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CHANGE IN CONTENT OF GLYCOSYLATED FLAVONOIDS IN SOYBEAN (*GLYCINE MAX* CV. NAMDAN) LEAVES UNDER APHID INFESTATION

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ABSTRACT

Changes in contents of glycosylated flavonoids in leaves of soybean (*Glycine max* (L.) Merr. cv. Namdan) in the flowering stage under infestation of cowpea aphid (*Aphis craccivora* Koch) were investigated. A strong decrease in the level of glycosylated flavonoids, e.g., genistein 7-O-glucoside, 2′OH genistein 7-O-glucoside, 2′OH genistein malonyl glucoside, after 48-96 hours post-infestation (hpi) of aphid was recorded. Levels of these compounds in aphid-infested leaves were remarkably lower than those in controls. Whereas, genistein and total flavonoids in the aphid-infested leaves were induced to higher contents than that observed in control. Activity of important enzymes regarding biosynthesis of flavonoids such as phenylalanine ammonia-lyase (PAL, EC. 4.3.1.5), chalcone synthase (CHS, EC 2.3.1.74) and chalcone isomerase (CHI, EC 5.5.1.6) in the aphid-infested leaves strongly increased during experimental time. An increase in activity of those enzymes was proportional with the induced decrease of the glycosylated flavonoids and an accumulated increase of genistein and total flavonoids. These results indicate that, changes in the glycosylated flavonoids occurred in Namdan soybean leaves infested by cowpea aphid may connect to a protective mechanism in this soybean cultivar in the flowering stage.

Keywords: Namdan soybean, cowpea aphid, glycosylated flavonoids, enzymes.

1. INTRODUCTION

Flavonoids have a variety of biological activities in plants, of which, the defensive functions to protect plants from different biotic and abiotic stresses were surely recorded [1]. The glycosylation of those antioxidants forming the glycosylated flavonoids impact several specific biochemical and physiological properties, which allows them to participate in plant defensive responses. The glycosylated flavonoids have been known to enable the plant defense mechanisms to better cope with biotic and abiotic stresses [2].

In our previous report [3], an accumulation of flavonoids was documented to participate in the defense responses of soybean (*Glycine max* (L.) Merr. cv. Namdan) to infestation of cowpea

aphid - *Aphis craccivora* Koch (Hemiptera: Aphididae). However, to date there has been lack of the available information of the insecticidal properties towards phloem-feeding insects of the glycosylated forms of flavonoids in this cultivar.

The aim of the present study was to examine whether genistein, a major component in soybean plants [4], and its glycosylation products such as genistein 7-O-glucoside, 2′OH genistein 7-O-glucoside and 2′OH genistein malonyl glucoside may be connected with responses of the Namdan soybean to effects of *A. craccivora* in the flowering stage. Simultaneously, post-infestion flavonoid contents were determined together with activity of enzymes regarding flavonoid metabolism such as phenylalanine ammonia-lyase (PAL), chalcone synthase (CHS) and chalcone isomerase (CHI).

2. MATERIALS AND METHODS

2.1. Materials

2.1.1. Plant

Plant used in this study is cultivar Namdan soybean (*Glycine max* (L.) Merr. cv. Namdan) that was cultured in Hoagland medium and placed in Plant physiology lab, Vinh University. The experimental conditions were such that: temperature of 23-25 ^{o}C, relative humidity of 70-75 %, light intensity of 110-130 µM photons.m^{-2}.s^{-1} and light period of 14 light/10 dark hours. Soybean plants in the R3 stage (flowering stage) were used to establish experiments.

2.1.2. Aphid

Aphid species used in experiments is cowpea aphid (*Aphis craccivora* Koch), which is supported by Department of Applied Entomology (Institute of Ecology and Biological Resources, Vietnam Academy of Science and Technology).

2.2. Experiment

Each soybean plant in stage R3 (the flowering stage) was treated by 30 wingless adults of *A.craccivora*. This number of cowpea aphid was observed to cause the most serious damages on soybean plants [3]. Control was soybean plants without aphid infestation.

Control and aphid-infested variants were separately put in clear glass boxes (100 cm × 50 cm × 50 cm) covered by nylon gauze and placed in the growth chamber, in which, the cultured environmental factors such as temperature, relative humidity, light intensity and light period were strictly controlled.

Leaves of soybean were carefully collected after 0, 24, 48, 72 and 96 hours post-infestation (hpi) of cowpea aphid, then were weighed and frozen in nitrogen liquid for subsequent analyses.

2.3. Methods of analysis

2.3.1. Chemicals

All analytical chemicals were purchased from Singapore supplier of Sigma-Aldrich (USA).

2.3.2. Analysis of glycosylated flavonoids

Prior to LC profiling of glycosylated flavonoids, the frozen soybean leaves (0.5 g) were homogenized in 80 % methanol (20 mL g^{-1}FW) and sonicated for 3 min using a Bransonic 5510 bath sonicator. The suspension was filtered through a Büchner funnel and concentrated under vacuum at 40 ^{o}C. The samples were concentrated by solid phase extraction (SPE) on cartridges containing a cation exchanger and RP C18 silica gel (Alltech, Carnforth, England) [5].

Quantitative analyses were performed using an L7000 Merck Hitachi HPLC pump, equipped with an L7450 diode array detector (Darmstadt, Germany) and a Superspher 100 RP-18 column (250 mm × 2 mm; Merck). The amount of 125 mM p-hydroxybenzoic acid was added to each analysed sample as an internal standard.

Content of genistein, genistein 7-O-glucoside, 2′OH genistein 7-O-glucoside, and 2′OH genistein malonyl glucoside content was quantified by integrating UV chromatograms normalized at 259 nm to the peak of the standard. Their concentrations were expressed as miligram per gram fresh weight (mg.g^{-1} fw) [6].

2.3.3. Analysis of total flavonoids

Content of total flavonoids in soybean leaves extract was determined by the aluminium chloride colorimetric method [7]. Total of 0.50 g frozen soybean leaves was extracted with 10 mL of 99.5 % ethanol under 200 rpm shaking for 24 hours. The filtrate was adjusted to 10 mL with 80 % ethanol and centrifuged at 10,000 × *g* for 10 min at 4 ^{o}C, the supernatant was collected and the precipitate was then extracted twice with 5 mL of 80 % ethanol.

A mixture of 200 µL extract and 150 µL of sodium nitrite ($NaNO_2$ 5 %, w/v), was incubated at room temperature for 6 min. Next, 150 µL of aluminium chloride hexahydrate ($AlCl_3.6H_2O$ 10 %, w/v) was added and incubated for 6 min, then 1.000 µL NaOH (10 %, w/v) solution was added. For blank, distilled water was used to replace the extract. The absorbance was measured at λ = 510 nm in the UV-Vis CARY 60 spectrophotometer (Agilent, USA).

2.3.4. Enzymatic assays

Activity of phenylalanine ammonia-lyase (PAL, EC 4.3.1.5) was determined using the spectrophotometric method [8]. 0.50 g frozen soybean leaves was ground and extracted at 4 ^{o}C using 5 mL of 100 mM Tris-HCl buffer, pH 8.9, which contain 10 mM mercaptoethanol and 30 mg Polyclar AT. Mixture was centrifuged at 15,000 × g for 30 min at 4 ^{o}C. A volume of 1.5 mL of incubation mixture contained 80 mM borate buffer (pH 8.9), 30 mM L-phenylalanine and 0.5 mL enzymatic extract. The reaction was proceeded for 1 h at 30 ^{o}C and was halted by the addition of an equal volume of 2N HCl. The product of the reaction was assessed at 290 nm using an Agilent Cary 60 UV-Vis spectrophotometer.

Chalcone synthase (CHS, EC 2.3.1.74) was determined using the spectrophotometric method [9]. 0.5 g of soybean leaves was extracted in 5 mL of 100 mM borate buffer (pH 8.8) added 1 mM 2-mercaptoethanol at 4 °C. Then, the solution was added 0.1 g Dowex resin and centrifuged at 15,000 × g for 10 min. Supernatant was transferred to a tube, added 0.2 g Dowex resin, and incubated for 20 min. Mixture was centrifuged at 15,000 × g for 15 min. A volume of 2 mL mixture including 100 µL supernatant, 10 mM potassium cyanide, Tris-HCI buffer (pH 7.8), 10 mg chalcone was mixed with enzymatic extract. The absorbance was measured at 370 nm in Agilent Cary 60 UV-Vis spectrophotometer.

Activity of chalcone isomerase (CHI, EC 5.5.1.6) was assayed by spectrophotometry based on the isomerization of naringenin chalcone and isoliquiritigenin at 390 nm [10]. The reaction was carried out at 25 ^{o}C using 50 μM substrate in DMSO, 50 mM phosphate buffer (pH 7.6) containing 1 mg bovine serum albumin, and an appropriate amount of enzymatic extract. The reaction was initiated by adding the enzyme and control reactions were carried out without enzyme. The rate of spontaneous reaction was subtracted from the total rate.

2.3.5. Statistical analysis

All analyses were performed in at least three replicates in three independent experiments. Analysis of variance (ANOVA) was applied to verify whether means from independent experiments within a given experimental variant were significant with level of significance $\alpha = 0.05$. Data shown in the figures are means and standard errors (s.e.) for each variant.

3. RESULTS AND DISCUSSION

3.1. Content of glycosylated flavonoids in leaves of Namdan soybean

Among various glycosylated flavonoids, genistein and its derivatives such as genistein 7-O-glucoside, 2′OH genistein 7-O-glucoside and 2′OH genistein malonyl glucoside are the important endogenous compounds because they may enhance bioactivity in plant responses to biotic and abiotic stresses [1, 2, 6]. Their chemical structures are shown in Figure 1.

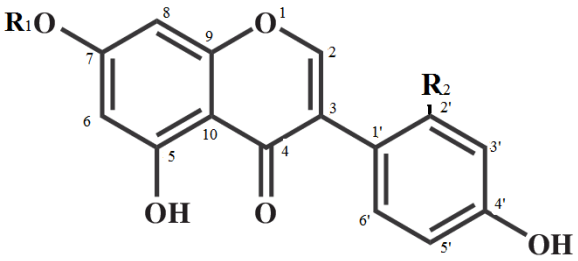

Figure 1. The structral scheme of genistein and its derivatives. Genistein ($R_1 = R_2 = H$); Genistein 7-O-glucoside (R_1 = glucose (glc); $R_2 = H$); 2′OH Genistein 7-O-glucoside (R_1 = glc; R_2 = OH); 2′OH Genistein malonyl glucoside (R_1 = (6″-O-malonyl)glc; R_2 = OH).

The change in content of those compounds in leaves of Namdan soybean under the different enviromental conditions are shown in Figure 2.

After 24 hours post-infestation (hpi) of cowpea aphid, a drop in content of glycosylated isoflavonoids (genistein 7-O-glucoside, 2′OH genistein 7-O-glucoside, 2′OH genistein malonyl glucoside) was particularly pronounced since 24 hpi (Fig. 2a-c). ANOVA showed that differences in the level of glycosylated flavonoids between aphid-infested leaves were highly statistically significant. In the control leaves, a different trend was observed. The levels of glycosylated flavonoids changed a little and always higher, whereas, genistein mostly was lower than that in the infested variant during experimental time.

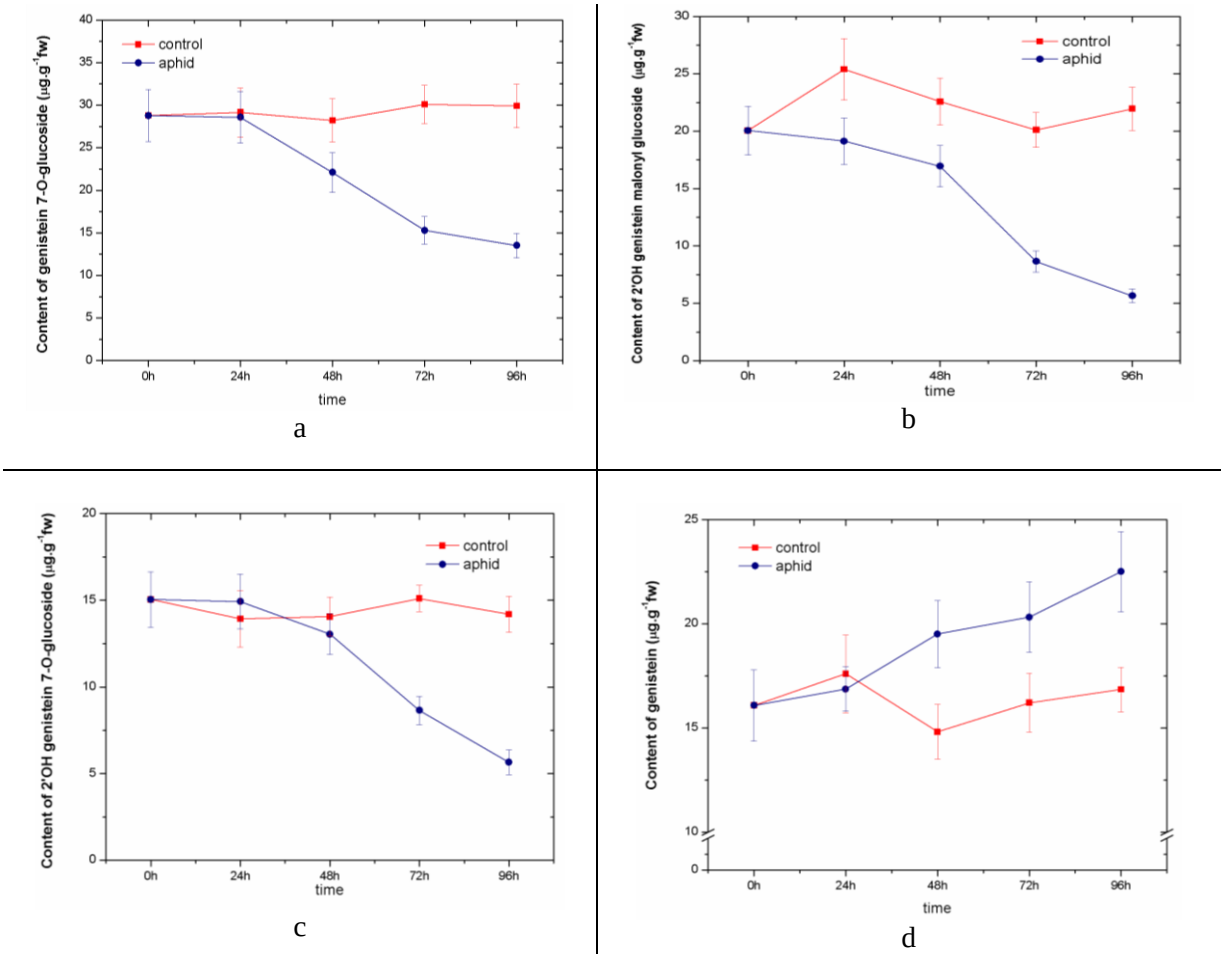

Figure 2. Content of glycosylated flavonoids (a-c) and genistein (d) in leaves of Namdan soybean infested by *A. craccivora* and control.

Interestingly, a significant decrease in contents of glycosylated flavonoids such as genistein 7-O-glucoside, 2′OH genistein 7-O-glucoside, 2′OH genistein malonyl glucoside, in the aphid infested leaves was probably a consequence of hydrolysis of the glycoside bonds in these molecules, leading to the release of genistein, a biologically active aglycone (Fig. 2d). Level of genistein in aphid infested leaves increased since 48 hpi as the glucosides strongly reduced.

Infestation of cowpea aphid can trigger oxidative burst in cells/tissues and cause serious damages to soybean plants [12]. Genistein of legume plants exhibits a wide range of biological activity, of which it play a significant role of antioxidant property, and thus counter the effects of oxidative stress. Therefore, metabolism of glycosylated flavonoids to form genistein may be connected with an enhancement the defense responses of *G. max* cv. Namdan to effects of *A. craccivora*.

3.2. Content of total flavonoids in leaves of Namdan soybean

In aphid-infested soybean leaves, level of total flavonoids were continuously increased and were always higher than that in the control during experiment (Fig. 3). The highest content of

flavonoids was 118.02 µg QE.g^{-1} dw at 96 hpi, which was by 2.08- and 1.76-fold higher than at the beginning of experiment and in control plants, respectively. Whereas, content of flavonoids in control was minor changed during experimental time. The significant differences between flavonoids levels in the aphid-infested leaves and control were recorded from 72-96 hpi ($P < 0.05$). That result provided additional evidence about the defensive function of flavonoids in the defense mechanism of Namdan soybean against *A. craccivora* [3].

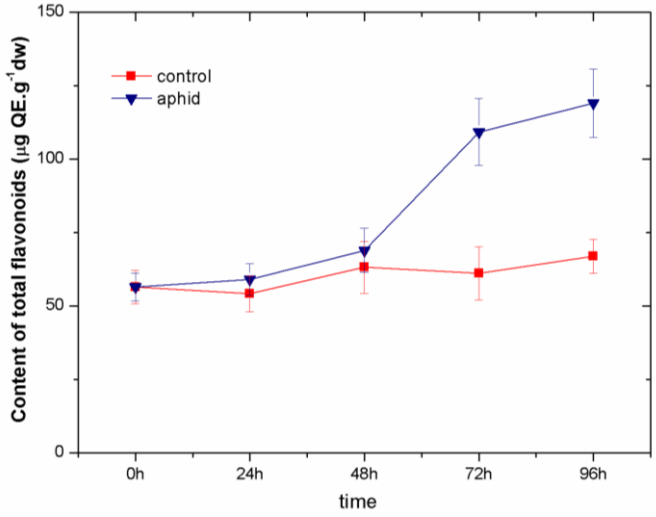

Figure 3. Content of total flavonoids in leaves of Namdan soybean infested by *A. craccivora* and control.

3.3. Activity of flavonoid-metabolic enzymes in leaves of Namdan soybean

During the experimental time, activity of phenylalanine ammonia-lyase (PAL, EC. 4.3.1.5), chalcone synthase (CHS, EC 2.3.1.74), and chalcone isomerase (CHI, EC 5.5.1.6) was observed to result in a strong increase in aphid-infested leaves of Namdan soybean, whereas, activity of those enzymes slightly changed in control plants (Fig. 4a). The highest activity of all enzymes was recorded at 96 hpi, which was from 2.08 (CHI)- and 2.51-fold (PAL) higher than that observed in control, respectively.

The biosynthesis pathway of flavonoids begins at precursors as phenylalanine catalyzed by PAL to form p-coumaroyl-CoA, which to yield chalcone (4',2',4',6'-tetrahydroxychalcone) catalyzed by CHS. The next step is isomerization of chalcone to flavanone by CHI. From this step onwards, the pathway branches to several different flavonoid classes including genistein and its glycosylate substances. An increase in activity of PAL, CHS, and CHI resulted in a strong increase in content of genistein (Fig. 2d) and total flavonoids (Fig. 3) in soybean leaves after aphid infestation, therefore, contributing to improve the defensive functions of those active substances in defense responses of *G. max* cv. Namdan to *A. craccivora*.

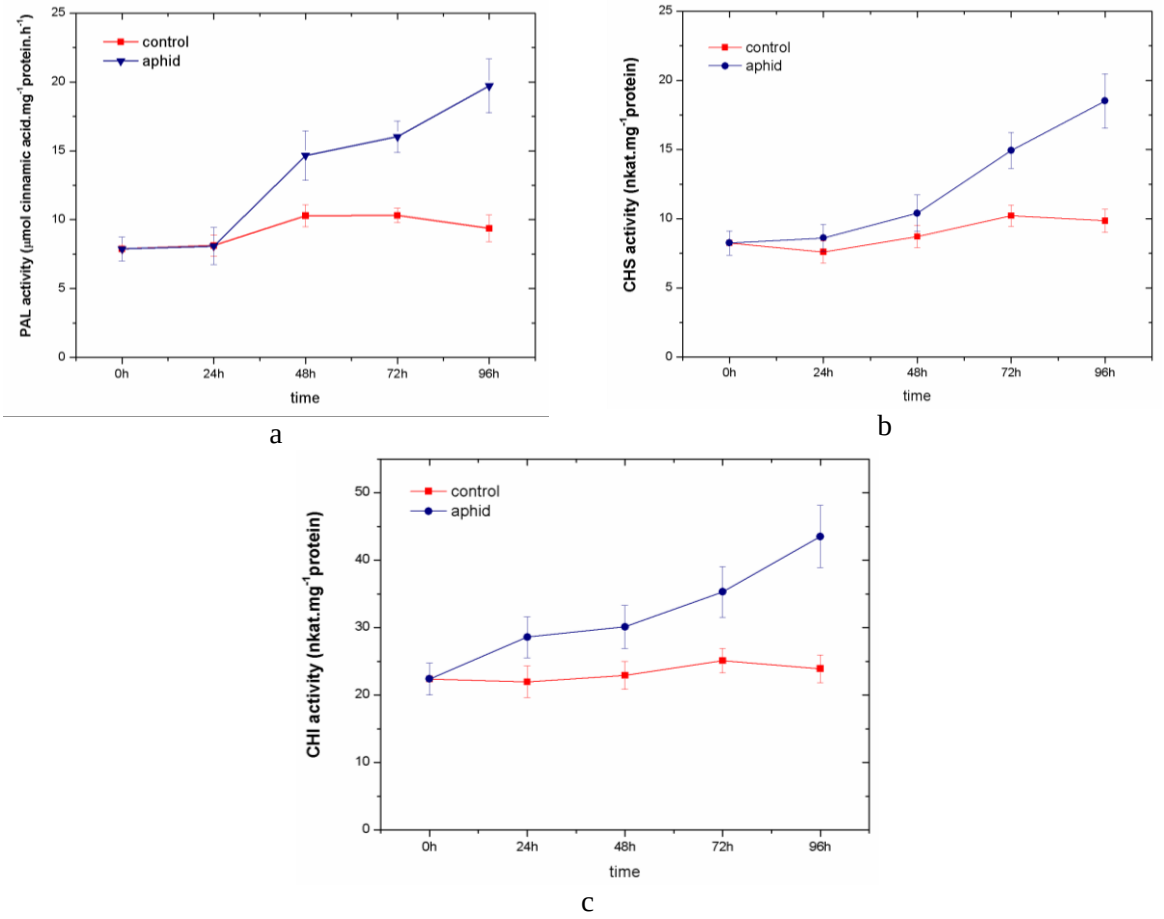

Figure 4. Activity of flavonoid-metabolic enzymes PAL (a), CHS (b) and CHI (c) in leaves of Namdan soybean infested by *A. craccivora* and control.

4. CONCLUSION

Infestation of *A. craccivora* induced the defense responses regarding flavonoids metabolism in leaves of soybean (*Glycine max* cv. Namdan) in the flowering stage. An increase in activity of important enzymes regarding biosynthesis of flavonoids such as PAL, CHS, and CHI was strongly connected with a strong decrease in the level of glycosylated flavonoids, e.g., genistein 7-O-glucoside, 2'OH genistein 7-O-glucoside, 2'OH genistein malonyl glucoside, and an accumulation of genistein and total flavonoids. Those changes in glycosylated flavonoids occurred in leaves infested by cowpea aphid were probably connected to the protection mechanism in Namdan soybean.

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