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Original Article

Reddish coloration in galls and its relationship with polyamines, phytohormones, and metal accumulation

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ABSTRACT

The reddish phenomenon in galls is commonly associated with anthocyanin accumulation triggered by different stimuli, including metal, which provides a more efficient antioxidant defense. Herein, we hypothesize that red galls have higher levels of polyamines (PAs) and phytohormones, especially those involved in stress production and dissipation, than green galls do. This, in turn, is likely associated with greater metal accumulation in galls induced by *Palaeomystella oligophaga* (Lepidoptera) on *Macairea radula* (Melastomataceae). Green and red galls were sampled and opened to select only those with the galling insect in the larval stage for later analyses of phytohormones, polyamines, and metals. The putrescine and spermine levels were greater in red galls than in green galls. The contents of salicylic acid (SA) and methyl jasmonate (MeJA) were also greater in red galls, as were the contents of Al, Fe, Pb, and Cu. We suggest a possible link between PAs, SA, and MeJA and the translocation of metals to reduce the toxicity caused by these chemical elements' accumulation, transforming them into less toxic forms and maintaining red coloration as a form of oxidative protection in galls.

Keywords: coloration; red gall; biotic stress; primary metabolites; metallic ions.

Introduction

The gall-inducing organism is a phenotype manipulator responsible for determining gall structure and metabolism, although the host plant tissue can impose constraints on gall development (Isaias *et al.*, 2014; Oliveira *et al.*, 2016). The biotic stress generated by gall-inducing insects is controlled by several metabolic pathways in the host tissue, allowing gall development (Arriola *et al.*, 2020; Naves *et al.*, 2021; Santos *et al.*, 2024). As a result of this parasitic interaction, galls exhibit great morphological and physiological diversity (Stone & Schönrogge, 2003; Ferreira *et al.*, 2019), including the accumulation of different pigments, especially anthocyanins, which are associated with red coloration in galls (Lev-Yadun, 2016).

Reddish coloration in galls is discussed based on distinct hypotheses, such as high light exposure leading to anthocyanin accumulation for photoprotection (Bomfim *et al.*, 2019), carbon and phytohormone accumulation inducing the upregulation of phenolic class biosynthesis in galls (Connor *et al.*, 2012), the predisposition of host tissues to produce these compounds at the site of induction (Cardoso *et al.*, 2022), and their defensive role in galls (Inbar *et al.*, 2010). Nevertheless, the reddish phenomenon in galls seems to be idiosyncratic, and investigating different physiological pathways and biomolecules involved in red color development may indicate promising ways to link these distinct hypotheses in the context of the adaptive value of plant galls. Phytohormones and polyamines, for example, are important molecules involved in plant responses to abiotic and biotic stresses and are associated with the accumulation of metals and phenolic compounds (Chen *et al.*, 2019; Tyagi *et al.*, 2023). However, the functions of PAs in the interaction between gall-inducing insects and host plants are poorly known, especially when they are associated with phytohormones, metal accumulation, stress dissipation, and indirect effects on gall coloration.

Polyamines (PAs) are low-molecular-weight aliphatic nitrogenous bases containing one or more amino groups that are widely distributed in the cells of living organisms (Liu *et al.*, 2015; Vuosku *et al.*, 2018). The main PAs found in plants are putrescine (Put), spermidine (Spd), and spermine (Spm), which are involved in the regulation of diverse morphological and physiological processes, such as the control of oxidative stress, adjustment of metal uptake, protection of membranes and proteins, and regulation of cell division (Mustafavi *et al.*, 2018; Spormann *et al.*, 2021). The cross-linking of PAs with phenolic derivatives is important not only for stress dissipation but also for plant morphogenesis (Falahi *et al.*, 2018; Kumar *et al.*, 2015). Gall development, for example, depends on tunable control of

oxidative stress when the galling organism manipulates the host plant (Isaias *et al.*, 2015). Phytohormones, PAs, and heavy metals can interfere with metabolism, development, and possibly gall color (Yadav, 2010; Malik *et al.*, 2022). Phytohormones, such as salicylic acid (SA) and methyl jasmonate (MeJA), are biosynthesized to regulate the defense mechanism of plants (Chamandoosti, 2019; Li *et al.*, 2019) and are directly associated with the metabolism of PAs and secondary metabolites, especially those related to colors such as chlorophylls, anthocyanins, carotenoids, and betalains (Li & Ahammed, 2023; Tyagi *et al.*, 2023). The manipulation of the cellular machinery by gall-inducing insects involves secondary metabolites such as phytoalexins, phenolic compounds, and phytohormones such as indole-3-acetic acid (IAA), which regulate gall growth (Bedetti *et al.*, 2014). This process occurs because secondary metabolites, for example, flavonoids, act synergistically with growth auxins and metals to control the dissipation of reactive oxygen species (Buer *et al.*, 2010; Murphy *et al.*, 2000; Peer & Murphy, 2007; Arriola *et al.*, 2020). Some metals, such as zinc (Zn), copper (Cu), manganese (Mn), and nickel (Ni), are essential for both plant and galling organism nutrition at low levels (Arriola *et al.*, 2024a), whereas others, such as cadmium (Cd), lead (Pb), and mercury (Hg), have no biological function (Angulo-Bejarano *et al.*, 2021). However, metal accumulation can have toxic effects on plants and impair gall development (Arif *et al.*, 2016; Sarwar *et al.*, 2017; Arriola *et al.*, 2024a). To avoid the effects of toxicity, they can biosynthesize polyamines and phytohormones that tend to bind to metals, forming complexes with antioxidant functions (Groppa & Benavides, 2008; Wybraniec *et al.*, 2013; Liu *et al.*, 2015).

In this study, we evaluated the red and green globoid galls induced by *Palaeomystella oligophaga* Becker and Adamski (Lepidoptera) on *Macairea radula* Bonpl. (Melastomataceae), which present two tissue compartments. The inner compartment, nutritive tissue, accumulates proteins and lipids adjacent to the larval chamber, and the outer compartment, storage tissue, accumulates structural carbohydrates as reserves (Rezende *et al.*, 2019; Naves *et al.*, 2021) and phenolic compounds such as flavonoids and anthocyanins, which may contribute to the red coloration of the gall (Kuster *et al.*, 2019; Cardoso *et al.*, 2022). In this study, we hypothesize that red galls accumulate higher polyamine (PA) and phytohormone levels, especially those involved in stress production and dissipation, than green galls do. This, in turn, is likely associated with increased metal accumulation, which explains the possible role of chelating and transforming these molecules into less toxic forms for the galls and maintaining red coloration as a form of oxidative protection.

Materials and Methods

Plant material

Galls induced by *Palaeomystella oligophaga* (Lepidoptera) on *Macairea radula* buds (Melastomataceae) (Figure 1) were sampled randomly from two populations of Cerrado vegetation located in the Estação Ecológica do Panga (19° 10'S, 48°24'W) and Araguari municipalities in the waterfall at the Irmâs site (19° 02' S, 49° 10' W). A voucher specimen of the fertile material (including flowers) was deposited in the Herbário Uberlandense (HUFU) under the accession number HUFU00059152. The galls were globoid-shaped and covered with a dense indumentum featuring long projections that varied in color. They have a diameter ranging from 10 to 15 mm when fully mature (Figure 1). The galls were considered mature when they reached their maximal size and when the gall-inducing insects were in the larval stage. The samples were collected during the second infestation period (July–August), immersed in liquid nitrogen, and stored in an ultrafreezer at -81 °C. The galls were then opened to select only those that were mature and contained galling insects in the larval stage. The larvae of the galling insect were not removed from the galls for subsequent analysis of phytohormones, polyamines, and metals (Figure 2).

Metal quantification

The samples with 200 mg of galls (n = 4, with three or four galls for each sample) were dried, crushed, sieved, and then digested in 8.75 ml of 2 mol/l nitric acid and 1.25 ml of hydrogen peroxide with the aid of a microwave (Ethos, Milestone) (Wu *et al.*, 1997). After digestion, the volume was increased to approximately 18 ml with the addition of Milli-Q water. The final dilution of the sample in the digestion step was approximately 90 times. In the digestion stage, a vial containing only the reagents used and a reference material (NIST 1515 - Apple Leaves) was also added. After each round with 24 bottles, the microwave was decontaminated. The samples were subsequently diluted in Milli-Q water according to the following dilution factors for each round of analysis: twofold for Cu, Pb, and Cd; fiftyfold for Al, Fe, and Zn; and one thousandfold for Mg. The concentrations of these elements were analyzed via mass spectrometry with inductively coupled plasma–mass spectrometry (ICP–MS) (Agilent 7700, Agilent Technologies, Santa Clara, USA).

Figure 1. Galls induced by *Palaeomystella oligophaga* Becker and Adamski (Lepidoptera) on buds of *Macairea radula* Bonpl. (Melastomataceae). A. Both green and red galls may occur in the same or different individuals. B. Details of a green gall. C. Details of red galls.
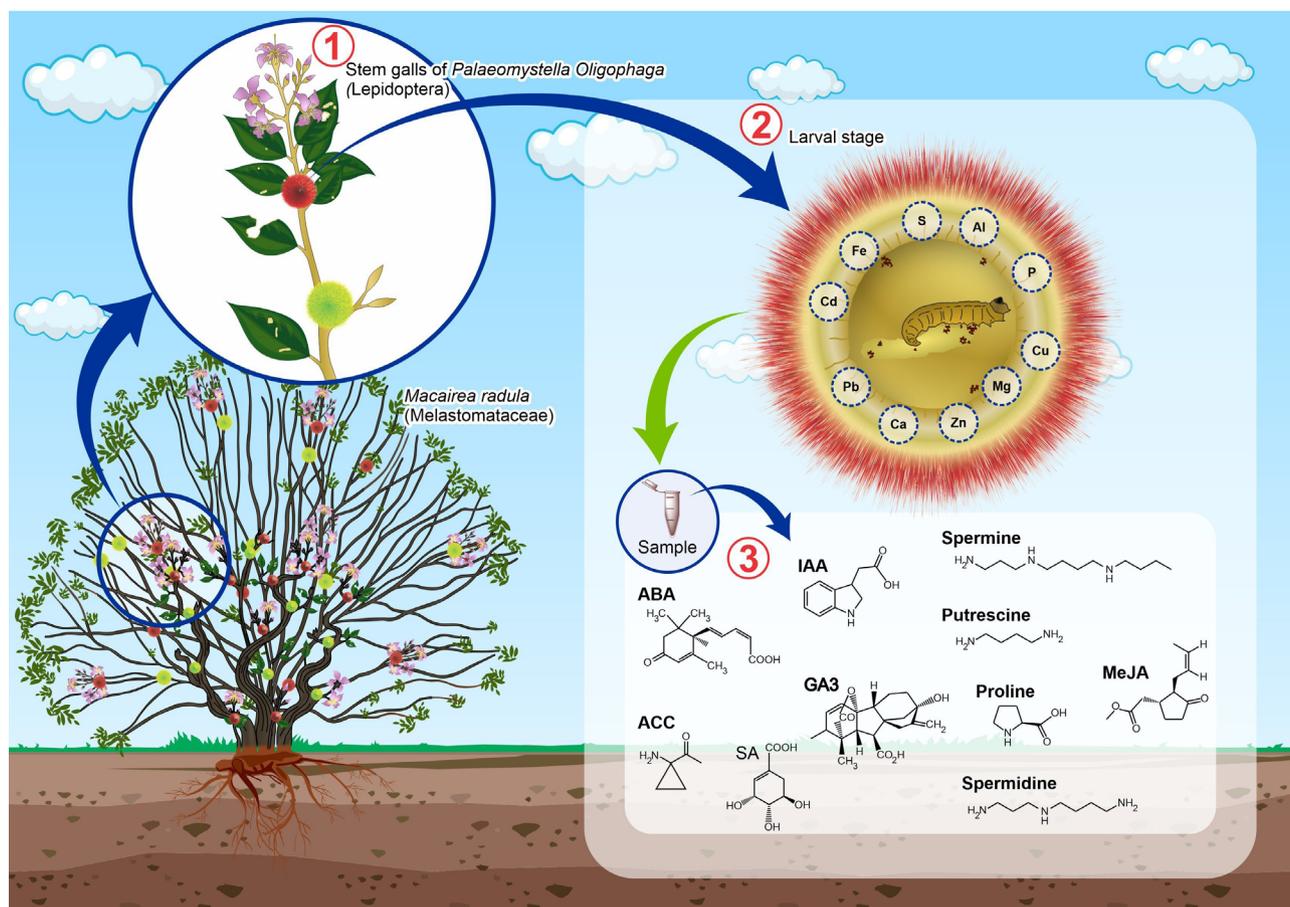
Figure 2. Schematic diagram of the study system showing galls induced by *Palaeomystella oligophaga* Becker and Adamski (Lepidoptera) on the buds of *Macairea radula* Bonpl. (Melastomataceae), along with the molecules analyzed in galls with gall-inducing in the larval stage, such as polyamines, phytohormones, and metals. 1) Green and red globoid galls; 2) open galls with galling insects in the larval stage with different metals; and 3) molecular structures of polyamines and phytohormones.

The concentrations of P, S, and Ca were determined via total reflection X-ray fluorescence (TXRF). Quartz glass disks 30 mm in diameter were used as sample carriers and were covered with 10 µL of silicon solution in isopropanol (ServaTM, Heidelberg, Germany). The digested sample (450 µL) and 50 µL of 100 mg/L gallium solution were mixed for the determination of the internal standard. These final solutions (10 µL) were applied to the siliconized disks, and the disks were dried at 60°C for 15 min and analyzed in an S2 PICOFOX™ (Bruker Nano GmbH, Berlin, Germany).

Phytohormone and polyamine analysis by liquid chromatography–mass spectrometry (LC–MS)

Approximately 110 mg of plant material—fresh galls (n = 3, with two or three galls for each sample)—was added to 1.5 ml Eppendorf tubes and macerated via glass or tungsten beads at 25 Hz/s for 3 minutes, with three replicates. Then, 400 µL of extraction solution (methanol:isopropanol: acetic acid – 20:79:1) was added, and the control without plant material (white) was added. Each sample was vortexed 4 times for 20 s on ice and sonicated for 15 minutes. The samples were placed on ice for 45 minutes, sonicated (15 minutes), and centrifuged at 13,000 × g for 10 minutes at 4°C. A total of 350 µL of the supernatant was pipetted into new 2.0 ml tubes (Eppendorf). Steps 2 and 3 were repeated for the remaining pellet, and the supernatants were added. Centrifuge again at 20,000 × g for 5 minutes and transfer approximately 600 µL into new 2.0 ml tubes. The samples were passed through filters (13 mm, 0.2 µm PVDF syringe filter, Hexis brand, AG5190-5261EST or HX0097-02623) to remove any particulate matter that could damage the LC–MS equipment. Three hundred microliters of each vial were added to the triple quadrupole (QQQ) (Forcat *et al.*, 2008).

The phytohormone and polyamine data obtained via liquid chromatography coupled with mass spectrometry (LC–MS-QQQQQ) were identified and quantified via Skyline software (Adams *et al.*, 2020). Liquid chromatography coupled with mass spectrometry (LC–MS-QQQQQ) of the Nucleus of Biomolecules Analysis (UFV) was also used.

Statistical analysis

Differences between means for treatments were tested via Student's t-test for polyamines, phytohormones, and chemical element quantification. All the data were subjected to the Shapiro–Wilk normality test with a significance level of p ≤ 0.05, which was performed via R software version 4.3.2 (2023).

Results

Red galls accumulated more Al, Fe, Pb, and Cu than green galls did. Specifically, red galls accumulate 75 % more Fe (t = -10.586; d.f. = 2.1007; *p* value = 0.007416), 64.4 % more Al (t = -12.75; d.f. = 2.1731; *p* value = 0.004406), 52.9 % more Pb (t =-6.8959; d.f. = 3.2044; p value = 0.005036) and 19.4 % more Cu (t = -13.129; d.f. = 3.8705; *p* value = 0.0002367) than do green galls. On the other hand, there were no significant differences in the accumulation of Cd (t = 3.4325; d.f. = 2.3242; *p* value = 0.06078), Ca (t = 1.5979; d.f. = 2.0504; *p* value = 0.2481), Mg (t = 1.2553; d.f. =2.0983; *p* value = 0.3311), P (t = 0.84509; d value = 2.3832; *p* value = 0.4744), S (t = 0.95429; d.f. = 2.2454; *p* value =0.4311) or Zn (t = 0.95, *p* value =0.3932) (Table 1) between red and green galls.

The putrescine content was 60 % greater in the red galls than in the green galls (t = -7.812; d.f. =2.0027; p value = 0.01593) (Figure 3A). Additionally, spermine (t = -4.8265; d.f. = 2.0009; *p* value = 0.04031) was more abundant in red galls than in green galls (Figure 3C). However, there was no difference in spermidine (t = 2.3186; d.f. = 3.9999; *p* value = 0.08126) or proline levels (t = -1.0664; d.f. = 2.0966; *p* value = 0.3935) (Figure 3B, D).

The contents of abscisic acid (ABA), gibberellic acid (GA3), and aminocyclopropane carboxylic acid (ACC) were greater in green galls than in red galls, whereas SA and MeJA were more abundant in red galls (Figure 4). There was 65 % more ABA (t = 7.11; d.f. = 2.00; *p* value = 0.01; Figure 4A), 69 % more ACC (t = 20.865; d.f. = 2.5756; p value = 0.0006075; Figure 4D) and 15.8 % more GA3 (t = 8.9908, df = 2.5071, p value = 0.005704; Figure 4B) in green galls than in red galls (Figure 4A and B). However, red galls presented 96 % more SA (t = 17.199; d.f. = 2.1478; p value = 0.002439; Figure 4E) than did green galls and 25 % more MeJA (t = -4.1218; d.f. = 3.8728; p value = 0.01559; Figure 4F) than did green galls (Figure 4D and E). The differences in IAA levels between green and red galls were not statistically significant. (t = 0.91878; d.f. = 2.0077; p value = 0.4549; Figure 4C).

Table 1. Metal quantification (mean ± SD) in mg kg^{-1} from green and red galls induced by *Palaeomystella oligophaga* Becker and Adamski (Lepidoptera) on buds of *Macairea radula* Bonpl. (Melastomataceae). Differences between means were tested via Student's *t* test and considered statistically significant differences when p ≤ 0.05.

Metals	Green gall	Red gall	t value	p value
Fe	*423 ± 38.31*	*1716 ± 241*	-10.58	**0.0074**
Cu	7.16 ± *0.16*	8.89 ± *0.20*	-13.12	**0.0002**
Zn	24.30 ± *5.62*	20.42 ± *5.84*	0.95	0.3932
Pb	0.30 ± *0.04*	0.65 ± *0.08*	-6.89	**0.0050**
Cd	0.10 ± *0.04*	0.03 ± *0.01*	3.43	0.0607
Al	794 ± *45.92*	2230 ± *220*	-12.75	**0.0044**
Ca	1562 ± *601*	1079 ± *67.46*	1.59	0.2481
Mg	3025 ± *2126*	2037 ± *333*	1.25	0.3311
P	702 ± *421*	516 ± *131*	0.84	0.4744
S	472 ± *258*	345 ± *64.07*	0.95	0.4311

Discussion

Several hypotheses have been proposed regarding the evolution, ecology, and functionality of red galls in plants. In the present study, the greater amounts of Al, Fe, Pb, and Cu found in red galls suggest a possible functional relationship between anthocyanin and metal accumulation in these structures (Mulyaningsih *et al.*, 2023). Anthocyanins may play a role in mitigating the oxidative stress caused by metals (Ahammed & Yang, 2022). The available form of aluminum for plants is trivalent (Al^{3+}), which is highly toxic to cells, tissues, and organs (Kochian *et al.*, 2015). When absorbed by cells, Al^{3+} indirectly induces oxidative stress, inhibits antioxidant enzymes, and promotes lipid peroxidation (Yamamoto *et al.*, 2002; Jones *et al.*, 2006; Ranjan *et al.*, 2021). Metals can also interact with phenolic compounds, leading to red coloration overexpression in plant tissues (Fedenko *et al.*, 2022).
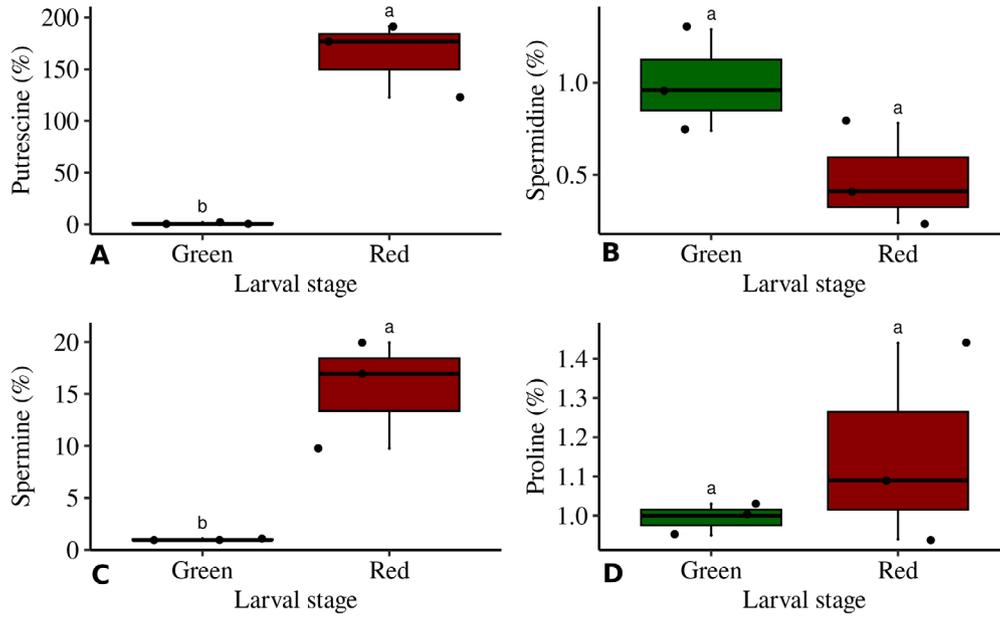
Figure 3. Polyamines quantification from red and green mature galls induced by *Palaeomystella oligophaga* Becker and Adamski (Lepidoptera) on buds of *Macairea radula* Bonpl. (Melastomataceae) with gall-inducing in the larval stage. (A) Putrescine, (B) spermidine, (C) spermine, and (D) proline. Differences between means were tested via Student's t-test, and statistically significant differences (p ≤ 0.05) were indicated by different letters.
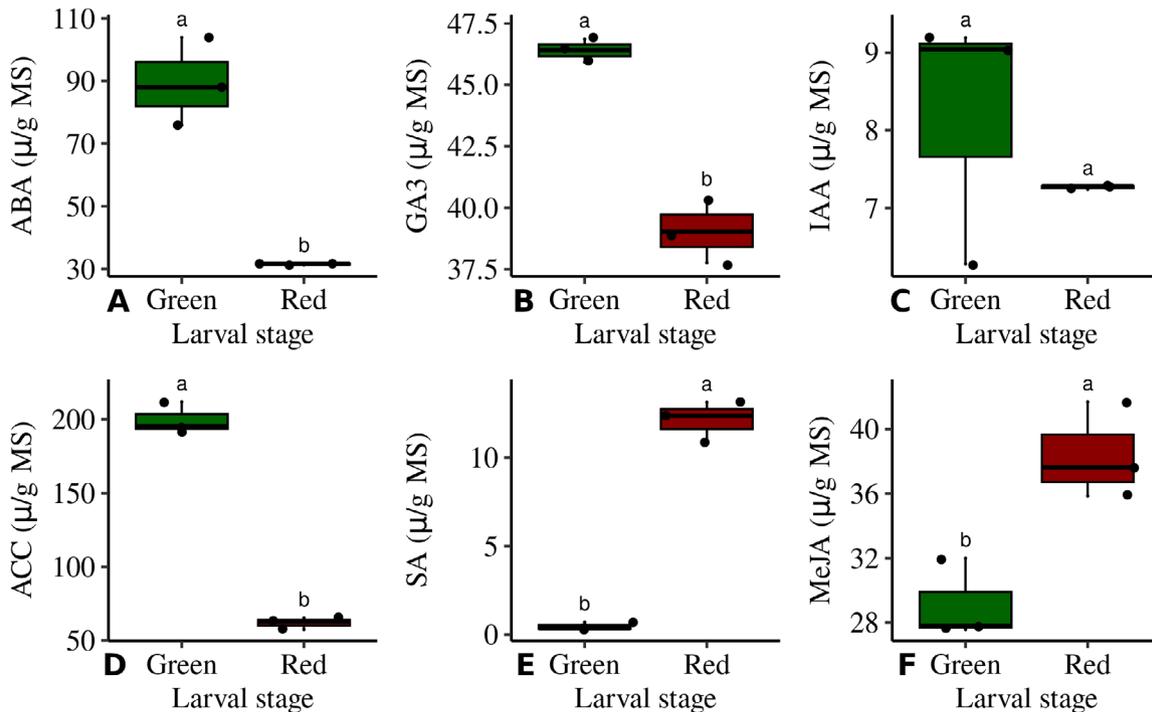

Figure 4. Phytohormone quantification from red and green mature galls induced by *Palaeomystella oligophaga* Becker and Adamski (Lepidoptera) on buds of *Macairea radula* Bonpl. (Melastomataceae) with gall induction in the larval stage. (A) Abscisic acid (ABA), (B) GA3, (C) indole acetic acid (IAA), (D) aminocyclopropane carboxylic acid (ACC), (E) salicylic acid (SA), and (F) methyl jasmonate (MeJA). Differences between means were tested via Student's *t-test*, and statistically significant differences (p ≤ 0.05) were indicated by different letters.

Plants possess various mechanisms to maintain the homeostasis of essential metals, and under stress, they undergo physiological and metabolic reactions leading to the accumulation of proteins, hormones, antioxidants, and stress-related signaling molecules, such as PAs (Alcázar et al., 2010). These molecules interact with plant hormones to act as signals for ROS production under both biotic and abiotic stresses (Wang et al., 2019; Diao et al., 2017; Napieraj et al., 2023). Most of the PAs evaluated in this study were more abundant in red galls than in green galls, which may indicate the relationship between these molecules and anthocyanins (Hudec et al., 2006). As phenolic compounds, anthocyanins accumulate in high amounts in galls induced by *P. oligophaga*, leading to red coloration (Cardoso et al., 2022). These molecules can scavenge free radicals produced under stress in plants and are important for antioxidant activity (Falahi et al., 2018; Li & Ahammed, 2023) and plant gall interactions (Guedes et al., 2022).

The higher putrescine and spermine levels in red galls may be related to the translocation of metals and the biosynthesis of chelating compounds to reduce their toxicity, stabilize ROS, increase the efficiency of antioxidant enzymes and biosynthesize photosynthetic pigments (Nahar et al., 2016; Balal et al., 2016). The high concentration of putrescine, for example, may also be due to stress caused by the accumulation of nonessential metals such as Al and Pb (Amist et al., 2017). On the other hand, proline is directly involved in insect nutrition and is connected to Fe metabolism and bioavailability (Arriola et al., 2024a). Compared with green galls, red galls accumulate high concentrations of both Al and Fe, which confirms previously discussed patterns of the association of these metals with the formation of red colors inside gall tissues (Arriola et al., 2024b). Polyamines can also reduce Cd toxicity in plants, as occurs in *Inula crithmoides* (Asteraceae) (Ghabriche et al., 2017), and protect cell membranes from oxidative damage through the formation of phospholipids and Fe^{2+} complexes (Velikova et al., 2000; Spormann et al., 2021). Thus, in red galls induced by *P. oligophaga* on *M. radula*, PAs can play an important chemical role in reducing Fe and in the control of Al accumulation and toxicity, facilitating the formation of metallo-anthocyanins in gall tissues, as previously reported for red galls induced on *Nothofagus obliqua* (Arriola et al., 2024b).

In galls induced on oak leaves, the PA content is determined by the activity of the enzymes lysine decarboxylase (LDC), tyrosine decarboxylase (TyDC), and ornithine decarboxylase (ODC) (Kot et al., 2019). The intensity of the changes in both PAs and associated enzymes may depend on the developmental stage of the gall and the gall-inducing insect (Kmiec et al., 2017). These proteins can help detoxify metals, forming chelates to prevent the excessive accumulation of nonessential metals in plant cells and transforming them into less toxic forms through complexation with primary and secondary metabolism molecules, such as hormonal regulators (Rahman et al.,

2023). Since PAs induce ROS production via NADPH oxidase and amine oxidase, the increase in PA content in red galls seems to be a response to the high level of metal accumulation and, consequently, increased capacity for ROS detoxification, as occurs in foliar plant tissues (Asija et al., 2023). Under low-temperature stress, for example, plants can accumulate MeJA, which significantly increases free Put and Spm, clearly indicating the role of PAs in stress signaling and the metabolism of phytohormones (González-Aguilar et al., 2000; Yoshikawa et al., 2007).

Phytohormones associated with responses to stress, such as SA and MeJA, were also found at high concentrations in red galls, corroborating their association with anthocyanin biosynthesis (Li & Ahammed, 2023). SA is a polyphenol compound synthesized in all plants through phenylpropanoid reactions and is a well-defined phytohormone that mediates responses to biotic stresses (Rahman et al. 2023) and protects plants against heavy metal stress (Jańczak-Pieniążek et al., 2022). In addition to SA, MeJA is a phytohormone that regulates several biological functions, especially those related to biotic and abiotic stress (Saini et al., 2021). Both SA and MeJA upregulate different enzymatic pathways associated with stress in plants under high concentrations of Cd, Pb, and Cu, among other heavy metals (Rahman et al., 2023). The high concentrations of certain metals in red galls may trigger increases in both SA and MeJA in response to toxicity.

Another phytohormone that modulates a broad array of plant responses, including stress and senescence, is ethylene (Sharma et al., 2012). The biosynthesis of this phytohormone competes with PAs for the same precursor, S-adenosyl-L-methionine (SAM) (Wang et al., 2020), and the increase in PAs can be associated with a decrease in ethylene in red galls, a possible metabolic advantage for controlling oxidative stress. GA3 and ABA are associated with different metabolic regulatory mechanisms in plants, but their relationships with PAs are limited to plant responses to salt stress (Ryu & Cho, 2015). In this context, green galls accumulate more ABA than red galls do, and our results may be associated with the negative feedback imposed by the high PAs and metal accumulation in red galls. An example of a relationship between phytohormones and metals is the toxic effects caused by Pb on a green microalga, *Acutodesmus obliquus*. In this microalga, Pb is associated with the downregulation of auxins, cytokinins, and gibberellins but may simultaneously increase the ABA content (Piotrowska-Niczyporuk et al., 2020). Lead also increases oxidative damage in plants, interfering with enzymatic activities and stomatal closure due to the induction of abscisic acid (ABA) (Alsokari & Aldesuquy, 2011). Compared with green galls, red galls presented high concentrations of Pb associated with lower levels of ABA, IAA, and ACC. Therefore, the increase in Pb in the red galls of *P. oligophaga* may be associated with an increase in oxidative stress and, consequently, an increase in the biosynthesis of phenolic compounds, such as anthocyanins and SA.

In summary, the accumulation of phenolic compounds, such as anthocyanins, can reduce the toxicity effects caused by the accumulation of Al, Fe, Pb, and Cu, transforming them into less toxic forms for galls and maintaining red coloration as a form of protection. Additionally, certain molecules, such as polyamines and phytohormones, particularly salicylic acid (SA) and methyl jasmonate (MeJA), are present at higher levels in red galls than in green galls. This response is part of a stress dissipation mechanism triggered by metal accumulation. Although reddish coloration in galls may appear idiosyncratic, investigating the metabolic pathways of polyamines, phytohormones, and associated metals provides promising insights into the role of these molecules in enhancing oxidative defense within gall tissues.

Acknowledgments

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