EFFECT OF COWPEA APHID ON THE BIOSYNTHETIC PATHWAY OF SALICYLIC ACID IN *Glycine max* cv. Namdan AT REPRODUCTIVE GROWTH STAGES

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Received on 12/3/2019, accepted for publication on 8/5/2019

Abstract: Cowpea aphid (*Aphis craccivora* Koch) infestation accumulated phytohormone salicylic acid (SA) in leaves of soybean (*Glycine max* cv. Namdan) as these soybean plants were at stages R1 (beginning to bloom) and R3 (beginning pod development). Activity of major enzymes involving biosynthesis of SA, such as phenylalanine ammonia-lyase (PAL), benzoic acid 2-hydroxylase (BA2H), was also enhanced under aphid effect. Changes in activity of these enzymes was closely correlated with the content of SA in the tissues. The enhancement of the SA biosynthetic pathway may reduce the effects of *A. craccivora* on *G. max* cv. Namdan plants at reproductive growth stages.

1. Introduction

Salicylic acid (SA) is a phytohormone functioned as an important signal molecule in plant defense mechanism. SA is mainly known to involve in plant systemic acquired resistance to pathogens. In a few studies on role of SA in the plant-aphid interaction, an accumulation of that phytohormone was recorded in barley [3], wheat [8], pea [6] and soybean [7] as resulted from aphid effects. The enhancement in SA content connects to the defense mechanisms of antibiosis or aphid repellence in resistant crops [10].

A recent study on the soybean-aphid interaction [12] presented that, cowpea aphid (*Aphis craccivora* Koch) is a serious pest of soybean (*Glycine max* (L.) Merr.) in the agricultural production in Nghe An Province and SA content was accumulated by infestation of cowpea aphid and triggered the inducible specific defensive reactions in leaves of *G. max* cv. Namdan at vegetative growth stages.

In the present study, we investigated the enhanced generation of SA in *G. max* cv. Namdan plants at reproductive growth stages under the effect of the different number of *A. craccivora* individuals. Besides, activity of main enzymes in SA biosynthesis such as phenylalanine ammonia-lyase (PAL) and benzoic acid 2-hydroxylase (BA2H) were assayed. Finally, changes in content of 4-hydroxybenzoic acid (4HBA)-another metabolic product from SA-precursor in the SA biosynthesis pathway were also examined.

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2. Materials and methods

2.1. Objectives and experiment

Soybean (*Glycine max* (L.) Merr. cv. Namdan) plants, whose seeds were supported by Nghe An Seed Center (Vietnam), were cultured in 15-cm-diameter plastic pots containing Hoagland medium.

Cowpea aphid (*Aphis craccivora* Koch) used in the infested experiment was supported by Department of Applied Entomology (Vietnam Academy of Science and Technology).

Each soybean plant at stage R1 (beginning bloom) was treated by 10, 20 or 30 wingless adults of *A. craccivora* that were carefully transferred to soybean leaves with a fine paintbrush. The control was soybean plants without treatment of aphid. Experiments were carried out in Plant Physiology Lab (Vinh University).

2.2. The analytical materials

Mature fresh leaves in control and aphid-infested plants were carefully collected at the R1, R3 (beginning pod development) and R5 (beginning of seed) stage. Leaves were weighed, immediately frozen in liquid nitrogen for subsequent analyses.

2.3. Methods of analysis

a. Measurement of salicylic acid

Content of salicylic acid (SA) was determined using HPLC based on method as described by Yalpani et al [13].

0.5g of frozen soybean leaves were ground in liquid nitrogen into a fine powder, then extracted twice with methanol 90% then centrifuged at 10,000×*g* for 15min at 4^{o}C. The selected supernatant was divided into two equal parts and then evaporated to dryness under a stream of nitrogen. Each part was extracted three times with the extractive mixture of ethyl acetate:cyclopentane:isopropanol (100:99:1, v/v/v).

After solvent evaporation, the dry residue of the extraction was dissolved in mobile phase (0.2M acetate buffer, pH 5.0 and 0.5mM EDTA) and analysed by a HPLC coupling with fluorometric detection. Chromatographic separation was on a Waters Spherisorb ODS2 column (3µm, 4.6×10mm). Fluorescence spectra were 295nm for excitation and 405nm for emission. Content of SA was expressed as nanograms per gram of fresh weight material (ng·g^{-1} FW).

b. Measurement of 4-hydroxybenzoic acid

Content of 4-hydroxybenzoic acid (4HBA) was measured by using the HPLC system [11]. 0.5g of frozen material were ground, extracted with methanol 90% and then centrifuged at 10,000×*g* for 15min at 4^{o}C. Supernatant was selected to use for analysis. 50µL of extract were injected onto a HPLC Column Agilent HC-C18 (5µm, 4.6 x 250 mm) with a flow rate of 1mL·min^{-1}. UV-absorbing compounds eluting from the column were monitored at 230nm and 254nm with a diode array detector. Content of 4HBA was expressed as nanograms per gram of fresh weight material (ng·g^{-1} FW).

c. Assay of phenylalanine ammonia-lyase

Phenylalanine ammonia-lyase (PAL, EC 4.3.1.24) activity was determined by using the spectrophotometric method [2].

0.5g of frozen leaves was homogenized at 4^{o}C in 4mL of 100mM Tris-HCl buffer (pH 8.9), 5mM β-mercaptoethanol and 0.050g PVP. The homogenate was centrifuged at 10,000×*g* for 25min at 4^{o}C to get the supernatant used for enzyme analysis. A volume of 1.5mL of the reaction mixtures contained extract, borate buffer 20mM, pH 8.9 and *L*-phenylalanine 10mM. Blank was sample without *L*-phenylalanine. After incubation at 30^{o}C for 24h, the substrate was added and the reaction was stopped with 1.5mL of 2N HCl. Activity of PAL was measured by the change of absorbance at 290nm using the UV-Vis CARY 60 spectrophotometer (Agilent, USA), and expressed as micro mole *trans*-cinnamic acid per milligram protein per hour (μmol *trans*-cinnamic acid·mg^{-1}protein·h^{-1}).

d. Assay of benzoic acid 2-hydroxylase

Benzoic acid 2-hydroxylase (BA2H) was measured by using HPLC based on method as described by León et al (1995) [5].

0.5 g of frozen leaves was ground and suspended in 2mL of extractive buffer [20mM HEPES, pH 7.0, 12.5mM 2-mercaptoethanol, 10mM sorbitol, 1% PVP, and 0.1mM PMSF]. The suspension was vortexed and then centrifuged at 10,000×*g* for 20min at 4^{o}C. The supernatant was used for enzyme assays. In a final volume of 0.5 mL the reaction mixture contained of 20mM of HEPES buffer (pH 7.0), 1μM of BA, 1μM of NAD(P)H, extractive buffer and enzyme extract in equal volume. The BA2H reaction mixture was incubated in the water bath for 30min at 30^{o}C. After centrifuging at 10,000×*g* for 15min, the supernatant was partitioned triple with 0.50mL of the extractive mixture of ethyl acetate:cyclopentane:isopropanol (100:99:1, v/v/v). The extract was transferred to a 2mL glass and evaporated to dryness under a stream of nitrogen. The sample was dissolved in 250mL mobile phase, thoroughly mixed, then transferred to Eppendorf tubes and centrifuged at 15,000×*g* for 2min. A volume of 200mL of extract was collected and placed in a glass vial fitted with a rubberized Teflon-sept and the contribution of PP and placed in the chamber of auto sampler. Activity of BA2H was expressed as nanograms of SA obtained as a reaction product extracted from one milligram of protein during one hour (ng SA.h^{-1}·mg^{-1} protein).

In all the enzyme analyses, protein content was determined following the method of Bradford (1976) using bovine serum albumin (Sigma-Aldrich) as a standard [1].

e. Statistical analysis

All analyses were performed repeatly in three independent experiments. Analysis of variance (ANOVA) was applied to verify whether means from independent experiments were significantly different at level of α=0.05. Data shown in figures are means and standard errors of triplicates for each treatment.

3. Results

3.1. Accumulation of salicylic acid in leaves of *Glycine max* cv. Namdan under effect of *Aphis craccivora*

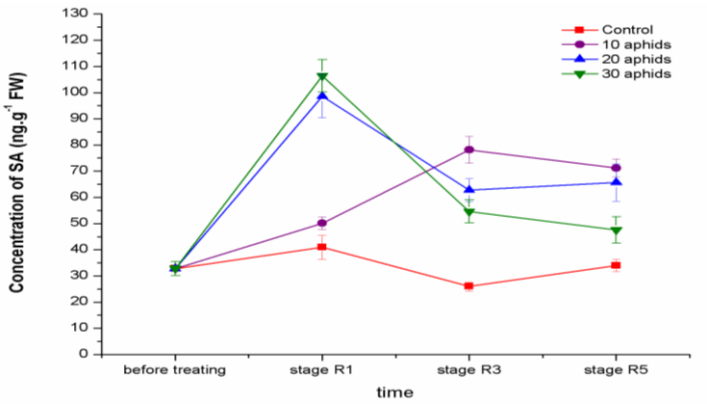

Fig. 1: *Effect of Aphis craccivora on SA content in leaves of Glycine max cv. Namdan. Values are means ± SE of three independent experiments*

Under the effect of 20 and 30 aphid individuals SA was remarkably accumulated at the R1 stage and then strongly reduced at R3 and R5, whereas this phytohormone in 10-aphid infested plants was lately induced to peak at R3 and maintained high level till R5 (Fig. 1). The highest content of SA obtained as 106.39ng·g^{-1} FW in 30-aphids infested leaves at R1, by 3.24-fold higher than that in the beginning of experiment (32.82ng·g^{-1} FW) and 2.61-fold higher than in the control plants (40.92ng·g^{-1} FW). The relation between the accumulated content of SA and aphid density was only recorded at R1.

Content of SA in the control plants maintained consistently at lower levels. The significant differences between concentration of SA in aphid-infested leaves and control were recorded in all points of investigated time.

3.2. Change in activity of SA biosynthetic enzymes in leaves of *Glycine max* cv. Namdan under effect of *Aphis craccivora*

a. Activity of PAL

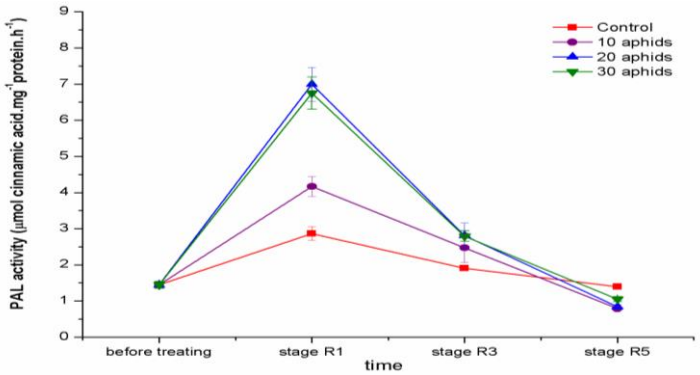

Fig. 2. *Effect of Aphis craccivora on PAL activity in leaves of Glycine max cv. Namdan. Values are means ± SE of three independent experiments*

There was a higher level of PAL activity in *G. max* cv. Namdan leaves infested by *A. craccivora* than that in the control at the R1 and R3 stages (Fig. 2). Activity of PAL in aphid-infested leaves strongly increased and reached to maximum level at stage R1, at which enzyme in 20- and 30-aphid infested leaves exposed to be greatly enhanced. The highest values (6.99µmol *trans*-cinnamic acid·mg^{-1}protein·h^{-1}) was recorded in 20-aphid infested leaves, by 2.43-fold and 4.85-fold higher than that in the control and before aphid treating, respectively. After reaching to peak, activity of PAL strongly reduced between R3 and R5 stages. In the control, this enzyme remained lower activity and little changed throughout the experiment.

b. Activity of BA2H

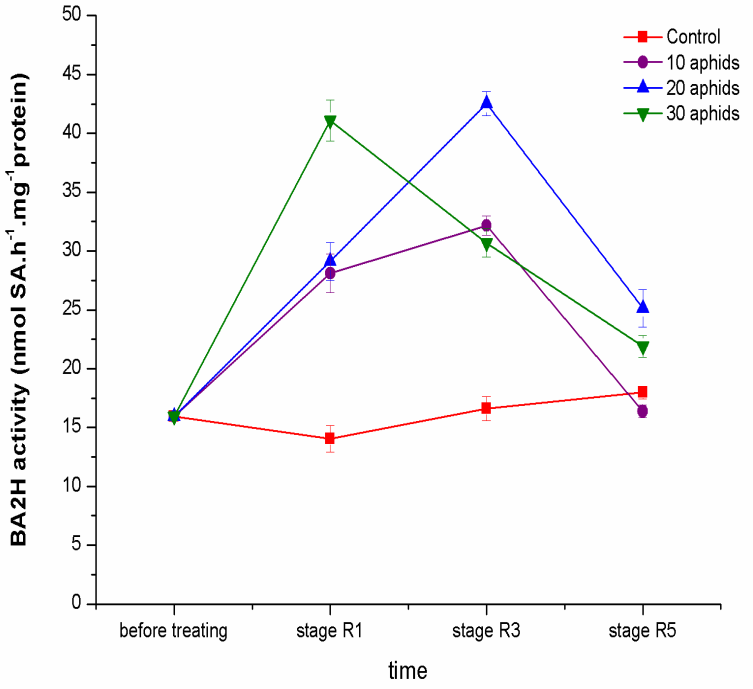

Fig. 3: *Effect of Aphis craccivora on BA2H activity in leaves of Glycine max cv. Namdan. Values are means ± SE of three independent experiments*

The BA2H activity in leaves of soybean Namdan differently accumulated under the effect of cowpea aphid (Fig. 3). 30-aphid infestation firstly induced BA2H activity that obtained the highest value (41.08ng SA·h^{-1}·mg^{-1} protein) at R1; then an enhancement of BA2H obtained in 10- and 20-aphid infested variants at the R3 stage. After reaching to peak, activity of BA2H in all aphid-infested leaves strongly decreased till to R5. Contrary, BA2H activity in control plant was minor changed in lower levels. The significant difference in activity of this enzyme between aphid-infested plants and control was recorded between R1 and R3 stages.

3.3. Change in content of 4-hydroxylase benzoic acid in leaves of *Glycine max* cv. Namdan under infestation of *Aphis craccivora*

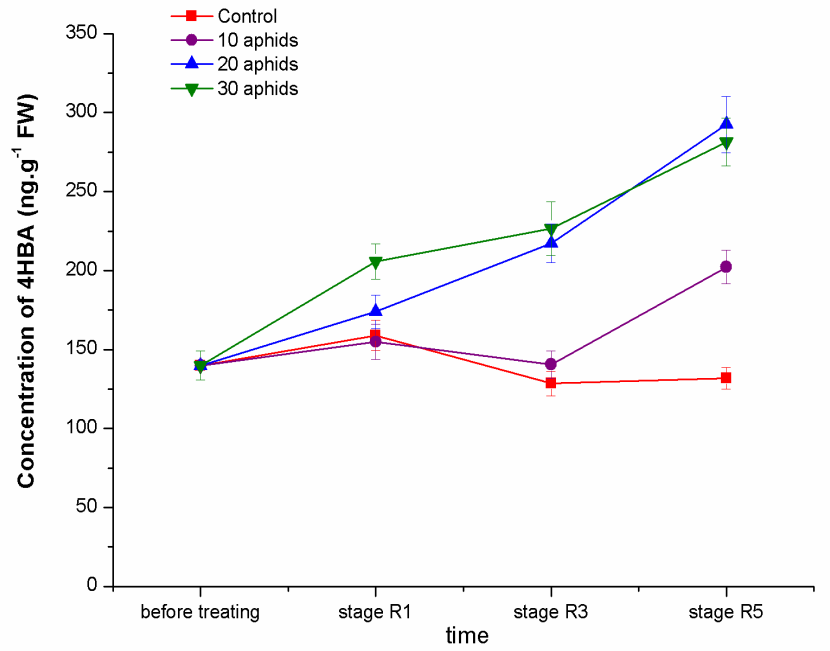

Fig. 4: *Effect of Aphis craccivora on 4HBA content in leaves of Glycine max cv. Namdan. Values are means ± SE of three independent experiments*

In leaves of soybean infested by cowpea aphid, content of 4HBA tended to increase continuously from the R1 to R5 stage and was mostly higher than that in the control plants (Fig. 4). The strongest increase was recorded in the 20- and 30-aphid infested soybean plants. The highest content of 4HBA was 292.51ng·g^{-1} FW, which was 2.09-fold higher than that as compared to what observed at beginning of the experiment (139.81ng·g^{-1} FW) and 2.21-fold higher than in the control plants (131.79ng·g^{-1} FW). Accumulation of 4HBA was proportional with infestation's intensity from different numbers of cowpea aphid at the R1 and R3 stages.

4. Discussion

In the most common pathway of SA synthesis in plants, the precursor -amino acid- phenylalanine, was converted to *trans*-cinnamic acid by PAL activity. Benzoic acid is synthesized by *trans*-cinnamic acid either via β-oxidation of fatty acids or a non-oxidative pathway. The hydroxylation of benzoic acid is catalyzed by enzyme BA2H to form SA. In the second branch of that pathway, cinnamate 4-hydroxylase (CA4H) catalyzes the conversion of *trans*-cinnamic acid to coumaric acid and then lead to forming 4-hydroxybenzoic acid (4HBA) (Fig. 5). Therefore, it was possible to be a contrary alteration in content of SA and 4HBA, of which the strong conversion of *trans*-cinnamic acid to SA resulted a low level of 4HBA and vice versa.

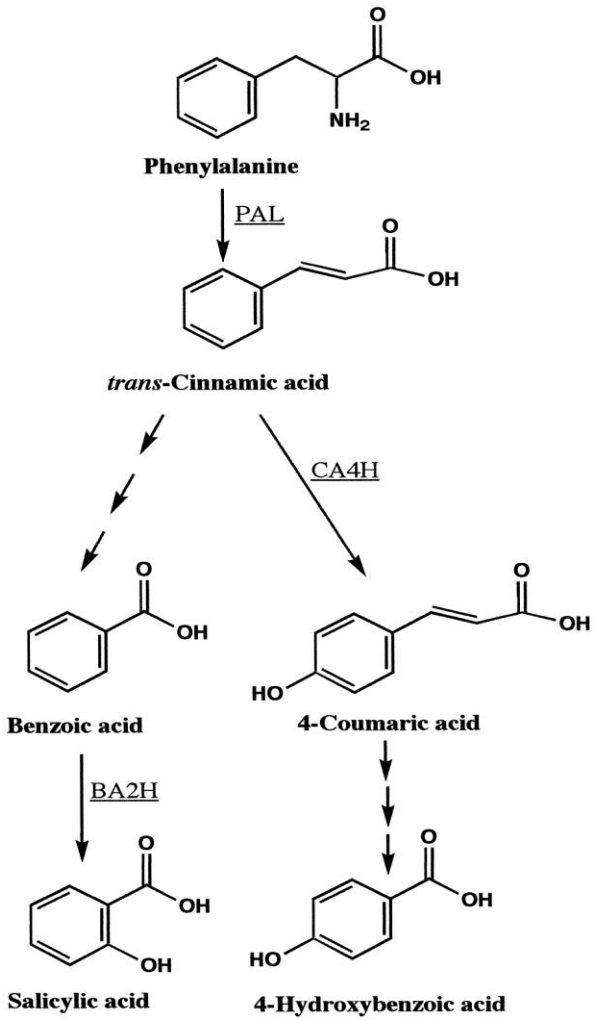

Fig. 5: *The phenylalanine pathway* [11]

Our study recorded that generation of SA was strongly decreased (Fig. 1) whereas 4HBA content tended to increase continuously from the R1 to R5 stage (Fig. 4). It is possible that *trans*-cinnamic acid mainly converted to SA in one branch of pathway at R1; whereas, at R3 and R5, metabolism of this unsaturated carboxylic acid was strongly turned to 4HBA in another branch.

As an important signal molecule in plant defense mechanism, SA has been known to accumulate strongly in response to aphids in resistant cultivars of legumes [6], [8]. Our previous study confirmed that, soybean Namdan was a resistant cultivar to cowpea aphid [12]. The accumulated content of SA is often associated with a build-up of reactive oxygen species that cause significant changes in cellular redox levels. These redox changes are sensed by the defense genes such as *PR* and *NPR1*, which directly involved in systemic acquired resistance, could influence plant defense against herbivores [9]. Some SA-defense-related genes in plants, e.g., *BGL*, *PR5*, *PR10*, *ICS1*, *ICS2*, have been induced by aphid feeding [4], [9]. Those evidences supplied an important link between SA-signaling and some different defense mechanisms in plants. Because infestation of

aphids early invoked SA-defensive genes, the strong accumulation of SA may be a critical step in the signaling of downstream responses of plants to aphids.

PAL, the first enzyme in the SA biosynthesis pathway, was strongly induced in *G. max* leaves after *A. craccivora* infestation (Fig. 2). A proportional relation between PAL activity and SA content recorded in the aphid-infested leaves of *G. max* cv. Namdan indicated that PAL induction is closely associated with SA accumulation in the SA-related signaling pathway induced upon aphid infestation. Similar results were previously found in other crops following aphid attack [4], [9], [14]. An increase in PAL activity was strictly connected to biosynthesis of SA, flavonoids and other antioxidants. Those compounds with deterrent, toxic and anti-nutritional properties were among the aphid repellents that may prevent aphids from infestation or settling on host plants.

Enzyme BA2H, whose major biochemical function is catalyzing the conversion of benzoic acid to SA, has related to the plant SA signaling. Early studies mainly reported function of BA2H in plants defense against pathogens [5], [13]. Limited information has mentioned an involvement of BA2H in plant response to herbivores. Previous studies presented that an accumulation of BA2H in *Pisum sativum* seedlings after pea aphid infestation [6] or in defense response of G. *max* cv. Namdan to cowpea aphid at vegetative growth stages [12]. Similarly, our study confirmed that *A. craccivora* resulted in an enhancement of BA2H activity in soybean Namdan at reproductive growth stages (Fig. 3). Furthermore, the inducible change in activity of BA2H was closely correlated with the alteration of the SA level (Fig. 1). Therefore, we suggested that enzyme BA2H probably participated in the SA-related signaling defense mechanism of soybean Namdan.

5. Conclusion

The SA-related signaling pathway is involved in the defense response of *G. max* cv. Namdan to *A. craccivora* at reproductive growth stages. Under effect of cowpea aphid, phytohormone SA in soybean Namdan leaves was accumulated to a high level since the R1 stage. Major enzymes in the SA biosynthesis such as PAL and BA2H were also elicited by the aphid; the change in activity of these enzymes was closely correlated with the induced content of SA. The accumulation of the SA signaling pathway may contribute to protect soybean Namdan plants from damage caused by cowpea aphid infestation.

Acknowledgements

The authors would like to thank Prof. I. Morkunas (Poznan University of Life Sciences) and her reseach group for the possibility to conduct analyses under HPLC system.

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TÓM TẮT

TÁC ĐỘNG CỦA RỆP HẠI ĐẾN SINH TỔNG HỢP AXIT SALICYLIC TRONG CÂY ĐẬU TƯƠNG NAM ĐÀN Ở GIAI ĐOẠN RA HOA KẾT QUẢ

Tác động của rệp muội đen (*Aphis craccivora* Koch) đã cảm ứng quá trình sinh tổng hợp axit salicylic (SA) trong lá cây đậu tương Nam Đàn (*Glycine max* cv. Namdan) khi cây bắt đầu ra hoa (R1) đến giai đoạn hình thành quả (R3). Hàm lượng hooc-môn thực vật này trong lá bị rệp phá hại luôn cao hơn so với trong cây không có rệp hại. Các enzym quan trọng trong quá trình sinh tổng hợp SA là phenylalanine ammonia-lyase (PAL), benzoic acid 2-hydroxylase (BA2H) cũng được cảm ứng tăng độ hoạt động; Sự biến đổi hoạt độ các enzym này tỷ lệ thuận với hàm lượng SA trong các công thức thí nghiệm. Sự tăng cường quá trình sinh tổng hợp SA là một trong những phản ứng tự bảo vệ của cây đậu tương Nam Đàn đối với tác động xấu của rệp muội đen trong giai đoạn ra hoa kết quả.