



# Purification, biochemical characterization, and antimicrobial activity of a new lipid transfer protein from *Coffea canephora* seeds

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**ABSTRACT.** Coffee, an agronomical crop of great economic

importance, is also among the most commonly traded commodities in worldwide markets. Antimicrobial peptides, which play a role in plant defense, have been identified and isolated particularly from seeds. We isolated and immunolocalized *Cc*-LTP2, a new lipid transfer protein (LTP) 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, which we named *Cc*-LTP2, was then subjected to amino acid sequencing. The analyses showed that it was similar to LTPs isolated from various plants. 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 inhibit the development of the phytopathogenic fungi *Colletotrichum lindemuthianum*, *Colletotrichum gloeosporioides*, *Fusarium solani*, *Fusarium lateritium*, and *Colletotrichum* sp, but did inhibit *X. euvesicatoria*. *Cc*-LTP2 also increased membrane permeability and induced endogenous production of reactive oxygen species in all the fungi tested.

**Key words:** Antimicrobial peptides; Phytopathogenic fungi; Chromatography; Membrane permeabilization; Reactive oxygen species

## 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*, *Colletotrichum gloeosporioides*, *Colletotrichum truncatum*, *Colletotrichum gossypii* var. *cephalosporioides*, frog-eye 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  $\alpha$ -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  $\alpha$ -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);  $\beta$ -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 (Zottich et al., 2011, 2013), have been isolated from coffee. In the present study, we report the characterization, immunolocalization, and antimicrobial activity of a new LTP called *Cc*-LTP2, which can be isolated from *C. canephora* seeds.

## MATERIAL AND METHODS

### 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.

## Microorganisms

Pathogenic fungi *C. lindemuthianum*, *C. gloeosporioides*, *Fusarium solani*, and *Fusarium lateritium* were cultured in Sabouraud agar and preserved in Laboratório de Fisiologia e Bioquímica de Microrganismo, at Universidade Estadual do Norte Fluminense Darcy Ribeiro (UENF), in 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 Laboratório de Melhoramento Genético Vegetal, at UENF in Campos dos Goytacazes, RJ, Brazil.

## 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)-Sephacrose 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 x 4.6, Shimadzu) [attached to a C8 pre-column (20 x 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 x 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.

## 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).

## Amino acid 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  $\mu$ L water, 400  $\mu$ L methanol with vortexing, and 100  $\mu$ L 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 x 4.6 mm) under isocratic conditions, according to the manufacturer instructions. Searches for sequence similarity were carried out using the BLASTp program.

### 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, USA) attached to a light microscope (Axioplan, Zeiss, Oberkochen, Germany).

### 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 x 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- $\mu$ m 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) attached to a light microscope (Axioplan, Zeiss).

### 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- $\mu$ L microplates in the presence of the *Cc*-LTP2 (200 and 400  $\mu$ g/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.

### **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  $\mu$ L) of the suspensions that had been grown in the presence of *Cc*-LTP2 (200  $\mu$ g/mL) were incubated with 0.2  $\mu$ M 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.

### **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  $\mu$ g/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  $\mu$ M, 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).

### **Effect of proteins on bacterial growth**

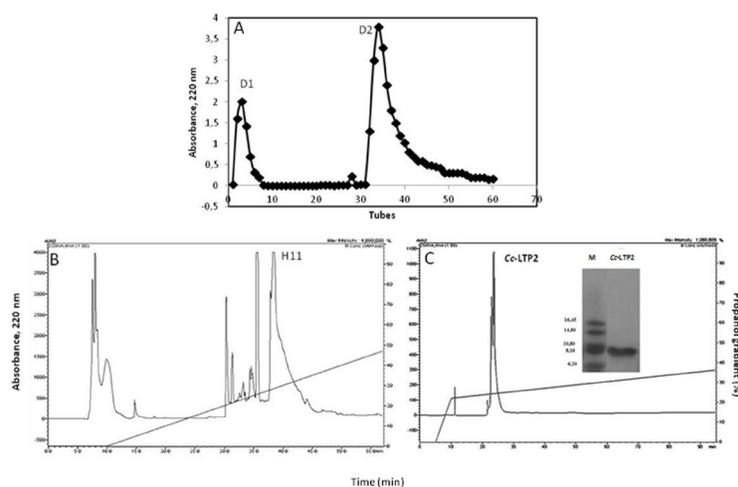
The method described by Filho and Romero (2009) with modifications was employed to evaluate the effects of *Cc*-LTP2 on the growth of the bacterium *X. euvesicatoria*. To evaluate the formation of inhibition zones at different times and concentrations, bacteria were grown in liquid DYGS medium (30 mL) and kept in a shaker (Tecnal TE420) under agitation for 24 h, at 28°C and 100 rpm. A bacteria-enriched aliquot (100  $\mu$ L) was then transferred to an Erlenmeyer flask

containing 0.8% semi-solid DYGS (30 mL), and heated to 40°C. The DYGS was poured onto Petri dishes. After medium solidification, two paper disks (approximately 6 mm in diameter) were loaded, one with 5  $\mu$ L (75  $\mu$ g/mL) *Cc*-LTP2 and the other with 5  $\mu$ L water (the control). The plates were evaluated for 48 h using a Stainless Hardened<sup>®</sup> digital caliper.

## RESULTS

### 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-Sephacrose resin, from which two fractions termed D1 and D2 were obtained (Figure 1A). 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 1B). 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 1C). The major peak was analyzed by denaturing tricine-SDS-PAGE and showed only one band (molecular weight approximately 9 kDa; Figure 1C).



**Figure 1.** Purification of *Cc*-LTP2 from *Coffea canephora* seeds. **A.** Chromatogram of the protein extract of *C. canephora* in diethylaminoethanol (DEAE)-Sephacrose 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).

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 2); in particular, there was 67% identity with *Musa acuminata* (XP009396871), 70% identity with *Solanum lycopersicum* (XP009798115), and 75% identity with *Nicotiana sylvestris* (XP009796551).

<i>Coffea canephora</i> (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
<i>Coffea canephora</i> (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		
<i>Musa acuminata</i>	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
<i>Solanum lycopersicum</i>	25	I	T	C	G	Q	V	D	A	N	L	A	P	C	V	P	F	L	T	-	Q	G	44	70	75
<i>Nicotiana sylvestris</i>	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 2.** 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: *C. canephora* (Zottich et al., 2011); *Musa acuminata* (XP009396871); *Solanum lycopersicum* (XP009798115); *Nicotiana sylvestris* (XP009796551). I = percentage of identical amino acids, P = 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.

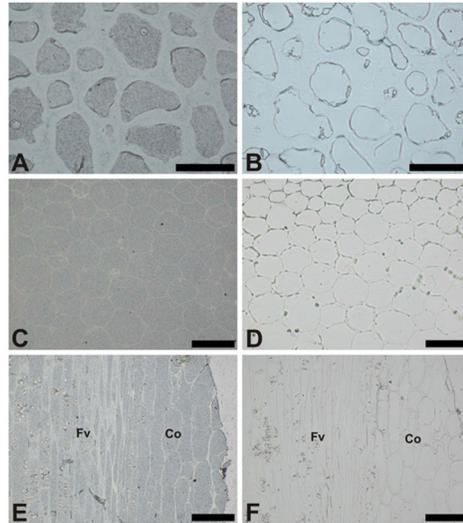
## Immunolocalization of Cc-LTP2

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 3A) as compared with the control (Figure 3B). It is noteworthy that the presence of LTPs from *C. canephora* was additionally detected in the vacuoles (Figure 3C) but not in the control of the root radicle sections (Figure 3D), and was apparent in the vascular bundle of the hypocotyl (Figure 3E) but not in the control root hair zone (Figure 3F).

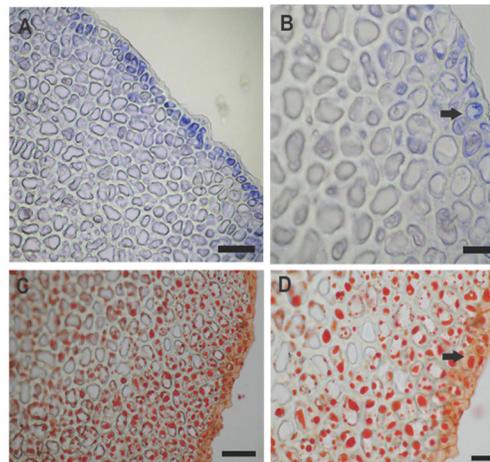
Proteins with a positive reaction (blue) occurred in all tissues with intracellular localization, and were more evident in tests on fibrous tissue (Figure 4A and B), which are known to be rich in oblique scores. The lipid substances present in all tissues analyzed by histochemistry are evident in the 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 4C and D).

## Antimicrobial activity against phytopathogenic fungi

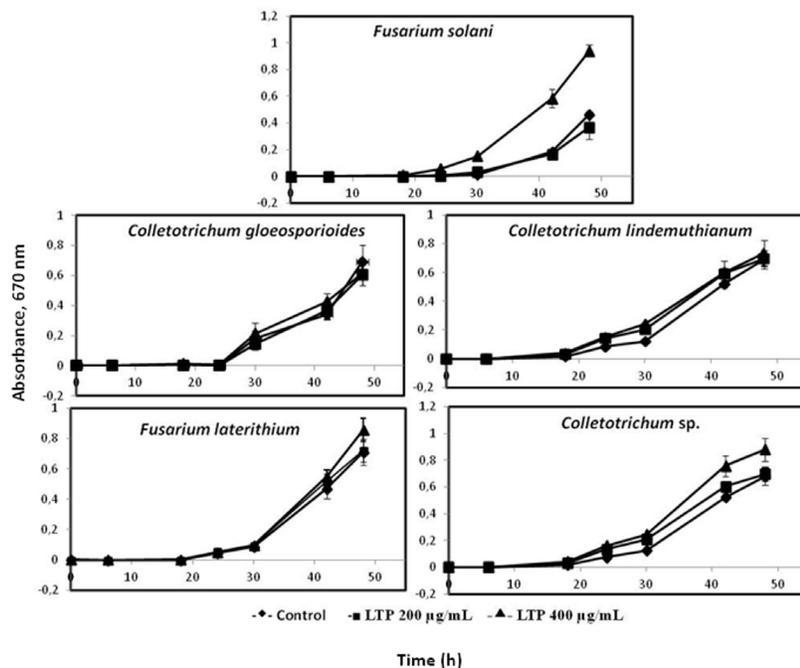
To analyze a possible antimicrobial role of Cc-LTP2, isolated in this work, antifungal assays were carried out with the pathogenic fungi *C. lindemuthianum*, *C. gloeosporioides*, *Colletotrichum* spp, *F. solani*, and *F. lateritium*, which are pathogens of several very important economic crops. The 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 5, the two concentrations of Cc-LTP2 tested were not able to inhibit the growth of fungi when compared with the control curves.



**Figure 3.** 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  $\mu\text{m}$ ; **C** and **D** 50  $\mu\text{m}$ . The results of immunolocalization are representative of one triplicate experiment.



**Figure 4.** Histochemical characterization of the seed endosperm in *Coffea 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.** lipid 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  $\mu\text{m}$ ; **C, D,** 20  $\mu\text{m}$ . The results of histochemistry are representative of one triplicate experiment.

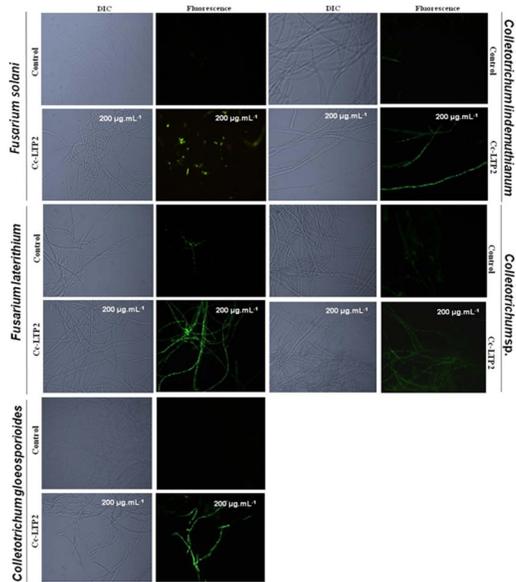


**Figure 5.** Growth inhibition assay of *Fusarium solani*, *Fusarium lateritium*, *Colletotrichum* spp *Colletotrichum gloeosporioides*, and *Colletotrichum lindemuthianum* in the presence of Cc-LTP2. The growth was observed for 48 h. Diamonds = Control; squares = 200 µg/mL; triangles = 400 µg/mL. Values are reported as means ± SD of triplicates. The results of growth inhibition assays are representative of one triplicate experiment.

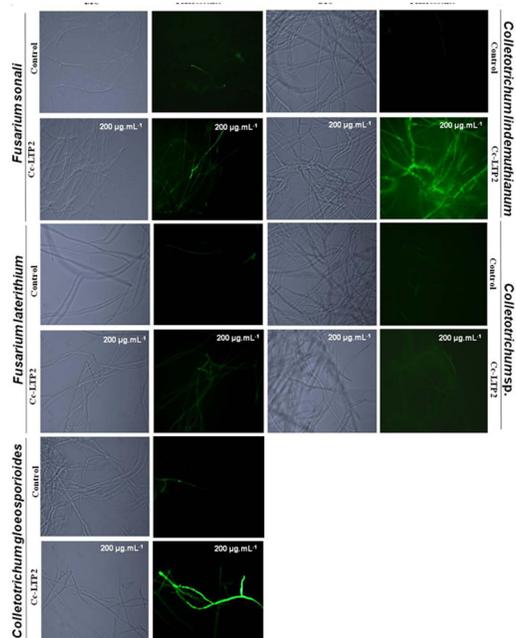
## Membrane permeabilization and ROS induction

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 6, 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 study 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 7).



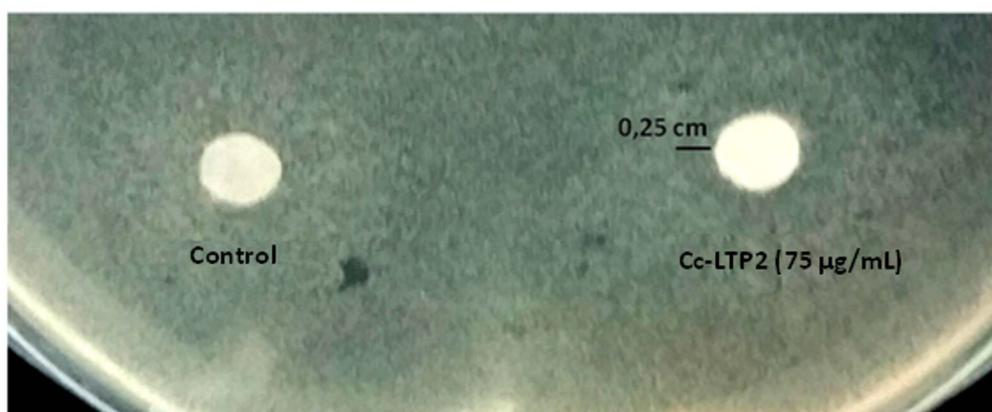
**Figure 6.** Membrane permeabilization assays of cells of different filamentous fungi previously incubated with *Cc*-LTP2 at 200 µg/mL for 24 h. Cells were treated with SYTOX green for 30 min to evaluate membrane permeabilization. 400X magnification. The results of membrane permeabilization are representative of one triplicate experiment.



**Figure 7.** 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.

### 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  $\mu\text{g}/\text{mL}$ , an inhibition zone of 0.25 mm diameter was formed, which was not observed in the control treatment (Figure 8). The LTP Ace-AMP1, isolated from onion seeds, was active against Gram-positive bacteria but apparently not toxic to Gram-negative bacteria.



**Figure 8.** Antibiogram of *Xanthomonas euvesicatoria* in the presence of 75  $\mu\text{g}/\text{mL}$  *Cc*-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.

### 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 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 annuum* 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 *Candida 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  $\alpha$ -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 *Saccharomyces cerevisiae* (Zottich et al., 2011). Another LTP called *Ca*-LTP1, isolated from the seeds of *C. annuum*, 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 *C. 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).

## 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.

## Conflicts of interest

The authors declare no conflict of interest.

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