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INSECT-INDUCED FOLIAR GALLS: A CROSS-TALK AMONG PHYTOHORMONES FOR TISSUE GROWTH AND ENDOGENOUS DEFENSE

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Abstract
Insect-induced gall tissue has a unique ability to influence its hosts' phenotypic expression. When plants are stressed by insects, phytochemical manipulations in galling tissue strengthen the plant's resilience to subsequent herbivore attacks, and as a result, the damaged plant tissue regenerates and rejuvenates. Gall tissue development and differentiation are initiated by changes in and modulation of a variety of phytohormones in the affected galling sites. Such hormonal changes ultimately boost the plant's ability to respond to herbivore invasions. In this study, the insect-induced gall tissues of three model plants—mature and immature galls and non-gall tissue—were evaluated for five phytohormone gradients. Phytohormone gradients are continually altered and compared from non-differentiated (non-gall) tissue to moderately (immature gall) and highly (mature gall) developed tissue. The results indicate that phytohormones serve a dual role in stimulating the plant's endogenous defense and promoting tissue growth, pointing to a complex chemogenesis process in galling tissue associated with developing neoplasm and plant defensive responses. Tissue abnormalities in galls are thought to have resulted from the herbivore's interactions with the plant on which it infests. Insects' ovipositing fluids or oral discharge may have also contributed to the accumulation of phytohormones in the stressed and wounded tissue. Foliar galls are thus a sign of manifestation of insects' adaptation since the herbivore and their hosts are likely to co-evolve in the context of chemical adaptation.

Keywords: Foliar gall, phytohormone gradients, plant-herbivore co-evolution.

Introduction

Insect-plant interaction is a dynamic process during which both sides often create defensive mechanisms to deceive one another (Mello & Silva-Filho, 2002). Plants and insects have evolved a number of defense strategies against one another over time (War et al., 2012; Agrawal & Konno, 2009). Plants can "directly defend" themselves by changing the herbivore's physiology and behavior or "indirectly defend" themselves by luring in the herbivore's natural predators (Tiku, 2021). According to War et al. (2012), the direct defense mechanism employs physical or chemical barriers to impede herbivores from grazing, resulting in immediate adverse consequences for the herbivores. On the other hand, the phenomenon of "indirect defense" diminishes the frequency of herbivore consumption by attracting natural competitors such as parasitoids and predators (Agrawal & Konno, 2009; Tiku, 2021). As a result, the involvement of external intervention leads to an augmentation of indirect defense mechanisms in plants, concurrently strengthening their inherent ability to withstand herbivorous attacks directly (Ullah et al., 2018).

The gall complex serves as a notable example of the co-evolutionary relationship between herbivores and plants (Meyer, 1987; Shorthouse & Rohfritsch, 1992; Winde & Wittstock, 2011; Melnyk, 2017; Minelli, 2018). As a consequence, the plant-generated galls accommodate the invaders; some of them are friends, but the majority of them are foes. Plant galls, or 'cecidia', develop into tumor-like organs where their cells lose adhesion to surrounding tissues and multiply faster than non-gall tissues. As a result, the development of galls can lead to new growths in various plant organs, which are typically the result of hypertrophic and hyperplastic processes. A variety of parasitic organisms known as parasitic gall-inducers, or cecidozoans, are responsible for this neoplasia (Orlovskis & Hogenhout, 2016). The gall-inducer stimulates plant tissue regeneration in response to damage by modifying the developmental pathway through physiochemical mechanisms (Pfunder & Roy, 2000; Goethals et al., 2001). The concept of the "resource sink"

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model (Larson & Whitham, 1991; Fay et al., 1993) describes gall tissue as a "dynamic reserve" that has the potential to exert substantial influence on plant growth and metabolism. Auxin (IAA) and cytokinin (CK) are two plant hormones that have been observed to enhance the growth of abnormal outgrowths in gall tissues (Hartley & Miles, 1999; Tooker & De Moraes, 2009; 2011; Jameson, 2000; Davies, 2004). When herbivores attack, jasmonic acid (JA), salicylic acid (SA), and ethylene (ET) elicit plant defense responses (Robert-Seilaniantz et al., 2007; Howe & Jander, 2008). This information shows a complicated mutualistic relationship between the injured plant and the herbivore during gall formation. Scientists have different opinions on the roles of phytohormones in gall development because the chemical physiology of gall tissue varies from plant to plant (Tooker & Helms, 2014). As a result, the role of phytohormones in promoting tissue growth at galling sites needs to be better known. It is, therefore, critical to determine whether galling tissue accumulates a higher concentration of phytohormones than non-galling tissue or whether they work independently. The current study focuses on how phytohormone gradients rush in galling tissue compared to non-gall tissue; as a result, quantitative adjustments of phytohormones at the galling site were compared to those of normal non-gall tissue. This research provides insight into how phytohormones promote cell aggregation in gall formation while avoiding plant defense.

Materials and methods:

Sample collection:

The comparison of phytohormone accumulation and depletion in galling tissue versus non-galling tissues was done using three tropical plant species: *Alstonia scholaris* L. (Apocynaceae) (Milkwood Pine), *Mangifera indica* L. (Anacardiaceae) (Mango), and *Ficus racemosa* L. (Moraceae) (Fig). Mango and Fig exude latex, while milkwood pine secretes resins as a defense chemical against herbivore attacks (Kim et al., 2003). Such plant species were chosen to investigate how herbivore infestations may occur despite the presence of defensive compounds in those plants. Three kinds of plant leaves were chosen: a) Non-Gall leaves: leaves that did not have any gall infestation (NG); b) Immature-Gall leaves: leaves that showed early gall formation (IG); and c) Mature-Gall leaves: leaves that showed only mature galls (MG). The proportions and arrangement of galls on a leaf were the basis for categorizing IG and MG leaves. Gall structures with a diameter of less than 1 mm were classified as IGs, while those with a 2–5 mm diameter were classified as MGs (Price & Clancy, 1986; Rehill & Schultz, 2001). Five plants of a species having both galled and non-galled leaves were collected during 2020–2021 from various locations in and around Kolkata, India, and returned to the laboratory for further studies. The NG samples were considered as 'control'. At least 20 leaves from various branches of each plant (of a species) were randomly picked for the NG, IG, and MG samples. Foliar tissue from IG and MG was removed from the leaves by fine blades, classified by plant species, and examined biochemically. Foliar NG tissues were collected at random from a non-gall leaf as the control sample.

An amount of 40 mg of plant tissue was collected for each tissue type (NG, IG, and MG) by extracting 2 mg of sample tissue from a leaf. For the biochemical study, specific tissue samples (either NG/IG/MG) from all leaves of an individual plant (40 mg) were mixed together. For the phytohormone analysis, there were five replicates for each tissue type, with each replicate consisting of 40 mg of tissue. An individual plant was considered a separate replicate. To minimize inaccurate calculations resulting from the potential uneven distribution or depletion of phytohormones in specific leaves, if any, all foliar tissues were homogenized for phytohormone analysis. Insects from each gall chamber were collected with soft forceps and needles for taxonomic identification.

Biochemical analysis:

For conducting phytohormone analysis, NG, IG, and MG foliar tissues were rinsed with distilled water and stored at -80ºC for 24 hours to eliminate any excess moisture. Following this, the tissues were subjected to drying in a micro-oven for 48 hours at 40ºC. Following previously established protocols (Yamaguchi et al., 2012; Tanaka et al., 2013; Silva et al., 2017), the following phytohormones were quantified for each tissue type (NG, IG, and MG) of each plant species: a) auxin, b) cytokinin, c) ethylene, d) jasmonic acid, and e) salicylic acid. Three separate runs of each test were conducted.

Tissue extraction:

Double volume cold extraction buffer [0.1M Tris-HCl, pH 8.0; 1μM ethylene diamine-tetra-acetic acid (EDTA); 0.5% polyvinyl pyrrolidone; 2% polyvinyl-poly-pyrrolidone; 10 ml 2-mercapto-ethanol and 10% SDS] was used to homogenise the finely ground foliar tissues during phytohormone assays (Dubois et al., 1956). Each assay sample (a-e) was extracted chemically, centrifuged (10000 rpm, 30 min, 4°C), and the supernatants were saved for future analysis.

Phytohormone estimation:

The concentrations of plant hormones like auxin (indole acetic acids, IAA) and cytokinins (CK) were quantified using stable isotope-labelled standards in each tissue type (NG, IG, and MG) following the protocols outlined by Yamaguchi et al. (2012) and Tanaka et al. (2013). Ethylene (ET) was quantified using Gas Chromatography-Mass Spectrometry (GC-MS) technology, as described by Tooker et al. (2009). The LC-electrospray ionisation (ESI)-MS/MS technique was employed to quantify the levels of Jasmonic acid (JA) and Salicylic acid (SA) by using a mass-mass-spectroscopy (Agilent 6410; ZORBAX Eclipse XDB-C18 column). Fresh tissue samples weighing approximately 100 mg were used for the analysis. The quantification process was carried out using the Mass Hunter v.B.01.02 spectrometer software (Agilent, Santa Clara, CA, USA), as reported by Tooker and De Moraes (2009). All measurements were taken in ng/g FW.

Statistical analysis:

The collected samples were represented as mean ± SD. The data were compared using parametric tests (one-way ANOVA) followed by Tukey's HSD post hoc test at 5% significance level. The R software (R Core Team, 2013) was used for all statistical analyses.

Results:

Gall-inducing insect:

The identified gall-inducing insects in the studied plants were: *Pauropsylla tuberculata* (Hemiptera: Psyllidae) from Milkwood Pine (Alstoniascholaris, Apocynaceae); *Indodiplosis mangifoliae* (Diptera: Cecidomyiidae) from Mango (*Mangifera indica*, Anacardiaceae); and *Horidiplosis mathuri* from Fig, (Ficus racemosa, Moraceae).

Auxin (IAA), Cytokinin (CK), Ethylene (ET), Salicylic acid (SA) and Jasmonic acid (JA):

Across all plant species tested (*A. scholaris, M. indica, and F. racemosa*), the auxin concentration (mean value in ng/g FW) showed a steady increase from non-gall tissues (NG) to immature gall tissues (IG), and then from immature (IG) to mature gall tissues (MG) (Figure 1). All of the plants under investigation demonstrated an approximately two-fold rise in auxin levels from newly formed non-gall (NG) to mature gall tissues (MG) (Fig. 2a). Overall, cytokinin levels were nearly three times higher in MG samples than in NG samples (Fig. 2b). Furthermore, the levels of ethylene and salicylic acid were found to be elevated by a factor of three to four in the MG group compared to the NG group. However, the increase in these compounds was only one to two times higher in the MG group compared to the IG group (Figs. 2c and 2d). The concentration of jasmonic acid (JA), a stress-responsive hormone, exhibited a gradual decline in all plant samples, starting from NG to IG and subsequently from IG to MG, in contrast to ethylene and salicylic acid (Fig. 2e). The Post-hoc (Tukey HSD) analysis results indicate that there were statistically significant increases in the levels of auxin, cytokinin, ethylene, and salicylic acid in all foliar tissues, specifically from the NG to the IG and from the IG to the MG stages. Conversely, the concentrations of jasmonic acid exhibited a significant decrease concurrently. IAA, CK, ET, and SA, which promote neoplastic growths in galling tissues, were observed in substantially higher quantities in the gall tissues of all model plants. The levels of different phytohormones in immature galled tissues (IG) exhibited an approximately two-fold elevation (ranging from 90–120%) in comparison to normal (NG) tissues. However, these levels may increase by as much as fourfold (about 200–300%) in mature gall tissues (MG). Furthermore, the study demonstrated that the levels of JA in IG and MG were significantly reduced by 35–45% and 65-75%, respectively, compared to the levels observed in normal tissues (NG). The data presented in Figure 1 indicates that the JA gradient was inverted across all plant species. The presented data in Table 1 illustrates the variations in phytohormone gradients across different tissue types (NG, IG, and MG) within individual plant species with statistical differences.

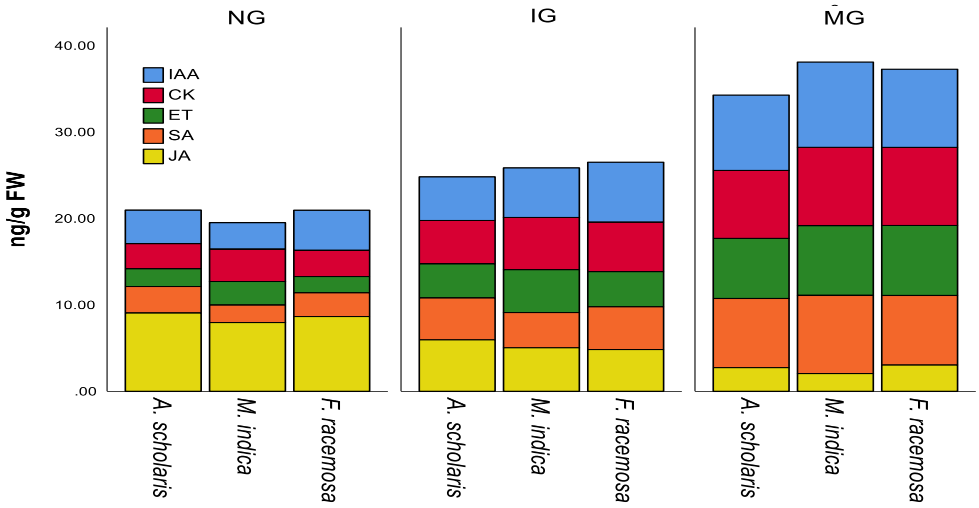

Figure 1. Phytohormone gradients among non gall (NG), immature gall (IG), and mature gall (MG) tissues for three model plants (*A. scholaris*, *M. indica* and *F. racemosa*). IAA: Indole acetic acid; CK: Cytokinine; ET: Ethylene; SA: salicylic acid; JA: Jasmonic acid

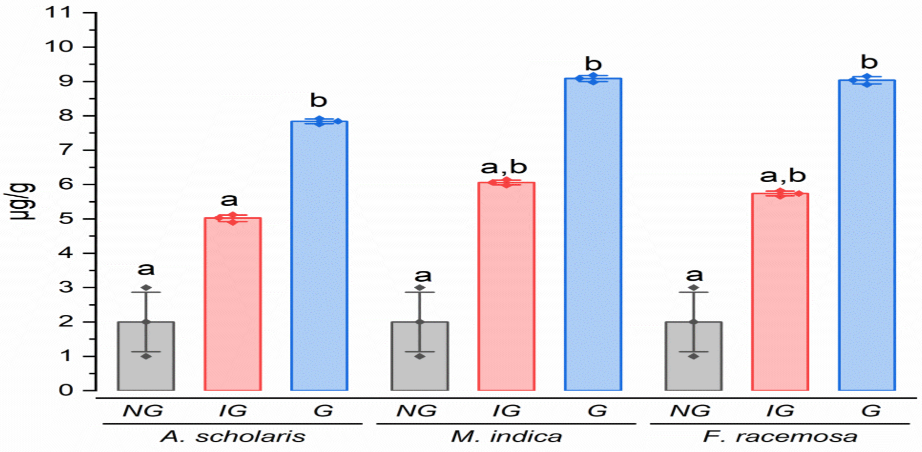

Figure 2a. Auxin (IAA) gradients from NG to IG and from IG to MG in foliar tissues for *A. scholaris*, *M. indica* and *F. racemosa*. Similar letters indicatenon-significant difference and non-similar letters indicate significant difference of phytohormone surge in three gall types for a plant species.

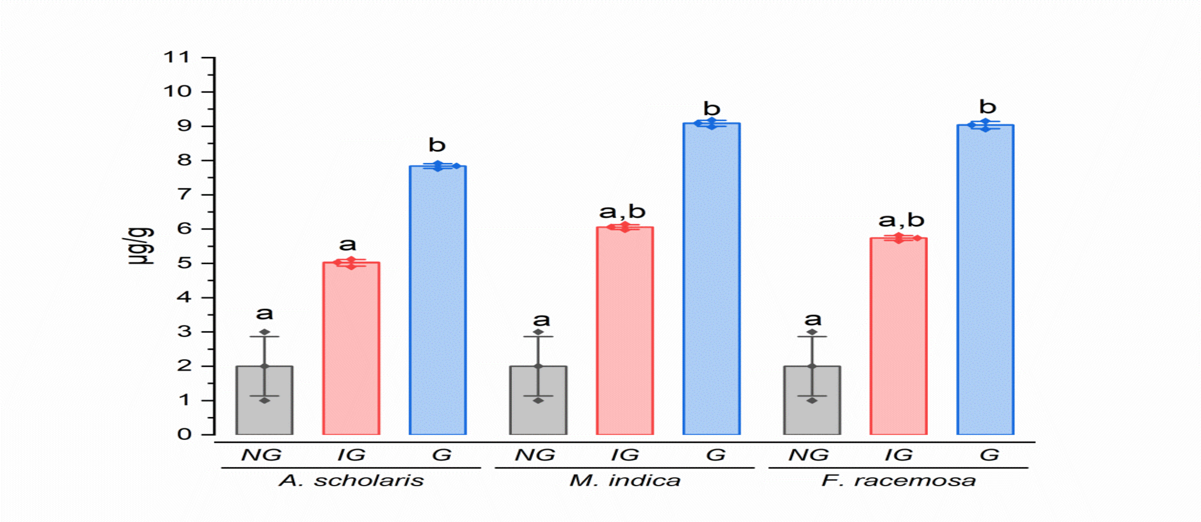

Figure 2b. Cytokin concentrations from NG to IG and from IG to MG in foliar tissues for *A. scholaris*, *M. indica* and *F. racemosa*. Similar letters indicate non-significant difference and non-similar letters indicate significant difference of phytohormone surge in three gall types for a plant species.

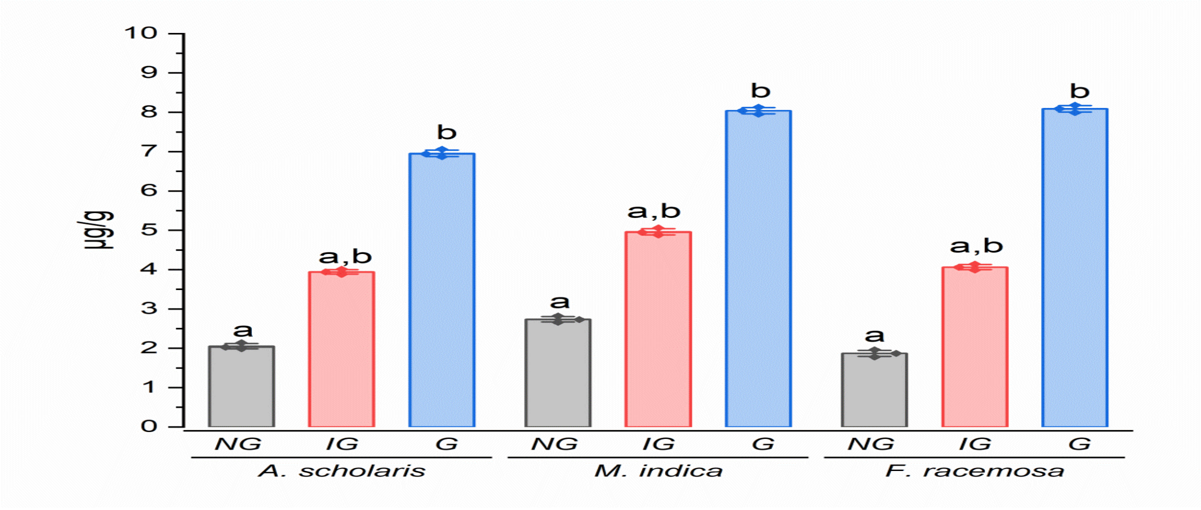

Figure 2c. Ethylene gradients from NG to IG and from IG to MG in foliar tissues for *A. scholaris*, *M. indica* and *F. racemosa*. Similar letters indicate non-significant difference and non-similar letters indicate significant difference of phytohormone surge in three gall types for a plant species.

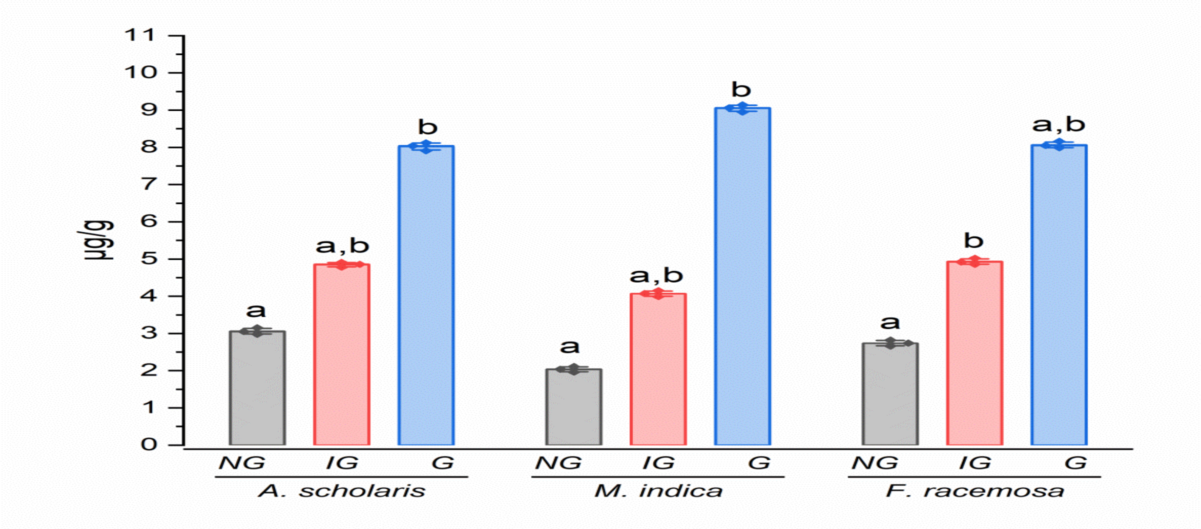

Figure 2d. Trends of phytohormone salicylic acid changes from NG to IG and from IG to MG in foliar tissues for *A. scholaris*, *M. indica* and *F. racemosa*. Similar letters indicate non-significant difference and non-similar letters indicate significant difference of phytohormone surge in three gall types for a plant species.

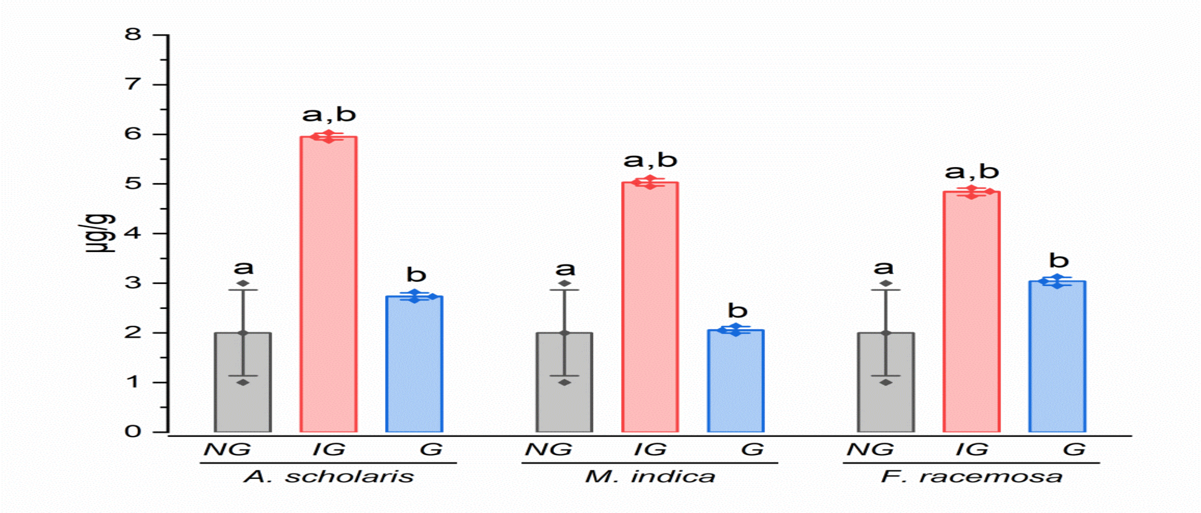

Figure 2e. Trends of Jasmonic acid changes from NG to IG and from IG to MG in foliar tissues for *A. scholaris*, *M. indica* and *F. racemosa*. Similar letters indicate non-significant difference and non-similar letters indicate significant difference of phytohormone surge in three gall types for a plant species.

Table 1. Phytohormone variations among non-gall (NG), immature gall (IG) and mature gall (MG) leaves of *Alstonia scholaris*, *Mangifera indica* and *Ficus racemosa* (*p* values are as obtained from ANOVA). Values followed by same letters (a, b, c) are statistically non-significant at 5% level by Tukey's post hoc analysis.

Plant	Tissue type	Phytohormone				
		Auxin (ng/g FW)	Cytokinin (ng/g FW)	Ethylene (ng/g FW)	Jasmonic acid (ng/g FW)	Salicylic acid (ng/g FW)
Alstoniascholaris	NG	3.9 ± 0.08^{a}	2.9 ± 0.08^{a}	2.05 ± 0.07^{a}	9.06 ± 0.08^{c}	3.06 ± 0.08^{a}
	IG	5.06 ± 0.11^{b}	5.01 ± 0.11^{b}	3.94 ± 0.06^{b}	5.95 ± 0.07^{b}	4.85 ± 0.06^{b}
	MG	8.73 ± 0.08^{c}	7.84 ± 0.08^{c}	6.95 ± 0.09^{c}	2.73 ± 0.08^{a}	8.02 ± 0.10^{c}
	$F_{(2,6)}$	2296.463	2131.721	2822.689	4872.255	2490.361
	p	0.000	0.000	0.000	0.000	0.000
Mangiferaindica	NG	3.05 ± 0.11^{a}	3.74 ± 0.08^{a}	2.73 ± 0.08^{a}	7.95 ± 0.10^{c}	2.03 ± 0.07^{a}
	IG	5.73 ± 0.08^{b}	6.06 ± 0.08^{b}	4.96 ± 0.16^{b}	5.03 ± 0.08^{b}	4.07 ± 0.08^{b}
	MG	9.86 ± 0.13^{c}	9.08 ± 0.10^{c}	8.04 ± 0.15^{c}	2.06 ± 0.07^{a}	9.05 ± 0.09^{c}
	$F_{(2,6)}$	2821.7	2722.383	2803.392	3264.363	5544.915
	p	0.000	0.000	0.000	0.000	0.000
Ficusracemosa	NG	4.64 ± 0.07^{a}	3.06 ± 0.10^{a}	1.87 ± 0.09^{a}	8.65 ± 0.10^{c}	2.74 ± 0.08^{a}
	IG	6.94 ± 0.07^{b}	5.74 ± 0.08^{b}	4.06 ± 0.07^{b}	4.84 ± 0.08^{b}	4.93 ± 0.08^{b}
	MG	9.05 ± 0.08^{c}	9.03 ± 0.12^{c}	8.08 ± 0.09^{c}	3.04 ± 0.09^{a}	8.06 ± 0.08^{c}
	$F_{(2,6)}$	2281.05	2525.436	3929.75	2786.502	3167.077
	p	0.000	0.000	0.000	0.000	0.000

Discussion:

The phenomenon of galling insects manipulating the metabolism of galling tissue to regulate the rate of growth and development of the affected tissue is widely acknowledged (Hartley & Lawton, 1992; Weis et al., 1988). Based on this perspective, the chemical interaction between gall and non-gall tissues is a multifaceted and varied phenomenon wherein numerous cellular metabolic processes facilitate the conversion of normal cells into gall tissue. The host plant and its associated organism engage in a mutually beneficial interaction, resulting in enhanced nutritional benefits and the accumulation of compounds that contribute to wound defense. While it may not be appropriate to construct an exhaustive framework specifically focused on the relationship between phytohormones and gall formation, it is plausible to hypothesise that galling sites exhibit an increased concentration of growth hormones that play a crucial role in facilitating tissue growth and differentiation (Hartley, 1998). Based on the findings of Wu and Baldwin (2010), it has been observed that an insect larva assimilates nitrogen compounds by entering the host plant's vascular system and consuming phloem fluids. The statement aligns with the findings of Abrahamson and McCrea (1986), who noted that the survival of larvae that induce gall formation is contingent upon their ability to obtain nitrogen from the host plant. Gall tissues have been described as "nitrogen sinks" or "physiological sinks" due to their ability to reduce the relative nitrogen content of adjacent leaves, thereby impeding nutrient transport to the leaves (Wu & Baldwin, 2010). The observation that the presence of gall causes a reduction in tissue nitrogen levels provides evidence supporting this perspective. The development of abnormal tissue growth at galling sites may be impacted by plant hormones or chemicals with similar hormonal properties, as suggested by Veselov et al. (2003). Turlings et al. (1993) also observed comparable findings, indicating that phytohormones play a predominant role in the induction of plant galls. The precise origins of the hormones in question, however, remain undetermined.

Gall-inducing insects may raise the concentration of phytohormones in galling tissues due to their activities and fluid output (saliva, reproductive fluids, etc.). Phytohormones probably play a dual role in the growth and prevention of galls caused by herbivores; plants are expected to boost their hormone synthesis and tissue expansion, resulting in a cascade of signal transduction in direct and indirect defensive systems. Phytohormones exhibit various interactions with both abiotic and biotic factors, functioning as signalling molecules that regulate the growth and development of plants. Plants may produce extra hormones in response to these obstacles. Multiple phytohormones at work in gall tissue have been hypothesised to allow for simultaneous growth and defense, as suggested by Dicke and Van Poecke (2002) and Kessler and Baldwin (2002). Hence, "cross-talk" between active and interfering phytohormones can be attributed to synergistic or antagonistic activities, depending on their respective mechanisms of action. The current investigation demonstrated a progressive elevation in growth-stimulating phytohormones across all plant specimens as they transitioned from non-galled to immature gall tissue and subsequently to mature gall tissue. The study results provide evidence in favour of the hypothesis that herbivore-induced galls promote the growth and differentiation of plant tissues by releasing phytohormones. Auxin (IAA) is believed to play a crucial role in the anomalous development of tissues, characterised by hypertrophy and hyperplasia, observed in galling regions (Byers et al., 1973; Dorchin et al., 2009). Tooker and Helms (2014) have posited that the presence of a defective auxin receptor gene can impede or halt the formation of gall tissue by diminishing the hormone's responsiveness at the site of gall formation. The results of this investigation align with previous research, which also reported elevated levels of indole-3-acetic acid (IAA) and IAA-oxidase in galls. Additionally, it was observed that the auxin concentration in galled tissues may be approximately 2.5 times greater compared to non-galled tissues (Byers et al., 1973; Dorchin et al., 2009). The elevated auxin levels in juvenile galls (IG), according to Tooker and De Moraes (2011), suggest that gall-inducing larvae may manage IAA levels in affected tissues. The precise role of cytokinin (CK) in plant defense remains uncertain. According to a theory proposed by Veselov et al. (2003), CK may regulate plant defense mechanisms against invasive gall-causing insects to promote neoplastic growth. The findings indicate that the levels of CK exhibit a greater increase, up to twice as much, compared to the levels of auxin in galling tissues. It suggests that CK might have a role in the differentiation of tissues and the formation of galls (Weiler & Spanier, 1981; Jameson, 2000). The higher levels of CK in gall locations may offer evidence that gall insects may have combined IAA and CK actions (Dorchin et al., 2009). The role of jasmonic acid (JA), salicylic acid (SA), and ethylene (ET) in plant defense is thought to be indirect, although the specific mechanisms by which they contribute to defense remain unclear (Erb et al., 2012; Zhang et al., 2015). The investigation revealed that the tissues of the present galling plants exhibited a threefold increase in salicylic acid (SA). This finding suggests that SA may be able to regulate plant growth and development, just like auxin and CK. There are two mechanisms through which an elevated concentration of SA can effectively enhance a plant's resistance against herbivore infestation: the production of volatile emissions and the attraction of natural enemies to the plant (Anand et al., 2008; Vidhyasekaran, 2015). Elicitors created by herbivores are thought to cause higher plant-herbivore resistance (Gatehouse, 2002; Howe & Jander, 2008; Schmelz et al., 2003). The current observation demonstrates that, regardless of the plant type, the levels of JA were roughly two times lower in galling tissue than in non-galling tissue. The empirical data and research findings from this analysis support the notion that JA and SA interact negatively, as observed previously (Tooker & De Moraes, 2009). According to Lee et al. (2009), the interaction known as "cross-talk" between jasmonic acid (JA) and salicylic acid (SA) can have a negative impact on plants, potentially increasing their susceptibility to insect infestation. In contrast, it has been observed that indirect plant defense mechanisms against herbivores exhibit synergistic interactions between jasmonic acid (JA) and salicylic acid (SA). Jasmonic acid (JA) and salicylic acid (SA) exhibit antagonistic properties, which necessitate an induced response after herbivorous consumption (Moran & Thompson, 2001; Morkunas & Gabry, 2011). In order to effectively synchronise the activation of the defensive mechanism in response to the elicited reaction, both entities may partake in a competitive process. According to Bari and Jones (2009), herbivore attacks boost ethylene (ET) production at galling sites. Similar to the current study, the "ET burst" phenomenon, which is triggered by insect oral secretions, has been seen in a variety of plant species (Akitt et al., 1980). The results of the present investigation suggest that ethylene production at galling sites may be more than twice that of non-galled tissues. This observation supports the notion that ET could potentially play a role in the indirect defense of plants by producing volatile blends (Schmelz et al., 2003). The ethylene (ET) production in galls has been observed to exhibit a threefold increase compared to non-galling tissues. The levels of jasmonic acid (JA) are commonly elevated by ethylene (ET), which acts as an inhibitor of salicylic acid (SA), a JA antagonist, thereby impacting the defensive reaction (Zander et al., 2010). Therefore, the interaction between jasmonic acid (JA) and ethylene (ET) is crucial for direct and indirect plant defense mechanisms in response to herbivore attacks. Recent investigations have shown that galling tissue can better regulate phytohormones than fresh, healthy leaves that do not gall. Phytohormone interaction was higher in mature gall tissues than in non-gall or immature gall tissues. According to this finding, galling tissues create multiple phytohormones and their various gradients to promote tissue growth and differentiation towards neoplasia or build a defense mechanism in response to the plants. The predominant phytohormones responsible for inducing gall formation exhibit dual functionality, as they facilitate tissue proliferation and activate internal defense mechanisms. The study highlights the impact of gall-inducing insects on delivering nutrients and chemicals to the host plant. The observed phenomenon can be attributed to the insects' ability to regulate phytohormones within injured tissues, thereby influencing plant tissue growth. As a result, the coevolutionary dynamics between gall-producing insects and their host plants have evolved along a convoluted evolutionary path.

Conclusion:

The gall-inducing insect initiates plant tissue differentiation by releasing chemical secretions that interact with the induced phytohormones produced by the plants. The natural formation of galls is attributed to the co-evolutionary process between gall inducers and their host plants, wherein both organisms undergo simultaneous adaptations. This adaptation is thought to have been influenced over time by changes in the production and release of several substances, including phytohormones.

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Conflict of Interest

The authors declare no conflict of interest.

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