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Structural Characteristics and Content of Polyphenolic Compounds in Healthy Organs and Galls Induced by *Allodiplosis crassa* Kieff. and Jörg (Cecydomyiidae, Diptera) on *Geoffroea decorticans* (Hook. and Arn.) Burkart (Leguminosae)

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Chañar (*Geoffroea decorticans*- Fabaceae) is a tree from South America that is normally infected with galls originated by insects. One of its parasites is *Allodiplosis crassa* (Cecidomyiidae, Diptera) which produces globular galls with sticky prolongations. Since this plant has medicinal uses in Argentina, its infestation could alter the quality of the plant drug. The surface of insect-induced galls usually contains defensive features such as trichomes, increased hardness and an increase in the content of polyphenolic compounds. The objective of this research is to assess the structural and histochemical features of the gall and to compare the content of polyphenolic metabolites in the gall, in the healthy leaf and in lignified stems of *G. decorticans*. The methanolic extract from the galls showed the highest amount of polyphenolic and proanthocyanidins and the lowest amount of hydroxycinnamic derivatives and flavonoids compared to the methanolic extract of the leaves. The photographs taken from the external surface of the gall showed that some prolongations have heads. The histochemical analysis showed that the prolongations have a high amount of proanthocyanidins and flavonoids; and that the heads are reactive to Sudan III. These phytochemical and histological characteristics may have a defensive role against harmful fungi and parasites that attack the larvae of the *A. crassa*. The results of this study show the presence of defensive features in an insect-induced gall of a medicinal plant with potential implications in the pharmacological activity of this species. This is the first report of a histochemical and phytochemical study in *G. corticans* galls.

Keywords: Insect induced galls, Geoffroea decorticans, polyphenols, Cecidomyiidae.

The *Chañar* (*Geoffroea decorticans*- Fabaceae) is a tree from the central-south region of South America. It can reach a height of 5 meters and it has an exfoliating bark in plaques, greenish trunk with intricate and thorny branches, leaves of 5 pairs of leaflets and a flower with a yellow papilionaceous shape (Cabrera and Zardini, 1978).

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This species has several medicinal uses: the fruit is used in the preparation of a fermented beverage, the "*aloja*" which has anti-coughing and anti-asthmatic properties, also helpful for colds and bronchitis. Candies and refreshing drinks are also prepared from it. The infusion of the bark is used as an expectorant medicine (Lahitte, 1998). Glycosides of apigenin and quercetin were found in the methanolic extract of the fruit (Silva et al., 1999), and a prenylated isoflavone was isolated from the bark. (Vila et al, 1998). *Wichi* indigenous people from Argentine Chaco used galls induced by insects, such as *Allodiplosis crassa* (Fig. 1) which was employed as a plant drug in labor and uterine bleeding in case of oxytocin activity.

Fig. 1. *A. crassa*-induced gall

It is worth noting that this is the only part of the plant used with such purpose (Suárez, 2014). Induced galls by insects are originated as a result of manipulation of the metabolism of a plant to produce a structure that provides appropriate nutrition and protection from predators and adverse environmental conditions (Shorthouse and Rohfritsch, 1992; Hartley and Lawton, 1992; Stone and Schönrogge, 2003; Tooker et al., 2008).

This stimulus generates hyperplasia, hypertrophy, and cell differentiation, with the formation of gradients of primary and secondary metabolites and enzymes related to the metabolism (Carneiro et al., 2015; Oliveira et al., 2016; Carneiro et al., 2014; Motta et al., 2005).

The elements involved in the protection may result in the development of sclerenchyma and changes in the structure of secondary metabolites (Braganca et al., 2016;

Nyman and Julkunen-Tiitto, 2000; Pascual-Alvarado et al., 2008; Hartley, 1998) such as flavonoids, hydroxycinnamic derivatives, and condensed tannins.

Galls induced by *A. crassa* can be seen in buds, and are globular and totally covered with subacute prolongations, which are lost in maturity. According to the classification of Isaias (Isaias et al., 2014), they are included in the globular type. During this stage, the galls are sticky and they attach to surfaces, even though no secretion can be observed at first sight.

Polanco and his collaborators have already recorded the anatomy of the galls induced by *A. crassa* (Polanco et al., 2000). They have mentioned the presence of cells with a high level of polyphenol in the content of the parenchyma as well as in the prolongations. The primary aim of this research is to know all the structural and histochemical characteristics of the gall, and to compare the content of polyphenolic metabolites in the gall, in the healthy leaf, and in the secondary stalk of *G. decorticans.*

Materials and Methods

Material Collection

Galls, leaves and stems were collected at Traslasierra, Córdoba province, Argentina. The material was identified using the taxonomic key of Cabrera and the article of Polanco (Polanco et al., 2000).

Obtention of Original Methanolic Extract (OME)

The process started with 500 mg of dry ground material from three pooled individuals. The extraction was performed with 10 mL of methanol 10% (methanol-water), at room temperature for 24 hours. Later, it was filtered and the frame was discarded.

Qualitative analysis of polyphenols (fingerprint of polyphenols)

It was performed by a two-dimensional chromatography (TLC-2D) in cellulose, according to the standard methodology (Mabry et al., 2012; Markham, 1982). For the two-dimensional analysis of the flavonoids and hydroxycinnamic acids, the solvent system TBA (tert-butanol, acetic acid, water 3:1:1) was used for the first dimension and 15% acetic acid for the second. Chromatograms were observed with UV light ($\lambda = 366$ nm) before and after exposure to ammonia vapors and revealed with natural product reagent (NPR) (Wagner et al., 1984) and subsequently observed at UV 366 nm. This was how the distribution pattern of the compounds for the study material was obtained, which will shed light on its qualitative composition.

Total phenol quantification

Total phenols were determined by the Folin-Ciocalteu method as described by Makkar et al. (1993). Aliquots (50 μL) of the extract were transferred to test tubes and diluted up to 500 μL with deionized water. Then, 250 μL of the Folin-Ciocalteu reagent and

1.25 mL of an aqueous solution of sodium carbonate (20%) were added. After 40 minutes, absorbance was measured at 725 nm. A calibration curve was generated with tannic acid. A stock solution of 0.1 mg/mL was used containing a range of 2-10 μg tannic acid in the final volume of the reagent. Total phenol content was expressed as equivalents of tannic acid (mg tannic acid / g dry material). All measurements were performed in triplicate.

Total flavonoids quantification

Aliquots of 0.1 mL of each extract were added to 1.4 mL of deionized water and 0.50 ml of flavonoid reagent (133 mg aluminum trichloride and 400 mg sodium acetate in 100 ml of solvent composed by 140 mL of methanol, 50 mL of water and 10 mL of acetic acid). After 30 minutes at room temperature, the absorbance was measured at 430 nm (Maksimovic et al., 2005). A rutin calibration curve was created, covering a concentration range between 10 and 50 μg/mL. The flavonoid content was expressed as rutin equivalents (mg rutin/g dry material). All measurements were performed in triplicate.

Quantification of condensed tannins (proanthocyanidins, PA)

Condensed tannins were determined through the reaction of proanthocyanidins following a methodology described by Waterman (Waterman and Mole, 1994). Aliquots of 0.50 mL of the extracts were transferred to test tubes and 3.0 ml of butane reactive HCI (butanol: HCI, 95:5 V/V) and 0.1 ml of 2% ferric reagent were added (2% ferric-ammonic sulfate in HCI 2 M). Tubes were shaken and heated for 60 minutes over a boiling water bath. When cool, absorbance was measured against a blank at 550 nm. Proanthocyanidins were expressed as absorbance at 550 nm. All measurements were performed in triplicate.

Total hydroxycinnamic acid quantification

It was determined by a modification of the methodology described by Dao and Friedman (1992). Aliquots 50 μL of each extract were taken to volume (2 ml) with absolute ethanol. Absorbance was determined at 328 nm. A calibration curve with chlorogenic acid (stock solution 1 mg/mL) was made, covering a range between 5 and 40 μg of chlorogenic acid in the final volume of the reaction. The values were expressed as equivalents of chlorogenic acid (mg of chlorogenic acid/g dry material). All measurements were performed in triplicate.

Observation through electron microscopy

Samples of leaves, galls, and metallic stalks with gold-palladium were analyzed under a Thermo VG Scientific metallizer. A Philips electronic microscope model XL30 TMP New Look was used.

Histochemical analysis of galls

The histochemical detection of proanthocyanidins was performed using a vanillin-chloride acid reagent (5% vanillin in ethanol – concentrated hydrochloric acid, 4:1);

flavonoids were detected with AEDBE reagent (1% of methanol) (Ricco et al, 2015), and the detection of lipids was carried out with Sudan III reagent (Argüeso, 1986) in gall tissue over cuts performed freely and in isolated prolongations.

Results

A higher concentration of total polyphenolic compounds in the gall extract in contrast with the leaves extract can be observed (Fig. 2). However, the concentration of flavonoids and hydroxycinnamic derivatives is higher in the latter (Figs 3, 4), whilst the gall has a higher concentration of proanthocyanidins (Fig. 5).

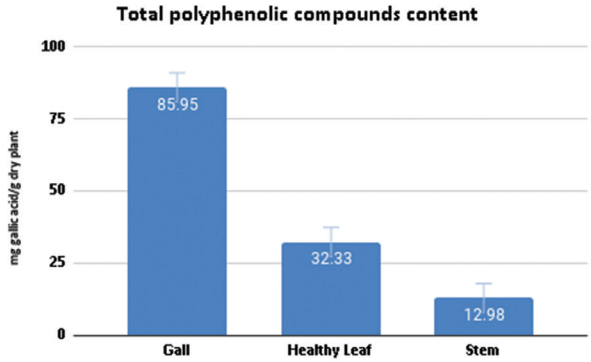

Fig. 2. Content of total polyphenolic compounds

This situation is exactly the opposite when comparing the chromatographic profile of flavonoids and hydroxycinnamic derivatives (Figs. 6, 7, 8). As regards the chromatographic profiles, the gall presents a stain of greater intensity with Rf close to 9 in the dimension eluded with TBA and Rf close to 0 in the dimension eluded with AcH 15% compatible with glycoside flavonoids, whereas the leaf has two stains of great intensity with Rfs close to 0.5 in the dimension eluded with TBA and close to 4 and 8 in the dimension eluded with AcH 15% compatible with flavonoid glycosides. The woody stem is the organ with less concentration and variety of polyphenolic secondary metabolites.

Images obtained with the scanning electron microscopy showed that some external prolongations have heads and bumps all over its length (Figs. 9, 10, 11).

In the histochemical analysis, it was observed that the proanthocyanidins are located mainly in the external prolongations of the gall (Fig. 12) and the flavonoids are located mainly in the bumps and heads (Figs. 13, 14).

Lipids are also numerous in this part (Figs. 15, 16). For these last two studies galls cut in half with binocular loupe were required since these prolongations are extremely fragile.

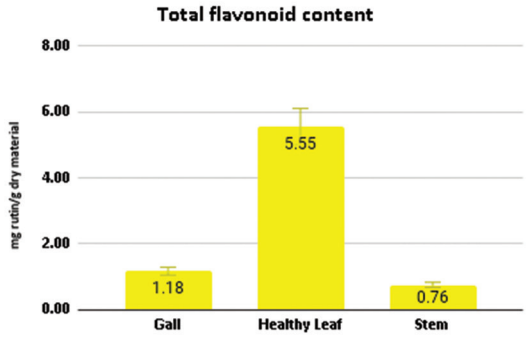

Fig. 3. Total flavonoid content

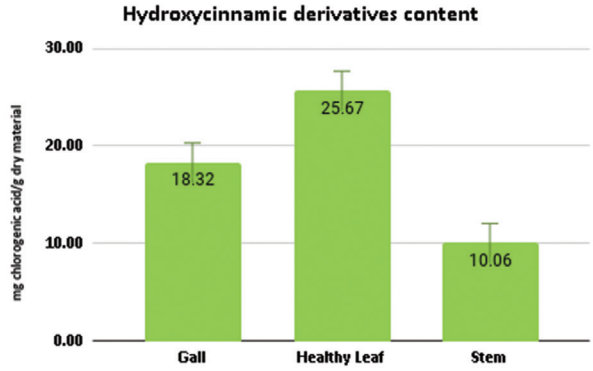

Fig. 4. Hydroxycinnamic derivatives content

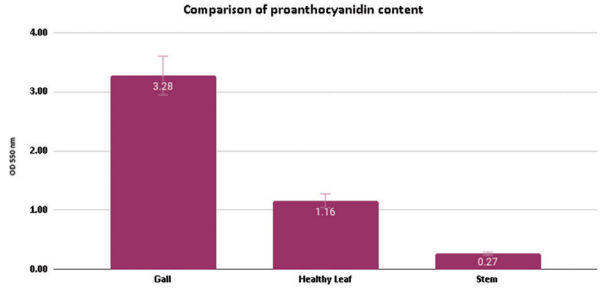

Fig. 5. Comparison of proanthocyanidin content

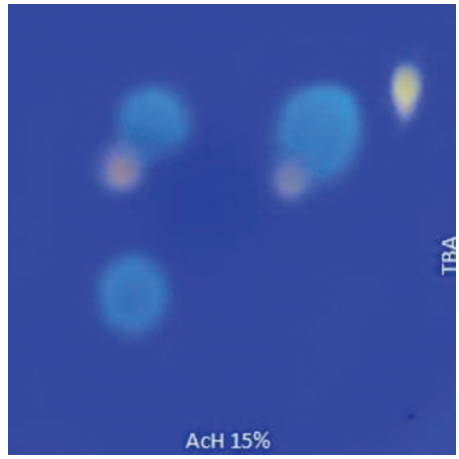

Fig. 6. TLC-2D of flavonoids and hydroxycinnamic derivatives of methanolic extract from the gall

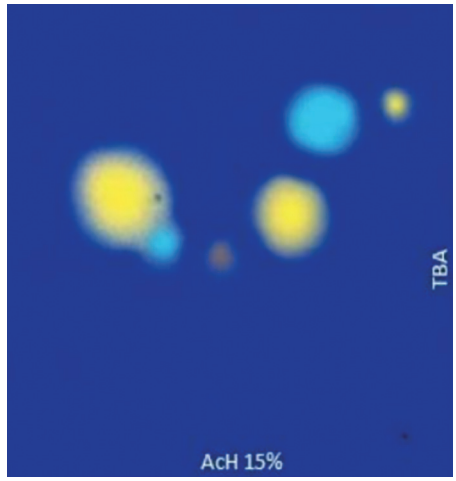

Fig. 7. TLC-2D of flavonoids and hydroxycinnamic derivatives of methanolic extract from the leaf

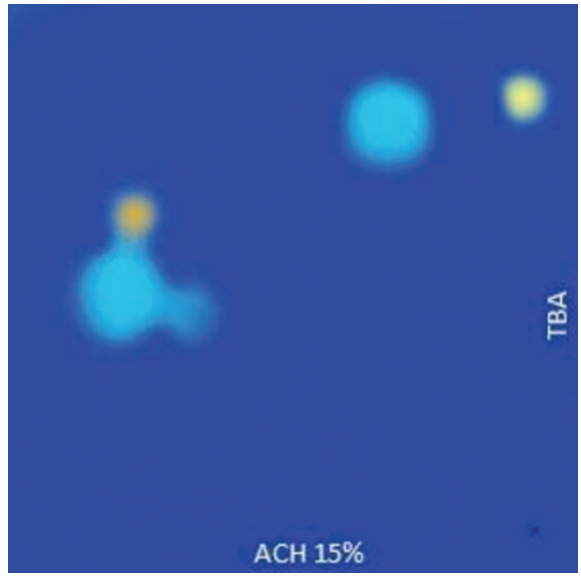

Fig. 8. TLC-2D of flavonoids and hydroxycinnamic derivatives of methanolic extract from the stem

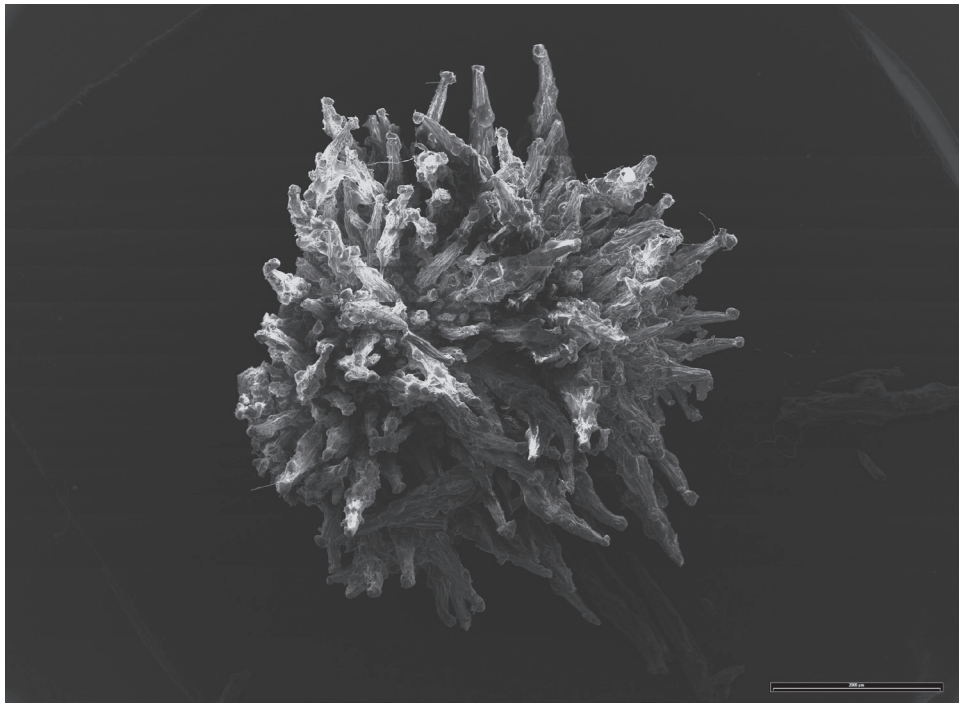

Fig. 9. Prolongation of the external surface of the gall isolated without head

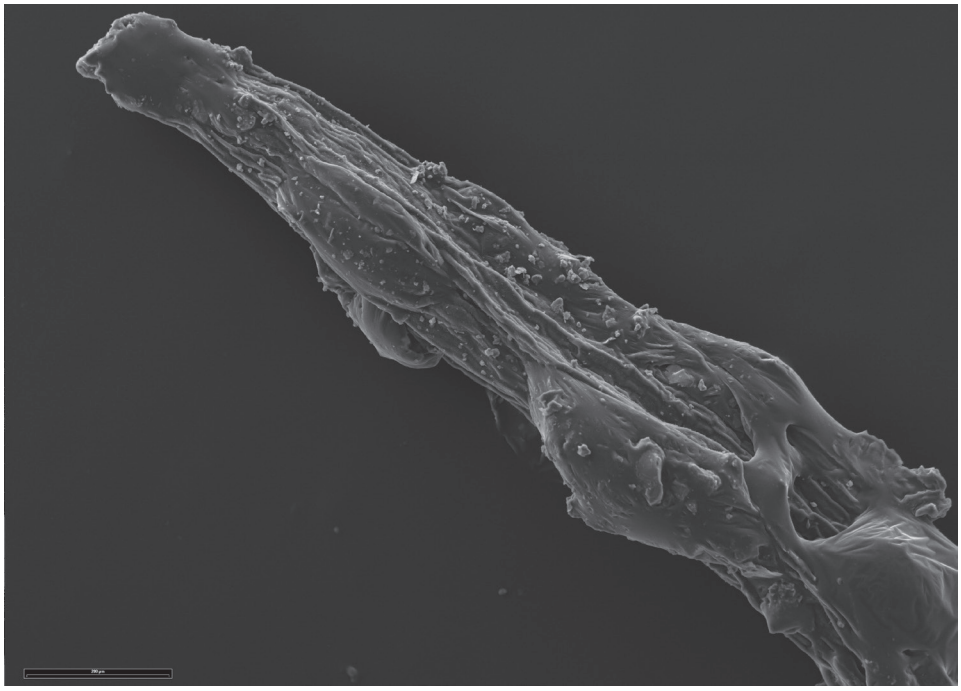

Fig. 10. External surface of the gall

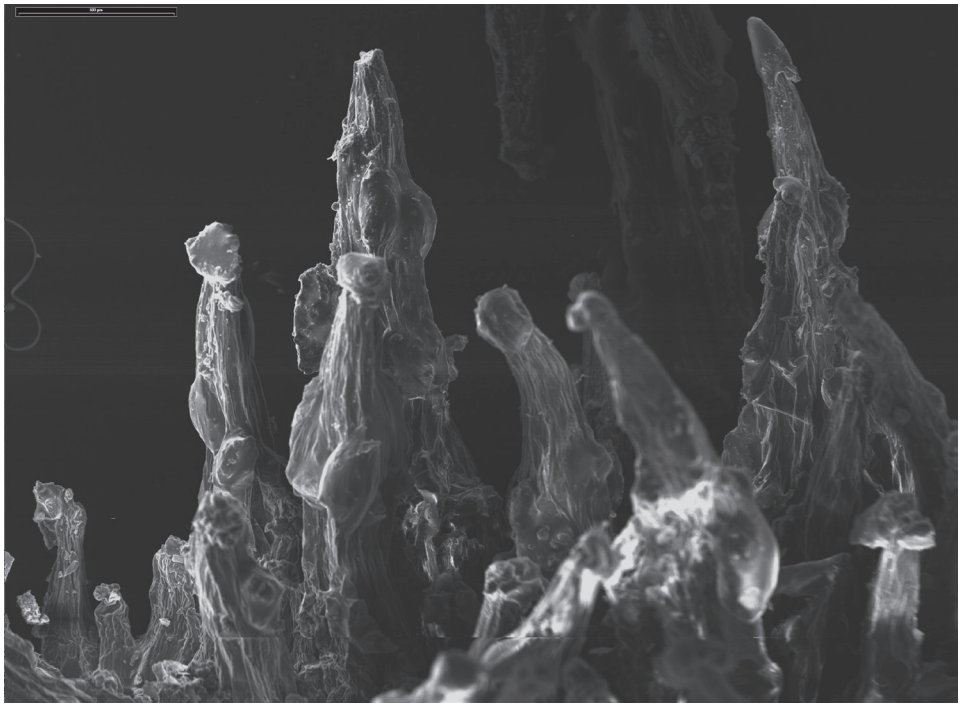

Fig. 11. Prolongations of the external surface of the gall with and without heads

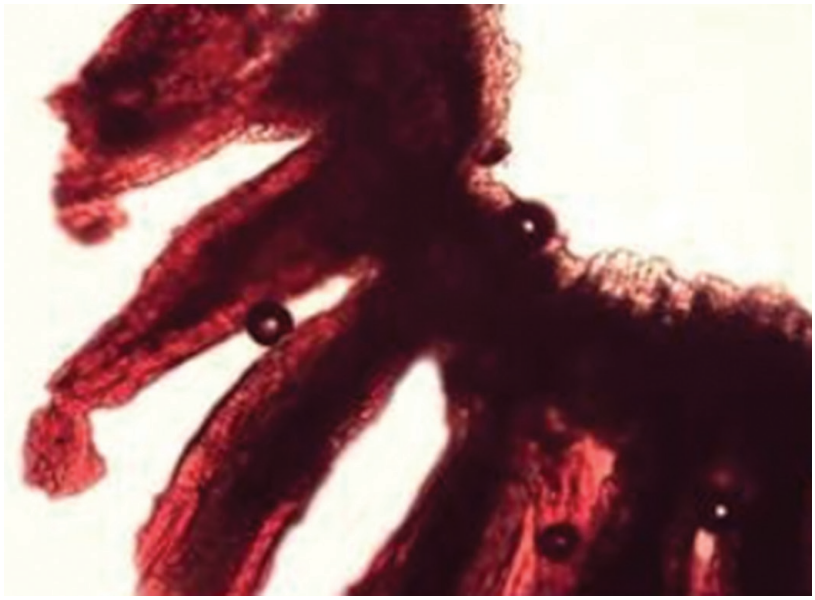

Fig. 12. Condensed tannin with vanillin/HCI reactive histochemistry (100×)

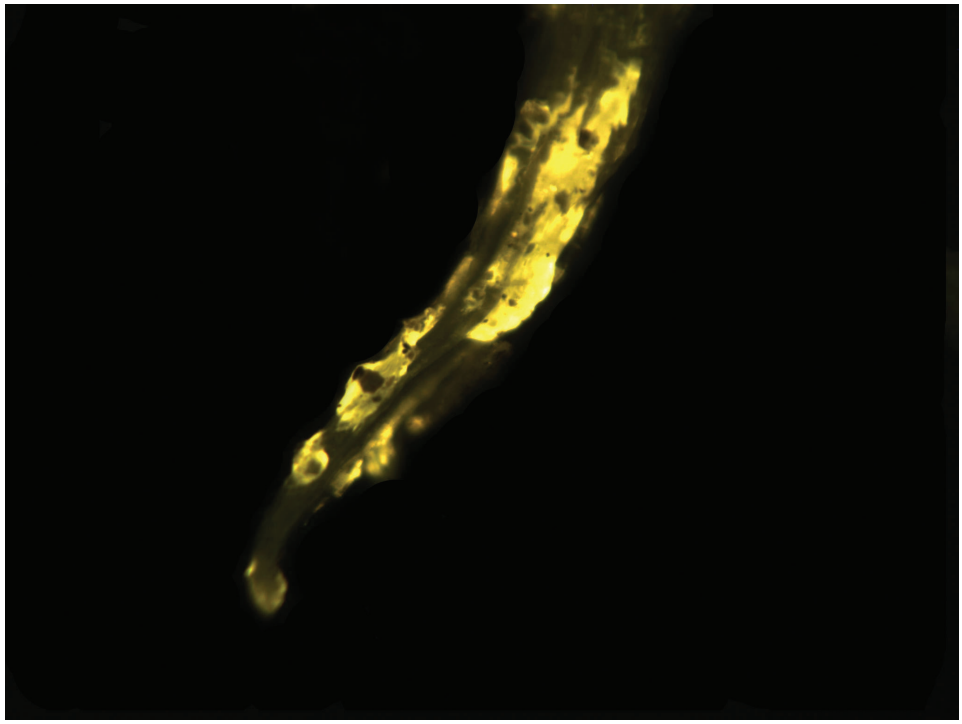

Fig. 13. Detail of fluorescence of the bumps of the prolongation without head with AEDBE reagent (100×)

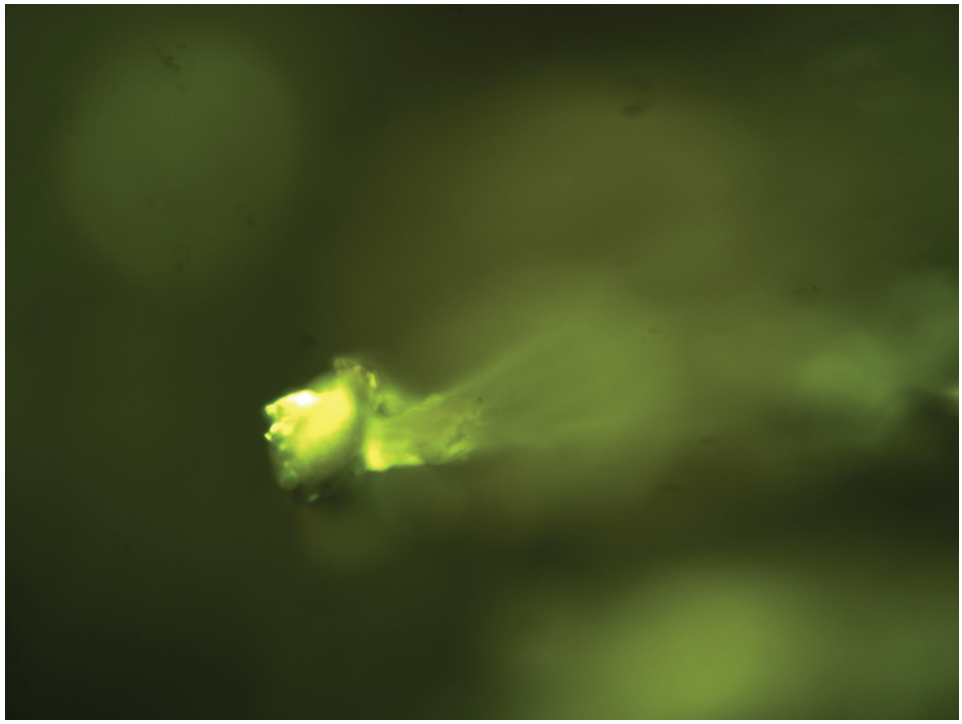

Fig. 14. Fluorescence Detail with head with AEDBE reagent (50×)

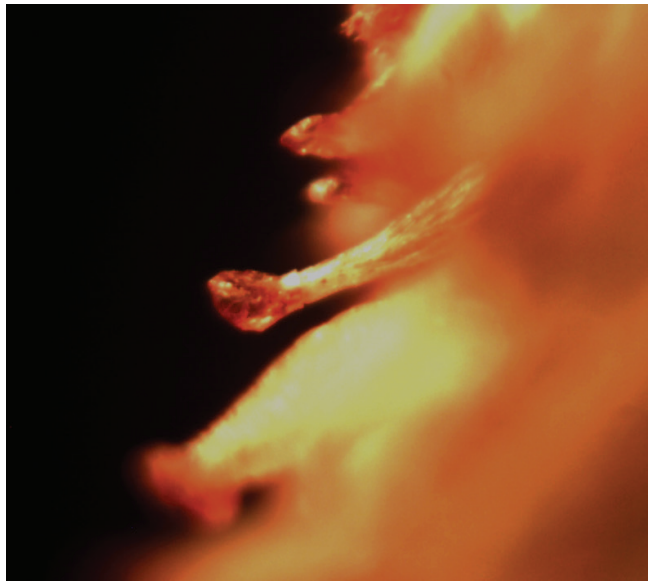

Fig. 15. Detail of Sudan III reaction in the head (50×)

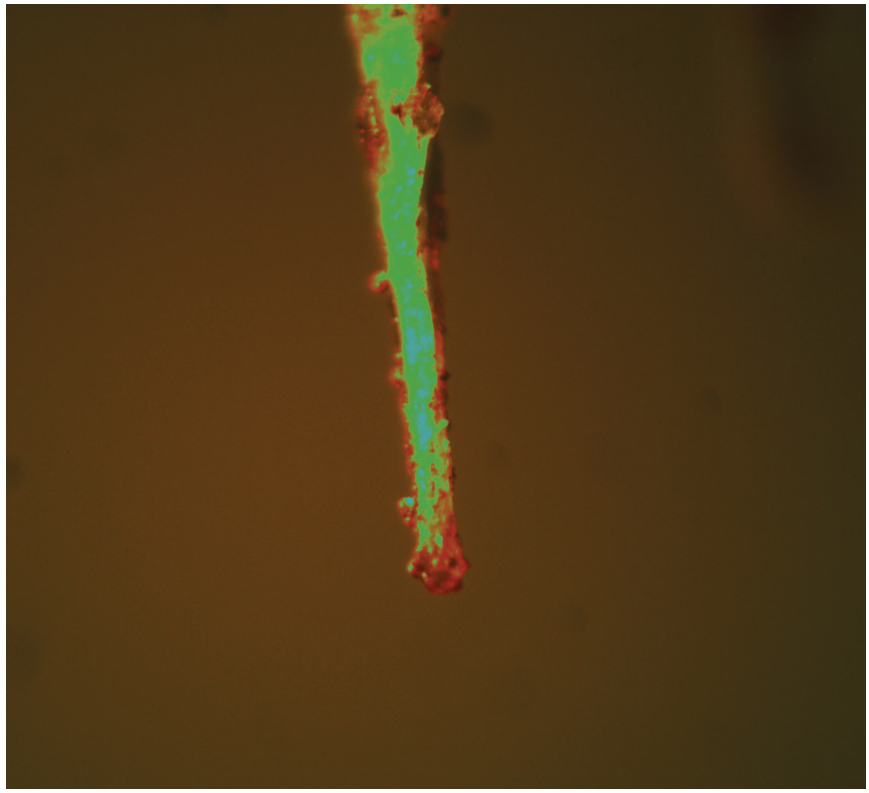

Fig. 16. Sudan III reaction detail in a prolongation without head (50×)

Discussion

Species known as "gall midges" are dipterous that belong to the Cecidomyiidae family which parasitize a wide diversity of vegetable species and generate a variety of structures, even on the same host (Oliveira et al., 2011). In the case of infections with *A. crassa* on *G. decorticans*, the gall has a higher polyphenol concentration compared to healthy leaves and stems. Besides, most of these compounds are condensed tannins since the gall is the organ with the higher amount of proanthocyanidins. The lowest concentration of flavonoids and hydroxycinnamic derivatives observed in the gall could correlate to a higher amount of condensed tannins since these metabolites are precursors in the proanthocyanidins synthesis (Zhou et al., 2015).

The leaf is the organ with the higher amount of hydroxycinnamic derivatives and flavonoids.

The results of this study should be analyzed in the light of the main hypothesis about the adaptive nature of insect-induced galls (Stone and Schönrogge, 2003). These structures, according to the above mentioned authors, have evolved to protect the early stages of development of the inducer against negative conditions of the environment such as desiccation and UV radiation (Microambient Hypothesis), against predator or patho-

gens (Enemy Hypothesis) and to improve nourishment of the inducer through the generation of a nutritive inner tissue (Nutrition Hypothesis).

Polyphenolic compounds perform numerous adapting functions under abiotic and biotic stress (Dixon, 1995; Matsuki, 1996; Quideau et al., 2011). Polanco and collaborators report polyphenolic substances in the prolongations and the cortical region of the gall and an amiliferous parenchyma without any of these metabolites in the larval chamber. This last result concurs with observations from other species of Cecidomyiidae and gives the idea of manipulation of defenses at a tissue-level to produce a nourishing tissue free from toxic substances. It has been observed that cecidogenous species in these families tend to produce a nourishing tissue with carbohydrates, different from galls produced by hymenopterous from the Cynipidae family, which generate a nourishing lipid tissue (Shorthouse and Rohfritsch, 1992).

The compounds observed in the chromatographic profiles are flavonoids (fluorescent orange spots) and hydroxycinnamic acids (light blue orange spots) (Wagner et al., 1984). Even though the aim of this research was not the identification of compounds, stains with intense orange fluorescence observed at higher Rf in the acetic acid 15% dimension in the chromatographic profile of the leaf, suggest that principal flavonoids are glycosylated. In the gall, the stain with higher intensity would correspond to flavonoid aglycones due to its poor mobility in the dimension created with acetic acid 15%. Studies of a higher complexity are needed to characterize and identify the compounds involved in this variation. However, the techniques employed in this study provide information about the phenolic metabolites as a whole.

It is known that cecidogeous insects can avoid and alter chemical defenses of those plants that lodge them for their benefit and that of their species. In this case, condensed tannins are known due to their fungicidal activity (Scalbert, 1991; Lattanzio et al, 2006), even in trichomes (Aziz et al., 2004; Kelsey et al., 1984; Li et al., 1996), and defense against herbivory (Forkner et al., 2004; Ceballos et al., 2002). Some authors claim that the digestion of tannins in the alkaline pH in the midgut of the insects may produce hydrogen peroxide. This compound produces oxidative stress (Salminen and Karonen, 2011) even though this characteristic may be linked to the pro-oxidant activity of hydrolysable tannins.

Nyman and Julkunen-Tiitto (2000) have reported the accumulation of phenolic compounds in general and condensed tannins in particular in the outer parts of galls induced by sawflies of the *Pontania* genus (Hymenoptera), with a decrease in the concentration of this family of secondary metabolites in the inner parts of the gall.

The results of our research are in concordance with the above mentioned study. The presence of condensed tannins in the prolongations present in the gall surface would be an adaptation for the protection of the gall against fungi and predators, and its biosynthesis would be favored over the synthesis of flavonoids and hydroxycinnamic acids.

Also, in general, polyphenolic compounds may act asinhibitors of the indole-acetic oxidase enzyme. This increases the duration of indole-acetic acid and contributes to the cellular hyperplasia and hypertrophy observed (Bedetti et al., 2014).

Many insects produce galls with physical and chemical defensive characteristics in their surface as prolongations and even nectaries (Nicholls et al., 2017). In galls induced by *A. crassa*, prolongations may have a defensive function, as they obstruct the oviposition of parasitoid wasps from the Apocrita suborder among other predators. The above mentioned research from Polanco and his collaborators explained the appearance of

diverse hymenoptera such as *Platygaster sociabilis* Kieff and Jog. (Hymenoptera: Platygasteridae) among others, inside the gall. This defensive character of physical nature has also a chemical component represented by the presence of areas with flavonoids in the bulbs and the heads; which, once they come apart, drop a hydrophobic substance, which hinders locomotion and oviposition of adult parasitoids in the external surface of the gall. The presence of zones reactive to Sudan III coloring provides support to this hypothesis. It is worth mentioning that handling the galls is difficult because the dry prolongations break and remain stuck to the fingers of the operator. Probably, this is why this character is hard to observe.

In conclusion, the gall contains a higher amount of total phenolic compounds, and condensed tannins are the substances present in a higher proportion. This group of secondary metabolites are present in the prolongations of the gall. However, it would not be the only chemical defense since there are hydrophobic compounds on top of these prolongations which could enhance the defense in a physical and/or chemical manner. These results support the idea of a defense against predators as was stated by the above mentioned Enemy Hypothesis.

It is necessary to carry out further histochemical and phytochemical research to understand the chemical nature of these substances and their role in the defense against predators, and to continue with the studies of galls induced by other insects on the same host.

Acknowledgment

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