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Metabolic profiling reveals an increase in stress-related metabolites in *Arabidopsis thaliana* exposed to honeybees

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Abstract Insects affect crop harvest yield and quality, making plant response mechanisms to insect herbivores a heavily studied topic. However, analysis of plant responses to honeybees is rare. In this study, comprehensive metabolic profiling of *Arabidopsis thaliana* exposed to honeybees was performed to investigate which metabolites were changed by the insect. A total of 85 metabolites—including chlorophylls, carotenoids, glucosinolates, policosanols, tocopherols, phytosterols, β-amyrin, amino acids, organic acids, sugars, and starch—were identified using high performance liquid chromatography, gas chromatography-mass spectrometry, and gas chromatography-time-of-flight mass spectrometry. The metabolite profiling analysis of *Arabidopsis* exposed to honeybees showed higher levels of stress-related metabolites. The levels of glucosinolates (glucoraphanin, 4-methoxyglucobrassicin), policosanols (eicosanol, docosanol, tricosanol, tetracosanol), tocopherols (β-tocopherol, γ-tocopherol), putrescine, lysine, and sugars (arabinose, fructose, glucose, mannitol, mannose, raffinose) in *Arabidopsis* exposed to honeybees were higher than those in unexposed *Arabidopsis*. Glucosinolates act as defensive compounds against herbivores; policosanols are components of plant waxes; tocopherols act as an antioxidant; and putrescine, lysine, and sugars contribute to stress regulation. Our results suggest that *Arabidopsis* perceives honeybees as a stress and changes its metabolites to overcome the stress. This is the first step to determining how *Arabidopsis* reacts to exposure to honeybees.

Keywords Environmental resistance · Herbivore · Honeybee · Metabolic profiling

Introduction

Insect herbivores and pollinators affect the growth and development of plants by changing primary and secondary metabolism in plants, which affects the yield and quality of harvest products [1,2]. Therefore, the metabolites and defense mechanisms against insect herbivores are relatively well documented. The gene expression patterns induced by *Pieris rapae* caterpillar attack in white cabbage (*Brassica oleracea* var. *capitata*) differ depending on the cultivar and timing of the analysis after feeding [3]. Aphid infestation induces the expression of transcripts associated with plant hormones, metabolites, and modification of host plant morphology [4]. Microarray analysis showed that transcripts related to hormone signalling pathways, terpenoid biosynthesis, cell wall remodeling, photosynthesis, and carbohydrate metabolism were altered in tobacco (*Nicotiana attenuate*) exposed to larvae of the tobacco hornworm (*Manduca sexta*) [5]. In wheat attacked by Hessian fly (*Mayetiola destructor*) larvae, and the genes encoding defense proteins or enzymes involved in phenylpropanoid, cell wall, and lipid metabolism pathway were altered by the larvae [6]. The 30 kinds of protein expression were changed in *Arabidopsis* by larvae of *Spodoptera exigua* feeding [7]. The 23 and 21 kinds of protein expression were altered in maize infested with the

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cotton leafworm (*Spodoptera littoralis*) and maize stalk borer (*Busseola fusca*), respectively [8].

Changes in primary and secondary metabolites in response to insect herbivory have been studied [9]. Primary metabolic profiling was performed in different parts of tomato after leaf herbivory by two caterpillars (*Helicoverpa zea*, *M. sexta*). The levels of simple phenolics, precursor amino acids (phenylalanine, tryptophan), sugars, and nitrogen transport-related metabolites (glutamine, glutamate, asparagine, aspartate) were altered by caterpillar herbivory [10]. Isoleucine was used to form jasmonic acid-isoleucine (JA-Ile) at the attack site in tobacco attacked by *M. sexta* [11]. Metabolites changed by *S. littoralis* herbivory were identified using ultra-high-pressure liquid chromatography time-of-flight mass spectrometry in maize [12]. Sucrose, glucose, and fructose acted as specific regulatory signals on wound-inducible gene expression in *Glycine max* [11]. Sucrose and nitrogen in plant were reallocated from damaged tissues into storage tissues when attacked by herbivores [13]. Spotted knapweed (*Centaurea maculosa*) was infected with *Agapeta zoegana* and, although nitrogen uptake was reduced in the plant, the nitrogen status of the entire plant was maintained by shifting nitrogen from the roots to shoots [14].

Secondary metabolites also change in response to cues from herbivores. Glucosinolates, representative defensive secondary metabolites, are converted into isothiocyanates by myrosinase when the plant is wounded or damaged by herbivores. The isothiocyanates have a pungent odor and are toxic to herbivores such as Lepidoptera, Coleoptera, Diptera, Homoptera, and Hymenoptera [15]. Glucosinolates accumulated in *Arabidopsis* fed to *Myzus persicae*, *Brevicoryne brassicae*, and *S. exigua* [16]. Herbivores also increased plant nicotine, alkaloid, benzoxazinoid, and terpenoid levels [9, 17]. Metabolic profiling of *Brassica oleracea* attacked by small cabbage white caterpillars (*Pieris rapae*) was performed and the metabolites of the caterpillars were analyzed to determine how they interact with the plant [18]. Metabolites against flea beetle larvae (*Phyllotreta nemorum*) were identified in winter cress (*Barbarea vulgaris*) by metabolite profiling using liquid chromatography-mass spectrometry (LC-MS) [19].

There are many genomic, proteomic, and metabolomic studies on how plant defense systems respond to various insect herbivores [9,20-21]. However, there are few studies about plant behaviors against honeybees, an important pollinator and herbivore. We hypothesized that, although honeybees do not act directly as a pollinator and an aggressor of the *Arabidopsis*, metabolic alterations in *Arabidopsis* may be induced by honeybee exposure. Behavior of honeybee (e.g. touch, flapping, landing, and stay) may elicit the response of *Arabidopsis*. In this study, we focused on metabolic changes in *Arabidopsis* caused by honeybees. We used comprehensive metabolic profiling to investigate how *Arabidopsis thaliana* responds to western honeybees (*Apis mellifera*). We used high-performance liquid chromatography (HPLC), gas chromatography-mass spectrometry (GC-MS), and gas chromatography-time-of-flight mass spectrometry (GC-TOFMS), to detect carotenoids, glucosinolates, policosanols, tocopherols, phytosterols, β-amyrin, amino acids, organic acids, and sugars in *Arabidopsis* exposed to honeybees for four days. The changes in metabolites exposed to honeybees will provide a better understanding of the response and defense mechanism in *Arabidopsis*. Since *Arabidopsis* is a model plant for biological and genetic research, it will provide valuable information for understanding the response mechanism in other plant species.

Materials and Methods

Exposing Arabidopsis to honeybees

Six of plastic net boxes (330×270×270 mm) were prepared to expose honeybees to *Arabidopsis*. The top of the net box was transparent, and the other sides were made of square, 3-mm holes (Fig. 1). Forager western honeybees (*Apis mellifera*) were randomly collected from one colony. One plastic pot (320×230×90 mm) with 32-days-old *Arabidopsis thaliana* (Colombia-0) that had flowered was kept with ten honeybees in one net box. Three boxes were prepared with honeybees (the experimental group) and the other three had no honeybees (control). Honeybee food (a sucrose:honey ratio of 6:1, w/w) was placed in each net box. The *Arabidopsis* of the experimental group was continuously exposed to honeybee for 4 days (October 17-21, 2019). The distance between groups was 12 m, and the position of each box was changed daily to minimize any effects of location. Metabolic change is typically observed about 1 h after the plant is exposed to herbivores [22]. However, in our previously study, *Arabidopsis* exposed to honeybees for 4 h did not show a significant metabolic change compared to the control (data not shown). Exposing time was chosen to give sufficient time for *Arabidopsis* and honeybees to interact and for secondary metabolites to accumulate before the plant needed water. The experimental sets were kept in green house. The average temperature and humidity was 30 °C and 18%, respectively. All the aerial parts of the plant were harvested after 4 days, immediately. The lyophilized samples were kept at –20 °C until metabolite profiling was performed.

Chlorophylls analysis

Chlorophylls analysis was performed using a spectrophotometer (Optizen POP, Mecasys Co., Daejeon, Republic of Korea) as previously described [23]. The 10 mg of freeze-dried sample was mixed with 1 mL of methanol and mixed at 70 °C for 30 min a mixing frequency of 1,200 rpm (Thermomixer Comfort, model 5355, Eppendorf AG, Hamburg, Germany). The mixture was centrifuged at 3,000 rpm for 10 min at 4 °C (model MX-307, TOMY, Tokyo, Japan). The absorbance of supernatant was measured at 666 and 653 nm. The chlorophyll content was calculated using the formula mentioned by Wellburn (1994) [24].

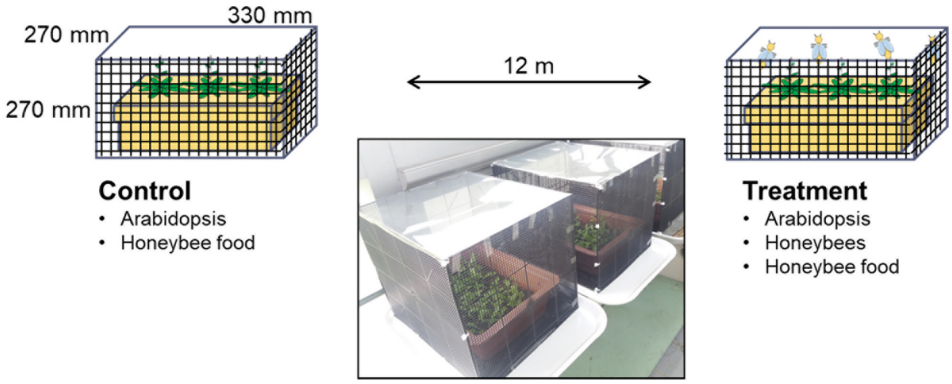

Fig. 1 The experimental set used in the present study

Carotenoids analysis

An ethanol containing 0.1% (*w/v*) ascorbic acid was used as carotenoids extraction solvent. The 10 mg of sample was mixed with the 3 mL of extraction solvent and incubated at 85 °C for 5 min. A 120 µL of 80% (*w/v*) potassium hydroxide (KOH) was added and incubated at 85 °C for 10 min. A 1.5 mL hexane and 1.5 mL deionized water were added to the cooled mixture. A 100 µL of β-apo-8'-carotenal (25 µg/mL in ethanol) was added as an internal standard (IS) and vortexed. The sample was centrifuged at 1,200× *g* for 5 min at 4 °C and the upper phase was pipetted into a new tube. A 1.5 mL of hexane was added to the pellet. The mixture was vortexed and centrifuged at 1,200× *g* for 5 min at 4 °C. The upper phase was combined in the tube. All the solvent was dried under nitrogen gas and reconstituted in 250 µL of 50:50 (*v/v*) dichloromethane:methanol. The reconstituted sample was filtered with a PTFE 0.50 µm hydrophobic filter (Advantec, Tokyo, Japan). The sample (20 µL) was injected and separated using Agilent 1100 series HPLC (Agilent, Massy, France) equipped with YMC Carotenoid S-3 µm column (250×4.6 mm; YMC Co., Kyoto, Japan) and photodiode array detector. A 92:8 (*v/v*) methanol:water with 10 mM ammonium acetate and methyl tert-butylether were used as gradient elution solvent A and B, respectively. The gradient used for elution was as follows: 0 min, 90% A/10% B; 20 min, 83% A/17% B; 29 min, 75% A/25% B; 35 min, 30% A/70% B; 40 min, 30% A/70% B; 42 min, 25% A/75% B; 45 min, 90% A/10% B; 55 min, 90% A/10% B. The solvent flow was 1.0 mL/min and column temperature was set at 40 °C. The chromatogram was obtained at 450 nm. The qualitative and quantitative analyses of carotenoids were performed using calibration curves made for corresponded standards.

Desulfoglucosinolates analysis

A 1.5 mL of boiling 70% (*v/v*) methanol was added to the 100 mg of lyophilized sample and incubated at 69 °C for 10 min. The supernatant was separated using centrifuge at 13,000× *g* for 10 min at 4 °C and pipetted into a new tube. The extraction step was repeated two more times. A disposable chromatography column (Bio-Rad Laboratories, Hercules, CA, USA) was filled with DEAE Sephadex A-25 (GE Healthcare, Uppsala, Sweden) using 0.5M sodium acetate and washed with deionized water. The collected supernatant was loaded onto the prepared disposable chromatography column. A 200 µL of 2.5 mM sinigrin was loaded onto another prepared column as external standard (ES). After loading, the column was washed with 3 mL of deionized water and 70 µL of purified sulfatase (23.3 mg/mL in deionize water; Sigma, St. Louis, MO, USA) was added. After incubating at 25 °C for 16 h, the desulfoglucosinolates were eluted with 2.4 mL of deionized water. The eluate was filtered with a PTFE 0.20 µm filter (Advantec) and 20 µL of the filtered eluate was injected for analysis. The desulfoglucosinolates were separated on a C18 column (250×4.6 mm, 5 µm, Inertsil ODS-3; GL Sciences, Tokyo, Japan) in Waters HPLC (e2695; Milford, MA, USA) equipped with a Waters 2998 photodiode array detector. A gradient elution was performed using third distilled water (solvent A) and acetonitrile (solvent B) at a flow rate of 1 mL/min. The gradient used for elution was as follows: 0 min, 99% A/1% B; 18 min, 80% A/20% B; 30 min, 80% A/20% B; 35 min, 70% A/30% B; 37 min, 99% A/1% B; 47 min, 99% A/1% B. The solvent flow was 1 mL/min and column temperature was set at 40 °C. The chromatogram was obtained at 227 nm. The peak identification of glucosinolates was done as described in previous study [23]. The calculation of glucosinolates content was performed using the response factor of each compound relative to that of sinigrin (ES) [25].

Policosanols, tocopherols, phytosterols, and β-amyrin analysis

Policosanols, tocopherols, phytosterols, and β-amyrin were analyzed using GC-MS (GCMS-QP2010 Ultra system, Shimadzu, Kyoto, Japan). The 50 mg of sample and 50 µL of 5α-cholestane (10 µg/mL in n-hexane) as an IS were added to 3 mL of ethanol containing 0.1% (w/v) ascorbic acid. After vortexing, the sample was placed in a water bath at 85 °C for 5 min and 120 µL of 80% (w/v) KOH was added. The sample was incubated at 85 °C for 10 min and placed on ice for 5 min. The deionized water (1.5 mL) and hexane (1.5 mL) were added and the vortexed sample was centrifuged at 1,200× *g* for 5 min at 4 °C. The hexane layer was

transferred to a separate tube and hexane (1.5 mL) was added to the pellet. The sample was vortexed and centrifuged at 1,200× *g* for 5 min at 4 ºC. The second hexane layer was combined in the tube. The collected hexane fraction was dried by nitrogen gas. For derivatization, 30 µL of *N*-methyl-*N*-(trimethylsilyl) trifluoroacetamide (MSTFA; Sigma) and 30 µL of pyridine were added and incubated at 60 ºC with shaking at 1,200 rpm for 30 min. The 1.0 µL of sample was injected to a Rtx-5MS column (30 m×0.25 mm, 0.25 µm; Restek, Bellefonte, PA, USA) with a split 10:1 ratio. Helium gas was used as carrier gas with a 1.0 mL/min flow rate. The injection, interface, and ion source temperatures were set at 290, 280, and 230 ºC, respectively. The initial oven temperature was set at 150 ºC for 2 min, followed by a temperature ramp of 15 ºC/min up to 320 ºC, with a hold time of 10 min. The qualitative and quantitative analyses of policosanols, tocopherols, phytosterols, and β-amyrin were performed using as previously described [26].

Primary metabolites analysis

Primary metabolites including amino acids, organic acids, sugars, and their derivatives were extracted as previously described [23], and the metabolites were analyzed using 7890B GC (Agilent) coupled to a Pegasus BT-TOF-MS (LECO, St Joseph, MI, USA). Briefly, the 10 mg of sample was mixed with 1 mL of 2.5:1:1 (*v/v*) methanol:water:chloroform and 60 µL of ribitol (200 µg/mL in methanol) as an IS. After incubation at 37 ºC for 30 min at 1,200 rpm, the mixture was centrifuged at 16,000× *g* for 3 min at 4 ºC. The 800 µL of supernatant was transferred to a separate tube and mixed with 400 µL of deionized water. After centrifuged at 16,000× *g* for 3 min at 4 ºC, The 900 µL of supernatant was transferred to a separate tube and dried by using a vacuum centrifuge dryer (VS-802, Vision, Daejeon, Republic of Korea) and freeze-dried (MCFD8512, IlShinBioBase, Dongducheon, Republic of Korea). For derivatization, 80 µL of methoxamine (MOX) reagent (Thermo Fisher Scientific, Waltham, MA, USA) was added and incubated at 30 ºC for 90 min with shaking at 1,200 rpm. A 80 µL of MSTFA was added and incubated at 37 ºC for 30 min with shaking at 1,200 rpm. The 1.0 µL of derivatized sample was injected into a CP-SIL 8 CB-MS column (30 m×0.25 mm, 0.25 mm, Agilent) with a split ratio of 1:25. A flow rate (helium gas) was set 1 mL/min and injection temperature was set at 230 ºC. The oven temperature was standardized to 2 min isothermal heating at 80 ºC, followed by an increase with a ramping rate of 15 ºC/min up to 320 ºC, with a hold time of 10 min. The transfer line and ion source temperatures were set at 280 and 250 ºC, respectively. The mass voltage was set at 1,700 and the mass range for scanning was 85 to 600. ChromaTOF software was used for component detection and automated deconvolution. The metabolites were identified using libraries (NIST, Wiley) and in-house libraries for standard chemicals. The relative level of each metabolite was calculated as peak area ratios relative to the IS peak area.

Starch analysis

The 50 mg of sample was used for estimating starch content. The starch was determined by the method of commercial Starch Assay kit (STA20; Sigma).

Statistical analysis

All the experiments were performed with six replicates. A PLS-DA was carried out with normalization (unit variance scaling) data using SIMCA (version 14.1, Umetrics, Umea, Sweden). The results of Student's *t*-test were obtained from the GraphPad Prism (GraphPad Software Inc., San Diego, USA).

Results

Metabolic profiling

To investigate how plants respond to honeybees, comprehensive metabolic profiling of *Arabidopsis* exposed to or not exposed to honeybees was performed using HPLC, GC-MS, and GC-TOFMS. Glucosinolates are representative defensive secondary metabolites which the building blocks for the secondary metabolites are derived from primary metabolism. Likewise, the complexity of plant defense responses requires an abundant supply of energy, mainly derived from primary metabolism. Thus, we have analyzed primary metabolites, including photosynthetic pigments, sugars, organic acids, and amino acids, as well as glucosinolates to determine *Arabidopsis* responses to honeybees. A total of 85 different types of metabolites were identified in *Arabidopsis*. Seven types of carotenoids and nine types of glucosinolates (five aliphatic and four indolic glucosinolates) were detected by HPLC (Fig. S1 and S2). Nine types of policosanols, three of tocopherols, five of phytosterols, and β-amyrin were detected by GC-MS (Fig. S3). A total of 48 different types of primary metabolites (22 amino acid groups, 12 organic acid groups, and 14 sugar groups) were identified using GC-TOFMS (Fig. S4). Chlorophyll a, chlorophyll b, and starch were analyzed using the spectrophotometer.

Partial least squares-discriminant analysis (PLS-DA)

The multivariate data obtained from 85 types of metabolites were subjected to PLS-DA. PLS-DA was used to select meaningful discriminative variables (metabolites) between *Arabidopsis* exposed to (treatment) and not exposed to (control) honeybees. The Q^2 and R^2 values indicate the quality of the model. Q^2 value indicates the goodness of prediction and Q^2 above 0.5 indicates a good prediction model. R^2 value indicates the goodness of fit and the R^2 value closer to 1.0 is desirable [27]. The PLS-DA model showed Q^2 of 0.814 and R^2Y of 0.976. It was indicated that this model had good prediction ability. The two groups were separated into two parts by PLS 1 (Fig. 2A). β-Carotene, 13*Z*-β-carotene, 9*Z*-β-carotene, zeaxanthin, lutein, and chlorophylls had negative

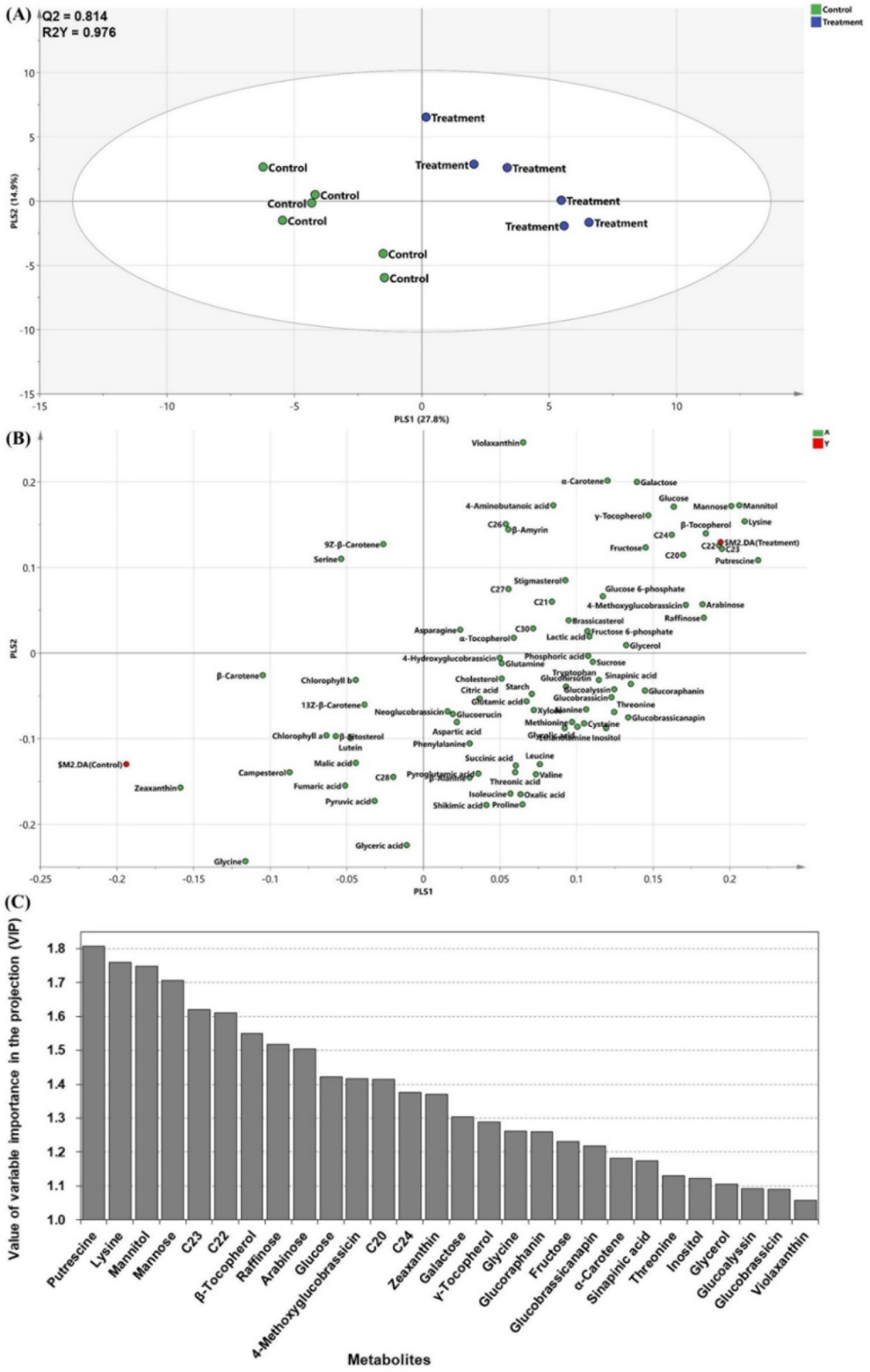

Fig. 2 (A) Partial least squares-discriminant analysis (PLS-DA) score plots and (B) loading plots obtained from metabolite data of *Arabidopsis thaliana*. The influence of variables used to distinguish *Arabidopsis* exposed to honeybees (treatment) from plants not exposed (control) for 4 days. (C) Metabolites with higher variable importance in the projection (VIP) values better explain the difference. Metabolites with VIP >1.0 were showed. Control, *Arabidopsis* not exposed to honeybees for 4 days; Treatment, *Arabidopsis* exposed to honeybees for 4 days; C20, Eicosanol; C21, Heneicosanol; C22, Docosanol; C23, Tricosanol; C24, Tetracosanol; C26, Hexacosanol; C27, Heptacosanol; C28, Octacosanol; C30, Triacontanol

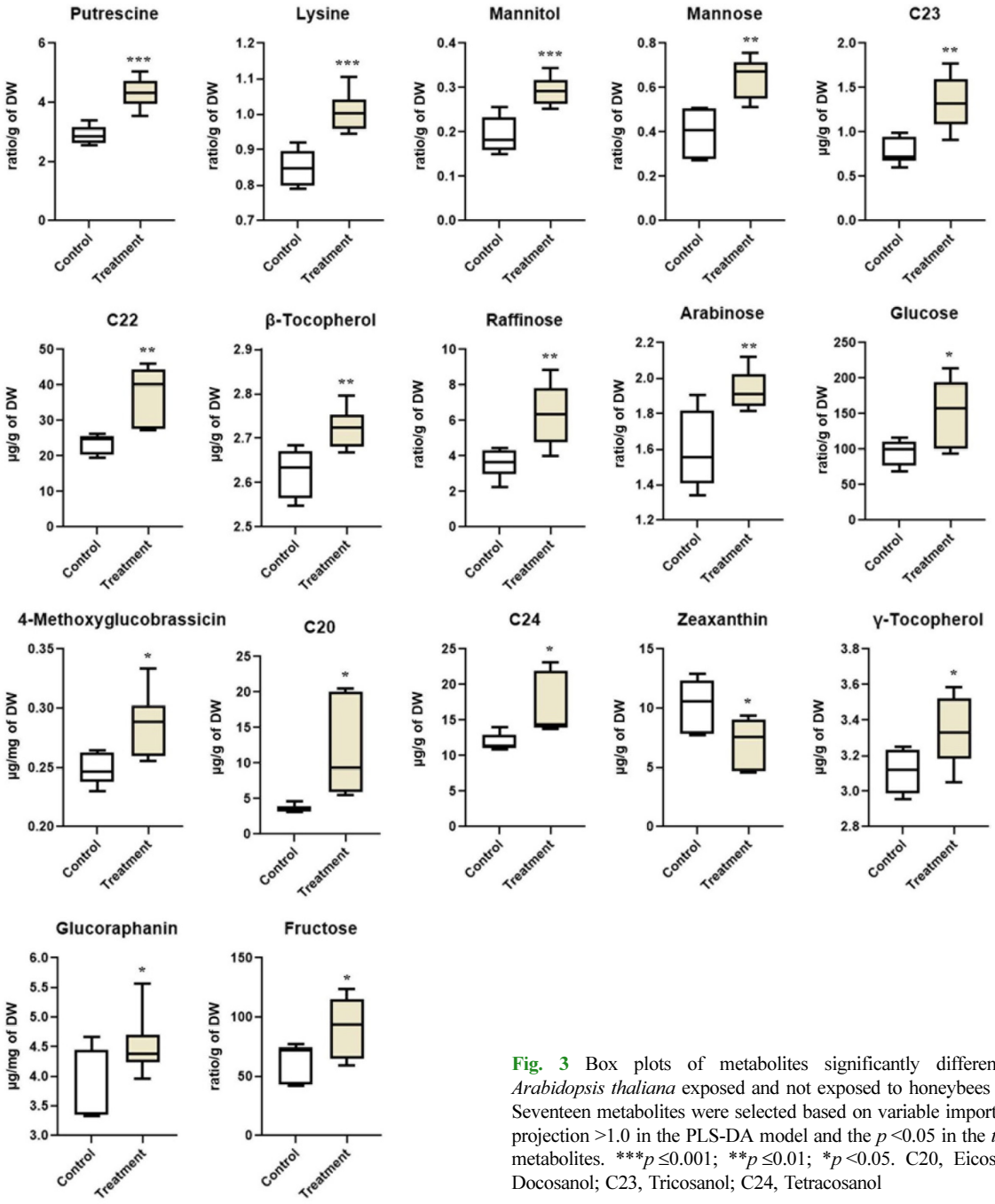

Fig. 3 Box plots of metabolites significantly different between *Arabidopsis thaliana* exposed and not exposed to honeybees for 4 days. Seventeen metabolites were selected based on variable importance in the projection >1.0 in the PLS-DA model and the p <0.05 in the t-test for all metabolites. ***p ≤0.001; **p ≤0.01; *p <0.05. C20, Eicosanol; C22, Docosanol; C23, Tricosanol; C24, Tetracosanol

eigenvectors of PLS 1. The treatment group had relatively lower levels of these metabolites than did the control (Fig. 2B). On the other hand, glucosinolates had positive eigenvectors of PLS 1, which indicated that the treatment group had relatively higher levels of glucosinolates than did the control. Tocopherols, sugars, and policosanols except for octacosanol (C28-ol) had positive eigenvectors of PLS 1. Most of the amino acids and their derivatives, including lysine and putrescine, had relatively high levels in the treatment group. Variable importance in the projection (VIP) value was used to find the metabolites responsible for separating the two groups. VIP values >1.0 indicated that the PLS-DA model effectively explained the data [27]. Twenty-eight types of metabolites were found to significantly explain the separation (Fig. 1C). The following among these were significantly different: putrescine, lysine, mannitol, mannose, tricosanol (C23-ol), docosanol (C22-ol), β-tocopherol, raffinose, arabinose, glucose, 4-methoxyglucobrassicin, eicosanol (C20-ol), tetracosanol (C24-ol), zeaxanthin, γ-tocopherol, glucoraphanin, and fructose (p-value <0.05) (Fig. 3).

Comparing the contents of metabolites between control and experimental Arabidopsis

As shown in the result of PLS-DA, the levels of most of photosynthetic pigments were lower in the treatment group, but only zeaxanthin was significantly different (p-value <0.05) (Table 1). The contents of zeaxanthin in control and treatment group were 10.30 μg/g dry weight (dw) and 7.10 μg/g dw, respectively. The total glucosinolates content in the treatment group (7.99 μg/mg dw) was higher than that in the control (6.91 μg/mg dw) (Table 1). Among them, glucoraphanin and 4-methoxyglucobrassicin showed significant differences between the experimental and control groups. The glucoraphanin was a predominant glucosinolate in *Arabidopsis*. The contents of glucoraphanin in control and treatment group were 3.75 and 4.50 μg/mg dw, respectively. The contents of 4-methoxyglucobrassicin in control and treatment group were 0.25 and 0.29 μg/mg dw, respectively.

Among policosanols, the contents of C20-ol, C22-ol, C23-ol, and C24-ol were significantly different (Table 2). The levels of C20-ol, C22-ol, C23-ol, and C24-ol were higher in the treatment group. On the other hand, the levels of hexacosanol (C26-ol), heptacosanol (C27-ol), C28-ol, and triacontanol (C30-ol) were lower in the treatment group, but they showed no significant differences. Total tocopherol content in the treatment group (140.61 μg/g dw) was slightly higher than that in the control (136.41 μg/g dw) (Table 2). However, α-tocopherol, type of tocopherol predominant in *Arabidopsis*, showed no significant differences between treatment and control groups (p-value ≥0.05). The levels of β-amyrin and starch showed no significant differences.

Lysine and putrescine showed only significant differences between the experimental and control groups among amino acids and its derivatives (Table 3). Lysine and putrescine levels in the treatment group were higher approximately 1.2 and 1.5 times those of the control, respectively. Organic acids, organic compounds, and inorganic acid showed no significant differences between the experimental and control groups. Sugar contents were higher in the treatment group than the control (Table 4). Among them, the levels of arabinose, fructose, glucose, mannose, mannitol, and raffinose were significantly different.

Discussion

Although various insect-inducible responses in plants have been previously described, the response elicited by exposing to honeybees was insufficient. Using metabolic profiling, this study revealed that stress-relative metabolites were altered by honeybee.

Table 1 Composition and abundance of chlorophylls, carotenoids, and glucosinolates in *Arabidopsis thaliana* not exposed (control) and exposed (treatment) to honeybees

Compounds[1)]		Control	Treatment
Chlorophylls	Chlorophyll a	8.12±0.31	7.97±0.25
(μg/mg dw)	Chlorophyll b	3.70±0.16	3.64±0.17
	Total	11.82±0.47	11.61±0.42
Carotenoids	Violaxanthin	1.14±0.04	1.17±0.07
(μg/g dw)	Lutein	449.56±15.19	442.42±20.93
	Zeaxanthin	**10.30±1.96**[2)]	**7.10±1.89**
	13*Z*-β-Carotene	99.74±3.44	98.49±4.46
	α-Carotene	23.10±1.23	24.25±0.81
	β-Carotene	579.52±29.01	558.39±13.75
	9*Z*-β-Carotene	102.27±6.22	101.08±4.87
	Total	1265.63±57.09	1232.91±46.78
Aliphatic glucosinolates	Glucoalyssin	0.15±0.03	0.19±0.03
(μg/mg dw)	Glucobrassicanapin	0.06±0.01	0.08±0.01
	Glucoerucin	0.46±0.06	0.47±0.07
	Glucohirsutin	0.46±0.07	0.51±0.06
	Glucoraphanin	**3.75±0.55**	**4.50±0.50**
	Total	4.88±0.72	5.75±0.67
Indole glucosinolates	4-Hydroxyglucobrassicin	0.22±0.02	0.23±0.04
(μg/mg dw)	Glucobrassicin	1.48±0.07	1.64±0.18
	4-Methoxyglucobrassicin	**0.25±0.01**	**0.29±0.03**
	Neoglucobrassicin	0.08±0.02	0.08±0.01
	Total	2.03±0.12	2.24±0.26

[1)]dw, dry weight. Each value is the mean of six replications ± standard deviation
[2)]Statistically significant mean scores (p <0.05) are in **boldface**

Table 2 Composition and abundance of policosanols, tocopherols, phytosterols, amyrin, and starch in *Arabidopsis thaliana* not exposed (control) and exposed (treatment) to honeybees

Compounds[1)]		Control	Treatment
Policosanols	C20-ol	**3.29±0.18**[2)]	**11.67±6.33**
(μg/g dw)	C21-ol	1.14±0.03	1.20±0.17
	C22-ol	**23.55±2.91**	**37.03±7.40**
	C23-ol	**0.79±0.10**	**1.23±0.25**
	C24-ol	**11.69±0.61**	**16.39±3.60**
	C26-ol	296.21±25.21	282.60±15.37
	C27-ol	16.98±1.86	16.81±0.85
	C28-ol	459.42±90.31	425.40±9.03
	C30-ol	439.15±83.49	421.44±45.80
	Total	1252.24±204.69	1213.77±88.80
Tocopherols	α-Tocopherol	130.72±1.97	134.64±5.75
(μg/g dw)	**β-Tocopherol**	**2.62±0.05**	**2.71±0.03**
	γ-Tocopherol	**3.07±0.12**	**3.26±0.19**
	Total	136.41±2.14	140.61±5.97
Phytosterols	Brassicasterol	18.43±0.56	20.35±1.53
(μg/g dw)	Campesterol	747.98±43.83	706.90±8.64
	Cholesterol	29.55±1.55	28.87±2.66
	β-Sitosterol	997.27±25.49	927.38±12.35
	Stigmasterol	16.84±2.47	19.21±2.40
	Total	1810.06±73.90	1702.71±27.57
Amyrin	β-Amyrin	18.52±1.46	18.33±1.56
(μg/g dw)			
Starch	Starch	24.19±3.94	25.95±1.34
(mg/g dw)			

[1)]C20-ol, Eicosanol; C21-ol, Heneicosanol; C22-ol, Docosanol; C23-ol, Tricosanol; C24-ol, Tetracosanol; C26-ol, Hexacosanol; C27-ol, Heptacosanol; C28-ol, Octacosanol; C30-ol, Triacontanol; dw, dry weight. Each value is the mean of six replications ± standard deviation
[2)]Statistically significant mean scores (p <0.05) are in **boldface**

The levels of most of photosynthetic pigments were lower in the treatment group than the control. On the other hand, the levels of glucosinolates, policosanols (C20-ol, heneicosanol (C21-ol), C22-ol, C23-ol, C24-ol), tocopherols, and sugars were higher in the treatment group.

Photosynthesis proteins were upregulated after feeding *Arabidopsis* to *S. exigua* larvae for 8 h [7]. On the other hand, leaves of parsnip (*Pastinaca sativa*) fed on by larvae of *Trichoplusia ni* for 24 h exhibited reduced rates of photosynthesis for 3 days after the caterpillars were removed [28]. Although *Arabidopsis* was not attacked by the honeybees directly, honeybees could affect photosynthesis within 4 days. Another hypothesis was that the carotenoids are used as a precursor for hormone. Carotenoids are used as precursor of abscisic acid. Mechanical stimuli resulted in an increase in abscisic acid in rice [29]. The abscisic acid plays a major role in the response to abiotic stress (drought, salt, wounding). In addition, Bodenhausen and Reymond (2007) provided that abscisic acid is related to resistance to insects in *Arabidopsis* [30].

The total glucosinolates content in the treatment group was higher than that in the control. Glucoraphanin and 4-methoxy-glucobrassicin showed significant differences between the experimental and control groups (p-value <0.05). Glucoraphanin is the predominant aliphatic glucosinolate in *Arabidopsis,* and 4-methoxyglucobrassicin accumulated with the application of defense hormones (salicylic acid, jasmonates) [15,31]. Glucosinolates is known to act as defensive compounds against herbivores [15-16]. *Arabidopsis* exposed to honeybees may accumulate the glucosinolates for defensive responses.

Among policosanols, the contents of C20-ol, C21-ol, C22-ol, C23-ol, and C24-ol in the treatment group were higher than those in the control. The micromorphology and chemical composition of epicuticular waxes influence herbivore behavior because insects search the surface of plants for food, spots to oviposit, and shelter [32]. Cell wall strengthening is a direct plant defense mechanism because it enhances plant barriers to outside forces [33]. In addition, cell wall associated protein genes were increased in touched *Arabidopsis* [34]. Policosanols are known as components of plant waxes and oils, and alterations in policosanols could be involved in cell wall modification [35].

Table 3 Composition and abundance (ratio/g of dry weight) of amino acids, organic acids, and their derivatives in the *Arabidopsis thaliana* not exposed (control) and exposed (treatment) to honeybees

Compounds[1)]		Control	Treatment
Amino acid and amino acid derivatives	Alanine	7.83±7.53	19.34±15.41
	β-Alanine	1.03±0.15	1.08±0.20
	4-Aminobutanoic acid	9.87±2.20	12.97±5.57
	Asparagine	0.42±0.15	0.46±0.19
	Aspartic acid	2.62±0.62	2.73±0.62
	Cysteine	0.10±0.02	0.12±0.02
	Ethanolamine	16.28±6.41	21.71±7.14
	Glutamic acid	32.81±5.78	36.56±7.45
	Glutamine	4.19±1.19	4.80±1.63
	Glycine	54.20±23.91	34.88±9.71
	Isoleucine	1.41±1.25	2.06±1.49
	Leucine	1.09±1.06	1.88±1.38
	Lysine	**0.85±0.05**[2)]	**1.01±0.05**
	Methionine	0.44±0.07	0.50±0.05
	Phenylalanine	1.55±0.34	1.61±0.17
	Proline	4.52±4.01	7.02±5.20
	Putrescine	**2.90±0.28**	**4.32±0.46**
	Pyroglutamic acid	4.57±1.03	5.10±2.33
	Serine	6.31±2.78	5.27±1.83
	Threonine	11.21±1.96	13.27±1.59
	Tryptophan	0.04±0.02	0.07±0.03
	Valine	3.03±2.93	5.07±3.59
Organic acids, organic compounds, inorganic acid	Citric acid	17.58±2.14	18.25±2.33
	Fumaric acid	509.32±78.01	474.48±87.31
	Glyceric acid	2.66±0.35	2.63±0.27
	Glycolic acid	2.57±0.43	3.08±0.69
	Lactic acid	1.77±0.90	2.57±0.75
	Malic acid	26.21±5.81	24.25±4.99
	Oxalic acid	4.66±1.68	5.72±2.27
	Phosphoric acid	2.06±0.61	2.87±1.03
	Pyruvic acid	0.59±0.16	0.56±0.07
	Shikimic acid	5.31±0.68	5.51±0.47
	Sinapinic acid	0.22±0.05	0.27±0.03
	Succinic acid	15.95±2.18	16.95±1.80

[1)]Each value is the mean of six replications ± standard deviation
[2)]Statistically significant mean scores (p <0.05) are in **boldface**

Total tocopherol content in the treatment group was slightly higher than that in the control. However, α-tocopherol, type of tocopherol predominant in leaves, showed no significant differences between treatment and control groups (p-value ≥0.05) [35]. The mechanical stress (rubbing with fingers) increased the formation of reactive oxygen species (ROS) in *Arabidopsis* [36]. Tocopherols have antioxidant activity and it relates with regulation of ROS in stress [37-38].

Lysine and putrescine levels in the treatment group were higher than the control, and these metabolites had the highest VIP values (Table 2 and Fig. 2C). Under some types of stress (osmotic stress, abscission), lysine is efficiently catabolized into glutamate via upregulation of lysine ketoglutarate reductase and saccharopine dehydrogenase genes. The glutamate is a precursor for proline, 4-aminobutanoic acid, and arginine. Those metabolites are stress-related compounds [39]. In this study, the levels of lysine, glutamate, proline, and 4-aminobutanoic acid in the treatment were slightly higher than those in the control, but lysine was the only compound that was significantly higher (p-value <0.05). Putrescine is also known to be related to stress tolerance. High

Table 4 Composition and abundance (ratio/g of dry weight) of sugars and their derivatives in the *Arabidopsis thaliana* not exposed (control) and exposed (treatment) to honeybees

Compounds[1)]		Control	Treatment
Sugars, Sugar acid, and Sugar alcohol	Arabinose	**1.60±0.20**[2)]	**1.93±0.10**
	Fructose	**63.46±14.80**	**91.51±23.17**
	Fructose 6-phosphate	0.07±0.01	0.10±0.030
	Galactose	0.90±0.08	1.19±0.28
	Glycerol	8.35±1.77	10.87±2.19
	Glucose	**95.31±16.46**	**152.48±43.16**
	Glucose 6-phosphate	0.07±0.01	0.09±0.03
	Inositol	19.34±0.70	20.77±1.69
	Mannitol	**0.19±0.04**	**0.29±0.03**
	Mannose	**0.40±0.10**	**0.65±0.08**
	Raffinose	**3.60±0.75**	**6.35±1.60**
	Sucrose	71.25±11.36	117.62±64.71
	Threonic acid	1.44±0.18	1.52±0.12
	Xylose	1.29±0.18	1.38±0.13

[1)]Each value is the mean of six replications ± standard deviation. [2] Statistically significant mean scores (p <0.05) are in **boldface**

putrescine levels enhance freezing and drought tolerance, and salt treatment increases putrescine levels in *Arabidopsis* [40]. Plant tissues infected with pathogens accumulate large amounts of putrescine, which inhibit the growth of bacteria and viruses. A previous study provided that plant cells infected with pathogens induced polyamine accumulation and polyamine oxidase activity [41].

Sugar contents were also higher in the treatment group than the control (Table 2). Sugars modulate vital processes, including responses to stress [11]. ROS scavenging capacity by mannitol was shown in tobacco. Increased concentration of raffinose resulted in an effective ROS scavenging capacity and oxidative stress tolerance in transgenic *Arabidopsis* plants [42]. Additionally, a recent study reported that treatment with mannitol strongly decrease wound-induced cell expansion and wound-triggered periclinal divisions in *Arabidopsis* root [43]. Several studies have reported extensive glucose control of abscisic acid biosynthesis and signalling genes that antagonizes ethylene signalling during seedling development under light. [44].

In this study, we identified 85 types of primary and secondary metabolites in *A. thaliana* exposed to honeybees and not exposed for the first time. The levels of metabolites involved in the stress response mechanism were higher in *Arabidopsis* exposed to honeybees than unexposed *Arabidopsis*. We hypothesized that *Arabidopsis* perceives honeybees as biotic or/and abiotic stresses and responds to them using metabolites. However, the changes in some of those metabolites were not significate (p-value ≥0.05) (Fig. S5). The defense response occurred in a second and altered or sustained for several days, so that the amount of time between attack and analysis is important [22,28]. The metabolic profiling of *Arabidopsis* exposed to honeybees at different hours is necessary to confirm changes in metabolites. In addition, the mechanical stimuli (touch, rubs, wind) and vibration caused by insect feeding led to the alteration of metabolite composition [24,45]. The comparison with the physical stimuli is also necessary to find the accurate stimulus (touch, vibration, flapping, or something). Plants respond to herbivores by reallocating their metabolites throughout the plant [13-14]. It is also necessary to divide analysist based on the parts of the plant. Above all, gene expression should be analyzed to support or refute our hypothesis. This study is the first step to investigate the behavior of a plant exposed to honeybees. It provides new insight for understanding general plant behavior in response to insects.

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