

Article

Effect of Cytokinins on Enzyme Activities, FRAP Antioxidant Capacity and Total Phenolic Content in Apple Tissue Cultures of Two Cultivars

Zsuzsa Máthéné Szigeti ^{1,*}, Richárd Kovásznai-Oláh ^{1,2} and Judit Dobránszki ^{1,*} 

¹ Center for Agricultural Genomics and Biotechnology, Faculty of Agriculture and Food Sciences and Environmental Management, University of Debrecen, 4400 Nyíregyháza, Hungary; richard.kovasznoi.olah@gmail.com

² Institute of Environmental and Natural Sciences, University of Nyíregyháza, 4400 Nyíregyháza, Hungary

* Correspondence: szigeti.zsuzsa@agr.unideb.hu (Z.M.S.); dobranszki@freemail.hu (J.D.)

Highlights

What are the main findings?

- First study on BAR, TOP, and the structurally distinct TDZ on apple antioxidants.
- Cytokinin-induced antioxidant responses are strictly genotype-dependent.
- Phenylurea-type TDZ altered ‘McIntosh’ antioxidant enzymes, indicating stress.

What are the implications of the main findings?

- Cytokinin potency depends on core structure and side-chains, limiting general use.
- Aromatic TOP successfully maintained the cellular physiological balance.

Abstract

Commonly used cytokinins in apple tissue cultures include 6-benzylaminopurine riboside (BAR), thidiazuron (TDZ), and meta-topolin (TOP). In this study, shoots of two distinct apple cultivars (‘Húsvéti rozmaring’ and ‘McIntosh’) were cultured on standard Murashige and Skoog medium supplemented with 4.5 μM of either BAR, TDZ, or TOP, alongside a cytokinin-free control medium. Since comprehensive comparative studies on these specific cytokinins and apple cultivars have not yet been conducted, we evaluated their individual effects on critical enzyme activities, including acid and alkaline phosphatase, β -glycerophosphatase, β -NAD diphosphatase, peroxidase (POD), and polyphenol oxidase (PPO), as well as total phenolic content (TPC) and ferric reducing antioxidant power (FRAP). Substantial changes in both enzymatic activity and antioxidant profiles were clearly detected across both cultivars after any cytokinin treatments in shoots. The highest acid phosphatase ($183.0\% \pm 8.72$) and β -glycerophosphatase ($191.9\% \pm 11.95$) activities, as well as the highest FRAP ($122.9\% \pm 8.16$), were observed in the TOP-treated HR plants. The highest alkaline phosphatase ($144.3\% \pm 5.51$) and polyphenol oxidase ($204.3\% \pm 5.07$) activities were measured in the BAR-treated HR plants. The highest β -NAD diphosphatase ($148.5\% \pm 5.29$) activity was determined in the TDZ-treated HR plants, while the highest peroxidase activity was measured in the control HR and McI plants. The highest TPC ($119.7\% \pm 2.03$) was determined in the BAR-treated McI plants. Distinct and notable variations were observed not only among the effects of individual cytokinins, but also between the two apple scions examined. In conclusion, TDZ exhibited consistent stress-like effects, such as the inhibition of shoot and root elongation, whereas TOP effectively maintained the overall physiological balance in the in vitro apple shoot cultures, keeping the photosynthetic pigment content similar to that of the control.



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Keywords: ‘McIntosh’ cultivars; ‘Húsvéti rozmaring’ cultivars; acid phosphatase; peroxidase; polyphenol oxidase; cytokinins; ferric reducing antioxidant power (FRAP); total phenolic content

1. Introduction

Apple (*Malus x domestica* Borkh.) is a member of the *Rosaceae* family and is one of the most highly consumed and economically important temperate fruits in the world [1]. The extensive diversity among more than 7500 cultivars cultivated worldwide is reflected not only in fruit morphology, texture and flavor, but also in their distinct biochemical profiles and metabolic responses [2].

Plant tissue culture techniques have been widely applied for the micropropagation and clonal multiplication of apple cultivars. This approach represents an important and extensively used method for large-scale plant production and germplasm conservation. Furthermore, in vitro cultures are highly useful for obtaining virus-free plant material, for cryopreserving genetic resources and for apple crop improvement through genetic transformation [3,4].

Cytokinins (CKs) were originally discovered for their ability to induce cell division in plant tissues; however, they are now known to modulate a wide range of developmental and metabolic pathways. They play an important role in plant adaptation to adverse environmental conditions, support chloroplast activity, inhibit degradation of chloroplasts, and induce shoot branching and growth of shoots and roots [5,6]. In tissue cultures synthetic cytokinin- and auxin-like compounds are used to induce shoot and root formation. TDZ is considered to be one of the most potent CK-like substances for enhancing shoot multiplication and has become one of the most commonly used CKs in apple cultures. However, it can exert adverse side-effects, such as inducing hyperhydricity and inhibiting both shoot and root elongation and subsequent root formation [5]. Aromatic CKs, such as BAR and TOP, which are derived from 6-benzylaminopurine (BA), can be used to minimize these adverse side-effects of TDZ. BAR is a biologically active N⁹-riboside derivative of BA, which causes better shoot growth in vitro in apple tissue culture than BA and TDZ. Similarly, TOP is considered one of the most effective aromatic CKs used in tissue cultures of certain plant species, as it does not induce the adverse side effects typically associated with TDZ and BA. The physiological effects of cytokinins are closely linked to the modulation of the plant’s antioxidant enzyme system [4].

In response to both abiotic and biotic stresses, plants modulate the activities of key metabolic and reactive oxygen species (ROS)-scavenging enzymes, such as phosphatases, while simultaneously elevating their overall antioxidant capacity. The activities of ROS-scavenging enzymes, including peroxidase, polyphenol oxidase and catalase, have already been studied in plant tissue cultures of apple in response to TDZ application [7]. Furthermore, catalase, ascorbate peroxidase and superoxide dismutase activities were determined in TOP-treated sugarcane and its preventive effect against oxidative stress was detected after TOP treatment [8]. TOP decreased superoxide dismutase and ascorbate peroxidase activities compared to BA treatment in sugarcane. Peroxidase activity increased in BA-treated *Brachypodium distachyon* (L.) P.Beauv. and blackberry [9,10]. In contrast superoxide dismutase and peroxidase activities were decreased in grape (*Vitis vinifera* L.) callus in a concentration-dependent manner following BA treatment [11]. Changes in acid phosphatase activity were also determined as a response to salt and drought stress in pigweed, alfalfa and wheat [12–14]. The non-enzymatic defense system of plants involves the synthesis and accumulation of secondary metabolites, such as polyphenols, to mitigate the effects of environmental stress. This non-enzymatic antioxidant network primarily com-

prises ascorbate, glutathione, phenolic compounds, flavonoids, curcumins, α -tocopherol, carotenoids and lycopene [15]. Further investigations were conducted using in vitro cultures of *Scutellaria altissima* (L.) supplied with BA and TDZ, as well as *Merwillia plumbea* (Lindl.) Speta treated with meta-topolin riboside (TOPR) and TDZ [16–18]. In both species, these cytokinin treatments significantly elevated the total phenolic content.

Since there is a lack of systematic and comparative studies on how different cytokinin types modulate enzymatic activity (acid and alkaline phosphatase, β -glycerophosphatase, β -NAD diphosphatase, peroxidase, polyphenol oxidase) and antioxidant profiles in apple, this study aimed to address this gap. To evaluate their impact on apple in vitro physiology, we tested specific aromatic cytokinins (BAR and TOP), alongside the phenylurea-derivative TDZ at a constant concentration of 4.5 μ M to ensure a direct molar comparison. While TDZ is typically effective at lower concentrations, this specific concentration (4.5 μ M) was selected to match that of the aromatic cytokinins, which are widely used at this level in in vitro apple shoot multiplication due to their ability to maximize shoot proliferation and break apical dominance [19,20]. To answer these questions, we formulated the following hypotheses: (i) TDZ application induces the activities of ROS-scavenging and stress-related enzymes; (ii) TOP treatment decreases these enzyme activities under in vitro conditions; (iii) BAR exerts a similar modulating effect on enzyme activities as TOP; and (iv) different CK types differentially affect the overall antioxidant content. Therefore, we systematically investigated the effects of BAR, TDZ and TOP on acid and alkaline phosphatase, β -glycerophosphatase, NAD diphosphatase, peroxidase, and polyphenol oxidase activities in ‘Húsvéti rozmaring’ and ‘McIntosh’ apple cultivars. Additionally, the influence of these CKs on ferric-reducing antioxidant power (FRAP) and total phenolic content was evaluated in both apple cultivars.

2. Materials and Methods

2.1. Plant Materials, Growth Conditions and Collecting of Plant Samples

Plant material was collected from shoots of four-week-old in vitro shoot cultures of two *Malus x domestica* Borkh. scion cultivars ‘McIntosh’ (McI) and ‘Húsvéti rozmaring’ (HR). Shoot cultures were maintained and subcultured monthly on Murashige and Skoog (MS) basal medium [21] supplemented with 2.22 μ M 6-benzylaminopurine (BA), 0.49 μ M indole butyric acid (IBA), 0.58 μ M gibberellic acid (GA_3), 3% (*w/v*) sucrose, and 0.7% (*w/v*) agar-agar. Before autoclaving for 20 min at 121 °C and 1.05×10^5 Pa, the pH of the medium was adjusted to 5.8. Each culture vessel contained 70 mL of medium and five shoot explants. Shoot cultures were grown at 23 ± 2 °C under a 16/8 photoperiod, at a light intensity of $106 \mu\text{mol m}^{-2} \text{s}^{-1}$. Prior to the experiments, shoots were grown in vitro on cytokinin-free medium to avoid the side effects of cytokinin in the experiments.

The 4-week-old in vitro shoots grown on CK-free medium were separated from in vitro mother shoots, cut into 20 mm long segments and placed on experimental media. Except for the CK content, the experimental media were the same as the medium used for maintenance described above. Three different CK-containing media were used during the experiments: medium without CK (control), and media containing 4.5 μ M thidiazuron (TDZ), 4.5 μ M 6-benzylaminopurine riboside (BAR) and 4.5 μ M meta-topolin (TOP), respectively. Culture conditions were the same as those applied for maintenance of shoot cultures. Shoots were collected from experimental cultures at the 4th week. Plant materials were collected from four different culture vessels from each CK treatment. Five shoots were transferred to each culture vessel. All the experiments were repeated three times.

Shoots were ground with a cold mortar and pestle with 1% quartz sand in a 0.05 M ice-cold TRIS-acetate buffer (pH 7.0) (4 mL of 0.05 M pH 7.0 TRIS-acetate buffer per 1 g of

tissue); crude extracts were stored at $-80\text{ }^{\circ}\text{C}$ until enzyme activity determination. All the measurements were carried out in triplicate.

2.2. Determination of Non-Specific Acid Phosphatase Activity

Non-specific acid phosphatase (ACP) activity was determined according to a modified method of McLachlan, 1980 [22]. Briefly, the crude extract was centrifuged for 10 min at $4\text{ }^{\circ}\text{C}$ and $4000\times g$ and 25 μL supernatant was added to 275 μL 0.2 M acetate buffer (pH 5.0) and mixed together with 100 μL 0.01 M para-nitrophenylphosphate (pNPP) dissolved in 0.2 M acetate buffer (pH 5.0). The reaction mixture was incubated for 15 min at $37\text{ }^{\circ}\text{C}$ in a water bath; the reaction was stopped with 100 μL 10 M NaOH and filled with 10 mL distilled water. Total reaction volume was 10.5 mL. The rate of change in absorbance at 405 nm was measured by a JENWAY 6705 UV/Vis spectrophotometer (Cole-Palmer, Stone, UK). Acid phosphatase activity was measured by determining the amount of para-nitrophenol produced ($a_{\text{p-NP}} = 16,000\text{ M}^{-1}\cdot\text{cm}^{-1}$). The levels of enzyme activity were expressed as the specific enzyme activity difference per minute per mg protein. Protein content was determined according to Bradford using bovine serum albumin (BSA) as a standard [23], and all the studied enzyme activity values were given in terms of protein.

2.3. Determination of β -glycerophosphatase Activity

Determination of β -glycerophosphatase (β -GP) was according to a modified method of Rao et al., 1960 [24]. 100 μL supernatant was added to 100 μL 0.2 M acetate buffer (pH 5.0) and mixed together with 200 μL 0.01 M β -glycerophosphate dissolved in 0.2 M acetate buffer (pH 5.0). The reaction mixture was incubated for 30 min at $37\text{ }^{\circ}\text{C}$ in a water bath; the reaction was stopped with 1000 μL 3% (w/v) sodium molybdate (dissolved in 0.2 M acetate buffer pH 5.0). A reducing agent, 0.12 M ascorbic acid, was added to get blue phosphomolybdate ($a_{\text{phosphomolybdate}} = 4000\text{ M}^{-1}\cdot\text{cm}^{-1}$) and filled with 5 mL distilled water. Total reaction volume was 6.5 mL. The rate of change in absorbance at 700 nm was measured by a JENWAY 6705 UV/Vis spectrophotometer. Absorbance values of each sample were corrected by subtracting the absorbance of the sample with buffer without substrate. The levels of enzyme activity were expressed as the specific enzyme activity difference per minute per mg protein.

2.4. Determination of Non-Specific Alkaline Phosphatase Activity

Non-specific alkaline phosphatase (ALP) activity was determined according to a modified method of Yamaya and Matsumoto, 1981 [25]. 100 μL supernatant was added to 200 μL 0.2 M TRIS-acetate buffer (pH 9.0) and mixed together with 100 μL 0.01 M para-nitrophenyl phosphate (pNPP) dissolved in 0.2 M TRIS- acetate buffer (pH 9.0). The reaction mixture was incubated for 15 min at $37\text{ }^{\circ}\text{C}$ in a water bath; the reaction was stopped with 100 μL 10 M NaOH and filled with 10 mL distilled water. Total reaction volume was 10.5 mL. The rate of change in absorbance at 405 nm was measured by a JENWAY 6705 UV/Vis spectrophotometer. Alkaline phosphatase activity was measured by determining the amount of para-nitrophenol produced ($a_{\text{p-NP}} = 16,000\text{ M}^{-1}\cdot\text{cm}^{-1}$). The levels of enzyme activity were expressed as the specific enzyme activity difference per minute per mg protein.

2.5. Determination of β -NAD Diphosphatase Activity

Determination of β -NAD diphosphatase (β -NADDP) was according to a modified method of Rao et al., 1960 [24]. 100 μL supernatant was added to 100 μL 0.2 M acetate buffer (pH 5.0) and mixed together with 200 μL 0.01 M β -NAD diphosphate dissolved in 0.2 M acetate buffer (pH 5.0). The reaction mixture was incubated for 30 min at $37\text{ }^{\circ}\text{C}$ in a water bath; the reaction was stopped with 1000 μL 3% (w/v) sodium molybdate (dissolved

in 0.2 M acetate buffer pH 5.0). A reducing agent, 0.12 M ascorbic acid, was added to get blue phosphomolybdate ($a_{\text{phosphomolybdate}} = 4000 \text{ M}^{-1} \cdot \text{cm}^{-1}$) and filled with 5 mL distilled water. Total reaction volume was 6.5 mL. The rate of change in absorbance at 700 nm was measured by a JENWAY 6705 UV/Vis spectrophotometer. Absorbance values of each sample were corrected by subtracting the absorbance of the sample with buffer without substrate. The levels of enzyme activity were expressed as the specific enzyme activity difference per minute per mg protein.

2.6. Determination of Peroxidase Activity

Determination of peroxidase (POD) activity was performed according to a method of Shannon et al., 1966 [26]. 50 μL supernatant was added to 2.1 mL distilled water, 420 μL 0.2 M phosphate buffer (pH 6.0), 320 μL 5% pyrogallol (dissolved in 0.2 M phosphate buffer (pH 6.0)), and mixed together with 160 μL 0.5% H_2O_2 in 0.2 M phosphate buffer (pH 6.0). Total reaction volume was 3.05 mL. The rate of change in absorbance at 420 nm was measured by a JENWAY 6705 UV/Vis spectrophotometer ($a_{\text{purporogallin}} = 2640 \text{ M}^{-1} \cdot \text{cm}^{-1}$). Absorbance values of each sample were corrected by subtracting the absorbance of the sample with buffer without substrate and substrate with buffer. The levels of enzyme activity were expressed as the specific enzyme activity difference per minute per mg protein.

2.7. Determination of Polyphenol Oxidase Activity

Determination of polyphenol oxidase (PPO) activity was performed according to a modified method of Kar and Mishra, 1975 [27]. 50 μL supernatant was added to 3.9 mL 0.2 M phosphate buffer (pH 6.8) 50 μL , and mixed together with 0.5% (*w/v*) pyrogallol (dissolved in distilled water). The total reaction mixture was 4 mL. The reaction mixture was incubated for 30 min at 40 °C in a water bath. The rate of change in absorbance at 420 nm was measured using a JENWAY 6705 UV/Vis spectrophotometer ($a_{\text{purporogallin}} = 2640 \text{ M}^{-1} \cdot \text{cm}^{-1}$). Absorbance values of each sample were corrected by subtracting the absorbance of the sample with buffer without substrate and substrate with buffer. The levels of enzyme activity were expressed as the specific enzyme activity difference per minute per mg protein.

2.8. Determination of Ferric-Reducing Antioxidant Power

Total antioxidant activity was determined by modified ferric-reducing antioxidant power (FRAP) assay of Benzie and Strain, 1996 [28]. The advantage of 2,2-dipyridyl compared to 2,4,6-tripyridyl-s-triazine is that the red color of the complex remains stable for a long period [29]. In this assay, in the presence of antioxidants, a red complex is formed when ferric ions (III) are reduced to ferrous ions (II). 450 μL freshly prepared FRAP α reagent (12.5 mL pH 3.6 buffer and 2.5 mL FeCl_3 solution ($270 \text{ mg FeCl}_3 \times 6 \text{ H}_2\text{O}$)) and 30 μL supernatant were mixed and incubated for 10 min at room temperature, then α' reagent (12.5 mL pH 3.6 buffer and 2.5 mL $\alpha\alpha'$ 0.03 M dipyridyl solution in ethanol) was added to this mixture and incubated for 30 min at room temperature. Total reaction volume was 0.93 mL. The rate of change in absorbance at 520 nm was measured by a JENWAY 6705 UV/Vis spectrophotometer. The results were evaluated by the standard calibration method. The levels of antioxidant content were expressed as μM ascorbic acid equivalent.

2.9. Determination of Total Phenolic Content

Total phenolic content (TPC) was determined by adapting the principle of detection of quinoidal form with thin layer chromatography combined with spectrophotometry [30]. The advantages of this method include the sensitivity of the reaction to the quinoidal form of polyphenols and the elimination of critical false-positive interferences caused by common biological, non-phenolic reducing agents (such as ascorbic acid and reducing sugars) [31,32].

In this assay, in the presence of pH 9.5 NaOH-borate buffer, polyphenols transfer to yellow quinoidal forms. 950 μ L pH 9.5 Na-borate buffer and 50 μ L supernatant were mixed, and absorbance was measured at 405 nm by a JENWAY 6705 UV/Vis spectrophotometer. The results were evaluated by the standard calibration method. The levels of total phenolic content were expressed as mg/ g fresh weight quercetin equivalent.

2.10. Statistical Analyses

Statistical analyses (normality test including Anderson-Darling test, D'Agostino and Pearson test, Shapiro–Wilk test and Kolmogorov–Smirnov test, ANOVA, and post hoc tests) were performed using GraphPad Prism 8 (GraphPad Software, Boston, MA, USA, [graphpad.com](https://www.graphpad.com)). When the data followed a normal distribution and significant differences were detected by one-way ANOVA, the Tukey–Kramer multiple comparisons test was used as a post hoc test. For data that did not follow a normal distribution, the Kruskal–Wallis non-parametric ANOVA test was performed, followed by Dunn's multiple comparisons test. Significant differences (at $p < 0.05$) were labeled with different letters. The strength and direction of the monotonic relationship between two continuous variables were measured using the Spearman correlation coefficient.

For the determination of the enzyme activity and antioxidant content data, each treatment group consisted of five culture vessels. Five shoots were in each vessel and pooled together to form a single biological sample per vessel. From these pooled samples, three individual measurements were taken in each independent experiment. Furthermore, these experiments were repeated independently three times. For statistical comparison, data were normalized relative to the corresponding controls (expressed as a percentage of the control group) at each of the three experiments to ensure an accurate assessment of treatment effects. Consequently, the plotted enzyme activity and antioxidant content values are expressed as percentages relative to the control group. The absolute measured values are provided in Table S1.

To account for potential block effects between experimental runs, data from each of the three independent experiments were normalized and expressed as a relative percentage compared to the mean of the corresponding control group within that specific run. The normalization was calculated using the following formula: Normalized Value (%) = (Value of Treatment/Mean Value of Control) $\times$ 100. The normalized data from all three independent runs were then pooled for normality tests and ANOVA. Consequently, the control group retains biological variance, represented by the standard error of the mean (SEM) and statistical lettering in Table 1.

Table 1. Effect of different cytokinin (BAR, TDZ, and TOP) treatments on acid and alkaline phosphatases, β -glycerophosphatase, β -NAD diphosphatase, peroxidase and polyphenol oxidase activity in the shoots of Húsvéti rozmaryng (HR) and McIntosh (McI) apple scions. Enzyme activities are expressed as a percentage of the control. Different letters in each row indicate statistically significant differences between the samples according to Kruskal–Wallis non-parametric ANOVA followed by Dunn's multiple comparisons test ($p < 0.05$). (Mean values and standard error of the mean are presented (SEM), $n = 15$).

	Húsvéti Rozmaryng (HR)				McIntosh (McI)			
	Control	BAR	TDZ	TOP	Control	BAR	TDZ	TOP
ACP activity	100 $\pm$ 3.57 a	124 $\pm$ 8.89 b	126.6 $\pm$ 5.65 b	183 $\pm$ 8.72 c	96.9 $\pm$ 3.10 a	121.2 $\pm$ 3.66 b	88.3 $\pm$ 3.67 c	117.7 $\pm$ 2.70 b
β -GP activity	104.8 $\pm$ 3.21 a	134.7 $\pm$ 6.39 b	126.2 $\pm$ 4.19 ab	191.9 $\pm$ 11.95 c	95.3 $\pm$ 5.59 a	227.8 $\pm$ 8.22 b	174.1 $\pm$ 9.05 c	189.8 $\pm$ 6.07 d

Table 1. Cont.

	Húsvéti Rozmaring (HR)				McIntosh (McI)			
	Control	BAR	TDZ	TOP	Control	BAR	TDZ	TOP
ALP activity	99.8 ± 4.33 a	144.3 ± 5.51 b	92.7 ± 3.69 a	130.5 ± 7.17 c	98.3 ± 4.31 a	109.4 ± 4.16 b	76.7 ± 4.05 c	96.1 ± 4.17 a
β-NADDP activity	100 ± 5.75 a	92.8 ± 3.46 a	148.5 ± 5.29 b	116.5 ± 4.75 c	101.1 ± 4.08 a	131.1 ± 5.16 b	121.6 ± 8.30 b	130.1 ± 5.43 b
POD activity	100 ± 1.19 a	97.6 ± 3.57 a	73.3 ± 2.7 b	47.5 ± 1.72 c	98.8 ± 1.70 a	59.1 ± 3.95 b	54.4 ± 3.52 b	61.0 ± 2.90 b
PPO activity	105.4 ± 2.95 a	204.3 ± 5.07 b	129.7 ± 5.09 c	98.2 ± 2.23 a	100 ± 1.77 a	122.7 ± 2.19 b	117.1 ± 8.50 b	118.9 ± 6.33 b

3. Results

All types of CKs significantly increased the ACP activity in the HR plants. In the BAR- and TDZ-treated HR plants (BAR: 124.0% ± 8.89, TDZ: 126.6% ± 5.65), ACP activities were similar and higher than the control. In the HR plants, the highest ACP enzyme activity was detected after TOP treatment (183.0% ± 8.72). In the HR plants, after BAR, TDZ and TOP treatments, ACP enzyme activities were higher than in the McI plants after the same treatments. In the McI plants, ACP activity increased in the presence of BAR (121.2% ± 3.66) and TOP (117.7% ± 2.70), while TDZ (88.3% ± 3.67) treatment decreased it. The highest ACP activity was measured in the TOP-treated HR plants and the lowest in the TDZ-treated McI plants (Figure 1A,B) (Table 1).

All types of CKs significantly increased the β-GP activity in the HR and McI plants. In the BAR- and TDZ-treated HR plants (BAR: 134.7% ± 6.39, TDZ: 126.2% ± 4.19), β-GP activity was similar and higher than the control. In the HR plants, the highest β-GP enzyme activity was detected after TOP treatment (191.9% ± 11.95). In the McI plants, after BAR (227.8% ± 8.22) and TDZ (174.1% ± 9.05) treatments, β-GP enzyme activities were higher than in the HR plants after the same treatments, while in the TOP-treated HR plants (191.9% ± 11.95) it was similar to the TOP-treated McI plants (189.8% ± 6.07). The highest β-GP activity was measured in the BAR-treated McI plants (227.8% ± 8.22) and the lowest in the TDZ-treated HR plants (Figure 1C,D) (Table 1).

BAR (144.3% ± 5.51) and TOP (130.5% ± 7.17) treatments increased ALP activity in the HR plants. In the HR plants, TDZ treatment had no effect on ALP enzyme activity. In the HR plants, after BAR, TDZ and TOP treatments ALP enzyme activities were higher than in the McI plants after the same treatments. The highest ALP activity was measured in the BAR-treated HR plants and the lowest in the TDZ-treated plants. In the McI plants, BAR (109.4% ± 4.16) treatment increased ALP activity, TDZ (76.7% ± 4.05) treatment decreased it, and TOP had no effect. ALP activity was highest in the BAR-treated HR plants and the lowest in the TDZ-treated McI plants (Figure 1E,F) (Table 1).

TDZ (148.5% ± 5.29) and TOP (116.5% ± 4.75) treatments increased β-NADDP activity in the HR plants. In the HR plants, BAR treatment had no effect on β-NADDP enzyme activity. In the HR plants, after TDZ treatment, β-NADDP activity was higher than in the McI plants treated with any type of CK. The highest β-NADDP activity was measured in the TDZ-treated HR plants and the lowest in BAR-treated ones. In the McI plants, all types of CKs increased β-NADDP activity to a similar level (BAR: 131.1% ± 5.16, TDZ: 121.6% ± 8.30, and TOP: 130.1% ± 5.43). The β-NADDP activity was highest in the TDZ-treated HR plants and the lowest in the BAR-treated HR plants (Figure 1G,H) (Table 1).

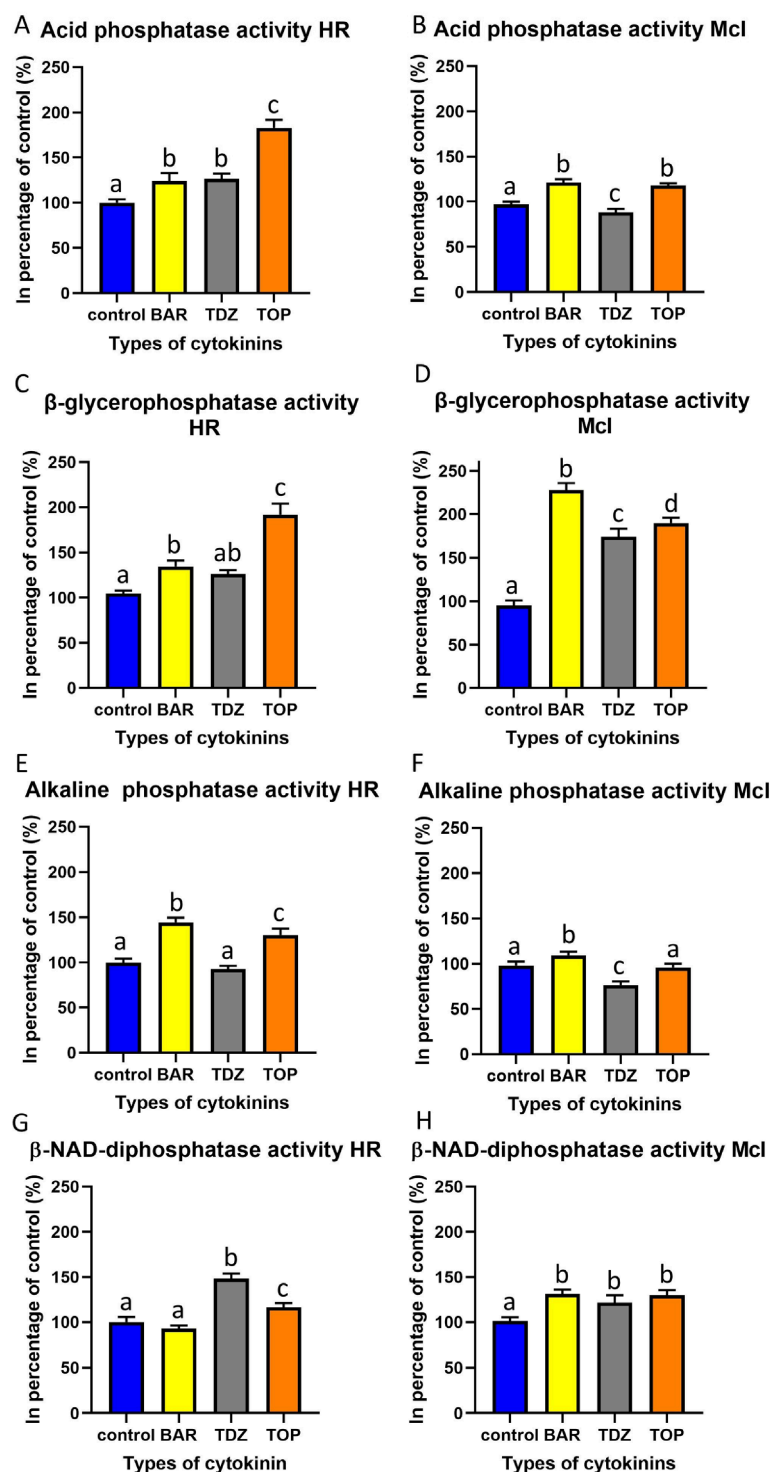


Figure 1. Effect of cytokinin (BAR, TDZ and TOP) treatments on (A) acidic phosphatase activity in HR, (B) acidic phosphatase activity in Mcl, (C) β -glycerophosphatase in HR, (D) β -glycerophosphatase activity in Mcl, (E) alkaline phosphatase activity in HR, (F) alkaline phosphatase activity in Mcl, (G) β -NAD diphosphatase activity in HR and (H) β -NAD diphosphatase activity in Mcl. Different letters indicate statistically significant differences between the samples according to the Kruskal–Wallis non-parametric ANOVA followed by Dunn’s multiple comparisons test ($p < 0.05$), ($n = 15$). Data are presented as mean $\pm$ standard error of the mean (SEM).

BAR treatment had no effect on POD activity in the HR plants. In the TDZ- and TOP-treated HR plants (TDZ: $73.3\% \pm 2.70$, TOP: $47.5\% \pm 1.72$), POD activities were significantly lower than in the control. In HR plants, the highest POD enzyme activity was detected in

the control, whereas the lowest was observed in plants after TOP treatment. In the Mcl plants, after BAR ($59.1\% \pm 3.95$) and TDZ ($54.4\% \pm 3.52$) treatments, POD enzyme activities were lower than in the HR plants subjected to the same treatments. Conversely, in the TOP-treated Mcl plants ($61.0\% \pm 2.90$), activity was higher than in the TOP-treated HR plants. All types of CKs decreased POD activity in the Mcl plants. Overall, the highest POD activity was measured in the control HR and Mcl plants (Figure 2A,B) (Table 1).

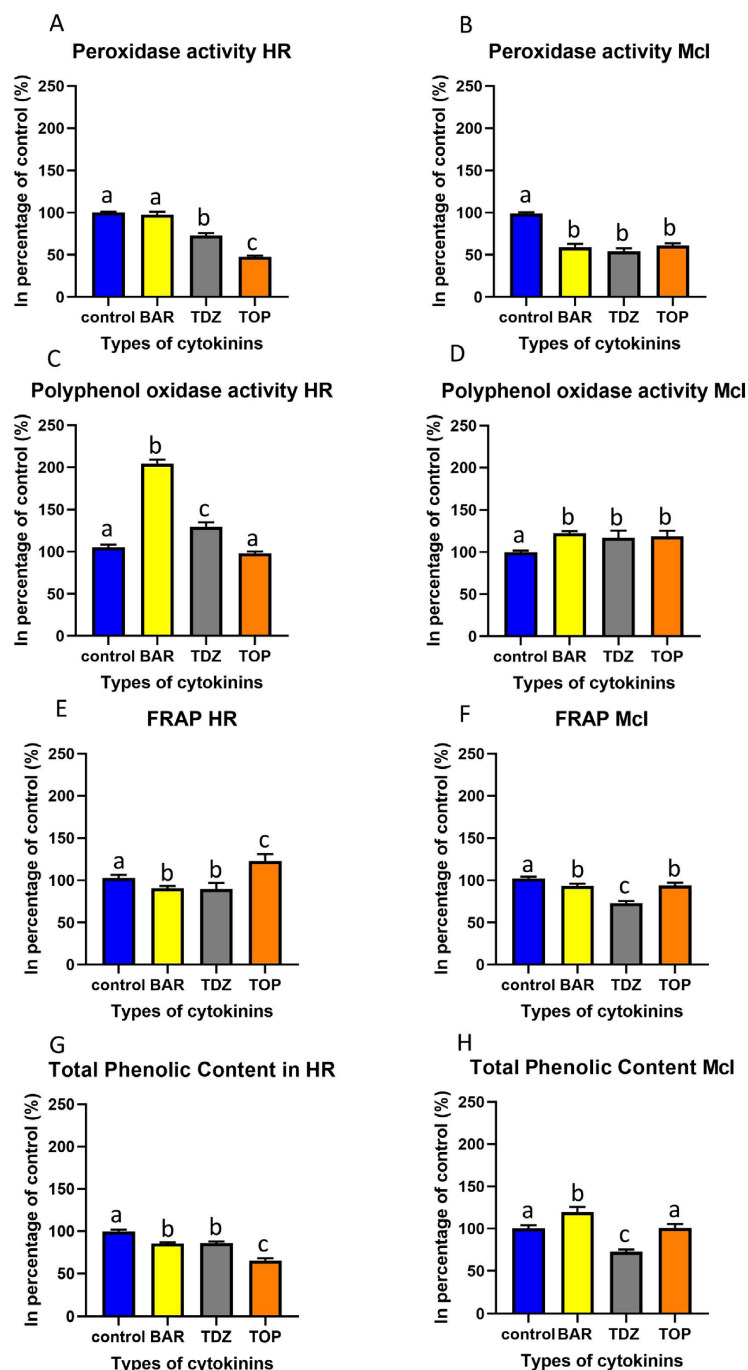


Figure 2. Effect of cytokinin (BAR, TDZ and TOP) treatments on (A) peroxidase activity in HR, (B) peroxidase activity in Mcl, (C) polyphenol oxidase activity in HR, (D) polyphenol oxidase activity in Mcl, (E) FRAP in HR, (F) FRAP in Mcl, (G) total phenolic content in HR and (H) total phenolic content in Mcl. Different letters indicate statistically significant differences between the samples according to the Kruskal–Wallis non-parametric ANOVA followed by Dunn’s multiple comparisons test ($p < 0.05$), ($n = 15$). Data are presented as mean $\pm$ standard error of the mean (SEM).

BAR ($204.3\% \pm 5.07$) and TDZ ($129.7\% \pm 5.09$) treatments significantly increased the PPO activity in the HR plants, while TOP treatment had no effect. In the HR plants, the highest PPO enzyme activity was detected after BAR treatment and the lowest after TOP treatment. In the Mcl plants, all types of cytokinins increased PPO enzyme activities. After BAR ($122.7\% \pm 2.19$) and TDZ ($117.1\% \pm 8.50$) treatments, PPO activities in the Mcl plants were lower than in the HR plants following the same treatments. In contrast, after TOP treatment ($98.2\% \pm 2.23$), activity in the HR plants was lower than in the TOP-treated Mcl plants ($118.9\% \pm 6.33$). The highest overall PPO activity was measured in the BAR-treated HR plants ($204.3\% \pm 5.07$) (Figure 2C,D) (Table 1).

TOP ($122.9\% \pm 8.16$) treatment increased FRAP in the HR plants, whereas BAR ($90.7\% \pm 2.64$) and TDZ ($89.9\% \pm 7.08$) treatments decreased it. In the HR plants, after TDZ and TOP treatments, FRAP was higher than in the Mcl plants following the same treatments (TDZ: $73.3\% \pm 2.17$, TOP: $94.0\% \pm 3.24$). In contrast, after BAR treatment, FRAP was similar in the HR ($90.7\% \pm 2.64$) and Mcl plants ($93.5\% \pm 2.37$). The highest FRAP was measured in the TOP-treated HR plants ($122.9\% \pm 8.16$), and the lowest in TDZ-treated Mcl plants ($73.3\% \pm 2.17$). In the Mcl plants all types of CKs decreased FRAP, but only TDZ treatment caused a significant decrease ($73.3\% \pm 2.17$) (Figure 2E,F) (Table 2).

Table 2. Effect of different cytokinin (BAR, TDZ, and TOP) treatments on FRAP value and total phenolic content in the shoots of Húsvéti rozmarín (HR) and McIntosh (Mcl) apple scions. FRAP and TPC values are expressed as a percentage of the control. Different letters in each row indicate statistically significant differences between the samples according to Kruskal–Wallis non-parametric ANOVA followed by Dunn’s multiple comparisons test ($p < 0.05$). (Mean values and standard error of the mean are presented (SEM), $n = 15$).

Húsvéti Rozmaring (HR)					McIntosh (Mcl)			
FRAP	102.9 ± 3.59 a	90.7 ± 2.64 b	89.9 ± 7.08 b	122.9 ± 8.16 c	102.3 ± 2.05 a	93.5 ± 2.37 b	73.3 ± 2.17 c	94 ± 3.24 b
TPC	100 ± 0.67 a	85.2 ± 0.50 b	86 ± 0.70 b	65.3 ± 0.88 c	100 ± 1.32 a	119.7 ± 2.03 b	72.4 ± 0.89 c	100.7 ± 1.61 a

All types of CK treatments decreased the TPCs in the HR plants. In the HR plants, BAR ($85.2\% \pm 0.50$) and TDZ ($86.0\% \pm 0.70$) treatments mildly decreased TPC, while after TOP treatment it decreased to $65.3\% \pm 0.88$ of the control. In the HR plants, TPC after BAR treatment was lower than in the Mcl plants, following either BAR or TOP treatments. The highest TPC was measured in the BAR-treated Mcl plants ($119.7\% \pm 2.03$) and the lowest in the TOP-treated HR plants ($65.3\% \pm 0.88$). In the Mcl plants, BAR ($119.7\% \pm 2.03$) increased and TDZ ($72.4\% \pm 0.89$) decreased TPCs, while TOP treatment had no effect on it (Figure 2G,H) (Table 2).

In the HR plants, BAR treatment increased ACP, ALP, β -GP, and PPO activities, whereas it decreased FRAP and TPC, and had no effect on β -NADDP and POD activities. In the HR plants treated with TDZ, ACP, β -GP, β -NADDP and PPO activities increased, but POD activity, FRAP, and TPC decreased; meanwhile, no effect was observed on ALP activity. In the HR plants, after TOP treatment, ACP, ALP, β -GP, and β -NADDP activities as well as FRAP increased. Conversely it decreased TPC and POD activity and had no effect on polyphenol oxidase activity.

In the Mcl plants, BAR treatment increased ACP, ALP, β -GP, β -NADDP, and PPO activities, as well as TPC, whereas it decreased POD activity and FRAP. In the TDZ-treated Mcl plants, β -GP, β -NADDP and PPO activities increased, but TPC, FRAP, ACP, ALP and POD activities decreased. In the Mcl plants, after TOP treatment, ACP, β -GP, β -NADDP and PPO activities increased, while it decreased POD activity and FRAP and had no effect on TPC and alkaline phosphatase activity.

In the BAR-treated HR plants, there was a strong negative correlation between acid phosphatase and FRAP ($r = -0.800$; $p = 0.0020$; $n = 15$). In the HR plants, after TDZ treatment, there was a strong negative correlation between β -NAD diphosphatase and polyphenol oxidase ($r = -0.713$; $p = 0.0083$; $n = 15$) (Table 3).

Table 3. Spearman correlation coefficient between the measured parameters (acid (ACP), alkaline (ALP) phosphatases, β -glycerophosphatase (β -GP), β -NAD diphosphatase (β -NADDP), peroxidase (POD) and polyphenol oxidase activity (PPO), FRAP value and total phenolic content (TPC)) in the shoots of cytokinin-treated (BAR, TDZ, and TOP) Húsvéti rozmarín (HR) and McIntosh (McI) apple scions. When it is strong or very strong, numbers are in bold letters. In all the treatment groups $n = 15$.

HR BAR	β -GP	ALP	β -NADDP	POD	PPO	FRAP	TPC
ACP	−0.159	−0.559	0.027	0.418	0.517	−0.676	0.569
β -GP		−0.198	−0.176	−0.036	−0.044	0.011	−0.109
ALP			−0.159	−0.691	−0.223	0.615	0.251
β -NADDP				−0.236	−0.250	0.005	0.067
POD					0.369	−0.800 ($p = 0.0020$)	−0.301
PPO						−0.592	0.536
FRAP							−0.159
HR TDZ	β -GP	ALP	β -NADDP	POD	PPO	FRAP	TPC
ACP	0.350	−0.275	0.275	−0.264	−0.088	−0.022	−0.100
β -GP		0.252	−0.154	0.182	0.252	0.014	0.500
ALP			−0.429	0.173	0.269	−0.060	0.217
β -NADDP				−0.500	−0.713 ($p = 0.0083$)	0.247	−0.433
POD					0.636	−0.436	0.067
PPO						−0.643	0.533
FRAP							0.317
McI BAR	β -GP	ALP	β -NADDP	POD	PPO	FRAP	TPC
ACP	−0.082	−0.165	0.258	0.155	0.225	−0.143	−0.417
β -GP		0.03	−0.313	−0.078	0.418	0.396	0.030
ALP			−0.093	−0.210	−0.154	0.203	0.350
β -NADDP				0.515	0.055	−0.577	−0.533
POD					−0.027	−0.838 ($p = 0.0020$)	−0.008
PPO						0.049	−0.433
FRAP							0.333
McI TDZ	β -GP	ALP	β -NADDP	POD	PPO	FRAP	TPC
ACP	−0.457	−0.498	−0.242	0.355	0.841 ($p = 0.0006$)	−0.703	−0.267
β -GP		0.591	−0.110	−0.474	−0.768 ($p = 0.0031$)	0.553	0.184
ALP			−0.253	0.068	−0.556	0.634	−0.042
β -NADDP				−0.227	−0.121	−0.022	−0.433

Table 3. Cont.

POD				0.373		−0.141	0.267
PPO						−0.713 (<i>p</i> = 0.0083)	−0.517
FRAP							−0.333
McI TOP	β-GP	ALP	β-NADDP	POD	PPO	FRAP	TPC
ACP	−0.063	−0.347	0.446	0.473	0.313	−0.368	0.209
β-GP		−0.554	−0.047	−0.366	−0.248	0.201	−0.391
ALP			−0.441	−0.165	−0.322	0.371	−0.259
β-NADDP				0.135	0.171	−0.198	0.714 (<i>p</i> = 0.0349)
POD					0.467	−0.659	−0.100
PPO						−0.841 (<i>p</i> = 0.0006)	0.251
FRAP							0.142

In the BAR-treated McI plants, there was a very strong negative correlation between POD and FRAP ($r = -0.838$; $p = 0.0020$; $n = 15$). In the McI plants, after TDZ treatment, a strong negative correlation was observed between β -GP ($r = -0.768$; $p = 0.0031$; $n = 15$) and PPO, as well as between PPO and FRAP ($r = -0.713$; $p = 0.0083$; $n = 15$), meanwhile there was a very strong positive correlation between ACP and PPO ($r = 0.841$; $p = 0.0006$; $n = 15$) (Table 3).

In the TOP-treated McI plants, there was a strong positive correlation between β -NADDP and TPC ($r = 0.714$; $p = 0.0349$; $n = 15$) and a very strong negative correlation between PPO and FRAP ($r = -0.841$; $p = 0.00006$; $n = 15$) (Table 3).

A very strong positive correlation in POD activity was found between the BAR-treated HR and McI plants ($r = 0.8702$). Similarly, a very strong positive correlation in FRAP was observed between the BAR-treated HR and McI plants ($r = 0.8242$), alongside a strong positive correlation in FRAP between the TDZ-treated HR and McI plants ($r = 0.7293$). Furthermore, very strong positive correlations were detected within the HR plants: PPO activity ($r = 0.8418$) and FRAP ($r = 0.8242$) between the BAR- and TDZ-treated plants, and in TPC ($r = 0.8452$) between the BAR- and TOP-treated HR plants.

4. Discussion

In this study we investigated the effects of the three types of cytokinins (BAR, TDZ and TOP) on the activities of acid and alkaline phosphatases, β -glycerophosphatase, β -NAD diphosphatase, peroxidase and polyphenol oxidase, as well as FRAP antioxidant capacity and total phenolic content in the shoots of two in vitro cultured apple scion cultivars ('Húsvéti rozmarín' and 'McIntosh'). During our experiments, we were able to reveal the cultivar dependence of CK effects at both enzymatic, FRAP values and total phenolic content levels.

β -glycerophosphatase activity significantly increased in the shoots of the HR and McI plants after CK treatments, which suggests a potential physiological response to exogenous hormonal stimulation. CKs increased β -glycerophosphatase in the CK-treated HR and McI plants, which was consistent with the findings in *Zea mays* L. under molybdenum deficiency, where β -glycerophosphatase levels increased [33], but under beryllium stress β -glycerophosphatase activity decreased [34]. Under stress conditions the availability of inorganic phosphate can decrease; increasing of β -glycerophosphatase activity after CK treatments may represent an important mechanism to sustain adequate phosphate pools during in vitro development (Figure 1C,D).

The significant increase in non-specific acid and alkaline phosphatase activities in the shoots of the CK-treated plants suggests a coordinated metabolic adaptation rather than a generalized environmental stress response. Cytokinins are fundamental triggers of *in vitro* shoot organogenesis and rapid cell proliferation. These intensive developmental processes heavily demand inorganic phosphate (P_i) for biomolecule synthesis, membrane formation, and ATP-driven energy metabolism. Therefore, the upregulation of these phosphatases likely serves as a crucial physiological mechanism to accelerate internal phosphate mobilization and sustain cellular phosphate homeostasis during hormone-stimulated growth.

Conversely, the distinct decline in both non-specific acid and alkaline phosphatase activities specifically observed in the TDZ-treated ‘McIntosh’ plants highlights a critical threshold. TDZ, as a highly potent phenylurea-type cytokinin, is known to exert strong physiological effects and inhibit endogenous cytokinin oxidase/dehydrogenase enzymes. This can cause an excessive accumulation of active cytokinins, which might trigger a potential intracellular redox disruption in sensitive genotypes [35,36]. In ‘McIntosh’, this TDZ-induced response appears to have surpassed the physiological tolerance limits, leading to potential enzymatic suppression or cellular exhaustion, which explains the sharp contrast to the responses seen under BAR and TOP treatments.

CK treatment increased β -NAD diphosphatase activity in the HR and McI plants, whereas BAR treatment in the HR plants had no effect on it. β -NAD diphosphatase maintains the energy balance in chloroplasts and mitochondria; besides this, it regulates the level of NADPH, which helps the antioxidant system to protect plants under stress. In *Arabidopsis thaliana* (L.) Heynh. under oxidative stress, activity of β -NAD diphosphatase increased, leading to concomitant decrease in NAD^+ levels [37]; we detected a similar tendency of enzyme activity changes. The opposite was found in *Pisum sativum* L. and *Triticum aestivum* L., where enzyme activity decreased under drought stress [38] (Figure 1G,H).

Peroxidase activity decreased in the cytokinin-treated HR and McI plants, whereas BAR treatment in HR had no effect on it. Peroxidase is a key enzyme involved in scavenging reactive oxygen species, such as hydrogen peroxide. CK treatments decreased peroxidase activity in HR and McI which was in accordance with the results in TOP- and TDZ-treated *Gentianella lutescens* subsp. *carpatica* (Beck) Holub. [39], decreasing of peroxidase activity means the culturing conditions are favorable for plants. The opposite was found in *Pelargonium hortorum* (L.H. Bailey) tissue cultures, where peroxidase activity increased after TOP treatment [40], as well as in *Malus x domestica* Borkh. seedlings under salt stress and hypoxia [41,42] (Figure 2A,B).

After CK treatments polyphenol oxidase activity increased, corresponding to the results in *Malus x domestica* Borkh. under abiotic stress, virus and fungal pathogens [43–45]. TOP treatment decreased it in the HR plants. Increasing of polyphenol oxidase activity may change traits in plants which can help the plants live in unfavorable environments and protect themselves against pathogens (Figure 2C,D).

Except in the TOP-treated HR plants, FRAP decreased after treatment with all the tested CKs in both the HR and McI plants. In contrast, FRAP was reported to increase in *Malus x domestica* Borkh. shoot cultures treated with NaCl, KCl, mannitol, or sorbitol [46], which strictly diverged from our findings. Specifically, these osmotic and saline stress-induced increases did not align with the results obtained in our the BAR- and TDZ-treated HR plants, nor in the McI plants treated with any of the tested cytokinins.

Background of decreasing of FRAP after CK treatment could be that polyphenol oxidase oxidizes and subsequently depletes the pool of phenolic compounds that act as primary drivers of FRAP antioxidant activity [45] (Figure 2E,F).

TPC decreased after treatment with all types of CKs in the HR plants and the TDZ-treated McI plants, whereas it increased in the BAR- and TOP-treated McI plants. In the BAR-treated McI plants increased total phenolic content was consistent with results in *Knautia sarajevensis* (Beck) treated with zeatin, *Brachystelma* species treated with 6-benzyladenin or TOP; *Merwillia plumbea* (L) treated with TOP; and *Malus x domestica* Borkh. cultivars under drought stress [17,47–49], while in *K. sarajevensis* treated with 6-benzyladenin, it decreased, which matched the results of the TDZ-treated McI plants and HR plants treated with all types of CKs [47]. Background of decreasing of total phenolic content after CKs treatment could indicate a metabolic shift, where plants allocate resources toward active growth and biomass production instead of the synthesis of polyphenolic secondary metabolites (Figure 2G,H).

The observed increase in polyphenol oxidase (PPO) activity, coupled with the simultaneous depletion of total phenolic content (TPC) and lower FRAP values across most cytokinin treatments, suggests a potential metabolic shift driven by aromatic and phenylurea cytokinins. Under exogenous hormonal stimulation, plants frequently undergo a metabolic shift, allocating resources away from secondary metabolites (such as protective polyphenols) and prioritizing primary metabolism for active growth and biomass production. The enhanced PPO activity appears to be associated with the oxidation of existing vacuolar phenolic compounds into quinones. This biochemical transition is correlated with a reduction in the pool of free phenolics that act as the primary drivers of non-enzymatic antioxidant capacity, as confirmed by our correlation analysis. This effect was most pronounced under TDZ application in ‘McIntosh’ shoots, where the drastic reduction in TPC and FRAP implies a genotype-specific sensitivity to potential oxidative stress when exposed to non-purine cytokinins.

A significant positive correlation was established between acid phosphatase and PPO. The plausible explanation is that CK increases active growth and acid phosphatase to ensure adequate availability of inorganic phosphate [50]. Conversely, a negative correlation was observed between peroxidase and FRAP, suggesting a complex interplay where enzymatic defense mechanisms might influence non-enzymatic antioxidant pools [45]. Furthermore, a negative correlation determined between polyphenol oxidase and FRAP is consistent with the hypothesis that PPO-mediated oxidation degrades the phenolic compounds that contribute to FRAP antioxidant capacity [45]. Additionally, a negative correlation was found between β -NAD diphosphatase and PPO. A likely mechanism is that CKs promote primary metabolism and stimulate NAD processing enzymes, meanwhile suppressing PPO synthesis and activity [51]. We have observed a positive correlation between β -NAD diphosphatase and total phenolic content. This relationship might be explained by the fact that phenolic compounds are synthesized via the phenylpropanoid pathway, which depends on NADPH as an electron donor [52] (Table 3).

We found that BAR treatment had a similar effect on peroxidase activity and FRAP in both cultivars; a similar phenomenon was observed on FRAP in the TDZ-treated HR and McI plants (Figure 2A,B,E,F). We observed that BAR and TDZ treatments had similar effects on PPO activity and FRAP in the HR plants (Figure 2C–F). We found that BAR and TOP treatments exhibited similar influences on TPC of the HR plants (Figure 2G).

The comparable influences exerted by BAR and TOP on several enzymatic and antioxidant parameters in ‘Húsvéti rozmarín’ can be chemically explained by their structural configuration, as both compounds share a common adenine-type benzylaminopurine backbone. However, their specific physiological potency is heavily side-chain dependent. Aromatic topolins, such as meta-topolin (TOP), possess a hydroxyl group on the N6-linked benzyl ring. This specific modification alters their interaction with cytokinin receptors and enables distinct metabolic pathways compared to standard ribosides like BAR [53,54]. This structural difference may explain why TOP was uniquely capable of elevating FRAP antiox-

idant capacity in ‘Húsvéti rozmaring’, showcasing that precise side-chain substitutions may dictate whether a cytokinin mitigates or amplifies tissue stress during in vitro propagation.

The limitations of this study include the evaluation of only two cultivars, the use of a single cytokinin concentration, a single sampling time, and the absence of direct oxidative stress markers. Our future plans include conducting experiments with additional cultivars, incorporating multiple sampling times, and determining direct oxidative stress markers, such as malondialdehyde (MDA) content, to assess lipid peroxidation.

5. Conclusions

In conclusion, application of TDZ increased polyphenol oxidase activity, but decreased peroxidase activity in both studied apple cultivars. In contrast, TOP decreased peroxidase activity in ‘Húsvéti rozmaring’ but did not significantly alter it in ‘McIntosh’. BAR exerted a similar effect to TOP on peroxidase activity, whereas its influence on polyphenol oxidase activity differed significantly. Regarding non-enzymatic antioxidants, only TOP treatment elevated FRAP values in ‘Húsvéti rozmaring’, while all the other cytokinin treatments across both cultivars resulted in a decline in this parameter. Similarly, total phenolic content (TPC) was enhanced exclusively by BAR and TOP in ‘McIntosh’, whereas a reduction was observed under all the other experimental combinations.

One plausible underlying mechanism driving these distinct enzymatic and antioxidant profiles is the different stress sensitivity exhibited by the two apple cultivars, coupled with their divergent metabolic responses, as evidenced by the dissimilar TPC trends. Although BAR and TOP exerted comparable effects on the enzyme activity and antioxidant contents of ‘Húsvéti rozmaring’ and ‘McIntosh’ shoots, which is consistent with the fact that both compounds share a common benzylaminopurine backbone, their physiological potency remains highly side-chain dependent. Specifically, hydroxylated topolin derivatives (TOP) are known to be markedly more active than ribosides (BAR) [53,54], thereby explaining the differences detected between the two aromatic cytokinins.

The principal novelty of our study lies in the comprehensive and comparative evaluation of BAR, TDZ and TOP on both the enzymatic system and non-enzymatic antioxidant pool in two distinct apple cultivars. In summary, TDZ treatment decreased the non-enzymatic antioxidant content and suppressed the majority of the studied enzymes in ‘McIntosh’, including acid and alkaline phosphatase, presumably because it triggered a higher level of oxidative stress in this genotype compared to ‘Húsvéti rozmaring’ and the other tested cytokinins [55,56]. These distinct responses among cultivars clearly demonstrate that in vitro culture protocols cannot be universally applied and must be used in a genotype-dependent manner [57–59].

From a practical standpoint, these findings provide a valuable framework for commercial laboratories to optimize shoot quality and multiplication rates by adjusting specific cytokinin applications to individual apple genotypes. While this study explicitly identifies these physiological shifts under controlled in vitro limitations, future investigations should focus on how these treatment-induced metabolic profiles influence the subsequent *ex vitro* acclimatization phase and long-term plantlet survival.

Supplementary Materials: The following supporting information can be downloaded at <https://www.mdpi.com/article/10.3390/horticulturae12080956/s1>: Table S1: Absolute values of enzyme activities, total phenolic content (TPC), and antioxidant capacity.

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Abbreviations

The following abbreviations are used in this manuscript:

HR	Húsvéti rozmaring
McI	McIntosh
ACP	Acid phosphatase
ALP	Alkaline phosphatase
G2PP	β-glycerophosphatase
β-NADDP	β-NAD diphosphatase
POD	Peroxidase
PPO	Polyphenol oxidase
FRAP	Ferric reducing antioxidant power
TPC	Total phenolic content

References

1. Lizárraga, A.; Fraga, M.; Ascasíbar, J.; González, M.L. In Vitro Propagation and recovery of eight apple and two pear cultivars held in a germplasm bank. *Am. J. Plant Sci.* **2017**, *8*, 2238–2254. [[CrossRef](#)]
2. Bhatti, S.; Jha, G. Current trends and future prospects of biotechnological interventions through tissue culture in apple. *Plant Cell Rep.* **2010**, *29*, 1215–1225. [[CrossRef](#)] [[PubMed](#)]
3. Teixeira da Silva, J.A.; Gulyas, A.; Magyar-Tábori, K.; Wang, M.R.; Wang, Q.C.; Dobránszki, J. In vitro tissue culture of apple and other *Malus* species: Recent advances and applications. *Planta* **2019**, *249*, 975–1006. [[CrossRef](#)] [[PubMed](#)]
4. Farkas, D.; Ambrus, V.; Király, A.; Abdalla, A.; Dobránszki, J. Transcriptomic response of apple (*Malus × domestica* Borkh. cv. Husveti rozmaring) shoot explants to in vitro cultivation on media containing thidiazuron or 6-benzylaminopurine riboside. *Plant Cell Tissue Organ Cult. (PCTOC)* **2025**, *161*, 29. [[CrossRef](#)]
5. Cortleven, A.; Schmölling, T. Regulation of chloroplast development and function by cytokinin. *J. Exp. Bot.* **2015**, *66*, 4999–5013. [[CrossRef](#)] [[PubMed](#)]
6. Oshchepkov, M.S.; Kalistratova, A.V.; Savelieva, E.M.; Romanov, G.A.; Bystrova, N.A.; Kochetkov, K.A. Natural and synthetic cytokinins and their applications in biotechnology, agrochemistry and medicine. *Russ. Chem. Rev.* **2020**, *89*, 787–810. [[CrossRef](#)]
7. Wang, S.Y.; Jiao, H.J.; Faust, M. Changes in the activities of catalase, peroxidase, and polyphenol oxidase in apple buds during bud induced by thidiazuron. *J. Plant Growth Regul.* **1991**, *10*, 33–39. [[CrossRef](#)]
8. de Souza, L.M.; de Araújo Silva, M.M.; Herculano, L.; Ulisses, C.; Camara, T.R. Meta-topolin: An alternative for the prevention of oxidative stress in sugarcane micropropagation. *Hoehnea* **2019**, *46*, e1072018. [[CrossRef](#)]
9. Mamedes-Rodrigues, T.C.; Batista, D.S.; Napoleão, T.A.; Fortini, E.A.; Cruz, A.C.F.; Costa, M.G.C.; Otoni, W.C. Regulation of cell wall development in *Brachypodium distachyon* in vitro as affected by cytokinin and gas exchange. *Plant Cell Tissue Organ Cult. (PCTOC)* **2019**, *136*, 207–219. [[CrossRef](#)]
10. Wei, Z.; Yang, H.; Duan, Y.; Fan, S.; Wu, W.; Lyu, L.; Li, W. Integrated physiological, hormonal and transcriptomic analyses reveal mechanisms of blackberry plants response to exogenous 6-benzylaminopurine. *Sci. Hortic.* **2024**, *332*, 113181. [[CrossRef](#)]
11. Ozden, M.; Karaaslan, M. Effects of cytokinin on callus proliferation associated with physiological and biochemical changes in *Vitis vinifera* L. *Acta Physiol. Plant* **2011**, *33*, 1451–1459. [[CrossRef](#)]
12. Ehsanpour, A.A.; Amini, F. Effect of salt and drought stress on acid phosphatase activities in alfalfa (*Medicago sativa* L.) explants under in vitro culture. *Afr. J. Biotechnol.* **2003**, *2*, 133–135. [[CrossRef](#)]

13. Cunhua, S.; Wei, D.; Xiangling, C.; Xinna, X.; Yahong, Z.; Dong, S.; Jianjie, S. The effects of drought stress on the activity of acid phosphatase and its protective enzymes in pigweed leaves. *Afr. J. Biotechnol.* **2010**, *9*, 825–833. [\[CrossRef\]](#)
14. Sharma, A.D.; Kaur, P. Drought-stress induced changes in the expression of acid phosphatases in drought tolerant and susceptible cultivars of wheat. *World J. Agric. Sci.* **2008**, *4*, 471–475.
15. Pandhair, V.; Sekhon, B.S. Reactive Oxygen Species and Antioxidants in Plants: An Overview. *J. Plant Biochem. Biotechnol.* **2006**, *15*, 71–78. [\[CrossRef\]](#)
16. Sen, A.; Alikamanoglu, S. Antioxidant enzyme activities, malondialdehyde, and total phenolic content of PEG-induced hyperhydric leaves in sugar beet tissue culture. *In Vitro Cell. Dev. Biol. Plant* **2013**, *49*, 396–404. [\[CrossRef\]](#)
17. Aremu, A.O.; Moyo, M.; Amoo, S.O.; Gruz, J.; Šubrťová, M.; Plíhalová, L.; Doležal, K.; Van Staden, J. Effect of a novel aromatic cytokinin derivative on phytochemical levels and antioxidant potential in greenhouse grown *Merwillia plumbea*. *Plant Cell Tissue Organ Cult. (PCTOC)* **2014**, *119*, 501–509. [\[CrossRef\]](#)
18. Grzegorzczak-Karolak, I.; Kuźma, Ł.; Wysokińska, H. The influence of cytokinins on proliferation and polyphenol accumulation in shoot cultures of *Scutellaria altissima* L. *Phytochem. Lett.* **2017**, *20*, 449–455. [\[CrossRef\]](#)
19. Dobránszki, J.; Teixeira da Silva, J.A. Micropopagation of apple—A review. *Biotechnol. Adv.* **2010**, *28*, 462–488. [\[CrossRef\]](#) [\[PubMed\]](#)
20. Abdalla, N.; Dobránszki, J. Meta-Topolin as an Effective Benzyladenine Derivative to Improve the Multiplication Rate and Quality of In Vitro Axillary Shoots of Húsvéti Rozmaring Apple Scion. *Plants* **2024**, *13*, 1568. [\[CrossRef\]](#) [\[PubMed\]](#)
21. Murashige, T.; Skoog, F. A revised medium for rapid growth and bioassays with tobacco tissue culture. *Physiol. Plant* **1962**, *15*, 473–497. [\[CrossRef\]](#)
22. McLachlan, K.D. Acid phosphatase activity of intact roots and phosphorus nutrition in plants. I Assay conditions and phosphatase activity. *Aust. J. Agric. Res.* **1980**, *31*, 429–440. [\[CrossRef\]](#)
23. Bradford, M.M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **1976**, *72*, 248–254. [\[CrossRef\]](#) [\[PubMed\]](#)
24. Rao, N.A.; Cama, H.R.; Kumar, S.A.; Vaidyanathan, C.S. Alkaline β -glycerophosphatase of green gram (*Phaseolus radiatus*). *J. Biol. Chem.* **1960**, *235*, 3353–3356. [\[CrossRef\]](#)
25. Yamaya, T.; Matsumoto, H. Properties of alkaline phosphatase in cucumber roots induced by calcium starvation. *Plant Cell Physiol.* **1981**, *22*, 1355–1365. [\[CrossRef\]](#)
26. Shannon, L.; Kay, E.; Lew, J. Peroxidase isozymes from horseradish roots. I. Isolation and physical properties. *J. Biol. Chem.* **1966**, *241*, 2166–2172. [\[CrossRef\]](#)
27. Kar, M.; Mishra, D. Catalase, peroxidase, and polyphenoloxidase activities during rice leaf senescence. *Plant Physiol.* **1976**, *57*, 315–319. [\[CrossRef\]](#) [\[PubMed\]](#)
28. Benzie, I.F.F.; Strain, J.J. The ferric reducing ability of plasma (FRAP) as a measure of “Antioxidant power”: The FRAP assay. *Anal. Biochem.* **1996**, *239*, 70–76. [\[CrossRef\]](#) [\[PubMed\]](#)
29. Naji, K.M.; Thamer, F.H.; Numan, A.A.; Dauqan, E.M.; Alshaibi, Y.M.; D’souza, M.R. Ferric-bipyridine assay: A novel spectrophotometric method for measurement of antioxidant capacity. *Heliyon* **2020**, *6*, e03162. [\[CrossRef\]](#) [\[PubMed\]](#)
30. Welcher, F.J. *Organic Analytical Reagents*, 4th ed.; D. Van Nostrand Co.: New York, NY, USA, 1948; pp. 396–402.
31. Singleton, V.L.; Orthofer, R.; Lamuela-Raventós, R.M. Analysis of total phenols and other oxidation substrates and antioxidants by means of Folin-Ciocalteu reagent. *Methods Enzymol.* **1999**, *299*, 152–178. [\[CrossRef\]](#)
32. Friedman, M.; Jürgens, H.S. Effect of pH on the Stability of Plant Phenolic Compounds. *J. Agric. Food Chem.* **2000**, *48*, 2101–2110. [\[CrossRef\]](#) [\[PubMed\]](#)
33. Agarwalac, S.C.; Sharmas, C.P.; Farooqa, S.; Chatterje, C. Effect of molybdenum deficiency on the growth and metabolism of corn plants raised in sand culture. *Can. J. Bot.* **1977**, *56*, 1905–1908. [\[CrossRef\]](#)
34. Shah, A.N.; Tanveer, M.; Hussain, S.; Yang, G. Beryllium in the environment: Whether fatal for plant growth? *Rev. Environ. Sci. Biotechnol.* **2016**, *15*, 549–561. [\[CrossRef\]](#)
35. Jones, M.; Yi, Z.; Murch, S.J.; Saxena, P.K. Thidiazuron-induced regeneration of *Echinacea purpurea* L.: Micropropagation in solid and liquid culture systems. *Plant Cell Rep.* **2007**, *26*, 13–19. [\[CrossRef\]](#) [\[PubMed\]](#)
36. Murch, S.J.; KrishnaRaj, S.; Saxena, P.K. Thidiazuron-induced morphogenesis of Regal geranium (*Pelargonium domesticum*): A potential stress response. *Physiol. Plant* **1997**, *101*, 183–191. [\[CrossRef\]](#)
37. Mahalingam, R.; Jambunathan, N.; Penaganti, A. Pyridine nucleotide homeostasis in plant development and stress. *Int. J. Plant Dev. Biol.* **2007**, *1*, 194–201.
38. Agava, T.; Ishikawa, K.; Haruda, K.; Fukusaki, E.; Yoshimura, K.; Shigeoka, S. Overexpression of an ADP-ribose pyrophosphatase, AtNUDX2, confers enhanced tolerance to oxidative stress in *Arabidopsis* plants. *Plant J.* **2009**, *57*, 289–301. [\[CrossRef\]](#) [\[PubMed\]](#)
39. Petrović, M.; Krstić-Milošević, D.; Stanišić, M.; Trajković, M.; Banjac, N. The effect of different cytokinins on in vitro shoot culture of an endemic and endangered *Gentianella lutescens* subsp. *carpatica* (Beck) Holub. *Plant Cell Tissue Organ Cult. (PCTOC)* **2025**, *160*, 72. [\[CrossRef\]](#)

40. Wojatnia, A.; Skrzypek, E. Effects of cytokinins on antioxidant enzymes in in vitro grown shoots of *Pelargonium hortorum* L. H. Bayley. *Acta Agrobot.* **2014**, *67*, 33–42. [\[CrossRef\]](#)
41. Abdelaziz, S.A.; Abdel-Rahim, E.A.; Rashed, M.M.; Mahmoud, R.A. In vitro assessment of salt tolerance in ‘Balady’ apple (*Malus domestica*) seedling clones. *Egypt. J. Bot.* **2025**, *65*, 341–351. [\[CrossRef\]](#)
42. Bai, T.; Li, C.; Ma, F.; Feng, F.; Shu, H. Responses of growth and antioxidant system to root-zone hypoxia stress in two *Malus* species. *Plant Soil* **2010**, *327*, 95–105. [\[CrossRef\]](#)
43. Skłodowska, M.; Gajewska, E.; Kuźniak, E.; Wielanek, M.; Mikiciński, A.; Sobiczewski, P. Antioxidant profile and polyphenol oxidase activities in apple leaves after *Erwinia amylovora* infection and pretreatment with a benzothiadiazole-type resistance inducer (BTH). *J. Phytopathol.* **2011**, *159*, 495–504. [\[CrossRef\]](#)
44. Demir, D.; Aksu, L.; Yilmaz, C.; Eken, C. Polyphenol oxidase activity in healthy and apple scar skin viroid infected apple (*Malus × domestica*) fruit. *Biol. Bull.* **2024**, *51*, 57–65. [\[CrossRef\]](#)
45. Zou, H.; Xiao, Q.; Li, G.; Wi, X.; Tian, X.; Zhu, L.; Ma, F.; Li, M. Revisiting the advancements in plant polyphenol oxidases research. *Sci. Hortic.* **2025**, *341*, 113960. [\[CrossRef\]](#)
46. Molassiotis, A.N.; Sotiropoulos, T.; Tanou, G.; Kofidis, G.; Diamantidis, G.; Therios, G. Antioxidant and anatomical responses in shoot culture of the apple rootstock MM 106 treated with NaCl, KCl, mannitol or sorbitol. *Biol. Plant.* **2006**, *50*, 331–338. [\[CrossRef\]](#)
47. Kirova, E.; Moskova, I.; Manova, V.; Koycheva, Y.; Tsekova, Z.; Borisova, D.; Nikolov, H.; Dimitrov, V.; Sergiev, I.; Kocheva, K. Exogenous cytokinin 4PU-30 modulates the response of wheat and einkorn seedlings to ultraviolet B radiation. *Plants* **2024**, *13*, 1401. [\[CrossRef\]](#) [\[PubMed\]](#)
48. Ijod, G.; Nawawi, N.I.M.; Sulaiman, R.; Khalid, N.I.; Anwar, F.; Adzahan, N.M.; Azman, E.M. Inactivation of polyphenol oxidase and peroxidase activity in mangosteen pericarp via blanching: Correlation between anthocyanins and enzyme activities. *Int. J. Food Sci. Technol.* **2025**, *60*, vvae010. [\[CrossRef\]](#)
49. Mihaljević, I.; Vuletić, M.V.; Šimić, D.; Tomaš, V.; Horvat, D.; Josipović, M.; Zdunić, Z.; Dugalić, K.; Vuković, D. Comparative study of drought stress effects on traditional and modern apple cultivars. *Plants* **2021**, *10*, 561. [\[CrossRef\]](#) [\[PubMed\]](#)
50. Karalija, E.; Zeljković, S.Č.; Tarkowski, P.; Muratović, E.; Parić, A. The effect of cytokinins on growth, phenolics, antioxidant and antimicrobial potential in liquid agitated shoot cultures of *Knaulia sarajevensis*. *Plant Cell Tissue Organ Cult. (PCTOC)* **2017**, *131*, 347–357. [\[CrossRef\]](#)
51. Hlophe, N.P.; Aremu, A.O.; Gruz, J.; Van Staden, J.; Finnie, J.F. Influence of different cytokinins on the phenolic acids and antioxidant activity of two *Brachystelma* species. *Plant Cell Tissue Organ Cult. (PCTOC)* **2021**, *145*, 689–699. [\[CrossRef\]](#)
52. Jiang, S.; Zhang, S.; Li, P.; Xue, N.; Chen, K.; Ji, X.; Ji, W. Response of dauciform roots to low phosphorus stress: Insights based on ecophysiology, transcriptomics, and metabolomics analysis. *Ind. Crops Prod.* **2025**, *234*, 121563. [\[CrossRef\]](#)
53. Araj, S.; Grammer, T.A.; Gertzen, R.; Anderson, S.D.; Mikulic-Petkovsek, M.; Veberic, R.; Phu, M.L.; Solar, A.; Leslie, C.A.; Dandekar, A.M.; et al. Novel roles for the polyphenol oxidase enzyme in secondary metabolism and the regulation of cell death in walnut. *Plant Physiol.* **2014**, *164*, 1191–1203. [\[CrossRef\]](#) [\[PubMed\]](#)
54. Sharma, A.; Shahzad, B.; Rehman, A.; Bhardwaj, R.; Landi, M.; Zheng, B. Response of phenylpropanoid pathway and the role of polyphenols in plants under abiotic stress. *Molecules* **2019**, *24*, 2452. [\[CrossRef\]](#) [\[PubMed\]](#)
55. Sakakibara, H. Cytokinins: Activity, Biosynthesis, and Translocation. *Annu. Rev. Plant Biol.* **2006**, *57*, 431–449. [\[CrossRef\]](#) [\[PubMed\]](#)
56. Aremu, A.O.; Bairu, M.W.; Doležal, K.; Finnie, J.F.; Van Staden, J. Topolins: A panacea to plant tissue culture challenges? *Plant Cell Tissue Organ Cult. (PCTOC)* **2012**, *108*, 1–16. [\[CrossRef\]](#)
57. Dobránszki, J.; Jambor-Benczúr, E.; Remenyi, M.L.; Magyar-Tábori, K.; Hudák, I.; Kiss, E.; Galli, Z. Effects of aromatic cytokinins on structural characteristics of leaves and their post-effects on subsequent shoot regeneration from in vitro apple leaves of ‘Royal Gala’. *Int. J. Hortic. Sci.* **2005**, *11*, 41–46. [\[CrossRef\]](#)
58. Chakrabarty, D.; Park, S.P.; Ali, M.B.; Shin, K.S.; Paek, K.Y. Hyperhydricity in apple: Ultrastructural and physiological aspects. *Tree Physiol.* **2005**, *26*, 377–388. [\[CrossRef\]](#) [\[PubMed\]](#)
59. Muniz de Oliveira, L.; Paiva, R.; Ferreira de Santana, J.R.; Alves, E.; Nogueira, R.C.; Pereira, F.D. Effect of cytokinins on in vitro development of autotrophism and acclimatization of *Annona glabra* L. *In Vitro Cell. Dev. Biol. Plant* **2008**, *44*, 128–135. [\[CrossRef\]](#)

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