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The role of phenolics in the control of auxin in galls of *Piptadenia gonoacantha* (Mart.) MacBr (Fabaceae: Mimosoideae)

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ABSTRACT

Gall inducers manipulate the biochemistry of plant cells, benefiting their own life cycles, and influencing on the growth of their host plants. Such manipulation may involve (poly)phenols and growth regulators, such as the indole-3-acetic acid (IAA). Some other molecules, the reactive oxygen species (ROS), may co-occur at the sites of (poly)phenols accumulation, indicating the generation of oxidative stress, which may trigger this accumulation. Herein we focused on the possible co-occurrence of ROS, (poly)phenols and IAA at the same gall tissues, and their involvement in growth and development of different gall morphotypes. This co-occurrence, together with indole-3-acetaldehyde (IAld), was confirmed in galls of *Piptadenia gonoacantha* (Mart.) MacBr. (Fabaceae) by histochemical tests. Developed color for commercial standards of IAA and IAld in TLC was used as controls. The presence of IAA and IAld in gall extracts was confirmed through ultraviolet–visible spectroscopy. The distinct compound is supposed to be auxin-(poly)phenol adducts, evidencing the metabolic interaction among ROS, phenolics and auxin. Current results suggest the associated role of (poly)phenols, ROS and auxin in gall development, and led to the conclusion that phenolics seem to act primarily as growth regulators and secondarily as a chemical defense against natural enemies in gall systems.

1. Introduction

Gall inducers manipulate the development of plant cells and tissues (Rohfritsch, 1992; Stone and Schnrögge, 2003) by altering their biochemical profiles (Hartley, 1998; Isaias, 1998; Oliveira et al., 2006). Consequently, galls accumulate primary metabolites used by the herbivores for their nutrition, which are sometimes located in a specialized nutritive tissue. Also, the concentration of (poly)phenols is usually higher in gall tissues as a result of an increased activity of phenylalanine ammonia-lyase (PAL) (Hartley, 1998, 1999). (Poly)phenols accumulation is commonly reported as a plant defense mechanism against galling herbivores and/or natural enemies (Westphal et al., 1981; Taper and Case, 1987). Nevertheless, the increasing concentration of these secondary metabolites in galls should benefit galling herbivores in alternative pathways (Abrahamson et al., 1991; Hartley and Lawton, 1992). It should reduce mortality rates provoked by fungi infestation (Taper et al., 1986),

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protect the galling herbivore from the attack of parasitoids and predators, and also help in the maintenance of gall structure as a response to the oxidative stress generated during gall development (Soares et al., 2000; Oliveira et al., 2011a, 2011b).

Besides chemical protection, phenolic compounds may be involved in determining plant growth by stimulating or inhibiting the activity of proteins and enzymes involved in plant hormone production (Hartley, 1999), such as indole-3-acetic acid (IAA), and its immediate precursor, the indole-3-acetaldehyde (IAld) (Zhao, 2010). Also, the biosynthesis of (poly)phenols should inhibit the activity of IAA oxidases (Hori, 1992), and consequently increase cell hypertrophy, a common process in the development of galls (Souza et al., 2000; Moura et al., 2008; Oliveira and Isaias, 2009, 2010). The molecular mechanisms and the control of IAA pathway are not completely elucidated (David et al., 2007). Some authors proposed its involvement with phenolics accumulation (Briggs and Ray, 1956; Leopold and Plummer, 1961) to restart cell cycles, which has been discussed in relation to gall development (Hori, 1992; Hartley, 1999; Moura et al., 2008).

One of the probable plant cell responses to galling stimuli is the formation of reactive oxygen species (ROS) (Pasqualini et al., 2003; Oliveira et al., 2010; Oliveira and Isaias, 2010) together with the accumulation of phenolics. This accumulation seems to be a secondary response and a strategy to scavenge ROS (Sousa et al., 2007), and block the activity of IAA oxidase, which end up increasing the levels of auxin in gall tissues. Oliveira and Isaias (2010) were the first to demonstrate a functional gradient established in the galls of *Aspidosperma australe* due to ROS generation. These authors related this gradient to the trigger for the differentiation of each gall morphotype. Based on these premises, current research focused on the investigation of the sites of accumulation of phenolics and auxin, and its relation with the growth and development of galls. Thus, pinnula lenticular and rachis fusiform galls of *Piptadenia gonoacantha* (Mart.) MacBr (Fabaceae:Mimosoideae) were analyzed with the purpose of answering the following questions: (i) does the accumulation of phenolic compounds overlap that of auxin and ROS? And (ii) is there any evidence of the metabolic interaction among ROS, phenolics and auxin?

2. Material and methods

2.1. Host plant species and sampling

Piptadenia gonoacantha is commonly known as "pau-jacaré" (=alligator stick), and occurs naturally in Brazil in the states of Rio de Janeiro (Borém and Oliveira-Filho, 2002), Goiás (Nascimento et al., 2004), Minas Gerais (Fagundes et al., 2007), and São Paulo (Yamamoto et al., 2005). Pinnula lenticular and rachis fusiform galls, herein nominated lenticular and fusiform, were collected in June 2012 at the Ecological Station of the Universidade Federal de Minas Gerais, Pampulha *campus*, Belo Horizonte, MG, Brazil.

2.2. Histochemical analyses

Fresh samples of young lenticular and fusiform galls were submitted to histochemical tests. The presence of phenols, flavonoid derivatives, IAA, IAld, and ROS was checked out in free-hand sections at room temperature. The accumulation of phenolics was demonstrated in samples fixed in 2% ferrous sulfate in 10% formalin (Johansen, 1940). Flavonoid derivatives were tested in sections incubated in 0.5% caffeine, 0.5% sodium benzoate and 90% butanol for 5 min, and immersed in 1% *p*-dimethylaminocinnamaldehyde (DMACA) in water, hydrochloric acid and ethanol (5:1:5) for 2 h (Feucht et al., 1986). For IAA and IAld detection, the sections were treated with Ehrlich (Leopold and Plummer, 1961) and DMAC (Schneider et al., 1972) reagents, respectively, for 5 min. The presence of ROS was checked by immersion of the sections in 0.5% 3,3′–diaminobenzidine DAB (Sigma–Aldrich, St. Louis, MO, USA) for 30 min, under dark conditions (Rossetti and Bonatti, 2001). Fusiform galls on senescent stage were used to validate the histochemical tests for auxin detection.

2.3. Phytochemical analyses

Samples of young lenticular and fusiform galls were processed for the extraction of IAA, and IAld. Fresh material was macerated in methanol at 4 °C (3 mL/1 g of fresh material) and incubated for 24 h (Schneider et al., 1972). The whole procedure occurred in the absence of light to avoid auxin degradation. Methanolic extracts were filtered, evaporated (~45 min) at 45 °C for volume reduction, and filtered again in Celite to remove precipitated chlorophyll. The pH of the concentrated methanolic portion was adjusted to 7.0, followed by three consecutive extractions with half-volume of distilled ethyl ether to obtain the IAld fraction. The pH of the remaining methanolic portion was further adjusted to 3.0, followed by three consecutive extractions with half-volume of distilled ethyl ether to obtain the IAA fraction (Schneider et al., 1972; modified). Aqueous solutions of commercial IAA (®Vetec) (pH 4.0) and IAld (®Aldrich Chemistry) (pH 7.0) were used at 1–8 mg/mL as standards, individually loaded in silica-based chromatographic plates, and eluted with CEF (chloroform/ethyl acetate/formic acid 5:4:1; v/v). After the development, silica plates were sprayed either with Ehrlich reagent (1% *p*-dimethylaminnobenzaldehyde in 1 M HCl; w/v) (Leopold and Plummer, 1961) or freshly prepared DMAC solution (5 g/L *p*-dimethylaminacinnamaldehyde in 90% acetone and 2% HCl) (Schneider et al., 1972). Plates were kept at room temperature until the development of colors. The best developing reagent (DMAC) was then used for TLC experiments with all extract fractions of galls, and the results were compared to those obtained with the commercial standards and the ones described by Schneider et al. (1972) and Harborne (1998) (Table 1). The resultant color after spraying plates with Ehrlich reagent was compared to those reported by Leopold and Plummer (1961).

Table 1
Histochemical tests on transverse sections of insect galls on *Piptadenia gonoacantha* under different developmental stages.

Gall morphotype/stage	Histolocalization	2% Ferrous sulfate in 10% formalin (phenolics)	DMACA (flavonoid derivatives)	Ehrlich reagent (IAA)	DMAC reagent (IAld)
Lenticular/Young	Outer epidermis	++	++	++	++
	Superior cortex (Parenchyma cells)	++	++	++	++
	Superior cortex (Sclerenchyma cells)	–	–	–	–
	Inferior cortex	+	+	+	+
	Nutritive tissue	–	–	+	+
Fusiform/Young	Outer cortex (Parenchyma cells)	++	++	++	++
	Outer cortex (Sclerenchyma cells)	–	–	–	–
	Inner cortex	+	+	+	+
	Nutritive tissue	–	–	+	–
Fusiform/Senescence	Outer cortex (Parenchyma cells)	+	+	+	+
	Outer cortex (Sclerenchyma cells)	–	–	–	–
	Inner cortex	–	–	–	–
	Nutritive tissue	–	–	–	–

(−) negative result, (+) weak reaction, (++) very intense reaction.

Methanolic and distilled ethyl ether extracts were analyzed in a Multiskan Microplate Reader (Thermo Scientific) to obtain the absorption spectra of IAA and IAld under ultraviolet–visible light.

3. Results

3.1. Histochemical analyses

The lenticular galls of *P. gonoacantha* are induced in pinnula lamina with a large increment of cells originated from the abaxial layers, evidenced by the curved design assumed by the vascular tissues (Fig. 1A). Phenolics were detected by the fixation with ferrous sulfate in formalin that yielded a brownish color (Fig. 1B). Flavonoid derivatives, evidenced by the blue color of the vacuoles content in sections submitted to DMACA, were concentrated on the top of the larval chamber (Fig. 1C–D). The green and pink colors obtained by histochemical tests with DMAC and Ehrlich reagents, respectively, evidenced the presence of IAld (Fig. 2A–B) and IAA (Fig. 2C–D) (Table 1). ROS were strongly overlapped in sites with high accumulation of (poly)phenols and IAA (superior and inferior cortices) (Fig. 2E–F).

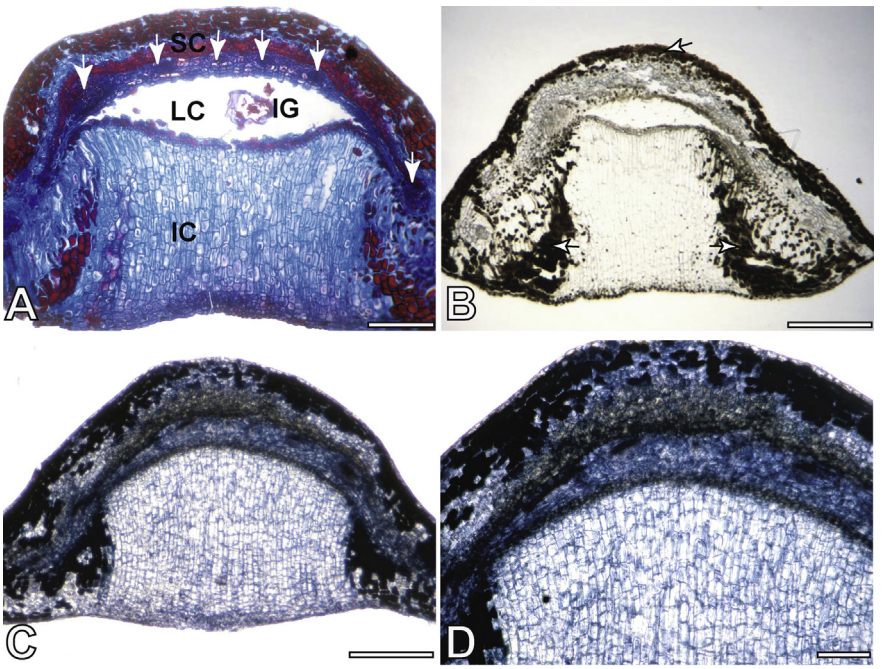

Fig. 1. Transverse sections of young lenticular galls on *Piptadenia gonoacantha* (Fabaceae). (A) Double staining with safranin-Astra blue. The vascular bundles (white arrow) follow the curvature of the gall structure. (B–D) Histochemical detections. (B) Phenolic compounds (black color) evidenced by fixation with ferrous sulfate in formalin (black arrows). (C–D) Flavonoids and derivatives (blue color) detected with DMACA. IG, Galling Insect; LC, Larval chamber; IC, Inferior cortex; SC, Superior cortex. Scale bars: 200 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

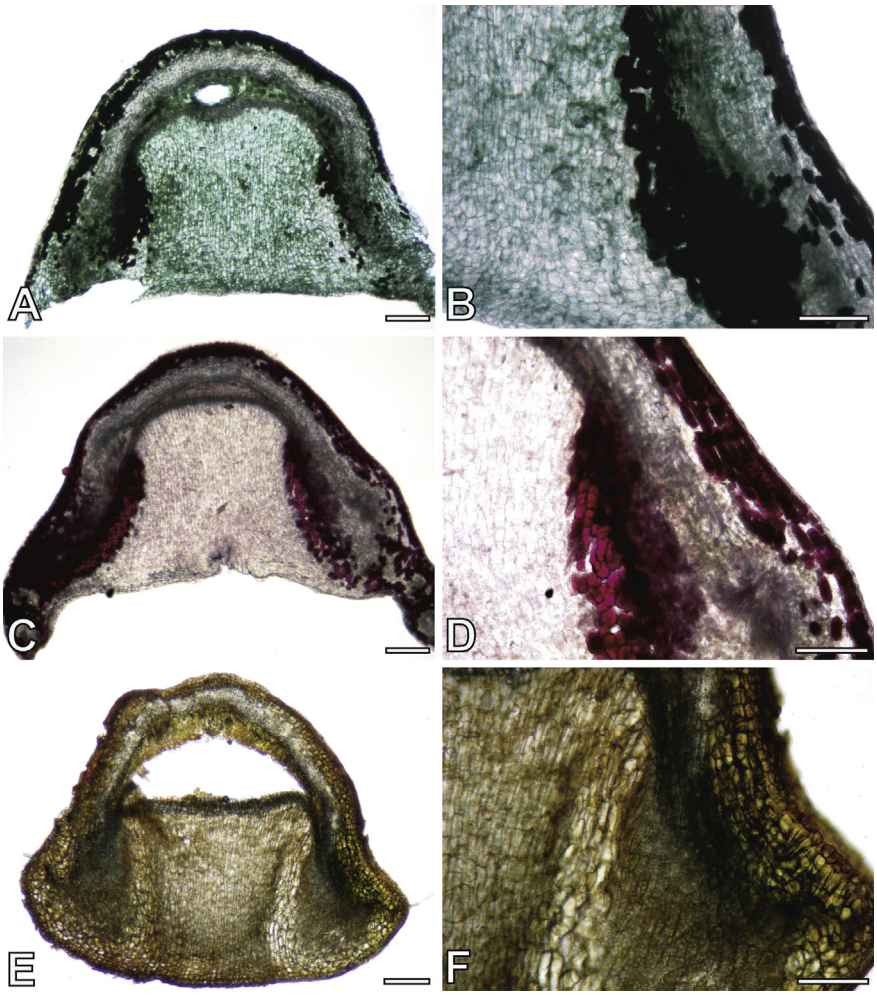

Fig. 2. Tranverse sections of young lenticular galls on *Piptadenia gonoacantha* (Fabaceae). (A–F) Histochemical detections. (A–B) IAld (green color) by using DMAC. (C–D) IAA (pink color) by using Ehrlich reagent. (E–F) ROS, particularly H_2O_2 (brownish color), by using diaminobenzidine tetrahydrochloride (DAB). Scale bars: 200 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

The fusiform galls of *P. gonoacantha* are induced in the rachis and presented processes of hyperplasia and cell hypertrophy at cortex and pith, with the larval chamber in central position (Fig. 3A). Phenolic compounds were detected by the formation of blackish color (Fig. 3B). In the outer cortex, parenchymatic layers of the inner cortex, and nutritive tissue, the green and the pink colors indicated the presence of IAld and IAA, respectively (Fig. 3C–D). All compounds were evidenced in the outer epidermis, and in the outer and inner cortical cells of young galls. As expected, such compounds were poorly detected in senescent galls (Table 1). The presence of low amounts of IAld, IAA (Fig. 3E–F), and phenolics, characterized by the formation of weak colors, was observed only in the outer cortical cells of senescent fusiform galls. The ROS accumulated in all tissue layers, except in the outer sclerenchymatic ones (Fig. 3G).

3.2. Phytochemical analyses

Characteristic pink spots were observed in TLC plates loaded with standard solutions of IAA immediately after development with Ehrlich reagent. Thirty minutes later, the color of the spots turned into purple (Supplementary Data Fig. S1), confirming the presence of IAA. The presence of IAld in TLC plates revealed with DMAC can be confirmed by the formation of green-blue spots. Neither IAA nor IAld was detected in the organic fractions obtained from gall extracts as attested by the absence of bands whose retention factors (Rfs) coincided with those obtained for the commercial standards (IAA = 0.87; IAld = 0.77). Spots of olive color (Rf = 0.96) were observed in both ethanolic fractions (pH 3.0 and 7.0) on TLC plates developed either with Ehrlich or DMAC reagents (Supplementary Data Fig. S1).

The ultraviolet–visible spectroscopy confirmed the presence of IAA and IAld in distilled ethyl ether extracts (Fig. 4). The major absorbance of the IAA was at wavelengths of 225, 235 and 290 nm, while that of IAld was at 345, 435 and 440 nm,

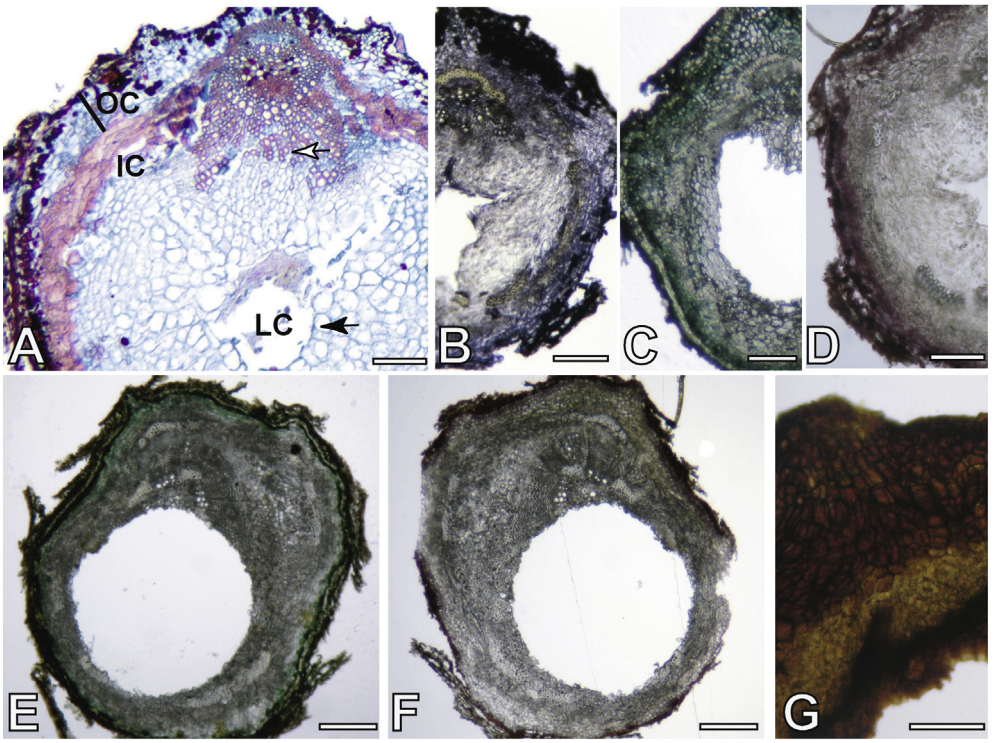

Fig. 3. Transverse sections of fusiform galls on *Piptadenia gonoacantha* (Fabaceae). (A–D) Galls on young stage. (A) Gall structure evidencing hypertrophy and hyperplasia of cortical and parenchyma cells of the vascular cylinder, mainly in the rachis abaxial portion. There is a disruption of the vascular system (white arrow), and installation of periderm. The outer cortex is formed by parenchyma cells, and the inner cortex by sclerenchyma and parenchyma cells. Nutritive tissue differentiates around the larval chamber (black arrow). (B–G) Histochemical detections. (B) Phenolic compounds (black color) evidenced by fixation with ferrous sulfate in formalin. The larval chamber is located at the central position of the structure. (C) IAld (green color) by using DMAC. (D) IAA (pink color) by using Ehrlich reagent. (E–G) Senescent fusiform galls. (E) IAld (green color) in the outer epidermis by using DMAC. (F) IAA (pink color) in outer epidermis by using Ehrlich reagent. (G) The ROS, detected by using diaminobenzidine tetrahydrochloride (DAB), accumulated in all tissue layers, except in the outer sclerenchymatic ones. LC, Larval chamber; IC, Inferior cortex; OC, Outer cortex. Scale bars: (A and G) = 200 μm, (B–F) = 300 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

confirming that these two different substances have been extracted (Fig. 4). In methanol extracts, the major peaks for IAA occurred at wavelengths 280 and 290 nm, while that of IAld was observed at 245 and 260 nm. These data, obtained for extracted fractions from galls, corroborated Schneider et al. (1972) and Harborne (1998) results for standard solutions of IAA and IAld scanned under UV–VIS light.

4. Discussion

Current study on *P. gonoacantha* galls evidences that histochemical together with phytochemical analyses have proved to be efficient to address the occurrence and influence of (poly)phenols and IAA during gall developmental processes on a topographical basis. Gall systems with their intricate metabolic requirements and recurrent accumulation of (poly)phenols seem to be interesting models for such kind of investigation.

The Ehrlich reagent is primarily used to identify indoles and was described by Steelink (1959) and Leopold and Plummer (1961) for the detection of free auxin (purple spot) and *in-vitro*-generated auxin-(poly)phenol adducts separated through Paper Chromatography technique. Herein, the Ehrlich reagent is used for the first time to histochemically detect the presence of auxin and/or auxin-(poly)phenol adducts in gall tissues.

Hori (1992) was the first to propose that the activation or inhibition of growth in galls should be influenced not only by water accumulation, but by an increase in the biosynthesis of phenolic compounds, via the inhibition of IAA oxidases activity and an increase in auxin levels. Nevertheless, his investigation do not include topographical proofs of this association, which can be evidenced by histochemical tests.

Histochemical tests detected IAA, IAld, phenolics, and ROS in cells of the outer cortex and nutritive tissue of the lenticular and fusiform galls on *P. gonoacantha*, sites of intense cell division and elongation. The high content of phenolics had been detected before in the middle stage of intensive cell growth and division in tobacco crown gall suspension cell culture (Chirek, 1990). Besides, our histochemical analysis showed a weak reaction for IAld, IAA and phenolics in the inner cortex of senescent galls, where cell division and growth had been arrested. This data is consistent with the observations of Mapes and Davies

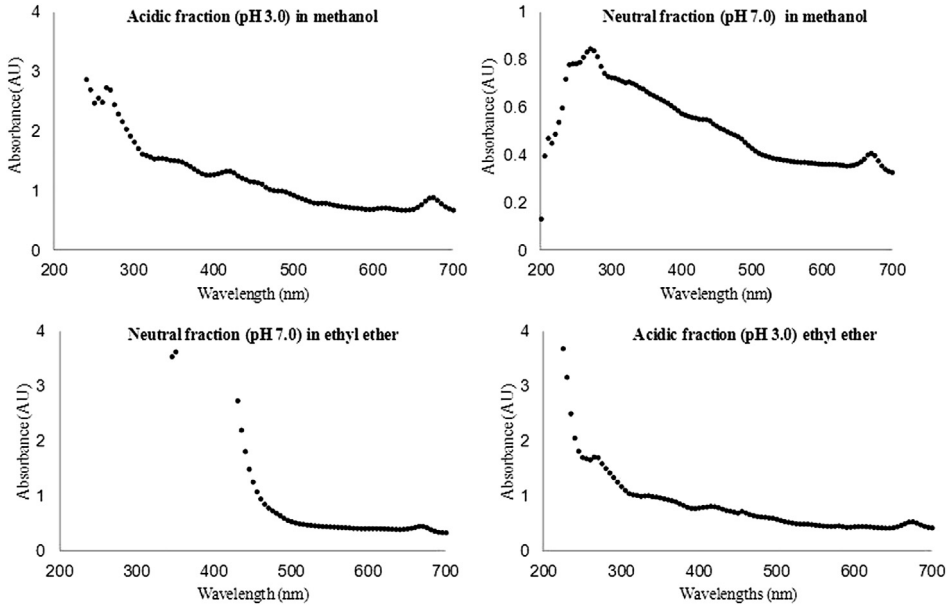

Fig. 4. Absorption spectra of acidic and neutral fractions obtained from crude extracts of *Piptadenia gonoacantha* lenticular galls. The major peaks found in acidic (IAA fraction) methanolic fractions were at 280 and 290 nm while the peaks in neutral (IAld fraction) methanolic fractions were at 245, 260 nm. As for distilled ethyl ether fractions, major peaks for the acidic ones were 225, 235 and 290 nm while neutral fractions exhibited λ_{max} equal to 345, 435 and 440 nm.

(2001) on the ball galls in *Solidago altissima*, in which the IAA concentrations were the highest in the early stages and decreased along the developmental phases.

Flavonoids have a variety of biological functions, such as the modulation of auxin transport and auxin-dependent responses (Murphy et al., 2000; Peer and Murphy, 2007), influencing plant architecture by signalizing molecular targets or controlling ROS levels (Buer et al., 2010). The histochemical overlapping detection of (poly)phenols, ROS, and auxin on gall tissues of *P. gonoacantha* points out to the influence of these compounds on auxin-dependent responses. Indeed, flavonoids were shown to induce auxin accumulation in plant roots during the formation of nodules resulting from the interaction with nitrogen-fixing bacteria (Mathesius et al., 1998). However, it was not tested for plant–insect interaction. The presence of flavonoids in gall tissues could minimize the oxidative stress via sequestration of ROS, whose formation has been already histochemically detected in gall tissues (Oliveira et al., 2011a, 2011b). So, in the galls of *P. gonoacantha*, the accumulation of phenolics, flavonoids and ROS detected in sites of intense cell division is another evidence of these compounds interaction in gall metabolism.

The lack of spots corresponding to free IAA or IAld in acid or neutral fractions of *P. gonoacantha* gall extracts separated on TLC may be explained by a loss of these compounds during the series of liquid–liquid extractions performed with distilled ethyl ether. Indeed, the concentration of hormones in tissues is usually low and should diminish to levels that are lower than those of the detection limit of the TLC technique. However, spectrophotometric analysis on UV–VIS light of these same fractions detected IAA and IAld as attested by the typical peaks reported in the literature for these substances. Leopold and Plummer (1961) have shown that the condensation product of IAA with catechol or caffeic acid turns into olive color in the presence of Ehrlich reagent. The conversion of (poly)phenols into the respective quinones favor the formation of IAA-(poly) phenol adducts as quinones are known to condense with nitrogen-containing substances (Beevers and James, 1948) to form the corresponding Michael addition adducts. Thus, olive-colored spots detected in the extract on TLC is evidence of some Michael-addition-adduct-type IAA-polyphenol formed in cells of *P. gonoacantha* galls. This presumption remains to be proved by more precise analytical techniques and indicates a new focus to be addressed in the elucidation of plant cell responses to galling herbivores' stimuli, as well as, to the role of the compounds involved on the interaction.

Topographical analyses of histochemical reactions revealed the presence of ROS, phenols, flavonoids, IAA and IAld in similar cell layers and led us to suggest the potential for the formation of auxin-(poly)phenol adducts. The formation of such adducts would primarily contribute for the development of gall structure in *P. gonoacantha* while the chemical defense function of (poly)phenols would comprise a secondary and opportunistic response to the stress imposed by the galling insect.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.bse.2014.02.016.

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