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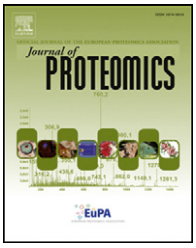

Proteomic analysis of responsive stem proteins of resistant and susceptible cashew plants after *Lasiodiplodia theobromae* infection

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ABSTRACT

Gummosis is an aggressive disease caused by the necrotrophic fungus *Lasiodiplodia theobromae* (Pat.) Griffon & Maubl that threatens commercial cashew orchads in Brazil. To study the molecular mechanisms involved in the cashew response to *L. theobromae*, a proteomic analysis of stems from the commercial cashew clone BRS 226 (resistant) was conducted at early times post-artificial infection. In addition, changes in the stem proteome profiles of gummosis resistant and susceptible cashew plants grown under field condition and naturally exposed to pathogen were also compared. After two-dimensional gel electrophoresis (2D-PAGE), 73 proteins showed statistically significant differences in spot abundance. Of these, 31 spots were identified in BRS 226 stems compared with mock-inoculated controls and 32 in stems collected from field-grown resistant and susceptible cashew plants. *L. theobromae*-responsive proteins were mainly involved in energy metabolism pathways, stress and defense, cell signaling and protein metabolism indicating modulation of various cellular functions upon fungal infection. As stress-inducing factors seem to be important for susceptibility to disease, the change in the abundance relative these proteins may possibly indicate an attempt to maintain cellular homeostasis, as resistance determinant factor, related with a possible role in the regulation of oxidative burst. These findings provide the first information about the cellular mechanisms acting in the *Anacardium occidentale* genotypes associated with the pathophysiological state of infection with *L. theobromae*.

Biological significance

Gummosis caused by *Lasiodiplodia theobromae*, a necrotrophic fungus, is the major disease of cashew plants in the semi-arid conditions of northeastern Brazil. Although various studies were carried out on this pathosystem, there is no information available on the molecular mechanisms of plant defense related to the incompatible interaction of cashew with *L. theobromae*. Therefore, this original study comprises a differential proteomic analysis of

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cashew stems from: (i) resistant dwarf clone BRS 226 mock-inoculated (control) and artificially inoculated with *L. theobromae*; and (ii) cashew plants bearing resistant and susceptible traits to gummosis, originated from open pollination of BRS 226 in a commercial orchard with high disease incidence. The contribution of the reprogrammed proteins to molecular events triggered in cashew plants challenged by *L. theobromae* has a great relevance in the identification of the host candidate proteins linked to biological pathways that respond to *L. theobromae* infection. Furthermore this study may contribute to improve breeding programs aimed at selecting resistant/tolerant cashew clones toward this pathogen.

1. Introduction

Cashew production has considerable social and economical importance in many countries, including Brazil, India and some African countries, particularly because the nuts and peduncles (pseudofruit) have numerous economic uses [1,2]. Brazilian cashew industry is one of the main sources of employment and income, contributing largely to Northeastern Brazil's economy [3].

The production of cashew is affected by diverse environmental factors particularly phytopathogens [4]. Currently gummosis caused by *Lasiodiplodia theobromae* (Pat.) Griff & Maubl., a necrotrophic fungus, is the major disease of *Anacardium occidentale* L. in the semi-arid conditions of northeastern Brazil. *L. theobromae* infects the stem leading to reduction in the transport of nutrients and water, with consequent reduction in photosynthesis and ultimately death [5]. Interestingly, resistance mechanism which cures the infected stem and suppresses the growth of fungi within the host tissue can occur when the factors inducing stress ceases [6], indicating a possible importance in the maintaining cellular homeostasis as resistance determinant factor. Although studies were carried out to evaluate the ultrastructural features of infection by *L. theobromae* [5], the effects of different combinations of graft and rootstock on gummosis incidence [7] and selection of resistant genotypes [6,8], there is no information available on the physiological and biochemical responses of cashew plants infected by *L. theobromae*. As genetic resistance remains the only practical means of controlling cashew gummosis in Brazil [9], genetic studies are still being initiated to determine these resistance genes within the cashew population [6].

The ability of plants to defend themselves against pests and disease is associated with a number of proteins that can be up- or down-regulated [10,11] after challenged. Thus, proteomic analysis to identify host's proteins and their changes in abundance linked to biochemical and cellular processes that control pathogen recognition, defense signal transduction and confer resistance [12,13] is of paramount importance. Therefore, as the molecular mechanisms of plant defense that make up the interaction of cashew with *L. theobromae* are unknown, we carried out a differential proteomic analysis of cashew stems from: (i) the resistant dwarf clone BRS 226 uninoculated (control) and artificially inoculated with *L. theobromae*; and (ii) cashew plants bearing resistant and susceptible traits to gummosis, originated from open pollination of BRS 226 in a commercial orchard with high disease incidence.

Differentially represented proteins in these conditions can generate knowledge about cell signaling pathways, providing supplementary information about the pathophysiological state of cashew plants challenged by *L. theobromae*. Furthermore, this proteomic approach is important for understanding the proteome changes of tropical fruit crop in response to pathogens, once these studies are very scarce.

2. Materials and methods

2.1. Plant material and *L. theobromae* inoculation

BRS 226, a commercial cashew clone resistant to *L. theobromae* [14] was obtained from a nursery of the experimental station of Brazilian Enterprise for Agricultural Research (EMBRAPA) Tropical Agroindustry, Pacajús, Brazil, where they were prepared as following. Branches randomly collected from trees of the cashew clone BRS 226 grown in an orchard where cross-pollination was only allowed in between specimens of the same clone, were grafted on one-month-old rootstocks obtained from plantlets developed from seeds of clone CCP 06. The grafted plantlets were kept in plastic tubes and ninety days post-grafting, they were transported to a greenhouse at the Laboratory of Phytopathology (EMBRAPA-Tropical Agroindustry, Fortaleza-Ceara, Brazil). Prior fungus artificial inoculation, plants were acclimated in the greenhouse for 1 week, with a 12:12-h light–dark photoperiod, photosynthetic active radiation (PAR) of 700 $\mu m\ m^{-2}\ s^{-1}$, 27 ± 1 °C day/30 ± 2 °C night cycle, 30–70% relative humidity and sprinkler irrigation to 8.60 mm/day per plant. *L. theobromae* cultures were obtained from the fungus collection of the Laboratory of Phytopathology of EMBRAPA, transferred to potato dextrose agar (PDA, Sigma-USA) and incubated under 25 °C, 12 h light/dark cycle, for 3 days, prior inoculation. Plantlets of cashew clone BRS 226, with 100 days post-grafting, were inoculated by introducing *L. theobromae* mycelium + PDA taken from a fresh colony inside a plant stem hole of about 0.5 mm deep made with the aid of a 1.6 mm diameter drill bit fitted in a small portable electric drill machine. Mycelium-free PDA was used for control plants (uninoculated). To ensure 100% humidity and avoid dehydration, after inoculation, holes were covered with plastic paraffin film (Parafilm®). *L. theobromae*- and mock inoculated plants were maintained in a greenhouse under the conditions previously mentioned. 12, 24, 48, 72 and 96 h post-inoculation (HPI), stem segments of 6 cm in length were harvested by cutting 3 cm above and below the inoculation point. These segments were stored at −80 °C and freeze-dried continuously during four entire days (FreeZone 6 benchtop freeze dry system, Labconco, Kansas City, USA). Next the dried cuts were milled to a fine powder and stored at −80 °C until protein extraction. A total of 4 stems were pooled for each