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The first assessment of the stress inducible defense of *Leucaena leucocephala* with acaricidal potential effect against *Rhipicephalus* (*Boophilus*) *microplus* (Acari: Ixodidae)

Primeira avaliação de extratos proteicos de *Leucaena leucocephala* induzidos por injúria mecânica com atividade carrapaticida sobre *Rhipicephalus* (*Boophilus*) *microplus* (Acari: Ixodidae)

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Abstract

Plants respond to wounding caused by mechanical stress or herbivory by synthesizing defense proteins. There are no studies reporting the action of induced plant proteins against ticks. The aim of this study was to investigate the effect of mechanically wounded *Leucaena leucocephala* leaves against *Rhipicephalus (Boophilus) microplus*. Initially, we carried out time course experiments to evaluate the impact of mechanical wounding on the protein content and the peroxidase, catalase and protease inhibitor activities in *L. leucocephala*. We then evaluated the acaricidal activity on *R. (B.) microplus* from protein extract collected from *L. leucocephala* after mechanical wounding. *L. leucocephala* leaves were artificially wounded, and after 6, 12, 24 and 48h, the leaves were collected for protein extraction. Quantitative and qualitative analyses of the proteins were performed. The protein content and peroxidase and protease activities increased 12h after wounding, and the acaricidal activity of this protein extract was evaluated using engorged *R. (B.) microplus* females. The protein extract obtained after wounding reduced egg production (8.5%) compared to those without wounding. Furthermore, the extract reduced egg hatching by 47.7% and showed an overall efficacy of 56.3% at 0.1 mgP/mL of the protein. We demonstrated that *L. leucocephala* defensive proteins could be effective against *R. (B.) microplus.*

Keywords: Mechanical stress, plant protein, induced defense, acaricide, tick.

Resumo

As plantas respondem a injúria causada por estresse mecânico ou por ataque de herbívoros através da síntese de proteínas de defesa. Não há estudos de proteínas induzidas de plantas contra carrapatos. O objetivo deste estudo foi verificar a atividade acaricida de extratos protéicos de folhas *Leucaena leucocephala* após injúria mecânica, sobre *Rhipicephalus (Boophilus) microplus*. Inicialmente foram conduzidos experimentos em diferentes intervalos de tempo para avaliar o impacto da injúria mecânica no conteúdo de proteína, atividade de peroxidase, catalase e inibidor de protease de *L. leucocephala.* Em seguida foi avaliada a atividade acaricida sobre *R. (B.) microplus* de um extrato protéico após injúria mecânica. Folhas de *L. leucocephala* foram artificialmente feridas e após 6, 12, 24 e 48h, as folhas foram coletadas para extração de proteínas. Análises quantitativas e qualitativas das proteínas foram realizadas. A quantidade de proteína e atividades de peroxidase e protease aumentaram 12h após a injúria. O extrato proteico obtido após injúria (12h) reduziu a produção de ovos (8,5%) em comparação com extratos de plantas sem injúria. O extrato reduziu 47,7% a eclosão de ovos e apresentou eficácia geral de 56,3% a 0,1 miligrama de proteína por mL (mgP/mL). Apresentamos que proteínas de defesa de *L. leucocephala* podem ter atividade sobre *R. (B.) microplus*.

Palavras-chave: Estresse mecânico, proteínas de planta, defesa induzida, acaricida, carrapato.

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Introduction

Throughout their evolutionary history, the constant exposure of plants to adverse conditions has led to the development of a complex system of defense responses to biotic and abiotic stressors; this system consists of diverse morphological, physiological, biochemical and molecular changes for the plants' acclimation (WANG et al., 2003; BONALDO et al., 2005). Plant defense can be constitutive, which occurs normally in the plant's metabolism, or it can be induced after stressful conditions (WAR et al., 2012).

The study of abiotic stress in plants has advanced considerably in recent years, and diverse studies have identified protein changes in response to different stress, such as cold, heat, drought, salinity and mechanical wounding (RIZHSKY et al., 2004; KOSOVÁ et al., 2011). Changes in protein abundance and the expression profiles of specific genes/proteins in response to wounding have been reported in plants, such as papaya (LOOZE et al., 2009; PAN & JIANG, 2014), rice (RANI & JYOTHSNA, 2010), turfgrasses (GULSEN et al., 2010), maize (LEWANDOWSKA-GNATOWSKA et al., 2011), and apple (BURON-MOLES et al., 2014). The changes in the proteome composition after the wound healing process are related to the increased expression of several proteins that are related to the stress response, such as protease inhibitors, pathogenesis-related proteins, peroxidases, chitinases and proteases (KOSOVÁ et al., 2011).

It is known that plants display bioactive molecules that can act in response to parasites. Several studies demonstrated the activity of plant compounds on different species of parasites, such as ticks (SOARES et al., 2010; LIMA et al., 2014; CASTRO et al., 2014; GEORGE et al., 2014; LAGE et al., 2015). To obtain natural bioactive compounds that are less harmful to the environment, animals and humans, the use of plant products has become an alternative method to synthesize chemicals for the control of ticks (AMARAL et al., 2002; ELLSE & WALL, 2014), including *Rhipicephalus (Boophilus) microplus*.

The cattle tick *R. (B.) microplus* is the most common parasite in livestock in tropical and subtropical regions (GRISI et al., 2014). Ticks are generally controlled with synthetic acaricides, but indiscriminate use has increased the frequency of resistance to these chemicals (RODRÍGUEZ-VIVAS et al., 2006; CASTRO-JANER et al., 2010; MILLER et al., 2013).

Native to Central America, *Leucaena leucocephala* can be found in many tropical and subtropical regions of the world (NEHDI et al., 2014) and is often used as forage for livestock (PANDEY & KUMAR, 2013). *L. leucocephala* is a tannin-rich plant with anti-parasitic activity against ticks and nematodes; almost all studies on this plant have focused on secondary metabolites (CUNHA et al., 2003; ALONSO-DÍAZ et al., 2008; AHMED et al., 2010; OLIVEIRA et al., 2011; FERNÁNDEZ-SALAS et al., 2011; HERNANDEZ et al., 2014; SOARES et al., 2015).

There are no studies indicating the possibility of acaricidal activity from proteins in *L. leucocephala* leaves nor are there any studies focused on the plant's induced proteins by mechanical wounding. Thereby, the aim of this study was to verify the acaricidal activity of the protein extract from *L. leucocephala* leaves after mechanical wounding against *R. (B.) microplus*.

Materials and Methods

Plant material, protein extraction and quantification

Mature seeds of the *L. leucocephala* plant were obtained commercially. After breaking dormancy with water (PASSOS et al., 1988), they were cultivated in plastic pots containing black soil and were grown in a greenhouse.

Leaves of plants at the three-leaf stage (15 days) were subjected to mechanical injuries using anatomical steel serrated forceps (140 mm). At specific intervals after the wounding (6, 12, 24 and 48 h), leaves of five plants per treatment at each time were harvested, frozen in liquid nitrogen and ground to a fine powder. Polyvinylpolypyrrolidone (PVPP) at 2% (w/w) was added for the removal of phenolics compounds. As the control group, *L. leucocephala* leaves from unwounded plants were subjected to the same procedure.

The proteins of the leaves were extracted with 100 mM potassium phosphate (pH 7.0) containing 75 mM NaCl in the ratio 1:10 (w/v). The slurry was centrifuged for 30 min. at 15,000 x g at 4 °C, and the supernatant was collected and centrifuged under the same conditions. The final supernatants were collected, classified as protein extracts of leaves (PELs) and stored at -20 °C for assays and protein analysis. All procedures were carried out in five replicates.

The soluble proteins of the PELs were quantified according to Bradford (1976), using bovine serum albumin (BSA) as the standard. The soluble protein content of the extracts was expressed as milligram of protein per gram of fresh mass (mgP/gMF) and was calculated using following equation: protein content (mgP/gMF) = [protein concentration (mg/mL)] x buffer volume (mL)/leaf weight (g).

Gel electrophoresis (SDS-PAGE)

SDS-PAGE (15%) was performed according to Laemmli (1970), and protein bands were revealed with silver nitrate. A Molecular Weight Marker kit (code 17044601 from GE Healthcare, Buckinghamshire, UK) was used as the protein markers (MW of 97, 66, 45, 30, 20 and 14 kDa).

Catalase, peroxidase, protease and protease inhibitor assays

The catalase activity (CAT) of the PELs was measured by the decrease in the absorbance at 240 nm for four min. every 10 seconds (HAVIR & MCHALE, 1987; PEIXOTO et al., 1999). A change of 0.01 absorbance unit per min. was considered a unit of catalase activity (1 UA). Catalase activity was expressed as the activity unit per gram of fresh mass (AU/gMF).

Peroxidase activity (POD) was determined following the methodology described by Urbanek et al. (1991). The variation of 1.0 absorbance unit per min. was assumed to be 1.0 unit of peroxidase activity. The peroxidase activity was expressed as the activity unit per gram of fresh mass (AU/gMF).

Total proteolytic activity of PELs was examined using azocasein as a nonspecific substrate (XAVIER-FILHO et al., 1989). One unit of activity (UA) was defined as the amount of enzyme capable of increasing absorbance by 420 nm at 0.01 mL^{-1} in 60 min.

Cysteine proteinase inhibitor activity in PELs was determined by measuring the inhibition of papain activity with benzoyl-DL-arginine-β-naphthylamide (BANA) as the substrate (ABE et al., 1992). One unit of inhibitory activity (UI) was defined as the decrease of 0.01 absorbance units at 540 nm/mL/min when compared with the control (papain activity in the absence of the inhibitor).

Adult immersion test (AIT)

The sensitivity of engorged *R. (B.) microplus* females to PELs was determined using the adult immersion test (AIT) described by Drummond et al. (1973). The PELs from the leaves collected at 12 hours after the initiation of the experiment were chosen because showed increased protein content, peroxidase and protease. Engorged *R. (B.) microplus* females were collected from artificially infested calves. Groups of ten engorged female ticks were each individually weighed to obtain groups with similar weights. The ticks were immersed for five min. in four mL of PELs from wounded and unwounded plants (control) at 0.1 mg/mL.

The engorged females were subsequently dried on a paper towel, placed in Petri dishes and maintained in a biochemical oxygen demand incubator at 27±1 °C with RH ≥80% for 15 days for further evaluation of oviposition and more 30 days to hatchability of their eggs. Hatchability was estimated from the average of three count of eggs and larvae by using a stereomicroscope.

The egg production index (EPI), the reduction in oviposition, and the efficiency of the extract (EP) were calculated according to the following formulas: EPI = (weight of eggs/ weight of engorged female)×100 (BENNETT, 1974); reduction in oviposition = (EPI control group – EPI experimental group/ EPI control group)×100 (ROULSTON et al., 1968); reproduction efficiency index (REI) = (egg mass weight × egg hatching (percentage)/ engorged females weight) × 20,000; EP = (REI control – REI treated)/ (REI control×100) (DRUMMOND et al., 1973).

Statistical analysis

The protein content and enzymatic activity of proteins related to the wounded plant response of the PELs were statistically analyzed for normality by a *Shapiro-Wilks* test, and the averages ± standard deviation were compared to the control group with a *Student's T*-test with the significance level of 5%.

Results

Quantitative and qualitative changes in the protein content were observed in the PELs of wounded plants (Figure 1). The average increase of protein content in the PELs after wounding was 4.47 mg protein per gram of fresh mass. Significant increases of protein content ($p \leq 0.05$) were observed from PEL groups at 6 (increase of 55.3%), 12 (increase of 46.4%) and 48 hours (increase of 43.6%) after wounding (Figure 1). The levels of two proteins increased in the 12, 24 and 48 hour groups.

Protease inhibitor and catalase activities were not detected in the PELs of *L. leucocephala*. Mechanical wounding increased the peroxidase activity in all analyzed time groups (Figure 2A). Proteolytic activity was significantly increased (54.0%) only in the PELs from the 12 h after wounding group (Figure 2B).

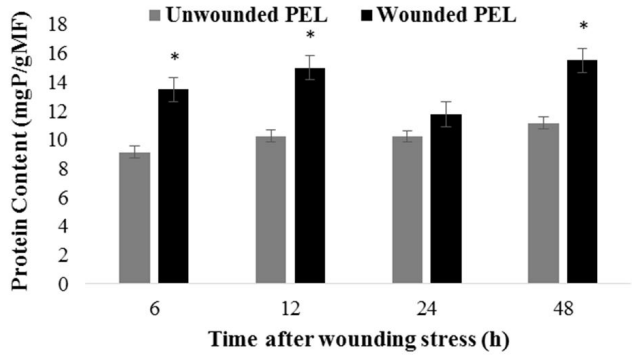

Figure 1. Assessment of protein content of the protein extracts of leaves (PELs) from *Leucaena leucocephala* plant. The unit mgP/gMF represents milligram of protein per gram of fresh mass. Time after wounding of the horizontal line corresponds to the periods that the leaves were harvested after the mechanical injury, forming the treatment group. Data are mean ± standard deviation of four independent experiments. *Data differ significantly ($p < 0.05$).

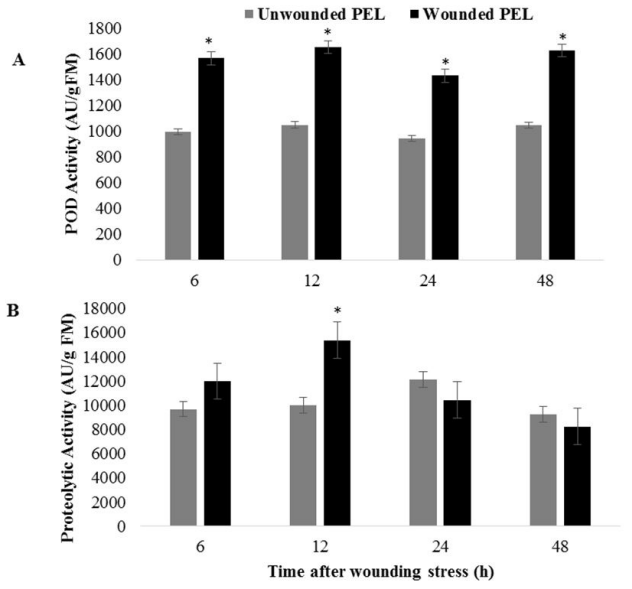

Figure 2. Peroxidase activity (POD) (A) and proteolytic activity (B) of the protein extracts of leaves (PELs) in response to mechanical wounding in a time course experiment (6, 12, 24 and 24 hrs after the wounding). Time after wounding of the horizontal line corresponds to the periods that the leaves were harvested after mechanical injury, forming the treatment group. The peroxidase and proteolytic activities were expressed as activity unit per gram of fresh mass (AU/gMF). Data are mean ± standard deviation of four independent experiments. *Data differ significantly ($p < 0.05$).

The PELs obtained from leaves collected 12 hours after wounding and from leaves of unwounded plants were used in AIT at 0.1 mg/mL. The protein extract obtained after wounding reduced egg production (8.5%) when compared to those without wounding. Furthermore, the extract reduced egg hatching by 47.7%, and showed an overall efficacy of 56.3%.

Discussion

Although peptides from poisons and fungi have demonstrated activity against ticks (MUKHERJEE et al., 2006; MORAIS-URANO et al., 2012), and plant defense proteins have been effectively used against phytophagous insects (MARTINEZ et al., 2016), there are no studies that have investigated the activities of plant defense proteins against ticks.

To associate induced *L. leucocehala* defense proteins with the potential for tick control, we performed a test on engorged *R. (B.) microplus* females with PELs obtained from leaves. PELs collected 12 h after wounding did not show a significant reduction in egg production; however, they showed a 47.7% inhibition of egg hatching and an overall efficacy of 56.3%. *L. leucocephala* leaf extract, rich in secondary metabolites, was observed to be effective against the larvae, but not against the engorged females of *R. (B.) microplus* (FERNÁNDEZ-SALAS et al., 2011).

Our results showed that the acaricide effect of PELs from wounded leaves coincided with high protease activity, however other studies are needed to confirm the acaricide properties of this enzyme. The peroxidase enzyme is an important component of the immediate response of plants to insect damage (RANI & JYOTHSNA, 2010; GULSEN et al., 2010). The same results have been observed for different types and classes of proteases; these enzymes have many different roles in the plant's defense against insect feeding, such as directly degrading proteins from the invader, releasing peptide-based toxins or activating enzymes from their precursor proteins (VAN DER HOORN & JONES, 2004).

In the present study, we verified changes in the protein content, pattern and enzyme activities of *L. leucocephala* leaves in response to mechanical wounding in a time course experiment. The protein content was increased, and the protein profile was altered after wounding. Although catalase and protease inhibitory activities were not detected, the activities of peroxidase and protease increased 6 to 48 hours and 12 hours after wounding, respectively. Similar alterations in response to herbivory and mechanical stress were observed in rice (RANI & JYOTHSNA, 2010) and apple (BURON-MOLES et al., 2014).

Mechanical stressors, such as injuries and wounding, can activate the defense mechanisms of the plant to induce local and/or systemic responses (KESSLER & BALDWIN, 2002). The defense mechanism is orchestrated by changes in protein expression, in which the plant is able to overexpress or inhibit the expressions or actions of molecules such as protease, protease inhibitor, catalase, peroxidase, chitinase and lipoxygenase (WANG et al., 2003; AHSAN et al., 2007; GULSEN et al., 2010; KOSOVÁ et al., 2011).

The increase of peroxidase activity in the wounded *L. leucocephala* plant can be attributed to the detoxification mechanism of peroxides, the healing induced by wounding and the defense mechanism against damage (ALMAGRO et al., 2009; RANI & JYOTHSNA, 2010). In this study, we evaluated peroxidase activity as a stress marker (DÍAZ & MERINO, 1998; TSCHARNTKE et al., 2001). Catalase is part of another enzyme group known to be antioxidant enzymes involved in the reduction of oxidative stress (WAR et al., 2013); however, we did not observe catalase activity in the protein extracts from the *L. leucocephala* plant. A similar finding was observed for the activity of protease inhibitors, which was not identified in our results.

The increase of proteolytic activity can be associated with the involvement of proteases in amino acids recycled for the synthesis of protective proteins and/or defense molecules against herbivory (GREEN & RYAN, 1972; PECHAN et al., 2000; VAN DER HOORN & JONES, 2004). In corn, an accumulation of a 33-kD cysteine protease called Mir1-CP has been observed in response to the feeding of lepidopterous larvae and the mechanical injury (PECHAN et al., 2000).

Hence, the induced wounding in leaves from the *L. leucocephala* plant causes enzymatic changes and alters the protein content. Furthermore, the protein extract of wounded leaves can suggest interferences in the *R. (B.) microplus* cycle by reducing the percentage of hatched eggs. The inducible proteins could be a novel approach for tick control. Further research should be conducted to discern the proteins which is involved in the acaricide effect.

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