Original Article

Ontogenetic variations in Salicylic acid and Jasmonic acid levels in the galls of *Garuga pinnata* Roxb. induced by *Phacopteron lentiginosum* Buckton

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Abstract

Galls are distinct structures formed as a result of interactions between host plants and gall-inducing insects. Gall inducers manipulate host plant physiology by altering the biochemical signalling pathways of the plant. The present study aims to find the ontogenetic changes in phytohormones, such as salicylic acid (SA) and jasmonic acid (JA), during gall development induced by *Phacopteron lentiginosum* on the leaves of *Garuga pinnata*. Galls with 3rd, 4th and 5th instars of the gall inducer were collected and the concentrations of SA and JA were analysed. Both phytohormones varied significantly across the developmental stages of galls. The highest concentrations of SA (147.67 ± 0.58 µg ml^{-1}) and JA (676.58 ± 25.48 µg ml^{-1}) were recorded in galls containing 3rd instar nymphs, followed by a decline in 4th and 5th stages of galls. Elevated SA and JA levels during early gall induction suggest a synergistic host plant defence mechanism, whereas their subsequent decline may be associated with gall development and maintenance. This study highlights the role of SA and JA in mediating insect–plant interactions.

Keywords: Cecidogenesis, gall inducer, *Garuga pinnata,* jasmonic acid, *Phacopteron lentiginosum,* plant-insect interactions, salicylic acid.

Introduction

Gall formation or cecidogenesis is one of the most specialized forms of plant-insect interactions.[1] Galls are abnormal plant growths characterized by tissue proliferation, differentiation, and structural reorganization induced by various organisms, including insects, mites, fungi, bacteria, and nematodes.[2] The gall inducing stimuli can be oviposition fluids, salivary secretions or mechanical stress imposed by gall-inducing organisms, triggering distinct morphological and physiological alterations in host plant tissues, ultimately resulting in gall development.[3] These elicitors bind to plant receptors, marking the earliest stage of gall initiation.[4,5]

Upon perception of gall-inducing stimuli, plants activate a range of defence responses. For successful gall development, the gall inducers must suppress or modulate these defences and redirect host tissues into specialized growth patterns.[6] The biotic stress induced by the elicitors are defended by the wide range of inducible defence mechanisms activated by the plant during the time of infestation.[6] Among these defence responses are the phytohormones jasmonic acid (JA) and salicylic acid (SA), both of which function as key signalling molecules.[7] In plant tissues, JA is synthesized from linolenic acid *via* octadecanoid pathway in response to wounding and herbivore attack.[8] Similarly, SA, a derivative of benzoic acid, is another important plant hormone regulating plant defence responses.[9] It functions as an endogenous growth regulator, influencing various metabolic and physiological processes associated with plant defence, growth, and development.[10] These compounds together coordinate complex signalling pathways that ultimately trigger the activation of plant immune systems and associated protective responses.[11,12] Previous studies have shown that hormonal signalling pathways are dynamically modulated, with SA and JA levels rising during early gall induction but declining sharply during later stages, suggesting a temporal modification of defence signalling that favours gall maintenance.[13]

The present study investigated the biochemical alterations in the levels of SA and JA, associated with gall formation in *Garuga pinnata* Roxb. (Burseraceae) induced by *Phacopteron lentiginosum* Buckton (Hempitera: Phacopteronidae) during different stages of gall development.

Materials and Methods

Gall system

The leaf galls induced by *P. lentiginosum* on *G. pinnata* are fruit-like, bilayered pouches (Figure 1) formed in response to the feeding activity of first-instar nymphs, with each gall harbouring 1–14 individuals.

Gall formation is closely linked to nymphal development. Initial feeding by 1st instar nymphs produces a localized swelling (first tier), within which nymphs moult to subsequent instars. Continuous feeding by these nymphs leads to the formation of a second tier of galls appearing as an inverted cup-shaped structure that serves as a protective shelter for the developing nymphs.[14] The 5th instar represents the final nymphal stage. The adult emergence occurs through a characteristic flower-like opening.

Figure 1: Galls induced on *Garuga pinnata* by *Phacopteron lentignosum*

Study area

Galls were collected from Pattambi, Kerala, India (10.8114°N, 76.1904°E) during 2019–2021. The stages of nymphal instars were identified based on morphometric parameters (body length, body width, head length, interocular distance and wing bud area), in accordance with Dyar's law[15]. Galls were dissected to remove inducers and parasitoids, pooled by stage (n=20 per stage), and the galls were subjected to LC-MS analysis. Ungalled leaves from uninfested plants served as controls.

Estimation of salicylic acid and jasmonic acid

The 3rd, 4th and 5th stages of galls containing the 3rd, 4th and 5th instars of the gall inducer respectively and ungalled leaves (control) were collected and immediately frozen in liquid nitrogen and stored at −80 °C until further analysis to prevent phytohormone degradation. Quantification of endogenous SA and JA in gall tissues and ungalled leaves was carried out using the external standard method. [16]

Preparation of SA and JA Standard Solutions: Stock solutions of SA and JA (0.1 mg ml^{-1}) were prepared by dissolving each compound in methanol and stored at 4°C. Working standard solutions were freshly prepared before analysis by diluting the stock solutions with the mobile phase to obtain the required concentrations.

Generation of Standard Curves for SA and JA: Standard curves were generated using a series of SA working solutions (10, 20, 30, 40, and 50 µg ml^{-1}) and JA working solutions (25, 50, 100, 150, and 200 µg ml^{-1}). Each concentration was injected into the LC-MS system, and peak areas were determined. Standard curves were constructed by plotting peak area against corresponding concentrations, and linear regression analysis was performed to obtain calibration equations for quantification.

Extraction of Endogenous SA and JA from different stages of galls and ungalled leaves: Endogenous SA and JA were extracted using the following procedure.[17] Frozen samples of different stages of gall tissues and ungalled leaves were separately ground to a fine powder in liquid nitrogen. Powdered tissue was extracted using 10% methanol containing 1% acetic acid. The samples were incubated on ice for 30 min, followed by centrifugation at 13,000 × g for 10 min at 4 °C. The supernatant was collected, and the pellet was re-extracted with additional extraction solvent. After 30 min incubation on ice, extracts were centrifuged again, and supernatants pooled. The extracts obtained from each maturation stage of the galls and from ungalled leaves were filtered through a syringe filter prior to analysis.

Analysis and Quantification: Analysis was performed using an Agilent 6100 Series Quadrupole LC/MS system. The mobile phase consisted of Solvent A: 100 % HPLC-grade water and Solvent B: 100 % HPLC-grade acetonitrile. Injection volume was 10 µl for all samples. Detection of SA and JA was done at 325 nm and 295 nm respectively. Peak areas obtained from sample chromatograms were compared with standard calibration curves to calculate hormone concentrations, expressed as µg ml^{-1}.

Statistical analysis

All biochemical data were organized using Microsoft Excel prior to statistical processing. Statistical analyses were conducted in R software (v4.3.3) using the R Studio user interface (2024-02-29 ucrt)[18] using R Studio as the integrated development environment. Data were presented as mean ± standard error (SE) unless stated otherwise.

Normality of data distribution was assessed using the Shapiro–Wilk test, while homogeneity of variances was evaluated using Levene's test. For comparisons between two groups (e.g., gall tissues *vs*. ungalled leaves) and for comparisons of different gall developmental stages, one-way analysis of variance (ANOVA) was used, followed by Tukey's honestly significant difference (HSD) post hoc test to identify pairwise differences.

Results

The present study showed that galls induced by *P. lentiginosum* on *G. pinnata* exhibit pronounced variation in the concentration of SA and JA across different developmental stages of galls and these profiles differ markedly from those of ungalled leaves (Table 1).

Salicylic acid content in different stages of galls and ungalled leaves

SA content differed significantly among gall developmental stages (3rd to 5th instars) and ungalled leaves (one-way ANOVA: F= 4048,

Table 1: Variation in biochemical parameters across different developmental stages of galls.

Stages of galls	SA (µg ml^{-1})	JA (µg ml^{-1})
L	0.26 ± 0.01	190.39 ± 10.26
G3	147.67 ± 0.58	676.58 ± 25.48
G4	91.10 ± 1.11	450.05 ± 8.07
G5	31.99 ± 0.54	140.75 ± 5.63

Values represent mean ± standard error (SE). L= ungalled leaf tissue; G3=Galls containing third instar nymphs of the gall inducer; G4=Galls containing fourth instar nymphs of the gall inducer; G5=Galls containing fifth instar nymphs of the gall inducer. JA=Jasmonic acid; SA=Salicylic acid; No. of observations=20.

$p < 0.001$). SA levels were the highest in 3rd stage galls (with 3rd instar nymphs) and declined progressively with gall maturation (Table 1). Ungalled leaves exhibited the lowest SA concentration and differed significantly from all gall stages (all $p < 0.001$) (Figure 2).

Jasmonic acid content in different stages of galls and ungalled leaves

JA content differed significantly among gall developmental stages (third to fifth instars) and ungalled leaves (one-way ANOVA: $F = 137.1, p < 0.001$). JA levels were the highest in galls containing 3rd nymphal instars compared to galls containing 4th and 5th instars and ungalled leaves (Table 1). JA levels were not significantly different in galls with 5th nymphal instar and ungalled leaves, both of which were significantly lower than 3rd and 4th developmental stage of galls (Figure 3).

Discussion

In the present study, we investigated the levels of salicylic acid and jasmonic acid across different developmental stages of galls induced by *P. lentiginosum* on *G. pinnata* in comparison with ungalled leaves. The results clearly demonstrate significant alterations in these phytohormone levels during gall development. Earlier reports have already established that the concentration of phytohormones varies across the developmental stages of the gall and the gall inducers.[19-21] These metabolic imbalances are likely the consequences of elicitors secreted by the gall inducer, which reprogram host plant metabolism and redirect normal organogenesis into the formation of a specialized structure.[22,23]

We observed a markedly higher concentration of SA and JA in galls with 3rd instar of the gall inducer, followed by a consistent decline as galls matured with the 4th and 5th nymphal instars. This suggests an activation of SA mediated defence response to counteract the tissue manipulation by the gall inducer. This pattern is consistent with the role of SA in activating systemic acquired resistance (SAR) against biotic stresses.[24] The significantly lower SA levels in ungalled leaves further indicates that gall induction enhances SA signalling as an immediate response to the elicitor released by the gall inducer.[25] JA is also associated with defence against herbivory[26], the suppression of which during the 5th stage could be due to the decline in elicitor levels required for the successful manipulation of gall formation and maintenance. In oak leaf galls induced by Cynipid wasps, gall tissues showed alterations in JA biosynthesis pathways compared to surrounding leaf tissue, consistent with our observations of JA levels in galled and ungalled leaves.[27]

As observed in our study, the concurrent decline of both SA and JA across gall maturation stages defines a coordinated plant defence signalling. The crosstalk between the two inducible hormones indicate that herbivory is associated with activation of SA defences and pathogenesis with activation of JA pathway.[28] However, phloem feeding is

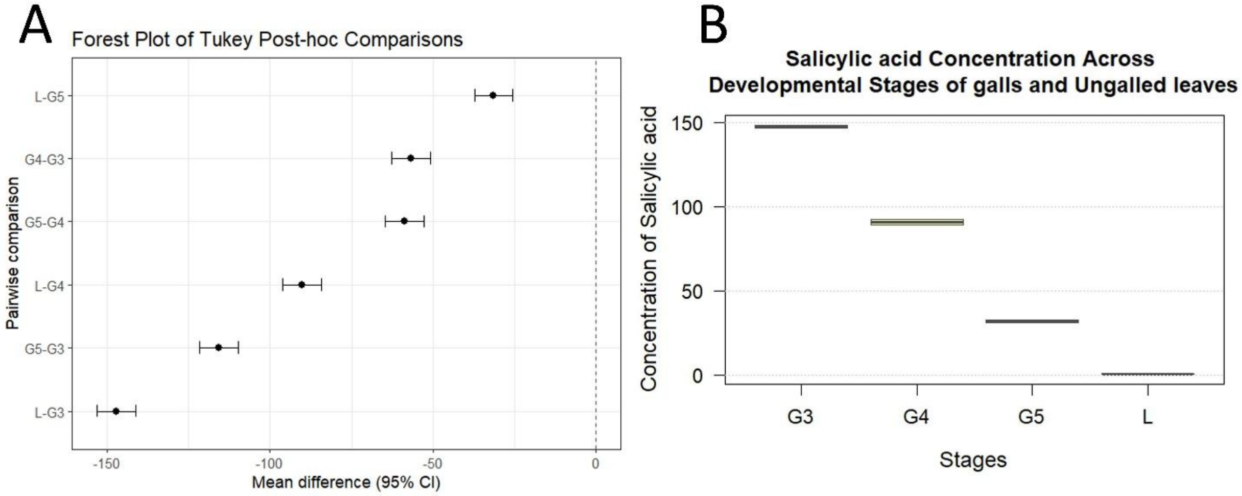

Figure 2: Variation in salicylic acid across different developmental stages of galls and ungalled leaves. (A) Forest plot showing Tukey-adjusted pairwise differences between groups. Horizontal lines represent 95% confidence intervals. (B) Boxplot showing SA concentration across different developmental stages of galls and ungalled leaves. G3, G4 and G5=galls with 3rd, 4th and 5th instar nymphs of the gall inducer respectively; L=ungalled leaves (control).

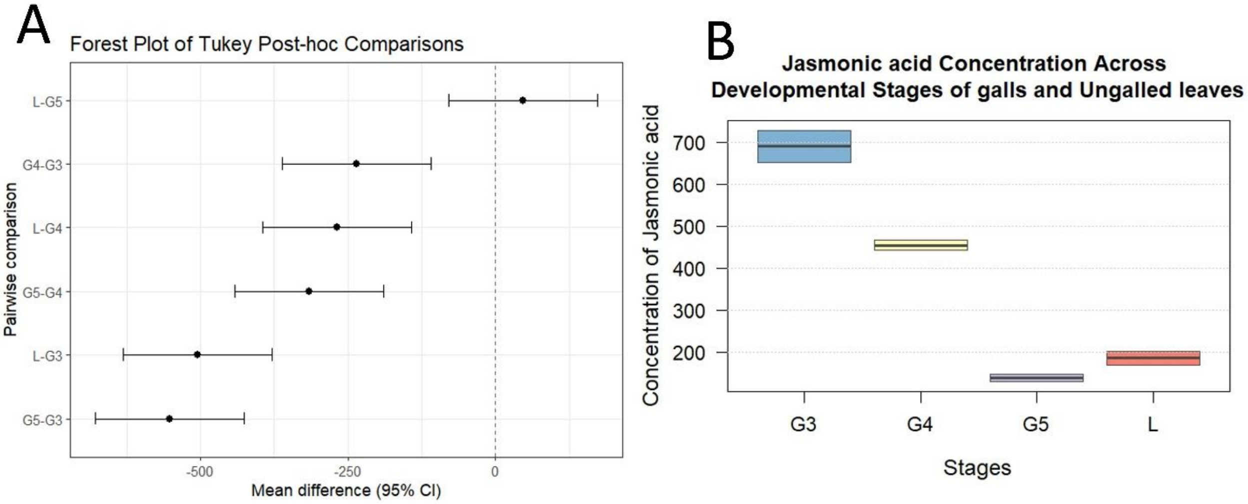

Figure 3: Variation in jasmonic acid across different developmental stages of galls and ungalled leaves. (A) Forest plot showing Tukey-adjusted pairwise differences between groups. Horizontal lines represent 95% confidence intervals. (B) Boxplot showing JA concentration across different developmental stages of galls and ungalled leaves. G3, G4 and G5=galls with 3rd, 4th and 5th instar nymphs of the gall inducer respectively; L=ungalled leaves (control).

considered as a special case, where the sap-sucking insects act as pathogen-like agents in plants and activate both SA and JA signalling pathways simultaneously in plants.[29] The synergistic effect of SA and JA is reported in the gene expression of Squash plants in response to Silverleaf whitefly feeding.[30] However, the root galls of *Vitis* spp. induced by *Daktulosphaira vitifoliae* showed an initial hike in JA concentration followed by a sharp decline due to subsequent SA induction; this simultaneous SA-JA crosstalk suppress JA mediated host responses.[31]

SA–JA crosstalk is often represented as a mutual antagonism.[32] The present study implies that gall inducers can modulate both pathways simultaneously to suppress host defences, thereby facilitating sustained gall growth, development and maturation. In contrast to the earlier mentioned antagonistic interaction between SA and JA, our findings suggest a synergistic interplay between the SA and JA pathways in protecting plants against biotic stress. When both pathways are activated together, plant resistance can become stronger and more effective as observed in *Nicotiana glutinosa*, where SA and JA work together to enhance disease resistance.[33] Thus, the present study provides insight into the mechanisms by which gall inducing insects overcome host defences and highlights the potential need to study whether synergism can lead to simultaneous resistance to insect attack.

Conclusion

Galls represent an extended phenotype where an inducing insect manipulates host plant gene expression to create a protective, nutrient-rich microenvironment. This study investigates the interaction between the host plant *Garuga pinnata* and the gall-inducer *Phacopteron lentiginosum*, focusing on the roles of SA and JA. SA is known to interact with auxins and cytokinins to drive tissue hypertrophy and hyperplasia, needed to physically build the gall. JA modulates stress responses and resource allocation. Its signalling is hijacked to modulate metabolic pathways and establish a nutrient sink for developing larvae. Contrary to the typical antagonism between these pathways, our findings reveal that the inducer elicits a synergistic, highly localized temporal wave of both SA and JA. This precise hormonal balancing may suppress the plant's lethal hypersensitive response while successfully directing the development of the gall structure.

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