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Influence of Exogenous Ethylene and Mechanical Damage on Gene Expression and Physiological Parameters of Maize Hybrids

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Abstract: This study investigated the responses of two maize hybrids, Armagnac and Desszert R-78, to exogenous ethylene and mechanical damage as stress treatments. The amounts of benzoxazinoids (BXDs) and malondialdehyde (MDA) and the activities of superoxide dismutase (SOD) and catalase (CAT) were examined 2 and 4 h after ethylene and mechanical damage treatments as well as at the age of 24 days, and the activity of genes encoding BXD biosynthesis and other stress-related genes was measured in shoots. In both hybrids, mechanical damage upregulated the genes responsible for the synthesis of BXDs (BX8 and BX9), the AOC1 gene encoding jasmonate, and the DEH gene encoding lipid biosynthesis enzymes. Significant genotype differences were found in the amounts of BXDs. In the case of the Desszert R-78 hybrid, the BXDs level was increased at 4 h after stress treatments compared to the control. In the case of the Armagnac hybrid, the amount of BXDs decreased in response to ethylene compared to the control. The absence/presence of a correlation between the activity of genes encoding BXDs and the amount of BXDs is thought to be due to the different rate/speed of the response in the two hybrids. Mechanical damage and ethylene treatments did not significantly affect the activities of SOD and CAT as well as the amount of MDA during the four-hour study period.

Keywords: benzoxazinoids; self-defence; gene expression; antioxidant enzymes

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1. Introduction

Within each plant species, significant differences may be observed in the stress tolerance of different genotypes. These differences are caused partly by morphological and partly by physiological characteristics. The stress metabolites produced in plants both reduce their susceptibility and help them to cope with stress with as little damage as possible. Benzoxazinoids (BXDs) are common stress metabolites in *Poaceae* family members. In addition to being indole derivatives, BXDs are plant chemical defence substances with a 2-hydroxy-2H-1,4-benzoxazin-3(4H)-one skeleton. These phytochemicals are widely distributed in grasses, including important cereals such as maize, wheat, and rye [1,2]. They have repellent, insecticidal, antimicrobial, and allelopathic activities [3]. Since their discovery, BXDs have been considered to act as a resistance factor in plants against herbivores, pathogens, and other plants [4]. The amount of BXDs differs between plant species and varieties as well as between tissues and developmental stages. For example, DIMBOA-Glc (4-hydroxy-7-methoxy-2-[3,4,5-trihydroxy-6-(hydroxymethyl)oxan-2-yl]oxy-1,4-benzoxazin-3-one) is the most abundant BXD in wheat and maize shoots [5], while HDMBOA-Glc (4,7-dimethoxy-2-[3,4,5-trihydroxy-6-(hydroxymethyl)oxan-2-yl]oxy-1,4-benzoxazin-3-one) is the most abundant BXD in maize roots [6]. The steps of the BXD biosynthesis process have been described in maize. The first step of biosynthesis is catalysis

by BX1, which converts indole-3-glycerol phosphate to indole. This enzyme is located in the chloroplasts. Following this first step, the free indole is converted to DIBOA through the incorporation of four oxygen atoms. These oxidations are carried out by four cytochrome P450-dependent monooxygenases, i.e., BX2–BX5, located in the endoplasmic reticulum. The DIBOA aglucone is converted to DIBOA-Glc by UDP-glucosyltransferases (UGT) BX8 and BX9. DIBOA-Glc can then be hydroxylated by the 2-oxoglutarate-dependent dioxygenase BX6 and further O-methylated by the O-methyltransferase BX7 to form DIMBOA-Glc. HDMBOA-Glc can then be formed from DIMBOA-Glc by an O-methylation reaction, catalysed by the three homologous O-methyltransferases BX10, BX11, and BX12 [7]. Although the steps of biosynthesis are known, little is known about its regulation [8]. The amount of BXDs formed is partly due to constitutive and partly due to inductive effects. Various stress factors activate biosynthesis through regulating its associated genes, and elevated BXD levels promote stress tolerance [9].

Plant hormones are involved in mediating stress factors of various origins. Ethylene is considered a stress hormone, and its production increases rapidly under abiotic and biotic stresses. Stress-induced ethylene inhibits leaf and shoot growth, can strengthen cell walls, and activates the expression of defence-coupled genes [10]. Ethylene, mainly through the AP2/ERF (APETALA2/ethylene response factor) transcription factor family, plays a key role in stress responses to both abiotic and biotic stresses, as has been demonstrated in *Arabidopsis* plants [11].

Antioxidant enzymes (e.g., SOD, CAT, etc.) are commonly used as stress markers to characterise stress effects/responses in plants. Furthermore, malondialdehyde (MDA)—a product of membrane injury—is a common marker of damage caused by ROS.

Our hypothesis is that mechanical stress and ethylene treatments generate genotype-dependent responses. These different responses potentially involve different activities of defence enzymes (e.g., SOD and CAT activities) and are accompanied by altered MDA and BXD contents as well as BXD biosynthesis gene expression in maize seedlings.

2. Materials and Methods

2.1. Plant Material, Treatments, and Sampling

Two maize (*Zea mays* L.) hybrids were used as test plants: Armagnac (Kite Zrt., Nádudvar, Hungary) is a fodder hybrid, while Desszert R-78 (Primag, Budapest, Hungary) is a sweet maize hybrid. The seedlings were sown in 9 cm diameter, 7 cm high plastic pots in commercially available potting soil. The plants were grown in a greenhouse at 22–26 °C during the day and 18–22 °C at night. Light was provided by natural sunshine, with the intensity that is typical for Hungary in May and June. The soil was watered and irrigated with tap water up to 60% of the water capacity during the growing period. At 24 days of age, when the fourth leaf was developed, the plants were treated. At the time of ethylene treatment, plants were sprayed with a 500 ppm solution of (2-chloroethyl)-phosphonic acid (Ethrel; Bayer Crop Science, Budapest, Hungary), to which a surfactant (Nonit; Arysta Life Sciences, Budapest, Hungary) was added for improved efficiency, and mechanical damage was achieved by applying an equal number of cuts to the fourth leaves. Control plants and plants in the mechanical injury treatment were sprayed with the same volume of distilled water containing surfactant as the ethylene treatment. The treatments were carried out separately, and there was no combined treatment. At each treatment and sampling time, four independent plants in separate pots were sampled. At two and four hours after the treatments, the fourth leaf was cut and divided into four parts. One side of the apical section was used for BDX determination and the other side for gene expression assays. Gene expression studies were performed only from samples taken 4 h after treatments. The bottom part was also divided into two parts—one for SOD and CAT activity and the other for MDA measurement. After fresh weight measurements, the samples were collected in liquid nitrogen and were stored in a deep freezer at −80 °C until required for measuring.

2.2. Method of BXD Isolation, Sample Preparation, and Measurement

Sample preparation and HPLC analyses were performed according to [12]. The DIMBOA standard was isolated from etiolated maize plants using the method developed by [13]. The HMBOA standard was synthesised and provided by Pierre Mateo (University of Bern, Institute of Plant Sciences). The DIMBOA standard was verified and identified by using its melting points and 1H NMR spectra recorded on CD3OD solutions. A Bruker 360 (360 MHz) spectrometer (Billerica, MA, USA) was used for this purpose. The chemical shifts were related to CH3OH (δ 3.31 ppm). The melting point of DIMBOA was 168.0–169.0 °C. A reversed-phase C18 column (Lichrospher RP18-5; 250 mm × 4.0 mm) was used for HPLC measurements in Jasco, UV/VIS HPLC instrument (Japan, Tokyo). The sample volume was 20 µL. Pure DIMBOA and HMBOA solutions were used as standards at the beginning and at the end of each series of measurements. Due to the mode of sample preparation, the determination of aglycones from glucosides produced by glucosidases was performed.

2.3. SOD Measurement Method

To prepare the enzyme extract, 0.3 g leaf sample was frozen in liquid nitrogen and ground in 3 mL extraction buffer (0.1 M phosphate buffer, pH 7.5, containing 0.5 mM EDTA). The homogenates were filtered through a cloth and centrifuged at 15,000× *g* for 20 min at 4 °C. The supernatant was collected and used for enzyme activity assay. All steps of enzyme extraction were performed at 4 °C [14]. The SOD activity was determined by adding 50 µL of enzyme extract to a solution containing 13 mM L-methionine, 75 µM nitroblue tetrazolium chloride (NBT), 100 µM EDTA, and 2 µM riboflavin in 50 mM potassium phosphate buffer (pH 7.8). All reagents were obtained from Sigma-Aldrich, Budapest, Hungary. The reaction was carried out under the illumination of an 18 W fluorescent lamp (Philips, Budapest, Hungary) at 25 °C. The reaction was started by switching on the fluorescent lamp and stopped after 30 min by switching off the lamp [15]. The blue formazan produced during photo-reduction in NBT was measured as an increase in absorbance at 560 nm. No enzyme extracts were present in the control reaction mixture. The blank solution contained the same complete reaction mixture but was kept in the dark. One SOD unit was defined as the amount of enzyme required to inhibit 50% of NBT photo-reduction, compared to cuvettes containing no plant extract, and expressed in units of enzyme activity (UA g^{-1} FW) [14].

2.4. CAT Measurement Method

From the enzyme extract prepared according to Sarker and Oba (2018) [14], 100 microliters was added to 2.9 mL of pH = 7 0.1 M phosphate buffer, which contained 0.102% H_2O_2. All reagents were obtained from Sigma-Aldrich, Budapest, Hungary. After the addition of the enzyme, the absorbance was measured at 240 nm and measured again after 1 min. The decrease was recorded. A unit of CAT activity (U) was defined as the amount of enzyme that caused a 0.001 decrease in absorbance over 1 min [16].

2.5. MDA Measurement Method

The malondialdehyde content was determined using the method of Heat and Packer (1968) [17]. Leaf tissue (0.1 g) was ground to powder with liquid nitrogen; then, the powder was placed into a tube containing 1 mL of 0.1% ($w\ v^{-1}$) trichloroacetic acid (TCA; Sigma-Aldrich, Budapest, Hungary) and mixed. The samples were then centrifuged at 10,000× *g* for 10 min, and the supernatant was transferred to a new tube. Then, 4 mL of 20% TCA containing 0.5% 2-thiobarbituric acid (TBA; Sigma-Aldrich, Budapest, Hungary) was added and mixed well. The mixture was boiled at 95 °C for 15 min and rapidly cooled on ice. The cooled mixture was centrifuged at 10,000× *g* for 5 min, and the supernatant was transferred to a new tube. The next step was to measure the absorbance of the obtained solution at 532 nm. The concentration of malondialdehyde was calculated by using an extinction coefficient of 155 mM^{-1} cm^{-1} [18].

2.6. Total RNA Isolation and cDNA Synthesis

The maize leaf samples were placed in liquid nitrogen and ground thoroughly to obtain a very fine powder with a mortar and pestle. The total RNA was extracted from maize leaf powder using a Plant Total RNA Extraction Miniprep System (Viogene BioTek, New Taipei City, Taiwan) with a DNase I (Sigma, St. Louis, MI, USA) following the manufacturers' protocols. The quantity and purity of isolated RNA were determined with a microplate reader (Synergy HT Multi-Mode Microplate Reader, BioTek Instruments, Winooski, VT, USA) using Gen5 microplate and imager software (BioTek version 3.03). The quality and integrity of RNA were checked by using a Qubit RNA IQ assay kit (Thermo Fisher Scientific, Waltham, MA, USA) with a Qubit 4 fluorometer (Invitrogen by Thermo Fisher Scientific, Waltham, MA, USA). The total RNA was reverse-transcribed into cDNA using LunaScript RT SuperMix Kit (New England Biolabs, Inc., Ipswich, MA, USA). cDNA samples were diluted 10-fold and stored at −20 °C.

2.7. qPCR Analysis

The maize cDNA samples were amplified according to the manufacturer's instructions using the 5× HOT FIREPol EvaGreen qPCR master Mix plus (Solid BioDyne, Tartu, Estonia). PCR reactions were run in triplicate, with a total volume of 10 µL consisting of 2 ng cDNA template, 5× HOT FIREPol EvaGreen qPCR mix plus 200 nM of each primer, and distilled water. Using an AriaMx Real-Time PCR system, we performed a real-time polymerase chain reaction (PCR) (Agilent Technologies-Applied Biosystems, Carlsbad, CA, USA). Intron-spanning forward and reverse primers were designed for maize by using the Oligo 7 software (Version number 7.6) and checked for target identity using the Primer-Blast tool of the National Centre for Biotechnology Information (NCBI) or were selected from previous gene expression studies. Primer sequences can be found in the Supplementary file. The PCR procedure included an initial denaturation at 95 °C for 12 min, 40 cycles of denaturation at 95 °C for 15 s, an annealing and extension step at 60 °C for 20 s, and a final elongation step of 72 °C for 20 s. The FPGS was selected as the reference gene among the four reference genes tested (LUG, EF1, and BTUB), and the stability of reference genes was analysed using three algorithms, namely ΔCt, NormFinder, and Best Keeper. The target gene mRNA expression was normalised with the selected reference gene, and the relative mRNA expression was calculated using the $2^{-\Delta\Delta Ct}$ models [19]. Relative expressions were determined as fold changes in the expression of the target gene in the treatment group compared with the control group.

2.8. Statistical Analysis

Statistical analysis was performed using SPSS version 23.0. After applying normality tests, the results were analysed using one- or two-way ANOVA and independent samples *t*-test, comparing the mean of the two treatments with the mean of the control group. The different groups were characterised using LSD, Duncan, and Tukey tests. When the distribution of results was not normal, a non-parametric test was used: Mann–Whitney and Kolmogorov–Smirnov tests. Data are presented as the mean ± standard error (SE) of the mean. Values with different letters are significantly different from each other at a minimum value of $p \leq 0.05$.

3. Results

3.1. Results of Benzoxazinoid (BXD) Measurements

Tables 1 and 2 show the amount of the two most abundant compounds of BXDs (DIMBOA and HMBOA) in the experimental samples and the sum of these two compounds. In the case of the Desszert R-78 (Table 1) hybrid, there was no difference in BXD content 2 h after treatments. However, in samples taken 4 h after treatments, the levels of DIMBOA (by 57.4 and 62.7%, respectively) and the total amount of DIMBOA and HMBOA (by 48.2 and 45.3%, respectively) were higher in the ethylene and mechanical damage treatments compared to the control. The sampling time had no significant effect on the BXD content in

the Desszert R-78 hybrid and on the average of treatments and when the treatments were considered separately.

Table 1. Benzoxazinoid (DIMBOA and HMBOA) content (mg kg^{-1}; n = 4; ±SE) of hybrid Desszert R-78, 2 and 4 h after the treatments.

Chemical Compound	Treatment	Sampling 2 h after Treatments	Sampling 4 h after Treatments
DIMBOA	Control	27.93 ± 8.56^{a1}	12.17 ± 2.79^{a1}
	Mechanical damage	34.62 ± 8.43^{a1}	32.62 ± 6.54^{b1}
	Ethylene	38.87 ± 9.08^{a1}	28.56 ± 5.27^{b1}
HMBOA	Control	12.36 ± 2.10^{a1}	11.90 ± 2.57^{a1}
	Mechanical damage	19.41 ± 2.01^{a1}	13.88 ± 2.55^{a1}
	Ethylene	15.00 ± 3.75^{a1}	15.43 ± 1.77^{a1}
DIMBOA+ HMBOA	Control	40.29 ± 7.75^{a1}	24.07 ± 5.27^{a1}
	Mechanical damage	54.04 ± 8.43^{a1}	46.50 ± 6.52^{b1}
	Ethylene	53.87 ± 8.79^{a1}	43.99 ± 4.99^{b1}

(Letters indicate the difference/similarity in the quantity of the different compounds for a given sampling time compared to the control, and numbers show the difference/similarity in treatments between the two sampling times in the same treatment; values with different letters are significantly different from each other at a minimum value of $p \leq 0.05$).

Table 2. Benzoxazinoid (DIMBOA and HMBOA) content (mg kg^{-1}; n = 4; ±SE) of hybrid Armagnac 2 and 4 h after the treatments.

Chemical Compound	Treatment	Sampling 2 h after Treatments	Sampling 4 h after Treatments
DIMBOA	Control	26.45 ± 6.76^{a1}	72.11 ± 7.87^{b2}
	Mechanical damage	15.07 ± 2.91^{a1}	44.51 ± 8.36^{ab2}
	Ethylene	26.87 ± 4.31^{a1}	36.43 ± 8.01^{a1}
HMBOA	Control	27.80 ± 9.19^{a1}	18.28 ± 3.03^{a1}
	Mechanical damage	55.47 ± 24.85^{a1}	36.84 ± 7.57^{ab1}
	Ethylene	23.72 ± 2.36^{a1}	23.32 ± 3.80^{a1}
DIMBOA+ HMBOA	Control	54.25 ± 7.69^{a1}	90.39 ± 6.47^{b2}
	Mechanical damage	70.53 ± 23.51^{a1}	81.35 ± 3.47^{ab2}
	Ethylene	50.59 ± 3.72^{a1}	59.75 ± 4.41^{a1}

(Letters indicate the difference/similarity in the quantity of the different compounds for a given sampling time compared to the control, and numbers show the difference/similarity in treatments between the two sampling times in the same treatment; values with different letters are significantly different from each other at a minimum value of $p \leq 0.05$).

Similar to the Desszert R-78 hybrid, in the case of the Armagnac hybrid, no differences were found in BXD content 2 h after the treatments. However, in samples taken 4 h after treatments, the DIMBOA content (by 49.5%) and the total amount of DIMBOA and HMBOA (by 36.1%) were lower in the ethylene treatment than in the control. Two-way ANOVA results showed that sampling time had a significant effect on the DIMBOA and HMBOA content of the Armagnac hybrid. In the control, the amount of DIMBOA and the total amount of DIMBOA and HMBOA were higher 4 h after the treatments than 2 h after the treatments. In the mechanical damage treatment, the amount of DIMBOA was higher (by 66.1%) 4 h after the treatments than 2 h after the treatments. In the ethylene treatment, there was no difference in the amount of BXDs 2 and 4 h after treatments.

3.2. Results of SOD Measurement

There was no difference in SOD activity between treatments of the tested hybrids (Figure 1). The SOD activity of the Desszert R-78 hybrid was significantly higher than that of the Armagnac hybrid (by 30.4% after 2 h of treatments and by 35.7% after 4 h of

treatments). The sampling time did not influence the SOD activity of hybrid Desszert R-78, but the SOD activity of hybrid Armagnac was higher (by 15.6%) at the first sampling time (Figure 2).

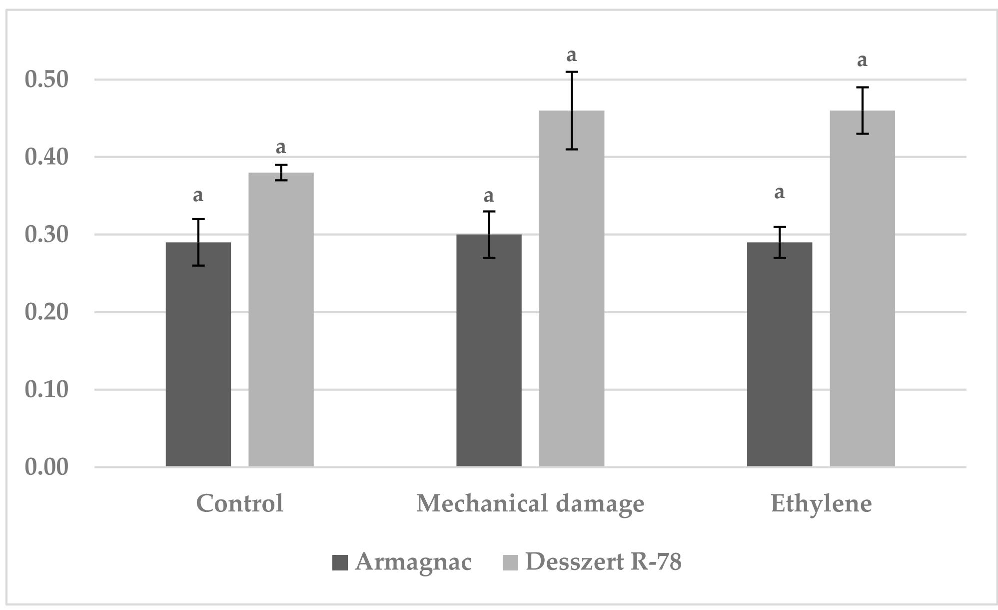

Figure 1. SOD activity (U mg^{-1} FW) of examined hybrids (Armagnac and Desszert R-78) in control, mechanical damage, and ethylene treatments on the average of the two sampling times (n = 8, ±SE). (The letters indicate the differences between the treatments belonging to a hybrid; values with different letters are significantly different from each other at a minimum value of $p \leq 0.05$).

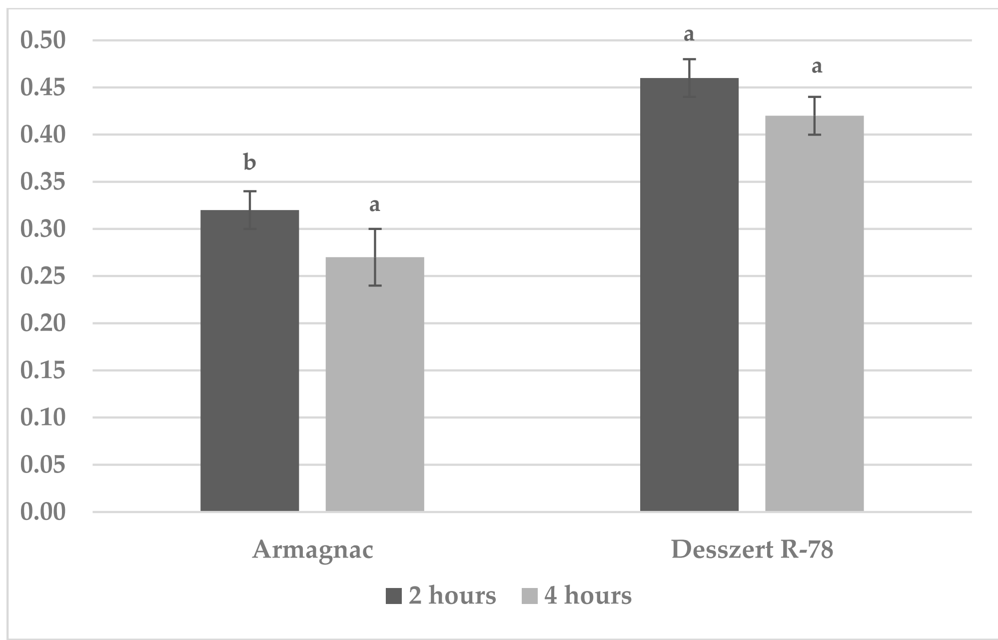

Figure 2. SOD activity (U mg^{-1} FW) of examined hybrids (Armagnac and Desszert R-78) at different sampling times (2 and 4 h after the treatments) on the average of all measured data (n = 12, ±SE). (The letters indicate the differences between the sampling times belonging to a hybrid; values with different letters are significantly different from each other at a minimum value of $p \leq 0.05$).

3.3. Results of CAT Measurement

The treatments had a significant effect on the CAT activity of the Armagnac hybrid (Figure 3). The activity of CAT measured in the ethylene treatment exceeded the control

by 61.9%. No significant effect of time elapsed relative to treatment on CAT activity was observed in the tested hybrids. In the case of the Desszert R-78 hybrid, there was no significant difference in CAT activity between treatments, and there was no difference in the effect of CAT activity between the hybrids on the average of treatments (Figure 4).

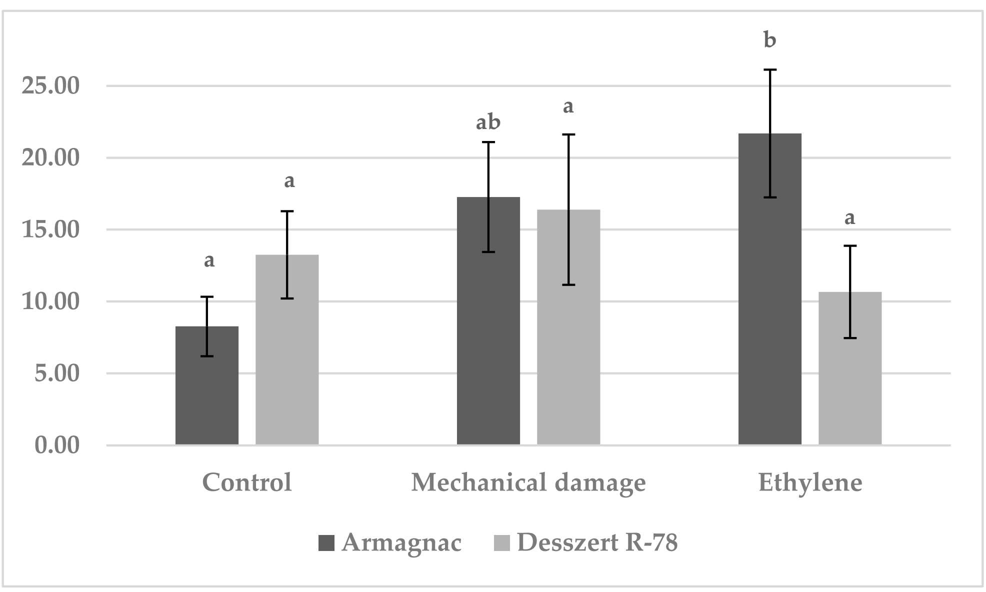

Figure 3. CAT activity (U g^{-1} FW) of examined hybrids (Armagnac and Desszert R-78) in control, mechanical damage, and ethylene treatments on the average of the two sampling times (±SE; n = 8). (The letters indicate the differences between the treatments belonging to a hybrid; values with different letters are significantly different from each other at a minimum value of $p \leq 0.05$).

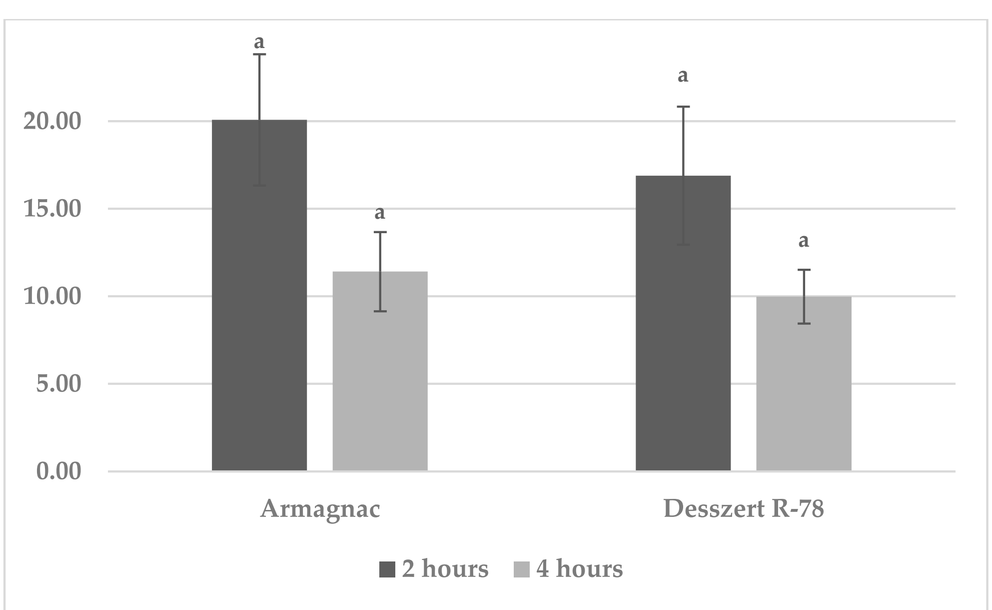

Figure 4. CAT activity (U g^{-1} FW) of examined hybrids (Armagnac and Desszert R-78) at different sampling times (2 and 4 h after the treatments) on the average of all measured data (±SE; n = 12). (The letters indicate the differences between the sampling times belonging to a hybrid; values with different letters are significantly different from each other at a minimum value of $p \leq 0.05$).

3.4. Results of MDA Measurements

Based on the results of the two-way ANOVA, it can be concluded that the sampling time and treatment have no detectable effect on the MDA content of the examined hybrids

(Figures 5 and 6). A comparison of the hybrids showed no differences in MDA content (Armagnac: 30.10 ± 1.98 µmol gram^{-1}; Desszert R-78: 30.79 ± 1.47 µmol gram^{-1}). Differences in MDA content were also not found when MDA content was examined between hybrids based on treatments.

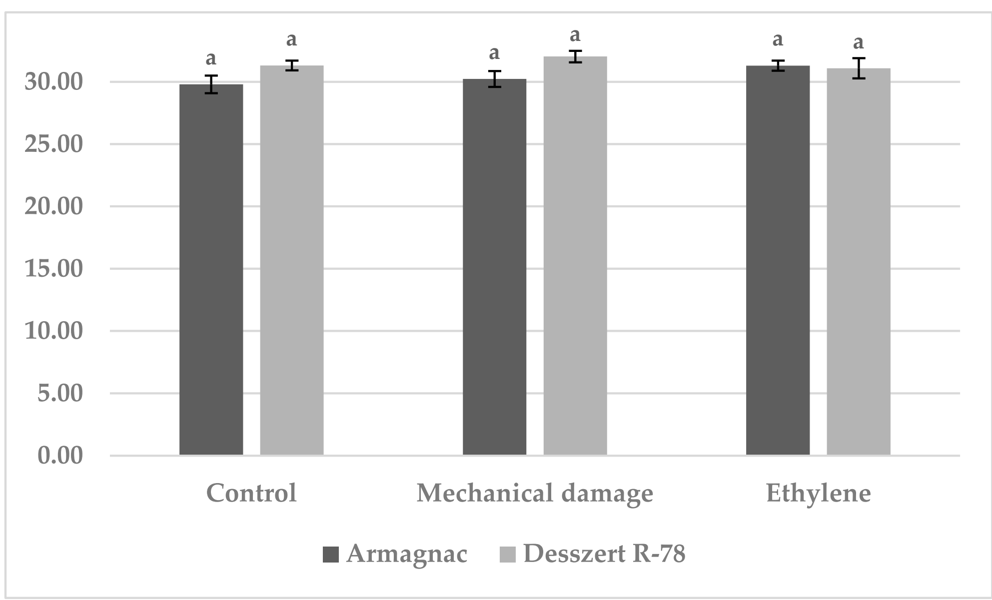

Figure 5. MDA content (µmol gram FW^{-1}) of examined hybrids (Armagnac and Desszert R-78) in control, mechanical damage, and ethylene treatments on the average of the two sampling times (±SE; n = 8). (The letters indicate the differences between the treatments belonging to a hybrid; values with different letters are significantly different from each other at a minimum value of $p \leq 0.05$).

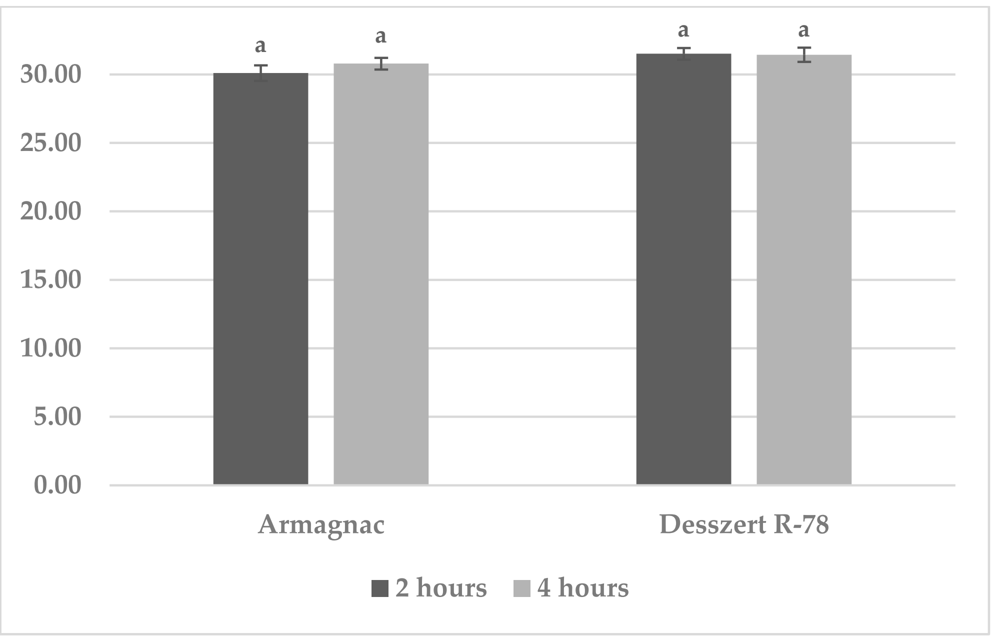

Figure 6. MDA content (µmol gram FW^{-1}) of examined hybrids (Armagnac and Desszert R-78) at different sampling times (2 and 4 h after the treatments) on the average of all measured data (±SE; n = 12). (The letters indicate the differences between the sampling times belonging to a hybrid; values with different letters are significantly different from each other at a minimum value of $p \leq 0.05$).

3.5. Results of Gene Expression Analysis

The gene expression results showed that mechanical damage increased the expression of the AOC1, BX8 and BX9, and DEH genes in both hybrids. Neither treatment modified the mRNA level of the HPL and LIP genes in the hybrids under study. Expression of the PX3 gene was affected by ethylene treatment in the hybrids tested. The opposite effect was observed for the two hybrids: Ethylene treatment downregulated PX3 gene expression in the Desszert R-78 hybrid and upregulated it in the Armagnac hybrid compared to the control group (Figures 7 and 8).

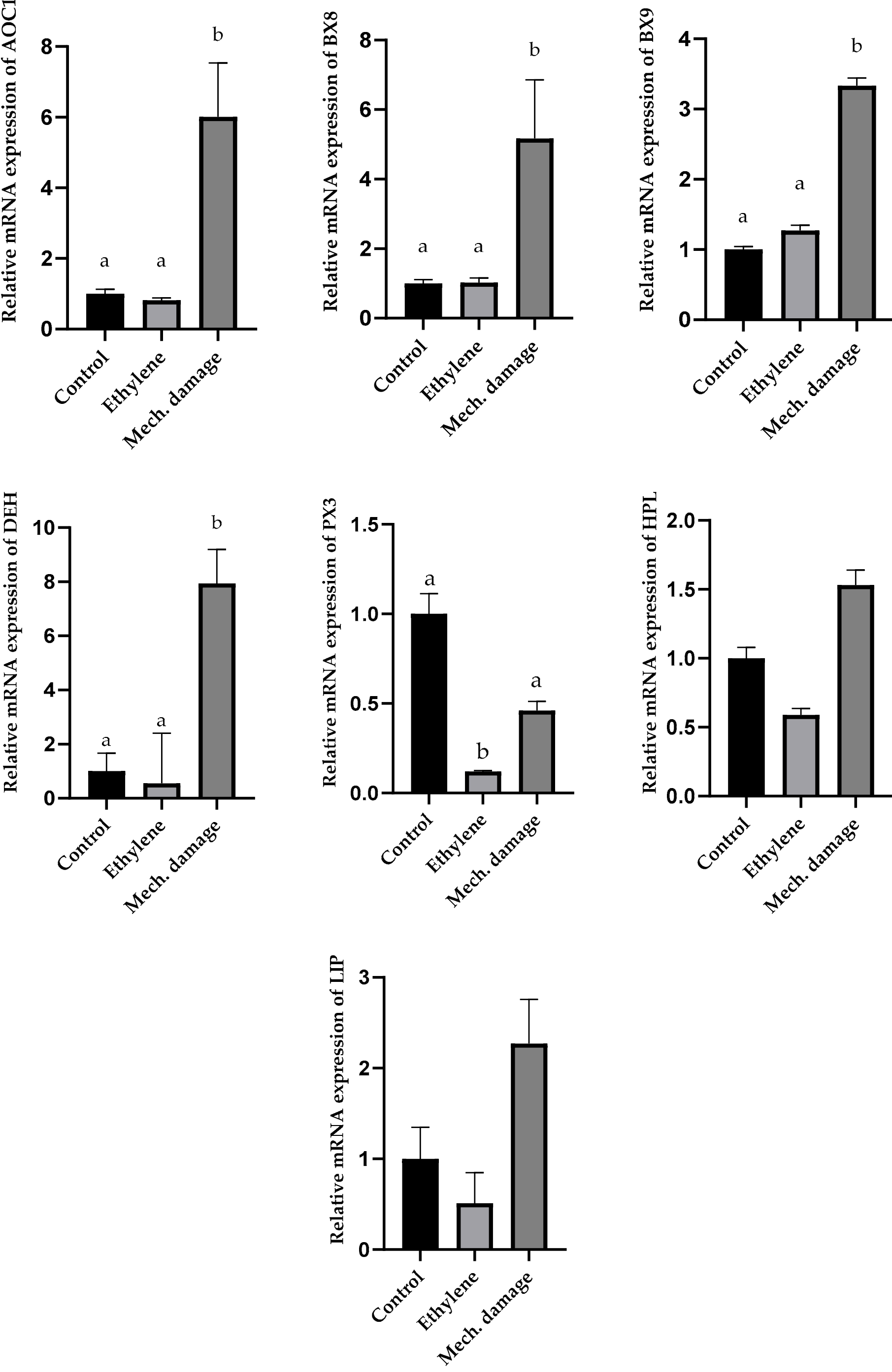

Figure 7. Effects of ethylene and mechanical damage (Mech. damage) on mRNA levels related to BXD biosynthesis and stress in Desszert R-78 hybrid. Data are presented as mean ±SE. (No significant difference between treatments in the sub-figures without letters; values with different letters are significantly different from each other at a minimum value of $p \leq 0.05$).

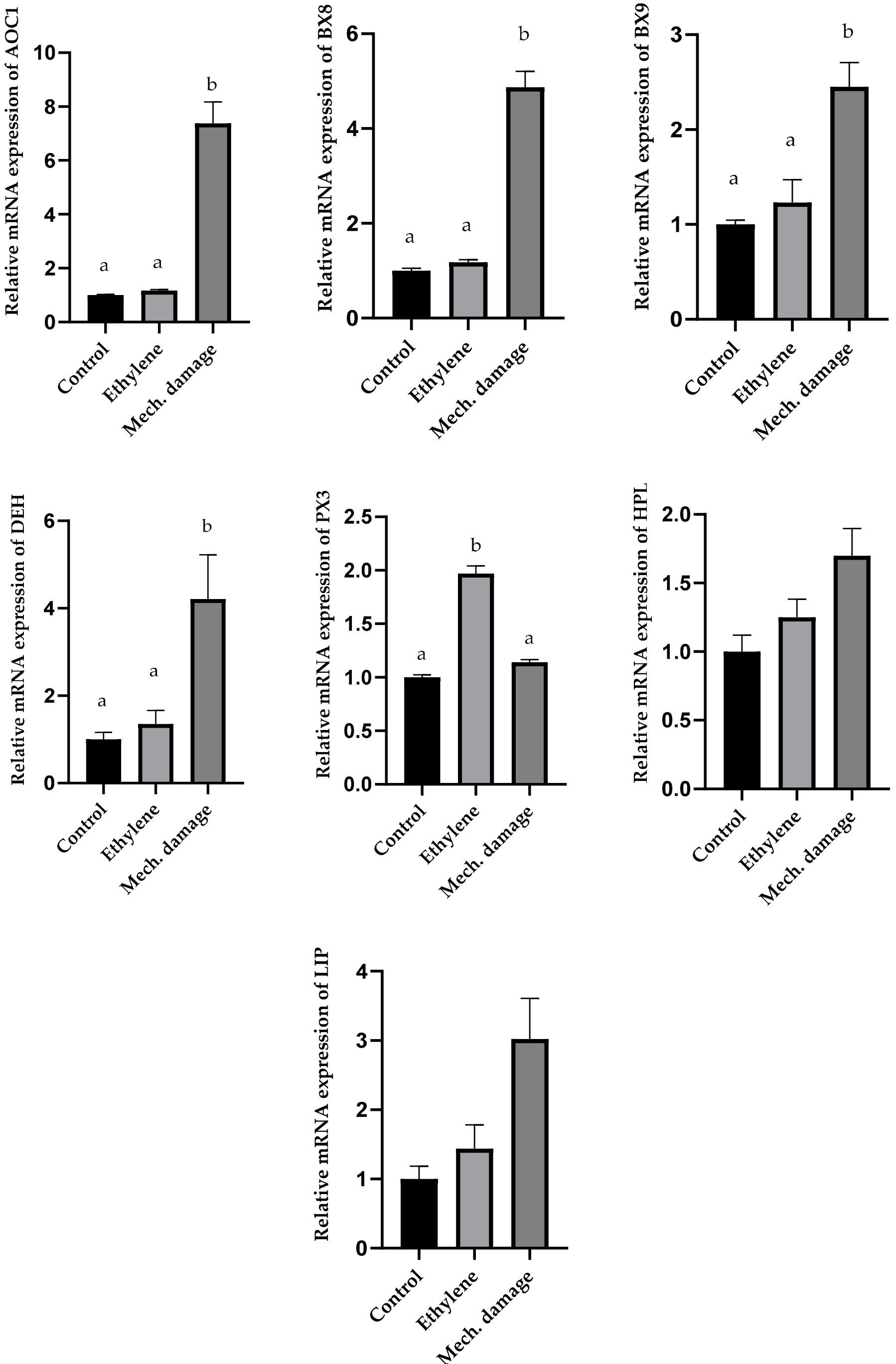

Figure 8. Effects of ethylene and mechanical damage (Mech. damage) on mRNA levels related to BXD biosynthesis and stress in Armagnac hybrid. Data are presented as mean ±SE. (No significant difference between treatments in the sub-figures without letters; values with different letters are significantly different from each other at a minimum value of $p \leq 0.05$).

4. Discussion

During the infection process, there is constant communication between the pathogen and the host plant. The effectiveness of the plant response determines the extent of infection/disease. To be able to track the steps of this communication, we need to be able to identify the exact onset of infection. This is not clear, even in the case of artificial inoculation, as it does not coincide with the injection of the pathogen. This is why the use of infection-induced effects can be effective in studying pathogen–host plant interactions. With hormone and/or mechanical injury treatments, the onset of action can be exactly de-

termined, allowing the processes to be monitored. The monitoring of BXDs in the examined hybrids showed the following results: In the case of the Desszert R-78 hybrid, in samples taken 4 h after treatments, the levels of DIMBOA and the combined amount of DIMBOA and HMBOA were higher in the ethylene and mechanical damage treatments compared to the control. In the case of the Armagnac hybrid, in samples taken 4 h after treatment, the DIMBOA content and the combined amount of DIMBOA and HMBOA were lower in the ethylene treatment than in the control. The latter result is in agreement with the findings of the authors of [8], who found that some components of the ethylene signalling pathway reduced the quantity of BDXs. Our results suggest that the effect of ethylene on the amount of BXDs is hybrid-dependent. The gene expression results of the Desszert R-78 hybrid confirm the BXD-enhancing effect of mechanical treatment, but the BXD-enhancing effect of ethylene is not reflected in the effect of ethylene in increasing the expression of BXD biosynthesis genes. It is likely that the influence of ethylene on gene activity occurred earlier than 4 h.

The BXD biosynthesis genes BX8 and BX9 encode UDP-glucosyltransferases that convert DIMBOA to DIMBOA-Glc. Their upregulation may allow an increase in the amount of BXDs and demonstrates that BXD biosynthesis is activated by mechanical damage. The AOC1 gene encodes allene oxide cyclase, a key enzyme in the biosynthesis of jasmonic acid (JA) [20]. It was demonstrated that whether necrotrophic pathogens, insects, herbivores, or mechanical damage cause tissue damage, the synthesis of jasmonates—which enhance stress resistance in plants in several ways—is induced [21]. Moreover, jasmonates induce the formation of a series of secondary metabolites that play a role in plant self-defence [22]. This explains why mechanical injury treatment in our experiment upregulated the AOC1 gene. Simultaneous increases in jasmonate and benzoxazinoid levels due to volatile organic compounds were reported previously [23]. DEH encodes 3-hydroxyacyl-CoA dehydratases, which are involved in the synthesis of cuticular waxes and membrane lipids (complex sphingolipids). The operation of these enzymes is essential to several developmental processes [24]. The increase in its mRNA level may be related to the healing of wounds caused by mechanical damage. The changes in the expression of ethylene-induced peroxidase (PX3) activity in the hybrids studied may not be due to a change in the activity of the other H_2O_2-degrading enzyme, CAT. Indeed, in the Armagnac hybrid, where a higher PX3 mRNA level was recorded, CAT enzyme activity was also enhanced. The expression of the LIP gene, which encodes transcription factors thought to play a regulatory role in abiotic stress [25], and the expression of the HPL gene, which encodes a lipid hydroperoxide-cleaving enzyme that cleaves hydroperoxide to produce signalling molecules [26], were not altered by our treatments.

Superoxide dismutases (SODs; EC 1.15.1.1) are metal-containing enzymes that catalyse the conversion of the superoxide radical to oxygen and H_2O_2. SODs play an important role in the defence against reactive oxygen species (ROS), which can be generated in a variety of ways, for example, by photosynthetic and mitochondrial electron transport [27]. Different stress effects always generate oxidative stress, which is associated with the generation of activated oxygen species, and therefore, SOD is considered to play an important role in plant stress tolerance [28]. The amount of SOD is partly given (native) and partly inducible. The greater the amount of SOD of two different origins in a plant, the more efficiently it can respond to stress [29]. Catalases (CAT, 1.11.1.6) are important antioxidant enzymes that directly catalyse the decomposition of H_2O_2 to non-toxic water, and O_2 plays a key role in the regulation of H_2O_2 levels in the cells [16,30]. Overexpression of CAT can increase plant resistance to abiotic and biotic stresses [31]. In addition to the harmful effects of H_2O_2, it has a function in signal transduction so that regulation of its quantity may be another function of CAT enzymes [32]. Malondialdehyde (MDA) is a natural product of membrane lipid peroxidation, traditionally used as an indicator of the extent of damage caused to cells by stress by reactive oxygen species (ROS) [33]. The effect of the different treatments on antioxidant enzyme activity in our experiment was not found to be significant. There was no difference in the SOD activity between treatments of the tested hybrids. The differences

we observed were as follows: The SOD activity of the Armagnac hybrid was higher at the first sampling time, and the CAT activity measured in the Armagnac hybrid was higher in the ethylene treatment than in the control. Changes in SOD activity in plant samples have been detected 24–48 h after the onset of stress in biotic stress [34]. Significant effects of low temperature on SOD activity have been demonstrated in pepper as early as 12 h after treatment initiation and on MDA amount and CAT activity 3 h after treatment initiation [35]. In maize hybrids, an increase in the activity of SOD and CAT was detected as early as 3 h after treatment and an increase in MDA 6 h after treatment in response to high temperature as a stress factor [36]. The magnitude of plant stress responses depends on the type of stress, its intensity and duration, as well as the plant species, cultivars, developmental stage, and organs under study [37]. This suggests that the effect of treatments used in our experiment did not reach a level that would have led to an increase in the activity of the antioxidant system or that the effect could be detected at a later point in time. This was also confirmed by the results of the MDA measurement, which showed no difference in MDA content between the two hybrids tested, and no significant differences were detected between treatments and sampling times. The MDA content is an indicator of plant stress tolerance [38]. This implies that plants with lower MDA content under stress are more tolerant to the given stress factor [39]. As no difference in MDA content was found between the two tested hybrids, even when this was examined on a treatment-by-treatment basis (no difference in control or in stress-inducing treatments), there was no difference in the level of tolerance between them, at least in terms of the impact of MDA content. The observed differences between the hybrids do not clearly support the susceptibility/heightened susceptibility of the common sweet hybrids observed in public cultivation, and therefore, we have to assume that there are other characteristics than those under investigation that explain these differences.

5. Conclusions

Pathogen-induced changes (mechanical damage, increase in stress hormones, etc.) in graminaceous species cause increases in the amounts of benzoxazinoids, which play a role in self-defence. The amount of benzoxazinoids formed and their rate and speed of induction differ between the hybrids/varieties of plant species producing these compounds. The amount of reactive oxygen species and MDA, which indicates membrane damage, and the level of enzymes that degrade reactive oxygen species will increase thereafter, depending on the efficacy of the plant's self-defence mechanisms. Not only could ethylene be an inducer of benzoxazinoid biosynthesis, but jasmonates may also potentially participate as primary messengers in the signalling pathway.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/agronomy14091950/s1, Table S1: Primer sequences.

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References

1. Makleit, P.; Szilvia, V.; Lóránt, S. Hazai Rozs (*Secale Cereale* L.) Fajták Ciklikus Hidroxámsav-Tartalma És Kiválasztása. *Növénytermelés* **2017**, *66*, 47–68.
2. Makleit, P.; Nagy, A.; Veres, S.; Fónagy, A. Cyclic Hydroxamic Acid Content and Its Temporal Changes in Five Commercial Maize (*Zea mays*) Hybrids. *Cereal Res. Commun.* **2018**, *46*, 686–696. [CrossRef]
3. Niemeyer, H.M. Hydroxamic Acids Derived from 2-Hydroxy-2*H*-1,4-Benzoxazin-3(4*H*)-One: Key Defense Chemicals of Cereals. *J. Agric. Food Chem.* **2009**, *57*, 1677–1696. [CrossRef]
4. Wouters, F.C.; Blanchette, B.; Gershenzon, J.; Vassão, D.G. Plant Defense and Herbivore Counter-Defense: Benzoxazinoids and Insect Herbivores. *Phytochem. Rev.* **2016**, *15*, 1127–1151. [CrossRef]
5. Cambier, V.; Hance, T.; De Hoffmann, E. Variation of DIMBOA and Related Compounds Content in Relation to the Age and Plant Organ in Maize. *Phytochemistry* **2000**, *53*, 223–229. [CrossRef] [PubMed]
6. Robert, C.A.M.; Veyrat, N.; Glauser, G.; Marti, G.; Doyen, G.R.; Villard, N.; Gaillard, M.D.P.; Köllner, T.G.; Giron, D.; Body, M.; et al. A Specialist Root Herbivore Exploits Defensive Metabolites to Locate Nutritious Tissues. *Ecol. Lett.* **2012**, *15*, 55–64. [CrossRef] [PubMed]
7. Frey, M.; Schullehner, K.; Dick, R.; Fiesselmann, A.; Gierl, A. Benzoxazinoid Biosynthesis, a Model for Evolution of Secondary Metabolic Pathways in Plants. *Phytochemistry* **2009**, *70*, 1645–1651. [CrossRef] [PubMed]
8. Zhang, C.; Li, J.; Li, S.; Ma, C.; Liu, H.; Wang, L.; Qi, J.; Wu, J. ZmMPK6 and Ethylene Signalling Negatively Regulate the Accumulation of Anti-insect Metabolites DIMBOA and DIMBOA-Glc in Maize Inbred Line A188. *New Phytol.* **2021**, *229*, 2273–2287. [CrossRef]
9. Ding, X.; Yang, M.; Huang, H.; Chuan, Y.; He, X.; Li, C.; Zhu, Y.; Zhu, S. Priming Maize Resistance by Its Neighbors: Activating 1,4-Benzoxazine-3-Ones Synthesis and Defense Gene Expression to Alleviate Leaf Disease. *Front. Plant Sci.* **2015**, *6*, 830. [CrossRef]
10. Iqbal, N.; Khan, N.A.; Ferrante, A.; Trivellini, A.; Francini, A.; Khan, M.I.R. Ethylene Role in Plant Growth, Development and Senescence: Interaction with Other Phytohormones. *Front. Plant Sci.* **2017**, *8*, 475. [CrossRef]
11. Müller, M.; Munné-Bosch, S. Ethylene Response Factors: A Key Regulatory Hub in Hormone and Stress Signaling. *Plant Physiol.* **2015**, *169*, 32–41. [CrossRef] [PubMed]
12. Lyons, P.C.; Hipskind, J.D.; Wood, K.V.; Nicholson, R.L. Separation and Quantification of Cyclic Hydroxamic Acids and Related Compounds by High-Pressure Liquid Chromatography. *J. Agric. Food Chem.* **1988**, *36*, 57–60. [CrossRef]
13. Hartenstein, H.; Lipmann, T.; Sicker, D. An Efficient Procedure for the Isolation of Pure 2,4-Dihydroxy-7-Methoxy-2H-1,4-Benzoxazin-3(4H)-One (DIMBOA) from Maize. *Indian J. Heterocycl. Chem.* **1992**, *2*, 75–76.
14. Sarker, U.; Oba, S. Catalase, Superoxide Dismutase and Ascorbate-Glutathione Cycle Enzymes Confer Drought Tolerance of *Amaranthus tricolor*. *Sci. Rep.* **2018**, *8*, 16496. [CrossRef] [PubMed]
15. Van Rossum, M.W.P.C.; Alberda, M.; Van Der Plas, L.H.W. Role of Oxidative Damage in Tulip Bulb Scale Micropropagation. *Plant Sci.* **1997**, *130*, 207–216. [CrossRef]
16. Alici, E.; Arabaci, G. Determination of SOD, POD, PPO and Cat Enzyme Activities in *Rumex obtusifolius* L. *Annu. Res. Rev. Biol.* **2016**, *11*, 1–7. [CrossRef]
17. Heath, R.L.; Packer, L. Photoperoxidation in Isolated Chloroplasts. *Arch. Biochem. Biophys.* **1968**, *125*, 189–198. [CrossRef]
18. Baryla, A.; Laborde, C.; Montillet, J.-L.; Triantaphylidès, C.; Chagvardieff, P. Evaluation of Lipid Peroxidation as a Toxicity Bioassay for Plants Exposed to Copper. *Environ. Pollut.* **2000**, *109*, 131–135. [CrossRef]
19. Livak, K.J.; Schmittgen, T.D. Analysis of Relative Gene Expression Data Using Real-Time Quantitative PCR and the $2^{-\Delta\Delta CT}$ Method. *Methods* **2001**, *25*, 402–408. [CrossRef]
20. Yang, L.; Sun, Q.; Geng, B.; Shi, J.; Zhu, H.; Sun, Y.; Yang, Q.; Yang, B.; Guo, Z. Jasmonate Biosynthesis Enzyme Allene Oxide Cyclase 2 Mediates Cold Tolerance and Pathogen Resistance. *Plant Physiol.* **2023**, *193*, 1621–1634. [CrossRef]
21. Han, G.-Z. Evolution of Jasmonate Biosynthesis and Signaling Mechanisms. *EXBOTJ* **2016**, *68*, 1323–1331. [CrossRef]
22. Sohn, S.-I.; Pandian, S.; Rakkammal, K.; Largia, M.J.V.; Thamilarasan, S.K.; Balaji, S.; Zoclanclounon, Y.A.B.; Shilpha, J.; Ramesh, M. Jasmonates in Plant Growth and Development and Elicitation of Secondary Metabolites: An Updated Overview. *Front. Plant Sci.* **2022**, *13*, 942789. [CrossRef] [PubMed]
23. Hu, L.; Ye, M.; Erb, M. Integration of Two Herbivore-induced Plant Volatiles Results in Synergistic Effects on Plant Defence and Resistance. *Plant Cell Environ.* **2019**, *42*, 959–971. [CrossRef] [PubMed]
24. Bach, L.; Michaelson, L.V.; Haslam, R.; Bellec, Y.; Gissot, L.; Marion, J.; Da Costa, M.; Boutin, J.-P.; Miquel, M.; Tellier, F.; et al. The Very-Long-Chain Hydroxy Fatty Acyl-CoA Dehydratase PASTICCINO2 Is Essential and Limiting for Plant Development. *Proc. Natl. Acad. Sci. USA* **2008**, *105*, 14727–14731. [CrossRef]
25. Kobayashi, F.; Maeta, E.; Terashima, A.; Kawaura, K.; Ogihara, Y.; Takumi, S. Development of Abiotic Stress Tolerance via bZIP-Type Transcription Factor LIP19 in Common Wheat. *J. Exp. Bot.* **2008**, *59*, 891–905. [CrossRef]
26. Bate, N.J.; Sivasankar, S.; Moxon, C.; Riley, J.M.C.; Thompson, J.E.; Rothstein, S.J. Molecular Characterization of an Arabidopsis Gene Encoding Hydroperoxide Lyase, a Cytochrome P-450 That Is Wound Inducible1. *Plant Physiol.* **1998**, *117*, 1393–1400. [CrossRef] [PubMed]
27. Bowler, C.; Van Camp, W.; Van Montagu, M.; Inzé, D.; Asada, K. Superoxide Dismutase in Plants. *Crit. Rev. Plant Sci.* **1994**, *13*, 199–218. [CrossRef]

28. Sen Raychaudhuri, S.; Deng, X.W. The Role of Superoxide Dismutase in Combating Oxidative Stress in Higher Plants. *Bot. Rev.* **2000**, *66*, 89–98. [CrossRef]
29. Berwal, M.K.; Ram, C. Superoxide Dismutase: A Stable Biochemical Marker for Abiotic Stress Tolerance in Higher Plants. In *Abiotic and Biotic Stress in Plants*; Bosco De Oliveira, A., Ed.; IntechOpen: London, UK, 2019; ISBN 978-1-78923-811-2.
30. Sharma, I.; Ahmad, P. Catalase. In *Oxidative Damage to Plants*; Elsevier: Amsterdam, The Netherlands, 2014; pp. 131–148. ISBN 978-0-12-799963-0.
31. Zeng, C.-Q.; Liu, W.-X.; Hao, J.-Y.; Fan, D.-N.; Chen, L.-M.; Xu, H.-N.; Li, K.-Z. Measuring the Expression and Activity of the CAT Enzyme to Determine Al Resistance in Soybean. *Plant Physiol. Biochem.* **2019**, *144*, 254–263. [CrossRef]
32. Mhamdi, A.; Queval, G.; Chaouch, S.; Vanderauwera, S.; Van Breusegem, F.; Noctor, G. Catalase Function in Plants: A Focus on Arabidopsis Mutants as Stress-Mimic Models. *J. Exp. Bot.* **2010**, *61*, 4197–4220. [CrossRef]
33. Ma, J.; Du, G.; Li, X.; Zhang, C.; Guo, J. A Major Locus Controlling Malondialdehyde Content under Water Stress Is Associated with Fusarium Crown Rot Resistance in Wheat. *Mol. Genet. Genom.* **2015**, *290*, 1955–1962. [CrossRef] [PubMed]
34. Diani, Z.; Yassine, O.; Aissam, S.; Hsissou, D.; El Modafar, C. Induction of Early Oxidative Events in Soft Wheat Leaves Inoculated with Septoria Tritici and Their Relationship to Resistance of Moroccan Cultivars. *Int. J. Agric. Biol.* **2009**, *11*, 351–359.
35. Guo, W.L.; Chen, R.G.; Gong, Z.H.; Yin, Y.X.; Ahmed, S.S.; He, Y.M. Exogenous Abscisic Acid Increases Antioxidant Enzymes and Related Gene Expression in Pepper (*Capsicum Annuum*) Leaves Subjected to Chilling Stress. *Genet. Mol. Res.* **2012**, *11*, 4063–4080. [CrossRef] [PubMed]
36. Hussain, I.; Ashraf, M.A.; Rasheed, R.; Iqbal, M.; Ibrahim, M.; Ashraf, S. Heat Shock Increases Oxidative Stress to Modulate Growth and Physico-Chemical Attributes in Diverse Maize Cultivars. *Int. Agrophysics* **2016**, *30*, 519–531. [CrossRef]
37. Zhang, H.; Zhao, Y.; Zhu, J.-K. Thriving under Stress: How Plants Balance Growth and the Stress Response. *Dev. Cell* **2020**, *55*, 529–543. [CrossRef]
38. Zhang, Y.; Luan, Q.; Jiang, J.; Li, Y. Prediction and Utilization of Malondialdehyde in Exotic Pine Under Drought Stress Using Near-Infrared Spectroscopy. *Front. Plant Sci.* **2021**, *12*, 735275. [CrossRef]
39. Wang, D.; Gao, Y.; Sun, S.; Lu, X.; Li, Q.; Li, L.; Wang, K.; Liu, J. Effects of Salt Stress on the Antioxidant Activity and Malondialdehyde, Solution Protein, Proline, and Chlorophyll Contents of Three *Malus* Species. *Life* **2022**, *12*, 1929. [CrossRef] [PubMed]

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