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A cotyledonary agglutinin from *Luetzelburgia auriculata* inhibits the fungal growth of *Colletotrichum lindemuthianum*, *Fusarium solani* and *Aspergillus niger* and impairs glucose-stimulated acidification of the incubation medium by *Saccharomyces cerevisiae* cells

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Abstract

The aim of this study was to test the hypothesis of the involvement of the cotyledonary *Lutzelburgia auriculata* agglutinin (LAA) in the plant defense against fungi. It was observed that LAA is localized at the periphery of the cotyledon and that it is released to the surrounding medium during seed germination when the plantlet is most vulnerable to phytopathogens. When LAA was included in the culture medium it inhibited the fungal growth of *Colletotrichum lindemuthianum*, *Fusarium solani* and *Aspergillus niger* compared to the same fungi cultured in the absence of LAA. To better understand the mechanism of action of LAA on fungal growth, *Saccharomyces cerevisiae* was used as a model. LAA reversibly bound to the yeast cells, inhibited its growth and promoted a 60% inhibition of the glucose-stimulated acidification of the incubation medium by *S. cerevisiae* suggesting its interference on the transport of intracellular protons to the external medium. In conclusion, the data indicate that LAA is possibly involved in the plant defense mechanisms against phytopathogen fungi.

Keywords: *Luetzelburgia auriculata*; Leguminous; Seed; Lectin; Phytopathogens; Defense

1. Introduction

Compared to other proteins, lectins are unique as they have one or more non-catalytic domains able to recognize and reversibly bind to free- and/or conjugate carbohydrates, without modification of their covalent structures [1]. They interact with various cellular structures, particularly cell walls and membranes, through which they can trigger several biological effects in and outside the plant. Although several hypotheses on the lectin functions have been put forward the widespread occurrence of plant lectins which recognize specific carbohydrate structures of potential plant enemies has led to the assumption that the principal role of these proteins is related to defense mechanisms against phytopathogenic bacteria, fungi, insects, and mammals [2–4]. This fact is of great interest because of the potential use in the field of plant genetic engineering for crop protection [5].

There is a body of experimental evidence showing that several lectins interact with and inhibit the growth of

Abbreviations: BSA, bovine serum albumin; LAA, *Luetzelburgia auriculata* agglutinin; FITC, fluorescein isothiocyanate; PBS, phosphate buffered saline; UDA, *Urtica dioica* agglutinin; WGA, wheat germ agglutinin

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phytopathogenic fungi. Broekaert et al. [6] showed that the fungi *Trichoderma hamatum*, *Botrytis cinerea* and *Phycomyces blakesleeanus* had their growth inhibited by *Urtica dioica* agglutinin (UDA). Ciopraga et al. [7] observed that the binding of wheat germ agglutinin (WGA) to the cell wall of *Fusarium graminearum* and *F. oxysporum* affected the growth of the germ tubes as revealed by the dramatic changes observed in fungal morphology: lysis of hyphal tips, swelling and vacuolation of cell content. A chitin-binding lectin from the corm of *Gastrodia elata* (Orchidaceae) displayed a strong in vitro inhibitory activity of the hyphal growth of the phytopathogenic fungi *Valsa ambien*, *Rhizoctonia solani*, *Gibberella zeae*, *Ganoderma lucidum*, and *B. cinerea* [8]. Inhibitory activity against different fungal strains such as *B. cinerea*, *F. culmorum*, *F. oxysporum*, *P. blakesleeanus*, *Pyrenophora triticirepandis*, *Pyricularia oryzae*, *Septoria nodorum*, and *T. hamatum* was also reported for hevein, a chitin-binding protein from rubber tree (*Hevea brasiliensis*) latex [9]. Does et al. [10] showed that genetically transformed tobacco plants expressing the gene of the isolectin UDA I produced a lectin which was correctly processed and inhibitory to the micelial growth of *B. cinerea*, *T. viride* and *Colletotrichum lindemuthianum*.

Despite the experimental evidence of the defense function of lectins few studies have deal with the mechanisms by which these proteins exert their anti-fungal effects. To explore the potential use of plant lectins as chemical weapons against fungi is essential a thorough understanding of the mechanisms by which these proteins act on these organisms.

Recently, a 123.5-kDa lectin was purified to homogeneity from *Luetzelburgia auriculata* cotyledons (named LAA) in our laboratory and it was verified that during the seed germination, when the plant is most vulnerable to pathogen attack, LAA was released to the surrounding medium [11]. Thus, to test the hypothesis of the involvement of this lectin in the plant defense against phytopathogenic fungi LAA was assayed regarding to its inhibitory activity against the conidial growth of the pathogenic fungi *C. lindemuthianum*, *Fusarium solani* and *Aspergillus niger*. Moreover, as an attempt to better understand the possible mechanism of action of LAA on fungi, its ability of binding to and inhibiting the cell growth of the yeast *S. cerevisiae* and also its effect on the glucose-stimulated acidification of the incubation medium by this organism were studied.

2. Materials and methods

2.1. Biological and chemical materials

Seeds of *L. auriculata* (L.) Ducke were collected from trees growing at the Ceará state, Brazil.

The filamentous fungi *C. lindemuthianum* (URM 3114), *F. solani* (URM 3708) and *A. niger* (URM 3292) were provided by the Departamento de Micologia of the Universidade Rural de Pernambuco, Recife, Brazil. The yeast *Saccharomyces cerevisiae* was from the Departamento de Biologia, Universidade Federal do Ceará, Fortaleza, Brazil. Three-month-old New Zealand rabbits were provided by the Departamento de Zootecnia, Universidade Federal do Ceará, Fortaleza, Brazil, and were used for production of the anti-*L. auriculata* lectin polyclonal antibodies and as donors of blood cells used in the hemagglutinating assays.

Acrylamide, bis-acrylamide, anti-rabbit IgG antibody coupled to alkaline phosphatase, bacto-peptone, β-mercaptoethanol, bovine serum albumin, coomassie brilliant blue (G and R), 5-bromo-4-chloro-3-indolyl phosphate (BCIP), D-galactose, nitro-blue tetrazolium (NBT), nitrocelulose membranes and Sabouraud dextrose agar were from Sigma Co., St. Louis, USA. Polyvinylidene difluoride membranes (PVDF, 0.45 μm) were from Millipore Corporation, Bedford, USA. Blotting protein bands were visualised by chimioluminescence using the kit (ECL) from Amersham International, UK.

2.2. Purification of L. auriculata agglutinin

A highly purified *L. auriculata* lectin (LAA) preparation devoid of contaminating proteins was obtained according to Oliveira et al. [11]. Mature seeds were dehulled and the cotyledons separated from the axes. Cotyledons were ground in a coffee grinder with the resulting flour treated with petroleum ether. Defatted flour (10 g) was extracted with 100 ml of 0.05 M sodium acetate buffer (pH 6.0) containing 0.15 M NaCl, for 4 h at room temperature under constant stirring. After centrifugation at 20,000 × *g*, 5 °C, 20 min (Sorvall RC-5B refrigerated centrifuge), the supernatant, denoted crude extract, was fractionated by saturation to 40–60% with solid ammonium sulphate. This protein fraction was resuspended with the extracting buffer, dialyzed against distilled water until free of NH_4^+ ions, and lyophilized. The 40–60% fraction was dissolved in 0.05 M sodium acetate buffer (pH 6.0) containing 0.15 M NaCl, the precipitate removed by centrifugation as above and the clear supernatant applied to an agarose-*N*-acetyl-D-galactosamine column. After elution of the unbound proteins from the column with the starting buffer, the lectin was eluted with 0.20 M D-galactose in the same buffer. Purity of LAA was checked by exclusion chromatography on Sephadex G-100, ion-exchange chromatography, on analytical isoelectric focusing (IEF) and denaturing gel electrophoresis as previously described [11]. To check for the presence of contaminating genuine anti-fungal proteins the LAA preparation obtained was assayed for chitinase and β-1,3-glucanase activities according to Xue et al. [8].

2.3. Exudation assay

L. auriculata seeds were surface-sterilized with 2% (v/v) sodium hypochloride solution for 5 min and washed