

# METHYL JASMONATE ELICITATION AFFECTS EXPRESSION OF GENES INVOLVED IN BIOSYNTHESIS AND TURNOVER OF 2-PHENYLETHYLAMINE IN MAIZE SEEDLINGS

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Received April 20, 2015; revision accepted February 14, 2016

The objective of the study was to assess the influence of methyl jasmonate (MJ) vapors on accumulation of 2-phenylethylamine (PEA), phenylacetic acid (PAA) and 2-phenylethanol (PE) in leaves and roots of maize (*Zea mays* L. subsp. *mays*, *saccharata* group, cv. Złota Karłowa) seedlings. Furthermore, we analyzed the expression patterns of eight genes (*ADH1*, *ADH2*, *AO2*, *CAO*, *PDC1*, *PDC2*, *PTA* and *LOX*, encoding alcohol dehydrogenase 1 and 2, primary amine oxidase, aldehyde oxidase 2, phenylalanine decarboxylase 1 and 2, phenylalanine (histidine) transaminase and lipoxygenase, respectively) involved in biosynthesis and turnover of PEA in maize tissues. In addition, the effect of MJ application on fresh biomass and growth of the tested seedlings was recorded. One-day MJ exposure increased the fresh weight of aerial parts and roots of *Z. mays* seedlings, whereas the opposite tendency occurred after 4-day of MJ treatment. One-day application of MJ resulted in an increase in the length of roots and its fluctuations in the aerial parts of maize plants, but extended exposure declined the growth of both parts of the seedlings. Methyl jasmonate elicitation caused various changes in the contents of PEA, PAA and PE in the maize seedlings. MJ treatments led to high upregulation of most genes, with the exception of three genes (i.e., *ADH1*, *ADH2* and *AO2*) whose expression was downregulated after a 4-day exposure.

**Keywords:** methyl jasmonate, 2-phenylethylamine, phenylacetic acid, 2-phenylethanol, gene expression, maize

## INTRODUCTION

Methyl jasmonate (MJ) is one of the most crucial plant signaling molecules that mediate both local and systemic responses against a wide spectrum of environmental stressing factors, such as wounding, ozone treatment, insect or pathogen attack (Thorpe et al., 2007; Pauwels et al., 2008; Loyola-Vargas et al., 2012). Thorpe et al. (2007) evidenced that exogenously applied MJ was exported from foliar tissues to phloem and xylem sieve tubes in tobacco (*Nicotiana tabacum* L.) plants. Additionally, MJ promoted translocation

of the photoassimilates as well as markedly influenced proton gradient in the plasma membrane. It has been also reported that MJ exposure led to enhanced generation of hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>), oxidative stress-related injuries in the mitochondrial membranes and declined ATP synthesis (Loyola-Vargas et al., 2012).

The potency of MJ to trigger accumulation of secondary metabolites in plants has been widely reported (Pauwels et al., 2008; Horbowicz et al., 2011; Ruiz-May et al., 2011; Concha et al., 2013). Transcriptomic analyses performed by Chen et al. (2013) demonstrated that MJ evoked rapid and

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circumstantial alternations in the expression pattern of genes involved in the biogenesis of indole alkaloids, terpenoids and putative phenylpropanoids pathways in the hairy roots of *Isatis indigotica* L. plants. Furthermore, Pauwels et al. (2008) uncovered the profound transcriptional reprogramming of various transcription factors (e.g., AZF2, ORA47, AP2/ERF, MYC2), and upregulation of genes participating in synthesis of phenylpropanoids, mono- and oligolignols in response to MJ application in *Arabidopsis thaliana* L. cells cultured under *in vitro* conditions.

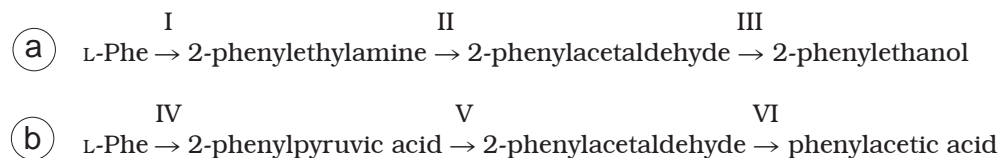
The presence of 2-phenylethylamine (PEA) has been confirmed in several species of plants (Smith, 1977; Horbowicz et al., 2011; Le Thi et al., 2014). Smith (1977) suggested the role of PEA in constituting the plant resistance towards a wide range of insect pests. Interestingly, Le Thi et al. (2014) documented the allelopathic potential of PEA derivative isolated from rice (*Oryza sativa* L.) against barnyard grass (*Echinochloa crusgalli* L.), cress (*Lepidium sativum* L.) and red sprangletop (*Leptochloa chinensis* L. Nees).

Phenylacetic acid (PAA) has been detected in the organs of many plant systems (Jerković and Marijanović, 2010; Kuš et al., 2013). Although PAA has been recognized to exhibit an auxinomimetic role in regulation of plant growth and development, detailed mechanisms of action of this compound remain unknown (Simon and Petrášek, 2011). The two-step biogenesis of PAA in plants consists in conversion of L-phenylalanine (L-Phe) to phenylpyruvate and glutamate, with subsequent decarboxylation and oxidation to phenylacetaldehyde (PAAld) (Tomè et al., 1975).

2-Phenylethanol (PE) is an aromatic alcohol that is a major floral scent ingredient emitted by rose flowers (Sakai et al., 2007; Chen et al., 2011). Furthermore, it has been identified in tissues of a wide range of plant systems, such as tomato, grape, apple, tamarind, jasmine, petunia, lily, hyacinth and narcissus (Kaminaga et al., 2006; Tieman et al., 2007; Jerković and Marijanović, 2010; Kuš et al., 2013). There have been described numerous biogenetic pathways of PE. Sakai et al. (2007)

reported that two biocatalysts (L-aromatic amino acid decarboxylase – AADC and phenylacetaldehyde reductase – PAR) are linked with biosynthesis of this compound from L-Phe via PAAld in the flower petals of *Rosa* ‘Hoh-Jun’ hybrid. Additionally, a turnover of PEA into PAAld by removal of the amine group, and further conversion to PE processed by two phenylacetaldehyde reductases in tomato fruits were described (Tieman et al., 2006; Tieman et al., 2007) (Fig. 1). Besides, Kaminaga et al. (2006) found that phenylacetaldehyde synthase (PAAS) isolated from *Petunia hybrida* plants catalyzes decarboxylation and oxidation of L-Phe to PAAld. Zarei et al. (2015) suggested that the isozyme MDAO2 (copper-containing amine oxidase 2) from *Malus domestica* Borkh. fruits may be responsible for PEA deamination process, the first step in PE biogenesis.

Methyl jasmonate stimulates a high-level accumulation of 2-phenylethylamine (PEA) in de-etiolated seedlings of common buckwheat (*Fagopyrum esculentum* Moench) (Horbowicz et al., 2011). This transformation is catalyzed by amino acid decarboxylase (AADC), wherein L-phenylalanine is used as a substrate. The results of our recent study indicated that PEA can be a possible intermediate in biosynthesis of 2-phenylethanol (PE) and phenylacetic acid (PAA) in common buckwheat plants (Horbowicz et al., 2015). We undertook genetic surveys consisting in expression evaluation of the genes involved in the pathways (i.e., L-phenylalanine decarboxylase, alcohol dehydrogenase, aldehyde oxidase, synthase of aromatic aldehydes, amine oxidase, and lipoxygenase). However, these studies did not bring the expected results. It was due to absence of DNA sequences of the mentioned genes relevant to buckwheat. The search of primers for the reaction real-time qRT-PCR in the available databases also failed. Importantly, the preliminary analyses with the use of HPLC-DAD-MS confirmed the presence of PEA, PE and PAA in leaves and roots of maize seedlings, and additionally, MJ-stimulated modulations in the contents of these compounds were demonstrated. Therefore, we decided to perform similar genetic studies on



**Fig. 1.** Scheme of 2-phenylethylamine and 2-phenylethanol (a), and phenylacetic acid (b) biogenesis in plants (Tomè et al., 1975; Zhang et al., 2014). I – Amino acid decarboxylase (AADC); II – monoamine oxidase (MOA); III – phenylacetaldehyde reductase; IV – amino acid transaminase (AAT); V – phenylpyruvic acid decarboxylase; VI – 2-phenylacetaldehyde oxidase. Expression of the genes encoding I, II and IV enzymes was investigated in the current study.

maize as a model plant species, whose genome has been sequenced.

There is no available information regarding the impact of MJ on accumulation of PEA, PE or PAA as well as the transcriptional reprogramming of the genes linked to biosynthesis and conversion of PEA in *Z. mays* seedlings. Moreover, the biogenetic routes of these metabolites in maize plants are highly unclear. Therefore, the objective of the study was to evaluate the effect of exogenously applied MJ on the content of PEA, PAA and PE, and expression of the selected genes (i.e., alcohol dehydrogenase 1 – *ADH1*, alcohol dehydrogenase 2 – *ADH2*, aldehyde oxidase 2 – *AO2*, primary amine oxidase – *CAO*, phenylalanine decarboxylase 1 – *PDC1*, phenylalanine decarboxylase 2 – *PDC2*, phenylalanine (histidine) transaminase – *PTA* and lipoxygenase – *LOX*), involved in biosynthesis and turnover of PEA and its metabolites in leaves and roots of *Z. mays* seedlings. The study was also aimed at establishing the impact of MJ treatment on fresh biomass and growth of aerial parts and roots of the maize plants.

## MATERIALS AND METHODS

### PLANT MATERIAL

Seeds of maize (*Zea mays* L. subsp. *mays*, *saccharata* group, cv. *Złota Karłowa*) were obtained from a local seed company (PNOS S.A., Ożarów Mazowiecki, Poland). They were surface sterilized for 2 min in 70% ethanol, and 3 min in 0.1%  $\text{HgCl}_2$ ; next, the seeds were washed five times for 30 s in sterile water. Maize seedlings were prepared by germination of seeds placed between two layers of wet filter paper, which were then rolled and inserted in a 2.5 dm<sup>3</sup> glass beaker containing ca. 200 cm<sup>3</sup> of tap water. The germination process was carried out in darkness at 24±1°C. After four days of germination, the seedlings were transferred to a growth room and conditioned for 24 h. The room temperature was maintained at 22±2°C/18±2°C (day/night: 16 h/8 h), whereas light (100 µM × m<sup>-2</sup> × s<sup>-1</sup>) was provided by high-pressure sodium lamps.

### METHYL JASMONATE TREATMENT

Healthy maize seedlings of similar height (length of the roots ca. 14.0 cm; length of the aerial parts ca. 7.5 cm) were treated with methyl jasmonate (MJ) vapors, and the experiments were performed in three independent replicates. Each series of the experiments included forty maize seedlings per each MJ vapor concentration and the same number of control plants. MJ solutions were prepared in ethyl alcohol, and 100 mm<sup>3</sup> of each solution was placed on a narrow ribbon of filter paper. Subsequently,

the solvent was evaporated at room temperature for 5 min, and then, the filter paper was inserted into a jar. Afterwards, the filter paper containing MJ was placed against the inner wall of the jar containing the rolls with maize seedlings, and the jar was immediately closed tightly with transparent silicon film. The concentrations of atmospheric MJ in these jars were calculated as 10<sup>-8</sup>, 10<sup>-6</sup> or 10<sup>-4</sup> M (with the assumption of its complete evaporation). After 1 or 4 days of growth in such conditions, the biomass and length of aerial parts and roots of the maize seedlings were measured. Additionally, the plant samples (leaves and roots) for genetic studies were immediately homogenized in liquid nitrogen using sterile and RNase-free ceramic mortar and pestle. For chemical analyses, leaves and roots of the seedlings were freeze-dried for 72 h in a freeze dryer ALPHA 1–2 LD Plus (Christ, Germany).

### QUANTIFICATION OF THE EXAMINED METABOLITES IN *Z. MAYS* SEEDLINGS

The freeze-dried powder (50 mg) of the respective organs of maize seedlings was homogenized with 2 cm<sup>3</sup> of methanol-deionized water solution (80%, *v/v*) using sonication (VC 750, Sonics & Materials, USA) for 60 s, and intensive vortexing for 30 s. Next, the samples were subjected to 24-h shaking (amplitude of 600 rpm) at 20°C, and centrifuged for 20 min at 13,000 × *g* (4°C). The supernatant was stored at -80°C until chemical analyses.

Determination of 2-phenylethylamine (PEA), phenylacetic acid (PAA) and 2-phenylethanol (PE), as well as identification of PAAld were accomplished with application of HPLC-MS/MS. The conditions for ion-spray voltage, ion-source gases, declustering potential, entrance potential, collision energy, and collision cell exit potential were applied as previously described (Horbowicz et al., 2015).

### EXTRACTION OF TOTAL RNA AND SYNTHESIS OF COMPLEMENTARY DNA

Isolation of total RNA from the maize seedlings was performed using the Spectrum Plant Total RNA Kit (Sigma-Aldrich, Poland), and then, trace amounts of DNA were hydrolyzed enzymatically with application of the On-Column DNase I Digestion Set (Sigma-Aldrich, Poland). Evaluation of quantity and purity of the obtained RNA samples was achieved with the use of Epoch UV-Vis microplate spectrophotometer (BioTek, USA). Subsequently, only high-quality total RNA samples were selected for performing cDNA synthesis using the High Capacity cDNA Reverse Transcription Kit with RNase Inhibitor (Life Technologies, Poland). Two types of negative controls (NT – no template, and NRT – no reverse transcriptase) were also prepared.

MEASUREMENT OF THE RELATIVE EXPRESSION  
OF THE INVESTIGATED GENES IN MAIZE SEEDLINGS

The amount of eight examined transcripts (*ADH1*, *ADH2*, *AO2*, *CAO*, *PDC1*, *PDC2*, *PTA* and *LOX*) in MJ-stressed and control (untreated) *Z. mays* seedlings was estimated using the quantitative real-time polymerase chain reaction (qRT-PCR). The obtained data were normalized to glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) gene. Primers and fluorescent probe for *GAPDH* gene were synthesized by Life Technologies (Poland), according to the sequences deposited in the GenBank probe database (accession no. Pr032251180). Relative expression of the four analysed genes (*ADH1*, *ADH2*, *CAO* and *LOX*) was measured with application of the TaqMan Gene Expression Assays (no. Zm04059111\_g1, Zm04059120\_g1, Zm04018249\_m1, and Zm04057709\_g1, respectively) that were provided by Life Technologies (Poland). The abundance of four other studied transcripts (*AO2*, *PDC1*, *PDC2* and *PTA*) was quantified using Custom TaqMan Gene Expression Assays (Life Technologies, Poland). Sequences of primers and fluorescent probes designed for these genes are presented in Tables 1–2. Designing of the primers and probes for the Custom TaqMan Gene Expression Assays was carried out on the basis of the reference sequence of *AO2* gene available in GenBank Database (accession no. D88452.1), whereas sequences of *PDC1* (ID: GRMZM2G451314), *PDC2* (ID: AC217811.3\_FGP002) and *PTA* (ID: GRMZM2G127308\_P01) were obtained from Maize Genetics and Genomics Database (<http://maizegdb.org>). Gene expression quantification was achieved using StepOne Plus Real-Time PCR System equipped with StepOne Plus Software v2.3 (Applied Biosystems, USA). A fast mode of DNA amplification in 96-well micro-

plates was chosen. The final volume (20 mm<sup>3</sup>) of the reaction mixture contained 4 mm<sup>3</sup> of the template (50 ng of cDNA), 10 mm<sup>3</sup> of 2 × TaqMan Fast Universal PCR Master Mix, 1 mm<sup>3</sup> of 20 × TaqMan Gene Expression Assay, and 5 mm<sup>3</sup> of PCR grade and RNase-free water. The measurement of the relative expression of the eight examined genes in *Z. mays* seedlings was performed using the comparative C<sub>T</sub> ( $\Delta\Delta C_T$ ) method of Livak and Schmittgen (2001). The results were presented as the mean of *n*-fold changes ( $\pm$  SD) in the abundance of the target transcripts in MJ-stressed maize seedlings in relation to the control plants. All the genetic analyses were conducted in four independent biological and three technical replications.

STATISTICAL ANALYSIS

Three-way analysis of variance (ANOVA) with Tukey's post-hoc test was applied to assess the significance of differences in the mean values of the examined parameters between MJ-treated and control (untreated) *Z. mays* seedlings. P-values smaller than 0.05 were considered significant. All calculations were performed using STATISTICA 10 software (StatSoft, Poland).

RESULTS

INFLUENCE OF METHYL JASMONATE ON BIOMASS  
AND GROWTH OF *Z. MAYS* SEEDLINGS

It was demonstrated that all the applied concentrations (10<sup>-8</sup>, 10<sup>-6</sup> and 10<sup>-4</sup> M) of methyl jasmonate (MJ) vapors increased the biomass of roots and aerial parts of *Z. mays* seedlings at 1 d, compared to the untreated control (Table 3). It was observed that 10<sup>-8</sup> M concentration of MJ led to ca. 23% and

TABLE 1. Sequences of the primers used for qRT-PCR amplification of *AO2*, *PDC1*, *PDC2* and *PTA* genes of maize

Target gene	Primer type	Primer sequence
<i>AO2</i>	F	AAGCAAGCCATCATTGAGTATAGCA
	R	CTGCTTCGTTCAATTGCATCTTCTA
<i>PDC1</i>	F	AGCAGCAAGAATGTACAGAATGGAT
	R	CAGCACAATCTATTTCCCCAGACAT
<i>PDC2</i>	F	AGTGGCGCCGACGG
	R	TCCCCATCACTATCTCCCTCTTC
<i>PTA</i>	F	CGTCAGCCGACAGTA
	R	GGCCTTGCCACGGA

F – forward primer, R – reverse primer.

TABLE 2. Sequences of *TaqMan* fluorescent probes used for qRT-PCR amplification of *AO2*, *PDC1*, *PDC2* and *PTA* genes of maize

Target gene	Primer sequence
<i>AO2</i>	FAM-CAGCCACCAATTCT-NFQ
<i>PDC1</i>	FAM-AAGGGTGTCAACTTTG-NFQ
<i>PDC2</i>	FAM-TTGGCAGCCTCCTCCC-NFQ
<i>PTA</i>	FAM-CCGCCGCCTCCACC-NFQ

FAM – 6-carboxyfluorescein, NFQ – 3'-non-fluorescent quencher.

7% increases in roots and aerial parts, respectively; higher concentration ( $10^{-6}$  M) of methyl jasmonate caused *ca.* 10% and 5% increments in the respective seedling parts, whereas the highest concentration ( $10^{-4}$  M) induced very similar elevations in the fresh weight (*ca.* 15% and 16%, respectively). The opposite tendency occurred after 4 days of continuous exposure of maize plants to MJ (Table 3). It was elucidated that both investigated parts of the seedlings were characterized by reduced biomass in relation to the non-stressed plants. Importantly, the strongest inhibitory effect was noted in the seedlings treated with the highest MJ concentration ( $10^{-4}$  M), whereas the smallest changes were noted at the lowest concentration ( $10^{-8}$  M). Furthermore, the roots were characterized by a greater decline in the fresh

weight (*ca.* 22–53%), compared with the aerial parts (*ca.* 2–21%). It was observed that a short-term (1 d) application of MJ ( $10^{-8}$  M) triggered comparable increases in the length of roots and aerial parts of the maize seedlings (*ca.* 8 and 9%, respectively) as compared to the untreated plants (Table 3). Higher concentration of MJ ( $10^{-6}$  M) evoked *ca.* 8% increment of root growth and a negligible decrease (1.7%) in the growth of aerial parts. Treatment with jasmonate at a concentration of  $10^{-4}$  M resulted in only slight alternations in the length of roots and aerial parts of the seedlings (4% increase and 2% decline, respectively). Longer MJ exposure (4 d) inhibited the growth of roots and aerial parts of the maize seedlings (*ca.* 14–42% and 3–31% decreases, respectively) (Table 3).

TABLE 3. Effect of methyl jasmonate (MJ) vapors on biomass (g FW) and length (mm) of aerial parts and roots of *Z. mays* seedlings

MJ treatment	Biomass		Length	
	1 day	4 days	1 day	4 days
<b>Aerial parts</b>				
Control (0)	2.84±0.26 <sup>c</sup>	4.35±0.71 <sup>a</sup>	76.6±6.30 <sup>i</sup>	131.7±8.87 <sup>f</sup>
$10^{-8}$ M	3.03±0.19 <sup>c</sup>	4.28±0.61 <sup>a</sup>	83.2±7.53 <sup>h</sup>	127.2±7.94 <sup>f</sup>
$10^{-6}$ M	2.99±0.33 <sup>c</sup>	3.97±0.81 <sup>ab</sup>	75.3±6.03 <sup>i</sup>	119.2±8.75 <sup>g</sup>
$10^{-4}$ M	3.29±0.27 <sup>bc</sup>	3.42±0.20 <sup>b</sup>	74.8±5.25 <sup>i</sup>	90.8±5.45 <sup>h</sup>
<b>Roots</b>				
Control (0)	0.71±0.06 <sup>fg</sup>	1.48±0.42 <sup>d</sup>	144.9±6.27 <sup>de</sup>	264.9±10.60 <sup>a</sup>
$10^{-8}$ M	0.87±0.13 <sup>f</sup>	1.15±0.22 <sup>de</sup>	157.0±7.58 <sup>d</sup>	228.1±10.70 <sup>b</sup>
$10^{-6}$ M	0.78±0.08 <sup>f</sup>	0.92±0.26 <sup>ef</sup>	155.9±6.32 <sup>d</sup>	196.1±7.50 <sup>c</sup>
$10^{-4}$ M	0.82±0.06 <sup>f</sup>	0.70±0.12 <sup>fg</sup>	150.0±6.80 <sup>d</sup>	154.4±4.90 <sup>d</sup>

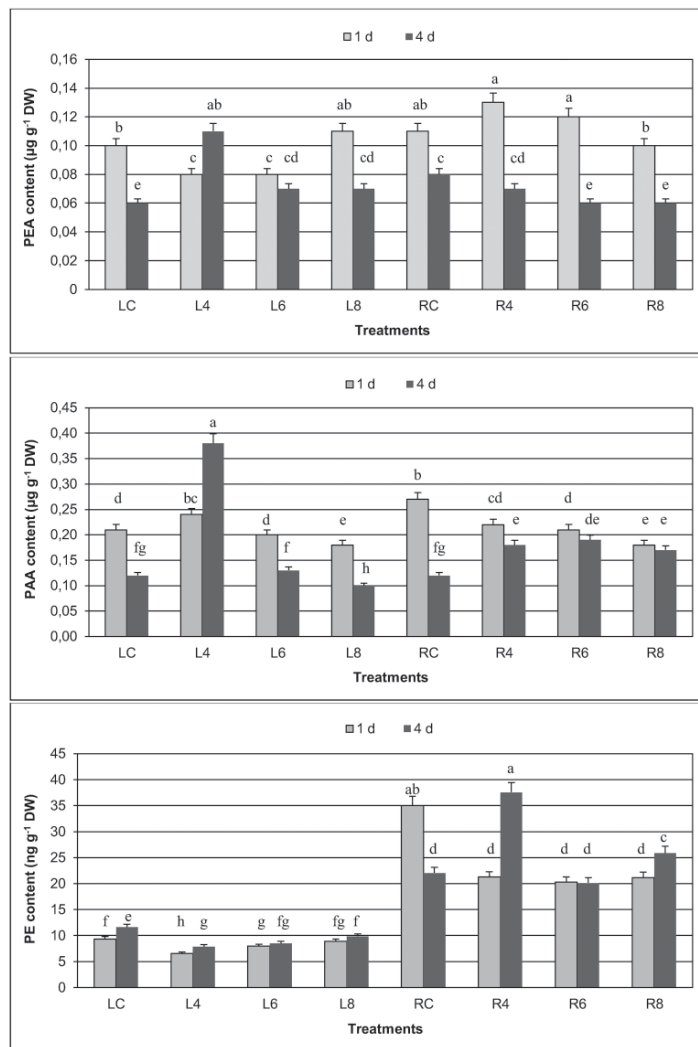
FW – fresh weight. All data are presented as the mean ± standard deviation (SD), and the experiments were performed in three independent replications. Each series of the biotests included forty maize seedlings ( $n=40$ ) per each MJ vapors treatment ( $10^{-8}$ ,  $10^{-6}$ , and  $10^{-4}$  M). The same number of control plants was untreated with MJ. Different letters indicate significant changes in the average values of biomass and length of aerial parts and roots of *Z. mays* seedlings ( $p<0.05$ ; post-hoc Tukey's test).

The results of the factorial analysis of variance confirmed significant effects ( $p < 0.05$ ) of the investigated parameters (seedling part, MJ treatment and exposure time) as well as the interactions affecting the biomass and growth of roots and aerial parts of the maize seedlings.

IMPACT OF METHYL JASMONATE  
ON THE CONTENT OF 2-PHENYLETHYLAMINE (PEA),  
PHENYLACETIC ACID (PAA)  
AND 2-PHENYLETHANOL (PE) IN *Z. MAYS* SEEDLINGS

It was found that 1-day exposure to MJ vapors affected circumstantial fluctuations in PEA levels in

leaves and roots of maize seedlings (Fig. 2). It was noted that the lowest MJ dose ( $10^{-8}$  M) resulted in ca. 9% decline in the content of PEA in the roots, while higher concentrations ( $10^{-6}$  and  $10^{-4}$  M) of MJ led to 9% and 18% increases, respectively. The opposite tendency was observed in the leaves. The use of  $10^{-8}$  M of MJ enhanced the level of PEA (ca. 10%), whereas two higher doses ( $10^{-6}$  and  $10^{-4}$  M) led to ca. 20% decrease in comparison with the control. Extended time of exposure (4 days) resulted in ca. 12–25% reduction of PEA content in the roots. In contrast, the leaves of MJ-treated maize seedlings contained about 17–83% higher levels of PEA in relation to the control.



**Fig. 2.** Effect of MJ vapors on content of 2-phenylethylamine (PEA), phenylacetic acid (PAA) and 2-phenylethanol (PE) in leaves and roots of maize seedlings. All data are presented as the mean ( $\pm$  SD). Different letters above SD bars indicate significant changes in the average content of the analysed compound in *Z. mays* seedlings ( $p < 0.05$ ; post-hoc Tukey's test). LC and RC – control leaves and roots of maize, respectively (untreated with MJ); L4, L6 and L8 – leaves of the maize seedlings treated with  $10^{-4}$ ,  $10^{-6}$  or  $10^{-8}$  M of MJ vapors, respectively; R4, R6 and R8 – roots of the seedlings exposed to the respective concentrations of MJ vapors; d – day.

It was found that 1-day MJ use resulted in declines of phenylacetic acid (PAA) content in the roots (Fig. 2). The levels of PAA were inversely proportional to the concentration of MJ (dose  $10^{-8}$  M caused ca. 33% decline in PAA content, whereas  $10^{-4}$  M evoked 18% decrease). Moreover, it was observed that the leaves of maize seedlings variably responded to the applied MJ concentrations. The lowest ( $10^{-8}$  and  $10^{-6}$  M) caused ca. 14% and 5% declines in PAA content in the leaves, respectively. In contrast,  $10^{-4}$  M of MJ led to 14% increase of PAA. Extended, 4-days exposure resulted in 42–58% increases in the levels of PAA, depending on the applied MJ concentration.

The results of the carried out experiments showed that 1-day exposure to MJ vapors declined the content of 2-phenylethanol (PE) in both examined organs of *Z. mays* seedlings (Table 3). The applied concentrations of MJ led to similar decreases (39–42%) in PE levels in the roots compared to the control. Also in the leaves, 5–30% declines in the PE content were observed. The extended exposure (4 d) of maize seedlings to the MJ caused 15–32% decreases in PE content in the leaves. At this time point, MJ concentration of  $10^{-6}$  M caused a small decline in PE level in the roots, whereas two other doses ( $10^{-8}$  and  $10^{-4}$  M) resulted in 17% and 70% increases, respectively.

Statistical analyses (factorial ANOVA) showed significant effects ( $p < 0.05$ ) of the tested indicators (seedling organ, MJ treatment and exposure time) as well as their interconnections influencing the content of the analysed compounds (PEA, PAA and PE) in *Z. mays* seedlings. The only exception was the effect of seedling organ on the level of PAA that was not significant ( $p = 0.124$ ).

#### IDENTIFICATION OF PHENYLACETALDEHYDE (PAALD) IN LEAVES OF MAIZE SEEDLINGS

Searching for a full picture of PEA metabolism, we made attempts to find intermediate metabolites in high-resolution mass spectra obtained from foliar extracts of *Z. mays* seedlings. In the spectra, there was observed an ion at  $m/z$  121.0591. The ion was fragmented to ion at  $m/z$  103.0529, which was attributed to the unit  $C_6H_5CH=CH^+$  (loss of  $H_2O$ ). Other peaks noted in the mass spectrum were the following:  $m/z$  77.0381 ( $C_6H_5^+$ ) and 91.0538 ( $C_6H_5CH^+$ ).

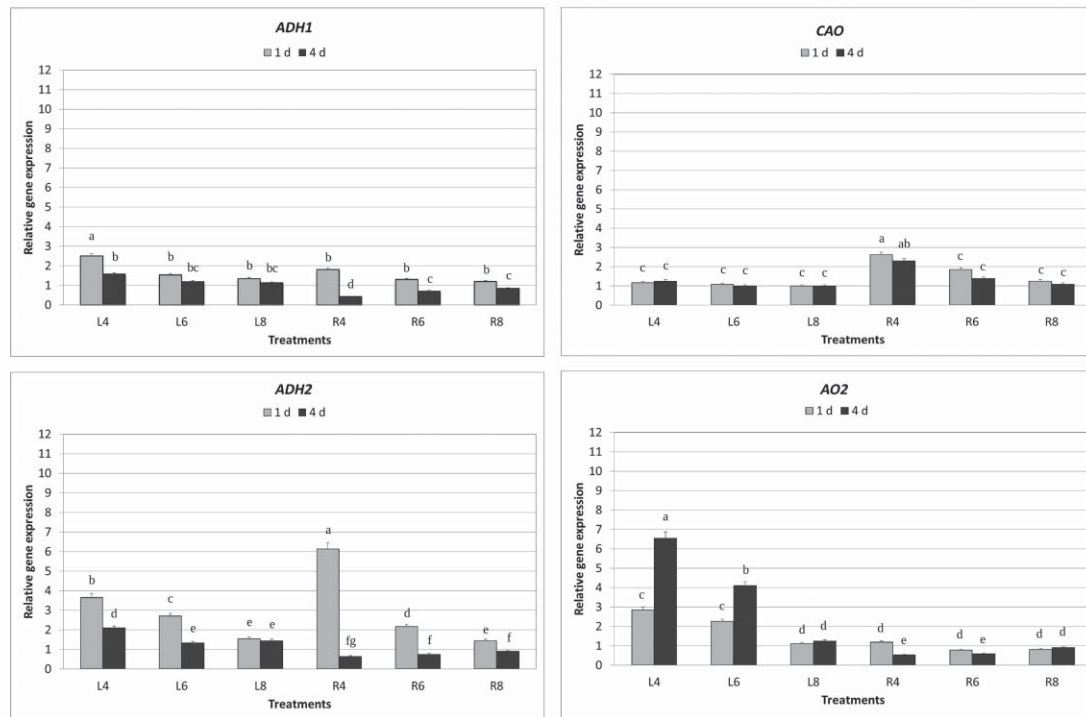
The obtained mass spectrum was very similar to phenylacetaldehyde shown in the DrugBank 4.2 Database (DrugBank ID: DB02178; [http://www.drugbank.ca/spectra/ms\\_ms/2479](http://www.drugbank.ca/spectra/ms_ms/2479)) (Law et al., 2014). Thus, we are certain of the presence of phenylacetaldehyde among the compounds in the foliar tissues of maize seedlings. We made an attempt to identify another possible metabolite of PEA, i.e., phenylpyruvic acid, but ions appropriate for this compound were not found among the studied spectra.

#### EFFECT OF METHYL JASMONATE ON EXPRESSION OF GENES INVOLVED IN BIOSYNTHESIS AND TURNOVER OF 2-PHENYLETHYLAMINE (PEA)

The results regarding MJ-triggered alternations in the levels of the relative expression of the eight tested genes (*ADH1*, *ADH2*, *AO2*, *CAO*, *PDC1*, *PDC2*, *PTA* and *LOX*) in leaves and roots of maize seedlings are presented in Figures 3–4. It was shown that 1-day treatment with MJ caused 35–150% and 20–80% increases in the amount of *ADH1* transcript in the leaves and roots of *Z. mays* seedlings, respectively (Fig. 3). Extended use of MJ led to increments in the leaves (13–57%), whereas in the case of roots 16–57% declines, in comparison to the control, were noted. A similar tendency was demonstrated in expression patterns of *ADH2* gene (Fig. 3). It was found that shorter exposure (1 d) to MJ led to 1.5–3.7-fold and 1.4–6.1-fold increases in the relative expression of *ADH2* gene in seedling leaves and roots, respectively. Four-day MJ treatment caused 1.4–2.1-fold increments and 8–36% decreases in the abundance of *ADH2* transcript in the respective organs of the maize seedlings.

The performed analyses showed that MJ vapors induced expression levels of *AO2* gene (encoding aldehyde oxidase 2) in the leaves of *Z. mays* seedlings (1d – 1.1–2.8-fold increases; 4 d – 1.3–6.5-fold increases in relation to the control) (Fig. 3). Additionally, the highest MJ concentration ( $10^{-4}$  M) stimulated expression of the gene (20%) in the roots at 1 d, but lower MJ doses repressed (17–22%) abundance of the target transcript. Extended exposure (4 d) of maize plants to MJ was associated with downregulation (8–47% decreases) of *AO2* gene in the roots. Moreover, it was shown that MJ slightly enhanced the expression of the primary amine oxidase (*CAO*) gene in the leaves (1 d – 18% and 9% increments at  $10^{-4}$  and  $10^{-6}$  M, respectively; 4 d – 26% increase at  $10^{-4}$  M) (Fig. 3). However, it should be noted that other MJ treatments ( $10^{-8}$  M at 1 and 4 days, and  $10^{-6}$  M at 1 d) did not affect any transcriptional reprogramming of *CAO* gene in the maize leaves when compared to the control. It was also found that MJ at all the applied concentrations stimulated upregulation of the target gene in the roots (1 d – 1.3–2.6-fold increases; 4 d – 1.1–2.3-fold increments).

MJ at all the concentrations triggered an increase in *PDC1* (phenylalanine decarboxylase 1) transcript abundance in *Z. mays* leaves (1 d – 1.3–10.2-fold increases; 4 d – 1.1–3.1-fold increments in relation to the control) (Fig. 4). Transcriptional responses of *PDC1* gene to MJ were markedly lower in the maize roots, compared with the levels recorded in the leaves. The lowest dose of MJ ( $10^{-8}$  M) after 4 days of treatment did not have an effect on the relative expression of *PDC1* gene in the



**Fig. 3.** Expression patterns of *ADH1*, *ADH2*, *AO2* and *CAO* genes in MJ-treated *Z. mays* plants. Relative gene expression is presented as the mean *n*-fold change ( $\pm$  SD) in abundance of the investigated transcript in MJ-stressed maize seedlings compared to the control (untreated) plants. Transcriptional data were normalized to glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) gene. All genetic analyses were conducted in four independent biological and three technical replications. Different letters above SD bars denote significant differences in the levels of the target gene expression between the tested groups of *Z. mays* plants ( $p < 0.05$ ; post-hoc Tukey's test). *ADH1* – alcohol dehydrogenase 1; *ADH2* – alcohol dehydrogenase 2; *AO2* – aldehyde oxidase 2; *CAO* – primary amine oxidase; d – day; L4, L6 and L8 – leaves of the maize seedlings treated with  $10^{-4}$ ,  $10^{-6}$  or  $10^{-8}$  M of methyl jasmonate (MJ) vapors, respectively; R4, R6 and R8 – roots of the seedlings exposed to the respective concentrations of MJ vapors.

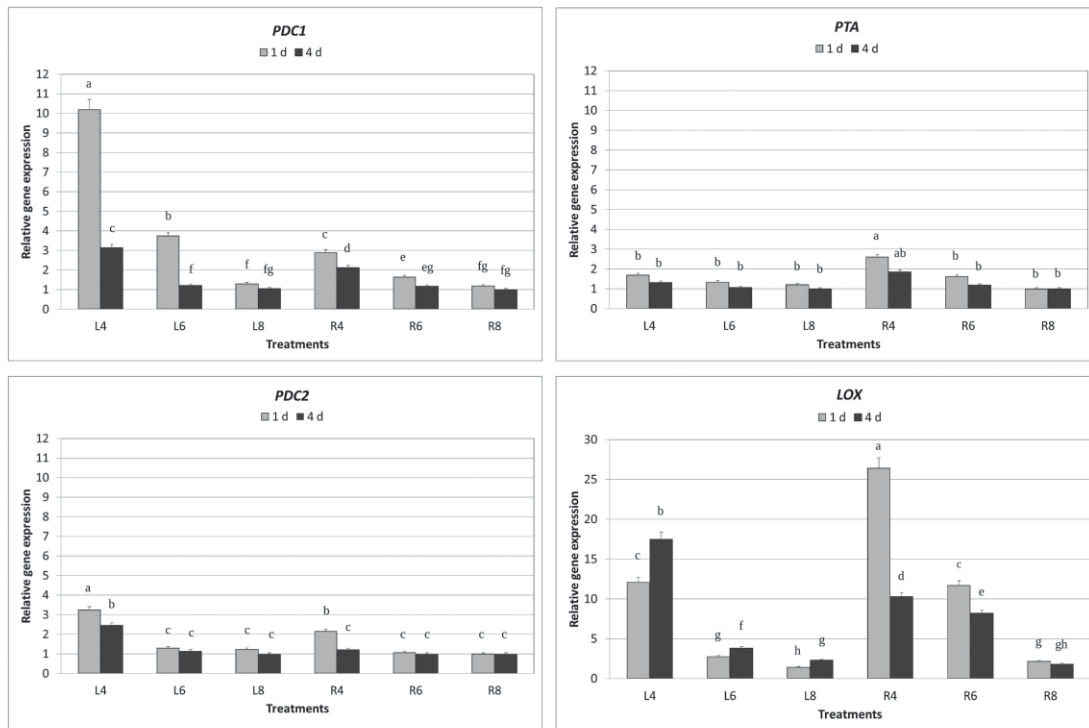
roots. All other experimental options were associated with a slight elevation in the transcriptional activity of the target gene (1 d – 1.2–2.9-fold increases, depending on the MJ concentration; 4 d – 2.1-fold and 1.2-fold increments at  $10^{-4}$  and  $10^{-6}$  M, respectively). Similar expression profiles of phenylalanine decarboxylase 2 (*PDC2*) gene in the maize seedlings subjected to MJ treatment were identified (Fig. 4). One-day MJ treatment ( $10^{-6}$  and  $10^{-4}$  M) stimulated 1.2–3.3-fold increases, whereas 4-day exposure led to 1.1-fold and 2.5-fold increments, respectively. The lowest dose of MJ ( $10^{-8}$  M) did not result in transcriptional alternations of the gene in the roots after 1 and 4 days of exposure, and in the leaves at 4 d. Additionally, no changes in the relative gene expression were noted in the roots of maize plants exposed to 1-day MJ treatment at  $10^{-6}$  M.

Short-term (1 d) application of MJ resulted in upregulation of the phenylalanine (histidine) transaminase (*PTA*) gene in the seedling leaves (1.2–

1.7-fold increases) and within the roots (2.6- and 1.6-fold increments at  $10^{-4}$  and  $10^{-6}$  M, respectively) (Fig. 4). However, the transcript amount remained unchanged in the roots of the seedlings treated with  $10^{-8}$  M of MJ. Furthermore, two highest MJ concentrations at 4 d of the exposure caused 32% and 8% increments, respectively, in *PTA* transcript abundance in the leaves, while the lowest one ( $10^{-8}$  M) at 4 d had no influence on relative expression of the gene.

Besides, MJ vapors caused a dramatic enhancement in the relative expression of the lipoxygenase (*LOX*) gene in both roots and leaves of maize seedlings (Fig. 4). One-day MJ treatment resulted in 1.5–12.1-fold and 2.2–26.4-fold increases in the levels of the *LOX* mRNA in leaves and roots, respectively. Long-term (4 d) exposure to MJ was also associated with 2.3–17.5-fold and 1.9–10.3-fold increases in the *LOX* transcript abundance in the seedling roots and leaves, respectively.





**Fig. 4.** Expression patterns of *PDC1*, *PDC2*, *PTA* and *LOX* genes in MJ-treated *Z. mays* plants. Relative gene expression is presented as the mean *n*-fold change ( $\pm$  SD) in abundance of the investigated transcript in MJ-stressed maize seedlings compared to the control (untreated) plants. Transcriptional data were normalized to glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) gene. All genetic analyses were conducted in four independent biological and three technical replications. Different letters above SD bars denote significant differences in the levels of the target gene expression between the tested groups of *Z. mays* plants ( $p < 0.05$ ; post-hoc Tukey's test). *PDC1* – phenylalanine decarboxylase 1, *PDC2* – phenylalanine decarboxylase 2; *PTA* – phenylalanine (histidine) transaminase; *LOX* – lipoxygenase; d – day; L4, L6 and L8 – leaves of maize seedlings treated with  $10^{-4}$ ,  $10^{-6}$  or  $10^{-8}$  M of methyl jasmonate (MJ) vapors, respectively; R4, R6 and R8 – roots of the seedlings exposed to the respective concentrations of MJ vapors.

Three-way ANOVA analyses proved a significant influence ( $p < 0.05$ ) of the studied parameters on the relative expression of the quantified genes in the maize seedlings. In addition, most of the tested interactions between the investigated indicators were statistically significant. However, a few interactions were not statistically confirmed, in the case of *ADH1* gene (seedling part  $\times$  MJ treatment,  $p = 0.09$ ; seedling part  $\times$  MJ treatment  $\times$  exposure time,  $p = 0.50$ ), for the *PDC2* gene (seedling part  $\times$  exposure time,  $p = 0.52$ ; seedling part  $\times$  MJ treatment  $\times$  exposure time,  $p = 0.15$ ), as well as for the *PTA* gene (seedling part  $\times$  exposure time,  $p = 0.20$ ).

## DISCUSSION

The potency of MJ to trigger an accumulation of diverse secondary metabolites in plants has been widely reported (Pauwels et al., 2008; Horbowicz

et al., 2011; Ruiz-May et al., 2011; Concha et al., 2013). The majority of these studies were related to the metabolism of phenylpropanoids, indole alkaloids and terpenoids (Pauwels et al., 2008; Chen et al., 2013). Although the presence of 2-phenylethylamine (PEA) has been confirmed in several plant species (Smith, 1977; Horbowicz et al., 2011; Le Thi et al., 2014), there is little information regarding biosynthesis and metabolic transformations of this amine.

The bioassays carried out in this study revealed that a short-time (1 d) exposure to MJ vapors increased the fresh biomass of primary roots and aerial parts of the maize seedlings, whereas 4-day treatment caused its significant reduction. Furthermore, a similar effect of exogenous application of MJ on growth of the major organs of *Z. mays* seedlings was noted. Although the suppression of leaf and/or root growth has been reported by several authors, biochemical and

molecular background of this influence is scarcely understood (Ruiz-May et al., 2011; Gharechahi et al., 2013). Ruiz-May et al. (2011) observed a profound growth inhibition of *Catharanthus roseus* (L.) G. hairy roots treated with 10, 100 and 250  $\mu\text{M}$  of MJ for 72 h. Moreover, it was shown that reduction in the dry weight was correlated with severe cell disorganisation and cell death of the root cap in the stressed plants. Some authors suggested that MJ-induced growth inhibition may be associated with impairments in the course of the primary metabolism (e.g., cellular respiration and mitochondrial efficiency), photosynthetic dysfunctions and programmed cell death (Zhang and Xing, 2008; Ruiz-May et al., 2011). Gharechahi et al. (2013) revealed that 0.1 mM of MJ led to a substantial inhibition of growth and cell disorganization in *Silybum marianum* L. hairy roots. These authors showed that the growth suppression was connected with elevations in the expression of profilin-1 gene encoding the actin binding protein.

The content of PEA in maize seedlings was considerably lower than in seedlings of common buckwheat (Horbowicz et al., 2011). PEA in buckwheat seedlings reached a level of several hundred micrograms per gram of dry weight, and elicitation by MJ enhanced this content several times. In organs of maize seedlings 1-day treatment with MJ had no effect on the level of PEA. A four-day use of MJ did not affect the content of PEA in the roots either, but  $10^{-4}$  M concentration of the elicitor increased the PEA contents in the maize leaves. These results are distinct from those observed in all organs (roots, hypocotyls and cotyledons) of buckwheat seedlings, in which considerable increases in PEA levels were found (Horbowicz et al., 2011; Horbowicz et al., 2015). The differences are probably due to the distinct role of PEA in both species. It is quite likely that PEA is responsible for allelopathic activity of common buckwheat. Le Thi et al. (2014) found the allelopathic influence of tyramine cinnamide in barnyard grass, cress and red sprangletop. Recently, the presence of a similar compound, 2-phenylethylamine cinnamide, was confirmed in common buckwheat tissues (Horbowicz et al., 2015). There is no known reason for accumulation of PEA in maize seedlings, so it is difficult to explain the slight impact of MJ on its biosynthesis. To the best of our knowledge, these data provide the first evidence of PEA presence in maize tissues.

Since plants contain many decarboxylases of L-aromatic amino acids (Facchini et al., 2000), the biogenetic pathway that begins with decarboxylation of L-Phe to PEA seems quite unclear. Monoamine oxidase (MAO) can oxidize PEA to phenylacetaldehyde (PAAld), and further reduction of PAAld to PE, catalyzed by phenylacetaldehyde reductase, was described in tomato (Tieman et al., 2006; 2007).

Tomato fruits accumulate only a small content of PAAld, which indicates its rapid conversion to PE.

PAA content in the examined tissues of maize seedlings exceeds 2–3 times the levels of PEA, and about 6–10 times the content of PE. This may mean that PAA is the target product of this pathway of L-Phe metabolism. MJ caused an increase in the content of PAA, which was particularly evident after 4 days of its use. The increment can be one of the putative plant responses to the MJ; however, PAA has a much weaker auxin activity than indoleacetic acid (IAA) (Wightman and Lighty, 1982).

PE content in the evaluated tissues of maize seedlings was relatively low. MJ caused a decline in the level of this compound in the leaves after 1 and 4 days of treatment. In the case of maize roots MJ caused a large decrease in PE content at 1 d, but after 4-day treatment this effect was reversed. Moreover, 4- and 7-day MJ treatments resulted in large increases in PE levels in tissues of buckwheat seedlings (Horbowicz et al., 2015). The biosynthetic pathway of PE in plants is not definitively established (Tieman et al., 2006). In rose flowers, PE is produced by both PPA and PAAld route as well as by phenyllactate pathway (Watanabe et al., 2002). Despite the suggestion that biosynthesis of PE in rose flowers and petunia leads through PPA, its presence was not found in these tissues (Watanabe et al., 2002; Maeda et al., 2010). Such results suggest that PPA is promptly converted to PAAld in the plants. Also, in our previous study, PAAld and PPA were not found in organs of buckwheat seedlings (Horbowicz et al., 2015).

The confirmed presence of PAE, PAAld, PE and PAA in the tissues of maize seedlings and the lack of PPA suggest quite likely occurrence of the following routes: L-phenylalanine  $\rightarrow$  2-phenylethylamine (PEA)  $\rightarrow$  phenylacetaldehyde (PAAld)  $\rightarrow$  2-phenylethanol (PE), and phenylacetaldehyde (PAAld)  $\rightarrow$  phenylacetic acid (PAA).

Different isoforms of alcohol dehydrogenase (ADHs; EC 1.1.1.1) in plants have been considered to participate in the reduction of phenylacetaldehyde (PAAld) to 2-phenylethanol (PE) (Peters and Frenkel, 2004). In the current study, similar patterns of *ADH1* and *ADH2* transcript accumulation profiles in MJ-treated maize seedlings were identified. Upregulation of these genes occurred in leaves (after 1 and 4 days) and roots at 1 d, while repression of relative expression was noted only in the roots at 4 d. Owen et al. (2004) noted a rapid increase in alcohol dehydrogenase 1 (*DcADH1*) transcript amount in carnation flowers (*Dianthus caryophyllus* L.) in response to hypoxia and anoxia conditions. Furthermore, Peters and Frenkel (2004) showed that alcohol dehydrogenase genes contributed to chilling tolerance in *Z. mays* plants. It was found that double null mutants *Adh1*<sup>-</sup> and *Adh2*<sup>-</sup> of

maize were characterized by suppressed activity of P-type H<sup>+</sup>-ATPase, as well as elevated levels of ATP and lipid peroxidation in the hypocotyls, compared to the control. Pathuri et al. (2011) observed that leaves of susceptible genotype of barley (*Hordeum vulgare* L.) infected by *Blumeria graminis* f.sp. *hordei* responded with upregulation of *HvADH1* gene, while no changes were noted in the resistant *mlo5* line.

Plant copper-containing primary amine oxidases (CAOs; EC 1.4.3.21) encompass homodimeric enzymes catalysing oxidization of primary amines to the corresponding amine aldehydes, with reduction of molecular oxygen to hydrogen peroxide (Klema and Wilmot, 2012). Pietrangeli et al. (2007) found that CAO isolated from *Lathyrus cicera* L. and *Pisum sativum* L. seedlings were able to utilize PEA as a substrate. The present study showed upregulation in CAO gene in the roots of maize seedlings exposed to MJ. In the case of maize leaves, only a small increase in CAO transcript amount was recorded at 1 d exposure to MJ, while higher upregulation was noted after 4 days. Planas-Portell et al. (2013) established that *A. thaliana* seedlings subjected to treatment with MJ responded by circumstantial elevations in CAO2 transcript abundance (about 12- and 5-fold increases, respectively). Two other copper-containing amine oxidase genes (CAO1 and CAO3) were also upregulated in response to MJ application, reaching the maximal levels (ca. 3.5-fold) at 1 d. Participation of CAOs in plant responses to various biotic and abiotic stresses has been reported (Planas-Portell et al., 2013).

In plant systems, aldehyde oxidases (AOs; E.C. 1.2.3.1) are involved in the final phase of ABA (abscisic acid) and IAA (indoleacetic acid) biosynthesis, and induction of ROS generation in response to adverse environmental stimuli (Yesbergenova et al., 2005; Zdunek-Zastocka and Sobczak, 2013). The present study demonstrated an elevation in the levels of AO2 transcript abundance in the maize leaves after 1 and 4 days of MJ treatments. Furthermore, an upregulation of the target gene in the roots exposed to 10<sup>-4</sup> M concentration of MJ was noted. On the other hand, low concentration of MJ downregulated AO2 gene in the roots of maize seedlings. There are no available reports regarding transcriptional reprogramming of the aldehyde oxidase genes in MJ-treated plants. However, upregulation of AO genes, high increase in the level of AO protein and exaggerated H<sub>2</sub>O<sub>2</sub> formation in leaves and roots of tomato plants under drought stress were shown (Yesbergenova et al., 2005).

There are some reports supporting the hypothesis referring to the involvement of pyridoxal 5'-phosphate (PLP)-dependent aromatic amino acid decarboxylases in bioconversion of L-Phe to PEA (Tieman et al., 2006; Sakai et al., 2007; Tieman

et al., 2007). The results of gene expression analyses performed by Pan et al. (2012) suggest that aromatic amino acid decarboxylase *VvAADC* from 'Vidal blanc' hybrid of *Vitis vinifera* × *Vitis riparia* possibly participates in the biogenetic route of 2-phenylethanol (PE). One of the enzymes linked to decarboxylation of L-Phe is phenylalanine decarboxylase (PDC; EC 4.1.1.53). We showed that both investigated phenylalanine decarboxylase genes (*PDC1* and *PDC2*) were upregulated by two highest concentrations of MJ in roots and leaves of maize seedlings. However, higher transcript accumulation was recorded in leaves of *Z. mays* in comparison with the roots. Additionally, a greater upregulation of both *PDC* genes at 1 d of MJ exposure compared to 4-day period was observed. To the best of our knowledge, there is no available information describing the effect of MJ treatment on the transcriptional responses of *PDC* genes in tissues of plant species.

In higher plants, amino acid transaminases, including phenylalanine (histidine) transaminases (PTAs; EC 2.6.1.58), participate in a wide range of metabolic processes, such as photorespiration, nitrogen assimilation and biosynthesis of amino acids and secondary metabolites (Gonda et al., 2010). They are pyridoxal-5'-phosphate-dependent enzymes catalysing the reversible transfer the amino group of the amino acid to  $\alpha$ -keto acid (Hirata et al., 2012). Interestingly, Gonda et al. (2010) evidenced that the product of catabolic conversion of L-Phe catalysed by the amino acid transaminase is utilized in the biosynthesis of amino acid-derived aroma volatiles in *Cucumis melo* L. fruit. Furthermore, Hirata et al. (2012) established that aromatic acid aminotransferase participates in biosynthesis of 2-phenylethanol (PE) in the isolated petal protoplasts of *Rosa*. In the present study, it has been elucidated that two higher concentrations (10<sup>-4</sup> and 10<sup>-6</sup> M) of MJ vapors led to slight increments in *PTA* transcript levels in tissues of the maize seedlings. The lowest dose of MJ (10<sup>-8</sup> M) did not affect the *PTA* transcript abundance or caused its negligible increase in the leaves at 1 d. So far, there are no published results concerning the effect of MJ treatment on the expression of *PTA* gene in plant tissues.

The majority of lipoxygenases (LOXs; EC 1.13.11.12) comprise non-heme and iron-containing dioxygenases responsible for oxygenation of polyunsaturated fatty acids to the corresponding hydroperoxides (Kim et al., 2003). Diverse physiological functions of these enzymes also include involvement in the biosynthetic route of jasmonates (Gharechahi et al., 2013). The present study showed that exogenous MJ vapors led to substantial increases in *LOX* transcript amounts in both roots and leaves of *Z. mays* seedlings. Kim et al.

(2003) identified the biphasic pattern of upregulation of the *LOX* gene expression (two peaks of *LOX* transcript accumulation at 6 and 24 h, respectively) in the maize seedlings exposed to MJ treatment. Similarly, Concha et al. (2013) also elucidated considerable increments in the levels of *LOX* gene expression in fruits of *Fragaria chiloensis* L. (Mill.) after 2- and 5-day exposure to MJ.

Our studies have evidenced that MJ vapors upregulated expression of both *PDC1* and *PDC2* genes (encoding phenylalanine decarboxylase 1 and 2, respectively) in leaves and roots of *Z. mays* seedlings. Elevated expression of these genes was not accompanied by increases in the content of PEA. This may indicate a rapid use of PEA as a substrate for biosynthesis of other metabolites, such as PAA. In support of this hypothesis, a higher content of PAA than PEA was found in maize seedlings. Additionally, the presence of phenylacetaldehyde (PAAld) and enhanced expression of *AO2* gene encoding aldehyde oxidase 2 appears to be strong evidence of prompt transformation of PEA into PAA. It may be assumed that another enzyme: CAO (primary amine oxidase) is probably involved in the transformation of PEA to PAA. The MJ-evoked upregulation of *CAO* gene seems to be another proof of the following pathway: PEA → PAAld → PAA in tissues of the maize seedlings. The studies have also shown that treatment of *Z. mays* seedlings with MJ vapors clearly increased the amount of *ADH1* and *ADH2* transcripts. The encoded ADH isoenzymes are involved in the reduction of phenylacetaldehyde (PAAld) to 2-phenylethanol (PE).

## AUTHORS' CONTRIBUTIONS

The idea and manner of carrying out the experiments: M.H., H.S. Experiments: H.S., W.W., T.S., J.M., D.K., J.S., M.S. Data analysis: H.S., W.W. Provision of reagents/materials/analysis tools: M.H., D.K., W.W., T.S. Writing the paper: H.S., M.H.

## ACKNOWLEDGEMENTS

The studies were supported by the National Science Centre (Poland), grant no. 2011/01/B/NZ9/02888, and as part of the statutory research activities of the Faculty of Natural Sciences at the Siedlce University of Natural Sciences and Humanities.

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