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Apple Fruit (*Malus domestica* Borkh.) Metabolic Response to Infestation by Invasive Brown Marmorated Stink Bug (*Halyomorpha halys* Stal.)

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Abstract: The brown marmorated stink bug (BMSB; *Halyomorpha halys* Stal.) has become a significant pest in Slovenia, especially in apple, pear, peach, and cherry orchards. In our study, apple fruit of the apple cultivar ‘Red Pinova’ were evaluated for visual injury and sugar, organic acid, and phenolic contents. The chemical composition of the area around the puncture wound, the uninjured part of the infested apple, was compared to, as a control, only uninjured apples. There was a significant response of the apple around the puncture wound, resulting in an 11.9 g/kg FW higher total sugar content, a 1.4 g/kg FW lower total organic acid content, and an 11.9 g/kg FW higher total phenolic content compared with control apples. A strong phenolic response in the puncture wound area, with high flavanol and hydroxycinnamic acid contents, with increases of 118% and 237%, respectively, compared with control apples, was detected. The brown marmorated stink bug induces a strong phenolic response in the injured area of the apple. The results of this study illustrated how apple fruit responds to the BMSB injury, not only sensorily (visual injury, odor), but also chemically in the form of metabolic responses.

Keywords: injury; insect; organic acids; pests; phenolics; sugars

1. Introduction

The apple (*Malus domestica* Borkh.) is one of the most important and widely grown fruits in the world, according to FaoStat [1]. Because apples are so widely grown around the world, they harbor a variety of pests and diseases. In recent years, farmers have discovered a new pest that attacks the apple fruit. The brown marmorated stink bug (BMSB; *Halyomorpha halys* Stal.) causes injury to apples and reduces yield and shelf life [2]. The BMSB is a polyphagous insect that can feed on various ornamental, fruit, and vegetable plants [3].

The BMSB can be recognized by its white and black banding on the antennae and abdominal margins. It also has white banding on tibiae, especially distinct in nymphs. Adult males have fork-shaped last sternite on the last ventral abdominal segment, which distinguishes them from females. The eggs, which are white, are laid on the underside of leaves in clusters [4]. Development into adults occurs at temperatures between 17 °C and 33 °C, although egg hatching can occur at 15 °C. Females can lay eggs every 4 days throughout their lifespan, and each clutch contains an average of 28 eggs [5]. In Europe, the BMSB has two generations per year and four to six in subtropical regions [6].

The BMSB is spreading rapidly throughout the world due to climate change and lack of predators [7]. Injury to apples can be detected by visual inspection, since the stink bug punctures and sucks the nutrient-rich apple juices [8]. On pome fruits, including apples and pears, feeding results in dented depressions on the surface and corky spots in the flesh, making the apple unappetizing. Feeding can also lead to a reduction in fruit set [2].


also acquire a noticeable odor typical of BMSBs, caused by their excretion of aldehydes [8]. Most injury to apples results from feeding by the adult insect in the later stages of apple development with up to 60% yield loss and with severe infestations resulting in 100% yield loss [9,10].

Fruit responds to insect attack with increased synthesis of phenolic compounds, which act as defense molecules to repel attackers or disrupt their reproductive and feeding cycles [11]. Phenolic compounds such as flavonoids and proanthocyanidins have cytotoxic and astringent effects. They act as feeding deterrents and prevent attacks by many insects [12].

In our study, we tested the apple cultivar ‘Red Pinova’. There have been studies that have looked at the metabolic response of apples to various pest and disease infestations, but there are no reports of BMSB injury to apples and apple fruit metabolic response to infestation. In this study, we examined how apple fruit respond to stink bug infestations in terms of tissue contents of sugars, organic acids, and individual phenolics. We also tested whether the entire apple was affected by the feeding of this invasive pest or only the part at which the puncture wound was located. The results of this study will better explain how the BMSB alters the metabolic profile of apples.

2. Materials and Methods

Apples infested by the BMSB were collected from an intensive apple orchard in Bilje, Slovenia (45°53′47.55″ N; 13°38′49.22″ E). The apple cultivar ‘Red Pinova’ was chosen for the experiment because it is severely and conspicuously affected by BMSB infestation. We identified the insect injury on apple fruit based on Nielsen and Hamilton [2] and Acebes-Doria, Leskey, and Bergh [10]. In our case, the injury on apples was late-season damage as described by Nielsen et al. (2008). Based on Acebes-Doria et al. (2016), the apples were injured 15 to 20 days before picking. The ‘Red Pinova’ cultivar was picked from 5–10 September 2020, so the feeding injury likely happened from 21–26 August 2020. The injured apples had depressed pits on the surface and corky areas in the flesh, which is typical of BMSB injury. Apples were picked at technological maturity in the second week of September 2020. Three treatments were established: (i) controls which consisted of uninjured apples; (ii) BMSB injured apples in which the visually injured areas on the apples were excised; and, (iii) the uninjured part of the BMSB-injured apples in which the uninjured parts of the injured apples were cut out. For each treatment, 15 apples were randomly picked from three apple trees, which were then divided into five replicates (each consisting of three apples) for each treatment. All apples had to be mature and cultivar-specific colored, with all being picked from the same spot on the branch. By distinguishing three treatments, we wanted to establish whether *H. halys* feeding affected apple metabolism in the part at which the actual puncture wound occurred, or if the entire infested apple was affected, compared to an uninjured apple. All treatment specimens were collected with a 10-mm biopsy punch, with each hole 3 mm deep with skin and cortex. If the injury area was smaller than 10 mm, a punch of sufficient size was used. The injury area was excised as precisely as possible, separating injured tissue from uninjured tissue. The uninjured portion of the BMSB apple was always excised with a punch at the exact opposite location of the injured area of the apple. All apples were harvested, sampled, and extracted on the same day. Analysis of sugars and organic acids was performed.

For the analysis of individual sugars and organic acids, 5 g of fresh apple tissue was extracted with 25 mL of double-distilled water. The samples were macerated and placed on an orbital shaker (Heidolph, Unimax 1010) for 30 min. After shaking, the samples were placed into a centrifuge set at 12,000×g for 10 min. The supernatant was then filtered through a 0.25 µm cellulose filter (Chromafil A-25/25; Macherey-Nagel, Düren, Germany) and stored at −20 °C until HPLC analysis. The HPLC settings for the analysis were based on Zamljen et al. [13]. The data for sugars and organic acids were expressed in g/kg fresh weight (FW). For shikimic and fumaric acid, the data were expressed in mg/kg FW.
2.1. Analysis of Individual and Total Phenolics

A fresh fruit sample (1 g) was extracted with 5 mL of 80 % MeOH. Samples were placed in a cooled ultrasonic bath (0 °C) for 1 hour. After the bath, samples were centrifuged at 10,000 × g for 6 min and then filtered through a 0.45 µm polyamide filter (Chromafil AO-45/25, Macherey-Nagel, Düren, Germany). Total phenolic contents were determined based on Singleton et al. [14]. The total phenolic contents were presented in g/kg GAE (gallic acid equivalents).

Individual phenolics were first determined by HPLC/MS based on Jakopic et al. [15]. The HPLC settings for the quantification of individual phenolics were based on Medic et al. [16]. All phenolics were calculated based on appropriate standards. Where no standard could be obtained, the data were calculated as equivalents of similar substances obtainable as standards. Procyanidin B2 was expressed as procyanidin B1 equivalent and phloretin-2-O-xylosylglucoside was expressed as phloridzin equivalent. All individual phenolics were expressed in g/kg FW.

2.2. Chemicals

The standards used in our study were: sucrose, glucose, and fructose (Sigma–Aldrich Chemie GmbH, Steinheim, Germany); citric acid, malic acid, shikimic acid, and fumaric acid (Sigma–Aldrich Chemie GmbH, Steinheim, Germany); and, procyanidin B1, catechin chlorogenic acid, epicatechin, 4-O-p-coumaroylquinic acid, phloridzin, quercetin-3-galactoside, quercetin-3-glucoside, and quercetin-3-rhamnoside (Sigma–Aldrich Chemie GmbH, Steinheim, Germany).

2.3. Statistical Analysis

Data were statistically processed using R program [17]. Where statistically significant differences among treatments were found using analysis of variance (ANOVA), Fisher’s Least Significant Difference (LSD) test was performed at p < 0.05.

3. Results and Discussion

3.1. Visual Injury

We examined all apples for visual injury from BMSB (Figure 1). Apples with BMSB injury had greenish-brown spots on the skin, and the spots felt harder than the rest of the apple. Each individual spot was 2 to 5 mm in diameter. The blemishes were 2 to 3 mm deep when cut. Apples infested with BMSB had a noticeable odor, due to the excretion of trans-2-octenal and trans-2-decenal aldehydes [18], making them unmarketable or lowering their quality according to Nations [19] standards.

![Figure 1. Uninjured apple (A) and brown marmorated stink bug-injured apple (B).](image-url)
3.2. Sugars

The individual and total sugar contents are shown in Table 1. Infestation with *H. halys* decreased the sucrose content by 13.0 g/kg FW compared with the control treatment. On the other hand, it increased the glucose content by 10.2 g/kg FW and fructose content by 13.5 g/kg FW compared with the uninjured control apples. The total sugar content of the apple injured by *H. halys* increased by 11.9 g/kg and 14.4 g/kg FW, respectively, compared with the control treatment. Sucking insects such as BMSB tend to feed more on sugars than amino acids [20]. The increase in glucose and fructose in biotically stressed plants is a common response, as previously reported by Lecompte et al. [21]. Glucose also serves as a signaling molecule in plants and can increase in abiotically and biotically stressed plants [22,23]. In this study, the stink bug increased the total sugar content in the apples, which is in contrast to Zhou et al. [24], who reported no noticeable reduction in sugar content between infested and uninfested blueberries.

| Treatment | Control | BMSB Injury Site | Uninjured Part of BMSB Apple |
|-----------|---------|-----------------|-------------------------------|
| Sucrose   | 43.2 ± 0.2 a² | 30.2 ± 0.2 b  | 40.2 ± 0.5 ab |
| Glucose   | 30.4 ± 0.3 b  | 40.6 ± 0.2 a  | 36.2 ± 0.2 ab |
| Fructose  | 69.8 ± 0.3 b  | 83.3 ± 0.3 a  | 79.4 ± 0.2 ab |
| Sorbitol  | 4.5 ± 0.2 a   | 5.7 ± 0.3 a   | 6.4 ± 0.3 a   |
| Total sugars | 148.0 ± 10.2 b | 159.9 ± 13.7 a | 162.4 ± 14.8 a |

²Different letters in a row denote statistically significant differences by Fisher’s Least Significant Difference (LSD) test at *p* < 0.05.

3.3. Organic Acids

Four organic acids were determined in the apple samples (Table 2). Citric acid was elevated only at the direct feeding site of BMSB, with 0.6 g/kg FW higher citric acid content compared with the control and the uninjured part of the injured apple. Malic acid, the most abundant organic acid in apples, decreased by 20.6% at the BMSB injury site compared with the control. Similarly, the content of fumaric acid also decreased in BMSB injury sites compared with the control. Total organic acid content decreased by 1.4 g/kg FW in BMSB injury sites as compared with the control.

| Treatment | Control | BMSB Injury Site | Uninjured Part of BMSB Apple |
|-----------|---------|-----------------|-------------------------------|
| Citric acid (g/kg FW) | 1.9 ± 0.1 b² | 2.5 ± 0.1 a  | 1.9 ± 0.1 b |
| Malic acid (g/kg FW)  | 9.2 ± 0.9 a  | 7.3 ± 0.4 b  | 8.3 ± 0.6 ab |
| Shikimic acid (mg/kg FW) | 22.3 ± 1.5 a | 22.6 ± 2.0 a  | 25.1 ± 3.0 a |
| Fumaric acid (mg/kg FW) | 66.1 ± 2.2 a | 53.2 ± 1.9 b  | 60.7 ± 5.1 a |
| Total organic acids (g/kg FW) | 11.3 ± 2.3 a | 9.9 ± 2.2 b  | 10.4 ± 2.7 ab |

²Different letters in a row denote statistically significant differences by Fisher’s Least Significant Difference (LSD) test at *p* < 0.05.

Organic acids are important to plants because they have important roles in various biochemical pathways and serve as key components in some plants for adaptation to nutrient deficiency and for metal tolerance [25]. Similar to our results, Weber et al. [26] reported a decrease in malic acid in strawberries infected with *Colletotrichum nymphaeae*, which is also a form of biotic stress. Interestingly, fumaric acid decreased compared with the control treatment, although Chia et al. [27] reported that it increases in stressed plants. Total organic acid decreased in BMSB injury sites compared with the control. One reason may be that the plant concentrates all metabolites and synthesizes phenolics around the puncture wound area, as previously reported by Selmar and Kleinwächter [28].
3.4. Phenolics

A strong phenolic response (Table 3) was observed in the parts of the fruit injured by BMSB. Procyanidin B1, procyanidin B2, and catechin increased in BMSB injury sites compared with the control, with increases of 0.95 g/kg, 3.98 g/kg, and 2.94 g/kg FW, respectively. The control apple and the uninjured portion of the injured apple did not differ in the contents of individual flavanols. The BMSB injury sites had 118.0 % and 103.5 % higher flavanol contents than the control and uninjured portion of the injured, respectively. Chlorogenic acid and 4-O-p-coumaroylquinic acid increased by 3.16 g/kg and 0.2 g/kg FW in BMSB injury sites compared with the control. Consequently, the content of hydroxycinnamic acids increased 3.5-fold due to BMSB infestation. The total content of the analyzed phenolics was higher in BMSB injury sites by 11.92 g/kg and 11.73 g/kg FW compared with the control and uninjured part of injured fruits, respectively.

Table 3. Individual and total phenolic contents (g/kg FW, mean ± SE) in apples.

| Phenolic Compound | Control | BMSB Injury Site | Uninjured Part of BMSB Apple |
|-------------------|---------|------------------|-----------------------------|
| Procyanidin B1    | 0.14 ± 0.02 b | 1.09 ± 0.27 a    | 0.23 ± 0.06 b               |
| Procyanidin B2    | 4.39 ± 0.30 b | 8.37 ± 1.40 a    | 4.71 ± 0.44 b               |
| Catechin          | 0.61 ± 0.10 b | 3.55 ± 0.62 a    | 0.52 ± 0.06 b               |
| Epicatechin       | 1.88 ± 0.09 a | 2.36 ± 0.35 a    | 2.09 ± 0.16 a               |
| Flavanols         | 7.03 ± 0.46 b | 15.39 ± 2.51 a   | 7.56 ± 0.66 b               |
| Chlorogenic acid  | 1.27 ± 0.07 b | 4.43 ± 0.41 a    | 1.27 ± 0.09 b               |
| 4-O-p-coumaroylquinic acid | 0.15 ± 0.02 b | 0.35 ± 0.06 a    | 0.22 ± 0.06 a               |
| Hydroxycinnamic acids | 1.42 ± 0.08 b | 4.79 ± 0.47 a    | 1.50 ± 0.14 b               |
| Phloretin-2-O-xylosylglucoside | 0.80 ± 0.17 a | 0.78 ± 0.29 a    | 0.64 ± 0.24 a               |
| Phlorizin         | 0.38 ± 0.04 a | 0.66 ± 0.14 a    | 0.36 ± 0.04 a               |
| Dihydrochalcones  | 1.19 ± 0.19 a | 1.44 ± 0.39 a    | 1.00 ± 0.28 a               |
| Quercetin-3-O-galactoside | 1.18 ± 0.27 a | 1.05 ± 0.39 a    | 0.91 ± 0.35 a               |
| Quercetin-3-O-glucoside | 0.75 ± 0.12 a | 0.81 ± 0.33 a    | 0.79 ± 0.31 a               |
| Quercetin-3-O-rhamnoside | 0.12 ± 0.03 a | 0.14 ± 0.03 a    | 0.12 ± 0.03 a               |
| Flavanols         | 2.06 ± 0.41 a | 2.01 ± 0.75 a    | 1.83 ± 0.70 a               |
| Total phenolic contents | 11.71 ± 1.01 b | 23.63 ± 3.95 a   | 11.90 ± 1.28 b |
| Total analyzed phenolics | 0.96 ± 0.04 b | 1.55 ± 0.23 a    | 1.02 ± 0.09 ab |

Different letters in a row denote statistically significant differences by Fisher’s Least Significant Difference (LSD) test at p < 0.05.

Plants synthesize phenolics as defense molecules against various biotic and abiotic stressors [29]. In our case, the response was concentrated in the area around the puncture wound, since the uninjured part of the BMSB-injured apple had similar phenolic levels to the control treatment, which is consistent with the findings of Eleftherianos et al. [30], who reported an increase in phenolics in aphid-infested Zea mays. A significant increase in flavanols was observed in BMSB injury sites compared with the other treatments. Similar results were also reported by Oszmiański et al. [31], who found an increase in procyanidin B1 and B2 in leaves of Aesculus parviflora Walt. and Aesculus glabra partially infested by leafminer (Cameraria ohridella). Flavanols such as procyanidin B1 commonly accumulate around the injured area in fruit [32]. Procyanidin polymers also act as feeding deterrents for Aphis craccivora (Koch) in groundnut [33]. Condensed tannins such as catechin act as antifeedants against some insects, such as Lymantria dispar L., Euproctis chrysorrhoea L. and Operophtera brumata L. [33], which would explain the significantly higher content in BMSB-injured sites in the apples. The content of hydroxycinnamic acids was higher in the apple part infested by stink bugs. Chlorogenic acid affects insect growth, as previously reported by Kundu and Vadassery [34], which would also be a defense mechanism.

4. Conclusions

The brown marmorated stink bug (Halyomorpha halys Stal.) has become an important pest in Slovenia and other parts of the world, especially in apple, pear, peach, and cherry
orchards. In our study, we evaluated the visual injury, sugar, organic acid, and phenolic content of the apple cultivar ‘Red Pinova’ of the area around the puncture wound, the uninjured part of the injured apple and, as a control, uninjured apples. We found a significant response of the apple around the puncture wound in content of individual sugars, organic acids, and especially phenols, showing that the area around the puncture wound is the most responsive to injury rather than the whole apple. There was a strong phenolic response in the puncture wound area. These results allow us to better understand how apple fruit responds to BMSB injury, not only sensorily (visual injury, odor), but also chemically in the form of metabolic responses. Based on market standards, infested apples are not marketable, resulting in large yield losses for farmers, thus raising awareness for monitoring and control of this invasive pest in apple orchards.

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