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Antioxidant responses of bushy host plants *Flemingia semialata* and *Flemingia macrophylla* on inoculation of two different strains of lac insect (*Kerria lacca* (Kerr))

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Abstract

Bearers of industrially important resinous material *shellac* secreted by Indian lac insects *Kerria lacca* (Kerr) grow and survive in a permanent association with certain plant species called host plants. These phloem-feeders display differential host suitability that is dependent on genotype of both plant and insect. Therefore, present study was conducted to evaluate induced biochemical responses in two host plants *Flemingia macrophylla* and *Flemingia semialata* upon inoculation by two infra sub-specific forms of this insect. Both host plants displayed differential responses locally in stem tissue and systemically in leaf to two different strains of lac insect. Systemic response is more intense in case of *rangeeni* inoculation on *F. macrophylla* and local antioxidant response is higher in *F. semialata* upon *kusmi* inoculation. *F. semialata* has almost twice constitutive H₂O₂ levels in its stem than *F. macrophylla* which decreased with time upon *kusmi* inoculation suggesting activated ROS detoxification in this species and absent in *F. macrophylla* in response to this strain. Basal level of H₂O₂ is high in leaves of *F. macrophylla* than *F. semialata* which is contrasting to that observed in stem tissues of both host plants. The intensity of defense response also varied with the strain of insect inoculated on the two host plants. Peroxidase levels in *F. macrophylla* in response to both strains suggest it is an important ROS detoxification system in this species that plays role in imparting specificity.

Keywords: Lac insect, host plants, stem herbivory

Introduction

Lac is an industrial product which is collectively an amorphous mix of majorly resin, wax and minor quantities of dye, volatiles and impurities obtained from hardened secretion from the body of female insect *Kerria lacca* (Kerr). The resin is used in paints, polishes, varnishes, lacquer, coating materials, electrical insulation, cosmetic products, pharmacology and is also recently being advocated as a food additive as a natural coloring agent (Srivastava and Thombare 2017)^[24]. *Kerria* species belong to Superfamily Coccoidea, a hemipteran (Sternorrhyncha) group of arthropods that are phytosuccivorous on selected host plant species. The crawlers settle on the stem of the plant, insert their proboscis into the phloem to suck the phloem sap and draw nutrients from it while completing their entire life cycle on the same spot. The fertilized female secretes this resinous secretion on its own body surface which is later scraped and purified industrially to obtain the industrial product devoid of impurities; shellac with its constituent fractions.

Kerria species is found associated with over 400 plant species belonging to different families that display differential potential in hosting this insect. Three major ones considered as traditional hosts are *Schleichera oleosa*, *Butea monosperma* and *Ziziphus mauritiana* (Sharma et al., 2016)^[23]. Increasing lac production would require more host plants planting to new agricultural or fallow land. The traditional host tree species have associated disadvantages of taking long gestation time to take up lac production, technical difficulty in climbing up tree branches for both broodlac inoculation as well as lac harvest. Thus bushy hosts are preferred for lac production owing to their quick establishment of growth and ease of management practices

suitable for lac cultivation. *Flemingia semialata* has recently been introduced as a potential quick growing bushy lac host producing lac @ 25-30 q/ ha/annum suitable for intensive and sustainable production of lac to newer areas (Sharma and Ramani 2010) [22]. *Flemingia* belongs to Papilionoidea family that fixes Nitrogen in soil with the rhizobia associated with its roots (Tribhuvan *et al.*, 2017) [26]. Using this as a host plant for lac cultivation would not only yield lac but also enrich the soil with the macronutrient.

Being one of its kind shellac is a unique product of commercial importance in whose production, India occupies the global leadership. Over 21 *Kerria* species exist that produce lac in the entire world. In India, two major infra sub-specific forms namely *rangeeni* and *kusmi* are predominantly exploited for lac production (Varshney 1977) [27]. These infra sub-specific forms differ in their host species preference, settlement behavior and quality as well as quantity of the resin produced (Mishra *et al.*, 1999; Sharma *et al.*, 2016) [17, 23]. Host plant- *Kerria* species interaction is highly specific and is genotype-dependent for both the interacting partners. *Rangeeni* insects can thrive well on *palas* (*B. monosperma*), *ber* (*Z. mauritiana*) *Flemingia macrophylla* as well as *Cajanus cajan* (Khichi *et al.*, 2023) but not on *kusum* (*S. oleosa*). Conversely, *kusmi* thrives well on *kusum*, *ber* and *Flemingia semialata* but not on *palas*.

Plants mount an inductive defense response when encountered with external stressor both biotic and abiotic that varies according to the plant genetic background, and the type of stressor (Nalam *et al.*, 2018) [18]. One of the early and prominent one being induction and accumulation of reactive oxygen species (ROS) that causes oxidative damage to cell and its constituents culminating in oxidative stress (Ebel and Mithöfer 1998) [5]. In order to maintain cellular homeostasis and cope with the detrimental effect of ROS, plants utilize an antioxidant mechanism of removing excess ROS from the cell. The antioxidant system includes ROS detoxifying proteins (e.g., superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), glutathione peroxidases (GPX), glutathione S-transferase (GST), peroxiredoxin (PRX)) and other antioxidant intermediate (Koch *et al.*, 2016) [12]. Reactive oxygen species (ROS) and its detoxification is an entire cellular system in itself which is employed by plants to signal and/or cope up with any sort of disturbance encountered. Induced activities of these enzymes have been associated with increased resistance in genotypes expressing them as in groundnut (War *et al.*, 2015) [28], Sorghum (Taggar *et al.*, 2012) [25], Soybean (Mai *et al.*, 2013) [15]. Multiple enzymes, metabolic intermediates and molecular players thus make up a robust system for ROS detoxification in plants.

Lac inoculation on host plants causes significant changes in host plant metabolism and physiology. *Rangeeni* inoculation on pigeonpea caused reduction in 100 seed weight as well as grain yield and also affected biochemical parameters such as reducing sugar, pigments such as chlorophyll 'a' and total chlorophyll (Ghosh *et al.*, 2014) [7]. In another study it was reported that *rangeeni* inoculation decreases seed soluble protein and starch in pigeonpea seeds and this varied genotypically (Lohot *et al.*, 2018) [14]. Genotypic variation of host plant was also recorded for physiological and morphological/biochemical characteristics in response to *kusmi* inoculation on *F. semialata*. Selection lines for drought tolerance that produced higher lac yield had increased peroxidase activity and lower catalase activity than local lines (Ghosh *et al.*, 2017) [6].

In order to increase lac production and select suitable host genotypes, the induced defense responses causing differential

behavior of hosts to different strains needs to be understood. Leaf is the primary organ of photosynthesis and majority of plant response is assayed in this organ. However, lac insect is a stem-dweller thus, local effect of its attachment on host needs to be deciphered apart from the systemic and SAR effect in long runs. Keeping in view of above points this study was conducted to identify the effect of two different strains of lac insect. This study tested the hypothesis that seedlings of *Flemingia* species exhibit varying levels of defense responses when exposed to different strains of lac insect under natural conditions. The research aimed to investigate how two species of *Flemingia* respond to lac infestation and evaluate their induced ROS detoxification system against various strains of *Kerria lacca*.

Materials and Methods

Experimental location, plant material and insect

The entire experiment has been conducted inside the greenhouse and polyhouse facility available at the Institute Research Farm of ICAR-NISA, Ranchi. 45 days old *Flemingia semialata* and *Flemingia macrophylla* seedlings were raised from seeds grown in autoclaved farm manure sourced from the IRF and grown in net house with best package of practices. Visually good-looking *kusmi* and *rangeeni* cells harvested from broodlac were tied at 300 mg on tender stem of each plant with the help of net cloth. *kusmi* and *rangeeni* strain of lac insect were used for both *F. semialata* and *F. macrophylla* during *aghani* and *katki* seasons.

Biochemical parameters

Three young leaves were sampled from 2 different plants and pooled to make one replicate and such three replicates were taken for each treatment for biochemical analysis. Three different enzymes namely superoxide dismutase (SOD) using NBT (Dhindsa *et al.*, 1981) [4], Guaiacol peroxidase (Pox) (Castillo *et al.*, 1984) [2] and H₂O₂ content using Potassium Iodide (Junglee *et al.*, 2014) [9] was estimated in extracts prepared from lac-inoculated seedlings and uninoculated ones at 12, and 24 hours post-inoculation along with uninfected control plants to understand the early responses.

Statistical Analysis

Data collected was analyzed using the standard techniques of 2-Factor Analysis of Variance (ANOVA) with the assistance of computer software Wasp2.0 maintained by ICAR-CCARI. "F" test with significance at a 5% level of significance ($P < 0.05$) is reported using critical difference (C.D) calculated to determine the significance of differences between two treatment means.

Result and Discussion

The susceptibility or resistance of plant is dependent upon the defense response mounted in response to an external agent such as an arthropod. The first line of induced defense in plants in response to damage by pests is the ROS generation and its homeostasis. This then defines the status of plant to be tolerant or susceptible to the stress encountered.

In order to understand the interaction between host-plant and a long-term associated lac insect, the induced biochemical responses in the host is necessary to be dissected. This will provide clues to get an insight into the specificity of host to a strain of insect which is not defined earlier for lac insect. In the present study this response has been captured and defined as the response of two similar but distinct host plants to two insect strains differing in specificity.

Local stress and antioxidant system

H₂O₂

Flemingia semialata (hereby referred as Fs) has higher constitutive H₂O₂ levels of almost 914.7 mM/g. fwt which is almost 2 times higher than the basal levels in *Flemingia macrophylla* (hereafter referred as Fm) amounting to 547.2 mM/mg f.wt as shown in Figure 1(a). Upon *kusmi* inoculation, the levels at both 12 and 24 hours post inoculation (hpi) was higher in Fs than Fm. At 12 h, the levels in Fs is 344.9 mM/mg f.wt while in leaves of Fm is almost 33% of it at 114.1 mM/mg f.wt. At 24 h, Fm has an elevated levels of 193.6 mM/mg f.wt H₂O₂ similar to its basal levels but Fs is still having low levels of 245.6 mM/mg.fwt. The result suggests that Fs had higher levels of H₂O₂ basally which might be responsible for its antibiosis effect for protection from other pest and pathogen. Higher constitutive H₂O₂ levels have been associated with increased resistance to aphid feeding in sorghum resistant genotypes (Shao *et al.*, 2019) [21]. But sharp decreasing level of H₂O₂ in *kusmi*

tolerant host Fs suggests that ROS detoxification system might be activated upon inoculation which leads to decline in its levels successively as time lapses. Thereby creating a non-stressed condition for host Fs to the insect while the feeding continues. Fm- a non-suitable host for commercial cultivation of *kusmi* strain shows lower levels constitutively and is only able to raise H₂O₂ levels at later time point of 24 hpi. H₂O₂ has dual role of both stress signaling factor at lower concentrations while at higher concentrations, it acts as a free radical and destroys cellular moieties (Gupta *et al.*, 2016) [8]. From the data it appears that Fm is starting to mount defense at later stage but at early stage is susceptible due to lower stress signal molecule H₂O₂ and therefore succumbs to the insect infestation and is not able to sustain the insect feeding. Differences in H₂O₂ levels in stem suggests that local defenses are activated in both host plants differentially.

H₂O₂

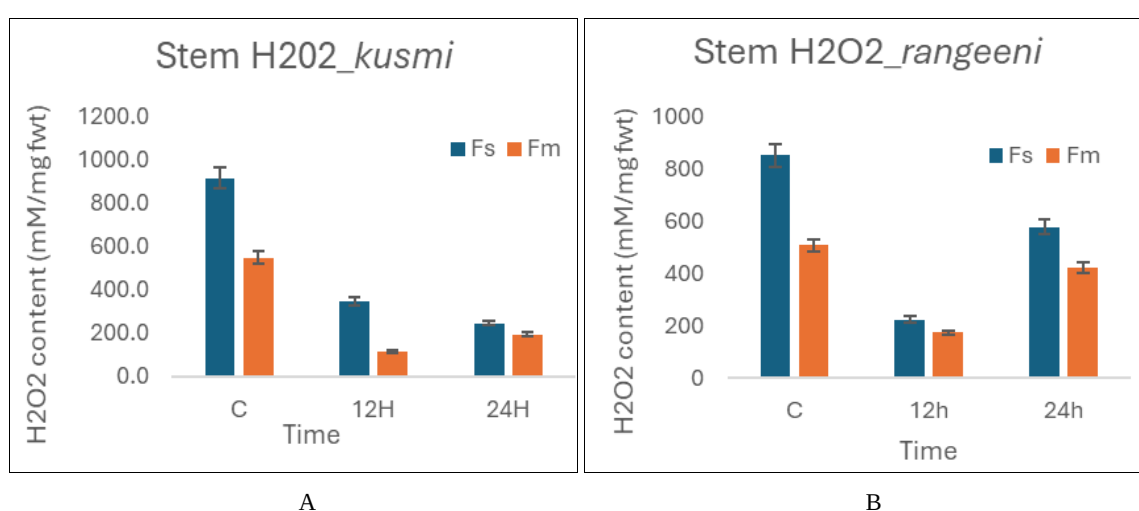


Fig 1: Variation in content of H₂O₂ upon (a) *kusmi* and (b) *rangeeni* strain inoculation on stem tissue of *Flemingia semialata* and *Flemingia macrophylla*

Upon *rangeeni* inoculation, the accumulation of H₂O₂ in Fs is almost 73% lower (224.3 mM/mg fwt) at 12 h than *kusmi* caused as in Figure 1(b). At 24 h both the host species accumulated similar levels of H₂O₂ 588.3 and 424.13 mM/mg fwt which did not differ significantly between them. At 24 h both the species accumulate higher levels of H₂O₂ than *kusmi* inoculation and that of Fm had higher at all levels than when *kusmi* was infected. This suggests that *rangeeni* inoculation in Fm is causing a less intense response of H₂O₂ accumulation which was abrupt upon *kusmi* inoculation. Host is becoming resistant progressively as feeding continues. While the non-suitable host, Fs is mounting defense response as the feeding continues. Although antixenosis and antibiosis dispel the insect pest since this is a life-long interaction of lac insect over host it must cause subtle changes in host to be able to survive the entire life. Such differential intensity of antioxidant response is also reported in wheat genotypes against aphid (Kaur *et al.*, 2017) [10]. Defense responses mounted in higher intensity to the insect would be deleterious for its survival.

ROS Detoxification

SOD catalyzes the dismutation of superoxide oxygen radical O₂⁻ to H₂O₂ and Oxygen molecule, O₂ (Caverzan *et al.*, 2016) [3].

When *kusmi* infects Fs there is an incremental increase in SOD levels at both 12 and 24 hpi as depicted in Figure 2(a). A small increase of over 12% occurs in Fm at 12 h which then declines by 50% at 24 h to 0.05 units/mg fwt. Increased SOD activity is reported in wheat tolerant to heat stress and resistant genotypes in soybean to stink bug (Kumar *et al.*, 2013; Sabljic *et al.*, 2020) [13, 20]. Fm did not have any difference in SOD activity levels at 12 h than its control however at 24 hpi it had a decreased SOD activity. This is in parallel to the increased H₂O₂ in its stem at 24 hpi and suggests that at 24 hpi the Fm stem samples start inducing defense responses to *kusmi* strain. *Rangeeni* inoculation caused a 24.6% increase in OD levels at 12 hpi in Fs which then plateaued at 24 hpi with 0.10 units/mg fwt. Fm did not show any significant change in SOD levels than control and 12 hpi but had a decreased SOD levels at 24 hpi by 32% to 0.04 units/mg fwt. Overall, the two species responded differently to the insect strain in SOD levels with Fs always having higher SOD levels than Fm suggesting genotypic differences causing the effect. SOD levels paralleled with H₂O₂ accumulation at 24 hpi in both the samples. The SOD activity is more pronounced in a species-specific manner to lac inoculation at later stage of infection (24 hpi) and not at early 12 hpi.

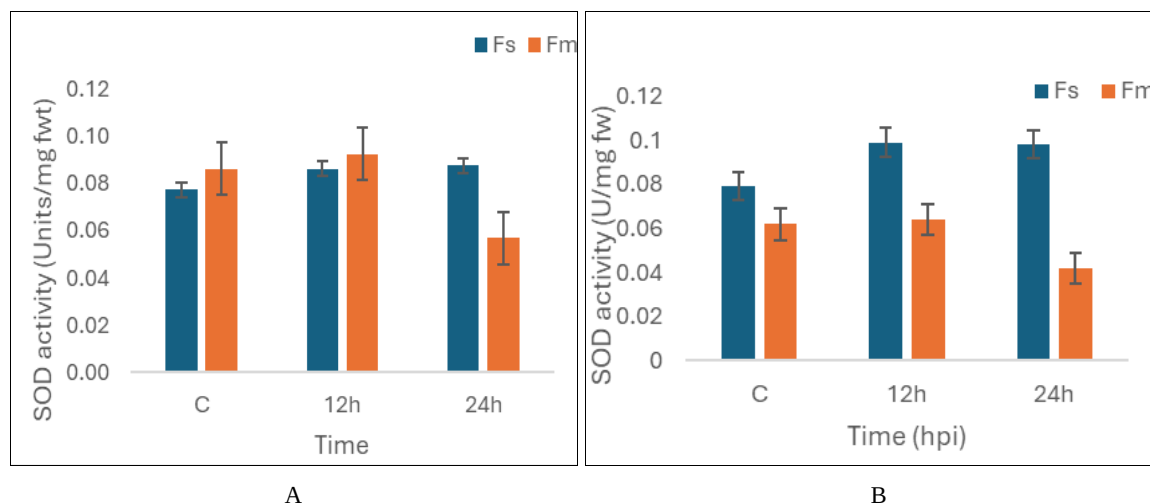


Fig 2: Variation in SOD activity levels upon (a) *kusmi* and (b) *rangeeni* strain inoculation on stem tissue of *Flemingia semialata* and *Flemingia macrophylla*

Peroxidase (Pox) levels fluctuated in response to *kusmi* inoculation. Fs had basal levels of 3178 units/mg fwt which declined by 57.7% to 1343.01 units/mg fwt at 12 h and then raised to 1905.1 units/mg fwt at 24 h. Fm had lower basal Pox activity in stem (1829 units/mg fwt) than Fs, almost 48% less. At 12 hpi the Pox activity declined to 1/3rd but increased significantly by 156% than its basal levels at 24 hpi. At 24 hpi both Fs and Fm showed significantly high differences in Pox levels with Fm displaying almost 2.5 times higher levels than Fs. Peroxidases catalyzes the detoxification of H₂O₂ to H₂O. Increased peroxidase activity upon whitefly infestation occurs in *Vigna* species and also in several plants (Taggar *et al.*, 2012)^[25]. Induced peroxides transcripts in resistant sorghum genotypes to aphid and greenbug infestation is reported to impart resistance

(Pant and Huang 2021)^[19]. Higher levels of Pox activity in Fm is suggestive of its resistance to *kusmi* strain. *Rangeeni* inoculation caused similar Pox levels suggestive that at local levels, this enzyme is not responsible for the differential response of Fs to the two strains. The other plausibility is that different isozymes existing in Fs might be involved which requires to be studied in a new set of experiments. Pox activity levels in Fm showed higher intensity upon *kusmi* inoculation and less on *rangeeni*. This suggests that some abnormalities exist in defense system in Fm to *kusmi* as is reported in resistance response of *Vigna* to whitefly (Taggar *et al.*, 2012)^[25]. Thus, Pox levels in Fm partially explains its specificity to both insect strains.

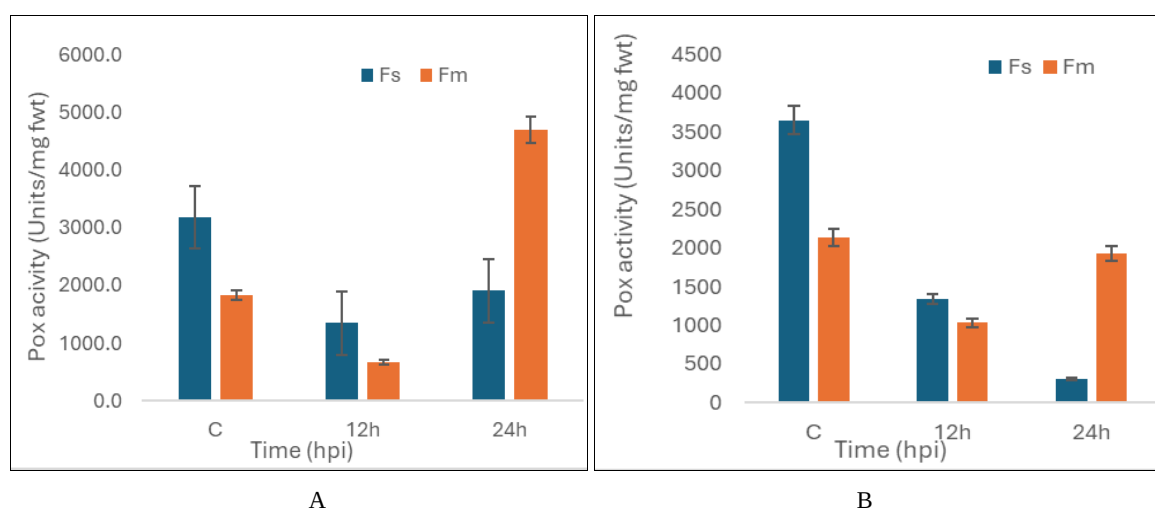


Fig 3: Variation in Pox activity levels upon (a) *kusmi* and (b) *rangeeni* strain inoculation on stem tissue of *Flemingia semialata* and *Flemingia macrophylla*

Systemic stress and antioxidant system

ROS also act as signaling molecule in modulating during growth, developmental as well as stress responses in plants. Lac insects are stem dwellers which feed on the phloem continuously from the host plants. Local cell/tissue response is crucial in the interaction between host-insect but the systemic responses of other plant parts specifically leaves, the photosynthetic organs must also not be undermined. The effect of lac insect feeding on leaf ROS and antioxidant system was thus investigated.

H₂O₂

The systemic accumulation of H₂O₂ levels in leaves of Fs was 3.5 fold higher than in stem which suggests that feeding of phloem sap by the insect is causing induction of stress as well as defense response to the leaves. H₂O₂ is a diffusible signal and can make consequential changes far from their site of origin (Anjum *et al.*, 2022)^[1]. Basal level of H₂O₂ is high in leaves of Fm than Fs which is contrasting to that observed in stem tissues of both the host plant. Inoculation by *kusmi* and *rangeeni* displayed differential results in Fs. While *kusmi* caused increase in H₂O₂ content in leaves of Fs, *rangeeni* caused a decrease in

H₂O₂ content which is strikingly different from Fm which accumulated decreasing levels of H₂O₂ upon both strain inoculation. Pea seedlings that were resistant to *Acyrtosiphon* feeding had increased H₂O₂ in their leaves upto 96 h which enhanced their defense potential to the herbivory (Mai *et al.*, 2013) [15]. In Fm, *kusmi* caused a decline by 47.5% over control

at 12 h and by 76.75% at 24 hpi while *rangeeni* inoculation caused 50% decline at 24 h which is less than that caused by *kusmi*. Thus, while basal levels of H₂O₂ is high in stem of Fs, leaves of species Fm has higher H₂O₂ levels suggesting genotypic differences causing differential responses to insect pest.

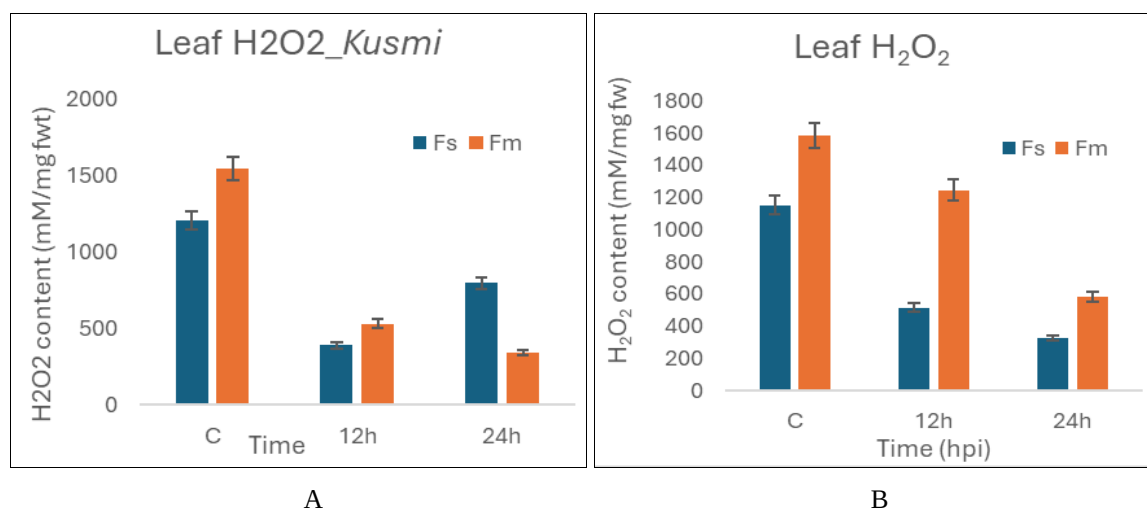


Fig 4: Variation in systemic content of H₂O₂ upon (a) *kusmi* and (b) *rangeeni* strain inoculation on leaf tissue of *Flemingia semialata* and *Flemingia macrophylla*

ROS detoxification

Rangeeni strain caused a 27.2% decrease in SOD activity levels at 12 h and a 76.8% decline at 24 h in Fm. This is in coherence with the declining H₂O₂ in its leaf. Fs displayed elevated SOD activity of 0.05 units/mg f.wt at 12 h which is 12% higher than control having 0.047 units/mg fwt but it declines at 24 hpi. *Kusmi* infection causes increase in SOD activity in Fm, with levels going to 0.04 units/mg fwt than 0.03 units/mg fwt of control which is opposing the trend upon *rangeeni* inoculation. Fs had higher basal SOD levels in leaf which might be responsible for detoxification of ROS generated by the aphid feeding.

Pox shows significantly different response in Fs to that in Fm leaves. The differential responses indicate multiple roles of peroxidases in tolerance and susceptibility in the *Flemingia* species. Such responses are also reported in soybean genotypes to aphid feeding (Werle *et al.*, 2014) [29]. Both the insect strain feeding causes an increase in Pox levels in Fs leaves. An increase of 1002 units/mg f.wt of Pox at 24 h upon *kusmi* infection and ~2x levels upon *rangeeni* inoculation suggest the distinctive role of Pox in Fs to both insect strains. A moderate increase in Pox levels upon *kusmi* inoculation suggest that Pox is involved in detoxifying the ROS generated in leaves by lac feeding. However, since Fs is not a good host for *rangeeni*, feeding by this strain is causing a very intense increase in Pox levels suggesting that Fs is mounting defense response to *rangeeni* feeding. Pox plays role in defense signaling by hormone signaling and also mediates cell wall strengthening (Kerchev *et al.* 2012) [11]. This result is suggestive that *rangeeni* feeding on Fs is inducing Pox levels that is activating the cell wall strengthening pathway which renders the host non-suitable for *rangeeni* lac survival.

Conclusion

The present work assessed local as well as systemic redox response of two distinct legume species belonging to same genera upon herbivory injury by a phloem-feeder lac insect. The

two species responded distinctly in their redox potential at both constitutive and induced levels. *Flemingia semialata* has higher constitutive H₂O₂ and sharp decline in its level upon *kusmi* inoculation suggest the activated ROS system in this species which is not evident in *kusmi* non-host Fm. Also, there was significantly differential local and systemic responses of both species to the two strains. The Pox content in this species paralleled the H₂O₂ content accumulation at early stages of infection at local stem levels which was aberrant in Fm. Similar accumulation of Pox with H₂O₂ levels occurs in cowpea infested with cowpea aphid (Mai *et al.*, 2016) [16]. This is a clue that peroxidases have crucial role in determining the host specificity by the lac insect. However, the different isozymes contributing to the level needs investigation.

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