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Cytological and histochemical gradients induced by a sucking insect in galls of *Aspidosperma australe* Arg. Muell (Apocynaceae)

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ABSTRACT

The storage of carbohydrates and lipids was previously investigated in nutritive tissues of galls of Cecidomyiidae and Cynipidae. Unexpectedly, starch accumulation has been detected in non-nutritive galls induced by Hemiptera, which feed directly from phloem bundles. Samples of non-galled leaves and galls induced by *Pseudophacopteron* sp. in *Aspidosperma australe* were processed for light and electron microscopy. Histochemical tests detected sites of ROS (reactive oxygen species), carbohydrates, and enzymes. PCD (programmed cell death) evidenced by plastoglobules and ROS formation also occurred. Phosphorylase and sucrose synthase activity indicated the steps of starch storage. The sites of glucose-6-phosphatase activity were related to the provision of sucrose for gall growth and nutrition of *Pseudophacopteron*. Acid phosphatase took part in the metabolism of starch and degradation of some organelles during the main trophic phase of the insect. The invertases were related to the sites of hyperplasia, and regulation of cell growth and intense respiration. The cytological and histochemical gradients validate the storage of starch as a pattern in galls induced by sucking insects. The detection of enzymes related to carbohydrate metabolism and sites of ROS production is described for the first time for galls induced by sucking insects in the Neotropical region.

1. Introduction

Insect galls commonly store substances that provide nourishment to the gall inducer, and take part in regulating the morphogenesis of the gall itself. These substances are located in specialized cells [1]. Lipids accumulate in the galls induced by Cynipidae; whereas carbohydrate accumulation prevails in galls induced by Cecidomyiidae [1]. Variations in these patterns may occur, as demonstrated by the detection of lipid droplets in nutritive tissues of galls induced by *Schismatodiplosis lantanae* (Cecidomyiidae) in *Lantana camara*, a lipid-producing Verbenaceae [2]. This may be evidence that the storage of lipids and carbohydrates is potentially constrained by the host plant metabolism. Studies of cellular differentiation and histochemical detection of reserve substances in galls induced by insects with sucking feeding habits are few, especially in the Neotropical region, and so, to the best of our knowledge, no pattern of reserve metabolism has been proposed for these gall systems.

Bronner [1] used histochemical techniques to propose reserve metabolism patterns in Cynipid and Cecidomyiid galls. Histochemical techniques are precise methods of localizing metabolites, and consequently of assessing the metabolism patterns of plant cells related to the nutrition of galling herbivores. Moreover, histochemical and cytological techniques may diagnose some stresses generated by the presence of the gall inducer within plant tissues [3,4], as well as the impacts of their different feeding habits. Among the host plant's responses to oxidative and respiratory stresses is the generation of reactive oxygen species (ROS) with the breakdown of membrane systems in chloroplasts and mitochondria, as well as the formation of plastoglobules [5,6]. The generation of ROS in gall tissues can be easily assessed by cytological analysis and confirmed by histochemical techniques [7].

Sucking gall-inducing insects are common in the Neotropical fauna [8], and their feeding site may be restricted to phloem cells where they introduce their stylets. This behavior may cause the deposition of "wound callose", which forms a physical barrier at the plasma membrane, and consequently may be the first step in the plant's reaction to the presence of the herbivore [9]. In the *Pseudophacopteron* sp. (Hemiptera)–*Aspidosperma australe* (Apocynaceae) gall system, due to the use of phloem as the feeding site [10], a nutritive tissue should be absent, and the storage of nutritional reserves and the enzyme activity should be related to maintaining the gall structure, as proposed by Oliveira et al. [11]

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for the *Euphalerus ostreoides–Lonchocarpus muehlbergianus* gall system. Because of the amplitude of the cecidogenic field [12,13], the highest metabolic activity should be expected in the tissues near the nymphal chamber, where respiratory and oxidative stresses are greater. These assumptions should be confirmed by cytological and histochemical analyses.

By studying the cytological and histochemical features of the *Pseudophacopteron* sp.–*A. australe* gall system, this investigation aimed to answer the following questions: (i) does the feeding activity of *Pseudophacopteron* sp. alter the storage of metabolites in its host plant? (ii) Is there a gradient of storage substances and enzymes in *A. australe* gall tissues? (iii) Do gall cytological features indicate oxidative stress? (iv) Do galls induced by *Pseudophacopteron* sp. in *A. australe* follow the patterns described in the literature for sucking gall inducers?

2. Materials and methods

2.1. Plant material collection

Tissue samples of non-galled leaves ($n \geq 12$) and of galls in three developmental stages, sorted by size, (immature galls 1.0 ± 0.3 mm wide, mature galls, 5.0 ± 0.5 mm wide, and senescent galls, 5.0 ± 0.7 wide and open) ($n \geq 12$ per stage), were collected from *A. australe* individuals ($n = 10$) located on the Pampulha campus of the Universidade Federal de Minas Gerais, Belo Horizonte, MG, Brazil. The collections were made from January through December 2008, at intervals of 2 months, and the samples obtained were submitted to cytological and histochemical analyses.

2.2. Cytological analysis

The samples were fixed in 4% Karnovsky in 0.1 M phosphate buffer (pH 7.2) for 24 h [14], post-fixed in 1% osmium tetroxide in 0.1 M phosphate buffer (pH 7.2), dehydrated in an ethanol series [15], and embedded in Araldite® [16]. The material was cross-sectioned in an Ultramicrotome Reichert-Jung – Ultracut, contrasted in uranyl acetate and lead citrate according to Reynolds [17], and examined in a Zeiss EM 109 transmission electron microscope.

2.3. Histochemical analysis

2.3.1. Nucleic acids

Samples of non-galled leaves and mature galls were fixed in Carnoy for 48 h, dehydrated in an ethanol series, embedded in Leica® historesin, and cross-sectioned (5–10 μm) in a Reichert Jung® rotating microtome [15]. The material was stained with 0.15% methyl green and 0.25% pyronin B in acetate buffer (pH 4.7) [18]. For the controls, RNA and DNA were extracted by incubating the slides in 0.1% ribonuclease (pH 6.8) for 1 h at 40 °C, and in 0.2 mg deoxyribonuclease ml^{-1} in 0.003 M magnesium sulphate (pH 6.5) for 1 h at 25 °C, respectively.

2.3.2. Primary metabolites

Handmade sections of fresh material were used for detection of proteins, lipids, starch, and reducing sugars. For proteins, the sections were stained in 0.1% bromophenol blue in a saturated solution of magnesium chloride in ethanol for 15 min, and then washed in acetic acid and water [19]. For starch detection, the sections were immersed in Lugol reagent (iodine potassium iodide) for 15 min [15]. For reducing sugars, sections were immersed in Fehling reagent [20]. For lipids, the sections were immersed in a saturated solution of Sudan III (CI 26100) in 70° GL ethanol [21]. For the controls, lipids were extracted with methanol:chloroform (1:1, v/v), and starch with salivary amylase. Blank sections were used for comparative analysis.

2.3.3. Callose

The samples were fixed in FAA for 48 h, washed in distilled water followed by 70% ethanol, and stained in 0.1% aniline blue (pH 9.5) for 10 min [15]. The material was mounted with the stain and observed in a scanning confocal laser microscope (Zeiss, LSM 510, Germany). The controls were treated in the same manner but without the stain.

2.3.4. Enzyme activity

Sections of fresh material were immersed in the appropriate detection solutions for acid phosphatase, phosphorylase, glucose-6-phosphatase, invertase, and sucrose synthase. For the detection of acid-phosphatase activity, the sections were incubated in 0.012% lead nitrate and 0.1 M potassium sodium glycerophosphate in 0.5 M acetate buffer (pH 4.5) for 24 h, at room temperature (25 °C). Subsequently, sections were washed in distilled water and immersed in 1% ammonia for 5 min [22]. For the control, the samples were incubated in the same solution without potassium sodium glycerophosphate. For the detection of phosphorylase activity, the sections were incubated in 1% glucose-1-phosphate in 0.1 acetate buffer (pH 6.0) for 2 h at room temperature [23]. After the incubation, the sections were immersed in Lugol reagent [15]. For the control, samples were not incubated in glucose-1-phosphate. For the detection of glucose-6-phosphatase activity, the sections were incubated in a solution containing 20 mg of potassium glucose-6-phosphate in 125 ml of 0.2 M Tris–maleate buffer (pH 6.7), and 3 ml of 2% lead nitrate in 7 ml of distilled water, for 15 min to 2 h, at 37 °C. Following the incubation, the material was washed in distilled water, immersed in 1% ammonium sulphate for 5 min, and mounted in glycerin jelly [23]. For the control, the samples were not incubated in potassium glucose-6-phosphate. For the detection of invertase activity, the sections were incubated in a neutral reaction buffered medium containing 0.38 mM sodium phosphate (pH 7.5), 0.024% tetrazolium blue, 0.014% phenazin metasulphate, 30 U of glucose oxidase, and 30 mM of sucrose at room temperature for 3 h [24,25]. For the control, an incubation medium without sucrose was used. For detection of sucrose synthase (SuSy) activity, handmade sections of fresh samples and of samples fixed in 2% paraformaldehyde with 2% polyvinylpyrrolidone 40 and 0.005 M dithiothreitol were placed in incubation medium for 30 min, at 30 °C. The incubation medium contained 5 μl of 150 mM NADH, 5 μl (1 U) of phosphoglucomutase from rabbit muscle, 5 μl of 3 mM glucose-1,6-biphosphate, 5 μl (1 U) of glucose-6-phosphate dehydrogenase from *Leuconostoc*, 5 μl (1 U) of UDPG–pyrophosphorylase from beef liver, 280 μl of 0.07% aqueous nitro-blue tetrazolium (NBT), 350 μl of buffer, and 50 μl of substrate. The buffer consisted of 100 mM HEPES, 10 mM $MgCl_2$, 2 mM EDTA, 0.2% BSA, and 2 mM EGTA at pH 7.4. The substrate contained 0.75 M sucrose, 15 mM UDP, and 15 mM pyrophosphate. For one of the controls, glucose-1,6-biphosphate and pyrophosphate were not used. In a second control, sucrose was not used [26].

2.3.5. Reactive oxygen species (ROS)

For DAB (3,3′-diaminobenzidine) staining, handmade sections of fresh material were immersed in 0.5% DAB (Sigma®) solution for 20–60 min, in the dark [7]. The intensity of the reaction was examined every 15 min.

3. Results

3.1. General features

The leaf gall induced by *Pseudophacopteron* sp. in *A. australe* forms a slight projection of the adaxial surface and a more prominent projection of the abaxial surface of its host leaf (Fig. 1a