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Nutritional Sink Formation in Galls of *Millettia pinnata* synergistically infected by *Myricomyia pongamiae* and *Eriophyes cheriani*

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Abstract
Investigation of source and sink relationships between galls and their host plants often invoke exploration of nutrition, environment and enemy hypotheses. According to the nutrition hypothesis, resource manipulation and endophagy are typical of insect-plant gall interaction, in which galls act as nutritional sinks providing insects with essential nutrients needed for their growth and development. The galling insect controls the nutrient levels in the gall for their own benefit. Insect galls (G), galled leaves (GL) and healthy leaves (HL) of Millettia pinnata infested synergistically by the galling insects Myricomyia pongamiae and Eriophyes cheriani were collected to study the various changes resulting from biotic stress challenged with insect feeding. There is mobilization of nutrients like reducing sugar, total soluble sugars (TSS), starch, and proteins into gall tissues from the ungalled region of the leaves. Higher concentrations of total phenols (TP) and ortho-dihydric phenols (OP) were reported in galled leaves. Biomarkers of stress such as H_2O_2, malondialdehyde, proline and ascorbic acid were reported to be in higher levels in galled leaf tissues. In addition, levels of reduced glutathione, a non-enzymatic antioxidant responsible for scavenging free radicals declined in infected leaves. Antioxidant enzymes like glutathione reductase, peroxidases and phenol oxidase were found to possess higher activities in galls and galled leaf when compared to healthy leaf tissue.

Keywords: Nutritional sink, galls, *Millettia pinnata*, phenolics, antioxidant, biotic stress

1. Introduction
Galls are formed as a result of parasitic attack by insects, mites, nematodes, fungi, bacteria, and viruses. These are outgrowths developing from rapid mitosis and morphogenesis of plant tissues resulting in hypertrophies and neoplasms [1]. Among various interactions, those involving galling insects and their host plants are believed to be the most intimate [2,3]. The galling insects are highly host and organ specific [4]. All plant tissues are susceptible to galling, however; the effect of gall-insect interaction is dependent on the developmental stage of the organ at the time of attack [5]. Some researchers attribute the induction of galls to plant-growth substances in the insect saliva that result in chemical changes in the plant tissues [5,6,7].
Millettia pinnata, popularly called Karanja is a medium sized glabrous tree, mainly distributed in tidal forests of India. It has been used in folkloric medicine, particularly in Ayurvedic and siddha systems of Indian medicine [8] for the treatment of varied human diseases like bronchitis, whooping cough, rheumatism, diarrhoea, dyspepsia, gonorrhoea, leprosy and even tumors. It has been increasingly used for producing oil used in biodiesel, lighting, as a raw material for soaps, varnishes and paints, to repel insects in storage instalments, and as a mosquito repellent [9,10]. The simultaneous infection by *Myricomyia pongamiae* and *Eriophyes cheriani* is essential for gall induction. The galls formed fuse to form complex, irregularly shaped, massive structures, covering the entire laminar area including the midrib, vein and veinlets. The mechanisms of gall formation by *M. pinnata* in response to the insect attack remain largely 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).

2. Materials and Methods
Millettia pinnata trees having frequent occurrence of galls on young leaves were selected at Vasanthnagar, Bengaluru, Karnataka (Fig 1). The galls and leaves (both galled and healthy) were sampled during November 2018 to February 2019. The plant and galling insect were authenticated at the Regional Research Institute (Ayurveda), Bengaluru. Chilled galls were quickly dissected, and the gall forming insects were removed from the galls. The frozen samples were homogenized in pestle and mortar using pre-chilled buffers as mentioned below. The homogenate was centrifuged at 8000 rpm for 15 min at 4 ºC. The supernatant was used to assay all parameters under consideration.

Fig 1: Insect galls on the laminar surface of *Millettia pinnata* leaf.

2.1 Effect of Galling on Establishment of Nutritional Sink

2.1.1 Carbohydrate content

Water-soluble polysaccharides (WSP), total soluble sugars (TSS) and starch was assayed by the phenol sulphuric acid method [11,12] and analyzed in a spectrophotometer. The plant material was dried in an oven and then macerated. The WSP were extracted with 10% ethanol [13], TSS with methanol: chloroform: water (12:5:3) [14] and starch with 30% perchloric acid [15].

2.1.2 Total soluble protein and free amino acid content

Total soluble protein content was determined according to the method of Lowry *et al* [16], using BSA as the standard. Free amino acids (FAA) was measured quantitatively according to the method of Moore and Stein [17]. Thin layer chromatography (TLC) was also performed to analyse the extract qualitatively using butanol: water (3:1) as solvent system. The TLC plate 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.

2.1.3 Metabolic enzyme activity

α -Amylase (AMY, EC 3.2.1.1) was measured using dinitrosalicylic acid reagent [18]. Invertase (INV, EC 3.2.1.26) activity was determined according to the reported method [19]. Acid phosphatase (AP, EC 3.1.3.2) activity against p-nitrophenyl phosphate was determined by monitoring the release of p-nitrophenol at 410 nm [20].

2.2 Effect of Galling on Indicators of Biotic Stress

2.2.1 Levels of stress response markers

Standard procedures were followed for the assay of hydrogen peroxide [21], reduced glutathione (GSH) [22], malondialdehyde (MDA) [23], total phenols [24] and o-dihydric phenols [25].

2.2.2 ROS scavenging antioxidant enzymes activity

Catalase (CAT, EC 1.11.1.6) was assayed by following the decline in optical density at 240 nm ($\varepsilon = 39.4\ M^{-1}\ cm^{-1}$) [26]. Peroxidase (POX, EC 1.11.1.7) was assayed by following an increase in optical density at 470 nm ($\varepsilon = 26.6\ mM^{-1}\ cm^{-1}$) [27]. Glutathione reductase (GR, EC 1.6.4.2) was determined by monitoring the oxidation of NADPH at 340 nm ($\varepsilon = 6220\ M^{-1}\ cm^{-1}$) [28].

2.3 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 MS Excel and the data expressed as mean ± SD.

3. Results and Discussion

3.1 Nutritional sink formation in galls of *Millettia pinnata*

Gall formation resembles the development of a new fruit or leaf on a plant. Fruits and leaves have a greater requirement for sugars and other nutrients, which is met through the phloem. This attraction of nutrients by the gall is called a sink. The first step in determining the mechanism of gall formation is by comparing the concentrations of different biomolecules found in the healthy leaf (HL), galled leaf (GL) and galls (G). This give us an idea about the possible role of these biomolecules in gall formation and hence an insight into the mechanism of induction of galls.

Carbohydrates levels in gall tissue are commonly manipulated by the galling herbivores and may confer distinct metabolic status between the galls and the non-galled tissues [11, 12, 21]. Carbohydrate contents were significantly different between the HL, GL and the G (Fig 2). The total soluble sugars (TSS) were higher in the HL (17.2 ± 2.8 mg g^{-1} FW) than in the GL (3.8 ± 0.70 mg g^{-1} FW) and G (13.4 ± 2.8 mg g^{-1} FW). This variation was in contrast to water-soluble polysaccharides (WSP), which were higher in the GL (1.78 ± 0.063 mg g^{-1} FW). Starch storage was significantly higher in G (0.8 ± 0.003 mg g^{-1} FW) when compared to GL (0.094 ± 0.0 mg g^{-1} FW) and HL (0.215 ± 0.03 mg g^{-1} FW). The TSS concentrated mainly in the non-galled leaves of *M. pinnata*, as the galls and galled leaves did not seem to perform photosynthesis to the same extent as the former. Nevertheless, the galls accumulate considerable amounts of starch and have water soluble polysaccharides for they are sinks of photoassimilates.

The concentration of total chlorophylls was 9-fold higher in the HL when compared to the G and 4-fold higher in GL (Table 1). Chlorophylls a and b, anthocyanins and carotenoids were higher in the HL than in the G and GL, but the chlorophyll a/b ratio was almost similar (Table 1). The analyses of galls induced in *M. pinnata*, together with the low content of photosynthesizing pigments, patterns of carbohydrates accumulation, and related enzyme activity indicate that these galls function as sinks of photoassimilates. The phenyl polyproponoid metabolism is linked to the import of carbohydrates into the galled leaf tissues. The relationship between galled leaf and galls was elaborated by Arnold and Schultz[2] by elimination of competing sinks and the generation of induced sink strength. A positive correlation was seen, that is, when the import of sugars increased, the phenolic content also increased (Fig 4). Thus, induced sink strength serves to provide resources for defense-response in the galled leaf. The decrease in starch content in GL and HL (Fig 2C) arises due to the activated amylase activity in these tissues (Fig 3). During infection, pathogens reallocate the sugars for their own needs forcing the plants to modify their sugar content and triggering their defense responses. Carbohydrates also 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 [29]. Current results partially corroborate the metabolic gradients previously described for other gall systems [30, 31, 32].

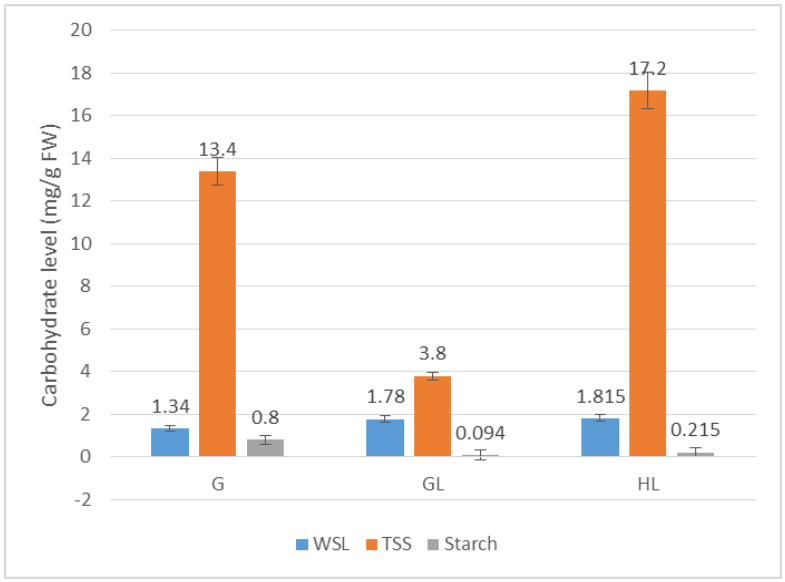

Fig 2: Levels of water-soluble polysaccharide (WSL), total soluble sugars (TSS) and starch in galls (G), galled leaves (GL) and healthy leaves (HL). Results are mean ± SE, obtained from three replicates**.**

Table 1: Content of photosynthetic and accessory pigments in healthy leaves (HL), galled leaves (GL) and galls (G) of *M. pinnata* (n = 3, mean ± SE; mg g^{-1} FW).

Pigments	Samples		
	HL	GL	G
Chlorophyll a	630.7 ± 25.4	157.6 ± 11.8	70.24 ± 8.6
Chlorophyll b	215.9 ± 13.9	53.7 ± 4.7	23.9 ± 2.1
Chlorophyll a/b	2.92 ± 0.03	2.93 ± 0.07	2.93 ± 0.06
Total chlorophylls	847.6 ± 40.9	209.4 ± 24.1	96.31 ± 12.5
Carotenoids	10.16 ± 0.3	7.61 ± 0.5	5.94 ± 0.2
Anthocyanins	22.34 ± 0.7	12.41 ± 1.8	3.54 ± 0.2

The activity of amylase in gall (G) was found to be lowest (11.49 ± 1.3 IU g^{-1} FW) when compared to HL (28.3 ± 4.3 IU g^{-1} FW) and GL tissues (19.25 ± 2.0 IU g^{-1} FW) (Fig 3). This relationship explains why the amount of starch is higher and reducing sugar is less in G (Fig 2). Conversely, activated enzyme activity in GL leads to breakdown of starch leading to accumulation of reducing sugars in them. The activity of invertase, sucrose hydrolyzing enzymes was found to be negligible in galls (0.076 ± 0.0 IU g^{-1} FW when compared to HL (0.37 ± 0.01 IU g^{-1} FW) and GL (0.55 ± 0.0 IU g^{-1} FW) (Fig 2). Within less than a month after the formation of the gall, a rise in the invertase activity around gall tissue has been documented [33]. Increased invertase activity underlies the enhanced sink strength commonly observed during the process of galling. The behaviour of invertase has been largely studied in developing crown galls caused by the bacterium *Agrobacterium tumefaciens* that injects its DNA into the host plant and codes for the growth hormone cytokinin. Cytokinin increases the activity of invertase in galls. With a sink created and localized on the adult leaf, cells again begin to grow and divide. Initial gall growth correlates positively with high INV activity, while later insect fecundity is dependent on reduced INV activity [31].

Activity of acid phosphatase was found to be highest in galls of *M. pinnata* (5.36 ± 0.3 IU g^{-1} FW) followed by galled leaf (4.12 ± 0.1 IU g^{-1} FW) and almost negligible in healthy leaf (0.96 ± 0.0 IU g^{-1} FW). This metabolic feature corroborates with the establishment of sinks in plant tissues and was previously cited to form a gradient towards the nymphal chamber in the galls of *A. austral* [34] breaking down starch to soluble sugars.

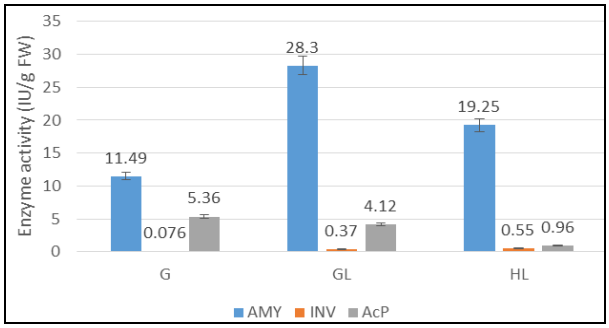

Fig 3: Enzyme activity of amylase (AMY), invertase (INV) and acid phosphatase (AcP) in galls (G), galled leaves (GL) and healthy leaves (HL). Results are mean ± SE, obtained from three replicates.

From the results obtained, galls were found to possess less amount of protein (12.34 ± 2.8 mg g^{-1} FW) than HL (19.89 ± 2.8 mg g^{-1} FW) and GL (26.29 ± 4.8 mg g^{-1} FW) (Fig 4). This may probably be due to increased amounts of free amino acid pool in the gall required by the galling insects for its survival [5]. GL has considerably significant amounts of total protein that can be correlated to defensive actions of the sink tissues to biotic stress, which causes them to release enzymes and certain defence proteins that increase the amount of total proteins in the affected leaves. Among all amino acids assayed, it was also found that histidine and methionine were present in excess in galls (Table 2). Hartley [35] proposed that proline and histidine serve as conditioners of gall induction by making the plant tissue more plastic and raising its sensitivity to the gall inducers.

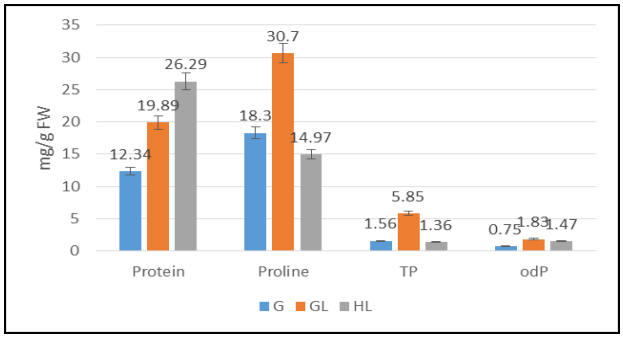

Fig 4: Total soluble protein, proline, total phenols (TP) and o-dihydric phenols (odP) in galls (G), galled leaves (GL) and healthy leaves (HL). Results are mean ± SE, obtained from three replicates.

Table 2: Qualitative estimation of amino acids by TLC.

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)	++	-	+

3.2 Effect of Galling on Indicators of Biotic Stress

Phenolic compounds are the most important non-enzymatic antioxidants. Levels of total phenols were found to be highest in GL (5.85 ± 0.2 mg g^{-1} FW), followed by almost similar levels in G (1.56 ± 0.08 mg g^{-1} FW) and HL (1.36 ± 0.1 mg g^{-1} FW) (Fig 4). Phenols are secondary metabolites for resistance against biotic stress and mainly possess antioxidant property. They are often produced and accumulated in the sub-epidermal layers of plant tissues exposed to stress and pathogen attack. They act as protective agents, inhibitors, pesticides against invading herbivores, nematodes, phytophagous insects and bacterial pathogens. Similarly, o-dihydric phenols (odP) were found to be highest in GL (1.83 ± 0.07 mg g^{-1} FW) in comparison to HL (1.47 ± 0.1 mg g^{-1} FW) and G (0.75 ± 0.0 mg g^{-1} FW) (Fig 4). odP play an active role in lignification, anti-auxin activities and resistance to the spread of diseases [36] in addition to serving as IAA oxidase inhibitors leading to hyperauxinity and thereby gall formation [37].

Subcellular levels of GSH and MDA, reactive oxygen species production, and antioxidative metabolism are considered valuable biotic stress indicators within plants during pathogen attack. When the defense system in plants stressed by insect gallers is overburdened, the reactive oxygen species (ROS) start to appear in excess and the antioxidative system is strongly activated. GL demonstrated maximum levels of H_2O_2 (0.150 ± 0.08 mg g^{-1} FW), followed by HL (0.138 ± 0.0 mg g^{-1} FW) and G (0.0815 ± 0.0 mg g^{-1} FW) (Fig 5). Reduced glutathione (GSH), the main non-enzymatic antioxidant and redox buffer was found to be higher in G (1.28 ± 0.03 mg g^{-1} FW) in comparison with GL (1.06 ± 0.01 mg g^{-1} FW) and HL (1.16 ± 0.0 mg g^{-1} FW) (Fig 5), a probable explanation for the reduced levels of H_2O_2 in galls. GSH is involved in the Halliwell-Asada cycle operative extensively for the protection of cells from the oxidative damage induced by environmental challenges.

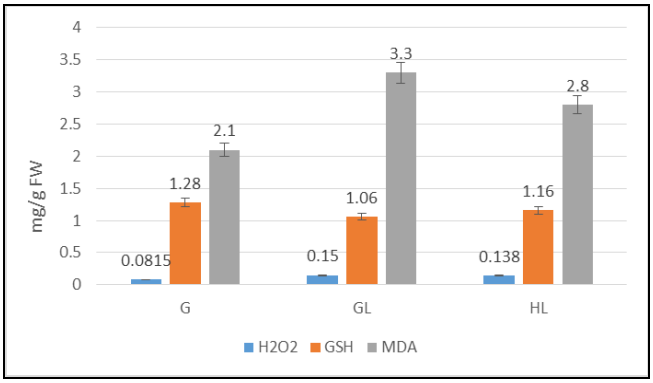

Fig 5: Levels of H_2O_2, reduced glutathione (GSH) and malondialdehyde (MDA) in galls (G), galled leaves (GL) and healthy leaves (HL). Results are mean ± SE, obtained from three replicates.

3.3 ROS scavenging antioxidant enzymes

Biotic stress has been found to enhance antioxidant enzymes such as CAT, SOD, APX and DHAR. These findings suggested that enzymes could be used as a biomarker for stress. Catalase (CAT), a heme-containing homotetrameric enzyme dismutates H_2O_2 into water and oxygen. It was found to be have similar levels of activity in all three tissues studied. This is indicative of the fact that CAT is not responsible for dismutation of H_2O_2 in *M. pinnata* (Fig 6). The brunt of reducing reactive oxygen species under galling conditions is taken over by peroxidase (POX) and glutathione reductase (GR), both of which actively participate in the Halliwell-Asada cycle. POX is involved in detoxifying H_2O_2, but at the expense of another substrate being oxidized such as ascorbate. The activity of POX was found to drastically increase in G (56.1 ± 4.3 IU g^{-1} FW) and GL (55.4 ± 5.6 IU g^{-1} FW). HL, however showed low levels of POX activity (15.6 ± 0.3 IU g^{-1} FW) (Fig 6). The activity of POX varies considerably depending upon plant species and stress condition. GR activity was found to be highest in HL (512.2 ± 10.6 IU g^{-1} FW), followed by G (384.4 ± 8.1 IU g^{-1} FW) and GL (256.1 ± 12.4 IU g^{-1} FW).

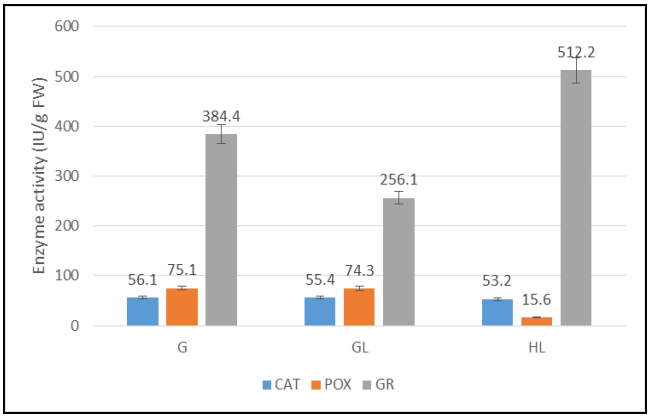

Fig 6: Activities of catalase (CAT), peroxidase (POX) and glutathione reductase (GR in galls (G), galled leaves (GL) and healthy leaves (HL). Results are mean ± SE, obtained from three replicates.

4. Conclusion

Plant and insects interact at different levels with the most interactive and deepest relationship being insect-induced gall formation. Such interactions possess high degree and specificity for the galling partners. Some galling insects are now to cripple plant growth and decrease crop yield significantly. Other interactions may not necessarily damage the host plant. Even in the later case, galls need to be mitigated for aesthetic reasons. In order to control such interactions, a thorough understanding of the plant-insect relationship at molecular and cellular level is needed. The results presented in this paper quantify a significant relationship between nutrients, metabolic enzymes, phenolics and antioxidants.

5. Acknowledgement

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