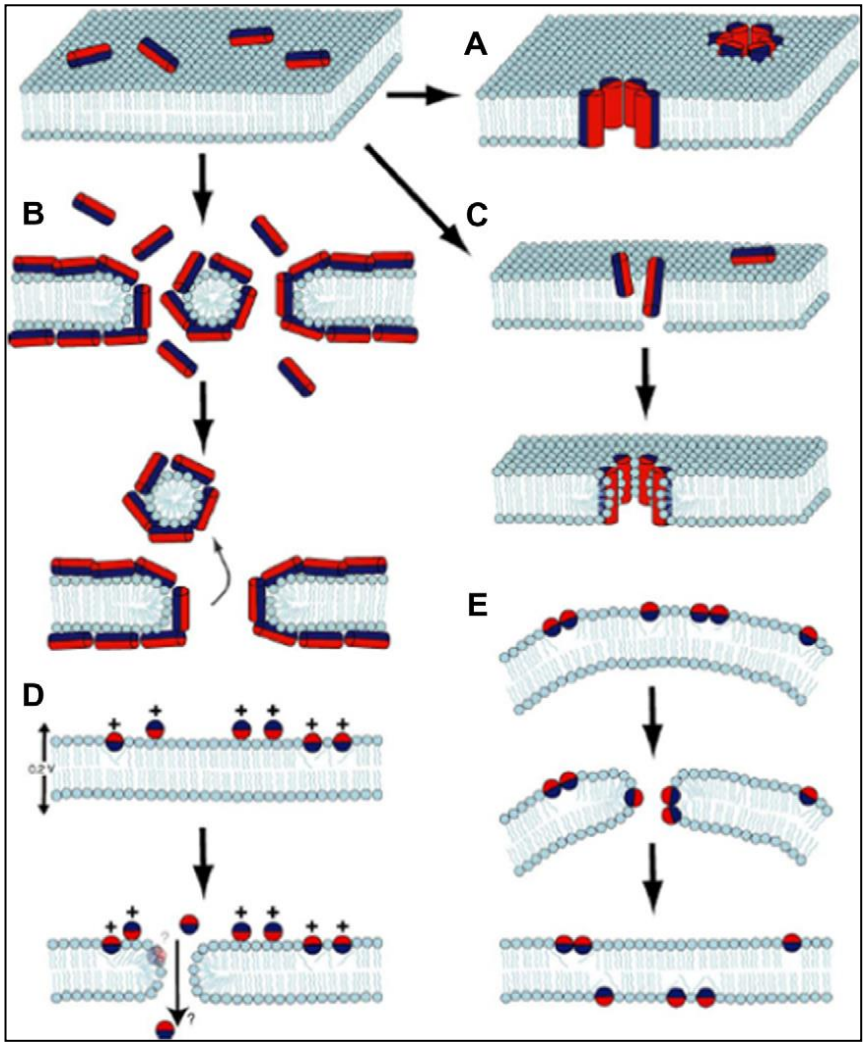


Figure 1 – Mechanisms of action proposed for antimicrobial peptides (Teixeira et al., 2012, adapted of Chan et al., 2006). A – Barrel-stave model; B – Carpet model; C – Toroidal pore model; D – Molecular electroporation model; E – Sinking raft model.

In addition to the ability to interact with membranes, some AMPs have intracellular targets. They can bind to DNA, RNA and proteins within the cell, inhibiting their synthesis; they may also act by inhibiting cell wall synthesis and peptide entry into the cell and induce accumulation of reactive oxygen species (ROS) within the cell (Li et al., 2012).

Often, for a better characterization of the mechanism of action of these AMPs, a high amount of these purified active molecules is required and the natural delivery of these peptides can sometimes be a slow and low yielding process. In addition to the advances in recombinant DNA technology, the cloning and overexpression of AMPs in eukaryotic and prokaryotic systems has been shown to be the most efficient method in terms of time and cost of production (Parachin et al., 2012).

3.3.3 – MATERIAL AND METHODS

3.3.3.1. – Seeds

The *Coffea canephora* Pierre Robusta Tropical (Emcaper - 8151) fruits were provided by the *Capixaba* Institute for Technical and Rural Assistance (INCAPER), located in Vitória - ES, Brazil. The fruits were kept in a freezer at –70 ºC and seeds were only removed at the time of RNA extraction to avoid contamination.

3.3.3.2 – Microorganisms

Escherichia coli cells, XL-10 strain [Relevant genotype: $Tet^R\Delta$*(mcrA)183* Δ*(mcrCB-hsdSMR-mrr)173 end A1 supE44 thi-1 recA1 gyrA96 relA1 lac* Hte [*F´ proAB* $lacI^q$ *Z*Δ*M15 Tn10 (*Tet^R*)* Amy Cam^R]^a were purchased from Agilent Technologies and maintained in glycerol stab (750 µL cell culture + 250 µL 50% glycerol).

3.3.3.3 – Molecular cloning of *Cc*-LTP2 and *Cc*-GRP from seeds of *C. canephora*

All molecular biology procedures used in this study were based on Sambrook and Russel (2001).

3.3.3.4 – Total RNA extraction of *C. canephora* seeds

Total RNA was extracted from *C. canephora* seeds using the RNeasy Plant Mini Kit (Qiagen®), according to the methodology described by the manufacturer. It is valid to point out that optional steps of this protocol were not performed and that only the RLT buffer provided by the kit was used during this extraction.

3.3.3.5 – Preparation of water treated with diethylpyrocarbonate (water-DEPC)

One milliliter (1 mL) DEPC (0.1% v/v) was added to 1 L ultra pure water and this solution was kept under strong stirring for approximately 16 h. At the end of this time, the water was autoclaved at 121 ºC for 15 min and stored at room temperature until used.

3.3.3.6 – Electrophoretic analysis of total RNA and quantification

To prepare the 1% agarose gel, 0.9 g agarose (Sigma) were weighed and 90 mL TAE buffer (40 mM Tris, 20 mM acetic acid, 1 mM EDTA and water-DEPC) were added thereto. The agarose was melted at 100 °C so that the solution did not boil, cooled to 50 °C and then 6.3 µL GelRed™ were added. The gel was homogenized and poured into a previously prepared form and washed with DEPC-water. The material was left to rest for 1 h after solidification before the use of the gel. Samples were prepared by mixing 4 µL of the total RNA extraction and 2.5 µL sample buffer (30 µL glycerol, 0.5 µL TAE 50X and 2 µL RNaseOUT™). The samples were homogenized and applied to the gel. The run was performed at a voltage of 4 $V.cm^{-1}$ gel, with the 1X TAE running buffer.

After the run, the gel image was obtained by ImageQuant™ LAS 500 (GE HealthCare). A 1-µL aliquot was used for the quantification of total RNA using the NanoDrop 2000c UV-Vis spectrophotometer (Thermo Fisher Scientific©).

3.3.3.7 – Obtaining the cDNA - Reverse transcription reaction

Complementary DNA (cDNA) was obtained using the GoScript™ Reverse Transcription System Kit (Promega©) according to the methodology described by the manufacturer. Two reverse transcription reactions were performed using two different primers provided by the kit: primer R (Random primer) and primer P (Oligo (dT) 15 primer) to obtain two cDNAs that we named cDNAr and cDNAp, respectively.

3.3.3.8 – Design of the degenerate primer for the N-terminal portion of Cc-LTP and Cc-GRP from *C. canephora* seeds

Two degenerate primers coding for the N-terminal sequence of the *Cc*-LTP peptides (obtained by Zottich et al., 2011) and *Cc*-GRP (obtained by Zottich et al., 2013) were designed. The primers were synthesized and commercially purchased (Invitrogen by Life Technologies). Sequences of these peptides were obtained by the Edman degradation method (Edman, 1950) and served as the basis for primer design, as shown below:

N-terminal sequence of Cc-LTP in a one-letter code for the amino acids:
I T C Q Q V T S E L G P C V P Y L T G Q G
ILTP degenerate initiator sequence
5'- A T I A C I T G Y C A R C A R G T I A C – 3'

N-terminal sequence of *Cc*-GRP in a one-letter code for the amino acids:
G N - - E G G G H G G H G G Y G G Y H H H H G G G G G Y

Degenerate initiator sequence for iGRP
5'- G G I A A Y G A R G G I G G I G G I C A Y G – 3'
Where I = inosine, Y = C or T, R = A or G\

3.3.3.9 – Polymerase chain reaction - Test of annealing temperature and primer concentration

For the polymerase chain reactions (PCRs) for amplification of the *Cc*-GRP fragment, the primers iGRP (sense primer) and oligo dT18 (antisense primer) were used. The polymerase chain reaction was initially performed at five different temperatures (69, 65, 61, 57 and 53 ºC), which varied around the primer annealing temperature provided by the manufacturer, using three different concentrations of the primers (iGRP and oligo dT18). The reaction was performed in a Veriti 96-well thermal cycler (PN 4375786) (Applied Biosystems®), which allows the formation of a temperature gradient. Three different mixtures were prepared by varying the ratio (iGRP/oligo dT18) at 1:1, 2:1 and 1:2, as shown below. Each condition was prepared with both cDNAr and cDNAp.

• Mix 1 (1:1) – 60.6 µL water, 12 µL 10 x Taq buffer [100 mM Tris-HCl, pH 9.0, and 500 mM KCl], 9.6 µL 25 mM $MgCl_2$, 19.2 µL 10 mM dNTP, 6 µL oligo dT18 10 pmol, 6 µL iGRP, 2,5 µL cDNAr or cDNAp and 3 µL Taq DNA polymerase (5 U/µL);
• Mix 2 (2:1) – 56.4 µL water, 12 µL 10 x Taq buffer [100 mM Tris-HCl, pH 9.0, and 500 mM KCl], 9.6 µL 25 mM $MgCl_2$ (1X), 19.2 µL 10 mM dNTP, 6 µL oligo dT18 10 pmol, 12 µL iGRP, 2.5 µL cDNAr or cDNAp and 3 µL Taq DNA polymerase (5 U/µL);
• Mix 3 (1:2) – 56.4 µL water, 12 µL 10 x Taq buffer [100 mM Tris-HCl, pH 9.0, and 500 mM KCl], 9.6 µL 25 mM $MgCl_2$, 19.2 µL 10 mM dNTP, 12 µL 10 pmol oligo dT18, 6 µL iGRP, 2.5 µL cDNAr or cDNAp and 3 µL Taq DNA polymerase (5 U/µL);

For the PCRs for amplification of the corresponding *Cc*-LTP fragment, the primers iLTP (sense primer) and oligo dT18 (antisense primer) were used. The polymerase chain reaction was initially performed at five different temperatures (54, 52, 50, 48 and 46 ºC) that varied around the primer annealing temperature provided by the manufacturer, using three different concentrations of primers (iLTP and oligo dT18). The reaction was carried out in a Veriti 96-well thermal cycler (PN 4375786) (Applied Biosystems®), which allows the formation of a temperature

gradient. Three different mixtures were prepared by varying the ratio (iLTP/oligo dT18) at 1:1, 2:1 and 1:2, as shown below:

• Mix 1 (1:1) – 60.6 µL water, 12 µL 10 x Taq buffer [100 mM Tris-HCl, pH 9.0, and 500 mM KCl], 9.6 µL 25 mM $MgCl_2$, 19.2 µL 10 mM dNTP, 6 µL 10 pmol oligo dT18,6 µL iLTP, 2.5 µL cDNAp and 3 µL Taq DNA polymerase (5 U/µL);

• Mix 2 (2:1) – 56.4 µL water, 12 µL 10 x Taq buffer [100 mM Tris-HCl, pH 9.0, and 500 mM KCl], 9.6 µL 25 mM $MgCl_2$, 19.2 µL of 10 mM dNTP, 6 µL 10 pmol oligo dT18, 12 µL iLTP, 2.5 µL cDNAp and 3 µL Taq DNA polymerase (5 U/µL);

• Mix 3 (1:2) – 56.4 µL water, 12 µL 10 x Taq buffer [100 mM Tris-HCl, pH 9.0, and 500 mM KCl], 9.6 µL 25 mM $MgCl_2$, 19.2 µL 10 mM dNTP, 12 µL 10 pmol oligo dT18, 6 µL iLTP, 2.5 µLl cDNAp and 3 µL Taq DNA polymerase (5 U/µL);

Because cDNAp PCRs presented better results for amplification of the *Cc*-GRP fragment, only cDNAp was used for amplification of the corresponding *Cc*-LTP fragment.

Each mixture was then aliquoted (20 µL) into PCR tubes and taken to the thermal cycler. For each reaction, a blank was made consisting of all reagents except cDNA (template).

The polymerase chain reaction was programmed for 40 cycles with the following sequential steps:

1) 95 ° C for 1 min, 2) 95 ° C for 35 s, 3) temperature gradient for 45 s, 4) 72 °C for 1.10 min, and back to step 2 forty times. At the end of the cycles, the samples were cooled at 4 °C in the thermocycler for up to 1 h.

After the PCR, the samples were visualized by 1% agarose gel electrophoresis according to Sambrook and Russel (2001) to confirm if there was amplification and the best condition for it to occur. In this case, the O'GeneRuler DNA ladder mix (Thermo Fisher Scientific©) marker was used, containing the following DNA fragments: 10000, 8000, 6000, 5000, 4000, 3500, 3000, 2500, 2000, 1500, 1200, 1000, 900, 800, 700, 600, 500, 400, 300, 200 and 100. This marker contains the strongest 3,000-, 1,000- and 500-bp bands for easy orientation.

3.3.3.10 – Purification of the fragments with the Wizard SV gel and PCR Clean-Up System kit

Once the amplification of the fragments was confirmed, a new PCR in larger quantity was run under the same predetermined optimum conditions and these samples were purified using the Wizard SV Gel and PCR Clean-Up System (Promega©) kit according to instructions of the manufacturer.

Initially, the samples were subjected to electrophoretic separation on 1% agarose gel containing GelRed™ and the DNA bands were visualized under ultraviolet light. The bands relating to the fragment of interest were identified, cut from the gel, transferred to microcentrifuge tubes and solubilized in the membrane binding solution (0.5 M potassium acetate, pH 5.0 and 4.5 M guanidine isothiocyanate) (1:1 ratio, mV; at 60 °C until complete dissolution of the gel). Subsequently, the mixture was transferred to a mini-column and left in contact therewith for 1 min at room temperature. The column was then centrifuged (10,000 x g for 1 min at room temperature) and the volume passed through it was discarded. Seven hundred microliters (700 µL) of the membrane wash solution were added (10 mM potassium acetate, pH 5.0, 80% ethanol and 16.7 µM EDTA, pH 8.0) to the column and it was centrifuged again (10,000 xg for 1 min at room temperature). Once again, the volume passed through the column was discarded and another 500 µL of the membrane wash solution were added. This time, the column was centrifuged at 10,000 x g for 5 min and at the end of the centrifugation the column was transferred to a new tube (collector tube). Fifty microliters (50 µL) of ultrapure water were added to the column and left in contact therewith for 1 min at room temperature. After this time, the column was centrifuged (10,000 x g for 1 min) and the collected material was stored at –20 ° C. All reagents and columns were supplied with the kit.

3.3.3.11 – Cloning the amplified fragment into the vector

The purified fragments were cloned into the pJET1.2/blunt vector according to the protocol provided by the manufacturer. To bind the vector correctly, prior to binding, the fragments were treated with the T4 DNA polymerase. A volume of 3.5 µL of the PCR product (~ 300 ng of the purified fragment), 5 µL 2x T4 DNA

polymerase (33 mM Tris-acetate, pH 7.0 66 mM sodium acetate, 10 mM magnesium, 1 mM DTT) and 0.5 µL of the T4 DNA polymerase were pooled in a microcentrifuge tube. This mixture was then homogenized and heated at 70 °C for 5 min in a dry bath and then cooled in an ice bath for 5 min.

To anneal the fragments to the vector, 9 µL of the solution prepared as mentioned above were mixed with 0.5 µL of the pJET1.2/blunt vector and 0.5 µL of the T4 DNA ligase. This mixture was incubated at 22 °C for 5 min. The resulting DNA construct was named pJET-GRP and pJET-LTP for the fragments amplified from the iGRP and iLTP primers, respectively. All material used was supplied by the CloneJET PCR cloning kit (Fermentas Inc.).

These constructs were used for the transformation of bacterial cells from *E. coli* strain XL-10.

3.3.3.12– Production of competent *E. coli* cells, XL-10 strain

A bacterial colony from a fresh culture of *E. coli*, strain XL-10, was inoculated into a tube containing 5 mL LB (Luria-Bertani) medium (Sigma®) (5 g/L yeast extract, 1 g/L tryptone, 1 g/L NaCl) and incubated at 37 °C for 16 h at 250 rpm (pre-inoculum). From this pre-inoculum, aliquots of 0.2, 0.4 and 0.8 mL were transferred to 50 mL of medium (LB) and incubated at 37 °C for 3 h at 250 rpm. After this period, spectrophotometer readings were taken at 600 nm. The culture having an optical density of about 0.5 was used for the preparation of the competent cells, transferred to an ice bath for 10 min and centrifuged at 3,500 x g for 10 min at 4 °C. The supernatant was discarded and the cell pellet was resuspended in 10 mL saline (10 mM PIPES, pH 6.7, 55 mM $MgCl_2$, 15 mM $CaCl_2$ and 250 mM KCl) and centrifuged (3,500 x g for 10 min at 4 ºC). The supernatant was discarded again and the cell pellet was suspended in 5 mL of the saline solution, plus 187.5 µL 100% DMSO. Cells were homogenized gently and incubated for 10 min in an ice bath. After this incubation, another 187.5 µL of 100% DMSO were added; the cell suspension was again homogenized and aliquots of 200 µL were transferred to sterile microcentrifuge tubes and immediately frozen in liquid nitrogen before being stored at –70 °C (Inoue et al., 1990).

3.3.3.13 – Transformation of competent *E. coli* cells (XL-10 strain) with the pJET-GRP and pJET-LTP constructs

Tubes containing 200 µL of competent cells stored at –70 °C were thawed in an ice bath and 5 µL and 10 µL of the pJET-GRP or pJET-LTP construct were added to the cells. Afterwards, the tubes were incubated for 10 min in an ice bath; subsequently, the tubes containing the cells with the pJET-GRP or pJET-LTP construct were transferred from the ice bath to a 42 °C water bath for 90 s and then again to an ice bath for another 10 min. After thermal shock, 800 µL of LB medium were added to the cells and then incubated at 37 °C for 1 h. After this time, the tubes were centrifuged at 10,000 x g for 1 min at room temperature. Excess medium was removed and the cells were plated on different Petri dishes containing LB medium plus ampicillin (100 µg/mL) (GE Healthcare); next, the plates were incubated at 37 °C for 16 h.

3.3.3.14 – Analysis of positive clones - extraction and digestion of vectors

To confirm whether positive clones actually had the fragment of interest, the vector was extracted from the XL-10 strain bacterial cells and subjected to Bgl II restriction enzyme digestion, which would release the bound fragment after digestion. The vectors were extracted from the bacterial cells using the following methodology: colonies were transferred to 5 mL liquid LB medium containing ampicillin (100 µg/mL) and incubated at 37 °C for 16 h at 250 rpm. After this growth period, 1500 µL of each culture were transferred separately to microcentrifuge tubes and centrifuged at 9,000 x g for 1 min. Supernatants were discarded, another 300 µL of TENS (0.01 M Tris-HCl, pH 8.0, 1 mM EDTA, 0.1 M NaOH and 25 mM SDS) were added to the pellets and the samples were shaken for 10 s for cell lysis. After lysis of the bacterial cells, 150 µL of 3 M sodium acetate pH 5.2 were added and again the samples were shaken for 10 s before being centrifuged at 13,200 x g for 3 min. The precipitates were discarded and the supernatants were transferred to fresh tubes and 1 mL of 100% ice cold ethanol was added. The samples were gently homogenized and incubated for 1 min at room temperature and again centrifuged under the same conditions. The supernatants were discarded and the precipitates were washed with 1 mL 70%

ice-cold ethanol. Samples were centrifuged under the same conditions; the supernatants were discarded and the precipitates were dried in a 37 °C dry bath for approximately 10 min. After drying, the precipitates were resuspended in 40 µL TE containing RNase A (0.01 M Tris-HCl, pH 8.0, 1 mM EDTA and 20 µg/mL RNase A) and incubated in that buffer at 37 °C for 1h. At the end of this procedure, the extracted vectors were subjected to digestion with the Bgl II restriction enzyme.

The pJET1.2/blunt vector has restriction sites for the Bgl II enzyme flanking the region where the fragment is inserted, so digestion with these enzymes opens the vector and at the same time releases the inserted fragment. The digestion reaction was performed as follows: 10 µL plasmid extraction, 2 µL 10x enzyme buffer [50 mM Tris-HCl pH 8.5 mM MgCl 2, 100 mM KCl, 0.02% Triton X-100, β 1 mM mercaptoethanol and 0.1 mg/mL BSA], 1 µL Bgl II (10 µL/µL) and 7 µL ultrapure water. The samples were homogenized and then incubated at 37 °C for 1 h. At the end of incubation, the digested vectors were analyzed by 1% agarose gel electrophoresis to confirm bacterial transformation. After confirmation of the transformation, the samples were purified using the Wizard SV gel and PCR Clean-Up System kit as described in item 4.3.2.2.7 and sent for nucleotide sequencing.

3.3.4 – RESULTS

Once the total RNA of the *C. canephora* seeds was extracted, it was subjected to 1% agarose gel electrophoresis to check its quality and integrity. The electrophoretic profile of the extracted total RNA can be observed in Figure 1, which shows that intact, high-quality (free of contamination) total RNA was obtained. The two bands visualized correspond to the major subunits of ribosomal RNA (arrows).

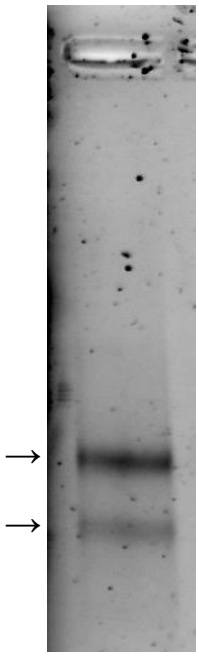


Figure 1 - Electrophoretic visualization on agarose gel (1%) of total RNA extracted from *C. canephora* seeds. Negative image of gel stained with GelRed™. The arrows indicate the major subunits of ribosomal RNA.

From the total RNA extracted, two reverse transcription reactions were performed using two different primers to obtain two cDNAs that were called cDNAr and cDNAp, which served as template for the amplification of the DNA fragments coding for the *Cc*-LTP and *Cc* -GRP from *C. canephora* seeds.

3.3.4.1 – Molecular Cloning of *Cc*-GRP

Various PCR conditions were attained using different temperatures and primer concentrations (iGRP/oligo dT18); all conditions were tested with both cDNAr and cDNAp.

For the first PCR, the iGRP/oligo dT18 primers were used at a 1:1 ratio and 12 reactions were assembled: one blank (without template DNA) and one at each temperature for each cDNA. The 1% agarose gel electrophoretic profile is shown in figure 2.

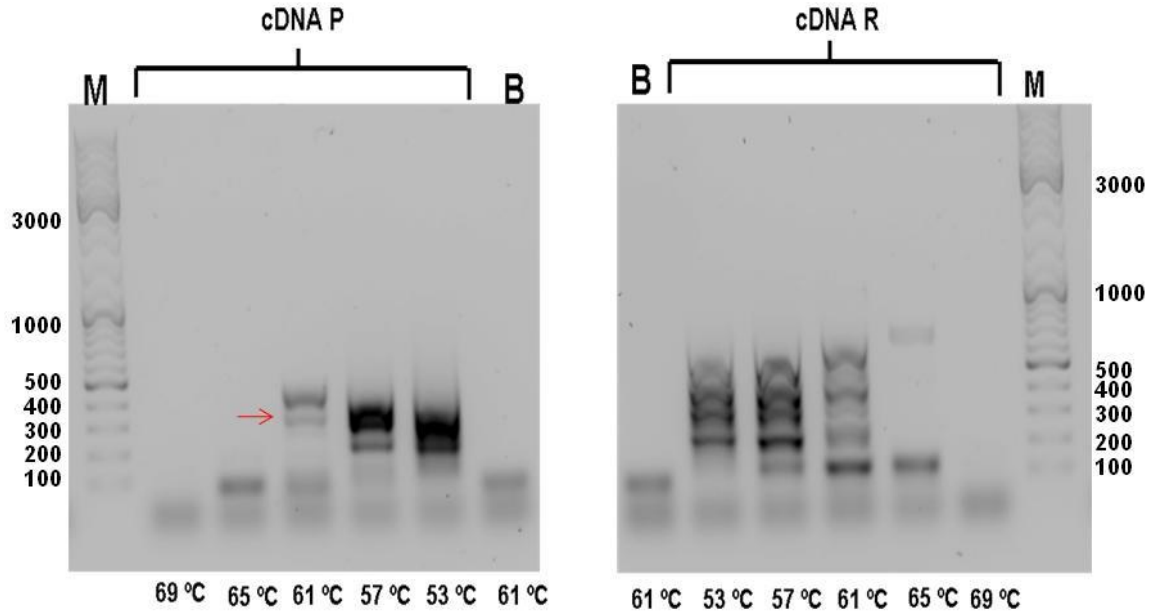


Figure 2 – Electrophoretic visualization on 1% agarose gel of samples obtained from the PCR using the iGRP/oligo dT18 primers at a 1:1 ratio, at different temperatures, using cDNAr and cDNAp as templates. M, molecular weight standard in bp. B, blank of the reaction from which the cDNA (template) was excluded. Negative image of gel stained with GelRed™. The red arrow indicates the fragment of interest that was cloned.

A second PCR was run, but this time iGRP/oligo dT18 primers were used at the ratios of 1:2 and 2:1. Twenty-four reactions were assembled: one blank (without cDNA (template)) and one at each temperature for each cDNA in both proportions. The 1% agarose gel electrophoretic profile is shown in figure 3 for the amplified fragments which had cDNAr as template and in figure 4 for the fragments whose template was cDNAp.

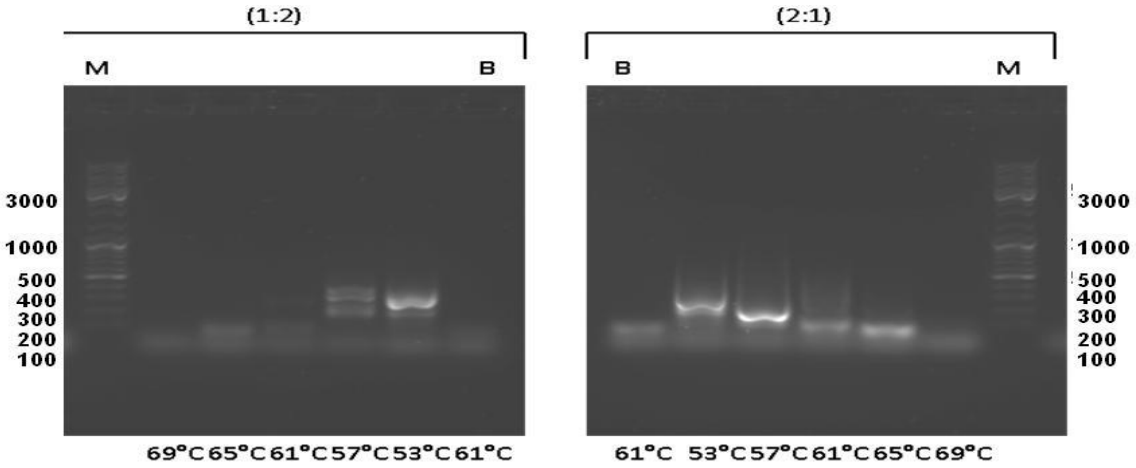
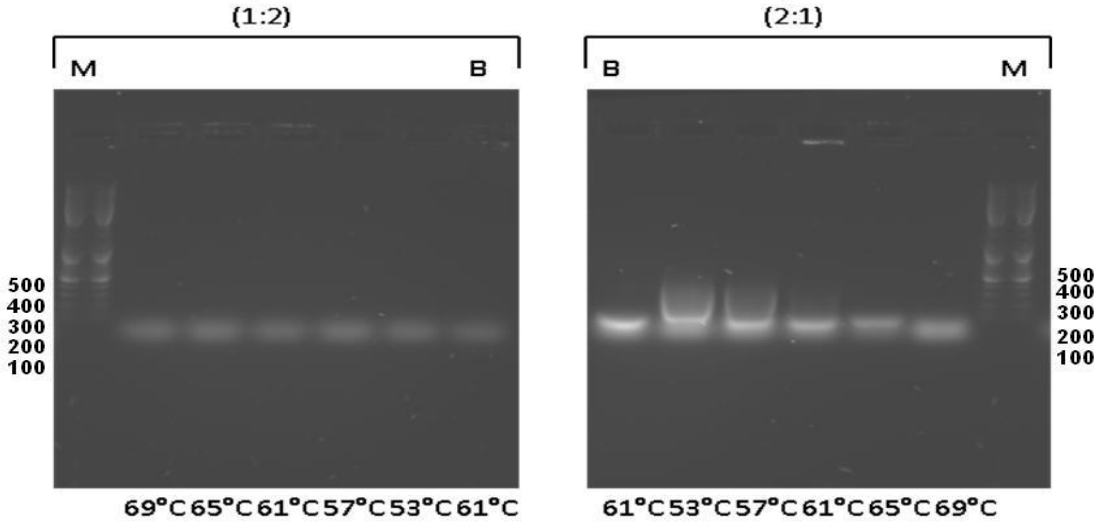


Figure 3 – Electrophoretic visualization on 1% agarose gel of samples obtained from PCR using iGRP/oligo dT18 primers at the ratios of 1:2 and 2:1, at different temperatures, using cDNAr as template. M, molecular weight standard in bp. B, blank of the reaction from which the template DNA was excluded. Image of gel stained with GelRed™.



.**Figure 4 -** Electrophoretic visualization on 1% agarose gel of samples obtained from PCR using iGRP/oligo dT18 primers at 1:2 and 2:1 ratios, at different temperatures, using cDNAp as template. M, molecular weight standard in bp. B, blank of the reaction from which the template DNA was excluded. Image of gel stained with GelRed™.

After analyzing the electrophoretic profile of all the PCR products at the different ratios of primers, temperatures and cDNAs, the reaction that had cDNAp as template was chosen, at the iGRP/oligo dT18 ratio of 1:1 and at the temperature of 61 °C. This condition was chosen because it had a well-defined

band around 400 bp (represented by the red arrow in figure 2), which would be the estimated size for the GRP, since the natural *Cc*-GRP is approximately 7 kDa. A new PCR under these chosen conditions was repeated in a six-fold volume for the accumulation of the fragment of interest (Figure 5), which was cut from the gel and stored in a freezer at –20 °C and then purified by the Wizard SV gel and PCR Clean-Up System kit (Promega©).

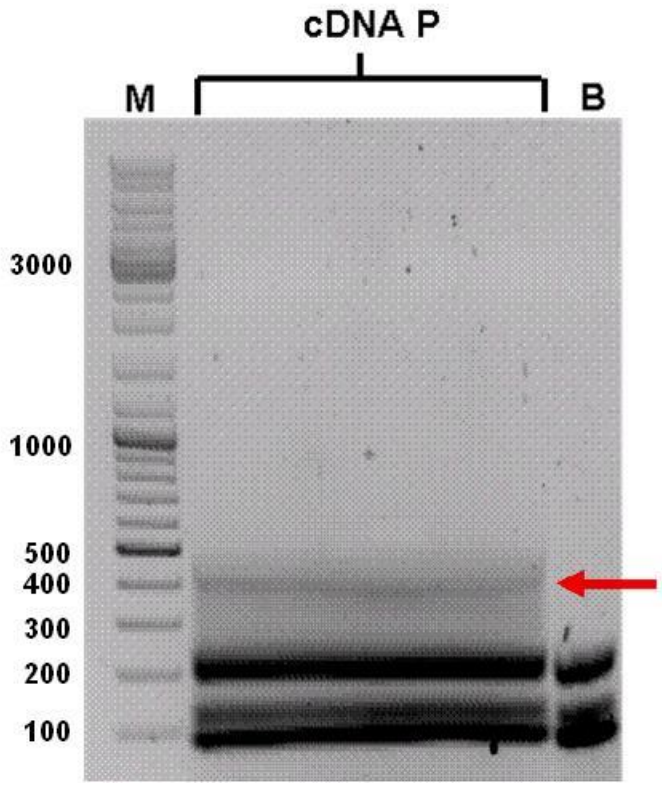


Figure 5 – Electrophoretic visualization on 1% agarose gel of samples obtained from the PCR using the iGRP/oligo dT18 primers at a 1:1 ratio, at the temperature of 61 °C, using cDNAp as template. M, molecular weight standard in bp. B, blank of the reaction from which the template DNA was excluded. Negative image of gel stained with GelRed™. The red arrow indicates the fragment of interest that was cloned.

3.3.4.2 – Transformation of competent *E. coli* cells (XL-10 strain) with the pJET-GRP construct

Once purified, the fragment of interest was ligated to the pJET 1.2/blunt vector. The pJET-GRP construct was then used for the transformation of *E. coli* competent cells (XL-10 strain). After transformation, different colonies were collected and subjected to analysis of positive clones by extraction and digestion of the restriction enzyme Bgl II vectors, which revealed the cloning of the fragment

of interest. After digestion, a fragment of approximately 400 bp corresponding to the fragment amplified with the iGRP primer was released (Figure 6). The best conditions were chosen, purified with the Wizard SV and Clean-up System kit (Promega©) and sent for nucleotide sequencing, but to date we have not obtained a quality sequence.

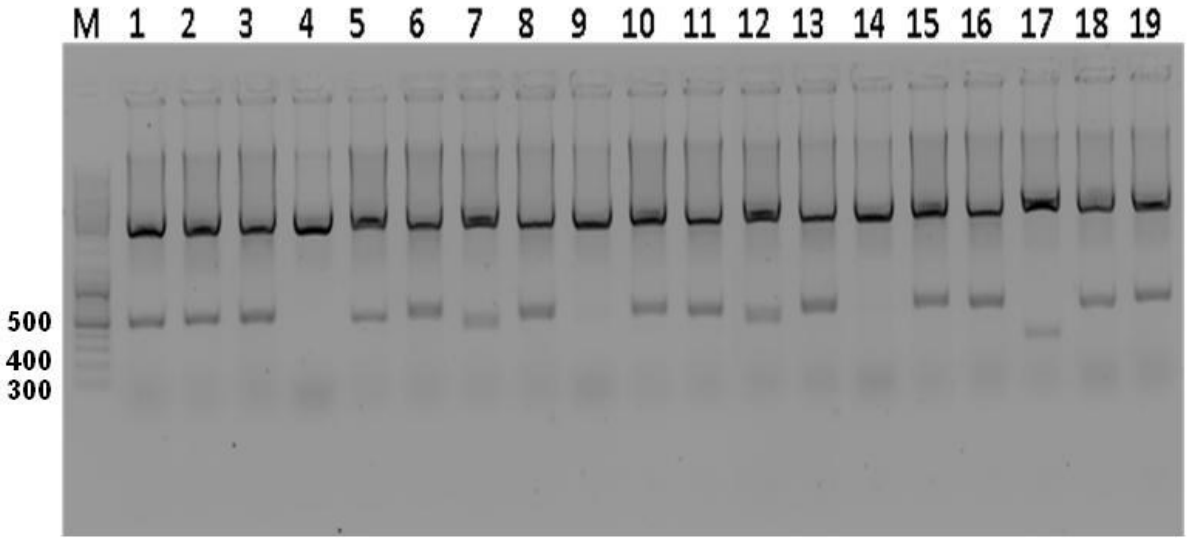


Figure 6 – Electrophoretic visualization on 1% agarose gel of samples obtained as a product of digestion of the pJET-GRP vector extracted from bacterial cells of the XL-10 strain with restriction enzyme Bgl II. (M) Molecular weight standard in bp. (1 to 19) result of the plasmidial extraction of the different chosen colonies. Negative image of gel stained with GelRed™.

3.3.4.3 – Molecular Cloning of *Cc*-LTP

For the cloning of *Cc*-LTP, only cDNAp was used as template, since it presented better results for the cloning of *Cc*-GRP.

Different PCR reactions were assembled using different temperatures and concentrations of iLTP and oligo dT18 primers. For the first PCR primers, iLTP and oligo dT18 were used at the ratios of 1:1, 1:2 and 2:1. Eighteen reactions were assembled: one blank (without template DNA) and one at each temperature for each ratio. The electrophoretic profile is shown in figure 7, where we can observe the amplification of a fragment of approximately 500 bp in all conditions tested.

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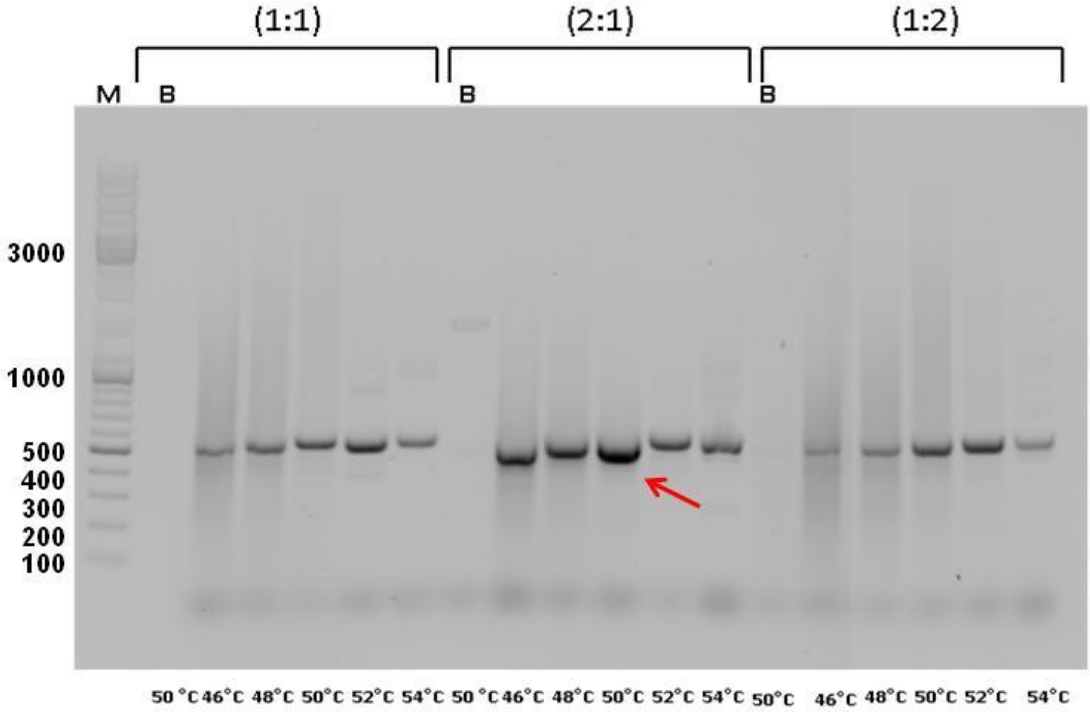

Figure 7 – Electrophoretic visualization on 1% agarose gel of samples obtained from the PCR using the iLTP/oligo dT18 primers at 1:1, 1:2 and 2:1 ratios, at different temperatures, using cDNAp as template. M, molecular weight standard (in bp). B, blank of the reaction from which the template DNA was excluded. Negative image of gel stained with GelRed™. The red arrow indicates the fragment of interest that was cloned.

After we analyzed the electrophoretic profile of all PCR products in the different proportions of primers and temperatures, we chose the reaction at the iLTP/oligo dT ratio of 18 2:1 at the temperature of 50 °C. This condition was chosen because it had a well-defined band around 500 bp (represented by the red arrow in figure 7), which would be the estimated size for LTP, since the natural *Cc*-LTP is approximately 9 kDa. A new PCR under these chosen conditions was repeated in a six-fold volume for the accumulation of the fragment of interest (Figure 8), which was cut from the gel, stored in a freezer at –20 °C and then purified using the Wizard SV gel and PCR Clean-Up System kit (Promega©).

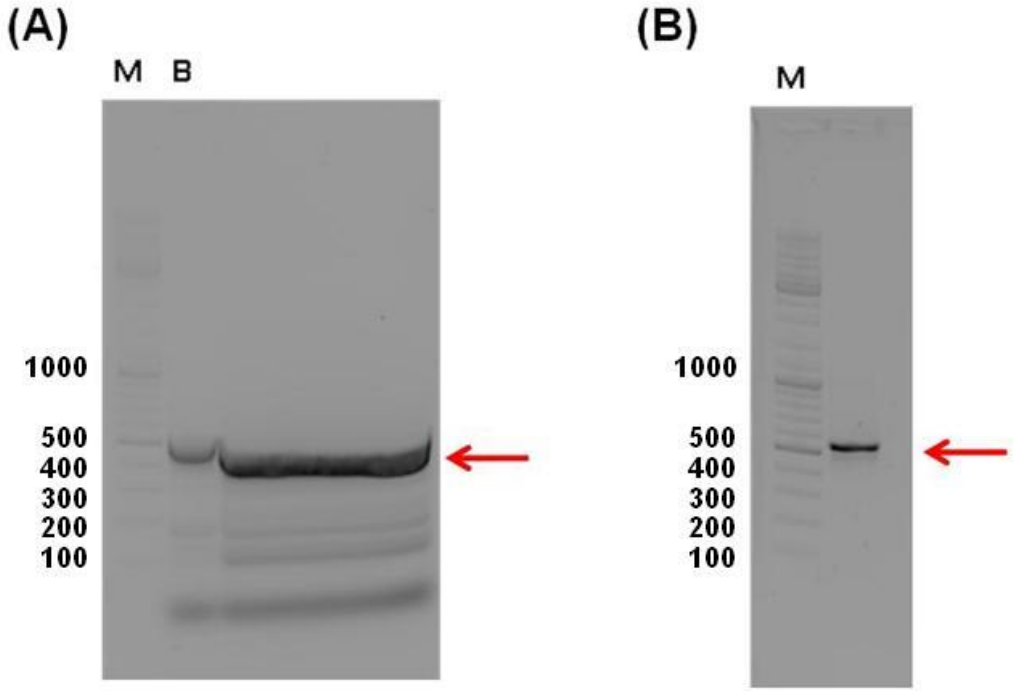

Figure 8 - (A) Electrophoretic visualization on 1% agarose gel of samples obtained from the PCR using the iLTP/oligo dT18 primers at a 2:1 ratio, at 50 °C, using cDNAp as template. (B) Purified fragment of approximately 500 bp. M, molecular weight standard in bp. B, blank of the reaction from which the template DNA was excluded. Negative images of gel stained with GelRed™. The red arrows indicate the fragment of interest prior to purification (A) and after purification (B), which was cloned

Once purified, the fragment of interest was ligated to the pJET 1.2/blunt vector. The pJET-LTP construct was then used for the transformation of *E. coli* competent cells (XL-10 strain).

After transformation, different colonies were collected and subjected to analysis of positive clones by extraction and digestion of the Bgl II restriction enzyme vectors, which revealed the cloning of the fragment of interest. After digestion, a fragment of approximately 500 bp corresponding to the fragment amplified with the iLTP primer was released. The best conditions were chosen, purified with the Wizard SVl and Clean-up System kit (Promega©) and sent for nucleotide sequencing; however, the sequence we obtained did not show homology with LTPs.

3.3.5 – DISCUSSION

Despite the great structural variability and difficulty of production on a large scale, with the advances in recombinant DNA technology, AMPs have been increasingly used in various sectors such as food, agribusiness, biotechnology, pharmaceutics and even cosmetics (Parachin et al., 2012).

In recent years, countless peptides have been cloned and expressed heterologously. Researchers from our group isolated and expressed *E. coli* heterologously in two common-bean (*Phaseolus vulgaris* L) (Mello et al., 2014) and string bean (*Vigna unguiculata* L) (Santos et al., 2010) defensins. The recombinant defensin PvD1r, cloned from a natural defensin isolated from common-bean seeds, retained all its antimicrobial activity when compared with its natural counterpart, with the ability to inhibit the growth of yeast *C. albicans* (Mello et al., 2014). The recombinant defensin of string bean seeds as well as its natural homologue were able to inhibit the activity of the alpha-amylase enzyme of the insect *Callosobruchus maculatus* (Santos et al., 2010)

Although the number of expressed recombinant AMPs is increasing, overexpressing a eukaryotic protein in a prokaryotic system is a task that requires specific care and strategies. Although bacterial systems appear to be useful for the expression of the eukaryotic protein, some limitations must be overcome, such as improper protein folding (the formation of its disulfide bonds may not occur), preferential codon use (protein may not even reach protein degradation from the host cell and the toxicity caused by the heterologous protein to the host (Jana and Deb, 2005; Tavares et al., 2012).In this work two fragments corresponding to the coffee pepetídeos, *Cc*-LTP2 and *Cc*-GRP were cloned in pJET1.2/blunt vector and the products of these clones were sent to nucleotide sequencing to confirm the sequence of the cloned peptides, to proceed the heterologous superexpression process in *E. coli*.

3.3.6 – CONCLUSION

The *Cc*-LTP2 and *Cc*-GRP peptides were cloned, but with the employed strategy, it was not possible to obtain the sequence of the cloned fragments.

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