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RESEARCH REPORT

Identification of enzyme inhibitors and antimicrobial activities from *Capsicum annuum* L. protein extracts against *Colletotrichum scovillei*

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Abstract
Diseases caused by phytopathogenic microorganisms are difficult to control and can affect plants at different stages of their development. Several resistance genes and antimicrobial peptides (AMPs) have been identified and related to the resistance process of *Capsicum*. In recent years, studies have shown that peppers, especially the accession UENF1381, present resistance against phytopathogenic microorganisms. This work aimed identify and characterize AMPs of the leaf and root from *Capsicum annuum* L. UENF1381 and to analyze the inhibitory activity of the AMPs on different enzyme families and valuated the inhibitory activity on *Colletotrichum scovillei.* Initially, self-fertilized pepper seeds were cultured for 45 days in glass flasks containing ½-MS medium. Then, leaves were inoculated with inoculum of *Xanthomonas euvesicatoria* (10^8 CFU mL^{-1}) or water (control). Leaf and root samples were collected at 12, 24 and 48 h after inoculation to extraction. The activity of extracts on different enzyme families was analyzed by incubating the enzymes (trypsin, α-amylase and β-1,3-glucanase) with 30 µg mL^{-1} of the obtained extracts. The effect of extracts on fungi was also tested. It was identified that protein of leaf and root extracts from *C. annuum* L. UENF1381 presented a majority of bands with a low molecular mass (6–14 kDa). We observed that all leaf and root extracts significantly inhibited trypsin and α-amylase activity and able to significantly inhibit *C. scovillei* growth. With this work we hope to contribute to the use of peptides as potential molecules in microbial control and in the development of new *Capsicum* cultivars resistant to microorganisms.

Keywords Pepper · Plant defense · Phytopathogens · Antimicrobial peptides

1 Introduction

Capsicum plants are part of the Solanaceae family, of which include sweet and chili peppers that have important nutritional and economic value (Moscone et al. 2007). Despite the increase in production of *Capsicum* plants, these crops are facing several problems, including diseases caused by phytopathogenic microorganisms (Carrizo García et al. 2016). Species of *Colletotrichum* and that *Xanthomonas* are important plant pathogens that cause disease and large losses in the production of *Capsicum* and other food crops (Schwartz et al. 2015; Suwannarat et al. 2017).

Capsicum annuum L. pepper accession UENF1381 is described as resistant to bacterial spot caused by *Xanthomonas* spp., anthracnose caused by *Colletotrichum* spp., and pepper yellow mosaic virus (PepYMV) (Bento et al.

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2017; Riva et al. 2004). In *C. annuum*, trypsin and α-amylase inhibitors have been identified. These inhibitors are mainly related to plant defence against insects and other phytopathogenic microorganisms (Antcheva et al. 2001; Srivastaval and Kumar 2013).

In Solanaceae plants, such as *Capsicum,* several peptide serine proteinase inhibitors and with β-1,3-glucanase activity have been characterized. These peptides are part of a diverse family of molecules responsible for maintaining the plant's physiological homeostasis, as they allow the delay of the disease or the penetration of phytopathogens (Bertoldo and Mazaro 2018; Hartl et al. 2010).

Plants produce also a range of antimicrobial peptides (AMPs) against their pathogens; these are components of the defense mechanism of plants and can be constitutively expressed or induced after pathogen infection (Campos et al. 2018; Egorov et al. 2005). AMPs are characterized by their small size (10–50 amino acid residues), most shows α-amphipathic helix conformation, they are cationic with two or more positive charges and have large percentage of hydrophobic amino acids (40–60%) (Strempel et al. 2014). It is observed that the presence of positively charged amino acids (lysine and arginine) and hydrophobic are characteristics important for antimicrobial and hemolytic activity because allows the interaction and insertion of peptides (positively charged) in the membranes negatively charged leading to the formation of pores and the loss of cellular homeostasis (Jiang et al. 2008; Lee et al. 2016; Oñate-Garzón et al. 2017).

In UENF1381, specifically, some AMPs have been identified in different parts of the plant, mainly seeds and fruits such as defensins, thionin and LTPs (Maracahipes et al. 2019; Santos et al. 2017; Silva et al. 2017; Taveira et al. 2018).

In this work, we identify and characterize AMPs of leaf and root from *C. annuum* L. UENF1381 and analyze the inhibitory activity of the AMPs on different enzyme families as well as the action of extracts on the growth of *Colletotrichum scovillei*. Thus, from the results obtained, we intend to identify molecular candidates capable of inhibiting the action of phytopathogenic microorganisms, which would be an important strategy for plant disease control.

2 Materials and methods

2.1 Plant material

Seeds from *Capsicum annuum* L. (UENF1381 accession), chili pepper, were provided by the Laboratório de Melhoramento Genético Vegetal (LMGV), Centro de Ciências e Tecnologias Agropecuárias (CCTA), Universidade Estadual do Norte Fluminense Darcy Ribeiro (UENF), Rio de Janeiro (RJ), Brazil.

2.2 Microorganisms

The microorganisms used in this research were supplied by the LMGV, UENF, RJ, Brazil. *Xanthomonas* euvesicatoria, isolate 4135, was cultured in DYGS medium (2 g L^{-1} glucose, 1.5 g L^{-1} bacteriological peptone, 1.5 g L^{-1} yeast extract, 0.5 g L^{-1} K_2HPO, 1.5 g L^{-1} glutamic acid, 0.5 g L^{-1} $MgSO_4$ and 18 g L^{-1} agar, pH adjusted to 7) at 28 °C. The phytopathogenic fungus *Colletotrichum scovillei* isolate 8.1 was cultured in BDA medium at 30 °C. These cultures were kept in the Laboratório de Fisiologia e Bioquímica de Microrganismos (LFBM), Centro de Biociências e Biotecnologia (CBB), UENF, RJ, Brazil.

2.3 Insect larvae

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

2.4 Plant cultivation

Capsicum annuum L. UENF1381 seeds were planted in a greenhouse and irrigated once a day. Cultivation was carried at 28 °C and 80% relative air humidity with photoperiod of 16 h. During the flowering periods the flowers were protected with paper bags. Due the possibility of cross-fertilization in plants of *Capsicum* self-fertilized seeds were used in this work. The seeds were initially washed in commercial liquid-detergent and rinsed in running water. After the seeds were immersed in 70% alcohol for 1 min and in 0.7% NaClO with three drops of Tween 20 added in an 80 ml volume for 15 min in an aseptic environment. Subsequently, seeds were rinsed for three times in deionized and autoclaved water.

The germination rate was assessed in the dark for 15 cultivation days in Petri dishes (90 × 15 mm) containing medium composed of half of the MS mineral (½ MS), White vitamin complex (Murashige and Skoog 1962), and 100 mg L^{-1} myo-inositol, without sucrose, with pH adjusted to 5.7 ± 0.1 and solidified in 2 g L^{-1} Phytagel (Sigma). The plants were transplanted into 350 ml bottles (125 mm × 60 mm) with the same medium with the addition of 20 g L^{-1} sucrose and maintained for approximately 30 days in a cultivation room in Laboratório de Fitotecnia, Setor de Horticultura, UENF, RJ, Brazil, at 27 ± 2 °C for 30 days under a 16 h light: dark photoperiod with irradiance of 50 µmol m^{-2} S^{-1} provided by

OSRAM® day-light fluorescence lamps (Walter et al. 2018). In vitro cultivation was used in this work to evaluate plants inoculated with *X. euvesicatoria* under controlled conditions and free from pathogen attack, unlike conditions observed under greenhouse conditions.

2.4.1 Plant infection

X. euvesicatoria was grown in DYGS culture medium for 36 h at 28 °C, and bacterial colonies were suspended in sterile water and adjusted to a volume of 10^8 CFU via a spectrophotometer using a wavelength of 600 nm. The bacterial inoculum was tested on a susceptible cultivar (Ikeda). Inoculation was performed after 45 days, using the method of suspended bacterial infiltration at the mesophyll (Bongiolo Neto et al. 1986; Costa et al. 2002; Riva et al. 2004). Uninoculated plants and plants inoculated with water were used as controls. The plants were collected at 12, 24 and 48 h after infection and were dissected into leaves and roots and were separately stored in aluminium foil bags, immersed in liquid nitrogen and immediately transferred to an ultra-freezer (− 70 °C) until subsequent use for protein extraction.

2.5 Extraction and characterization of proteins

2.5.1 Extraction of leaves and roots from *C. annuum*

Plant tissue extractions (leaves and roots) were performed according to Granier (1988). In Brief total soluble protein content was determined by Bicinchoninate (BCA) method and bovine serum albumin (BSA) was used as a protein standard. The samples were named as described in Table 1.

2.5.2 Gel electrophoresis

Sodium dodecyl sulfate (SDS)-tricine gel electrophoresis was performed according to Schägger and Von Jagow (1987). An ultra-low ranger molecular weight marker was used (Sigma-Aldrich).

2.5.3 Amino acid sequencing by mass spectrometry analysis

The sequencing was performed in partnership with Laboratório de Bioquímica Marinha (BioMar-Lab), Departamento de Engenharia de Pesca, Universidade Federal do Ceará (UFC), Ceará, Brazil. For sequencing of the peptides *L1* and *R1* present in the LC_{48} and RC_{48} extracts, respectively, after separation by gel electrophoresis, the protein bands of interest were extracted from the gel. Then, the peptides were digested by trypsin according Shevchenko et al. (2006) and subjected to mass spectrometry evaluation. The instrument used was a hybrid mass spectrometer (ESI-Q-ToF) (Synapt HDMS, Waters Corp, MA, USA). The machine parameters were adjusted as described by Carneiro et al. (2013). The sequences of the peptides were compared with the sequences reported in NCBI-BLAST amino acid databases and were presented for automatic alignment using the Clustal Omega program online.

2.6 Assay for residual trypsin activity

Proteinase inhibitory activity was determined by measuring the residual hydrolytic activity of porcine trypsin towards the substrates N-benzoyl-DL-arginyl-p-nitroanilide (BAPNA) at

Table 1 Samples (leaves and roots) derived from control (uninoculated and water-inoculated) or inoculated *C. annuum* L

Genotypes	Feature	Samples	Identification
UENF 1381	Resistant to bacterial spot, anthracnose and pepper yellow mosaic virus (PepYMV)		
1		Leaf control not inoculated	L_{NC}
2		Leaf control inoculated (12)	LC_{12}
3		Leaf control inoculated (24)	LC_{24}
4		Leaf control inoculated (48)	LC_{48}
5		Inoculated leaf (12)	IL_{12}
6		Inoculated leaf (24)	IL_{24}
7		Inoculated leaf (48)	IL_{48}
8		Root control not inoculated	R_{NC}
9		Root control inoculated (12)	RC_{12}
10		Root control inoculated (24)	RC_{24}
11		Root control inoculated (48)	RC_{48}
12		Inoculated roots (12)	IR_{12}
13		Inoculated roots (24)	IR_{24}
14		Inoculated roots (48)	IR_{48}

UENF1381with the bacterium *Xanthomonas euvesicatoria* at intervals of 12, 24 and 48 h after inoculation

pH 8.0 after pre-incubation with 30 µg mL^{-1} of different extracts from *C. annuum* L. UENF1381 (Table 1).

The proteolytic activity was measured using synthetic peptide derivates of *p*-nitroanilide (0.0625 Mm each) in 50 mM Tris–HCl buffer, pH 8.0, 37 °C, in a final volume of 200 µl. The reaction was interrupted by adding 100 µl of 30% acetic acid (v/v). The substrate hydrolysis was followed by measuring the absorbance of released *p*-nitroaniline by absorbance at 405 nm (Macedo et al. 2007) using a spectrophotometer (EZ Read 400).

The intestinal α-amylase activity of *Tenebrio molitor* larvae in the presence of leaf and root extracts from *C. annuum* L. UENF1381 was performed as previously described by Bernfeld (1955). Initially, the reaction mixture for determination of enzymatic activity was assayed in water at 37 °C for 30 min using 1% starch (Sigma-Aldrich Co.) as the substrate and α-amylase (2 µl). EDTA was used with positive control.

The 3,5-dinitrosalicilic acid (DNS) reagent solution was prepared by combining 30 ml of solution 1 (4.5% NaOH) + 88 ml of solution 2 (1% DNS + 25.5% potassium/sodium tartrate) + 10 ml of solution 3 (2.2 ml of 10% NaOH + 1 g of phenol + water q.s.p. 10 ml).

After the addition of DNS, the reaction was stopped by heating to 100 °C for 5 min, 400 µl of water was added, and the absorbance at 540 nm was monitored (UVVIS-1203, Shimadzu). One unit of α-amylase activity was defined as the quantity of enzyme that increased the absorbance at 540 nm by 0.1 absorbance units over 30 min. α-amylase inhibitory assays with the extracts were carried out as described for the determination of α-amylase activity in units, with leaf and root extracts (30 µg mL^{-1}). All inhibition assays were performed in triplicate.

2.7 Determination of β-1,3-glucanase activity

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

2.8 Effect of total extracts on fungal growth

To assay the effect of extracts on *Colletotrichum scovillei* growth, the conidia (20.000 cells mL^{-1} in 1 ml of saline solution) were incubated at 30 °C in microplates in the presence of the different extracts at 100 and 200 µg mL^{-1}. Optical readings at 620 nm were taken (Plate chameleon™ V, Mikrowin program) at 48 h to evaluate fungal growth. Cell growth without the addition of extracts was also determined. The entire assay was performed in triplicate and under aseptic conditions in a laminar flow hood (Trox technic®) (Broekaert et al. 1990).

2.8.1 Effect of extracts on membrane permeabilization

Plasma membrane permeabilization was measured by SYTOX Green uptake (Thevissen et al. 1999) with some modifications. One hundred microliter aliquots of the suspensions of *C. scovillei* that had been grown in the presence of leaf and root extracts (100 µg mL^{-1}) for 24 h, period for fungus growth, were incubated with 0.2 µM SYTOX Green in 96-well microplates for 30 min at 25 °C under periodic agitation and then were further observed using a DIC microscope (Axioplan Imager.A2, Zeiss) equipped with a fluorescence filter (excitation wavelengths, 450–490 nm; emission wavelength, 500 nm). Negative (no protein extract added) controls were also run to evaluate the baseline membrane permeability. The images were viewed through software Axiovision version 4.0 (Zeiss). All fluorescence images were taken with the same exposure time.

2.8.2 Intracellular ROS induction assay

After fungal growth inhibition assays, 50 µl of these cells grown in the absence and presence of the leaf and root extracts (100 µg mL^{-1}) for 24 h were incubated with 200 µM of the fluorescent probe 2′,7′-dichlorofluorescein diacetate (H2DCFDA). After 30 min incubation at room temperature with constant shaking, the cells were analysed according to item 2.8.1 (Mello et al. 2011).

2.8.3 Mitochondrial functionality determination assay

Following the growth inhibition assay in presence of the leaf and root extracts (100 µg mL^{-1}) for 24 h, mitochondrial functionality was assessed by the fluorescent dye Rhodamine 123 (Sigma-Aldrich). The fungal cells were resuspended and incubated with 10 µg mL^{-1} Rhodamine 123 after 15 min of incubation at room temperature with constant shaking, and the cells were analysed according

to item 2.8.1 with modifications in the fluorescence filter (excitation wavelength, 506 nm; emission wavelength, 530 nm) (Taveira et al. 2018).

2.9 Statistical analysis

All data were obtained from experiments performed in triplicate and 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 Results and discussion

3.1 SDS-tricine-PAGE of leaf and root extracts from *C. annuum*

Diseases caused by phytopathogenic microorganisms affect the commercialization of important crops for food (Buttimer et al. 2017; Dias 2010). The methods traditionally adopted to minimize diseases caused by phytopathogenic microorganisms, are based on the use of pesticides in the soil, seeds and plants during their development. However, the cultivation areas of *Capsicum* have been affected by salt due to the frequent use of mandatory pesticides and fertilizers for the cultivation of this culture (Hahm et al. 2017).

Currently, research is focused on the identification of secondary plant metabolites, for example, alkaloids, glycoalkaloids, terpenoids, organic acids and alcohols, possible tools in the control of pests and microorganisms. The Solanaceae family contains several species that produce toxic substances for insects and microorganisms (Chowanski et al. 2016). With the increase in the restriction of the use of pesticides and the search for new strategies for the control of diseases in plants, AMPs have shown to be a promising strategy in the control of diseases in plants. In recent years, have been growing reports of the presence of antimicrobial peptides from various plants species including the genus *Capsicum* (Moguel-Salazar et al. 2011).

In this work, we studied the induction of proteins and peptides from *Capsicum annuum* inoculated with the bacterium *Xanthomonas euvesicatoria*. The protein profile of the leaf and root extracts from *C. annuum* was determined by SDS-tricine-PAGE. Electrophoresis revealed that protein present in leaf and root extracts are rich in low molecular weight proteins. Both extracts present major proteins above 14 kDa and the presence of proteins of approximately 6 KDa is observed (Fig. 1).

It was observed in the extracts the differentiated expression of low molecular weight bands. In leaf exctracts, a band named *L1* of approximately 6 kDa was induced in both control plants (uninoculated and water-inoculated plants) and in plants inoculated with *Xanthomonas euvesicatoria*. Although protein is present in all leaf extracts only in the

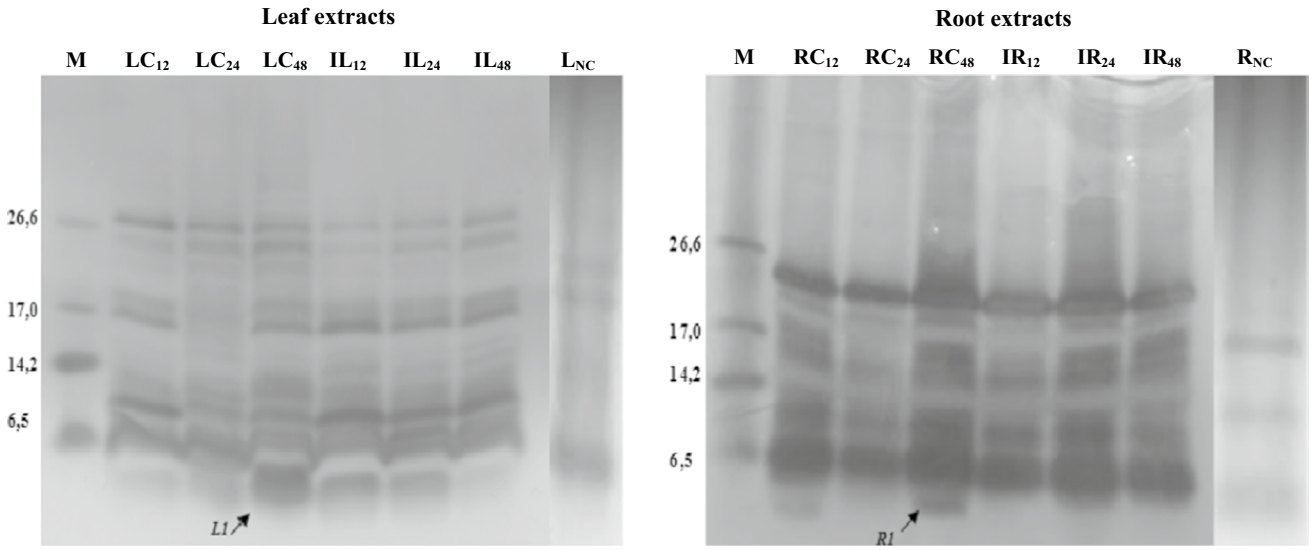

Fig. 1 Electrophoretic visualization of proteins of leaf and root extracts from *Capsicum annuum* L. UENF1381 (30 µg mL^{-1}), subjected to reverse-phase chromatography, by SDS-tricine-PAGE. L_{NC}—Leaf control not inoculated; LC_{12}—Leaf control inoculated (12); LC_{24}—Leaf control inoculated (24); LC_{48}—Leaf control inoculated (48); IL_{12}—Inoculated leaf (12); IL_{24}—Inoculated leaf (24); IL_{48}—Inoculated leaf (48); R_{NC}—Root control not inoculated; RC_{12}—Root control inoculated (12); RC_{24}—Root control inoculated (24); RC_{48}—Root control inoculated (48); IR_{12}—Inoculated roots (12); IR_{24}—Inoculated roots (24); IR_{48}—Inoculated roots (48). *L1*—non-specific-transfer protein; *R1*—ethylene-responsive proteinase inhibitor; M—low molecular mass markers (kDa). The protein bands were stained with silver nitrate

LC_{48} and IL_{12} extracts, the protein induction peak occurs. In the leaf samples inoculated with the bacteria, a decrease in protein concentration was observed (Fig. 1). In root extracts, a band named *R1* of approximately 5, 6 kDa was present only in the RC_{12} and RC_{48} extracts (Fig. 1). Pathogens express effector proteins to infect plants. These molecules are of fundamental importance for pathogen development in plant tissue. This fact suggests that proteins suppression is important for pathogen development, allowing recognition of pathogen virulence genes by plant resistance genes to establish a defence response (Kushalappa et al. 2016; Villamil et al. 2019).

3.2 Mass spectrometry analysis

L1 and *R1* bands (selected from the LC_{48} and RC_{48} extracts, respectively) had their expression suppressed in plants inoculated with *X. euvesicatoria*. The bands were submitted to mass spectrometry to identify aninoacid residues. The *L1* sequencing resulted in a fragment of 24 amino acid residues (Fig. 2a), and the *R1* sequence resulted in a fragment of 42 amino acid residues (Fig. 2b). The sequences of *L1* and *R1* were compared with the sequences reported in NCBI-BLAST amino acid databases, and the sequences were subjected to automatic alignment using the Clustal Omega program. For the alignment, only the mature proteins were used, and the percentage of identity and positive amino acids was calculated.

Band *L1* showed similarity with sequences of non-specific transfer protein: 79% identity and 87% positivity with a sequence of Non-specific lipid-transfer protein (nsLTP) from *C. baccatum* (Sequence ID: PHT38497.1); 75% identity and 83% positivity with a sequence of nsLTP from *C. chinense* (Sequence ID: PHU22162.1); 75% identity and 83% positivity with a sequence of $nsLTP_1$—like from *C. chinense* plant (Sequence ID: XP_016565683.1); and 75% identity and 83% positivity with a sequence of nsLTP from *C. annuum* plant (Sequence ID: PHT86247.1) (Fig. 2a).

Several plant defense genes encoding for LTPs have already been identified in *C. annuum* (Do et al. 2004), for example, the CALTPI and CALTPIII genes, which show differences in structure and sequence, are transcriptionally activated in pepper tissues by pathogen infection, abiotic and environmental stresses. In seeds and fruits of *Capsicum* species, peptides homologous to LTPs were characterized, however there are no reports of the presence of this peptide

A

Identification	10 20 30 40 50 60 70 80	P(%)	I(%)
L1	----------TLNGQATTTPDRS--------AAGSIGGINVR----------		
nsLTP (*C. baccatum*)	ISCGEVISKLTPCIKYVTGRGVVTPECCGGIK*TLNGQATTTPDR*QMACKCLKSAAG*AISGIN*LALASGLPSKCGVNLPYKISPSIDCS	87	79
nsLTP (*C. chinense*)	ISCGQVISKLSPCINYVRGSGGVTPACCSGIKA*LNGQATTTPDR*QMACKCIKSAAG*TISGIN*LGFASTLPSKCGVNLPYKISPSIDCS	83	75
$nsLTP_1$-like (*C. annuum*)	ISCGEVIAKLSPCINYVRGVGVVSPKCCEGIKA*LNGQATTTPDR*QMACKCIKSAAG*TISGIN*LALASALPRKCGVNLPYEISPSIDCS	83	75
nsLTP (*C. annuum*)	ISCGGVISKLSPCINYVRGVGVVSPKCCDGIKALNGQATTTPDRQMACKCIKSAAG*TISGIN*LGLASGLPSKCGVNLPYKISPSIDCS	83	75

B

	10 20 30 40 50 60	P(%)	I(%)
R1	-EKWPELLGTPAKFAQQIIQKENPKLTNVPSVLNGSPVTADFR----------		
Ethylene-responsive proteinase inhibitor 1-like (*C. annuum*)	K*EKWPELLGTPAKFAQQIIQKENPKLTNVV*T*VLNGGPVTE*DLRCNRVRLFVNLLDFVVQTPQVG	90	88
Ethylene-responsive proteinase inhibitor 1 (*C. baccatum*)	K*EKWPELLGTPAKFAQQIIQKENPKLTNVV*T*VLNGGPVTE*DLRCNRVRLFVNLLDFVVQTPQVG	90	88
Trypsin inhibitor 1 (*N. attenuate*)	K*ETWPEL*I*GVPAKFA*RE*IIQKENSKLTNVPSVLNGSPVTK*DFRCNRVRLFVNVLDFVVQIPRVG	90	83
Trypsin inhibitor 1 (*N. sylvestris*)	K*ETWPEL*I*GVPAKFA*RE*IIQKENSKLTNVPSVLNGSPVTK*DFRCERVRLFVNVLDFVVQIPRVG	90	83

Fig. 2 Alignment of amino acid residues from peptides of leaves (*L1*) and roots (*R1*) of *Capsicum annuum* (UENF1381) from LC_{48} and RC_{48} samples, respectively. The sequences were obtained from Blast and aligned by Clustal Omega. **a** The leaf peptide named *L1* with approximately 6 kDa showed similarity with the following sequences: Non-specific lipid-transfer protein (nsLTP) from *Capsicum baccatum* (Sequence ID: PHT38497.1); nsLTP from *Capsicum chinense* (Sequence ID: PHU22162.1); $nsLTP_1$–like from *C. chinense* plant (Sequence ID: XP_016565683.1); and nsLTP from *C. annuum* plant (Sequence ID: PHT86247.1). **b** The root peptide named *R1* with approximately 5.6 kDa showed similarity with the following sequences: predicted sequence of ethylene-responsive proteinase inhibitor 1-like from *C. annuum* (Sequence ID: XP_016567356.1); ethylene-responsive proteinase inhibitor 1 from *C. baccatum* (Sequence ID: PHT57973.1); predicted sequence of trypsin inhibitor 1 from *Nicotiana attenuata* (Sequence ID: XP_019254743.1); and predicted sequence of trypsin inhibitor 1 from *Nicotiana sylvestris* (Sequence ID: XP_009774947.1). P% indicates the percentage of positive residues (that present the same physic-biochemical features) and are written in gray. I% indicates the percentage of identical residues and are written in italics. Gaps (-) were introduced for better alignment

in leaves of these plants (Cruz et al. 2010; Diz et al. 2011; Maracahipes et al. 2019; Santos et al. 2020).

Band showed *R1* similarity with the sequence of ethylene-responsive proteinase inhibitor 1: 90% identity and 88% positivity with the predicted sequence of ethylene-responsive proteinase inhibitor 1-like from *C. annuum* (Sequence ID: XP_016567356.1); 90% identity and 88% positivity with the sequence of ethylene-responsive proteinase inhibitor 1 from *C. baccatum* (Sequence ID: PHT57973.1); 83% identity and 90% positivity with the predicted sequence of trypsin inhibitor 1 from *Nicotiana attenuata* (Sequence ID: XP_019254743.1); and 83% identity and 90% positivity with the predicted sequence of trypsin inhibitor 1 from *Nicotiana sylvestris* (Sequence ID: XP_009774947.1) (Fig. 2b).

The presence of protease inhibitors in different *Capsicum* species was specified by Silva et al. (2017). The seeds of *Capsicum annuum* (UENF1381 access), *C. baccatum* (UENF 1732 access), *C. baccatum* (UENF 1496 access), *C. chinense* (UENF 1498 access) and *C. frutescens* have shown the ability to inhibit trypsin activity.

3.3 Trypsin inhibitory activity of extracts

The inhibitory potential of leaf and root extracts from *C. annuum* on trypsin enzyme activity was evaluated. The leaf and root extracts were able for inhibiting trypsin activity at a concentration of 30 µg mL^{-1} (Fig. 3). The leaf extracts showed an inhibition of over 90% of the enzyme activity (Fig. 3a), and the root extracts showed an inhibitory range of 56.62–94.6% (Fig. 3b).

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

3.4 Amylase inhibitory activity of extracts

The α-amylase (*Tenebrio molitor*) inhibitory activity of leaf and root extracts was measured using starch as a substrate (Fig. 4). The leaf extract, at the tested concentration of 30 µg mL^{-1}, was able to inhibit over 90% of the enzyme activity (Fig. 4a). The root extracts showed an inhibitory range of 84.3–96.7% (Fig. 4b). It was observed that only L_{NC} and R_{NC} extracts at the same concentration were able to inhibit 100% of the enzyme activity (Fig. 4a, b).

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

3.5 Determination of β-1,3-glucanase activity

The activity of β-1,3-glucanase was detected in leaf and root extracts (Fig. 5a, b). The enzyme activity was low, although all extracts were significant when compared to the control. Better results were observed with R_{NC} and IR_{48}. In these

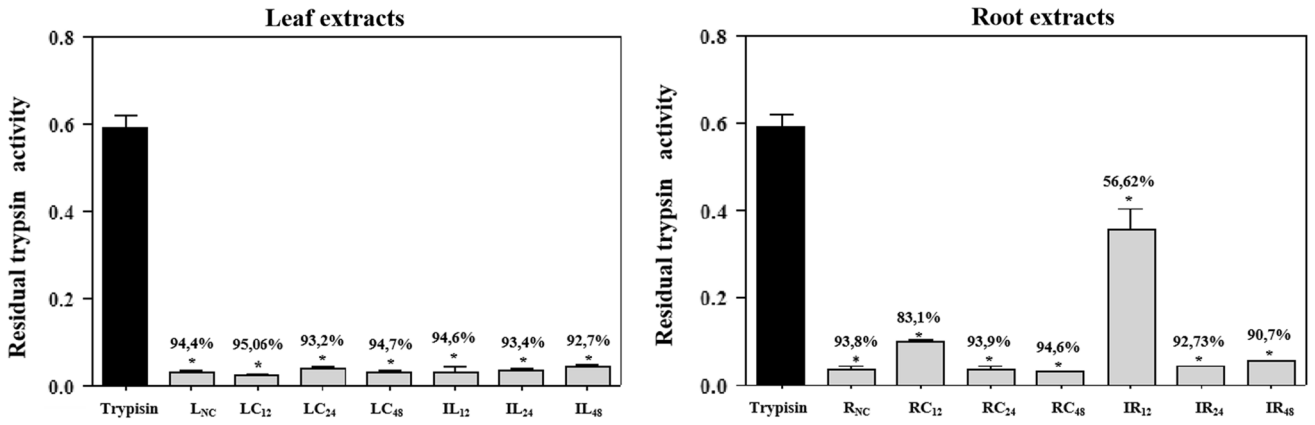

Fig. 3 Inhibitory activity of trypsin in the presence of 30 µg mL^{-1} of leaf and root extracts from *C. annuum*. The assays were performed in water and at 37 °C. The values are the means (± SD) of triplicate experiments. Asterisks indicate significant differences ($p < 0.05$) between each experimental treatment and the control and above the bars are show the inhibits percentage

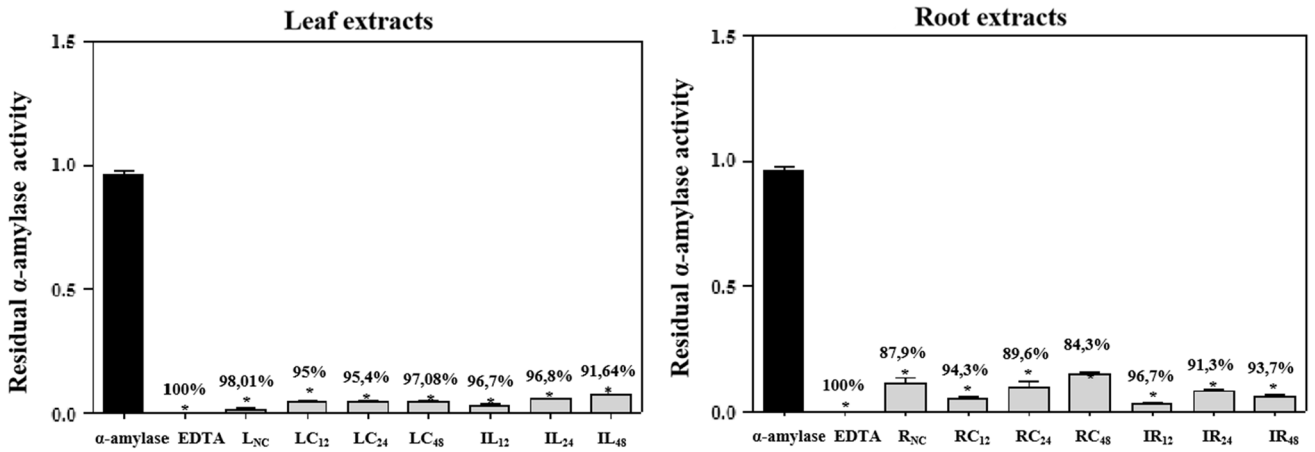

Fig. 4 Inhibitory activity of α-amylase (*Tenebrio molitor*) in the presence of 30 μg mL^{-1} of leaf and root extract from *C. annuum*. The assays were performed in water and at 37 °C. The values are the means (± SD) of triplicate experiments. Asterisks indicate significant differences ($p < 0.05$) between each experimental treatment and the control and above the bars are show the inhibits percentage

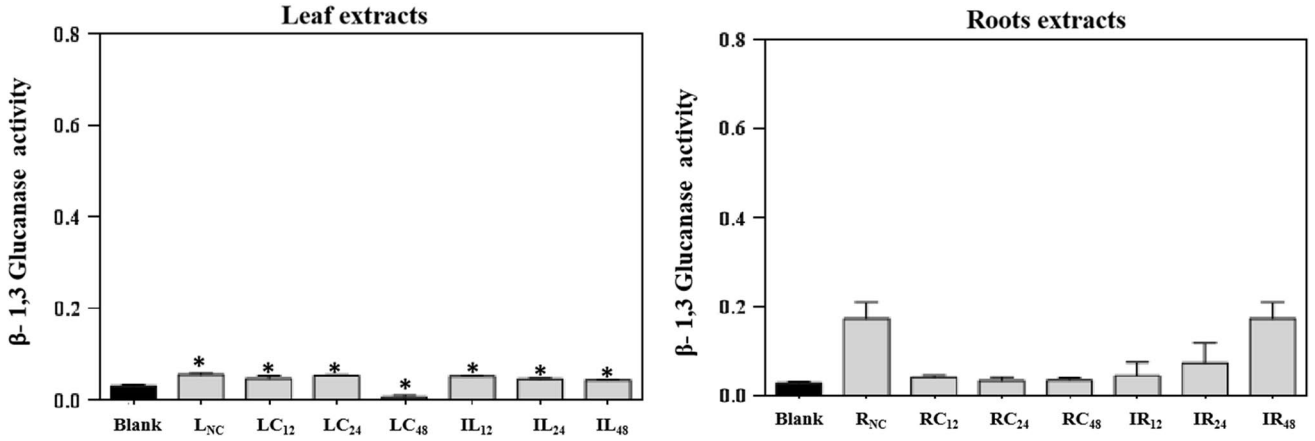

Fig. 5 Determination of β-1,3-glucanase activity in the presence of 30 μg mL^{-1} of leaf and root extracts from *C. annuum.* The values are the means (± SD) of triplicate experiments. Asterisks indicate significant differences ($p < 0.05$) between each experimental treatment and the control

extracts, the β-1,3-glucanase activity was higher than that of the control (Fig. 5b).

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

3.6 Effect of extracts on fungal growth

To evaluate the effect of leaf and root extracts from *C. annuum* on *C. scovillei* growth, the fungus was first grown for a period of 48 h in the presence of 200 μg mL^{-1} of leaf and root extracts (Fig. 6). The leaf extracts L_{NC}, LC_{12}, LC_{24}, LC_{48}, IL_{12} and IL_{48} were able to inhibit 100% of the fungus growth, and the IL_{24} extract inhibited approximately 96.7% (Fig. 6a). Only the root extracts R_{NC} and RC_{48} were able to inhibit 100% of the fungus growth. Moreover, the RC_{12} extract inhibited approximately 9.6% of the fungus growth, the RC_{24} extract inhibited approximately 61.09%, the IR_{12}

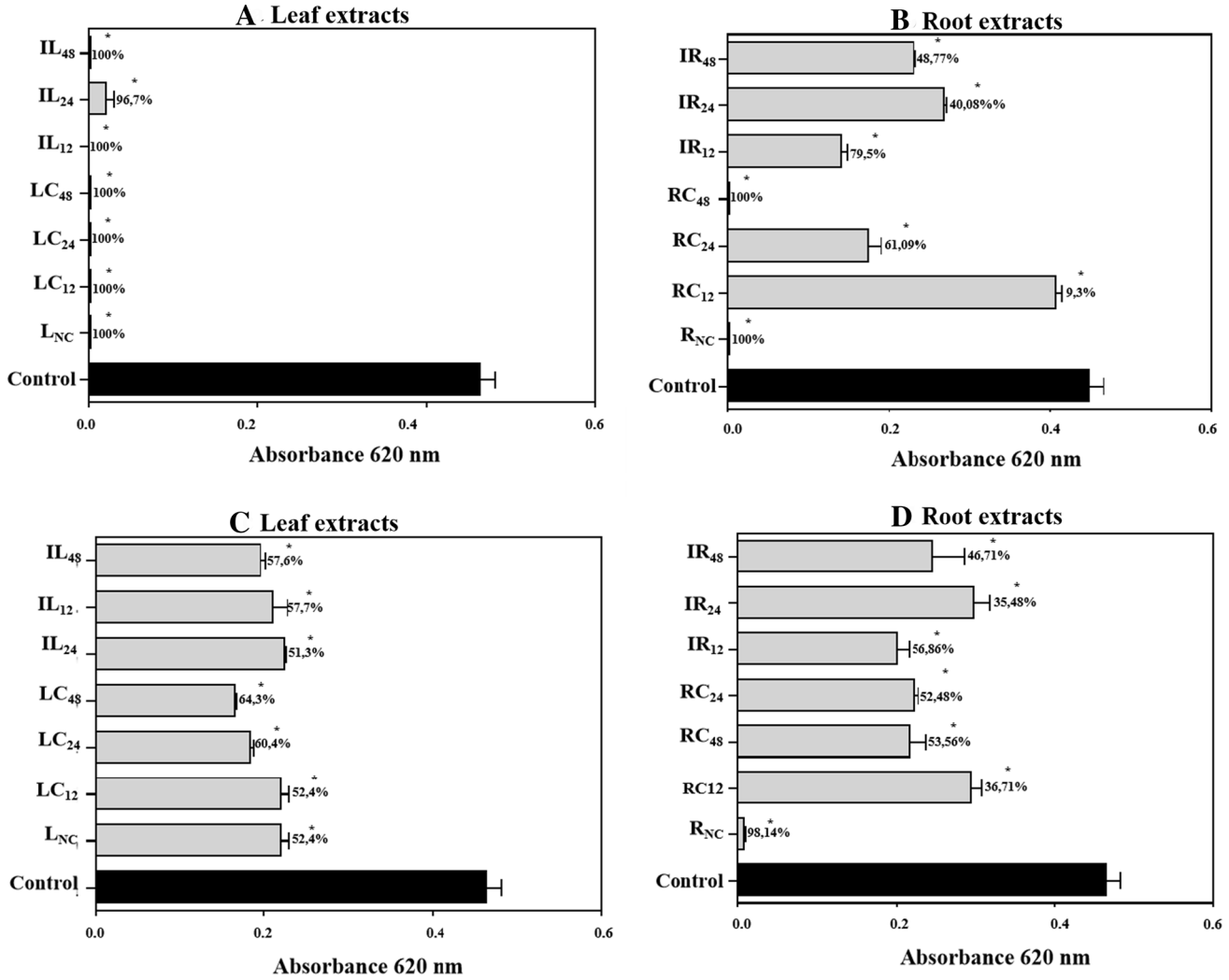

Fig. 6 Growth inhibition assay of *C. scovillei* in the presence of 200 µg mL^{-1} of leaf (**a**) and root (**b**) extracts and in the presence of 100 µg mL^{-1} of leaf (**c**) and root (**d**) extracts. The growth was observed until 48 h. The values are the means (± SD) of triplicate experiments. Asterisks indicate significant differences ($p < 0.05$) between the experimental treatment and control at 48 h of the experiment and the control and above the bars are show the inhibits percentage

extract inhibited approximately 79.5%, the IR_{24} extract inhibited approximately 40.08% and the IR_{48} extract inhibited approximately 48.77% (Fig. 6b).

In the next experiment, a lower concentration (100 µg mL^{-1}) of all extracts was used. The leaf extracts L_{NC}, LC_{12}, LC_{24}, LC_{48}, IL_{12}, IL_{24} and IL_{48} were able to inhibit from 51.3 to 64.3% of the fungus growth (Fig. 6c). The root extracts RC_{12}, RC_{24}, RC_{48}, IR_{12}, IR_{24} and IR_{48} were able to inhibit from 35.48 to 52.48% of the fungus growth, and the R_{NC} extract was able to inhibit 98.14% (Fig. 6d).

Capsicum plants have several biological activities, for example, antifungal activity. *Capsicum* extracts in low concentrations have been able to inhibit fungal growth. Soumya and Bindu (2012) demonstrated the antifungal efficacy of leaf and fruit extracts from *C. frutescens* L. against the fungi *Aspergillus flavus*, *A. niger*, *Penicillium* sp. and *Rhizopus* sp. The leaf extract inhibited 88.06% of the growth of the *A. flavus*, while the fruit extract inhibited 88.33% of the *A. niger*, with the concentration using 10 mg mL^{-1} of both extracts. Maracahipes et al. (2019) identified different AMPs in *Capsicum annuum* fruits such as defensin, LTP and protease inhibitor. In their studies, it was found that *C. annuum* fruit extracts are potent growth inhibitors of the fungus *C. gloeosporioides*. Using a concentration of 200 µg mL^{-1}, total inhibition of the pathogen was verified. Pereira et al. (2018) identified that leaf and root extracts from *C. annuum* inhibit the growth of the fungus *C. lindemuthianum.* Root extracts were also able to inhibit the total growth of the fungus *Colletotrichum gloeosporioides* at a concentration of 1000 µg mL^{-1}. The mechanism of action used by these

molecules present in the extracts was membrane permeabilization of the fungus.

3.6.1 Plasma membrane permeabilization

To evaluate whether the of leaf and root extracts were able to permeate the membranes of *C. scovillei* and thus begin to understand the mechanism of action of these peptides, we incubated the fungus treated with 100 µg mL^{-1} of extracts after 24 h with the fluorescent dye SYTOX Green and observed the fluorescence by microscopy. Figure 7 shows *C. scovillei* in the control with normal morphology. The LC_{12} and RC_{24} were able to permeabilize the membrane of *C. scovillei.* The leaf extracts L_{NC}, LC_{24}, LC_{48}, IL_{12}, IL_{24}, IL_{48} and the root extracts R_{NC}, RC_{12}, RC_{48}, IR_{12}, IR_{24} and IR_{48} were not able to permeabilize the membrane because they did not exhibit fluorescence (data not shown).

Most AMPs are cationic and amphipathic, which allows for greater interaction with negative charges from the microorganism's membranes. Thus, there is a change in the conformation of the peptides, which can act by several mechanisms. The mechanism of action of AMPs being highly influenced by the structure of the peptide and by the properties of the lipid membrane of the microorganism (Bechinger and Gorr 2017).

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

3.6.2 Intracellular ROS induction

Evaluation of the leaf and root extracts showed that they could induce increase in reactive oxygen species levels. *C. scovillei* was treated with 100 µg mL^{-1} of extracts and then incubated for 30 mim with the probe 2′, 7′-dichlorofluorescein diacetate (H2DCFDA) and observed by fluorescence microscopy. In Fig. 8, fungal was observed to have normal morphology in the control. The LC_{24} and IR_{12} extracts caused changes in fungus morphology and an increase in reactive oxygen species levels, as observed by an increase in fluorescence. The leaf extracts L_{NC}, LC_{12}, LC_{48}, IL_{12}, IL_{24}, IL_{48} and the root extracts R_{NC}, RC_{12}, RC_{48}, IR_{24} and IR_{48} did not induce increase reactive oxygen species levels (data not shown).

Studies suggest that membrane permeability is the main mechanism that would lead to the death of microorganisms. But recent studies have suggested membrane permeabilization with an event that comes after endogenous growth in the production of reactive oxygen species. An example and an isolated defensin of seeds of *Adenanthera pavonina* L. which in interaction with yeast *Saccharomyces cerevisiae* caused the drastic ROS leading to the permeabilization of the plasma membrane, chromatin condensation and death by activation of caspase apoptosis via (Koprivnjak and Peschel 2011; Malanovic and Lohner 2016; Soares et al. 2017). Gebara et al. (2020) shows some fractions of *C. annuum* fruit extract induced changes in the membrane of some strains of yeasts of the species *Candida*, leading to permeabilization and observed that production of reactive oxygen species was induced by fractions in some yeast strains. In *C. annuum* fruits, Maracahipes et al. (2019) noted that 200 µg mL^{-1} of a fraction called F7 was able to permeate the membrane of *C. gloeosporioides* and induce an increase in ROS. In addition, loss of mitochondrial viability was observed.

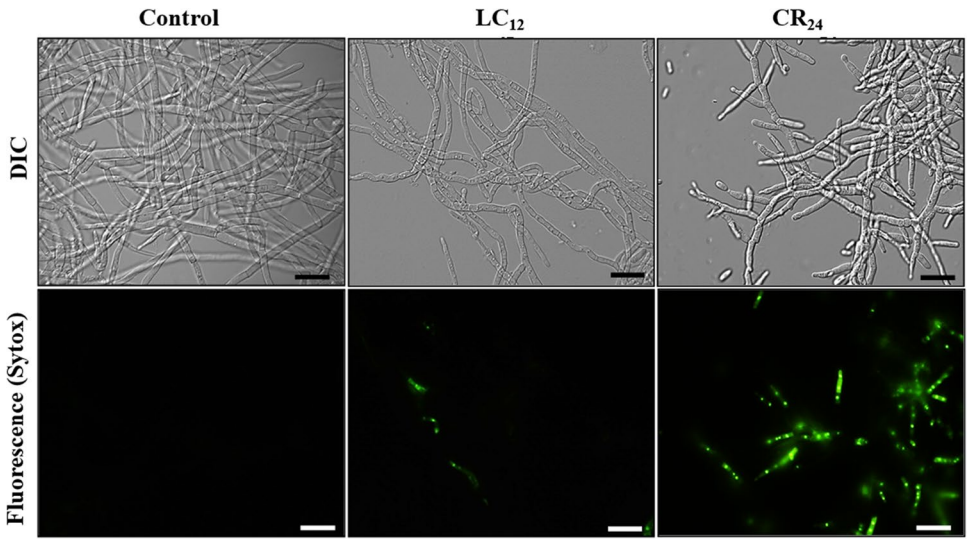

Fig. 7 Membrane permeabilization assay of *C. scovillei* cells by fluorescence microscopy using the fluorescent probe SYTOX Green. Cells were treated with 100 µg mL^{-1} of extracts and then assayed for membrane permeabilization. Control cells were treated only with the SYTOX Green probe. Bars = 20 µm

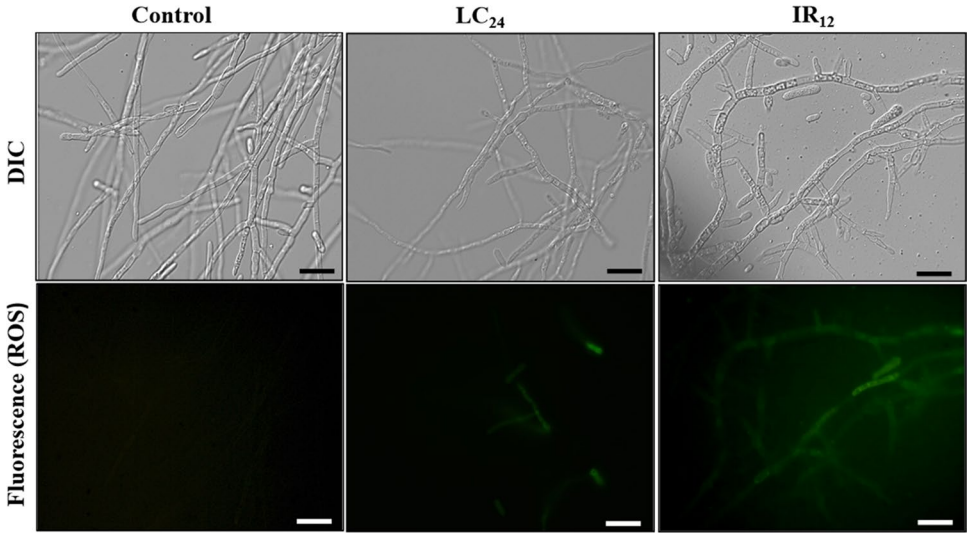

Fig. 8 Cells of *C. scovillei* after ROS induction assay by fluorescence microscopy using the H_2DCFDA probe. Cells were treated with 100 µg mL^{-1} of extracts for 24 h and then assayed for oxidative stress. Control cells were treated only with H_2DCFDA. Bars = 20 µm

3.6.3 Mitochondrial functionality determination assay

Figure 9 shows the results to the mitochondrial functionality of *C. scovillei* after 24 h of incubation with 100 µg mL^{-1} of different extracts. In leaf extracts L_{NC}, LC_{12}, LC_{24}, LC_{48} and the root extracts R_{NC}, RC_{12}, RC_{24}, IR_{12} and IR_{24}, the cells had decreased mitochondrial activity compared to control cells (without extract treatments), that was observed by the low fluorescence signal of Rhodamine 123 dye.

It is reported that some of the cationic AMPs interact with fungal organelles, such as mitochondria, leading to the death of the fungus. It is observed that cationic AMPs and rich in amino acids such as histidine have high activity against fungal cells. These peptides can interact with intracellular mitochondria, lead to the efflux of ATP without cell lysis, block mitochondrial respiration and oxidation of phospholipids and macromolecules, which can lead to damage to the mitochondrial membrane and the plasma membrane, and

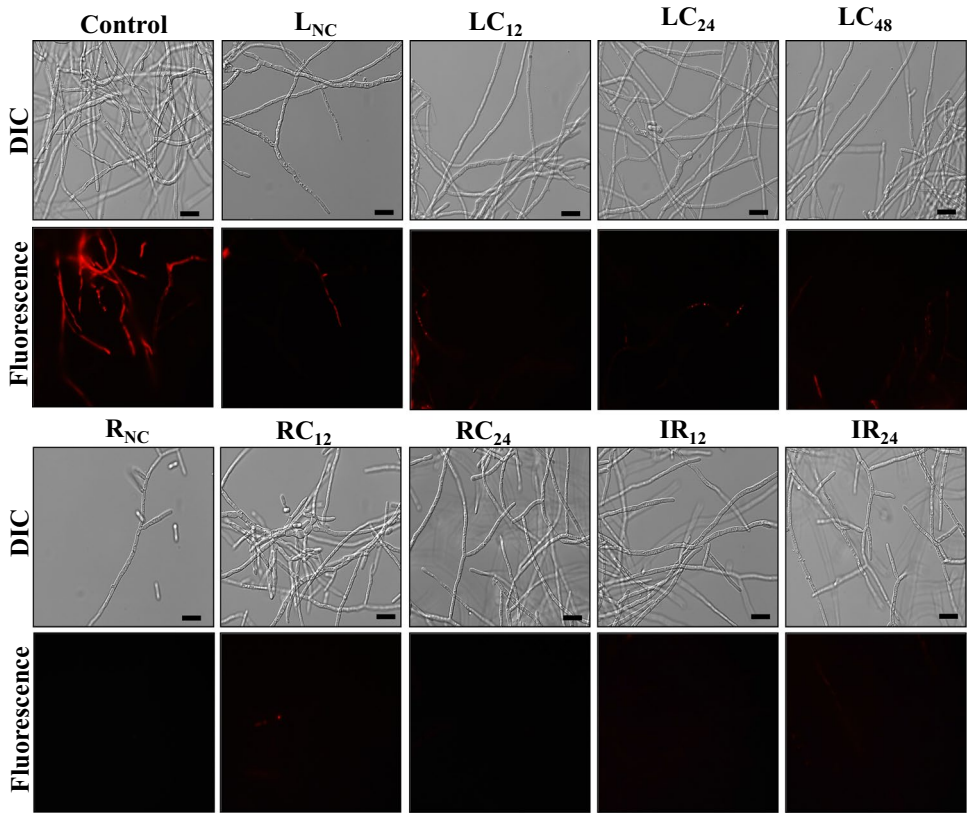

Fig. 9 Cells of *C. scovillei* after mitochondrial functionality assay by fluorescence microscopy using the fluorescent probe Rhodamine 123. Cells were treated with 100 µg mL^{-1} of extracts for 24 h and then analysed for mitochondrial functionality. Control cells were treated only with Rhodamine 123 probe. Bars = 20 µm

trigger ions efflux and cell death (Li et al 2018; Taniguchi et al. 2019).

Large losses in agriculture are caused by phytogenic microorganisms, and these microorganisms threaten agriculture and food production. Many strategies used to control microorganisms involve the use of chemicals, which are becoming ineffective, causing resistance to microorganisms. There is a strong interest in reducing the use of chemicals in agriculture for both health and environmental issues (Moguel-Salazar et al. 2011). In this paper we present an overview of the potential of leaf and root extracts from *C. annuum*, rich in AMPs, these interactions with microorganisms can be used to create pepper resistant cultivars and develop models for any pathosystem.

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Author contributions TAMS contributed with the fungal assay and microscopy. RW and VSC designed of the plant cultivation in vitro. LAS performed the experiment of the trypsin inhibitory activity. ACM contributed with the experiment of the determination of β-1,3-glucanase activity. GBT contributed with sequences analysis. CSN and RPC performed the amino acid sequence. RR and CPS provided the plant material and contributed with methodology of the plant infection. VMG and AOC designed the experiment and supervision.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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