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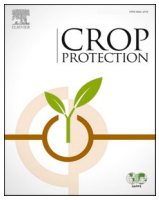

# Quantitative and qualitative damage caused by *Halyomorpha halys* (Hemiptera: Pentatomidae) on soybean crop at different growth stages

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ABSTRACT

The brown marmorated stink bug *Halyomorpha halys*, an invasive species native to East Asia, is now present and abundant on soybean throughout Europe where it has become a major pest damaging the crop. Because a better understanding of the impact of *H. halys* is crucial to implement effective and sustainable pest management, the present study aimed to assess the stages at which soybean is most susceptible to *H. halys* attacks, and the qualitative and quantitative alterations caused by its feeding on soybean seeds. Therefore, soybean plants were exposed to stink bug adults for 2 weeks at different development stages and were examined at harvest for damage. Stay-green syndrome occurred most severely as a result of *H. halys* attacks at soybean development stages R4-R5. In the same period, the bug feeding activity significantly reduced the number of seeds per pod, thus indicating a higher damage rate at the R4-R5 stage when soybean surveillance should be intensified in order to properly target pest management strategies. The lower number of seeds per pod corresponded to an increase in the seed weight due to plant compensation mechanisms, leading to grain yields that did not differ in relation to the time of attack or the rate of damaged seeds. However, while not causing overall a loss of weight production, *H. halys* attacks were shown to cause qualitative damage to soybean seeds, especially by altering protein content and mobilizing several primary metabolites from storage macromolecules, which will have to be considered depending on the intended use of the production.

## 1. Introduction

Soybean, *Glycine max* (L.) Merr. (Fabales: Fabaceae), is an important grain crop worldwide for its potential to produce high protein food and feed, providing approximately 10% of the global edible oil and 66% of the global protein for livestock (FAO, 2023). Modern soybean cultivars produce seeds that contain about 17% oil and 35% protein (Duan et al., 2023). Moreover, the soybean quality becomes even more important when it is produced for human consumption (e.g., edamame, soymilk). In this case, all factors that may affect the overall appearance of the product, such as discoloration and distortion of the seed, or the fat and protein quality, which could cause off-flavor quality specifications for the consumer, must be considered (McPherson et al., 2008). Some species belonging to Pentatomidae (Hemiptera), commonly known as stink bugs, are among the most harmful pests of soybean in producing areas worldwide, and can cause important losses in yield, quality, and germination potential of soybean (Corrêa-Ferreira and De Azevedo, 2002; Sardoy et al., 2021). They are piercing-sucking insects: to feed, they insert stylets into the plant tissue and inject watery saliva rich in enzymes. The extent of the damage varies depending on the target tissue, the feeding strategy, the enzymatic content of saliva, and the plant response (Giacometti et al., 2020; Panizzi et al., 2021a, 2021b).

The main damage on soybean is recorded when stink bugs feed on seeds, resulting in empty pods, or affecting seed quality and germination, or giving wrinkled and deformed seeds (Owens et al., 2013). Stink bugs use the cell rupture feeding strategy in seed endosperm, where lacerate and macerate-and-flush tactics are used simultaneously. As a result of these actions, the involved cells in the seed endosperm are destroyed mechanically (laceration due to movements of the stylets) and chemically (maceration due to digestive enzymes injected via watery saliva), allowing the degraded cell content to be ingested (Lucini and Panizzi, 2018; Panizzi et al., 2021a, 2021b). Stink bug feeding on soybean plants during the pod elongation and filling stages can cause a delay in plant senescence, resulting in stay-green leaves and stems at

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harvest, and consequently making harvesting operations difficult due to high plant moisture (Boethel et al., 2000; Vyavhare et al., 2015). This symptom complex is commonly referred as soybean stay-green syndrome (Rice et al., 2014; Wei et al., 2023), and can also be caused by other factors, both biotic and abiotic ones (Harbach et al., 2016; Cheng et al., 2002; Wang et al., 2022). However, insects are often reported among these factors, such as *Riptortus pedestris* (F.) (Hemiptera: Alydidae) in China (Li et al., 2019; Wei et al., 2023), and *Nezara viridula* (L.) and *Piezodorus guildinii* (Westwood) (Hemiptera: Pentatomidae) in Louisiana and in Texas, respectively (Boethel et al., 2000; Vyavhare et al., 2015).

Species composition and abundance of stink bugs on soybean were investigated especially in North and South America resulting in a variable complex of species depending on the survey area. In recent decades, the brown marmorated stink bug, *Halyomorpha halys* (Stål) (Hemiptera: Pentatomidae), has been added to the species already known on soybean; accidently introduced from Asia to various areas, including North America and Europe, it has become one of the dominant bug species causing significant economic losses on soybean crop (Hoebeke and Carter, 2003; Nielsen et al., 2011; Leskey and Nielsen, 2018). The establishment of the new species has been particularly serious for soybean crop in Europe, where before its arrival the stink bugs were not considered highly damaging pests.

Management of *H. halys* on soybean in Europe relies mainly on the application of the few broad-spectrum insecticides registered on the crop against this pest. Moreover, it should be considered that this species is highly polyphagous and moves widely from crops to wild plants and vice versa, following fruit presence and ripening (Leskey and Nielsen, 2018; Bosco et al., 2020). Therefore, its chemical control requires frequent insecticide applications which are not always effective, and lead to secondary pest outbreaks and disruption of integrated pest management practices now widely adopted (Masetti et al., 2023). Consequently, it is critical to know the appropriate timing for insecticide application to protect the crop from damage: targeted treatments when needed are more effective and safer for human and environmental health (Morrison et al., 2019; Leskey et al., 2020; Ribeiro et al., 2021).

To implement effective and sustainable pest management, it is essential to know at what time and what damage the feeding activity of *H. halys* causes on the soybean crop. Therefore, the objective of the present study was to assess i) the stages at which soybean is most susceptible to attacks by *H. halys*, and ii) the qualitative and quantitative alterations caused by *H. halys* feeding on soybean seeds.

## 2. Materials and Methods

### 2.1. Insect collection and rearing

In summer 2021 and 2022, *H. halys* nymphs were collected from different host plants in the Piedmont region, north-western Italy, and transferred to the laboratory where they were reared in net cages (930 × 475 × 475 mm; MegaView Science Co., Ltd, Taichung City, Taiwan). Broad bean (*Vicia faba* L.) seedlings, fresh fruit (e.g., apples), and shelled hazelnuts were provided and periodically replaced as food sources. Adults emerged in the laboratory rearing were used in field experiments. Mass rearing was performed in climatic chambers at 24 ± 1 °C and RH 65 ± 5%, with an L/D of 16:8 h.

### 2.2. Field trials

The experiment was carried out at the experimental Center of the University of Turin in Carmagnola (44°53′07″ N, 7°41′01″ E, 240 m a.s.l.), north-western Italy, in a field of 30 × 100 m of irrigated soybean crop during two growing seasons (2021 and 2022). Meteorological data were collected in an agrometeorological station located 150 m from the experimental field. The accumulation of the growing degree days (GDD) from the time the crop was sown, over a base temperature of 10 °C, was calculated using the following formula for each year: Σ[(Tmax + Tmin)/2], where Tmax and Tmin were the daily maximum and minimum temperatures, respectively, in °C; negative values were not included in the summation. The semideterminate soybean variety PR92M22 (Pioneer®, Corteva, Indianapolis, IN, USA), belonging to maturity Group I, was sown on June 10 and 9, in 2021 and 2022, respectively, and harvested on October 15 and 23, in 2021 and 2022, respectively. The crop was sowed at a plant density of 45 plant $m^{-2}$ in both years and resulted in an effective plant density of 35 and 32 plants $m^{-2}$ in 2021 and 2022, respectively. In both years, metribuzion 35% (Medor 35 Class, Gowam, Faenza, Italy) was applied for pre-emergence weed control at the label dose.

**Table 1**
Periods of bug insertion and removal inside cages and corresponding development stage of soybean in 2021–2022.

| Period | Insertion | | | Removal | | |
|---|---|---|---|---|---|---|
| | | Date | | | Date | |
| | Stage | 2021 | 2022 | Stage | 2021 | 2022 |
| R4-R5 | R4 | 17 Aug | 12 Aug | R5 | 31 Aug | 26 Aug |
| R5-R6 | R5 | 31 Aug | 26 Aug | R6 | 14 Sept | 9 Sept |
| R6-R7 | R6 | 14 Sept | 9 Sept | R7 | 28 Sept | 23 Sept |

To evaluate the effects of *H. halys* feeding on soybean, plants were exposed to insects by introducing them into cages at the following soybean stages of development (Fehr et al., 1971; Fehr and Caviness, 1977): R4 (full pod), R5 (beginning seed), and R6 (full seed), while other plants were never exposed to insects during the growing season as control. Soybean plants were exposed to the stink bugs during these development stages corresponding to the crop colonization by *H. halys*, that is, during pod and seed development (Nielsen et al., 2011). For this purpose, at the crop developmental stage V3–V4 (Fehr et al., 1971; Fehr and Caviness, 1977), i.e., on July 19 in 2021 and on July 23 in 2022, soybean plants grown on a surface of 0.25 $m^2$ (corresponding to 8–10 plants) were isolated into net cages to prevent possible bug attacks (28 cages per year). The cages consisted of a wooden frame (0.5 m width, 0.5 m length, and 1.5 m height), covered with anti-insect net (1 mm × 1 mm mesh), and equipped with an opening in the upper part closed with a hook-and-loop fastener. The perimeter of the cages was buried 0.3 m deep to provide stability and to prevent stink bugs from escaping, following Owens et al. (2013). The experiment was set up over a Randomized Complete Block Design (RCBD) in which the field was divided into seven blocks to obtain replications of each treatment (one in each block). In each year, the 28 cages were divided into seven blocks randomly distributed in the field. Each block included four cages, i.e., one cage per treatment, corresponding to the following treatments: exposure to *H. halys* feeding at the R4-R5 stage, at the R5-R6 stage, or at the R6-R7, or no exposure to *H. halys* feeding during the whole growing season (Control). Therefore, for each treatment (R4-R5 stage, R5-R6 stage, R6-R7 stage, Control), seven cages (i.e., replications) were used.

For each exposure period (R4-R5, R5-R6, R6-R7 stages), 20 *H. halys* adults emerged from laboratory colonies were introduced into one cage per block (20 adults $cage^{-1}$ × 7 cages = 140 adults $period^{-1}$), and were removed after two weeks (Table 1). At each bug removal, the dead adults were counted, all egg masses found in the cages were removed, and lambda-cyhalothrin (Karate Zeon 1.5, Syngenta, Basilea, Switzerland) was applied at the label dose into the cages by a costal sprayer. The insecticide was applied, and repeated at each survey, to eliminate any nymphs hatched from the eggs laid during the two-week period, which might have escaped visual inspection and could have affected the damage rate later in the season (not as 1st-instar nymphs that do not feed on the plant). The insecticide was applied at each survey also in the Control cages. At the harvest time, at full seed maturity (R8) on October 15, 2021, and October 23, 2022, all cages were removed and the whole plants were manually harvested, separately per each cage.

### 2.3. Plant and seed evaluations

All plants harvested inside the cages were transferred to the laboratory. For each cage, mature and still green plants were divided and counted. The plants that had not reached the point of maturity (R8) (Fehr et al., 1971; Fehr and Caviness, 1977) and exhibited evident delayed senescence compared with uninfested plants in the Control cages (Rice et al., 2014), i.e., that still had green leaves attached to the stem, green pods and green stems, were considered as green plants. Stems were separately weighed fresh and after oven drying at 80 °C for 48 h to evaluate dry matter (DM) content. Two fresh sub-samples from each cage of 50 pods (each corresponding to around 120 seeds) were examined for symptoms of stink bug damage (i.e., starting from the point of insertion of the stylets, darkened seeds, alterations of the seed tissue, such as whitish or brownish spots and/or wrinkled and deformed seeds), and healthy and damaged seeds were divided and counted. Furthermore, the weight of 1000 seeds was determined by randomly selecting seeds from each replicate. Moreover, three samples of 50 seeds damaged by *H. halys* (seeds showing symptoms from cages with insects) and three samples of 50 healthy ones (seeds from Control cages without insects) per each cage were weighted to assess the damage caused by the stink bug.

All the seed samples were weighted fresh and after oven drying at 80 °C for 48 h to evaluate DM content.

### 2.4. Compositional analyses of seeds

Samples of seeds damaged by *H. halys* (seeds showing symptoms from cages with insects) and of healthy ones (seeds from cages without insects) were analyzed each year for starch, fat, fibre, and protein content. The samples were oven-dried at 65 °C to constant weight, and air equilibrated. The air equilibrated samples were ground in a Cemotec laboratory grinder (CM290 Foss, Hilleroed, Denmark) to pass a 1 mm screen. The samples were analyzed for: i) total nitrogen (TN) (Dumas method, method number 992.23; AOAC, 2005), using a Primacs SN nitrogen analyser (Skalar, Breda, The Netherlands); ii) crude protein (CP) (total N × 6.25); iii) ether extract (EE) (Soxhlet method, method number 920.39; AOAC, 2005); iv) starch concentrations (method number 996.11; AOAC, 2005). Neutral detergent fibre (NDF) was analyzed using a Raw Fiber Extractor (FIWE, VELP Scientifica, Usmate Velate, Italy), with the addition of heat-stable amylase (A3306, Sigma Chemical Co., St. Louis, MO, USA), and was expressed on a DM basis, including residual ash, as described by Van Soest et al. (1991).

### 2.5. Primary and volatile metabolome profiling

Seeds damaged by *H. halys* (seeds showing symptoms from cages with insects) and healthy ones (seeds from cages without insects) were stored at −80 °C and they were analyzed for primary and volatile metabolites quali-quantitative distribution. Soybean primary metabolites (free amino acids, sugars, organic acids, and low molecular weight polar metabolites) were profiled by comprehensive two-dimensional gas chromatography coupled to time-of-flight mass spectrometry (GC × GC-TOF MS) after defatting, extraction, and derivatization according to an optimized protocol (Stilo et al., 2020). Polar metabolites were converted in trimethylsilyl derivatives and analyzed in a single run by GC × GC-TOF MS to establish their relative distribution within samples. Quality controls and internal standardization were adopted as strategies to check method performance parameters and normalize response data (Cialiè Rosso et al., 2020).

Soybean volatile metabolites were directly profiled on ground material (0.500 g) placed in 20 mL glass vials by headspace solid phase microextraction (HS-SPME) coupled to GC × GC-TOF MS. Sampling was optimized for temperature (40 °C) and time (50 min) to achieve a balanced coverage of all volatiles. The multi-component SPME device consisted in a 2 cm Divinylbenzene/Carboxen/Polydimethylsiloxane (DVB/CAR/PDMS) fiber of $d_f$ 50/30 μm from Merck (Milan, Italy). Analyses were carried out on a system equipped with a SPR auto sampler for GC (SepSolve-Analytical, Llantrisant, UK) installed on an Agilent 7890B GC chromatograph (Agilent Technologies, Wilmington, DE, USA) coupled with a Markes BenchTOF Select™ mass spectrometer featuring tandem ionization (Markes International, Llantrisant, UK).

For primary metabolites, the GC transfer line was set at 270 °C, the TOF MS was tuned for single ionization at 70 eV and the scan range was set between 45 and 650 m/z with a spectrum acquisition frequency of 100 Hz. The thermal modulator was a loop-type, two-stage KT 2004 (Zoex Corporation, Houston, TX, USA) cooled with liquid nitrogen and controlled by Optimoide, v2.0 (SRA Instruments, Cernusco sul Naviglio, Milan, Italy). The modulation period ($P_M$) was set at 3 s, while the hot-jet pulse duration was set at 350 ms. The cold-jet stream at the mass flow controller (MFC) was programmed to linearly reduce the total flow from 35% to 8% across the analytical run. The column set consisted of a $^1$D apolar column HP5 (5% phenyl-methylpolysiloxane; 60 m × 0.25 mm $d_c$ × 0.25 μm $d_f$) coupled with a $^2$D VF-17MS column (50% phenyl-methylpolysiloxane; 1.3 m × 0.10 mm $d_c$ × 0.10 μm $d_f$), both supplied by Agilent Technologies (Wilmington, DE, USA). A fused silica capillary loop (1.0 m × 0.1 mm $d_c$) was used in the modulator slit. SilTite™ μ-unions (Trajan Scientific and Medical, Melbourne, Australia) were used to connect the columns with the capillaries. The oven temperature program was set as follows: from 60 °C (2 min) to 120 °C at 10 °C min$^{-1}$ and to 300 °C (10 min) at 4 °C min$^{-1}$; the GC split/splitless injector port was set at 290 °C and operated in pulsed-split mode (250 kPa overpressure applied to the injection port until 2 min) with a 1:20 split ratio. Helium was used as the carrier gas at a nominal flow of 1.3 mL min$^{-1}$.

For volatile metabolites, the system was set as follows: GC transfer line 270 °C, the TOF MS operating in single ionization at 70 eV in a range 35–350 m/z with an acquisition frequency of 100 Hz. The $P_M$ was set at 3.5 s, and the hot-jet pulse was set at 300 ms. The MFC connected at the cold jet stream was programmed to linearly reduce the total flow from 40% to 8% across the analytical run. The column set consisted of a $^1$D HeavyWax™ column (100% polyethylene glycol – PEG; 30 m × 0.25 mm $d_c$ × 0.25 μm $d_f$) coupled with a $^2$D DB17 column (50% phenyl-methylpolysiloxane; 1.0 m × 0.10 mm $d_c$ × 0.10 μm $d_f$) supplied by Agilent Technologies. The fused silica capillary loop was the same as for primary metabolites setting. The oven program was set from 40 °C (2 min) to 260 °C (10 min) at 3.5 °C min$^{-1}$; the GC injector was set at 260 °C and operated in pulsed-split mode (250 kPa overpressure applied to the injection port until 2 min) with a 1:20 split ratio. Helium was used as the carrier gas at a nominal flow of 1.3 mL min$^{-1}$.

### 2.6. Statistical analyses

To evaluate plant and seed damage, percentage data were arcsine square-root-transformed. Obtained data were analyzed using a linear mixed model according to the following statistical model:

$$Y_{ijk} = \mu + T_i + B_j + P_k + \varepsilon_{ijk},$$

where μ is the overall mean, $T_i$ is the fixed effect of the bug insertion period treatment (i = 1 to 4), Bj is the effect of the block (j = 1 to 7), $P_k$ is the random effect of the year (k = 1 to 2), and $\varepsilon_{ijk}$ is the residual error. Significant effects were declared at $P < 0.05$. When the calculated values of F were significant, the Bonferroni post-hoc test ($P < 0.05$) was used to interpret any significant differences among the mean values.

For compositional analysis, differences between seeds damaged by *H. halys* and healthy ones were evaluated by means of unpaired *t*-test for weight of 50 seeds, starch, fat, neutral detergent fibre (NDF) and protein content after verification of the normal distribution of the data for each year of the trial. These statistical analyses were performed using the R software (R CoreTeam, 2020).

Metabolite distributions (2D chromatographic peaks absolute and % response indicators) were analyzed with non-parametric testing (i.e.,

**Table 2**
Weight of one stem, percentages of stem moisture and of green plants, with the insertion of *Halyomorpha halys* adults into the cages in the three periods (R4-R5, R5-R6, R6-R7) or no insertion of insects along the season (Control), in two-year semi-field trials. In each column, values followed by the same letter are not significantly different (Bonferroni test, $P < 0.05$, under linear mixed model procedure).

| Treatment | Weight of one stem (g) | Stem moisture (%) | Green plants (%) |
|---|---|---|---|
| R4-R5 | 8.81 | 59.93a | 37.08a |
| R5-R6 | 8.49 | 46.02b | 19.68b |
| R6-R7 | 7.93 | 42.09b | 19.01b |
| Control | 8.67 | 40.45b | 12.12b |
| P-value | 0.80 | <0.001 | <0.001 |
| SE | 0.43 | 2.16 | 9.39 |

SE: Standard error.

**Table 3**
Weight of 50 pods, weight of 1000 seeds, number of seeds per pod, rate of damaged seeds, and grain yield, with the insertion of *Halyomorpha halys* adults into the cages in the three periods (R4-R5, R5-R6, R6-R7) or no insertion of insects along the season (Control), in two-year semi-field trials. In each column, values followed by the same letter are not significantly different (Bonferroni test, $P < 0.05$, under linear mixed model procedure).

| Treatment | Weigh 50 pods (g) | Weight 1000 seeds (g) | N seeds per pod (g) | Damaged seeds (%) | Grain yield (kg DM/m$^2$) |
|---|---|---|---|---|---|
| R4-R5 | 22.5 | 169.7a | 2.14b | 16.32 | 0.286 |
| R5-R6 | 22.8 | 157.6b | 2.40a | 11.76 | 0.266 |
| R6-R7 | 23.4 | 161.2b | 2.40a | 9.44 | 0.284 |
| Control | 23.5 | 162.4 ab | 2.41a | – | 0.270 |
| P-value | 0.08 | <0.001 | <0.001 | 0.28 | 0.18 |
| SE | 0.17 | 1.88 | 0.03 | 1.41 | 0.005 |

For damaged seeds the statistical analysis was performed for the three treatments (R4-R5, R5-R6, R6-R7) excluding the Control with no damage.
DM: dry matter; SE: Standard error.

Kruskall-Wallis with Bonferroni correction and post-hoc Dunn's test), Fisher discriminant value (F value), and multivariate statistics (i.e., principal component analysis, PCA, and partial least-square discriminant analysis, PLS-DA) to highlight meaningful signatures in the metabolite distribution ascribable to the *H. halys* damage. Statistical analysis and chemometrics were performed using Matlab R2021a (The MathWorks, Inc., Natick, MA, USA) with the following packages: PCA toolbox (v1.5) (Ballabio, 2015) and classification toolbox (v6.0) (Ballabio and Consonni, 2013), and XLSTAT statistical and data analysis solution software (Addinsoft, 2020; New York, NY, USA).

## 3. Results

The two growing seasons, from June to October, were characterized by different climatic conditions: in 2021 the maximum temperature ranged from 9.6 to 33.8 °C, while in 2022 from 14.5 to 35.8 °C. The cumulative rainfall in these months was of 236.6 mm in 2021 and 153 mm in 2022, therefore, irrigation interventions were applied twice in 2021, while five times in 2022 because of both the high temperature and the drought. The meteorological data, the GDD for soybean and the amount of water through irrigation are reported in Supplementary Table 1. During trials, the survival of adult stink bugs did not appear to be strongly affected by the cages, and several females managed to oviposit, as evidenced by the egg masses found upon removal of the adults. Overall, *H. halys* adult mortality across treatments and replications was highly variable, with a mean value of 25.48% ± 3.01 between the two years and the three treatments (data not shown).

**Table 4**
Weight of 50 damaged and 50 healthy seeds and rate of starch, fat, neutron deterged fiber (NDF) and protein in samples of soybean seeds damaged by *Halyomorpha halys* and healthy ones, compared via *t*-test, $P < 0.05$.

| Sample | Seed weight (g) | Starch (%) | Fat (%) | NDF (%) | Protein (%) |
|---|---|---|---|---|---|
| damaged | 5.44b | 0.8 | 15.6 | 20.2 | 42.6a |
| healthy | 8.37a | 0.6 | 16.7 | 20.1 | 40.5b |
| P-value | <0.001 | ns | ns | ns | <0.05 |
| SE | 0.16 | 0.09 | 0.37 | 0.71 | 0.60 |

SE: Standard error.

### 3.1. Plant and seed damage evaluations

The results of stem weight, stem moisture and green plant percentages are shown in Table 2. No significant differences between the treatments were found for stem weight, while the presence of *H. halys* in the first period (R4-R5 stage) significantly increased the percentage of stem moisture and of green plants compared to values from the Control and the other two periods (R5-R6 and R6-R7 stages). The weight of 50 pods, weight of 1000 seeds, number of seeds per pod, rate of damaged seeds and grain yield are given in Table 3. No significant differences were found for the weight of 50 pods, whereas the weight 1000 of seeds was significantly lower when the bugs were inserted in the second and third periods (R5-R6 and R6-R7 stages) than in the first period (R4-R5 stage), but never significantly different from the Control. The significantly lower number of seeds per pod, corresponding to the higher weight of 1000 seeds, was observed when the bugs were caged on the soybean in the first period (R4-R5 stage) compared to number of seeds per pod and weight in the other two periods (R5-R6 and R6-R7 stages) as well as the Control. In contrast, no significant differences were found between the treatments in the percentage of damaged seeds and in the grain yield.

### 3.2. Compositional analyses of seeds

Because no significant differences between the years were found, the data were shown as the average between the two years (2021 and 2022). The weight of healthy seeds was significantly higher compared to the damaged seeds. No significant differences in starch, fat and NDF content in percentage were found between damaged and healthy seeds, while the protein content was significantly higher in damaged seeds (Table 4).

### 3.3. Primary and volatile metabolome profiling

The detectable metabolome (primary metabolites and volatiles) showed significant differences, in terms of overall semi-quantitative distribution, between healthy and damaged soybean seeds.

#### 3.3.1. Primary metabolome changes

Detectable features in the primary metabolome fraction (signal-to-noise ratio S/N > 150) were 657, of which 72 were putatively identified by combining retention data (i.e., linear retention index $I^T$ along the $^1$D ± 10 units compared to NIST reference) and EI fragmentation pattern similarity (against reference spectra collected in the NIST, 2022 database) (i.e., direct match factor DMF > 950). Table 5 lists primary metabolites detected in the soybean extracts together with their $^1$D and $^2$D retention times, % relative standard deviation (% RSD) on retention times, experimental and tabulated $I^T$s, and mean 2D peak responses (absolute and % response) accompanied by F value (Fisher discriminant ratio – classes damaged *vs*. healthy). For 585 features, it was possible to confidently track their presence across samples thanks to their retention times ($^1t_R$ and $^2t_R$) in the two chromatographic dimensions and mass spectral similarity above a DMF of 750 (Reichenbach et al., 2019; Cialiè Rosso et al., 2020). Untargeted features are listed in Supplementary Table 2 together with $^1$D and $^2$D retention times, % relative standard

**Table 5**
List of primary metabolites detected in the sample extracts together with their $^{1}$D retention time ($^{1}t_R$), $^{2}$D retention time ($^{2}t_R$), experimental retention index (Exp. $I^T$), and reference retention index (Ref. $I^T$) according to NIST database. RSD% (% relative standard deviation refers to the n = 24 replicated samples). TMS = trimethylsilyl derivative.

| Compound Name | $^{1}$D | | $^{2}$D | | $^{1}I^T$ | | 2D peak response | | 2D peak % response | |
|---|---|---|---|---|---|---|---|---|---|---|
| | $^{1}t_R$ (min) | % RSD | $^{2}t_R$ (sec) | % RSD | Exp. $I^T$ | Ref. $I^T$ | Mean | F Value | Mean | F Value |
| 3-Pyridinol TMS | 15.1 | 0.05 | 0.8 | 4.67 | 1032 | 1034 | 8.63E+06 | 0.1 | 7.95E-02 | 6.8 |
| Diacetone alcohol TMS | 15.3 | 0.03 | 0.7 | 7.51 | 1036 | 1040 | 3.24E+05 | 1.2 | 2.99E-03 | 0.1 |
| Lactic Acid 2TMS | 15.9 | 0.00 | 0.6 | 4.55 | 1051 | 1057 | 1.28E+07 | 5.1 | 1.17E-01 | 2.7 |
| Glycolic acid 2TMS | 16.6 | 0.03 | 0.7 | 7.28 | 1068 | 1072 | 1.10E+06 | 0.2 | 1.01E-02 | 0.2 |
| Pyruvic acid, 2TMS | 16.9 | 0.03 | 0.6 | 4.17 | 1098 | 1110 | 1.73E+06 | 1.0 | 1.59E-02 | 2.7 |
| Alanine 2TMS | 17.9 | 0.00 | 0.6 | 5.10 | 1098 | 1110 | 1.07E+06 | 0.0 | 9.84E-03 | 1.7 |
| Oxalic acid 2TMS | 19.2 | 0.03 | 0.9 | 3.16 | 1123 | 1125 | 2.71E+07 | 6.2 | 2.49E-01 | 8.1 |
| Leucine TMS | 20.6 | 0.05 | 0.8 | 5.32 | 1151 | 1155 | 8.61E+06 | 3.5 | 7.93E-02 | 5.7 |
| Isoleucine TMS | 21.6 | 0.08 | 1.1 | 4.14 | 1173 | 1178 | 1.40E+06 | 3.7 | 1.29E-02 | 4.1 |
| Phenol, 4-methyl, TMS | 21.7 | 0.00 | 0.6 | 1.61 | 1150 | 1153 | 2.35E+06 | 9.1 | 2.04E-02 | 10.2 |
| Malonic acid 2TMS | 22.9 | 0.00 | 0.9 | 2.52 | 1197 | 1205 | 7.61E+05 | 2.6 | 7.01E-03 | 0.2 |
| Valine 2TMS | 23.5 | 0.00 | 0.7 | 6.50 | 1210 | 1208 | 6.37E+06 | 2.9 | 5.87E-02 | 7.2 |
| Benzoic Acid TMS | 25.3 | 0.00 | 1.2 | 4.81 | 1244 | 1248 | 8.43E+05 | 3.5 | 7.76E-03 | 5.6 |
| Glycerol 3TMS | 26.5 | 0.00 | 0.6 | 6.75 | 1268 | 1266 | 2.83E+07 | 0.0 | 2.60E-01 | 1.6 |
| Threonine 2TMS | 27.4 | 0.02 | 0.7 | 4.83 | 1286 | 1292 | 2.37E+05 | 0.2 | 2.18E-03 | 0.1 |
| Proline 2TMS | 27.6 | 0.03 | 0.8 | 3.94 | 1289 | 1282 | 3.15E+06 | 0.0 | 2.90E-02 | 0.6 |
| Glycine 3TMS | 28.3 | 0.11 | 0.7 | 5.77 | 1302 | 1310 | 2.80E+06 | 1.5 | 2.58E-02 | 3.3 |
| Succinic acid 2TMS | 28.6 | 0.10 | 1.0 | 3.98 | 1308 | 1313 | 2.01E+07 | 8.1 | 1.85E-01 | 11.8 |
| Fumaric acid 2TMS | 30.5 | 0.00 | 0.9 | 4.04 | 1345 | 1348 | 1.61E+07 | 7.3 | 1.48E-01 | 12.3 |
| Threonine 3TMS | 32.3 | 0.06 | 0.8 | 1.22 | 1380 | 1367 | 1.23E+06 | 1.5 | 1.13E-02 | 5.8 |
| 2-Deoxyribose 3TMS | 33.2 | 0.11 | 0.9 | 4.09 | 1397 | 1404 | 1.82E+07 | 0.5 | 1.67E-01 | 0.3 |
| Aspartic acid 2TMS | 34.2 | 0.04 | 1.1 | 7.33 | 1419 | 1413 | 1.26E+07 | 7.2 | 1.16E-01 | 23.2 |
| Erythrose 3TMS | 34.6 | 0.03 | 0.8 | 1.36 | 1426 | 1431 | 8.52E+06 | 0.5 | 7.85E-02 | 3.9 |
| Decanoic acid TMS | 36.0 | 0.04 | 0.9 | 3.88 | 1455 | 1450 | 3.05E+05 | 0.0 | 2.81E-03 | 0.3 |
| Malic acid 3TMS | 37.4 | 0.02 | 0.9 | 9.45 | 1483 | 1480 | 1.07E+08 | 0.4 | 9.90E-01 | 3.2 |
| Erythritol 4TMS | 38.0 | 0.02 | 0.7 | 4.23 | 1496 | 1505 | 1.04E+06 | 3.8 | 9.64E-03 | 9.5 |
| Hexanedioic acid 2TMS | 38.2 | 0.00 | 1.0 | 4.22 | 1500 | 1498 | 6.02E+05 | 0.0 | 5.54E-03 | 0.7 |
| Deoxyribopyranose 3TMS | 38.4 | 0.08 | 0.7 | 3.48 | 1504 | 1502 | 5.48E+05 | 0.8 | 5.05E-03 | 3.9 |
| 5-Oxoproline 2TMS | 38.9 | 0.00 | 1.3 | 4.05 | 1516 | 1520 | 1.06E+07 | 3.4 | 9.74E-02 | 11.1 |
| Aspartic acid 3TMS | 39.4 | 0.03 | 0.8 | 4.21 | 1527 | 1522 | 3.26E+06 | 1.4 | 3.00E-02 | 7.9 |
| Pyroglutamic acid 2TMS | 39.4 | 0.06 | 1.1 | 4.21 | 1527 | 1521 | 6.96E+06 | 2.5 | 6.41E-02 | 12.1 |
| Phenylalanine TMS | 40.7 | 0.00 | 0.8 | 4.43 | 1555 | 1559 | 2.13E+06 | 0.2 | 1.96E-02 | 0.0 |
| α-Hydroxyglutaric acid 3TMS | 41.4 | 0.07 | 0.9 | 4.17 | 1569 | 1576 | 4.66E+05 | 2.5 | 4.29E-03 | 3.6 |
| β-Hydroxy-β-methylglutaric acid 3TMS | 42.4 | 0.00 | 0.8 | 2.07 | 1596 | 1606 | 1.60E+06 | 0.8 | 1.48E-02 | 0.0 |
| Ornithine 3TMS | 42.6 | 0.03 | 1.1 | 4.37 | 1607 | 1610 | 1.65E+06 | 0.7 | 1.52E-02 | 0.3 |
| Ribofuranose 4TMS | 43.0 | 0.00 | 0.8 | 1.02 | 1610 | 1625 | 4.03E+06 | 1.5 | 3.64E-02 | 1.2 |
| Glutamic acid 3TMS | 43.2 | 0.22 | 1.0 | 5.28 | 1612 | 1626 | 2.35E+07 | 1.5 | 2.16E-01 | 6.9 |
| Dodecanoic acid TMS | 44.5 | 0.04 | 0.8 | 4.30 | 1648 | 1650 | 3.05E+05 | 0.4 | 2.81E-03 | 2.4 |
| Xylitol 5TMS | 47.4 | 0.04 | 0.7 | 1.32 | 1715 | 1710 | 5.67E+06 | 3.6 | 5.17E-02 | 3.1 |
| Citric acid 4TMS | 51.1 | 0.03 | 0.9 | 3.59 | 1811 | 1816 | 6.26E+07 | 0.2 | 5.77E-01 | 0.0 |
| Pinitol 5TMS | 51.6 | 0.00 | 0.7 | 3.94 | 1827 | 1826 | 1.68E+05 | 1.5 | 1.54E-03 | 1.4 |
| Myristic acid TMS | 52.2 | 0.00 | 1.0 | 3.35 | 1844 | 1840 | 2.45E+06 | 2.1 | 2.26E-02 | 2.3 |
| 3-Deoxyhexonic acid 4TMS | 52.6 | 0.06 | 0.8 | 1.59 | 1854 | 1855 | 4.46E+07 | 0.1 | 4.16E-01 | 0.7 |
| anti-Fructose 5TMS | 53.0 | 0.04 | 0.8 | 4.30 | 1866 | 1867 | 1.57E+07 | 14.4 | 1.46E-01 | 20.7 |
| syn-Fructose 5TMS | 53.4 | 0.07 | 0.8 | 4.55 | 1876 | 1878 | 1.93E+08 | 26.6 | 1.80E+00 | 35.0 |
| Galactose 5TMS | 53.7 | 0.00 | 0.8 | 3.38 | 1889 | 1896 | 6.30E+07 | 1.3 | 5.80E-01 | 10.8 |
| Glucose 5TMS | 53.9 | 0.03 | 0.8 | 4.30 | 1891 | 1897 | 2.36E+08 | 11.5 | 2.19E+00 | 32.1 |
| Mannitol 6TMS | 55.3 | 0.04 | 0.7 | 1.22 | 1932 | 1928 | 1.19E+07 | 4.6 | 1.10E-01 | 35.9 |
| Pentadecanoic acid TMS | 55.7 | 0.04 | 1.0 | 1.21 | 1943 | 1943 | 1.26E+06 | 2.6 | 1.16E-02 | 2.8 |
| Scyllo-inositol 6TMS | 56.0 | 0.03 | 0.7 | 6.18 | 1952 | 1972 | 1.81E+08 | 0.6 | 1.68E+00 | 4.1 |
| Glucono-1,4-lactone 4TMS | 56.7 | 0.08 | 0.8 | 3.60 | 1972 | 1980 | 2.62E+07 | 0.7 | 2.41E-01 | 0.7 |
| Gluconic acid 6TMS | 57.4 | 0.00 | 0.8 | 7.80 | 1995 | 1997 | 2.06E+07 | 12.6 | 1.89E-01 | 15.2 |
| Galactaric acid 6TMS | 58.8 | 0.00 | 0.8 | 4.40 | 2037 | 2050 | 1.47E+07 | 2.0 | 1.35E-01 | 5.8 |
| Palmitic acid TMS | 58.9 | 0.13 | 1.1 | 3.25 | 2041 | 2041 | 1.05E+07 | 0.9 | 9.69E-02 | 3.6 |
| Myo-inositol 6TMS | 60.4 | 0.00 | 0.8 | 2.60 | 2087 | 2096 | 7.48E+07 | 1.8 | 6.92E-01 | 22.6 |
| 3-Deoxyarabino-hexaric acid 5TMS | 60.8 | 0.04 | 0.9 | 4.34 | 2100 | 2092 | 3.08E+07 | 0.1 | 2.84E-01 | 2.8 |
| (Z,Z)-9,12-Octadecadienoic acid TMS | 64.0 | 0.04 | 1.2 | 9.70 | 2206 | 2208 | 2.36E+07 | 0.7 | 2.18E-01 | 0.3 |
| (Z)-9-Octadecenoic acid TMS | 64.2 | 0.13 | 1.2 | 4.61 | 2212 | 2215 | 1.05E+07 | 0.9 | 9.69E-02 | 3.6 |
| (E)-9-Octadecenoic acid TMS | 64.3 | 0.08 | 1.2 | 5.67 | 2218 | 2217 | 2.74E+06 | 1.3 | 5.00E-02 | 3.1 |
| Stearic acid TMS | 64.9 | 0.00 | 1.1 | 2.94 | 2238 | 2239 | 7.94E+05 | 0.6 | 7.31E-03 | 0.5 |
| Tryptophan 2TMS | 65.3 | 0.02 | 1.1 | 1.38 | 2260 | 2257 | 2.98E+05 | 2.7 | 2.75E-03 | 11.4 |
| Threitol 4TMS | 66.8 | 0.05 | 1.1 | 1.13 | 2302 | 2304 | 1.94E+06 | 0.2 | 1.78E-02 | 0.7 |
| Galacturonic acid 5TMS | 68.4 | 0.00 | 1.0 | 3.06 | 2363 | 2370 | 1.67E+07 | 0.0 | 1.54E-01 | 0.1 |
| 1-Monopalmitin, 2TMS derivative | 73.2 | 0.04 | 2.1 | 4.41 | 2600 | 2606 | 2.53E+06 | 11.4 | 2.32E-02 | 11.1 |
| Sucrose 8TMS | 75.4 | 0.14 | 1.0 | 7.60 | 2627 | 2623 | 1.50E+09 | 6.1 | 1.39E+01 | 0.3 |
| 2-α-Mannobiose 8TMS | 77.0 | 0.00 | 1.0 | 5.34 | 2693 | 2700 | 2.58E+08 | 0.0 | 2.39E+00 | 4.5 |
| 3-α-Mannobiose 8TMS | 77.5 | 0.05 | 1.0 | 3.63 | 2715 | 2722 | 2.97E+06 | 0.1 | 2.74E-02 | 0.0 |
| Glycerol monostearate 2TMS | 78.9 | 0.00 | 1.2 | 4.36 | 2798 | 2806 | 4.54E+07 | 0.7 | 4.22E-01 | 9.0 |
| Catechin 5TMS | 80.5 | 0.08 | 1.1 | 7.41 | 2871 | 2861 | 1.40E+06 | 1.3 | 1.29E-02 | 0.4 |
| Galactinol 9TMS | 81.3 | 0.00 | 0.9 | 2.23 | 2924 | 2926 | 8.33E+06 | 0.8 | 7.67E-02 | 0.3 |
| Maltose 8TMS | 84.0 | 0.02 | 1.1 | 4.23 | 3062 | 2748 | 3.55E+07 | 10.4 | 3.31E-01 | 25.0 |
| β-Sitosterol TMS | 88.2 | 0.13 | 2.1 | 4.10 | 3246 | 3249 | 2.37E+05 | 0.4 | 2.18E-03 | 2.0 |

**Table 6**
List of volatile metabolites detected in the sample headspace together with their $^1$D retention time ($^1t_R$), $^2$D retention time ($^2t_R$), experimental retention index (Exp. $I^T$), and reference retention index (Ref. $I^T$) according to NIST database. RSD% (% relative standard deviation refers to the n = 24 replicated samples). Fisher discriminant value refers to class analysis (damaged *vs.* healthy). A variable is considered significant for the class discrimination if the $F_{calc} > F_{crit}$, with $F_{crit}$ (2,12) = 6.927 (α = 0.01).

| Compound Name | $^1$D | | $^2$D | | $^1I^T$ | | 2D peak response | | 2D peak % response | |
|---|---|---|---|---|---|---|---|---|---|---|
| | $^1t_R$ *(min)* | *RSD %* | $^2t_R$ *(sec)* | *RSD %* | Exp. | Ref. | Mean | F Value | Mean | F Value |
| Carbon disulfide | 3.13 | 0.27 | 0.86 | 0.90 | >700 | 745 | 7.43E+05 | 13.07 | 0.03 | 179.0 |
| Dimethyl sulfide | 3.33 | 0.00 | 0.68 | 0.59 | >700 | 777 | 1.88E+06 | 110.70 | 0.07 | 28.7 |
| Octane | 3.92 | 0.00 | 1.18 | 0.57 | 800 | 800 | 1.29E+06 | 26.98 | 0.05 | 13.1 |
| 2-Propanone | 4.08 | 0.00 | 0.63 | 0.70 | 817 | 821 | 2.60E+08 | 0.47 | 10.67 | 4.4 |
| 1-Octene | 4.50 | 0.00 | 1.17 | 1.22 | 839 | 842 | 2.62E+05 | 48.56 | 0.01 | 11.4 |
| Furan, 2-methyl- | 5.33 | 0.00 | 0.73 | 1.18 | 843 | 846 | 6.21E+06 | 1.25 | 0.28 | 27.7 |
| Acetic acid, ethyl ester | 5.37 | 0.22 | 0.78 | 1.05 | 851 | 854 | 1.57E+06 | 20.20 | 0.06 | 3.1 |
| 2-Butanone | 5.58 | 0.00 | 0.76 | 0.95 | 906 | 909 | 2.74E+06 | 4.67 | 0.12 | 16.8 |
| Butanal, 2-methyl | 5.88 | 0.27 | 0.88 | 1.20 | 914 | 916 | 7.36E+05 | 15.53 | 0.03 | 15.8 |
| Butanal, 3-methyl | 6.00 | 0.00 | 0.85 | 0.85 | 928 | 929 | 7.54E+05 | 13.41 | 0.03 | 15.3 |
| 2-Propanol | 6.20 | 0.19 | 0.57 | 1.10 | 930 | 927 | 1.49E+07 | 14.10 | 0.69 | 32.7 |
| Ethanol | 6.36 | 0.47 | 0.55 | 2.07 | 937 | 932 | 2.66E+08 | 14.30 | 11.26 | 3.1 |
| Furan, 2-ethyl | 6.88 | 0.00 | 0.90 | 0.57 | 952 | 950 | 2.00E+06 | 54.25 | 0.08 | 67.3 |
| 2-Pentanone | 7.59 | 0.19 | 0.90 | 0.59 | 972 | 975 | 1.42E+07 | 109.53 | 0.54 | 66.7 |
| Acetonitrile | 8.34 | 0.10 | 0.60 | 0.55 | 1001 | 997 | 1.80E+07 | 0.07 | 0.79 | 47.2 |
| 1-Penten-3-one | 8.87 | 0.10 | 0.85 | 0.80 | 1018 | 1019 | 8.69E+05 | 26.80 | 0.03 | 10.9 |
| 2-Butanol | 8.97 | 0.18 | 0.62 | 1.60 | 1039 | 1041 | 6.65E+05 | 14.70 | 0.03 | 44.2 |
| Toluene | 9.38 | 0.09 | 1.03 | 0.51 | 1053 | 1056 | 4.78E+06 | 0.32 | 0.21 | 3.7 |
| 2-Butenal, 2-methyl, (2E) | 10.32 | 0.34 | 0.91 | 3.41 | 1089 | 1092 | 7.98E+05 | 17.97 | 0.03 | 11.4 |
| Hexanal | 10.92 | 0.08 | 1.00 | 0.60 | 1095 | 1098 | 6.50E+07 | 26.37 | 2.21 | 31.8 |
| 1-Propanol, 2-methyl- | 11.49 | 0.40 | 0.60 | 8.53 | 1099 | 1101 | 2.94E+06 | 1.86 | 0.13 | 0.7 |
| 2-Propanone, 1-methoxy | 11.68 | 0.36 | 0.74 | 6.20 | 1103 | 1104 | 6.03E+05 | 111.94 | 0.02 | 65.4 |
| 3-Pentanol | 11.74 | 0.38 | 0.57 | 0.94 | 1107 | 1110 | 1.34E+07 | 147.52 | 0.54 | 28.0 |
| 2-Pentanol | 12.40 | 0.47 | 0.66 | 7.93 | 1126 | 1121 | 1.50E+06 | 5.42 | 0.06 | 0.2 |
| 2-Pentenal, (E)- | 12.63 | 0.42 | 0.84 | 1.03 | 1129 | 1127 | 3.25E+06 | 66.67 | 0.12 | 63.7 |
| 2-Propanol, 1-methoxy- | 12.72 | 0.44 | 0.65 | 11.09 | 1130 | 1133 | 3.84E+07 | 6.96 | 1.56 | 1.4 |
| Ethylbenzene | 12.82 | 0.43 | 1.22 | 0.65 | 1132 | 1129 | 2.97E+05 | 1.23 | 0.01 | 5.9 |
| 1-Penten-3-ol | 13.75 | 0.27 | 0.61 | 5.22 | 1161 | 1164 | 3.31E+06 | 4.03 | 0.14 | 0.0 |
| 1-Butanol | 14.17 | 0.53 | 0.60 | 2.89 | 1148 | 1146 | 5.35E+06 | 3.27 | 0.19 | 2.3 |
| α-Phellandrene | 14.27 | 0.10 | 1.82 | 0.72 | 1157 | 1160 | 4.56E+05 | 1.92 | 0.02 | 75.8 |
| Benzene, 1,3-dimethyl- | 14.35 | 0.39 | 1.17 | 1.19 | 1147 | 1143 | 3.58E+05 | 0.56 | 0.02 | 9.3 |
| 2-Heptanone | 14.53 | 0.24 | 1.05 | 0.90 | 1183 | 1184 | 7.98E+06 | 314.46 | 0.28 | 374.0 |
| Limonene | 14.54 | 0.26 | 1.61 | 4.35 | 1197 | 1200 | 5.36E+05 | 1.15 | 0.02 | 1.1 |
| 1-Butanol, 2-methyl | 15.41 | 0.29 | 0.62 | 7.09 | 1205 | 1208 | 3.34E+07 | 10.12 | 1.39 | 0.4 |
| 1,8-Cineole | 15.44 | 0.23 | 1.76 | 1.12 | 1211 | 1213 | 5.40E+05 | 0.01 | 0.02 | 29.3 |
| 2-Hexenal, (E)- | 15.83 | 0.05 | 0.89 | 0.57 | 1217 | 1220 | 2.22E+07 | 51.57 | 0.79 | 51.2 |
| 2-Hexanol | 15.93 | 0.16 | 0.68 | 2.66 | 1223 | 1226 | 7.10E+05 | 0.44 | 0.04 | 0.8 |
| Furan, 2-pentyl- | 16.26 | 0.26 | 1.24 | 2.01 | 1232 | 1235 | 5.25E+06 | 49.80 | 0.19 | 58.0 |
| Hexanoic acid, ethyl ester | 16.51 | 0.25 | 1.26 | 3.19 | 1234 | 1236 | 4.25E+05 | 144.09 | 0.02 | 97.5 |
| γ-Terpinene | 16.66 | 0.16 | 1.59 | 2.89 | 1245 | 1246 | 3.89E+05 | 0.16 | 0.02 | 13.5 |
| 1-Pentanol | 16.94 | 0.61 | 0.61 | 1.09 | 1253 | 1250 | 5.34E+07 | 208.18 | 2.03 | 395.1 |
| 3-Octanone | 17.19 | 0.36 | 1.16 | 1.92 | 1261 | 1256 | 1.47E+08 | 52.80 | 5.92 | 3.0 |
| p-Cymene | 17.60 | 0.26 | 1.36 | 1.16 | 1269 | 1272 | 1.43E+06 | 4.10 | 0.06 | 3.8 |
| Acetic acid, hexyl ester | 17.91 | 0.18 | 1.15 | 0.75 | 1275 | 1278 | 2.20E+06 | 665.93 | 0.08 | 97.0 |
| 2-Octanone | 18.28 | 0.15 | 1.08 | 1.49 | 1286 | 1287 | 9.39E+06 | 703.16 | 0.34 | 160.6 |
| 2-Butanone, 3-hydroxy- (Acetoin) | 18.37 | 0.25 | 0.59 | 1.43 | 1287 | 1284 | 2.36E+06 | 19.03 | 0.08 | 19.4 |
| Octanal | 18.44 | 0.11 | 1.12 | 1.29 | 1294 | 1289 | 5.22E+05 | 31.94 | 0.02 | 38.7 |
| 4-Penten-1-ol | 18.54 | 0.27 | 0.61 | 1.26 | 1297 | 1299 | 7.10E+05 | 13.98 | 0.03 | 1.3 |
| 3-Heptanol | 18.57 | 0.26 | 0.74 | 3.98 | 1292 | 1290 | 2.03E+05 | 46.73 | 0.01 | 33.5 |
| 1-Octen-3-one | 18.84 | 0.22 | 1.02 | 1.22 | 1297 | 1300 | 1.76E+07 | 23.11 | 0.62 | 23.3 |
| 6-Octen-2-one, (Z)- | 19.09 | 0.18 | 1.01 | 0.87 | 1313 | 1316 | 3.57E+06 | 213.82 | 0.13 | 65.9 |
| 2-Penten-1-ol, (E)- | 19.11 | 0.11 | 0.58 | 1.96 | 1316 | 1312 | 5.33E+05 | 2.00 | 0.02 | 9.6 |
| 2-Penten-1-ol, (Z)- | 19.38 | 0.07 | 0.59 | 1.53 | 1317 | 1318 | 3.51E+05 | 6.61 | 0.01 | 0.8 |
| 2-Heptanol | 19.42 | 0.04 | 0.70 | 2.57 | 1317 | 1320 | 3.47E+06 | 66.87 | 0.13 | 30.2 |
| 2-Heptenal, (E)- | 19.66 | 0.18 | 0.93 | 0.81 | 1321 | 1323 | 6.34E+06 | 22.55 | 0.22 | 26.3 |
| 2-Hexenal, 2-ethyl- | 20.28 | 0.18 | 1.13 | 1.01 | 1330 | 1333 | 6.87E+05 | 594.96 | 0.02 | 192.2 |
| 5-Hepten-2-one, 6-methyl- | 20.32 | 0.06 | 1.00 | 2.87 | 1335 | 1338 | 3.71E+05 | 104.94 | 0.01 | 87.6 |
| 1-Hexanol | 20.58 | 0.20 | 0.63 | 0.57 | 1353 | 1355 | 5.45E+08 | 399.53 | 20.75 | 335.2 |
| 3-Hexen-1-ol, (E)- | 20.91 | 0.13 | 0.63 | 1.61 | 1366 | 1367 | 6.96E+06 | 72.64 | 0.28 | 2.5 |
| Nonanal | 22.00 | 0.06 | 1.13 | 2.19 | 1384 | 1387 | 9.26E+05 | 19.95 | 0.03 | 25.1 |
| 3-Hexen-1-ol, (Z)- | 22.06 | 0.12 | 0.62 | 0.89 | 1385 | 1382 | 1.27E+07 | 46.62 | 0.53 | 1.3 |
| 3-Octanol | 22.10 | 0.04 | 0.76 | 0.53 | 1390 | 1393 | 3.04E+07 | 16.55 | 1.41 | 71.2 |
| 2-Hexen-1-ol, (Z)- | 22.11 | 0.15 | 0.67 | 2.27 | 1392 | 1395 | 2.45E+05 | 14.13 | 0.01 | 0.2 |
| 2-Nonanone | 22.22 | 0.12 | 1.09 | 2.44 | 1395 | 1390 | 5.41E+05 | 210.45 | 0.02 | 49.3 |
| 2-Hexen-1-ol, (E)- | 22.34 | 0.08 | 0.62 | 0.89 | 1402 | 1405 | 5.18E+06 | 148.76 | 0.21 | 5.5 |
| 3-Octen-2-one | 22.53 | 0.12 | 0.96 | 0.94 | 1409 | 1411 | 4.16E+06 | 37.91 | 0.14 | 57.3 |
| 2-Hexen-1-ol, (Z)- | 22.63 | 0.13 | 0.60 | 2.03 | 1415 | 1416 | 2.91E+05 | 65.40 | 0.01 | 0.2 |
| 2-Octanol | 22.84 | 0.05 | 0.72 | 1.23 | 1415 | 1412 | 3.24E+06 | 63.79 | 0.12 | 27.5 |
| 2-Octenal, (E)- | 23.30 | 0.15 | 0.95 | 2.30 | 1434 | 1432 | 3.68E+06 | 32.44 | 0.13 | 46.8 |
| α-Thujone | 23.45 | 0.22 | 1.17 | 1.22 | 1435 | 1430 | 1.05E+08 | 10.87 | 4.48 | 7.6 |

(*continued on next page*)

**Table 6** (*continued*)

| Compound Name | $^1$D | | $^2$D | | $^1I^T$ | | 2D peak response | | 2D peak % response | |
|---|---|---|---|---|---|---|---|---|---|---|
| | $^1t_R$ *(min)* | *RSD %* | $^2t_R$ *(sec)* | *RSD %* | Exp. | Ref. | Mean | F Value | Mean | F Value |
| β-thujone | 23.80 | 0.07 | 1.13 | 1.55 | 1441 | 1444 | 7.42E+06 | 5.02 | 0.32 | 8.5 |
| 1-Octen-3-ol | 23.89 | 0.10 | 0.66 | 1.42 | 1449 | 1450 | 3.29E+08 | 99.28 | 15.41 | 528.1 |
| Acetic acid | 23.98 | 0.15 | 0.43 | 1.09 | 1453 | 1449 | 1.67E+07 | 11.27 | 0.69 | 0.0 |
| 4-Hepten-1-ol | 24.21 | 0.00 | 0.69 | 0.63 | 1484 | 1487 | 1.44E+06 | 1.52 | 0.06 | 30.3 |
| Decanal | 24.34 | 0.03 | 0.96 | 5.17 | 1488 | 1490 | 2.81E+05 | 53.31 | 0.01 | 24.8 |
| 2-Octynoic acid | 24.38 | 0.10 | 0.80 | 1.60 | 1496 | 1499 | 2.71E+05 | 34.83 | 0.01 | 38.0 |
| 3,5-Octadien-2-one, (E,E)- | 25.08 | 0.08 | 0.79 | 1.05 | 1510 | 1513 | 3.30E+06 | 31.00 | 0.12 | 34.3 |
| 2-Hepten-1-ol, (E)- | 25.75 | 0.03 | 0.61 | 0.70 | 1514 | 1517 | 1.01E+06 | 43.58 | 0.04 | 0.8 |
| Benzaldehyde | 26.16 | 0.07 | 0.70 | 0.69 | 1517 | 1520 | 4.59E+06 | 29.75 | 0.17 | 35.3 |
| 2-Nonenal, (E)- | 26.62 | 0.13 | 0.83 | 1.12 | 1532 | 1534 | 4.39E+06 | 20.92 | 0.16 | 19.4 |
| 1-Octanol | 27.04 | 0.03 | 0.60 | 1.17 | 1556 | 1557 | 4.16E+06 | 363.07 | 0.16 | 377.6 |
| γ-Pentalactone | 28.46 | 0.11 | 0.58 | 1.54 | 1592 | 1589 | 2.50E+06 | 57.93 | 0.10 | 4.0 |
| γ-Butyrolactone | 28.88 | 0.06 | 0.55 | 0.84 | 1597 | 1595 | 1.46E+07 | 12.74 | 0.66 | 81.1 |
| 2-Octynoic acid, methyl ester | 29.39 | 0.12 | 0.70 | 2.13 | 1658 | 1658 | 3.57E+07 | 13.50 | 1.54 | 41.1 |
| 1-Nonanol | 29.49 | 0.10 | 0.60 | 1.71 | 1662 | 1660 | 2.39E+06 | 47.43 | 0.10 | 12.1 |
| γ-Hexalactone | 30.51 | 0.05 | 0.57 | 1.52 | 1674 | 1670 | 1.27E+07 | 303.17 | 0.47 | 114.1 |
| 3-Nonen-1-ol, (Z)- | 30.61 | 0.03 | 0.54 | 1.12 | 1679 | 1682 | 4.87E+06 | 0.33 | 0.21 | 84.9 |
| Hexanoic acid | 32.92 | 0.00 | 0.39 | 1.51 | 1843 | 1846 | 6.07E+06 | 105.74 | 0.23 | 45.4 |
| Benzyl alcohol | 33.46 | 0.00 | 0.46 | 0.88 | 1861 | 1864 | 4.07E+06 | 15.57 | 0.17 | 2.4 |
| Butyl benzoate | 33.46 | 0.05 | 0.65 | 0.86 | 1869 | 1871 | 6.29E+06 | 7.58 | 0.27 | 56.0 |
| Dimethyl sulfone | 33.89 | 0.11 | 0.41 | 2.55 | 1900 | 1903 | 3.44E+05 | 0.00 | 0.02 | 7.1 |
| Phenylethyl Alcohol | 34.04 | 0.00 | 0.47 | 1.22 | 1903 | 1906 | 8.41E+06 | 11.57 | 0.34 | 0.4 |
| γ-Octalactone | 34.36 | 0.13 | 0.54 | 4.47 | 1947 | 1950 | 1.37E+06 | 193.92 | 0.05 | 45.9 |
| γ-Nonalactone | 36.00 | 0.00 | 0.53 | 1.40 | 2048 | 2050 | 8.23E+06 | 200.73 | 0.31 | 59.9 |
| Octanoic acid | 36.13 | 0.00 | 0.38 | 1.85 | 2071 | 2072 | 5.07E+06 | 10.59 | 0.21 | 1.2 |
| Nonanoic acid | 37.54 | 0.00 | 0.38 | 1.59 | 2174 | 2171 | 6.96E+06 | 5.48 | 0.29 | 0.4 |
| Decanoic acid | 38.87 | 0.04 | 0.40 | 2.19 | 2281 | 2276 | 3.20E+06 | 15.95 | 0.14 | 3.5 |

deviation (% RSD) on retention times, experimental I$^T$s, and mean 2D peak responses (absolute and % response) accompanied by F value (Fisher discriminant ratio – classes damaged *vs.* healthy).

An explorative principal component analysis (PCA) based on normalized responses for the 72 targeted features tracked over all analyzed samples is illustrated in Supplementary Fig. 1. The natural clustering of samples is consistent with the bug damage, the first two PCs explain about 53% of the total variance with a fairly good discrimination along the PC1. A significant difference in the relative distribution was evidenced for glucose, fructose, maltose, glycerol, and glycerol monostearate detected in higher amounts in damaged seeds (Supplementary Fig. 1). Lactic acid, on the contrary, resulted more abundant in healthy seeds.

### 3.3.2. *Volatile metabolome changes*

The volatile metabolome accounted for 568 features (S/N > 150), of which 97 reliably identified by $I^T$ and MS similarity *vs.* reference in databases. Table 6 lists targeted volatile metabolites together with their $^1$D and $^2$D retention times, % relative standard deviation (% RSD) on retention times, experimental and tabulated I$^T$, and mean 2D peak responses (absolute and % response) accompanied by F value (Fisher discriminant ratio – classes damaged *vs.* healthy). Untargeted volatile features (n = 461) are listed in Supplementary Table 2.

Unsupervised exploratory statistics, i.e., PCA, confirmed the natural clustering of samples according to the bug damage. For this fraction (i.e., 568 detected features), class distinction is fairly good, with 64% of the total variance explained by the first two PCs. The PCA loading plot based on the normalized responses of volatile metabolites is reported in Fig. 1, while differences between damaged and healthy soybean seeds of some selected volatile metabolites (alcohols, lactones, aldehydes, and methylketones) are reported in Fig. 2.

## 4. Discussion

Soybean is one of the most important protein crops in the world, and provides 10% of global edible oil (FAO, 2023), therefore it is crucial to know when the crop is more susceptible to attack by stink bugs, specifically *H. halys*, in order to better address the pest management program in Europe. In this work, soybean was shown to respond with delayed maturity to *H. halys* feeding activity during the creation of pods and the initial development of seeds (growing stage of soybean R4-R5). The stay-green syndrome was already observed as a result of *H. halys* feeding activity on soybean (Owens et al., 2013; Koch and Rich, 2015), but without relating it to the period of bug attack, as was previously assessed for *N. viridula* and *P. guildinii* (Boethel et al., 2000; Vyavhare et al., 2015). In our study, soybean was confirmed to be more susceptible to stay-green syndrome when *H. halys* fed on plants at the stages R4-R5, during pod elongation and initial seed development, than in the last stages. Similarly, in North America, stages R3-R5, from pod elongation to the onset of pod filling, were more susceptible to this damage than other stages when attacked by *N. viridula* (Boethel et al., 2000), while the feeding activity of *P. guildinii* during a 10-day period in stages R4 and R5 also triggered a delay in soybean maturity, although without comparison with other stages (Vyavhare et al., 2015). The different climatic conditions between the two years did not affect the role of *H. halys* on stay-green. However, it is worth considering that other factors may also contribute to the soybean stay-green syndrome; for example, recent studies have correlated this syndrome with geminivirus infection (Wang et al., 2022) or other soybean pathogens (Harbach et al., 2016). In any case, stink bug activity in the R4-R5 stage has been confirmed here to contribute to the increase of stem moisture and green plants.

In North America seed damage and pod loss occurred most severely when soybean was attacked by *H. halys* at the R4 stage compared to R2 and R6 stages (Owens et al., 2013). Similarly, in our study the number of seeds per pod was significantly lower following *H. halys* attacks at the R4-R5 stage. In contrast, the percentage of damaged seeds showed no significant differences in relation to the attack periods of *H. halys*, consistent with what was observed when soybean was attacked by *N. viridula* from R2 to R6 stages (Boethel et al., 2000). However, while the rate of damaged seeds did not vary in relation to the time of attack, the feeding activity of *H. halys* on soybean in the first period significantly reduced the number of seeds per pod, thus indicating a higher damage rate by bugs at the R4-R5 stage. The lower number of seeds per pod corresponded, however, to an increase in the 1000 seed weight due to plant compensation mechanisms, leading to pod weights and grain yields that did not differ among the compared treatments. Similar results

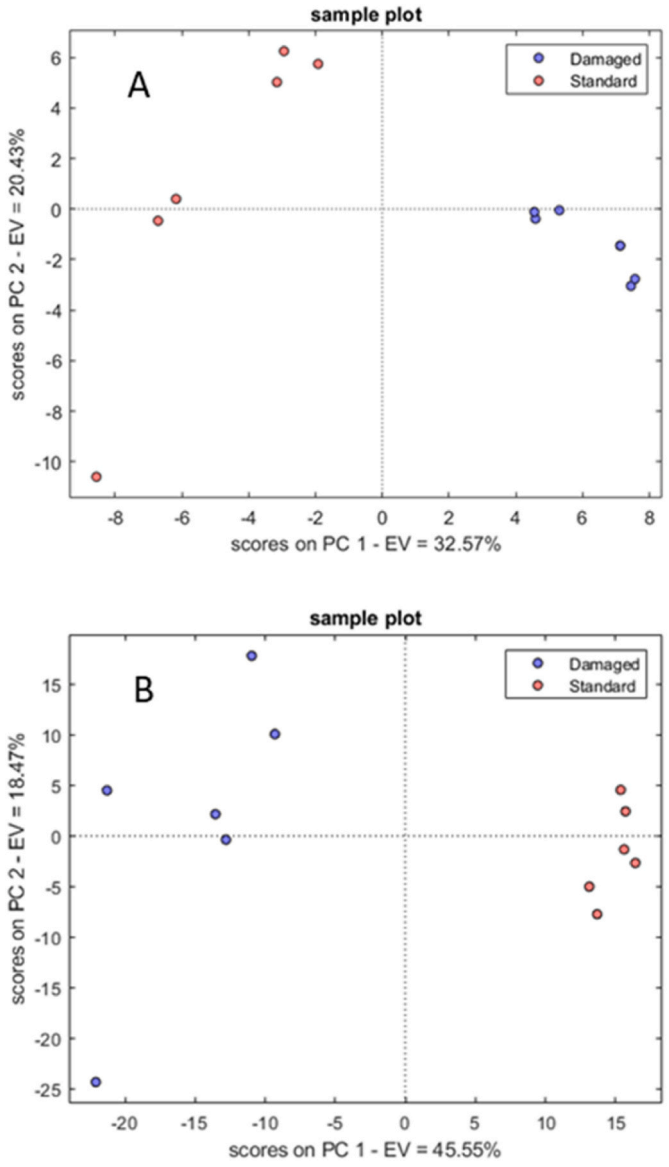


**Fig. 1.** Loading plots obtained by considering normalized chromatographic responses from (A) targeted primary metabolites (n = 110); and (B) all detected volatile metabolites (n = 570) for damaged and healthy soybean seeds. Normalized response data were pre-processed by autoscaling.

were obtained in a previous study in which seeds from plants attacked by *H. halys* weighed more than healthy ones, due to the reduced number of seeds per pod resulting in an increase in the weight of remaining seeds (Koch and Rich, 2015).

While not causing overall a loss of weight production, the seeds damaged by *H. halys* showed qualitative differences compared to healthy ones; in particular, damaged seeds showed a significantly higher protein content, as already reported after feeding on dry soybean seeds (Bae et al., 2014), which could be due to salivary compounds injected during feeding activity. Similarly, increase in protein and decrease in oil content were also observed in seeds damaged by other stink bugs, but not in seeds damaged by *H. halys* in North America (Koch et al., 2017). However, the negative correlation between oil and protein contents is already known; in fact, when oil concentration decreases, the protein content increases (Liu et al., 2022). The stink bug attack is definitely more relevant to soybean for human consumption (e.g., edamame, soymilk, soybean oil). Indeed, the bug feeding affects both the product appearance, and the product quality (McPherson et al., 2008).

Metabolomic data confirmed that bug damage induced metabolic derangements in seeds with an impact on the primary metabolite fingerprint. While Giacometti et al. (2016) observed a damage-induced jasmonic acid accumulation and ethylene emission in developing seeds after *N. viridula* attack, reference data on molecular derangements are not yet available. Our data suggest enzymatic activation at different levels with the mobilization of primary metabolites, mostly from starch and polysaccharides (up-regulation of glucose, fructose, and maltose), and from glycerides (up-regulation of glycerol and glycerol monostearate). According to Peiffer and Felton (2014) amylases, and lipases/esterase are likely activated and/or transferred to seeds by the bug's saliva. Lipoxygenase activation, under the trigger of jasmonic acid induced by *N. viridula* attack in soybean, was studied by Barneto et al. (2024). The volatile metabolome, for which data are not available in literature, likely validated the actual impact of *H. halys* on the lipid fraction. Most of the volatiles, which were detected in higher relative abundance in damaged seeds, are products of lipid oxidation both regulated by LOXs and hydroperoxide-liases pathways or by autoxidation. Saturated aldehydes (hexanal and octanal), mono-unsaturated aldehydes (*E*-2-hexenal and *E*-2-heptenal), and methyl-ketones (2-heptenone, 2-octanone, 3-octen-2-one) are known products of fatty acids hydroperoxides degradation. Their presence suggests an extensive oxidation of the major unsaturated fatty acids (i.e., linoleic acid ≈54%, oleic acid ≈23%, and linoleic acid ≈8%) (Boué et al., 2003; Squara et al., 2022). These unsaturated fatty acids determine the quality of soybean fat fraction and are known to play a favorable role in the health and stability of the product as well as in improving human health (Liu et al., 2022; Duan et al., 2023).

The biochemical alterations that occur in plants, especially in fruits and seeds, as a result of the action of stink bug saliva are still poorly known, and further studies will help to better understand the interaction between pest and host, and the plant defence reaction involved. The results could be helpful to better describe and evaluate the damage in seeds, or the whole-plant reaction as in case of the stay-green syndrome, but also to better address pest management strategies to promote pest control at the right time. This study may be preparatory to a future evaluation to determine the action threshold for stink bug control on soybean in the Po Valley to ensure sustainable management of soybean cultivation, as already proposed in other countries (Sosa-Gómez et al., 2020).

In our study, we evaluated the damage caused by *H. halys* on soybean in relation to its attack period, confirming that it can pose a threat to the crop. Regardless of the attack period, and under our experimental conditions, the stink bug has been shown to have limited effect on overall grain yield but to cause alterations in seeds quality, which will have to be considered depending on the intended use of the production. However, if the percentage of damaged seeds did not vary in relation to the attack period, the stay-green syndrome occurred particularly when *H. halys* was caged at the R4-R5 stage. Therefore, the early stages of pod and seed development are the stages at which soybean is the most susceptible to the stay-green syndrome, which compromises mechanized harvesting (Boethel et al., 2000; Vyavhare et al., 2015); consequently, both monitoring and pest management strategies should focus mainly on this period. Another strategy to contain the stink bug attacks that cause stay-green syndrome could be the use of early maturity varieties, which could escape attacks at R4-R5 stage by maturing before stink bug populations reach high levels on the crop (Panizzi and Slansky, 1985; Gore et al., 2006), although further research in this area is needed.

## 5. Conclusions

Stay-green syndrome occurred most severely as a result of *H. halys* attacks at soybean development stages R4–R5, a period when soybean surveillance should be intensified, in order to properly target pest management strategies. Moreover, *H. halys* attacks were shown to cause qualitative damage to soybean seeds, especially by altering protein

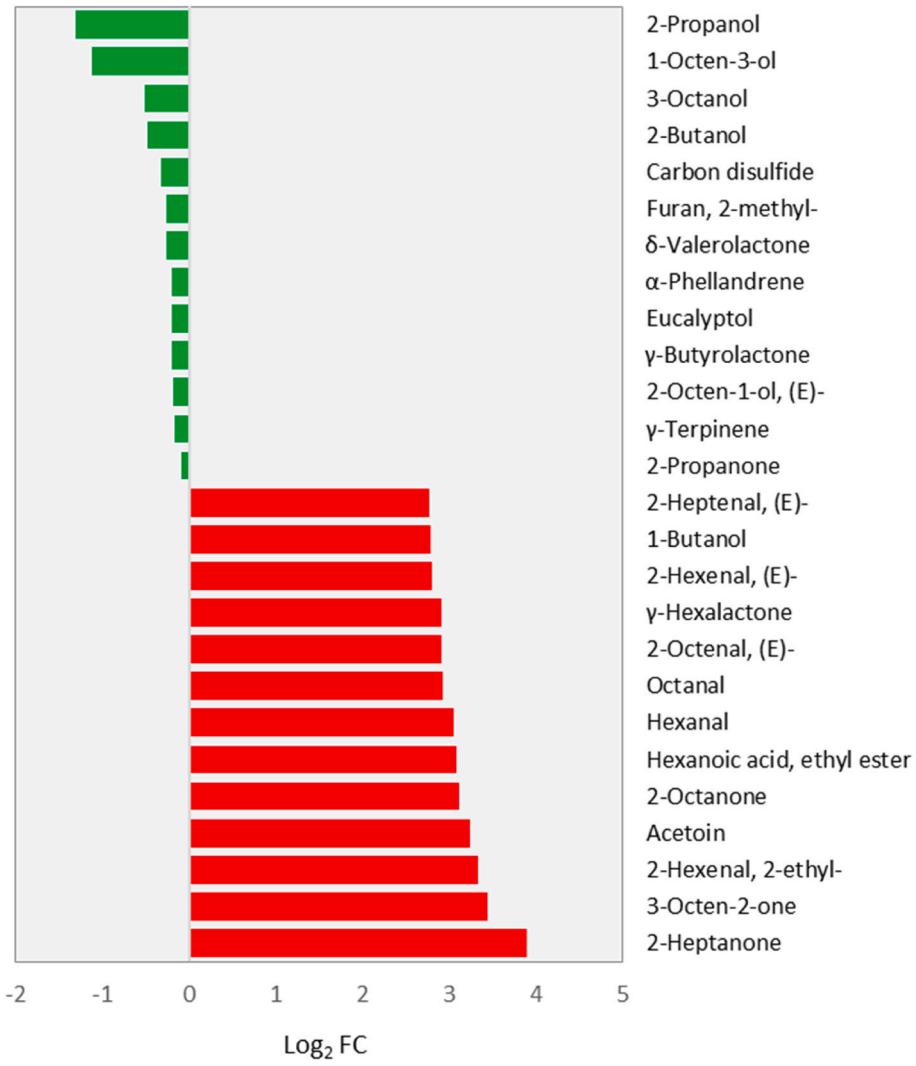


**Fig. 2.** Histogram illustrating the log2 fold change (FC) of selected volatile metabolites. Green bars correspond to volatiles more abundant in the healthy seeds; red bars are for analytes more abundant in damaged seeds. Analytes variations between classes (damaged vs. healthy) were significant as resulting by Kruskal-Wallis test after Bonferroni correction.

content and mobilizing several primary metabolites from storage macromolecules. Further studies on seed alterations and salivary composition of *H. halys* could better explain the interaction between plant and insect, and provide data on compositional and nutritional quality.

## CRediT authorship contribution statement

**Gabriele Rolando:** Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis, Conceptualization. **Silvia Teresa Moraglio:** Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis, Conceptualization. **Andrea Caratti:** Methodology, Investigation, Formal analysis. **Chiara Cordero:** Writing – review & editing, Methodology, Investigation, Formal analysis. **Giorgio Borreani:** Writing – review & editing, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Conceptualization. **Luciana Tavella:** Writing – review & editing, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Conceptualization.

## Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cropro.2024.106987.

## Data availability

Data will be made available on request.

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