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Full Length Research Paper

Perioxidases play important roles in abscisic acid (ABA)-simulating photosystem II (PSII) thermostabilty of apple tree rootstock leaves

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Leaf photosynthetic activity limited by summer heat stress represents large constraint to production process of fruit trees. To cope with this problem, we tested photosystem II (PS II) thermostability in clonal apple tree rootstocks with different growth intensity - semi-vigorous-MM106 and dwarfing-J-TE-F experiencing summer heat stress, and related antioxidative activity and phytohormonal balance. For this purpose, expanded leaves were collected and utilized for determination of 3-indoleacetic acid (IAA), cytokinins (CKs), abscisic acid (ABA) concentrations, and superoxide dismutase (SOD), total peroxidases (POX), catalase (CAT), total antioxidative activity (TAA) and PS II thermostability before and after 30 min dark exposition to temperature of 42°C (because of relatively-stable PS II). Leaves from dwarfing-J-TE-F showed higher PS II thermostability than that from semi-vigorous-MM106 as indicated by higher maximal quantum yield of primary photochemistry (Φ_{Po}), excitation transfer efficiency to electron transport chain (ψ_o) , electron transport yield (Φ_{Eo}) and lower thermal dissipation yield (Φ_{Do}). From antioxidant parameters, genotypic specifity can be seen in total peroxidases activity and only J-TE-F leaves predominated. Despite higher CKs concentration in leaves of MM106, active CKs did not show any difference between two rootstocks. IAA also exhibited balanced level, but ABA concentration was four times higher in J-TE-F leaves than MM106 ones. We can conclude that in PS II heat-hardening of apple tree rootstock leaves, which is at least partly provided by enhanced peroxidases activity, ABA plays the central role and J-TE-F rootstock can be recomended to heat stress in the prone regions.

Key words: Photosystem II thermostability, antioxidant activity, phytohormones, heat stress, oxidative stress, apple tree rootstocks, global climate change.

INTRODUCTION

Summer light, temperature and water distribution often exhibit suboptimal courses amplified by global warming, and rising abiotic environmental stress imposes more constraint to photosynthetic production in many crop and tree species (Greer et al., 2005; Repková et al., 2009; Ogaya et al., 2011). Fruit trees with deeper root system suffer mainly from heat stress with complex negative effects on photosynthetic processes. Approaching a temperature of 40°C, stomatal and mesophyll conductivity to CO_2 decreases substantially (Bernacchi et al., 2002; Cui et al., 2006), rubisco activation fails (Law and Crafts-Brandner, 1999) and thylakoid membrane integrity (Liu and Huang, 2002) and photosynthetic electron transport become impaired (Lu and Zhang, 1999; Toth et al., 2005). Disruption of cellular homeostasis coupled with signal sensing and transduction leads to activation of numerous protective mechanisms, providing

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new homeostasis (Wang et al., 2004; Vinocur and Altmen, 2005; Shao et al., 2005d, 2008a, b, c). Heat stress induces changes in membrane fluidity, protein functions and reactive oxygen species (ROS) generate related signals based on MAPK cascade, chaperonins and ROS themselves (Sairam et al., 2000; Wahid et al., 2007; Shao et al., 2008a, b, c, d, e), which provide antioxidant system up-regulation (Hare et al., 1998), compatible solutes (Iba, 2002; Larkindale and Huang, 2004; Shao et al., 2008d) and heat shock protein accumulation (Shao et al., 2008d, e).

Heat stress also causes phytohormonal change. It has been showed that phytohormone participated in signal transduction processes. Larkindale and Huang (2004), Shao et al. (2008a, b, c, d, 2011) and Yan et al. (2011) showed that heat stress-stimulated antioxidative defense and reduced-tissue damage are involved in calcium, abscisic acid (ABA), ethylene and salicylic acid (SA) (Webster, 1995; Goncalves et al., 2006). In fruit trees, rootstock has substantial influence on scion. Webster (1995) determined: (1) the amount and/or ratio of promoting and inhibiting endogenous hormones circulating within tree plant, particularly between the root system and above-ground tree parts; (2) the movement of assimilates (example sugars and amino acids) or mineral elements between the scion and rootstock; and (3) the amount of water taken up and moved through the rootstock to scion. Except growth regulation, rootstocks can determine stomatal conductance, photosynthetic pigment concentration, net CO_2 assimilation and maximum photochemical efficiency of leaves (Lliso et al., 2004), assimilate distribution (Fallahi et al., 2002), fruiting precocity, fruit yield and quality, as well as tolerance to adverse environmental conditions (Sircelj et al., 2005, 2007).

There are some information of drought stability of fruit tree photosynthetic apparatus (Rieger and Duemmel, 1992), but no work is dedicated to heat tolerance of apple tree rootstocks. Therefore, we focused on how the different phytohormonal supply to apple tree leaves provided by rootstocks of different growth intensity and modified by summer heat stress, regulates the photosystems II thermostability and what participation of antioxidative system in it can be expected. For this purpose simplified-model of rootstock leaves deprived of grafting, light regime and fruit proximity effects, was utilized.

MATERIALS AND METHODS

Plant material and experimental site

Three-year-old apple tree (*Malus domestica* Borkh.) rootstock plants with different growth intensity (semi-vigorous-MM106, bred in East Malling, United Kingdom and dwarfing-J-TE-F, bred in Techobuzice, Czech Republic), cultivated by Central Controlling and Testing Institute in Agriculture, Testing Station of Velke Ripnany in east-west oriented rows (3 m far), were cut just above ground in the early spring and allowed to grow naturally. During the vegetation period, standard tillage, fertilization, weed, pest and disease regulation were ensured. Experimental base in Velke Ripnany is localized in Danube uplands on clayey brown soil with ground water depth of 18 m. Local climate is defined with yearly rainfall of 582 mm and yearly temperature of 9.7°C. Figure 1 shows the daily air temperatures and daily rainfall in summer months of 2007.

Photosystems II thermostability analysis

After summer period, with air temperatures approaching 40°C, fifth expanded leaves from the top of upright rootstock shoots were collected in the morning (9:00 a.m.) on a cloudy day. In the laboratory, they were submitted to phosystem II (PS II) thermostability test. The rated parameters were as follows: 1) 30 min dark adaptation, 2) chlorophyll *a* fluorescence kinetics measurement, 3) 30 min dark exposition to temperature of 42°C (because of relatively stable PS II) and 4) chlorophyll *a* fluorescence kinetics measurement.

Chlorophyll *a* fluorescence kinetics were determined using portable fluorometer Handy-PEA (Hansatech Instruments Ltd., UK) and thereafter JIP-test was applied to them (Biolyzer 4HP v. 3.06 software, created by Ronald Rodriguez). Processing of the fluorescence data by JIP-test gained parameters concretely describing bioenergetic state of leaf PS II (Strasser et al., 2004): maximal quantum yield of primary photochemistry (Φ_{Po}), exciton transfer efficiency to electron transport chain (ψ_o), electron transport yield (Φ_{Eo}) and thermal dissipation yield (Φ_{Do}).

Leaf antioxidant activity measurement

Approximately, 0.1 g of frozen sixth leaves from the top of upright rootstock shoots were ground in liquid nitrogen, homogenized with 2 mM ascorbate and 2 mM EDTA in 1 ml 0.1 M Na-phosphate buffer (pH 7.8) and centrifuged at 14000 g for 20 min. Every operation was made at 2 to 4°C. Obtained supernatant was filtered by low protein-binding-syringe filter of 0.22 µM pore size (Millex GV, Millipore Corp., USA) and utilized for total soluble protein concentration, superoxide dismutase (SOD), catalase (CAT) and peroxidases (POX) activity determination.

Concentration of total soluble protein was ascertained according to Bradford (1976). For SOD activity characterization, instructions in determination kit SOD-525 (Bioxytech, Oxis Inc., USA) were followed. POX activity was determined according to Chance and Maehly (1955). In the course of 90 s, guajacol (0.2%) consumption, provided by peroxidases in the presence of hydrogen peroxide (0.2%) and 0.1 M Na-acetate buffer (pH 5.4), was observed by spectrophotometer at 470 nm. CAT activity was defined in the reaction mixture of 0.1 M Na-phosphate buffer (pH 7.3), 30 mM hydrogen peroxide and the sample, for 60 s lasting absorbance measurement at wavelength 240 nm, describing hydrogen peroxide consumprion rate (Aebi et al., 1984). Total antioxidative activity (TAA), as an ascorbate concentration equivalent, was ascertained using determination kit AOP-490 (Bioxytech, Oxis Inc., USA). For this purpose, samples without ascorbate supplement were prepared.

Leaf phytohormonal composition and analysis

Frozen fifth leaves from the top of upright rootstock shoots (0.5 to 1.0 g) were homogenized and extracted overnight in cold methanol-water-formic acid (15:4:1, v/v/v). Following centrifugation (15 000 g, 4°C, 20 min), part of lipids were removed from the supernatant by filtration through Sep-Pack C18 cartridge. After evaporation to near dryness, the residue was dissolved in 5 ml 1 M formic acid and applied to an Oasis MCX column (150 mg reversephase cation-

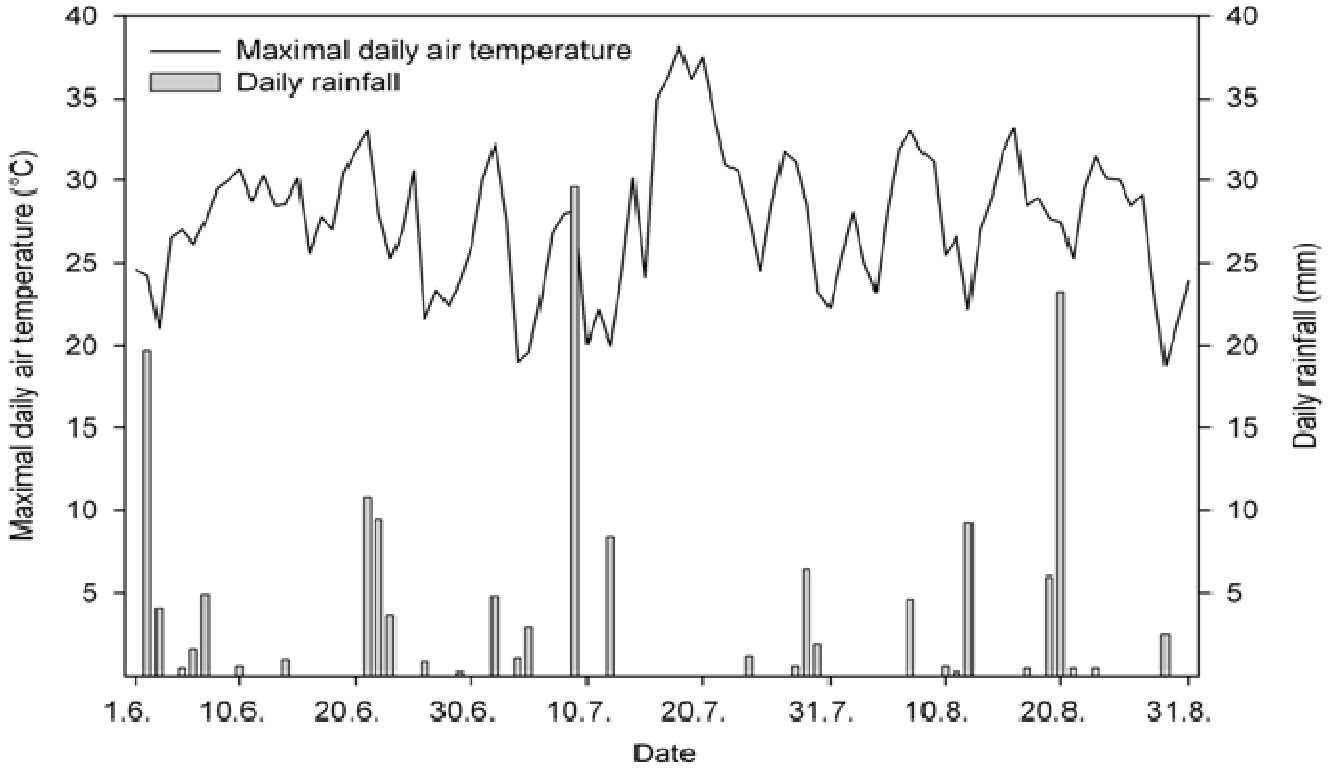

Figure 1. Maximal daily air temperatures and daily rainfall in summer months 2007 in the experimental site of Central Controlling and Testing Institute in Agriculture-Experimetal Base for Velke Ripnany, Slovakia.

exchange sorbent). After washing the column with 5 ml of formic acid, abscisic acid (ABA) and 3-indoleacetic acid (IAA) were eluted with 5 ml of methanol, cytokinin (CK) nucleotides were eluted with 5 ml 0.35 M NH_4OH, while CK bases, ribosides, and glucosides were eluted with 5 ml 0.35 M NH_4OH in 60% (v/v) methanol. The eluates containing ABA, IAA, CK bases, ribosides and glucosides were evaporated to dryness using a Speed-Vac. The eluate containing CK nucleotides was evaporated in the same way to 2 to 3 ml to remove the ammonia. The CK nucleotides were dephosphorylated to nucleosides by incubation with alkaline phosphatase (Sigma).

The CKs were quantified by HPLC linked to an Ion Trap Mass Spectrometer Finnigan LCQ equipped with an electrospray interface using a RP column (Phenomenex, AQUA, 250 mm). Linear gradient of acetonitrile (B) in 0.0005%, v/v, acetic acid in water (A): 10 % B for 5 min, to 17% B in 15 min and to 50% B in 35 min were used at a flow rate of 0.2 $ml.min^{-1}$. Detection and quantification were carried out by using a Finnigan LCQ operated in the positive ion, full-scan MS/MS mode using a multilevel calibration graph with [2H] labeled CKs as internal standards. Each analysis was repeated twice.

Moreover, ABA and IAA extract were purified by HPLC and the proprietary fraction was collected. This purified fraction was methylated by diazomethane. ABA and IAA were quatified by GC linked to an Ion Trap Mass Spectrometer PolarisQ-equipped with an EI using a column DB-5MS (30*0.25*0.25). Temperature gradient was as followed: 60°C for 5 min, to 160°C for 1 min and to 160°C in 13 min was used at a flow rate of 2 $ml.min^{-1}$. Detection and quantification were carried out using a PolarisQ operated in the positive ion, full-scan MS/MS mode using a multilevel calibration graph with [2H] labeled ABA and [13C] labeled IAA as internal standards. Each analysis was repeated twice.

RESULTS

Photosystem II thermostability

Chlorophyll *a* fluorescence transients (OJIP curves) in leaves from the rootstocks obtained before high temperature treatment (30 min at 42°C in the dark) showed differences only at the J and P step, with lower fluorescence values in leaves from semi-vigorous-rootstock MM106 (Figure 2). Leaf exposition to high temperature had a significant impact on chlorophyll *a* fluorescence kinetics. Relatively higher fluorescence values were at the O step, formation of K step and lower fluorescence at the J step were followed by faster appearence of the I step of markedly lower fluorescence values and significant decreases of fluorescence at the P step.

Rootstock comparison revealed larger extremes in leaves from semi-vigorous-MM106. Parameters derived from chlorophyll *a* fluorescence transients brought more concrete information of bioenergetic fluxes on the PS II level. Before the high temperature treatment reactions of maximal quantum yield of primary photochemistry (Φ_{Po}), exciton transfer efficiency to electron transport chain (ψ_o), electron transport yield (Φ_{Eo}) and thermal dissipation yield (Φ_{Do}) did not showed any significant difference between rootstocks (Figure 3). High temperature caused marked

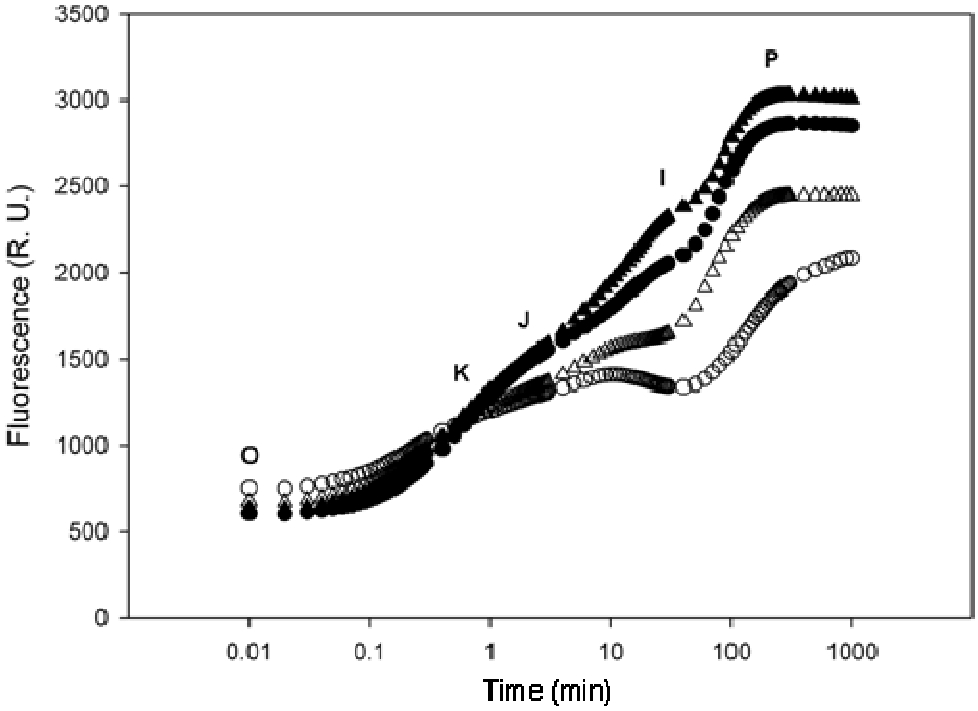

Figure 2. Rapid chlorophyll a fluorescence kinetics (OJIP curves) in fifth leaves of upright rootstock shoots. Circles – Semi-vigorous MM.106, triangles – very dwarf J-TE-F, full symbols – before, and empty symbols – after high temperature treatment (30 min at 42°C in the dark). Transients are mean of 50 measurements.

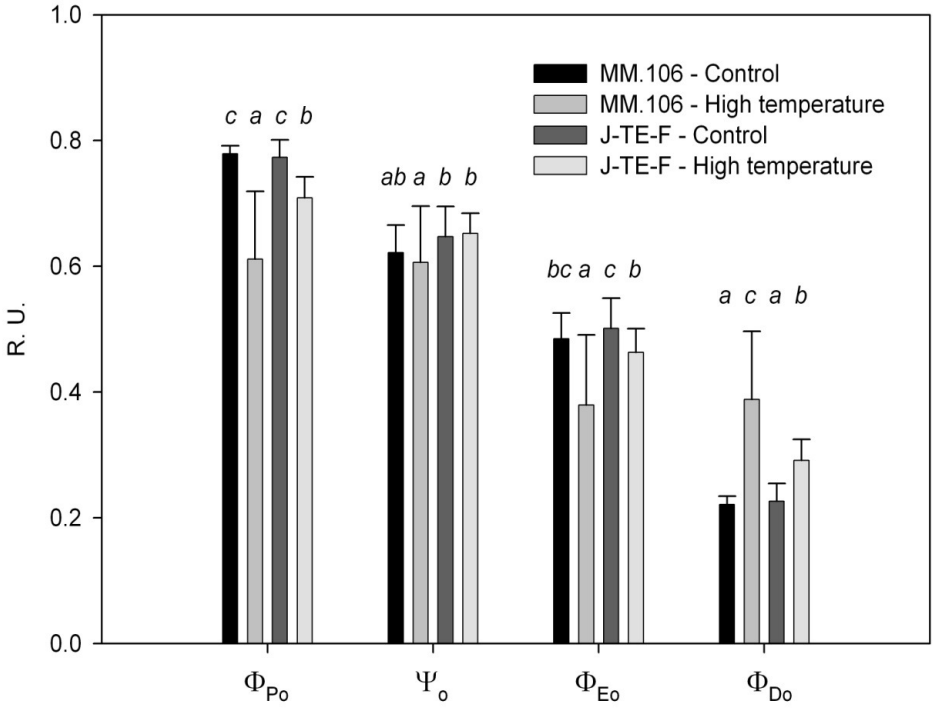

Figure 3. Maximal quantum yield of primary photochemistry (Φ_{Po}), exciton transfer efficiency to electron transport chain (ψ_o), electron transport yield (Φ_{Eo}) and thermal dissipation yield (Φ_{Do}) in fifth leaves of upright rootstock shoots before and after high temperature treatment (30 min at 42°C in the dark). Letters indicate on statistically significant difference at P=0.01.

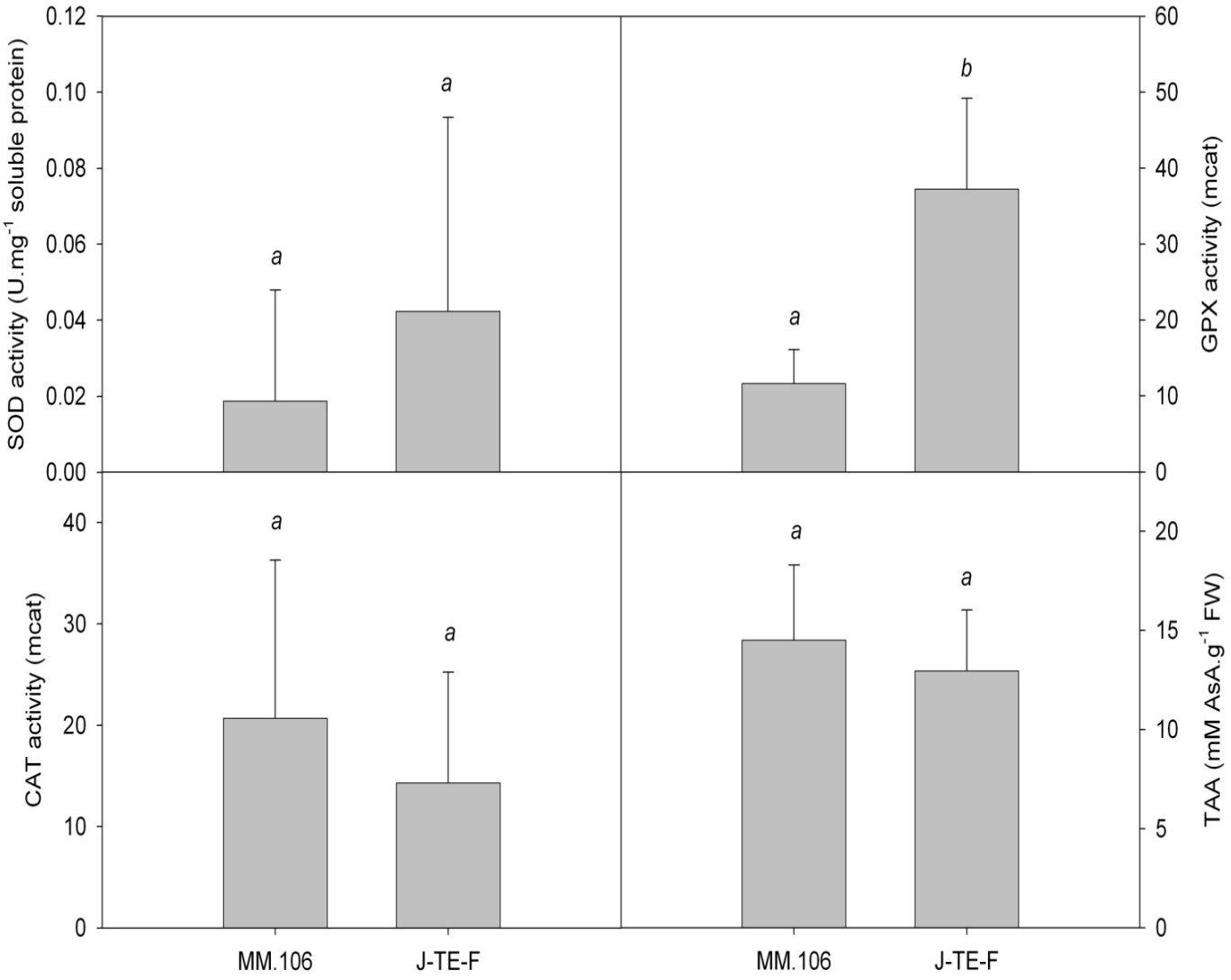

Figure 4. Basal superoxid-dismutase (SOD), peroxidase (POX) and catalase (CAT) activity, and total antioxidant activity (TAA) in sixth leaves of upright rootstock shoots. Results are means of 6 determinations. Letters indicate no statistical significant difference at P = 0.01.

decrease of Φ_{Po} and Φ_{Eo}, and increase of Φ_{Do} in leaves of both rootstocks, but they were stronger in semi-vigorous rootstock MM106. ψ_o also remained relatively stable in both genotypes.

Leaf antioxidant defense

Superoxid-dismutase (SOD) and catalase (CAT) determined in leaves of rootstock MM106 and J-TE-F was low and relatively balanced (Figure 4). Similar values were obtained in the case of total antioxidant activity (TAA) as well. The only one antioxidant parameter, which exhibited genotype differences, was total peroxidases (POX) activity. In this characteristics, leaves from dwarfing rootstock J-TE-F dominated.

Phytohormonal balance of rootstock leaves

Leaves from the rootstocks with different growth intensity contained approximately equal amount of 3-indoleacetic acid (IAA) and biologically active cytokinines (*trans*-zeatin-9-riboside, dihydrozeatin-9-riboside, *cis*-zeatin-9-riboside and isopentenyladenine-9-riboside), but the total cytokinines and abscisic acid (ABA) detected strongly predominates in semi-vigorous rootstock MM106 and dwarfing J-TE-F, respectively (Figure 6). Therefore, we found no significant difference in Act.CKs/IAA ratio between rootstocks, but ABA/Act.CKs and ABA/IAA ratio

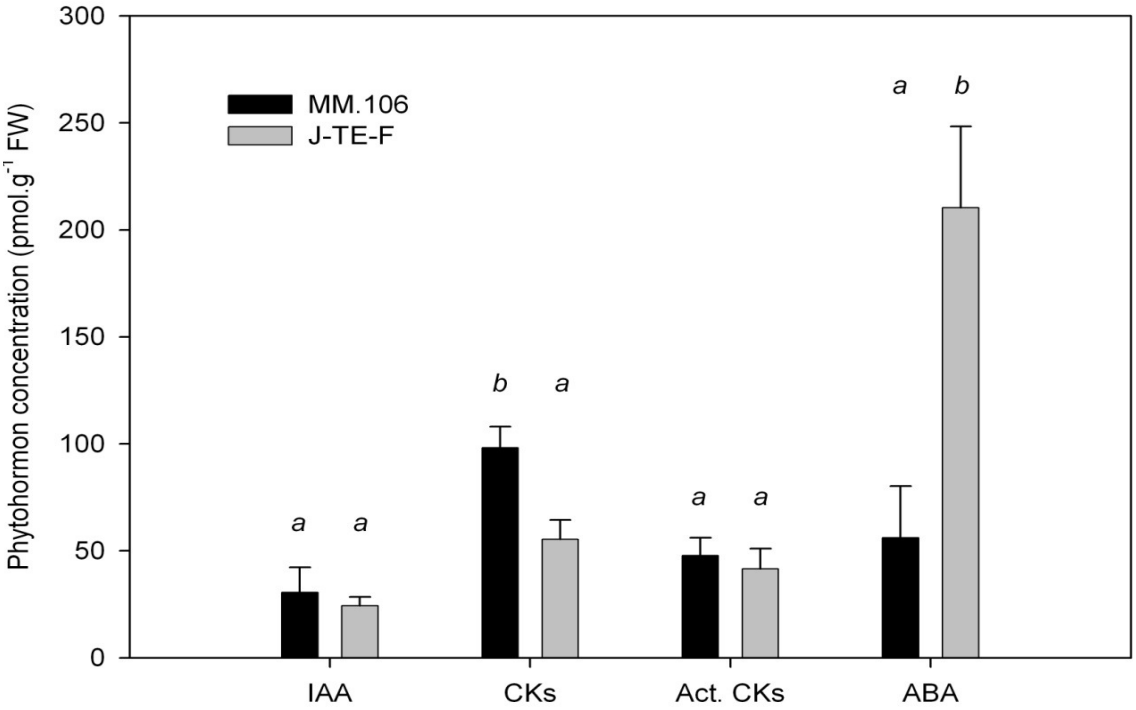

Figure 5. Concentration of 3-indoleacetic acid (IAA), total found cytokinines, biologically active cytokinines (*trans*-zeatin-9-riboside, dihydrozeatin-9-riboside, *cis*-zeatin-9-riboside and isopentenyladenine-9-riboside) and abscisic acid (ABA) in fifth leaves of upright rootstock shoots. Results are means of 6 determinations. Letters indicate no statistical significant difference at P = 0.,01.

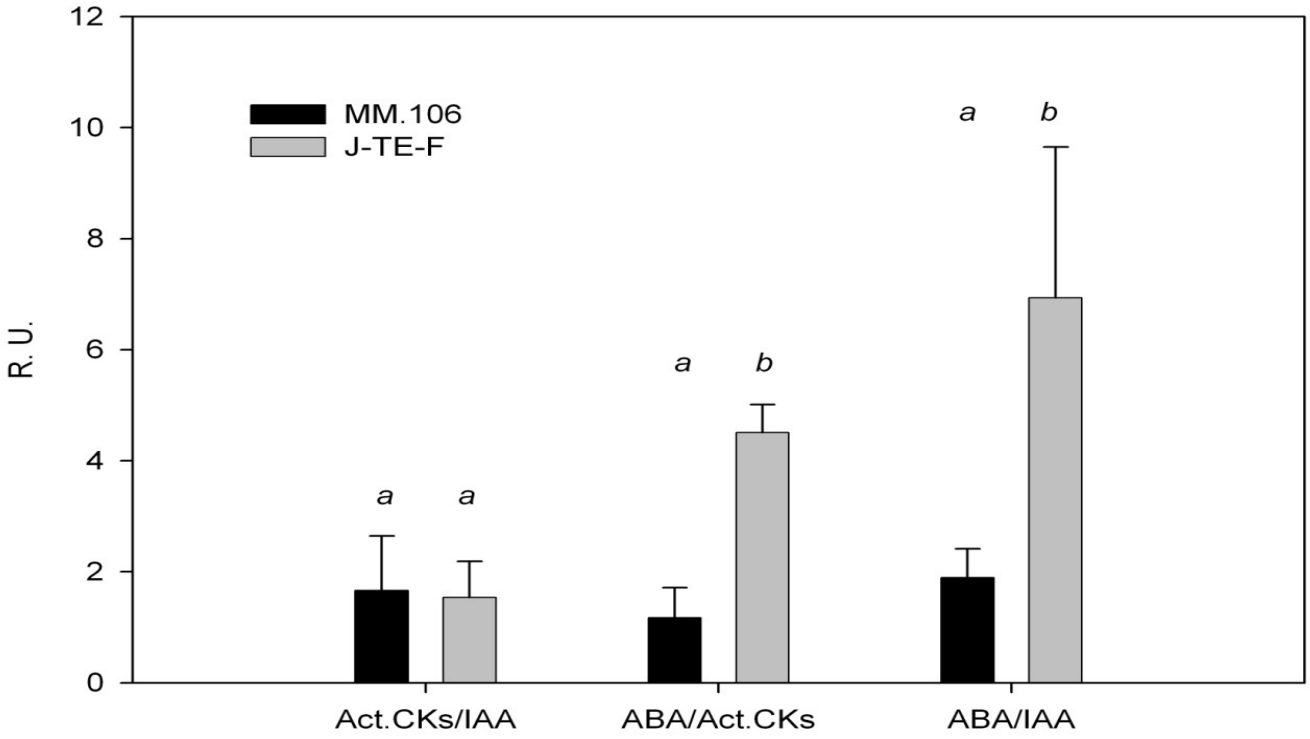

Figure 6. Active cytokinines (Act.CKs) to 3-indoleacetic acid (IAA), abscisic acid (ABA) to active cytokinines, and abscisic acid to 3-indoleacetic acid ratio in fifth leaves of upright rootstock shoots. Results are means of 6 determinations. Letters indicate no statistically significant difference at P = 0.01.

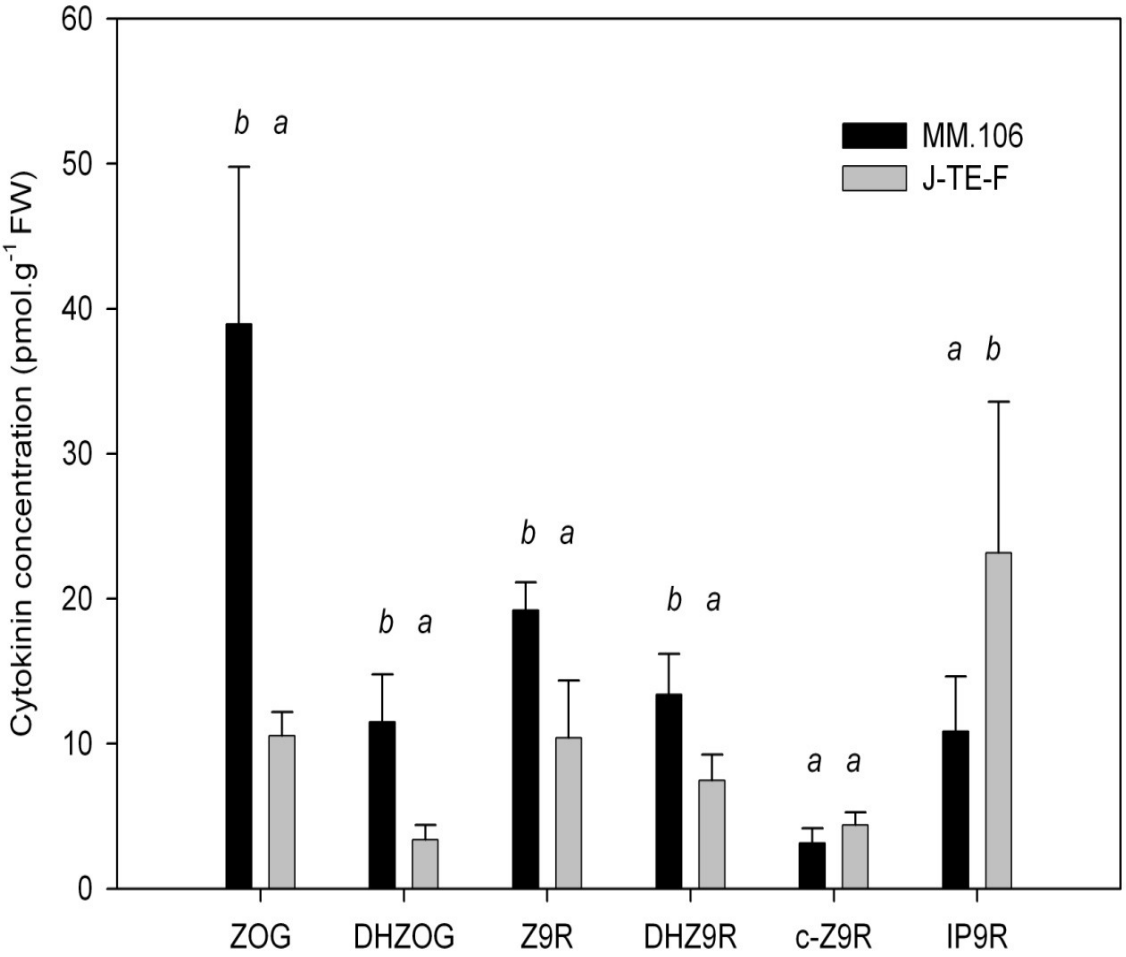

Figure 7. Cytokinin composition in fifth leaves of upright rootstock shoots: ZOG - *trans*-zeatin-O-glucoside, DHZOG - dihydrozeatin-O-glucoside, Z9R - *trans*-zeatin-9-riboside, DHZ9R - dihydrozeatin-9-riboside, *c*-Z9R - *cis*-zeatin-9-riboside and IP9R - isopentenyladenine-9-riboside. Results are means of 6 determinations. Letters indicate no statistically significant difference at P = 0.01.

in leaves from J-TE-F were almost four times higher than in leaves from MM106 (Figure 4). From a wide scale of cytokinins, only 6 forms were reliably detected (Figure 7). *Trans*-zeatin-O-glucoside (ZOG), *trans*-zeatin-9-riboside (Z9R) and dihydrozeatin-9-glucoside (DHZ9R) were the major representatives in MM106 leaves. On the other hand, in J-TE-F leaves isopentenyladenine-9-riboside (IP9R) showed the highest concentration.

DISCUSSION

Rootstock genotypes markedly influence phytohormonal composition of the scion part in fruit trees. According to Kamboj et al. (1999 a, b), shoot bark of dwarfing rootstocks (M.27 and M.9) exhibited significantly higher ABA content than more vigorous rootstocks MM106 and MM111. Concentrations of 3-indolelacetic acid (IAA) showed opposite features though not significant. Strong possitive relationship with rootstock invigoration was also observed in cytokinin concentration determined in scion xylem sap (Kamboj et al., 1999b). What was interesting to note was that rootstock modifies cytokinins composition in dwarfing rootstocks, producing predominated zeatin on more vigorous one zeatine riboside.

It has been observed that extreme temperature fluctuations usually impairs leaf phytohormonal picture. However, little is known about heat stress effects on leaf auxin concentrations, although it was reported that drought decreased IAA concentration in tobacco leaves (Pustovoitova et al., 2000; Tounekti et al., 2011). On the other hand, many reports showed cytokinin loss and that ABA concentration rises in relation to heat stress (Talanova et al.,2003; Li et al.,2003; Xu and Huang, 2007). Apple trees at the experimental site of Velke Ripnany usually experience heat stress. In 2007, maximal daily air temperature oscilated between 20 and 35°C, but in the middle of July it approached 40°C (Figure 6).

Despite reduced root growth of dwarfing rootstock J-TE-F (Bianco et al., 2003; Yao et al., 2006; Kuroda and Chiba, 2006), trees did not suffer from water stress; thanks to good soil capillarity and summer rainfall, which was relatively frequent at the beginning of June and July, and in the middle of August (Figure 7) .

At the end of summer, leaves from the rootstocks of different growth intensity, MM106 and J-TE-F, exhibited marked differences in phytohormonal composition (Figures 5 to 7). Except cytokinin composition, our results fully correspond with observations of Kamboj et al. (1999a, b), although obtained in different plant organs. In Southern Slovakia regions more prone to high temperature stress than East Malling experimental base in United Kingdom where the experiments were conducted, leaves from semi-vigorous rootstock MM106 passing through heat stress probably changed the balance of the active form (ribosides) to the stored cytokinins (glucosides) in favour to storing ones, and active cytokinins appear at the same level as in the J-TE-F rootstock leaves. This idea is supported also by Chou et al. (2000), observing high temperature induced accumulation of glucoside cytokinins and reduction in free base and riboside cytokinins in *Phalenopsis* leaves. Such cytokinin composition movement in J-TE-F rootstock leaves must then be minimal or almost none, and it can be supposed that this is characterized by a heat sensitivity feature. Of course, differences in sample-taking time as well as genotype specifity can also be taken into account (Nováková et al., 2005). More also, soil types and physical-chemical property should be considered (Ma et al., 2011)

One of the key mechanisms stabilizing photosynthetic processes under stress conditions is antioxidative complex (Cheng and Ma, 2004). As described by Sircelj et al. (2007, 2005), drought stimulates ascorbate, glutathione, β-carotene, zeaxanthin and α-tocopherol synthesis in apple tree leaves, but there is no work describing reactions of antioxidative system to heat stress. In many other species, heat tolerance of photosynthetic apparatus provided by antioxidative activity is associated with higher cytokinin level and also with ABA signaling (Gong et al., 1998; Zhao et al., 2008; Song et al., 2008; Shao et al., 2008e; Yan et al., 2010). Our results indicate that ABA is related to elevation of POX activity, and there is only one antioxidant characteristics showing significant difference between rootstocks (Figures 4 and 5). Enzymatic and non-enzymatic antioxidative defense activity (Lopéz-Gomez et al., 2007) in apple fruits and leaves follows more or less diurnal changes in light intensity and air temperature (Yan et al., 2010). Despite the morning sample-taking time, POX showed relatively high activity and therefore can be taken as a component of early antioxidative response of apple tree leaves, or a component of permanent antioxidative watch (Türkan and Demiral, 2009; Srinivasan et al., 2011). On the other hand, balanced total antioxidative activity (TAA) in both rootstock leaves suggests a higher activity of secondary antioxidant enzymes or higher concentration of non-enzymatic water soluble antioxidants in MM106 leaves.

Thermotest imposed stress to photosynthetic apparatus and revealed photosystem II stability/injury as related to sum of internal stabilizing factors. Less pronounced changes in chlorophyll *a* fluorescence transients and parameters derived from them - maximal quantum yield of primary photochemistry (Φ_{Po}), exciton transfer efficiency to electron transport chain (ψ_o), electron yield (Φ_{Eo}) and thermal dissipation yield (Φ_{Do}) after thermotest pointed to a higher PS II thermotability in leaves of very dwarfing rootstock J-TE-F (Figures 1 and 2). Interestingly, balanced PS II thermostability was obtained in our former work on scion (cv. Idared) engrafted to semi-vigorous rootstock MM104 and dwarfing rootstock M.9 (recently submitted). in contrast however, Goncalves et al. (2006) stated that cherry cultivars grafted to dwarfing rootstocks had lower midday maximal photochemical efficiency (F_V/F_M) than invigorating ones. More work is therefore needed for the full understanding the complex regulation of PS II thermostability in fruit trees.

Conclusion

Nevertheless, we can conclude that like the work of Li et al. (2003) in cucumber leaves, PS II of J-TE-F leaves are more thermostabile because of ABA-stimulated POX activity. Activity of other antioxidant enzymes or concentration of non-enzymatic water soluble antioxidants was not essential for PS II stabilization. More probably, compatible solutes and/or protein chaperones (Shao et al., 2008a, b, c, d; 2011; Yan et al., 2010) can take part in it. Therefore, the dwarfing-rootstock J-TE-F is recommended for utilization in heat stress-prone environments and regions of the world.

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Abbreviations

ABA, Abscisic acid; **CAT,** catalase; **CK,** cytokinin; ***c-***

Z9R, *cis*-zeatin-9-riboside; **DHZOG,** dihydrozeatin-O-glucoside; **DHZ9R,** dihydrozeatin-9-riboside; **IAA,** 3-indoleacetic acid; **IP9R,** isopentenyladenine-9-riboside; **POX,** peroxidases; **PS II,** photosystem II; **SOD,** superoxid-dismutase; **TAA,** total antioxidative activity; **ZOG,** *trans*-zeatin-O-glucoside; **Z9R,** *trans*-zeatin-9-riboside; Φ_{Do}, thermal dissipation yield; Φ_{Eo}, electron transport yield; Φ_{Po}, maximal quantum yield of primary photochemistry; ψ_o, exciton transfer efficiency to electron transport chain.

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