INNSPUB

International Journal of Agronomy and Agricultural Research (IJAAR)
ISSN: 2223-7054 (Print) 2225-3610 (Online)
http://www.innspub.net
Vol. 11, No. 1, p. 51-59, 2017

RESEARCH PAPER OPEN ACCES

Interactive defense responses of *Arabidopsis thaliana* to phloem feeding and leaf chewing insects

Robert O. Okayo[*1], George O. K'Otuto[2], Fredrick O. Ouma[2]

[1]*Department of Plant Animal and Food Sciences, Jaramogi Oginga Odinga University of Science and Technology, Bondo, Kenya*

[2]*Department of Applied Plant Sciences, Maseno University, Kenya*

Article published on July 31, 2017

Key words: *Arabidopsis thaliana*, Crosstalk, Phytohormones, *Pluetella xylostella, Brevicoryne brassicae*

Abstract

Plants activate defense signaling pathway depending on the feeding guild of the attacking herbivores. Usually two signaling pathways namely, Salicylic acid (SA), Jasmonic acid (JA), mediate plant responses to insect attacks. When attacked by multiple herbivores, the two signaling interact in ways whose outcome may be complicated. To investigate this cross talk phenomenon we infested wild type and SA insufficient mutant plant types of *Arabidopsis thaliana* L. with only *Pluetella xylostella* L. caterpillars, only adult *Brevicoryne brassicae* L. aphids and with both caterpillars and aphids. We analyzed the insect performances and the underlying defense mechanisms in the plant via expression levels of the marker genes for JA (*LOX2*) and SA (*PR1*). The caterpillar accumulated higher biomass in dual infested wild type and SA treated wild type plants in comparison to the rest of the treatments. The higher biomass of caterpillar in these treatments was attributed to suppression of plants' JA mediated defense systems by SA. The SA antagonism of JA mediated defense system was supported by lower expression levels of *LOX2*. In dual infestations, aphids performed better under SA insufficient mutants compared to wild type plants pointing to possible activation of SA mediated defense in the wild type plants in response to attacks by aphids. Our results show that the phloem feeder weakened JA-related plant defenses, thus facilitating the growth and development of the leaf chewers. We conclude that such facilitative inter-guild interactions may present serious challenges to beneficially harnessing the natural plant defense systems against herbivory.

* **Corresponding Author:** Robert O. Okayo ✉ robbymoloben@gmail.com

Introduction

Plants deploy various defense mechanisms when attacked or on perception of attack from herbivorous insects (Pieterse *et al.*, 2012). They produce toxic, anti-nutritive and anti-digestive compounds that directly affect the attacking herbivore (Mithofer and Boland, 2012). This is in addition to pre-existing physical and chemical barriers to attacking insects such as trichomes, toxic metabolites and secondary metabolites (Howe and Jander, 2008). Plants can also employ indirect defense mechanisms which comprise of emissions of volatiles to recruit predators or parasitoids of the attacking herbivores (Mithofer and Boland, 2012).

Advance studies in molecular biology have provided valuable insight in the functioning of induced plant defenses. The defensive responses of the plants to attacking insects are mediated by phytohormones. The plant recognition of insect herbivores and/or insect attack leads to activation of phytohormone signaling pathways (Pieterse *et al.*, 2009) of the many phytohormone signaling pathways only limited numbers of interactive pathways are activated in response to wide variety of attackers (Bari and Jones, 2009). In particular, salicylic acid (SA), jasmonic acid (JA) and ethylene (ET) are the critical signaling molecules that underlie the activation of plant defenses. Among these three, SA seems to play a major role in plant defense against biotrophic and hemi-biotrophic pathogens and phloem-feeding insects while, JA and ET play a role in the defense against necrotrophic pathogens and leaf chewing insects such as caterpillars (Bari and Jones, 2009).

The leaf chewing insects induce the plants to produce JA which intern regulates the production of secondary metabolites such as insect proteinase, nicotine, active phenolics and phytoelexins that deter feeding or inhibit digestion of these insects (Balbi and Devoto, 2008, Creelman and Mullet, 1995). JA is synthesized from linoleic acid through octadecanoid pathway (Pan *et al.*, 1998). Linoleic acid is converted to hydroperoxy-Octadecatrienoic acid by lipoxygenase. Hydroperoxy-octadecatrienoic acid undergoes reactions catalyzed by allene oxide synthase (AOS) and allene oxide cylose (AOC) to yield phytodienoic acid which is then oxidized to form Jasmonic acid (Creelman and Mullet, 1997, Pan *et al.*, 1998). The JA formed then regulates the expression of defense genes.

SA mediated pathway induces the production of pathogenic resistance genes. These genes initiate the hypersensitive response and synthesis of phytoalexins and pathogenic related proteins (Laoke and Grant, 2007). In addition, the two phytohormone signaling pathways (JA and SA) interact in mediating plant responses to attacking insects. Salicylates, synthesized or exogenously applied to plants, inhibit the JA synthesis through inhibition of allene oxide synthase activity in JA biosynthesis (Fig. 1) in a phenomenon referred to as JA/SA cross talk (Soler *et al.*, 2012). This limits the production of Jasmonic acid and its secondary metabolites (Raskin, 1992, Pan *et al.*, 1998). The antagonistic effect seems to be dependent on concentration and timing of the induction of the phytohormones (Mur *et al.*, 2006, Pieterse *et al.*, 2009). Other than the three phytohormone pathways, studies have also suggested the participation of other hormones that can modulate the defense signaling networks (Pieterse *et al.*, 2012). For instance, Abscisic acid (ABA) can act synergistically with distinct JA and ET regulated responses but antagonize the SA responses. Growth hormones such as Auxin, gibberellins, and cytokinins can prioritize growth of the plant at the expense of defense (Pieterse *et al.*, 2012).

Insects feeding on plants are often grouped according to their feeding guilds, such as phloem feeders, leaf chewers, leaf miners or root feeders. Plants respond differently and thus engage different signal transduction pathways to the various insect feeding guilds (Howe and Jander, 2008). Leaf chewers, such as caterpillars, mainly induce the JA pathway whereas phloem-feeders, such as aphids, induce the SA pathway (Howe and Jander, 2008). Under natural conditions, plants may be attacked simultaneously by an array of insects with different feeding guilds, which elicit different defense mechanisms mediated via different transduction pathways (Poelman *et al.*, 2012).

Given the complex interactions among phytohormones including the JA/SA cross talk, the ultimate plant response to multi-insect infestations may not be obvious. Generally the amount and sequence of attack modifies the JA/SA cross talk (Pieterse *et al.*, 2009) and may result in the prioritization of one defense mechanism over the other(s) and consequently loss of effectiveness of the unprioritised defense(s).

Whereas the induction of plant defenses under attack by most common insect feeding guilds in seclusion is relatively well studied (Soler, 2009), plants responses to attacks by multiple insects with varied feeding guilds typical of their natural habitats is still largely unexplored. In particular, how effects of different attackers impact on those (effects) of others remains unclear (Pieterse *et al.*, 2009). Whereas the cross talk allows the plant to fine tune their defense activation network to optimize induced defense to attackers (Pieterse and Dicke, 2007), the mechanism of how the plants fine tune their defense response under multiple attackers with different feeding guilds remains elusive. The aim of this study is to examine the interactive response to caterpillars (leaf chewers) and aphids (phloem-feeders) on *Arabidopsis* plant and to whether the interaction is reflected at the molecular level.

Materials and methods

Plant and insect materials

The Seeds of wild type and SA mutant *Arabidopsis thaliana* L. plants were sourced from Arabidopsis Resource Centre and sown in a soil mixture that was autoclaved at 80°C for 4 hours. After 10 days, the seedlings were transplanted into growing pots in the growing chamber and grown for five weeks at RH 50–70%, 8 hours light and 16 hours dark cycle at 23°C.

Insect rearing

Two specialist insects: aphids (*Brevicoryne brassicae* L.) and caterpillars (*Plutella xylostella* L.) were used in the experiment. Adult aphids and second stage (L2) caterpillars were used to infest the plants. Aphids were reared in a greenhouse at a temperature of approximately 23° C and RH 50–70% while the caterpillars were reared in cages in a climate room at a temperature of 21° C and RH 50 – 70%. They were both reared on *Brassica oleracea* plants.

Two independent experiments were conducted namely: insect development Assays and gene expression analyses.

Insect development assays

Experimental design

Five treatments were assigned randomly to thirty plants in a completely randomized design. The five treatments were as follows: (1) Wild type plants infested with 2 caterpillars, (2) Dual infested wild type infested with two caterpillars and five aphids, (3) SA treatment wild type plants in which the plants were dipped in a solution of 0.015% Silwet L 77 containing 0.5mM SA and infested with 2 caterpillars. (4) SA insufficient mutant plants infested with 2 caterpillars (5) SA insufficient mutants plants infested with two caterpillars and five aphids. SA treated plants were first placed under about 100% RH to open up their stomata and keep them erect. Plants infestation with the insects was done in the growing chamber and in dual infestation, the herbivores were placed on the same leaf simultaneously. The development of the insects was determined by taking weights of the caterpillars after the third and fifth day. This experiment was repeated after 14 days.

Gene expression analysis

Four treatments replicated three times were used in gene expression analysis: (i) Non-infested plants as controls; (ii) Plants infested with 2 caterpillars on each plant; (iii) Plants infested with 5 aphids on each plant; (iv) Plants infested with both 2 caterpillars and 5 aphids on each plant. Twenty four hours after infestation, the leaves with highest number of herbivore were harvested. Two leaves were harvested from each replicate and pooled together for the RNA isolation. The collected materials were immediately frozen in liquid nitrogen and stored at -80°C for RNA isolation.

Total RNA was extracted using an RNeasy plant mini kit (www.qiagen.com). The RNA quality and quantity were measured using a NanoDrop ND-100 (NanoDrop Technologies, Wilmington, DE, USA) spectrophotometer (all samples with $OD_{260/280}$ = 1.9-2.3 were used in the experiment). For cDNA synthesis, 1 µg of total RNA was reverse-transcribed using M-MLV reverse transcriptase (Invitrogen) in a final volume of 25 µl.

Quantitative RT-PCR analysis

To gain further insight in the interaction between JA and SA signaling, underlying multiple insect attacks, the expression of SA-responsive marker gene *PR1* and JA-responsive marker gene *LOX2* were analyzed.

A RT-PCR analysis was used to evaluate the expression profiles of selected genes involved in the SA and JA signaling pathways. This was performed in a CFX96 Touch Real-Time PCR Detection System (Bio-Rad) machine. The amplification reactions were performed in 25 µl final volume containing 2.5 µl of cDNA, 0.1 µM of each primer, 0.03 µlM of reference dye, and 2 x Brilliant II Fast SYBR® Green QPCR Master Mix (Agilent, www.agilent.com).

All RT-PCR experiments were performed in duplicate and average values were used in the analyses. All the RT-PCR reactions were performed under the following conditions: one cycle of initial denaturation at 95°C for 3 minutes followed by 40 cycles of subsequent denaturation for 15 seconds, annealing at 65°C, elongation at 72°C and finally hold at 4°C.

A normalization factor was calculated by geometrically averaging the threshold cycle (Ct) values from the constitutively expressed gene Elongation Factor-1-α (EF1-α). Ct values were normalized for differences in cDNA synthesis by subtracting the Ct value of the normalization factor from the Ct value of the gene of interest. Normalized gene expression was then calculated using $2^{-\Delta\Delta Ct}$ method.

Statistical analysis

SPSS 20.0 software was used for all the analyses. The normality and homogeneity of data were checked before the analyses could be done. Where the assumptions were violated, a Kruskal-Wallis test was used. Where the assumptions were confirmed, ANOVA was undertaken and LSD used as a post-hoc test.

Results

Insect development assays

Caterpillar performance

There was no significant difference in the weights of the caterpillars in the two experiments ($p>0.05$) (Fig. 2). By the 5th day, the dual infested wild type plants and SA treated plus *P. xylostella* infested plants had higher accumulation of caterpillar biomass compared to *P. xylostella* only treated wild type, dual infested mutants and *P. xylostella* infested mutant plants (Fig. 2). The dual infested wild type plants and SA treated plus *P. xylostella* infested plants seemingly had reduced resistance to caterpillar damage.

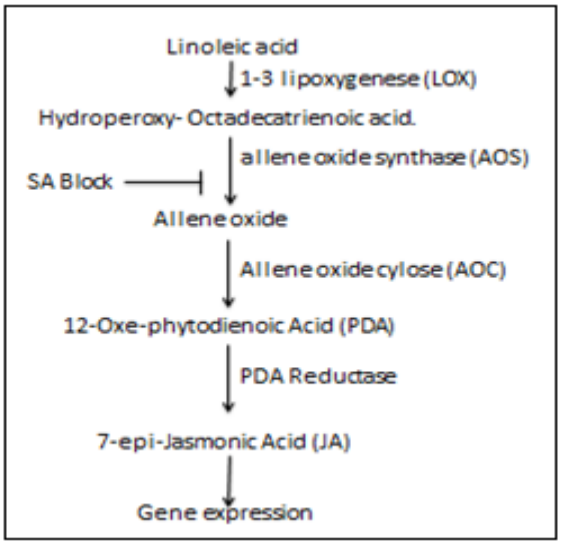

Fig. 1. Jasmonic Acid biosynthesis pathway indicating a point of inhibition by SA in a cross talk as adapted from Pan *et al.* (1998).

Aphid performance

There was significant difference ($P= 0.045$) in the total number of aphids between the wild type and the mutant plants infested with both caterpillars and aphids (Fig 3). In addition to the higher number of aphids, the aphid nymphs on dual infested mutant plants also reached a second stage earlier than those on the control wild type plants (data not presented).

Gene expression analysis

Caterpillars' infestation significantly affected the expression of *LOX2* (Fig. 4).

Plants infested with *P. xylostella* had a significant higher *LOX2* expression compared to the other treatments ($p<0.05$). *LOX2* expression in dual infested plants though not significantly different from SA + xylostella, was higher than in the control and *B. brassicacea* infested plants.

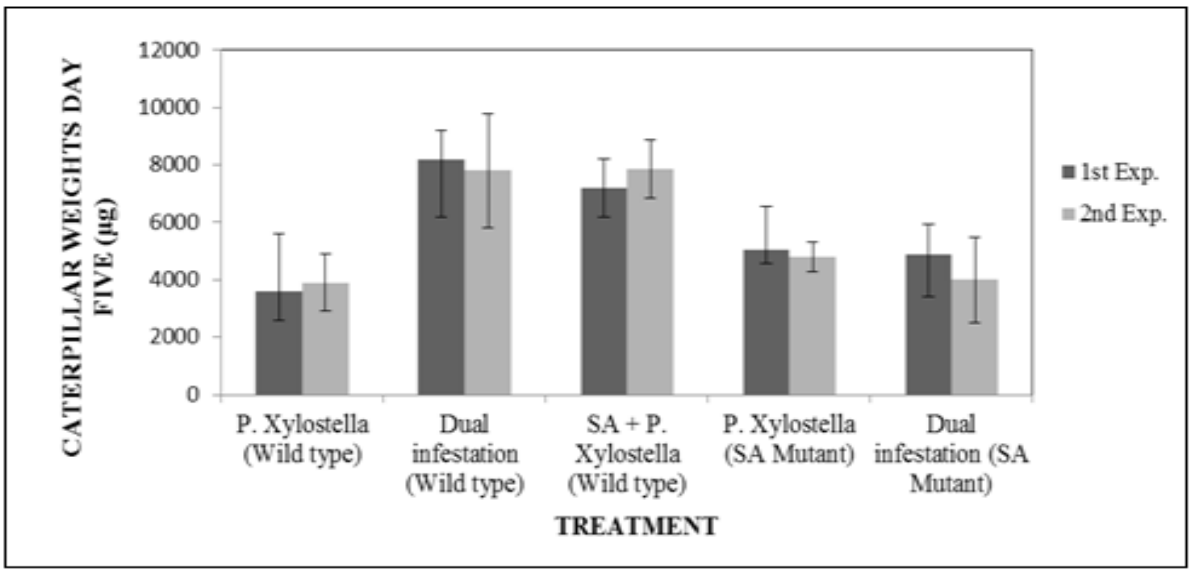

Fig. 2. Mean weight (µg) of *P. xylostella* caterpillars 5 days after infestation in the two independent experiments: Values are means ± error bars.

Discussion

Caterpillars had better development and thus accumulated higher biomass on wild Arabidopsis plants co-infested with aphids (dual infestation) and those treated with SA (Fig. 2). Leaf chewers such as caterpillars are generally known to induce the plants to produce JA which intern regulates the production of secondary metabolites such as insect proteinase, nicotine, active phenolics and phytoelexins that deter feeding or inhibit digestion leading to a reduced biomass accumulation (Balbi and Devoto, 2008; Creelman and Mullet, 1995). The induction of JA signaling pathway is supported by the higher expression of JA marker gene LOX2 in the caterpillar-infested wild plants compared to those not infested with caterpillars (Fig. 4). Such JA-mediated anti-feeding effects on caterpillars would account for the lower biomass accumulation on the wild type plants infested with caterpillars.

The higher caterpillar biomass accumulation on the dual infested and the SA-treated plants may be attributed to suppression of the JA mediated defenses by SA.

Exogenously applied SA in SA-treated wild type plants and the SA induced by the feeding action of aphids in the dual infested wild type plants may have antagonized the activation of JA defense responses.

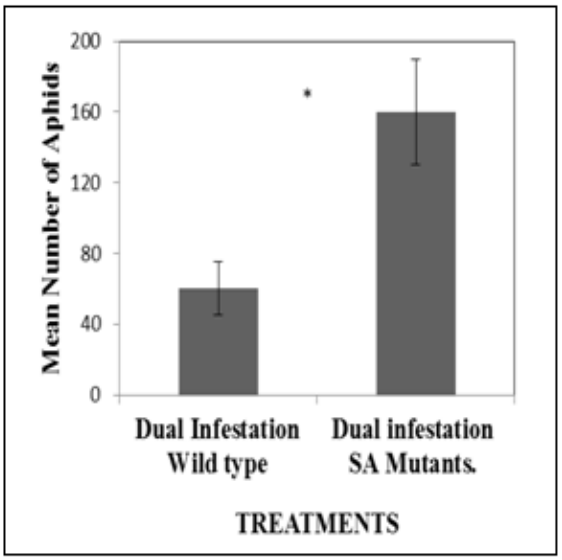

Fig. 3. Mean number of Aphids in the two treatments. Values are means ± error bars.

This antagonism of JA signaling pathway is evident in the molecular analysis which shows the dual infested and SA-treated plants having lower expression levels of *LOX2* gene (Fig 4).

This is in agreement to the work of Zhang *et al.* (2009) who reported that effectors contained in the saliva secretions of phloem feeders activated SA induction pathway that then actively suppressed the JA-mediated defense systems.

Such suppressors may inhibit positive regulation of JA inducible gene expression by interfering with it or induce transcription of suppressive factors that directly bind the promoter of JA responsive gene to repress their expression (Does *et al.*, 2013).

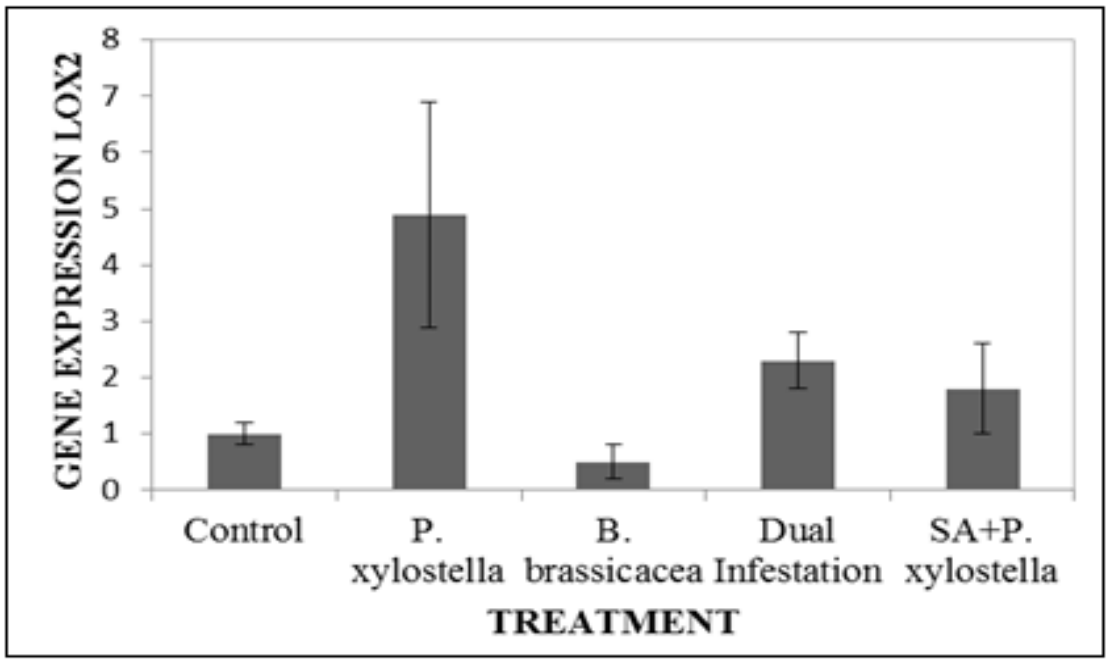

Fig. 4. Expression levels of *LOX2* genes in plant leaves 24 hours after infestation. Values are means ± error bars No significant difference in *PR1* expression was found between the different treatments (p =0.08; Fig. 5).

One such saliva secretion is glucose oxidase activity, present in oral secretions of lepidopteran herbivores and aphids, that has been reported to induce salicylic acid (SA, 2-hydroxy benzoic acid) signaling (Eichenseer *et al.*, 2010), leading to the suppression of JA-dependent defences.

In the SA-treated wild plants the exogenously applied SA is absorbed and antagonizes the JA biosynthetic pathway leading to reduced defense against the caterpillars. These results are consistent with our model where SA blocks the production of allene oxide synthase (AOS), which is a necessary enzyme in the production of jasmonic acid (JA) through the octadecanoid pathway (Fig. 1). With JA defense impaired, the plants are unable to produce the necessary defense compounds (such as polyphenol oxidase, peroxidase, lipoxygenase and proteinase inhibitors) and become susceptible to the herbivore (Stout *et al.*, 1998).

On the other hand, the lack of signal for SA production in wild type plants only infested with *P. xylostella*, and the natural inability to produce SA in the mutant plants (dual infested mutants and *P. xylostella* infested mutant plants) prohibits any SA inhibition of the JA defense responses. These plants are therefore capable of synthesizing JA as evident by higher expression levels of LOX2 gene in them (Fig. 4). With higher LOX2 expression, these plants are capable of producing defense proteins against, and deter the feeding of, the caterpillars and hence the lower caterpillar biomass (Fig 2). Our findings concur with earlier research works reporting the suppression of JA by exogenously applied SA. Doherty *et al.* (1988) noted suppressed JA-induced wounding response in tomato by application of exogenous SA and its acetylated form, aspirin (while Imanishi *et al.* (2000) also reported similar antagonistic relationship in Tobacco where exogenously applied SA suppressed the expression of JA marker genes.

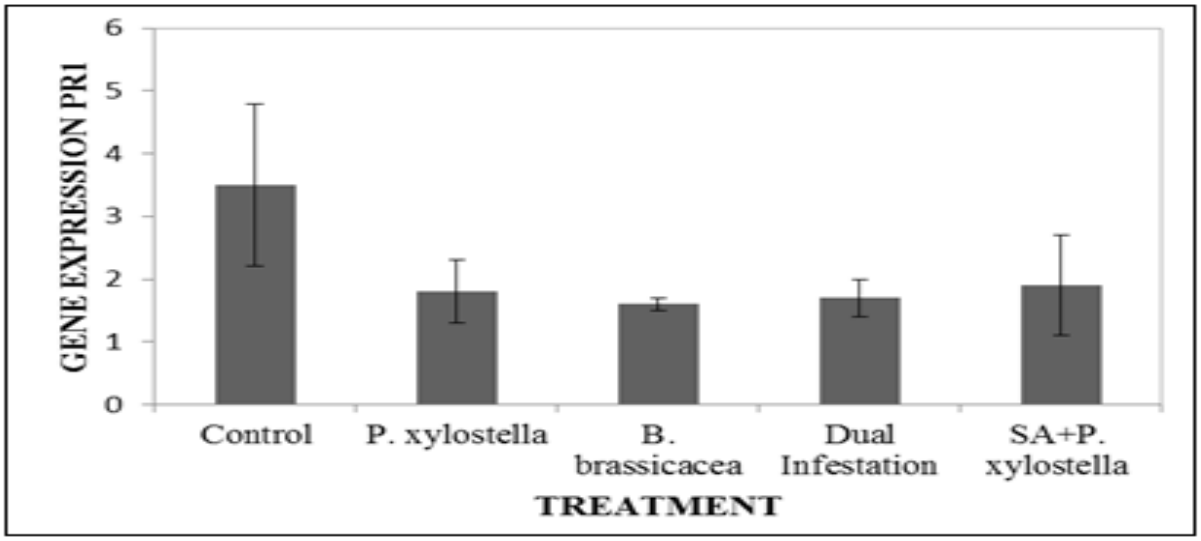

Fig. 5. Expression levels of *PR1* genes in plant leaves 24 hours after infestation. Values are means ± error bars.

Dual infested mutant plants had higher number of aphids than dual infested wild type plants (Fig 3) pointing to a better performance of the aphids in the SA mutant plants. Aphid infestation possibly activated the SA-mediated defense mechanism (Glazebrook, 2005; Howe and Jander, 2008). This may have been via glucose oxidase activity on the oral secretions of feeding aphids inducing the salicylic acid signaling (Eichenseer *et al*., 2010), thus activating SA defence response in our wild type plants leading to the suppression of aphid infestation and development in the wild type plants. However in the SA insufficient mutants plants, inability to produce SA inhibited SA mediated suppression of aphid feeding and development and hence the higher numbers of aphids. However, the analysis of the SA marker gene *PR1* did not show significant difference in expression levels between the two treatments (Fig. 5). Instead, trend pointed to a down regulation of the gene in all the other treatments in comparison to the control. This is contrary to our expectation of an up regulation of *PR1* gene in plants attacked by *B. brassicae*. This anomaly in *PR1* levels may be attributed to its natural degradation that depends on the levels of SA.

Fu and Dong (2013) in their review pointed out that the activity of *PRI* is a master transcription co-regulator of SA-dependent genes in the SA pathway. The abundance of *PRI* transcripts in an Arabidopsis under attack is regulated by SA-dependent modifications.

Recent findings have suggested that *PRI* has two homologues *PR3* and *PR4* which are SA receptors and participates in SA modifications (Fu *et al*., 2012; Wu *et al*., 2012). These homologues act as *CUL3* ligase adaptor proteins in proteasome mediated degradation of *PRI* and they differ in their ability to bind SA and capacity to degrade *PRI*. The SA level is therefore critical for the determination of degradation of *PRI*. When the SA level is critically low, the *PR4* binds with *PR1* leading to its degradation while on high SA levels, *PRI* is degraded through its reaction with *PR3*. This degradation at high and low levels prevents excessive or untimely activation of *PRI* and is thought to help in the activation of programmed cell death, of which *PRI* is a negative regulator. Intermediate levels of SA alleviates the interaction between *PR3* and *PR4* with *PR1* allowing the plants to activate SA-dependent defenses (Fu *et al*., 2012). The low level of *PRI* transcripts in our experiment may have been due to its degradation either at high or low level of SA. To unravel this hypothesis I would recommend the expression analysis of these genes alongside *PR1* to determine whether there was degradation at high levels or low levels.

Conclusion

Our findings suggest that the leaf chewer and phloem feeder interact not via competition as would be expected from interspecific herbivores but instead via facilitation; the aphids weakened JA-related plant

defenses, thus facilitating the growth and development of the caterpillars. Even though the interactions between JA and SA signaling pathways have been considered useful in allowing plants to fine-tune their defenses, frequent occurrences of facilitations in interspecific inter-guild interactions among herbivores, as we have reported herein, may on the contrary represent important constraints to plant defenses.

It is however necessary to be cautious of comprehensive generalizations when making conclusions about plant defense responses. There are plenty of variations in plants in response to herbivore defense. Our work was done in *Arabidopsis thaliana,* but we are aware that other plant species may control hormone signaling differently. The complexity and dynamism of feeding guild interactive responses has to be appreciated and plant species specific studies conducted. Lastly, we would recommend the analysis of other genes such as JA-responsive transcription factor, myrosinase and cycteine proteinase inhibitor associated with the JA. On SA we would recommend the inclusion of pathogenic related genes and phenylalanine ammonia-lyase to investigate how they interact upon insect attack.

References

Bari R, Jones J. 2009. Role of plant hormones in plant defence responses. Plant Molecular Biology **69(4),** 473-488.

Balbi V, Devoto A. 2008. "Jasmonate signalling network in Arabidopsis thaliana: crucial regulatory nodes and new physiological scenarios." NewPhytol **177,** 301–318.

Poelman H, Kamp A, Hemerik L, Prekatsakis G, Dicke M. 2012. "Plants under multiple herbivory: consequences for parasitoid search behaviour and foraging efficiency." Animal Behaviour **83(2),** 501-509.

Doherty M, Selvendran R, Bowels J. 1988. "The wound response of tomato plants can be inhibited by aspirin and related hydroxy- benzoic acids." Physiol MolPlant Pathol **33,** 377–384.

Eichenseer H, Mathews C, Powell S, Felton W. 2010. "Survey of a Salivary Effector in Caterpillars: Glucose Oxidase Variation and Correlation with Host Range." J Chem Ecol **36,** 885–897 [PubMed].

Fu Z, Yan Q, Saleh S, Wang A, Ruble W, Oka J. 2012. "NPR3 and NPR4 are receptors for the immune signal salicylic acid in plants." Nature **486,** 228–232. http://dx.doi.org/10.1038/nature11162

Fu Q, Dong X. 2013. "Systemic acquired resistance: Turning local infection into global defense." Annu. rev. plant biology **64,** 839-863.

Imanishi S, Nakakita M, Yamashita K, Furuta A, Utsuno K, Muramoto N. 2000. "Aspirin and salicylic acid do not inhibit methyl jasmonate-inducible expression of a gene for ornithine decarboxylase in tobacco BY-2 cells." Biosci Biotech Biochem **64,** 125–133.

Howe G, Jander G. 2008. "Plant immunity to insect herbivores." Annual Review of Plant Biology. **59,** 41-66.

Mithofer A, Boland W. 2012. "Plant Defense Against Herbivores: Chemical Aspects. Annual Review of Plant Biology, S.S. Merchant **63,** 431-450.

Creelman R, Mullet J. 1995. "Jasmonic acid distribution and action in plants: regulation during development and response to biotic and abiotic stress. Proc Natl Acad Sci USA **92,** 4114-19.

Loake G, Grant M. 2007. "Salicylic acid in plant defence – the players and protagonists." Curr Opin Plant Biol **10,** 466–472.

Mur J, Kenton P, Atzorn R, Miersch O, Wasternack C. 2006. "The outcomes of concentration-specific interactions between salicylate and jasmonate signaling include synergy, antagonism, and oxidative stress leading to cell death. Plant Physiology **140(1),** 249-262.

Pieterse J, Van der Does D, Zamioudis C, Leon-Reyes A, Van Wees M. 2012. "Hormonal Modulation of Plant Immunity." Annual Review of Cell and Developmental Biology, Vol 28. R. Schekman. Palo Alto, Annual Reviews. **28,** 489-521.

Pieterse J, Dicke M. 2007. Plant interactions with microbes and insects: from molecular mechanisms to ecology. Trends Plant Sci **12,** 564–56.

Pieterse J, Leon-Reyes A, Van Wees M. 2009. "Networking by small-molecule hormones in plant immunity. Nature Chemical Biology **5,** 308–316.

Pan Z, Camara B, Gardner W, Backhaus A. 1998. Aspirin inhibition and acetylation of the plant cytochrome P450, allene oxide synthase, resembles that of animal prostaglandin endoperoxide H synthase. J. Biol Chem **273**, 18139-45.

Raskin I. 1992. Salicylate a new plant hormone. Plant Physiol **99,** 799-803.

Soler R, Badenes-Pérez R, Broekgaarden C, Zheng S, David A, Boland W, Dicke M. 2012. "Plant-mediated facilitation between a leaf-feeding and a phloem-feeding insect in a brassicaceous plant: from insect performance to gene transcription. Functional Ecology **26(1),** 156-166.

Stout J, Workman V, Bostock M, Duffey S, 1998. Stimulation and attenuation of induced resistance by elicitors and inhibitors of chemical induction in tomato (*Lycopersicon esculentum*) foliage. Entomol Exp et Appl **86,** 267-79.

Wu Y, Zhang D, Chu JY, Boyle P, Wang Y, Brindle ID. 2012. The Arabidopsis NPR1 protein is a receptor for the plant defense hormone salicylic acid. Cell Rep. **1,** 639–647.
http://dx.doi.org/ 10.1016/j.celrep.2012.05.008

Zhang J, Zheng J, van Loon A, Boland W, David A, Mumm R, Dicke M. 2009. Whiteflies interfere with indirect plant defense against spider mites in Lima bean. Proceedings of the National Academy of Sciences of the USA, **106**, 21202–21207.