



## Research Article

# A chemically sulfated derivative galactomannan from *Adenanthera pavonina* seeds elicits defense-related responses in cowpea and confers protection against *Colletotrichum gloeosporioides*

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**ABSTRACT:** This work was conducted to assess whether or not a natural occurring polysaccharide from *Adenanthera pavonina* seeds (PLSap) and its chemically sulfated derivative galactomannan (SPLSap), which did not act as fungicides, induce defense proteins and protect a susceptible cowpea (*Vigna unguiculata*) genotype (BR3-Tracueteua) against the phytopathogenic fungus *Colletotrichum gloeosporioides*. Twelve-day-old plants were sprayed to run-off with PLSap or SPLSap, both at 100 and 200 mg L<sup>-1</sup>, dissolved in 0.01% Triton X-100, which served as control. The primary leaves were collected at 0, 6, 12, 24, and 48 h after the carbohydrate treatments and the activities of guaiacol peroxidase (POX),  $\beta$ -1,3-glucanase ( $\beta$ GLU), and chitinase (CHI) were determined to verify the ability of the studied galactomannans to induce alterations in their kinetics. In addition, the primary leaves of a plant group previously sprayed with SPLSap and 6 h later inoculated with *C. gloeosporioides* were collected at 0, 6, 12, 24, and 48 h after the polysaccharide treatment, the enzyme activities measured, and the severity of the fungal disease evaluated. SPLSap induced more prominently POX,  $\beta$ GLU, and CHI activity than PLSap. Importantly, the combined treatment of SPLSap and *C. gloeosporioides* was even more effective as enzyme inductor than SPLSap alone, and the necrotic lesions of the anthracnose disease caused by the fungus were much less severe compared to control plants. The results indicate that SPLSap induces plant defense proteins and increases the cowpea tolerance to *C. gloeosporioides*. These findings indicate that SPLSap merits further investigation as a promising alternative or a supplemental environmentally friendly protector agent against anthracnose.

**KEY WORDS:** *Colletotrichum gloeosporioides*, cowpea, defense response, sulfated galactomannan, *Vigna unguiculata*

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## INTRODUCTION

A wide range of pathogens can attack and affect cowpea (*Vigna unguiculata* [L.] Walp.) productivity and quality. Due to its great importance as a nutritious foodstuff, particularly in the arid and semi-arid regions of the world, and the crescent demand for food, improved agronomic practices are required to increase cowpea output. These include the use of natural products to combat pathogens and pests in place of toxic pesticides that are harmful to humans and other living things, and pollute the environment (Mostafalou and Abdollahi, 2013). To reduce these damaging effects, considerable efforts have been made to develop alternative protection strategies. Plants can defend themselves against

pathogen attacks by triggering defense reactions that include physical, chemical, and enzymatic responses. Therefore, induction of plant defense responses based on the use of environmentally friendly elicitors constitutes a good strategy. Induction of plant defense mechanisms can be triggered by elicitors from the pathogen (exogenous elicitors) or from the plant itself (endogenous elicitors) (Thakur and Sohal, 2013). Once these elicitors are recognized by plant cells through interaction with specific receptors on plasma membranes the elicitor-receptor complexes formed transmit intracellular signals that will provoke the differential expression of plant defense related genes, involved with reactive oxygen species (ROS) generation, cell wall reinforcement, biosynthesis of

secondary metabolites, lytic enzymes and pathogenesis related proteins (PR-proteins), amongst other defensive compounds (Mishra *et al.*, 2012; Thakur and Sohal, 2013). Many types of elicitors of plant defense have been characterized such as fungal cell wall fragments, oligosaccharides, lipopolysaccharides, peptidoglycans, glycoproteins, and proteinogenic amino acids (Mishra *et al.*, 2012). Therefore, elicitor compounds from various biological sources such as plants, bacteria, oomycetes, and fungi as well as their potential for crop protection have been extensively investigated (Wiesel *et al.*, 2014)

Diverse biological activities have been attributed to natural and chemically sulfated derivative polysaccharides such as anticoagulation, anti-thrombotic, antiviral, antioxidant, antitumor, anti-inflammatory and immunomodulatory, antiatherosclerotic, anti-adhesive, antipeptic, antiulcerogenic, antilipidemic, and inhibition of microbial growth (Raposo *et al.*, 2013; Caputo *et al.*, 2019). In addition, it has been reported that sulfation of polysaccharides, which can turn the molecules more negatively charged and alter their three-dimensional structure, are important to induce systemic resistance in plants and increased resistance responses against pathogens (Caillot *et al.*, 2012; Trouvelot *et al.*, 2014). In consonance with the search for new environmentally friendly elicitors, which activate chemical defense in plants, the aim of this current work was to assess whether or not a natural galactomannan isolated from *Adenanthera pavonina* seeds (PLSAp) and its chemically sulfated derivative galactomannan (SPLSAp) induce plant defense-related proteins in a susceptible cowpea genotype (BR3-Tracuateua) and confer protection against *Colletotrichum gloeosporioides* disease. *A. pavonina* (Fabaceae), popularly known as Carolina, dragon's eye, and red-bead tree, is common in Brazil, where it has been used for ornamental purposes, forestation, and shading. Importantly, it was scientifically demonstrated that the bark and seeds of *A. pavonina* contain compounds that are antioxidant, anti-inflammatory, analgesic, antihypertensive, anthelmintic, antibacterial, antifungal, and antiviral (Ara *et al.*, 2010; Soares *et al.*, 2012; Godoi *et al.*, 2014).

## MATERIALS AND METHODS

### Materials

Cowpea (*Vigna unguiculata* (L.) Walp.) genotype BR3-Tracuateua (thereafter referred as BR-3) seeds were provided by the Empresa Brasileira de Pesquisa Agropecuária (Brazilian Enterprise for Agricultural Research), EMBRAPA - Meio Norte (Piauí State- Brazil) and surface sterilized with sodium hypochlorite solution (0.05% [v/v] active chlorine) for 3 min, rinsed thoroughly and soaked in distilled water for 10 minutes. Seeds were sown in 0.5-L pots containing 300 g autoclaved (120 °C,  $1.5 \times 10^5$  Pa, 30 min) river sand previously washed thoroughly with tap

water. After planting, the pots were kept in a greenhouse where average temperature varied from 24 °C (night) to 35 °C (day) and Relative Humidity (RH) varied from 55% (day) to 80% (night). The pots were exposed to natural light with Photosynthetic Photon Flux Density (PPFD) varying from 300-650  $\mu\text{moles m}^{-2} \text{s}^{-1}$  (190SA quantum sensor, LI-COR, USA) and 12 h photoperiod. During the first 3 days after sowing, irrigation was done daily with 50 mL per pot of 10x diluted proper nutrient solution (Silveira *et al.*, 2001) and, thereafter, irrigated with the undiluted solution.

The naturally occurring galactomannans from *Adenanthera pavonina* seeds (PLSAp) and its chemically sulfated derivative polysaccharides (SPLSAp) were prepared in the Laboratory of Polymer at Federal University of Ceara, Brazil. Seeds were first boiled in distilled water for 20-30 min and kept at room temperature until the seeds doubled their original size. Next the hulls and germs were removed and the endosperms reserved. The galactomannan was extracted from the seed endosperms using hot (85-100 °C) distilled water for 3 h. The gelatinous material obtained was passed through nylon net, filtered through celite, and lyophilized (Vieira *et al.*, 2007). To prepare (Godoi *et al.*, 2014) the chemically sulfated derivative galactomannan (SPLSAp), the lyophilized material was swollen in pyridine:N,N-dimethylformamide (50:10, v/v) at 25 °C under stirring until a finely dispersed suspension was obtained. After cooling at 4 °C, chlorosulfonic acid was slowly added and the suspension left under stirring for 24 hours. Next, saturated aqueous  $\text{NaHCO}_3$  was added to neutralize the material, which was dialyzed (dialysis tube with 12 kDa cutoff) for 120 h against distilled water to allow lyophilization of SPLSAp. SPLSAp presents 13.6% sulfate content percentage (S%), 1.21 degree of sulfation (DS), which represents the average number of sulfates on monomer residues and  $7.0 \times 10^5 \text{ g mol}^{-1}$  molecular mass (Godoi *et al.*, 2014).

*Colletotrichum gloeosporioides* stock cultures were from the Laboratory of Phytopathology of EMBRAPA Tropical Agroindustry (CNPAT), Fortaleza, CE, Brazil, maintained on Potato-Dextrose-Agar (PDA) and incubated at  $28 \pm 1^\circ\text{C}$ , with a photoperiod of 16 h light/8 h dark, for 7 days for mass-production. Fourteen-day-old cultures of *C. gloeosporioides* were flooded with sterile distilled water, scraped with a sterile glass rod, and the slurry obtained filtered through a four-layers of sterile cheesecloth to remove fungal mycelia and other debris. The spore suspension (filtrate) was counting in a Neubauer chamber under a microscope (Olympus System BX60) and diluted with sterile distilled water to  $4 \times 10^5$  spores  $\text{mL}^{-1}$ .

### Treatment of cowpea with PLSAp and SPLSAp

To assess the ability of the studied galactomannans in

inducing plant defense responses, visually healthy cowpea plantlets were selected twelve days after sowing and the whole canopy of a group of plants was sprayed (100 or 200 mg L<sup>-1</sup>) to run-off with PLS*Ap* and other group with SPLS*Ap*, both prepared in 0.01% (v/v) Triton X-100, which was used to spray a third group of control plants. Plants were placed in a highly humid chamber (RH > 80%, 22 ± 1 °C) overnight, after which they were submitted to a 12 h light/12 h dark photoperiod. The primary leaves were collected at 0, 6, 12, 24, and 48 h after the carbohydrate treatments, immediately conditioned in plastic bags, and kept at -85 °C until analyses. These consisted in determining the alterations on the kinetic of the enzyme activity of guaiacol peroxidase (POX), β-1,3-glucanase (βGLU), and chitinase (CHI), taken as plant defense-related proteins and used as molecular markers of the ability of the studied galactomannans in inducing plant defense responses. To evaluate whether SPLS*Ap* confer any protection to the susceptible cowpea genotype against the studied fungus, a plant group was previously sprayed with SPLS*Ap* and other with 0.01% (v/v) Triton X-100 (control), as above. Six hours later they were inoculated with *C. gloeosporioides* by applying microdrops (100 μL) of the fungus suspension (4 × 10<sup>5</sup> spores mL<sup>-1</sup>) on the adaxial cowpea leaf blade. These plant groups were placed in a highly humid chamber (RH > 80%, 22 ± 1 °C) overnight, after which they were submitted to a photoperiod of 12 h light/12h dark regime, RH kept > 60%, and temperature at 24 ± 1 °C. The appearance of any disease (anthracnose) signal in leaves was evaluated 12 days after *C. gloeosporioides* inoculation and severity determined according to the rating scale proposed by Suryanto *et al.* (2014): 0 = no symptom; 1 = 0 to 20%; 2 = 20 to 40%; 3 = 40 to 60%; 4 = 60 to 80%; and 5 = 80 to 100% disease severity range. The enzyme activity of ascorbate peroxidase (APX), POX, βGLU, and CHI were also evaluated at 0, 6, 12, 24, and 48 h after the carbohydrate treatments. Every treatment was conducted in triplicates, each consisting of six plants in individual pots. The experiment was thrice repeated under the same above conditions.

#### Preparation of enzyme extracts from primary leaves of cowpea

The extraction of soluble proteins was performed by macerating the primary cowpea leaves collected from each experiment, and for each time point, with 0.05 M sodium acetate buffer (pH 5.2) containing 0.50 M NaCl (in a relation of 1 g of leaves per 5 mL of buffer), for 15 min, using a pre-chilled mortar and pestle kept on ice bath. The suspension was filtered through one layer of cheesecloth and centrifuged at 10,000 g for 5 min at 4 °C. The supernatant was recovered and dialyzed against the extracting buffer for 24 h (six changes with ten times the supernatant volume, at a 12-h interval) at 4 °C. The dialyzed leaf extract (thereafter DLE) obtained from each treatment and time point was utilized for

protein content and enzymatic activity measurements.

#### Protein content determination

Total soluble protein content of DLE was quantified spectrophotometrically (Bradford, 1976). To 100 μL DLE 2.5 mL Bradford reagent were added and 10 min later the absorbance readings of triplicates were measured at 595 nm. The protein content was calculated on the base of a standard curve built with known concentrations of Bovine Serum Albumin (BSA).

#### Enzyme assays

The enzymatic activities of DLE were conducted using an amount of protein in the linear range of the assays. Three enzymatic independent assays for each time point were carried out using three independent DLE samples, each one obtained from 3 plants.

POX activity was measured as previously described (Urbanek *et al.*, 1991). A 10 μL aliquot of DLE was added to 990 μL of 50 mM sodium acetate buffer (pH 5.2), 500 μL of 60 mM H<sub>2</sub>O<sub>2</sub>, and 500 μL 20 mM guaiacol (Sigma Chemical Company). The reaction mixture was incubated at 30°C and the increase in absorbance at 480 nm recorded during 2 min at 20 s intervals. The variation of 1.0 absorbance unit mL<sup>-1</sup> min<sup>-1</sup> (ΔAbs<sub>480nm</sub> mL<sup>-1</sup> min<sup>-1</sup>) was taken as one POX activity (1 AU), which was expressed per gram of leaf fresh mass (AU g<sup>-1</sup> FM).

βGLU activity was measured by determining the amount of glucose liberated from laminarin (Sigma-Aldrich) used as substrate (Boller, 1992). Laminarin was dissolved in ultrapure water (Milli-Q grade), heated to 60 °C for 10 min, and dialyzed exhaustively against ultrapure water (Milli-Q) for removal of free glucose. The assay was conducted with 0.1 mL of sample incubated with 0.9 mL of laminarin (2.0 mg mL<sup>-1</sup>) at 50 °C for 30 min. After addition of the appropriate reagents to the reaction mixture, according to Boller (1992), absorbance readings were taken at 520 nm. The quantity of glucose monomers released from laminarin was determined using a standard curve built with known amounts (42.2 to 1332.1 nM) of the corresponding sugar. βGLU activity was expressed in nanokatal per gram fresh mass (nkat g<sup>-1</sup> FM). One nkat is equivalent to 1.0 nmol of D-glucose released mL<sup>-1</sup>s<sup>-1</sup>.

CHI activity assay was conducted by measuring free N-acetyl-D-glucosamine (GlcNAc) released from non-radioactive colloidal chitin (Molano *et al.*, 1977) used as substrate by the combined hydrolytic action of cowpea chitinases and β-glucuronidase (Sigma-Aldrich), as previously described (Boller, 1992). GlcNAc content was assessed using a colorimetric method (Reissig *et al.*, 1995), at 585 nm, based

on a standard curve of commercial GlcNac (Sigma–Aldrich), ranging from  $1 \times 10^5$  to  $6 \times 10^5$  nM concentrations. Both assays and the standard curve were developed in 50 mM sodium acetate buffer (pH 5.2). Chitinase activity was expressed in nanokatal per gram of leaf fresh mass (nkat  $g^{-1}$  FM). One nkat is equivalent to 1.0 nmol of GlcNac released  $mL^{-1}s^{-1}$ , at 37 °C.

APX activity was determined by adding 0.1 ml of DLE to 0.8 ml of a solution containing 0.5 mM L-ascorbic acid diluted in 50 mM potassium phosphate buffer, pH 6.0, and 0.1 mL of 2 mM  $H_2O_2$ . The decrease in absorbance at 290 nm was monitored during 2 min at 20 s intervals at 30 °C (Koshiba, 1993; Nakano and Asada, 1981). One APX unit of activity (1 UA) was defined as the decrease of 1.0 absorbance unit  $mL^{-1}min^{-1}$  ( $\Delta Abs_{290nm}$   $mL^{-1}min^{-1}$ ) and expressed as UA per gram of leaf fresh mass (UA  $g^{-1}$  FM).

### Effect of SPLS*Ap* on spore germination and vegetative growth of *Colletotrichum gloeosporioides*

To assess whether SPLS*Ap* have direct effects on both *C. gloeosporioides* spore germination and mycelial growth the methodology described by Freire *et al.* (2002), with modifications, was employed using 96-well ELISA plates. Inhibition of *C. gloeosporioides* spore germination was assayed as follow: in each well 100  $\mu$ L of Yeast Potato Dextrose (YPD) medium received 10  $\mu$ L of the spore suspension ( $2 \times 10^5$  cells  $mL^{-1}$ ), and 100  $\mu$ L SPLS*Ap* at 100 and 200 mg  $L^{-1}$  concentrations in 0.01% (v/v) Triton X-100, previously filtered through Millex GV (0.22  $\mu$ m) filter. After 48 h, spores were examined under a light microscope (Olympus BX 60 Microscope System). A spore was considered germinated when the germination hypha length emitted corresponds to at least twice the length of the largest spore diameter (Paul *et al.*, 1992). To examine the possible inhibitory effect on the fungus mycelial growth the spore suspension was added to the wells as above to allow spore germination and 12 h later SPLS*Ap* at 100 and 200 mg  $L^{-1}$  concentrations in 0.01% (v/v) Triton X-100, previously filtered through Millex GV (0.22  $\mu$ m) filter, were added. Absorbance readings were taken at 630 nm in an ELISA reader (EL<sub>x</sub> 800 Bio-Tek Instruments Inc.) at 12 h intervals, up to 96 h after the carbohydrate or control incubation with the fungus. Triton X-100 (0.01%) was used as the negative and hydrogen peroxide (100 mM) as the positive controls.

### Statistical analysis

Data were subjected to one-way analysis of variance (ANOVA) and the significance of differences ( $p \leq 0.05$ ) between means evaluated using the Tukey's test.

## RESULTS AND DISCUSSION

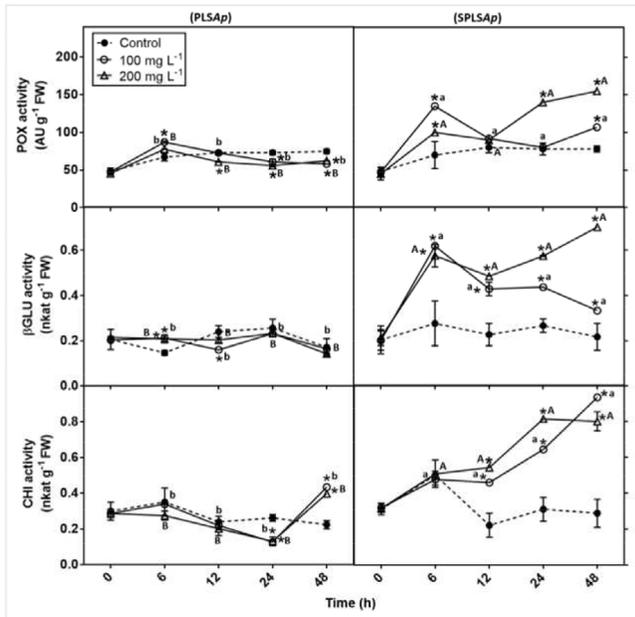
### SPLS*Ap* induced biochemical defense responses in cowpea

The chemically sulfated derivative galactomannan (SPLS*Ap*) induced the PR-proteins POX,  $\beta$ GLU, and CHI (Fig. 1) more prominently than the natural galactomannan (PLS*Ap*) in comparison to the respective control plants. SPLS*Ap* treatment, at 100 mg  $L^{-1}$  increased ( $p \leq 0.05$ ) POX activity at 6 h ( $\approx 97\%$ ) and 48 h ( $\approx 28\%$ ). Increased values ( $p \leq 0.05$ ) of POX was also perceived after treatment with SPLS*Ap* at 200 mg  $L^{-1}$  at 6 h ( $\approx 35\%$ ), 24 h (79.5%), and 48 h ( $\approx 81\%$ ) (Fig. 1). PLS*Ap* also increased POX activity at 100 mg  $L^{-1}$ , but only at 6 h ( $\approx 37\%$ ), whereas at 24 h and 48 h there were decreases in POX activity around  $\approx 36\%$  and  $\approx 15\%$ , respectively. At 200 mg  $L^{-1}$ , PLS*Ap* also diminished POX activity at 12 h ( $\approx 25\%$ ), 24 h (36%), and 48 h ( $\approx 15\%$ ) post carbohydrate treatment.

Induction ( $p \leq 0.05$ ) of  $\beta$ GLU activity (Fig. 1) by 100 mg  $L^{-1}$  SPLS*Ap* was  $\approx 136\%$ ,  $\approx 84\%$ , 63.7%, and 53.2%, respectively, at 6 h, 12 h, 24 h, and 48 h after the carbohydrate treatment over that of control plants. At 200 mg  $L^{-1}$ , SPLS*Ap* augmented ( $p \leq 0.05$ )  $\beta$ GLU activity by  $\approx 114\%$ ,  $\approx 108\%$ ,  $\approx 114\%$ , and  $\approx 232\%$ , at 6 h, 12 h, 24 h, and 48 h after SPLS*Ap* treatment, respectively, in relation to the respective control plants (Fig. 1). In contrast, PLS*Ap* treatment exerted little effect on  $\beta$ GLU activity.

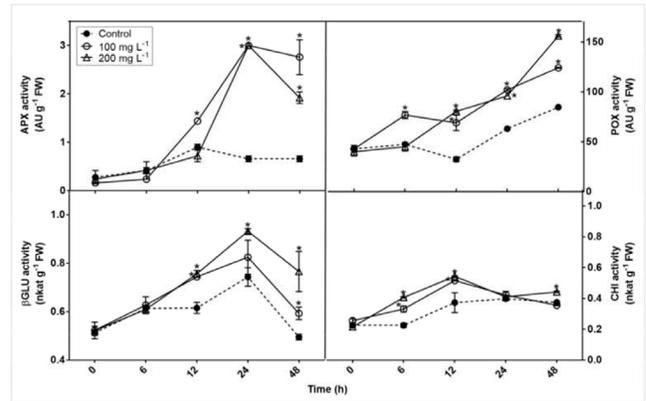
Analysis of CHI activity profiles of cowpea plants sprayed with SPLS*Ap* at 100 mg  $L^{-1}$  revealed significant ( $p \leq 0.05$ ) increases in activity over those of control plants by around 92%, 100%, and 207% at 12 h, 24 h, and 48 h, respectively. Application of SPLS*Ap* at 200 mg  $L^{-1}$  also provoked increases of approximately 117%, 150%, and 160% over those of the corresponding control plants at 12 h, 24 h, and 48 h, respectively. In regard to PLS*Ap*, the tendency of the CHI activity values was to be similar or below those of the respective control plants, from 6 h to 24 h after the treatment at both carbohydrate concentrations. However, at 48 h, the CHI activity increased by 109% for 100 mg  $L^{-1}$  and 73% for 200 mg  $L^{-1}$ . In summary, comparative examination of the enzyme kinetics tested within every time point studied after the carbohydrate treatments revealed overall higher activity ( $p \leq 0.05$ ) in plants treated with SPLS*Ap* over those treated with PLS*Ap* (Fig. 1).

As SPLS*Ap* promoted the highest increased in POX,  $\beta$ GLU, and CHI compared to PLS*Ap* (Fig. 1), the combined treatment in which the carbohydrate (100 or 200 mg  $L^{-1}$  concentrations) was applied and 6 h later the plants were inoculated with *C. gloeosporioides* was carried out exclusively using SPLS*Ap*. Under these combined conditions, APX, an important antioxidant enzyme, increased in activity (Fig. 2), particularly at 24 h (354.5% and 354.5% applying 100 and 200 g  $L^{-1}$ , respectively) and 48 h (318.2% and 190.9%, applying 100 and 200 g  $L^{-1}$ , respectively) after



**Fig. 1** Antioxidant enzymes and PR-protein kinetics in cowpea leaves treated with the natural (PLSAp) and chemically sulfated derivative galactomannan (SPLSAp). POX,  $\beta$ GLU, and CHI activities were measured in the primary leaves sprayed until dripping (~ 10 mL in each plant) with 100 and 200 mg L<sup>-1</sup> of PLSAp or SPLSAp, prepared in 0.01% (v/v) Triton X-100, and evaluated 0, 6, 12, 24 and 48 h after the carbohydrate treatments. Control plants were sprayed with 0.01% (v/v) Triton X-100. Asterisk (\*) indicates significant difference between the treatments and control. Lowercase letters represent significant differences ( $p \leq 0.05$ ) between the treatments with PLSAp and SPLSAp at 100 mg L<sup>-1</sup> concentration and capital letters represent differences ( $p \leq 0.05$ ) between the treatments at 200 mg L<sup>-1</sup>, calculated by the Tukey's test at a confidence level of 5% ( $p \leq 0.05$ ).

the SPLSAp treatment in relation to control plants treated with 0.01% (v/v) Triton X-100. Higher significant increases ( $p \leq 0.05$ ) in POX activity were also observed at 6 h (63%) after applying 100 g L<sup>-1</sup> SPLSAp and after 12 h (95.2% and 125.2%, applying 100 and 200 g L<sup>-1</sup>, respectively), 24 h (39.5% and 53.2%, applying 100 and 200 g L<sup>-1</sup>, respectively), and 48 h (48.2% and 85.4%, applying 100 and 200 g L<sup>-1</sup>, respectively) in comparison with those of control plants (Fig. 2). Likewise, the plants previously treated with SPLSAp, at 100 mg L<sup>-1</sup>, followed 6 h later by *C. gloeosporioides* inoculation had also much higher  $\beta$ GLU activity at 12 h (56.2%) and 48 h (93.3%) after the SPLSAp treatment, over that of control plants (Fig. 2). Using SPLSAp at 200 mg L<sup>-1</sup> concentration, followed 6 h later by *C. gloeosporioides* inoculation, the  $\beta$ GLU activity significantly increased ( $p \leq 0.05$ ) at 12 h (64.0%), 24 h (55.3%), and 48 h (257.5%) in comparison with the control plants. CHI activity also increased ( $p \leq 0.05$ ) in cowpea plants treated with SPLSAp, at 100 and 200 mg L<sup>-1</sup>, followed by *C. gloeosporioides* inoculation (Fig. 2),



**Fig. 2** Antioxidant enzymes and PR-protein kinetics in cowpea leaves treated with the chemically sulfated derivative galactomannan (SPLSAp) and 6 h later infected with *Colletotrichum gloeosporioides*. APX, POX,  $\beta$ GLU, and CHI activities were measured in the primary leaves sprayed until dripping (~10 mL in each plant) with 100 and 200 mg L<sup>-1</sup> SPLSAp prepared in 0.01% (v/v) Triton X-100 and 6 h later inoculated with *C. gloeosporioides*. Control plants were sprayed with 0.01% (v/v) Triton X-100 and 6 h later inoculated with *C. gloeosporioides*. All samples were evaluated 0, 6, 12, 24, and 48 h after the carbohydrate treatments. Error bars indicate standard deviations. Asterisk (\*) indicates significant difference between the treatment and control, calculated by the Tukey's test at a confidence level of 5% ( $p \leq 0.05$ ).

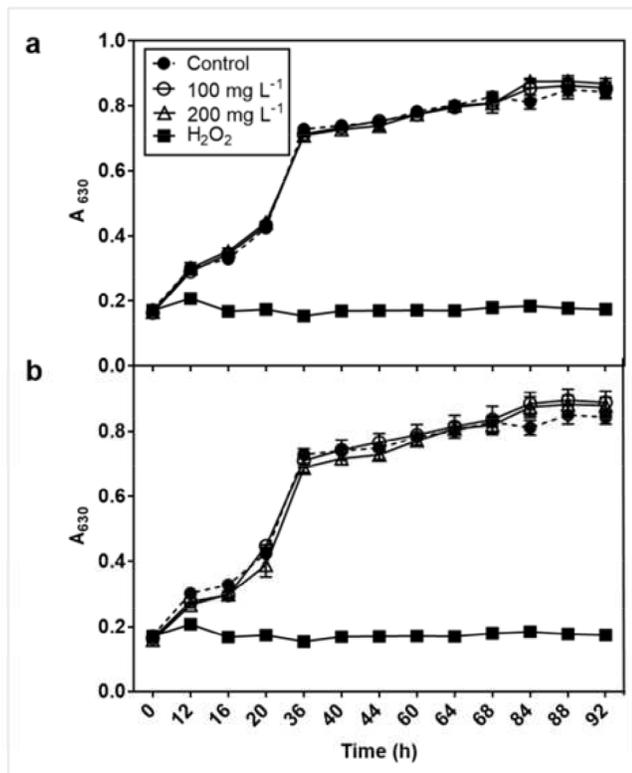
particularly at 6 h (36.4% and 77.3% for 100 and 200 mg L<sup>-1</sup> SPLSAp treatment, respectively) and 12 h (38.4% and 46% for 100 and 200 mg L<sup>-1</sup> SPLSAp treatment, respectively) in relation to control plants. However, at 48 h, only application of 200 mg L<sup>-1</sup> SPLSAp followed 6 h later by *C. gloeosporioides* inoculation showed a significant ( $p \leq 0.05$ ) augment (27.0%) respective to control plants. Importantly, it was not found any significant differences at 0 h, for all enzyme activities tested between the SPLSAp-treated (100 and 200 mg L<sup>-1</sup>) and controls plants.

**SPLSAp had no effect on the *Colletotrichum gloeosporioides* spore germination and mycelial growth**

SPLSAp used as elicitor of plant defense in this study did not inhibit *in vitro* the *C. gloeosporioides* spore germination or mycelial growth (Fig. 3), at any of the concentrations (100 and 200 mg L<sup>-1</sup>) tested. This result indicates that SPLSAp has no direct antifungal action on *C. gloeosporioides*.

**SPLSAp protected cowpea leaves against *Colletotrichum gloeosporioides* infection**

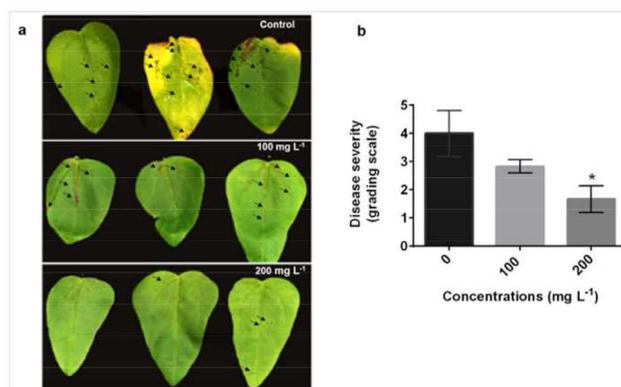
The macroscopic examination of the primary leaves (Fig. 4a) inoculated only with the fungus (control) showed intense and numerous necrotic regions, 10 days after infection (Fig. 4a). Similarly, the primary leaves of cowpea plants previously treated with 100 mg L<sup>-1</sup> SPLSAp and challenged with the fungus also showed necrotic lesions,



**Fig. 3** Effect of SPLSAp on the germination (a) and growth (b) of *Colletotrichum gloeosporioides*. Control: 0.01% (v/v) Triton X-100; Treatments: 100 and 200 mg L<sup>-1</sup> SPLSAp prepared in 0.01% (v/v) Triton X-100; Positive control: 100 mM H<sub>2</sub>O<sub>2</sub> in ultrapure Milli-Q grade water. Vertical bars represent standard deviation. Each assay was carried out in triplicate.

but at much less extent (Fig. 4a) compared with the control cowpea plants although not quantitatively significantly different (Fig. 4b). However, the primary leaves of cowpea plants previously treated with 200 mg L<sup>-1</sup> SPLSAp followed by inoculation with *C. gloeosporioides* spores presented very few necrotic lesions (4a) that characteristically possessed very low signal intensity and a lower ( $p \leq 0.05$ ) disease severity (Fig. 4b) relative to the control plants solely inoculated with the fungus (Fig. 4b).

The use of natural and derivative inducers of resistance in plants for pest and pathogen management have become a promising option for triggering plant immunity and plant protection because they can reduce the residues left in fruits and vegetables after application of pesticides (Caillot *et al.*, 2012; Llorens *et al.*, 2017). In this present work we report that SPLSAp, a chemically sulfated carbohydrate derived from a natural galactomannan (PLSAp) from *A. pavonina* seeds, has potential to be used as a plant defense elicitor that protects cowpea (genotype BR-3) against *C. gloeosporioides*. Indeed, in parallel to induce changes in POX,  $\beta$ GLU, and CHI (Fig. 1) SPLSAp mitigated the severity of the anthracnose symptoms caused by the hemibiotrophic fungus



**Fig. 4** Protective effect of SPLSAp on cowpea plants against *Colletotrichum gloeosporioides* evaluated 10 days after the fungus infection. (a) Average disease development and (b) disease severity in cowpea primary leaves infected with *C. gloeosporioides*. Plants were sprayed until dripping (~10 mL in each plant) with 100 or 200 mg L<sup>-1</sup> SPLSAp prepared in 0.01% (v/v) Triton X-100 and 6 h later inoculated with *C. gloeosporioides* spores. Control plants were sprayed with 0.01% (v/v) Triton X-100 and 6 h later also inoculated with the fungus. The mock- inoculated plants were treated with Milli-Q grade water. Error bars represent standard deviation. Asterisk (\*) indicates significant differences between treatment and control, calculated by the Tukey's test at a 5% confidence level ( $p \leq 0.05$ ). Grading scale: 0 = no symptom; 1 = 0 to 20% disease severity; 2 = 20 to 40% disease severity; 3 = 40 to 60% disease severity; 4 = 60 to 80% disease severity; 5 = 80 to 100% disease severity.

*C. gloeosporioides* (Fig. 4). Enhanced defense responses and reduction of disease symptoms have been reported for other carbohydrate elicitors such as the yeast saccharide (Yu *et al.*, 2012), oligo-carrageenans (Vera *et al.*, 2012), and chitosan (Chen *et al.*, 2014) among others. Sangha *et al.*, (2011) showed that carrageenans have differential effects on *Arabidopsis* resistance to *Trichoplusia ni* and that the degree of sulfation of the polysaccharide chain mediates this effect. Recently, it was reported that application of ulvan, a water-soluble polysaccharide from green seaweeds, in bean (*Phaseolus vulgaris*) leaves was effective in reducing locally and systemically the anthracnose severity in a *Colletotrichum lindemuthianum* susceptible genotype (Freitas and Stadnik, 2012).

In our work, SPLSAp sprayed alone at 100 and 200 mg L<sup>-1</sup> concentrations (Fig. 1) and in combination with *C. gloeosporioides* inoculation (Fig. 2) induced considerable increases in the leaf POX activity of cowpea plants. POX are well studied enzymes in plant/pathogen interaction. They participate in the reaction of H<sub>2</sub>O<sub>2</sub> dependant polymerization of hydroxycinnamyl alcohols for lignin biosynthesis and deposition that reinforce the plant cell wall (Voxeur *et al.*, 2015) in opposition to the entry of pathogens. Increased POX activity

in plant tissues can arise from the contact with pathogens, mechanical damages, and chemicals, which distinguish this enzyme as a marker of resistance. Recently, Nikraftar *et al.* (2013) demonstrated that the resistance of tomato cultivars to *Rhizoctonia solani* isolates is related to antioxidant defense mechanisms that involve APX and POX. The data presented in our work showed increased POX activity in the cowpea leaves after SPLSAp treatment (Fig. 1), particularly at 200 mg L<sup>-1</sup> concentration. The POX activity was even higher when the SPLSAp treatment (100 and 200 mg L<sup>-1</sup>) was combined with *C. gloeosporioides* inoculation (Fig. 3). This suggested an added inducing effect probably by pathogen-/microbe associated molecular patterns (PAMPs/MAMPs) from *C. gloeosporioides* and/or damage-associated molecular patterns (DAMPs) elicitors from cowpea, which were recognized by plant pattern recognition receptors (PRRs) and triggered defense responses (Boutrot and Zipfel, 2017). The foliar spray of ulvan increased POX activity in inoculated susceptible bean (*P. vulgaris*) and reduced in 60 and 40% the anthracnose severity caused by *C. lindemuthianum* at the site of infection and systemically, (Freitas and Stadnik, 2012). Previously, we have noticed, in a time-course study, association of a rapid increase in the PR-protein POX activity during an incompatible interactions of a cowpea genotype (TE 97-411-1E) with *C. gloeosporioides* (Oliveira *et al.*, 2013).

Another important enzyme that participates in the process of plant defense is APX, which is involved in the response to oxidative stress. Increased activity of APX in response to different stresses has been previously reported (Zeng *et al.*, 2010; Jindrichová *et al.*, 2011). In our work, SPLSAp sprayed in combination with *C. gloeosporioides* inoculation induced considerable increases in the leaf APX activity of the cowpea plants (Fig. 2). The foliar spray of *Artemisia annua* with chitosan induced a peak of APX activity and other enzymes related to plant defense, 24 h after application (Lei *et al.*, 2011). For instance, one of the main events that occur at the onset of plant pathogen infection is the generation of ROS, particularly hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) (Waszczak *et al.*, 2018). Accumulation of free radicals after pathogen attack can cause oxidative damage, deregulation of several metabolic pathways and physiological processes (Foyer and Noctor, 2016). Therefore, antioxidant enzymes as APX have fundamental roles in the establishment of cell homeostasis and prevention of plant cell death, which could favor the necrotrophic stage of the *C. gloeosporioides* life cycle (Elloy *et al.*, 2015). Actually, APX is considered one of the most important H<sub>2</sub>O<sub>2</sub>-scavenging enzymes in the chloroplasts and cytosol. During reaction, APX uses ascorbate as a reducing agent, which is regenerated through the glutathione-ascorbate cycle (Waszczak *et al.*, 2018). Zeng *et al.* (2010) suggested that chitosan treatment could induce disease resistance in navel orange (*Citrus sinensis*) fruit by regulating the H<sub>2</sub>O<sub>2</sub>

levels, antioxidant enzyme and ascorbate-glutathione cycle.

βGLU activity also increased when the cowpea leaves were treated with SPLSAp (Fig. 2). Similarly, to POX and APX activity, cowpea plants pre-treated with SPLSAp and 6 h later inoculated with *C. gloeosporioides* showed higher increases in βGLU activity (Fig. 3) in comparison with plants that received solely the SPLSAp spray (Fig. 2). Elicitor-inducing effects of SPLSAp together with recognition of MAMPs/PAMPs from *C. gloeosporioides* and/or DAMPs from cowpea by PRRs (Boutrot and Zipfel, 2017) might have contributed to this higher βGLU response. βGLU belongs to the PR-2 family of PR-proteins and catalyzes the breakdown of endo-β-1,3-glycolytic bonds present in β-1,3-glucans, abundant in the cell wall of fungi (Feofilova, 2010). Usually, the βGLU content is low in healthy plants. However, in pathogen infected plants or in elicitor induced plants βGLU activity increases rapidly and, in general, this implies that the Systemic Acquired Resistance (SAR) was established since induction of this PR-protein is a marker of active defense (Bargabus *et al.*, 2004).

In relation to CHI, increased activity in cowpea leaves treated with SPLSAp at both concentrations (100 and 200 mg L<sup>-1</sup>) (Fig. 2) was noticed soon after 12 h exposition. In the group of plants pre-treated with SPLSAp and post-inoculated with *C. gloeosporioides*, there was also a significant increase in CHI activity in relation to control plants, but at the first hours (6-12 h) after the SPLSAp treatment (Fig. 3). This earlier induction in the combined treatments compared with the treatment of plants solely with SPLSAp without fungal post-inoculation might also be an added inducing effect of MAMPs/PAMPs from *C. gloeosporioides* and/or DAMPs from cowpea (Boutrot and Zipfel, 2017) that led to a more quickly response to the fungal aggression. For instance, PR-proteins are well defined as proteins that are induced by pathogens and elicitors (Van Loon *et al.*, 2006; Boutrot and Zipfel, 2017). βGLU and CHI are believed to contribute to plant defense in two ways. Directly, by degrading the β-1,3-glucan and chitin, respectively, from the fungal cell walls and destroying the pathogenic organisms integrity, or indirectly by releasing elicitor-active oligosaccharide from the fungal cell wall (Ji and Kuć, 1996). These released elicitors can be detected by receptors of the host plant where they trigger gene activation, production of antimicrobial compounds, and possibly Programmed Cell Death (PCD) that led to both local and systemic disease resistance (Gururania *et al.*, 2012). Indeed, PR-proteins such as βGLU and CHI have been shown to be associated with induced resistance to pathogens (Falcón-Rodríguez *et al.*, 2011; Caillot *et al.*, 2012). For example, transgenic plants expressing one or more PR-proteins showed enhanced resistance to infection

by fungi (Kishimoto *et al.*, 2002; Wang *et al.*, 2003; O’Kennedy *et al.*, 2011).

The necrotic lesions presented in the primary leaves of cowpea inoculated with *C. gloeosporioides* were large in size and numerous and spread along the entire leaf blade (Fig. 4), resembling the typical lesions of anthracnose (bronze-colored to brown, circular, deepened, and moistened) as reported for other cowpea varieties (Bailey *et al.*, 1990). However, the plants previously treated with SPLSAp and next inoculated with *C. gloeosporioides* showed weak disease severity with lesions reduced in size and quantity, mainly in the cowpea plants treated with the highest carbohydrate concentration (200 mg L<sup>-1</sup>). This means that the pre-treatment with SPLSAp enhanced resistance of cowpea to anthracnose (Fig 5). Importantly, we found that SPLSAp had no *in vitro* deleterious effects on the germination or mycelial growth of *C. gloeosporioides*. Good defense response inducers are those devoid of direct fungicidal action *per se* (Terry and Joyce 2004; Bautista-Baños *et al.*, 2006; Zhang *et al.*, 2013). This trait for elicitors is advantageous because they do not act directly on non-target organisms holding exciting promise for their application in agriculture. However, for SPLAAp to become commercially acceptable and available several questions have to be addressed (Favel, 2005): (a) does SPLAAp control anthracnose under field conditions? (b) is SPLAAp effective alone or it has to be combined with biological chemicals or biological control agents?; (c) is the cost of SPLAAp production compatible with an efficient induction of resistance?; (d) is SPLAAp effective to be used in multiple locations and seasons?; (e) is the large scale production of SPLAAp limited by the restrictive number of seeds available to extract its precursor galactomannans?; (f) as a product, how long it can be stored for commercialization?. In summary, many challenges need to be overcome before SPLAAp becomes an alternative elicitor of direct plant defence reactions.

In conclusion, the chemically sulfated derivative galactomannan (SPLSAp) produced from the naturally occurring galactomannan of *A. pavonina* seeds has the potential to be used as an elicitor toward enhancing cowpea resistance to anthracnose. SPLSAp induced cowpea defense reactions by changing the kinetic patterns of some PR-proteins and increasing the tolerance of cowpea against the fungus *C. gloeosporioides*. Thus, SPLSAp constitutes an elicitor that may be recognized by receptors of the cowpea cells and lead to molecular events that trigger plant defense reactions. Accordingly, SPLSAp merits further investigation as a promising alternative or supplemental environmentally friendly elicitor to protect cowpea against anthracnose.

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## AUTHOR CONTRIBUTIONS

All authors contribute equally with this manuscript.

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