Tropical Ecology **55**(1): 129-136, 2014 ISSN 0564-3295

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Nutritional sink formation in galls of *Ficus glomerata* Roxb. (Moraceae) by the insect *Pauropsylla depressa* (Psyllidae, Hemiptera)

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Abstract: Nutritional resource manipulation is typical of insect-plant gall interactions. Galls act as physiological sinks providing insects with essential nutrients needed for their growth and development. Insect galls, galled (diseased) leaves and un-galled (healthy) leaves of *Ficus glomerata* infested by the gall making insect *Pauropsylla depressa* were collected to study the different changes resulting from the biotic stress caused by insect feeding. The first instar nymph initiates gall formation during the feeding stage by secreting saliva rich in proteins and lytic enzymes. This leads to localized cancerous growth causing mobilization of nutrients such as reducing sugars (RS), total soluble sugars (TSS), starch, free amino acids (FAA), proline and protein to the gall from the un-galled region of the leaf. A corresponding decrease of these nutrients was noted in the latter. Higher levels of total phenols (TP) and ortho-dihydric phenols (OP) were observed in galls when compared to the galled leaf. In addition, activities of amylase (AMY) and invertase (INV) were found to be higher in galls than in the galled leaf.

Resumen: La manipulación de recursos nutricionales es típica de las interacciones insecto-planta en las que intervienen agallas. Las agallas actúan como resumideros fisiológicos que proporcionan a los insectos nutrimentos esenciales necesarios para su crecimiento y desarrollo. Se recolectaron agallas, hojas agalladas (enfermas) y hojas no agalladas (sanas) de *Ficus glomerata* infestadas por el insecto formador de agallas *Pauropsylla depressa*, con el fin de estudiar los diferentes cambios que resultan del estrés biótico causado por la actividad de alimentación del insecto. La ninfa del primer estadio larvario inicia la formación de agallas durante la fase de alimentación al secretar saliva rica en proteínas y enzimas líticas. Esto resulta en un crecimiento canceroso localizado que causa la movilización de nutrientes tales como las azúcares reductoras, las azúcares solubles totales, el almidón, los aminoácidos libres, la prolina y la proteína hacia la agalla desde una región no agallada de la hoja. En esta última fue notable un decremento correspondiente de estos nutrientes. Se observaron niveles altos de los fenoles totales y los fenoles ortodihídricos en las agallas en comparación con la hoja agallada. Además, se encontró que las actividades de la amilasa y la invertasa fueron mayores en las agallas que en la hoja agallada.

Resumo: A manipulação de recursos nutricional é típica das interações inseto-planta-galha. As galhas atuam como sumidouros fisiológicas fornecendo aos insetos nutrientes essenciais necessários ao seu crescimento e desenvolvimento. Para estudar as diferentes mudanças resultantes do estresse biótico causado pela alimentação do inseto, colheram-se galhas de insetos, folhascom galhas (doentes) e folhas sem galhas (saudáveis) de *Ficus glomerata* infestadas pelo inseto *Pauropsylla indutor* da formação de galhas. O primeiro instar de ninfa inicia a formação da galha durante a fase de alimentação secretando saliva rica em proteínas e enzimas líticas. Isto conduz ao crescimento canceroso localizado, causando a mobilização de nutrientes, como açúcares redutores (RS), açúcares solúveis totais (SST), amido, aminoácidos

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livres (FAA), prolina e proteínas para a galha a partir da região não afectada da folha. A diminuição correspondente destes nutrientes foi observado nesta última. Nas galhas foram observadas níveis mais altos de fenóis totais (TP) e de orto-dihidro-fenóis (OP), em comparação com a folha com galhas. Além disso, as atividades da amilase (AMY) e invertase (INV) mostraram-se mais elevadasnas galhas do que na folha com galhas.

Key words: Amylase, galls, invertase, phenols, proline, sugars.

Introduction

Galls are induced by a great variety of organisms including insects, mites, nematodes, fungi, bacteria, and viruses. These are outgrowths that develop from rapid mitosis and morphogenesis of plant tissues resulting in hypertrophies (cell expansion) or neoplasms (tumorous outgrowths) (Byers *et al.* 1976). Galls and other symptoms of disease that develop on host plants under the influence of insects have inspired many entomologists and botanists to conduct investigations into the causes of gall formation. All plant organs are subject to galling including roots, stems, leaves, flowers and fruits. The effect of the gall-insect attack on different plant organs varies depending not only on the type of the organ attacked but also on the developmental state of the organ at the time of attack (Birch 1974). Some investigators ascribe induction of galls to the plant-growth substances in the insect saliva that result in chemical changes in the plant tissues (Birch 1974; Giuntoli 1985; Shorthouse *et al.* 1992).

Ficus glomerata Roxb. (Moraceae), popularly called Cluster fig tree or Goolar fig is a large deciduous tree common in South India, Punjab, Bihar, Orissa, West Bengal and Rajasthan. The tree is 18 m in height; leaves are ovate or elliptic, sub-acute, entire and petiolate. Figs are subglobose or pyriform, red when ripe, borne in large clusters, on short leafless branches emerging from the trunk and the main branches (Paarakh 2009). A plant bug, *Pauropsylla depressa* Crawf (Psyliidae, Hemiptera) induces galls on the leaves of the plant. The leaf gall thus formed is an epiphyllous (growing on leaves), simple, fleshy, globose (spherical), sessile (attached), perfoliate (near the stem), unilocular (single chamber), pouch gall, or they are multilocular agglomerate (clustered) masses, with large spherical or convex bulges representing individual galls that have become fused into a composite mass. The color of the gall is usually yellow, orange, reddish or reddish-brown and almost completely devoid of chlorophyll. The size is ~5 - 10 mm in diameter.

Specific interactions between insects and plants are very common in nature; among these interactions those of galling insects and their host plants are believed to be the most intimate (Fernandes 1990). Gall forming insects have the ability to alter the development of plant tissues to cause the formation of tumor-like growths that surround the insect to protect it from the environment and supply it with a source of food (Ananthakrishnan 1998). However, the mechanisms of gall formation by plants in response to the insect attack remain largely unknown. In addition, the stimuli which trigger gall formation are also unknown. This work was undertaken to begin the study of the initial events of gall formation, particularly the generation of a nutritional sink by first identifying the nutrients in the gall (G) that differ from those in the galled leaf (GL) and healthy leaf (HL).

Materials and methods

Ficus glomerata trees having frequent occurrence of galls on young leaves induced by *Pauropsylla depressa* were selected at Bengaluru, Karnataka. The galls and leaves (both galled and healthy) were sampled during October and November 2006. The plant and galling insect were authenticated at the Regional Research Institute (Ayurveda), Pune. Chilled galls were quickly dissected, and the gall forming aphids were removed from the galls. The frozen samples were homogenized with pre-chilled 50 mM sodium phosphate buffer (pH 7.0) containing 5 mM ß-mercaptoethanol and 1 mM EDTA using pestle and mortar. The homogenate was centrifuged at 3000 rpm for 15 min at 4 ºC. The supernatant was used to assay all parameters under consideration.

Estimation of carbohydrates

Reducing sugars (RS): Reducing sugars were estimated according to Miller (1972). 500 mg of plant material was treated with 10.0 ml of 80 % ethyl alcohol. 3.0 ml of DNS (3, 5-dinitro-salicylic acid) reagent was added to 3.0 ml of the alcoholic extract. The mixture was heated for 5 min in a boiling water bath. After colour development, 1.0 ml of 40 % Rochelle salt was added.The tubes were cooled under running tap water. Absorbance was recorded using a spectrophotometer at 520 nm. The amount of reducing sugar was calculated using a standard curve prepared from glucose. The quantity of reducing sugar was expressed as mg g^{-1} fresh weight of tissue.

Total soluble sugars (TSS): The amount of total soluble sugars was estimated by a Phenol sulphuric acid reagent method (Dubois *et al.* 1951). 500 mg of each plant material was homogenized with 10.0 ml of 80 % ethanol. Each sample was centrifuged at 2000 rpm for 20 min. 1.0 ml of 5 % phenol solution was added to 1.0 ml of the supernatant. Then 5.0 ml of 96 % sulphuric acid was added rapidly. Each tube was gently agitated and allowed to stand in a water bath at 30 ºC for 20 min. The optical density (OD) was measured in a spectrophotometer at 490 nm against the blank. A standard curve was prepared by using known concentration of glucose. The quantity of TSS was expressed as mg g^{-1} fresh weight of tissue.

Starch: Estimation of starch was carried out by the method of McCready *et al.* (1950). The residual mass obtained after the extraction of soluble sugar was suspended in 5.0 ml of water and subsequently 6.5 ml of 52 % perchloric acid was added to the residue, the contents were centrifuged for 20 min at 2000 rpm. The supernatant was decanted and collected and the whole procedure was repeated thrice. The supernatant of each step were then poured into a standard flask and the total volume was made up to 100 ml with distilled water. The mixture was then filtered through Whatman filter paper (No. 42). 1.0 ml aliquot of this filtrate was analyzed for starch content following the same procedure as that of total soluble sugar. Quantity of starch was calculated in terms of glucose equivalent and factor 0.9 was used to convert the values of glucose to starch. The quantity of starch was expressed in terms of mg g^{-1} fresh wt. of tissue.

Estimation of amino acids and protein

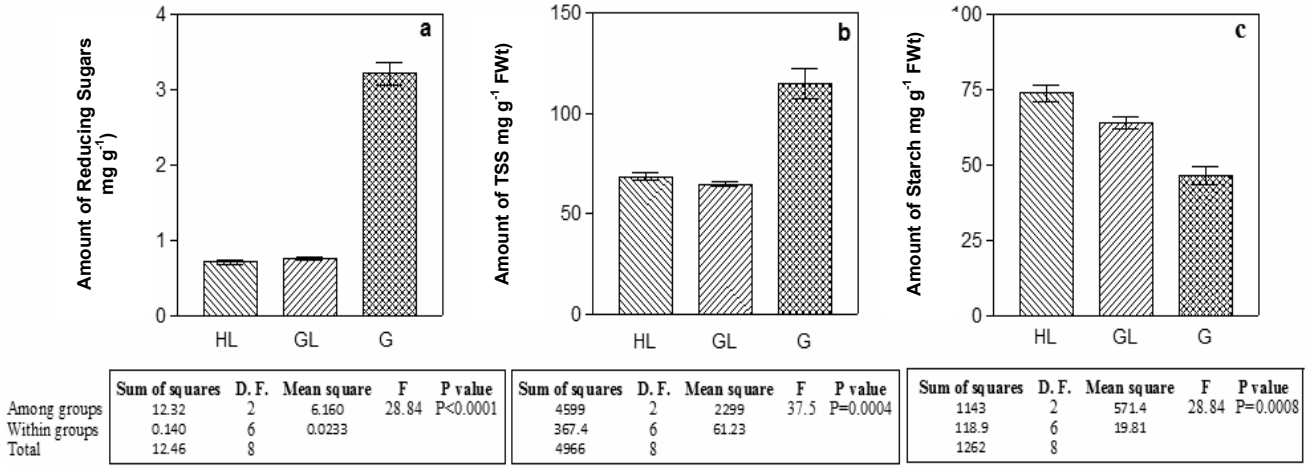
Total protein: The extract was mixed with an equal volume of 10 % tri-chloro acetic acid and again centrifuged at 3300 rpm for 10 min at 4 ºC. The pellet was washed with water 2 - 3 times and then dissolved in 0.1 N NaOH. Proteins were quantified by the protein dye binding method of Bradford (1976) using Bovine Serum Albumin (BSA) as a standard.

Proline: The estimation was carried out by the method of Bates *et al.* (1973). 500 mg of the plant material was homogenized in 10.0 ml sulpho-salicylic acid (3 %) and the extract was filtered through Whatman no.1 filter paper. 0.2 ml of the filtrate was mixed with 2.0 ml of acid ninhydrin reagent. The contents were boiled for 1 h in a boiling water bath and cooled rapidly in an ice bath. 4.0 ml of toluene was added to each test tube and vigorously shaken for a few seconds. The organic layer was separated and OD recorded at 520 nm against a toluene blank. A standard curve of proline was prepared for different concentrations for proline.

Free amino acids (FAA): FAA was measured quantitatively according to the method of Moore & Stein (1948). Chromatographic separation was also performed to analyze the extract qualitatively. All three tissue types were fixed in 70 % ethanol to extract free amino acids. The insects were removed before fixing the material to avoid any possible extraction of amino acids from the insects. The fixed materials were then crushed and centrifuged to obtain clear extracts which were then transferred to beakers and dried in an oven at 30 - 40 ºC. The residue thus obtained was weighed each time before dissolving in absolute alcohol to obtain equal concentrations. A known volume of the extracts was used for spotting so that visual comparison for amino acids was possible on the chromatogram. Both paper and thin layer chromatography (TLC) were employed to separate FAA using butanol : acetic acid : water (4:1:5) and butanol: water (3:1) as solvent systems, respectively. In both instances, the plate or paper was air-dried, sprayed with 0.3 % ninhydrin in 90 % butanol and kept at 60 ºC. The intensity of colour developed was compared with the standard amino acid chromatograms.

Estimation of phenols

Total phenols (TP): Total phenolic contents were estimated according to Slinkard & Singleton (1977) using gallic acid as standard. 0.1 ml of the extract was taken in a volumetric flask, 46.0 ml distilled water and 1.0 ml Folin-Ciocalteu reagent was added and the flask was shaken thoroughly.

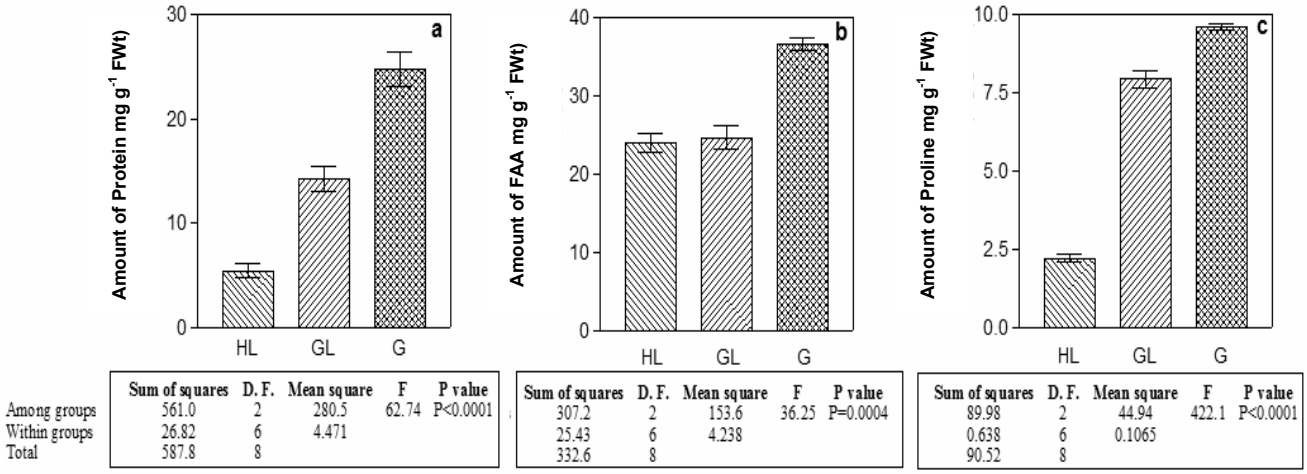


	Sum of squares	D. F.	Mean square	F	P value
Among groups	12.32	2	6.160	28.84	P<0.0001
Within groups	0.140	6	0.0233		
Total	12.46	8			

	Sum of squares	D. F.	Mean square	F	P value
	4599	2	2299	37.5	P=0.0004
	367.4	6	61.23		
	4966	8			

	Sum of squares	D. F.	Mean square	F	P value
	1143	2	571.4	28.84	P=0.0008
	118.9	6	19.81		
	1262	8			

Fig. 1. Levels of reducing sugars (a), total soluble sugars (b), and starch (c), of healthy leaves, galled leaves and galls. Results are mean ± SE, obtained from three replicates.



	Sum of squares	D. F.	Mean square	F	P value
Among groups	561.0	2	280.5	62.74	P<0.0001
Within groups	26.82	6	4.471		
Total	587.8	8			

	Sum of squares	D. F.	Mean square	F	P value
	307.2	2	153.6	36.25	P=0.0004
	25.43	6	4.238		
	332.6	8			

	Sum of squares	D. F.	Mean square	F	P value
	89.98	2	44.94	422.1	P<0.0001
	0.638	6	0.1065		
	90.52	8			

Fig. 2. Levels of total protein (a), free amino acids (b), and proline (c) of healthy leaves, galled leaves and galls. Results are mean ± SE ($P < 0.05$), obtained from three replicates.

After 3 min, 3.0 ml of 2 % Na_2CO_3 solution was added and the mixture was allowed to stand for 2 h with intermittent shaking. Absorbance was measured at 760 nm. The same procedure was repeated for obtaining the standard curve of gallic acid.

Ortho-dihydric phenols (OP): OP was estimated according to Johnson & Schaal (1957). The supernatant was mixed with 0.05 N HCl, Arnow's reagent, and 1 N NaOH. The absorbance was read at 515 nm. Standard curve was prepared using pyro-catechol.

Enzyme activities

α-amylase (*AMY, E.C. 3.2.1.1*): α-amylase was determined by colorimetric procedure of Bernfeld (1955). A total of 500 mg fresh weight of each tissue sample was crushed with 5.0 ml of 0.01 M phosphate buffer at pH 7.0. The homogenate was centrifuged at 2500 rpm for 20 min. The supernatant was used to determine the enzyme activity. The reaction mixture consisted of 0.5 ml of enzyme extract and 0.5 ml of substrate solution (2 % starch). The reaction mixture was incubated at room temperature for 10 min and the reaction was stopped by adding 1.0 ml of DNS reagent. The tubes were heated in a boiling water bath for 15 min. 10.0 ml of distilled water was added to each tube. OD of the yellow colour developed was read at 540 nm against a zero min blank. The activity was expressed in terms of IU mg^{-1} fresh weight of tissue.

Invertase (*INV, E.C. 3.2.1.26*): Invertase activity was measured according to Sridhar & Ou

(1972). 500 mg plant material was homogenized in 5.0 ml of 0.1 M sodium acetate buffer (pH 5.5). In a test tube 1.0 ml of 2.5 % sucrose was added. To this reaction mixture, 0.5 ml enzyme extract was added followed by 2.0 ml of buffer. After incubation at 30 ºC for 30 min, 1.0 ml of DNS reagent was added. Tubes were boiled in water bath for 10 min and then diluted to 10.0 ml by adding distilled water. OD was taken at 560 nm. Invertase activity was expressed as IU mg^{-1} fresh weight tissue.

Statistical analysis

The experiment was performed using a randomized design. All data are expressed as means of triplicate experiments unless mentioned otherwise. Comparisons of means were performed using GraphPad Prism version 3.02. Data were subjected to a one-way analysis of variance (ANOVA), and the mean differences were compared by Least Significant Difference (LSD) test. Comparisons with $P \leq 0.05$ were considered significantly different.

Results and discussion

Galls are sinks for photoassimilates and mineral nutrients (Bagatto & Shorthouse 1994). By altering patterns of allocation, gall formers can cause major changes in the architecture and ecology of their hosts (Fay *et al*. 1996). In some cases, galls can be very strong sinks leading to the death of other parts of the plant. The sink strength establishes the success of galling insects. Galls are formed due to biochemical changes occurring in leaves as a result of induction by the insect. The first step in determining the mechanism of gall formation is by comparing the concentrations of different biomolecules found in the healthy leaf, galled leaf and galls. This gives us an idea about the possible role of these biomolecules in gall formation and hence an insight into the mechanism of induction of galls. Galls exhibited a considerable increase in reducing sugars (3.7-fold) (Fig. 1a) and total soluble sugars (~1.7 fold) (Fig. 1b) when compared to healthy and galled leaves. This accumulation may involve the translocation of sugars from neighbouring tissues to the gall in order to sustain the developing galling insect (Shaw & Samborski 1956). An increased rate of synthesis due to enhanced gene expression as a result of certain inducers and transcription factors produced by the galling insect may also contribute to the development of a sink (Stotz *et al.* 1999). A decrease in starch content in galls (Fig. 1c) arose due to the activated amylase activity in this tissue. Similar results were reported by Shekhawat *et al.* (1978) and Rao (1989). Carbohydrates play a dual role as a source of carbon and structural components needed for plant cells and tissues, as well as for the provision of energy for the developing galling insect (Marmit & Sharma 2008).

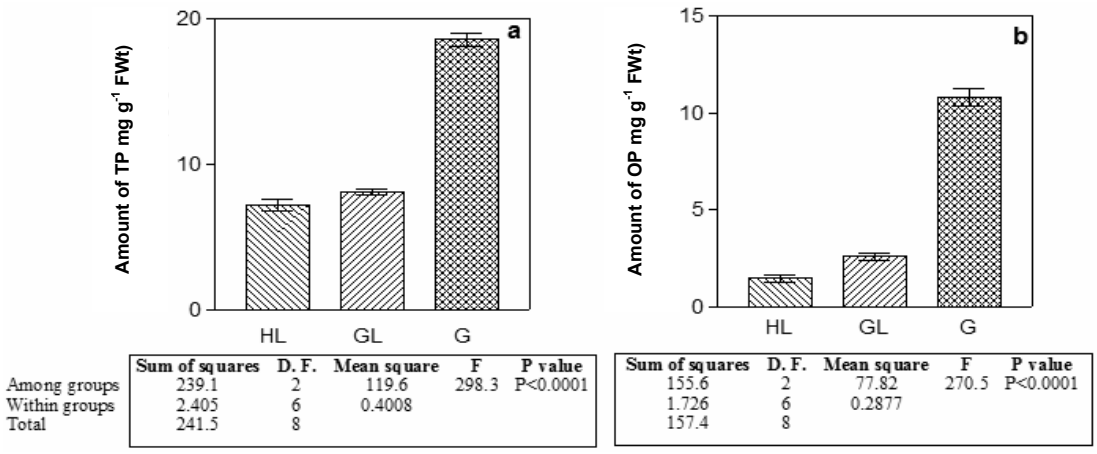
The protein content in galls was found to be higher compared to healthy and galled leaves (Fig. 2a). This increase is contributed by insect-induced increases in host gene expression or by the plant as a means of defense against the gall insect (Birch 1974). The gall forming insect also secretes certain enzymes or enzyme-like substances, proteins and growth regulators during gall formation (Decleene *et al*. 1980). It was also found that the free amino acid content in galls was almost three fold when compared to healthy and galled leaves (Fig. 2b). The presence of free amino acids in galls provides building blocks for protein synthesis in the galling insect (Birch 1974). Among all amino acids assayed, it was also found that histidine and methionine were present in excess in galls (Table 1). Hartley (1998) proposed that these two amino acids serve as conditioners of gall induction by making the plant tissue more plastic and raising its sensitivity to the gall inducers. The amino acid, proline is significantly higher in galls and galled leaves than healthy leaves (Fig. 2c). This indicates that the process of galling has a stressful effect on infested leaves and that excess proline might play a role in oxidative stress adaptation within the cell (Bates *et al.* 1973). Proline is also involved in biosynthetic processes, energy production, stabilization of cellular membranes and signaling (Nayer & Reza 2008).

Phenolic compounds occur in a variety of simple and complex forms. During the present investigation phenolics in galls were higher as compared to healthy and galled leaves. Similar findings were recorded in leaf gall of *Ziziphus mauritiana* and *Ficus mysorensis* (Purohit *et al*. 1979). A significant increase in total phenols and orthodihydric phenols (Fig. 3a, b) was noted in galls. Polyphenols play an active role in lignification, anti-auxin activities and resistance to the spread of disease (Sgherri *et al.* 2004). O- and p-dihydroxyphenols and polyphenols also act as IAA-oxidase inhibitors leading to hyperauxinity and then gall formation (Shekhawat *et al.* 1978).

In addition to the growth-stimulating chemicals, many insects produce enzymes that convert the plant's starch into sugar, causing some of the galls

Table 1. Qualitative estimation of amino acids by TLC and paper chromatography.

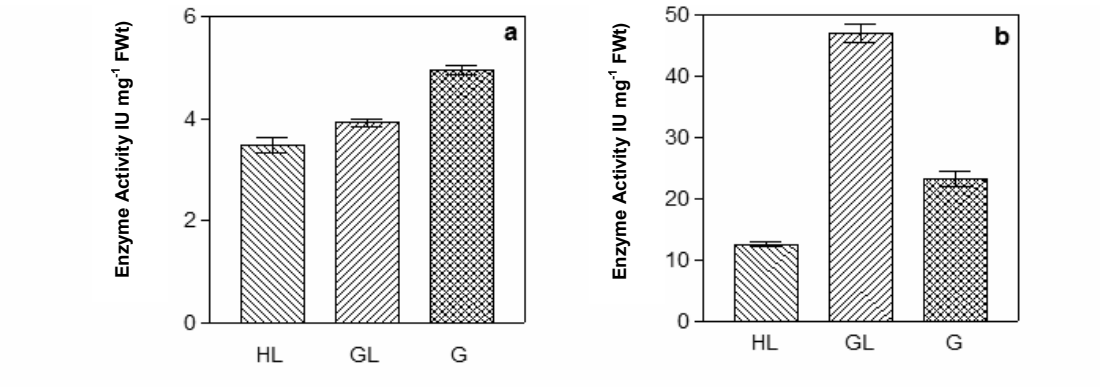
Amino acid	Healthy Leaf (HL)	Galled Leaf (GL)	Gall (G)
Arginine (Rf = 0.12)	++	-	++
Glycine (Rf = 0.17)	++	+	+++
Alanine (Rf = 0.22)	++	+	++
Valine (Rf = 0.45)	++	+	++
Leucine (Rf = 0.42)	+	+	+
Isoleucine (Rf = 0.54)	+	+	+
Methionine (Rf = 0.40)	-	-	+
Cysteine (Rf = 0.23)	+++	-	+
Proline (Rf = 0.29)	++	+++	+++
Tryptophan (Rf = 0.47)	-	-	-
Lysine (Rf = 0.10)	++	-	+
Histidine (Rf = 0.07)	-	-	++
Aspartate (Rf = 0.12)	+++	-	++



	Sum of squares	D. F.	Mean square	F	P value
Among groups	239.1	2	119.6	298.3	P<0.0001
Within groups	2.405	6	0.4008		
Total	241.5	8			

	Sum of squares	D. F.	Mean square	F	P value
	155.6	2	77.82	270.5	P<0.0001
	1.726	6	0.2877		
	157.4	8			

Fig. 3. Levels of total phenols (a) and orthodihydric phenols (b) of healthy leaves, galled leaves and galls. Results are mean ± SE, obtained from three replicates.



	Sum of squares	D. F.	Mean square	F	P value
Among groups	3.441	2	1.720	45.78	P=0.0002
Within groups	0.2255	6	0.03758		
Total	3.666	8			

	Sum of squares	D. F.	Mean square	F	P value
	0.00169	2	0.000847	121.0	P<0.0001
	0.00004	6	0.000007		
	0.00173	8			

Fig. 4. Activities of amylase (a) and invertase (b) of healthy leaves, galled leaves and galls. Results are mean ± SE, obtained from three replicates.

to accumulate large amounts of sugar (Joshi *et al.* 2009). Amylase (AMY) activity was found to be highest in galls than in healthy and galled leaves (Fig. 4a) thus contributing to the nutritional sink of galls. Invertases (INV) are sucrose hydrolyzing enzymes often associated with plant tissues acting as physiological sinks. INV was found to be greater in galled leaves than in healthy leaves or galls (Fig. 4b) which underlies the enhanced sink strength commonly observed in galls. Initial gall growth correlates positively with high INV activity, while later insect fecundity is also dependent on the INV activity.

Plants and insects interact at various levels; some of the most exciting and deepest relationships are seen in insect-induced gall formation. Our results quantify a significant relationship between nutrients and phenolics found within galls. The degree and specificity of the interactions are also finely tuned and represent a well-developed co-evolutionary relationship. While most of the insect galls do not cause serious damage to crop yield, they nevertheless must be mitigated for aesthetic reasons. Control of such diseases is possible only by studying the plant-insect relationship at molecular and cellular level. Here we provide a significant first step in understanding this relationship.

Acknowledgment

The authors wish to thank Dr. S. Sundarajan, Director, Centre for Vrikshayurveda, a Division of Center for Advanced Studies in Biosciences, Jain University, for his guidance and expertise in this field and Wingene Biotech Research Laboratory for providing us the facilities for conducting research in their labs.

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(Received on 31.12.2011 and accepted after revisions, on 22.11.2012)