

Article

Apple Autotetraploids—Phenotypic Characterisation and Response to Drought Stress

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Abstract: Polyploidization is an important source of variability for plant breeding. Polyploids are often characterised by increased resistance to biotic and abiotic stresses. Since drought and pathogen attack are the main threats to apple cultivation, obtaining new sources of resistance is an important issue for apple breeding. The newly obtained autotetraploid clones of apple cv. ‘Redchief’ showed superior resistance to fire blight. The aim of the presented research was the in-depth phenotypic characterisation of ‘Redchief’ tetraploids and assessment of their response to drought at the physiological and genetic level. The growth of own-rooted five-year-old trees of ‘Redchief’ tetraploids was poor compared with diploids; all growth parameters—the number and length of current season shoots, the total length of current season shoots per tree and the cross-section area of the trunk—were reduced in tetraploid clones. Grafting on M9 rootstock improved the growth characteristics of ‘Redchief’ tetraploids. Compared with diploid plants, the leaves of tetraploids were thicker, with altered shape, higher chlorophyll content, and larger stomata, but the stomatal density decreased. The leaf anatomical structure of tetraploids was changed, the adaxial and abaxial epidermis and both types of mesophyll were significantly thicker than in diploids. Moreover, the pollen grains of tetraploids were larger, but their viability and germination were reduced. Under conditions of limited water supply, the reduction in growth parameters was smaller and the physiological parameters were higher in the ‘Redchief’ tetraploid clone 4x-25 than in diploid plants. The expression of *APX* gene was higher in tetraploids than in diploids 15 days after drought stress induction. The results suggest the enhanced drought tolerance of the studied ‘Redchief’ autotetraploid clone compared with its diploid counterpart.

Keywords: apple; autotetraploid; phenotypic characteristics; drought tolerance



Citation: Wójcik, D.; Marat, M.; Marasek-Ciołakowska, A.; Klamkowski, K.; Buler, Z.; Podwyszyńska, M.; Tomczyk, P.P.; Wójcik, K.; Treder, W.; Filipczak, J. Apple Autotetraploids—Phenotypic Characterisation and Response to Drought Stress. *Agronomy* **2022**, *12*, 161. <https://doi.org/10.3390/agronomy12010161>

Academic Editor:
Hirokazu Takahashi

Received: 22 November 2021

Accepted: 8 January 2022

Published: 10 January 2022

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1. Introduction

The apple tree (*Malus × domestica* Borkh.) is one of the most important fruit crop plants, widely cultivated in the temperate climate zones of North America, Europe, and Asia. The global production of apples in 2019 reached over 87 million tonnes, while Poland, with a production exceeding 3 million tonnes, is the fourth producer of this fruit in the world (United Nations, Food and Agriculture Organization, FAOSTAT database, <https://www.fao.org/faostat/>, accessed on 27 December 2021). The main factors that threaten apple cultivation and cause losses in plant productivity and fruit quality are unfavourable environmental conditions (including limited water resources) and the attack of pathogens; therefore, one of the main goals of apple breeding programs is to obtain cultivars with increased tolerance/resistance to biotic and abiotic stresses [1–3]. The most

devastating bacterial pathogen of apple trees is *Erwinia amylovora*, the causative agent of fire blight disease. Due to its high harmfulness and difficulty in control of this pathogen, *E. amylovora* is considered a quarantine organism in all European Union countries [4,5]. Among the fungal diseases, the most destructive is apple scab caused by the pathogen *Venturia inaequalis* (Cooke) Wint.; yield losses caused by this disease reach up to several dozen percent [6].

Drought affects many physiological and biochemical processes in plants, adversely reducing the growth and production of many crops, including apple, which is considered a drought-stress-sensitive plant [7–11]. Water stress causes stomatal closure, decline in tissue water potential, reduction of transpiration and photosynthesis, and alterations in assimilate partitioning [9–13]. Moreover, under drought, the hormonal balance of the plant is disturbed; increased production of abscisic acid (ABA) is observed; and decreased levels of auxins, gibberellins, and cytokinins are observed [10–12,14]. The effect of these changes is stunted growth manifested by a reduction of shoot elongation, leaf size, stem extension, and root proliferation [10,12,14]. Changes in leaf anatomy, stomatal size, and frequency and retard in the development of plant reproductive organs have been also reported [12,15,16].

Drought, similar to other abiotic and biotic stress factors, triggers oxidative stress, in which an increased production of reactive oxygen species (ROS) is observed. Elevated level of ROS in plant tissues leads to peroxidation of lipids, proteins, and nucleic acids, and consequently, to structural damage of cell membranes [7,17]. Plant response to oxidative stress includes, among others, an increase in the activity of the major antioxidant enzymes acting as ROS scavengers: superoxide dismutase (SOD), catalase (CAT) and peroxidase (POX), and enzymes from the ascorbate–glutathione pathway (ascorbate peroxidase APX, glutathione peroxidase GPX) [10,18–20]. The expression level and activity of these enzymes are commonly studied parameters in the analysis of plant responses to biotic and abiotic stress factors, including water deficit [14,20–22]. The proteins that play a key role in water balance and water use efficiency are aquaporins (AQPs)—the large superfamily of integral membrane proteins involved in the transcellular transport of water [10,23]. There are five subfamilies of AQPs, the most abundant are the plasma membrane intrinsic proteins (PIPs) and the tonoplast membrane intrinsic proteins (TIPs). In apple genome, 42 genes encoding putative AQPs, belonging to all five subfamilies, were identified and characterised [23]. Overexpression of aquaporin genes in transgenic plants enhanced tolerance to drought and salinity in banana, tomato, and *Arabidopsis thaliana* [23–25].

Polyploidization is an important source of variability for plant breeding. Both allopolyploidy and autopolyploidy significantly alter the genotype and phenotype of the plant [26–32]. Genome duplication results in a change in the structure of DNA, not only does it cause doubling of the number of the same genes, but it is also the source of other types of mutations—chromosomal such as deletions, translocations, and inversions or point mutations, it also causes changes in the DNA methylation pattern [33,34]. Polyploids are widely used in breeding programs of many crop plant species due to their better phenotypic features, such as, for example, vigorous growth, larger flowers and fruits, compact habit, and often increased adaptability to biotic (pathogens and pests) and abiotic stress factors [35].

Recently, research on the generation and characterisation of apple tetraploids has been carried out in several research centres [36–41]. The obtained tetraploids have been shown to possess many favourable phenotypic traits. Tetraploids of ‘Hanfu’ and ‘Gala’ showed much lower susceptibility to leaf blight caused by *Alternaria alternata* and anthracnose caused by *Colletotrichum gloeosporioides* than their diploid counterparts [39]. It was also found that the autotetraploids of these two apple cultivars possess better drought and salt tolerance than diploids; under stress, they had higher relative water content (RWC) and higher chlorophyll fluorescence, but lower levels of malondialdehyde (MDA) and proline compared with diploids [37,38]. Differences in the expression pattern of key aquaporin genes, *MdPIP1;1* and *MdTIP1;1*, between the tetraploid and diploid ‘Hanfu’ and ‘Gala’ were also observed when the plants were exposed to salt and drought stress [37,38]. Moreover,

tetraploids of ‘Hanfu’ were characterised by increased volume and size of the flowers, fruits and seeds, and soluble solids content of the fruits compared with their diploid counterpart [40]. Tetraploid apples are maintained in genetic resources mainly for crossing with diploids to obtain triploid genotypes, considered to be optimal ploidy for commercial apple cultivars [42,43]. Apple triploids account for about 10% of all currently grown apple cultivars; they are characterised by many valuable traits such as large, attractive fruits; high level of productivity; and more regular fruit-bearing [42,43].

In recent years, an effective method of in vitro apple polyploidization was developed at the Department of Applied Biology of the National Institute of Horticultural Research (NIHR), Skierniewice, Poland. As a result, numerous autotetraploid clones of six apple cultivars were obtained [44,45]. Advanced research is currently underway to characterize these clones at the phenotypic and genetic levels. Some tetraploid clones of cultivar ‘Free Redstar’ showed enhanced resistance to apple scab caused by *Venturia inaequalis* [46]. In response to pathogen inoculation, the expression of several disease-resistance-related genes (*PR1*, *WRKY29*, *CDPK*, *MPK4*) was significantly enhanced in ‘Free Redstar’ tetraploids compared with diploids [46]. Among the generated apple tetraploids, promising results indicating the superior resistance to fire blight were obtained for tetraploid clones of the cv. ‘Redchief’ during multiple in vitro assays [47]. The cultivar ‘Redchief’ is considered to be highly/moderately resistant to *E. amylovora* infection [47,48], it can be assumed that genome duplication enhanced this trait in tetraploids. The tetraploids of ‘Redchief’ were selected for further evaluation as promising genotypes that could be used for breeding.

The tetraploid ‘Redchief’ clones differed in the level of resistance to *E. amylovora* infection [47], it can be assumed that the reasons for these differences were genetic changes occurring during individual polyploidization events. The aim of the presented research was the in-depth phenotypic characterisation of several tetraploid clones of apple cv. ‘Redchief’, including comparison of the growth characteristics of the own-rooted and M9-grafted plants growing in the orchard. For one of the clones, 4x-25, showing a high level of resistance to fire blight, an assessment of the drought response was also performed at the physiological and genetic levels. Since the possibility of crossing the obtained tetraploids with diploid apple genotypes is an important issue for breeding reasons, pollen characterisation was also performed.

2. Materials and Methods

2.1. Phenotypic Observations and Measurements

2.1.1. Plant Material

The plant material for the presented study was the diploid apple cultivar ‘Redchief’ and several clones of its synthetic autotetraploids, obtained using in vitro technique at NIHR [44]. The tetraploidy of the obtained clones was confirmed using flow cytometry several times during in vitro shoot propagation, after acclimatisation to ex vitro conditions and during further plant growth [44]. AFLP analysis showed genetic variability between ‘Redchief’ tetraploids and its diploid counterpart [49]. The phenotypic measurements and observations were carried out on 5-year-old own-rooted and M9-grafted trees growing in an experimental orchard of NIHR in Dąbrowice. Each genotype was represented by 3–5 plants, both own-rooted and grafted. Standard agrotechnical measures for the species were used to maintain the experimental plots.

2.1.2. Growth Parameters

Growth parameters of own-rooted and M9-grafted trees of ‘Redchief’ and its four tetraploid clones—4x-11, 4x-23, 4x-24 and 4x-26—were compared. Measurements included the number and length of current season shoots and the diameter of the trunk at a height of 30 cm above the ground. Based on the results, the total length of current season shoots per tree and the cross-section area of the trunk were calculated. Measurements were made at the end of the growing season.

2.1.3. Leaf Characteristics

Leaf Morphological Traits

For morphological characteristics, the leaves from M9-grafted trees of ‘Redchief’, diploid, and its three tetraploid clones—4x-11, 4x-25, and 4x-26—were taken. From each genotype, 15 fully grown leaves were collected from the middle part of the shoots. The leaf area, length, and width were measured with a planimeter (Area Meter AM350, ADC BioScientific Ltd., Hoddesdon, UK). Based on the measurements, the ratio of the length to the width of the leaves was calculated. The relative chlorophyll content (CCI) of the leaves was measured with the CCM-200 Chlorophyll content meter (Opti-Sciences Int., Hudson, NH, USA) on 20 leaves from each genotype.

For the evaluation of stomata, abaxial epidermis was isolated from the middle part of the leaves with a transparent Scotch-type adhesive tape and dyed with 2% toluidine blue according to the procedure of Dyki and Habdas [50]. For each genotype, the length of the stomata ($n = 3$ replicates \times 100 stomata per genotype) and their density per 1 mm² ($n = 3$ replicates per genotype) were determined. Microscopic observations and measurements were carried out under an Eclipse 80i light microscope (Nikon, Tokyo, Japan) using an image analysis system (NIS-Elements Basic Research; Nikon Instruments Inc., Tokyo, Japan).

Anatomical Structure of the Leaves

Analysis of leaf anatomical structure was made for the diploid apple cv. ‘Redchief’ and its tetraploid clone 4x-25. From each genotype, 5 leaves (eighth leaf from the tip of the shoot) of M9-grafted trees were collected. For anatomical observations, fragments of 10 \times 15 mm were cut out of the leaves and fixed in chromoacetoformalin (CrAF) for 48 h at room temperature, dehydrated in graded series of ethanol (70, 80, 90, and 100%), embedded in paraffin according to the method reported previously by Marasek-Ciolakowska [51], and then cut into 10- μ m sections on a rotary microtome (Leica, Wetzlar, Germany). Slides were stained in 1% aqueous Safranin solution and 1% fast green (prepared in 95% ethanol). The observations and measurements were carried out under an Eclipse 80i light microscope (Nikon, Tokyo, Japan) using an image analysis system (NIS-Elements Basic Research). For each genotype, the thickness of the leaves, abaxial and adaxial epidermal layer, and palisade and spongy mesophyll were determined. Three replicates of 20 measurements were performed for each genotype.

2.1.4. Pollen Characteristics

In 2021, the first M9-grafted trees of two tetraploid clones, 4x-14 and 4x-24, began flowering. Pollen for the study was collected from flowers of diploid ‘Redchief’ and its tetraploid clone 4x-14. A mixed sample of pollen collected from anthers taken from 5 flowers of each genotype was stained according to Alexander [52]. Pollen viability was assessed on the basis of staining level—red-coloured pollen was considered viable and colourless as nonviable. Pollen germination was tested on microscope slides on media with 15% solution of sucrose after 24 h of incubation at room temperature [53]. The measurements of pollen length and observations of pollen germination and viability were performed using a microscope as described for stomata measurements (see above) at 400 \times magnification.

2.2. Testing for Drought Tolerance

2.2.1. Plant Material

For drought tolerance, M9-grafted, 4-month-old plants of diploid ‘Redchief’ and its tetraploid clone 4x-25 were tested. Plants were grafted in the early spring. The grafts were stored for one month in a cold chamber (4 °C) to improve callus formation; then, the grafts were planted in 2 L pots filled with a mixture of soil substrate and sand at a ratio of 4:1; at the same time, 2 g of the slow-release fertilizer Osmocote Exact 5–6 M Standard (ICL Specialty Fertilizers, ICL Group, Tel Aviv, Israel) was added to each pot. The plants were placed in a greenhouse, and then, when the shoots and leaves had developed, they were transferred outside under a transparent cover to protect them from rain. The plants were

divided into 2 groups, control and drought-stressed, each experimental combination was represented by 9 plants from each genotype.

The plants were watered with a drip irrigation system. Control plants were irrigated to maintain water potential of the growing medium at the level of approx. (-)10 kPa (optimal irrigation). Water stress was imposed by limiting irrigation (water potential was kept below (-)30 kPa. The moisture and water potential of the growing medium was monitored with dielectric probes (Teros 12 and MPS-6, METER, Pullman, WA, USA). The stress intensity was additionally monitored by measurements of midday leaf water potential. Measurements were made on 5 leaves from each combination by a means of psychrometric method (C-52 sample chambers and PSYPRO datalogger, Wescor, Logan, UT, USA).

2.2.2. Physiological Parameters

Measurements of physiological parameters were performed twice during the experimental period, 15 and 29 days after drought induction. The gas exchange rate (net photosynthesis and transpiration rate) was measured on 20 young, fully expanded leaves from each combination using an LCpro+ portable photosynthesis system (ADC BioScientific Ltd., Hoddesdon, UK). Temperature, CO₂ concentration, and irradiance in the leaf cuvette during analysis were set to approximate ambient conditions.

2.2.3. Growth Parameters

The growth parameters of diploid and tetraploid plants, optimally irrigated and subjected to water stress, were compared. Height of main shoot and main shoot diameter above the grafting site were measured before applying stress and at the end of plant growth. The increase in the height of the main shoot and in the diameter of the shoot were calculated. Leaf area was measured with a planimeter (Area Meter AM350, ADC BioScientific Ltd., Hoddesdon, UK) at the end of plant growth, the area of 10 leaves of each experimental combination was measured.

2.2.4. Gene Expression Analysis

Gene expression analyses were performed using the quantitative real-time PCR technique (qRT-PCR). Expression of 7 apple genes was analysed, encoding aquaporins (PIP1;1, PIP2;1, PIP2;3, and TIP1;1) and enzymes related to oxidative stress response: ascorbate peroxidase (APX), superoxide dismutase (SOD), and catalase (CAT). As reference, genes encoding actin (AC11) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were used [54,55]. Primers for qRT-PCR were taken from the literature or were designed basing on cDNA sequences collected in GeneBank (NCBI, National Center for Biotechnology Information; <http://www.ncbi.nlm.nih.gov>, accessed on 20 November 2021) using Primer3 program, online version 0.4.0 (<https://bioinfo.ut.ee/primer3-0.4.0/>, accessed on 20 November 2021). Primers' sequences and accession number of APX cDNA sequence or references are presented in Table 1.

For gene expression analysis, leaf samples were taken from diploid and tetraploid 'Redchief' plants subjected to drought at four time points, before drought and 7, 15, and 29 days after stress induction. Plant material was harvested in triplicate from each experimental combination, 2–3 young leaves from 3 plants were collected for each sample. Leaves were collected directly into liquid nitrogen, ground in liquid nitrogen, and stored at -80 °C. Total RNA from leaf samples was extracted using Plant/Fungi Total RNA Purification Kit (Norgen Biotek Corp., Thorold, ON, Canada). DNA traces were removed from RNA samples by digestion with RQ RNase-Free DNase (Promega, Madison, WI, USA). Concentration and purity of obtained total RNA was examined spectrophotometrically (Epoch spectrophotometer, BioTek, Highlan Park, VT, USA). From each RNA sample, 1 µg was reverse transcribed using AffinityScript QPCR cDNA Synthesis Kit (Agilent Technologies, Santa Clara, CA, USA).

Table 1. Primer sequences used for qRT-PCR analysis.

Gene	Primer Sequence (5'-3')	GenBank Accession No/Reference
<i>CAT</i>	Forward: GACTTCTTCTCCCACCATCCAG Reverse: TTGGTAGCGTGGCTGTGATTAG	Li et al. [56]
<i>SOD</i>	Forward: GGGAGATGGCCCAACTACTG Reverse: TTGCCAAGGTCATCAGGGTC	Li et al. [56]
<i>APX</i>	Forward: GCTTTCTTTGCTGACTATGCTGAA Reverse: ATTGGCTTCATCCCTTGTAGTTCT	XM 008346624
<i>PIP1;1</i>	Forward: AGCTGACGTCATGGTCGTTCTACA Reverse: TGCACTTCGACTTGGACTTGACGA	Zhang et al. [38]
<i>PIP2;1</i>	Forward: CCTTCTACCACCAATACATTC Reverse: TGATTATCTACAATTCCATAGCC	Liu et al. [23]
<i>PIP2;3</i>	Forward: CAAGAGGAGTGCTAGAGAC Reverse: GCCAAGTGGACAATGAAC	Liu et al. [23]
<i>TIP1;1</i>	Forward: TCGACCGTCCAATCATCTCAACCA Reverse: ATCGAGATGAACTCGGCTAACGCA	Zhang et al. [38]
<i>AC11</i>	Forward: GCTGTTCTTTCCCTCTACGC Reverse: GCATGGGGAAGAGCATATCC	Perini et al. [34]
<i>GAPDH</i>	Forward: GCTGCCAAGGCTGTTGGAA Reverse: CAGTCAGGTCAACAACGGAAAC	Vergne et al. [55]

Real-time PCR was performed using KAPA™ SYBR® qPCR Kit (Kapa Biosystems, Amsterdam, The Netherlands). The reaction mixture of a total amount of 20 µL contained 10 µL of KAPA™ 2 × Master Mix, 300 nM of each gene-specific primer, and 2 µL of 10-fold diluted reverse transcription reaction. The reaction was carried out in Rotor-Gene 6000 (Corbett Research, Bath, UK). At the end of each PCR, the melting curve of amplification product was also analysed at 72–95 °C, with temperature raised by 1 °C/5 s. Four tenfold dilutions of cDNA were run together with analysed samples to calculate PCR efficiency and generate the standard curve (correlation coefficient > 0.99). A control reaction with no cDNA was performed for each analysis. All qRT-PCR reactions were performed in three technical replications. Amplification products were additionally analysed by electrophoresis in agarose gel and commercially sequenced to confirm their identity/homology with original sequences (Genomed, Warsaw, Poland).

For relative quantification, the standard curve method was applied [57]; the relative mRNA level of studied genes was normalised with respect to *AC11* and *GAPDH* reference genes. The data analysis was conducted using the Rotor-Gene 6000 Series Software 1.7 (Corbett Research, Bath, UK).

2.3. Statistical Analysis

All data were statistically elaborated by an analysis of variance followed by Duncan's multiple range test at $p = 0.05$ (Statistica 13.1, StatSoft Inc., Tulsa, OK, USA).

3. Results

3.1. Phenotypic Observations and Measurements

3.1.1. Growth Parameters

Observations of 5-year-old 'Redchief' trees growing in the orchard showed that diploids and tetraploids differed significantly in their growth parameters (Table 2). Moreover, significant differences were also observed between the own-rooted and M9-grafted plants. Diploid own-rooted trees were characterised by the highest values of all estimated parameters; in turn, the tetraploid own-rooted trees had the worst growth parameters (Table 2, Figures 1 and 2). Summing up the results for own-rooted trees, the total length of current season shoots per tree was 4.8 times greater in diploids, diploids had 3.5 times more current season shoots, their trunk cross-section area was 4.5 times greater, and their current season shoots were longer by approx. 40% compared with tetraploid plants (Table 2). Graft-

ing on the M9 rootstock caused a dwarfing effect in diploids, while improved the growth parameters of tetraploid plants (Table 2, Figure 1). Compared to own-rooted diploid trees, all growth parameters were significantly reduced in M9-grafted diploids (Table 2). In the case of tetraploids, M9-grafted plants had 80% higher total length of current season shoots, they had 60% more current season shoots, the current season shoots were longer by 14% and their trunk cross-section area was greater by 50% than in own-rooted tetraploid plants (Table 2). When the M9-grafted trees were compared, the differences between diploids and tetraploids were smaller than in own-rooted plants. M9-grafted diploid trees had significantly greater total length of current season shoots (by 60%) and had more current season shoots (by approx. 50%) than M9-grafted tetraploids, while the other two growth parameters were comparable for M9-grafted diploids and tetraploids (Table 2, Figure 1C,D). No significant differences in growth parameters between sibling tetraploid clones were recorded (Figure 1).

Table 2. Growth parameters of the own-rooted and M9-grafted five-year-old trees of diploid and tetraploid apple cv. ‘Redchief’ growing in an experimental orchard at the National Institute of Horticultural Research (Skierniewice, Poland).

Trait	Own-Rooted		M9-Grafted	
	Diploid	Tetraploid *	Diploid	Tetraploid *
Total length of current season shoots per tree (m)	14.1 ± 3.3 a **	2.9 ± 0.9 d	8.4 ± 0.7 b	5.2 ± 1.3 c
Number of current season shoots per tree	56.8 ± 12.9 a	16.3 ± 4.6 d	38.7 ± 5.5 b	26.0 ± 6.5 c
Mean length of one current season shoot (cm)	24.7 ± 0.7 a	17.6 ± 1.9 c	21.8 ± 1.6 b	20.0 ± 2.2 b
Trunk cross section area (cm ²)	14.3 ± 4.4 a	3.1 ± 1.0 b	5.0 ± 0.8 b	4.7 ± 1.0 b

* Average of four tetraploid clones: 4x-11, 4x-23, 4x-24 and 4x-26. ** Means for each trait marked with the same letter do not differ significantly at $p = 0.05$; Duncan’s test.

3.1.2. Leaf Characteristics

Leaf Morphological Traits and Chlorophyll Content

The leaves of the tetraploids and diploids differed from each other. Tetraploid leaves were shorter and wider than those of diploids, which made their shape more rounded, as evidenced by the length-to-width ratio, which was significantly lower in all tetraploid clones than in diploids (Table 3, Figure 3). The stomata of tetraploids were longer than those of diploids, while the frequency of the stomata were significantly higher in diploids (Table 3, Figure 4a,b). Leaves of tetraploids had higher chlorophyll content (CCI) compared with diploids (Table 3).

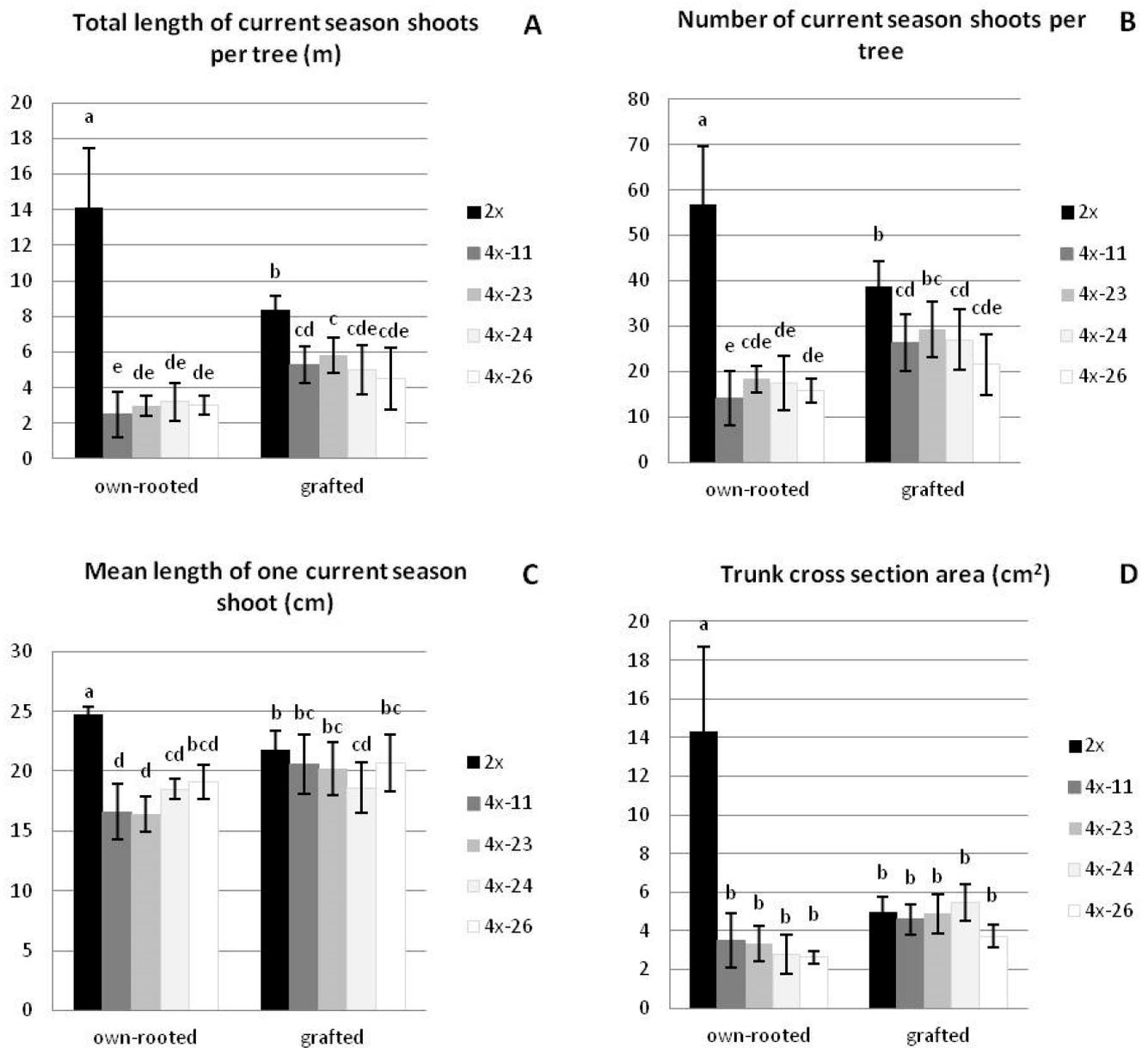


Figure 1. Growth parameters of the own-rooted and M9-grafted five-year-old trees of diploid (2x) and tetraploid clones (4x-11, 4x-23, 4x-24 and 4x-26) of the apple cv. ‘Redchief’ growing in an experimental orchard at the National Institute of Horticultural Research (Skierniewice, Poland): (A) total length of current season shoots, (B) number of current season shoots per tree, (C) mean length of one current season shoot, (D) trunk cross-section area. Bars marked with the same letter do not differ significantly at $p = 0.05$; Duncan’s test.



Figure 2. Own-rooted five-year-old diploid (2x) and tetraploid (4x) trees of the apple cv. ‘Redchief’ growing in an experimental orchard at the National Institute of Horticultural Research (Skierniewice, Poland).

Table 3. Differences in leaf morphological traits and chlorophyll content between M9-grafted five-year-old trees of diploid (2x) and tetraploid clones 4x-11, 4x-25, and 4x-26 of the apple cv. ‘Redchief’.

Trait	2x	4x-11	4x-25	4x-26
Leaf area (cm ²)	32.1 ± 6.0 a *	33.9 ± 5.8 a	31.0 ± 5.1 a	33.7 ± 4.5 a
Leaf length (cm)	9.3 ± 1.5 a	8.5 ± 1.1 ab	8.4 ± 0.8 b	9.0 ± 0.7 ab
Leaf width (cm)	4.3 ± 0.7 b	5.1 ± 0.8 a	5.0 ± 0.5 a	5.0 ± 0.6 a
Leaf length/width	2.18 ± 0.29 a	1.70 ± 0.18 b	1.71 ± 0.16 b	1.81 ± 0.13 b
Stomata length (µm)	26.2 ± 1.2 b	34.2 ± 1.6 a	33.8 ± 0.6 a	34.1 ± 0.8 a
Stomata frequency (no. mm ⁻²)	279.3 ± 10.8 a	186.7 ± 11.1 b	177.0 ± 5.0 b	152.7 ± 17.6 c
Chlorophyll content index (CCI)	21.2 ± 3.4 b	27.2 ± 6.4 a	24.4 ± 3.6 ab	25.1 ± 5.1 a

* Means for each trait marked with the same letter do not differ significantly at $p = 0.05$; Duncan’s test.

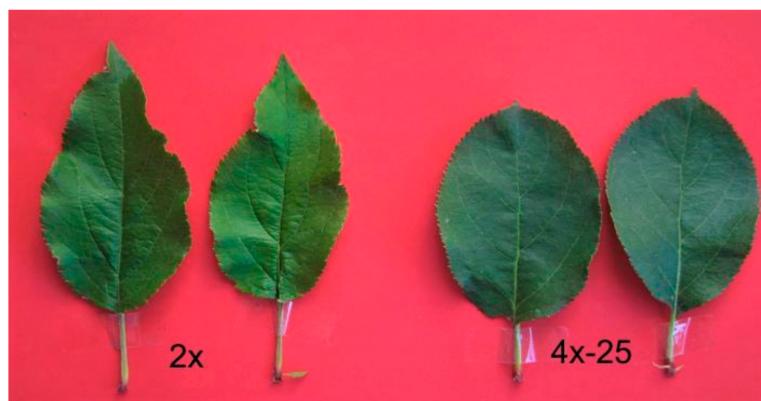


Figure 3. Leaves of diploid and tetraploid clone 4x-25 of the apple cv. ‘Redchief’.

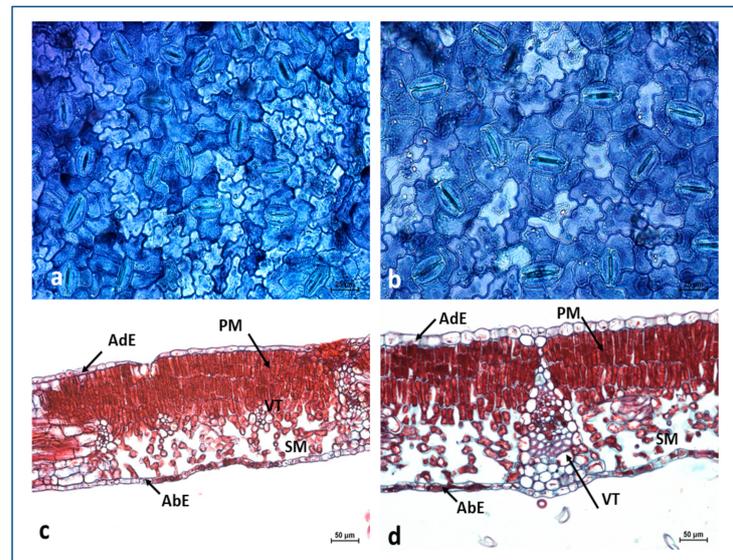


Figure 4. Stomata and leaf cross-section of diploid (a,c) and autotetraploid clone 4x-25 (b,d) of the apple cv. 'Redchief'; AdE—adaxial epidermis, AbE—abaxial epidermis, PM—palisade mesophyll, SM—spongy mesophyll, VT—vascular tissue.

Anatomical Structure of the Leaves

Microscopic analysis of the leaf cross-sections showed that both the leaves, the adaxial and abaxial epidermis, and both types of mesophyll were significantly thicker in the 4x-25 tetraploid clone compared with its diploid counterpart (Table 4, Figure 4).

Table 4. Anatomical structure of the leaves of diploid and tetraploid clone 4x-25 of the apple cv. 'Redchief'.

Thickness (μm)	Diploid	Tetraploid
leaf blade	204.1 \pm 10.3 b *	273.2 \pm 14.7 a
spongy mesophyll	78.0 \pm 7.2 b	116.5 \pm 11.0 a
palisade mesophyll	95.3 \pm 10.1 b	108.6 \pm 10.7 a
adaxial epidermis	10.1 \pm 1.9 b	17.0 \pm 3.1 a
abaxial epidermis	10.6 \pm 2.4 b	14.2 \pm 2.3 a

* Means for each trait marked with the same letter do not differ significantly at $p = 0.05$; Duncan's test.

3.1.3. Pollen Characteristics

The five-year-old own-rooted 'Redchief' tetraploids did not have flowers yet, while a few flowers appeared on two tetraploid clones, 4x-14 and 4x-24, grafted on M9 rootstock. Diploid 'Redchief' trees, both own-rooted and grafted, were flowering profusely. Microscopic observations showed that the pollen of the 4x-14 tetraploid clone was significantly larger than that of diploid plants (Table 5, Figure 5A). It was also observed that the viability and germination of pollen was reduced in the tetraploid compared with the diploid (Table 5, Figure 5B).

Table 5. Pollen grains characteristics of diploid and the tetraploid clone 4x-14 of the apple cv. 'Redchief'.

Trait	Diploid	Tetraploid
Pollen grain length (μm)	32.9 \pm 3.2 b *	41.2 \pm 3.6 a
Pollen viability (%)	91	48
Pollen germination (%)	77	48

* Means for each trait marked with the same letter do not differ significantly at $p = 0.05$; Duncan's test.

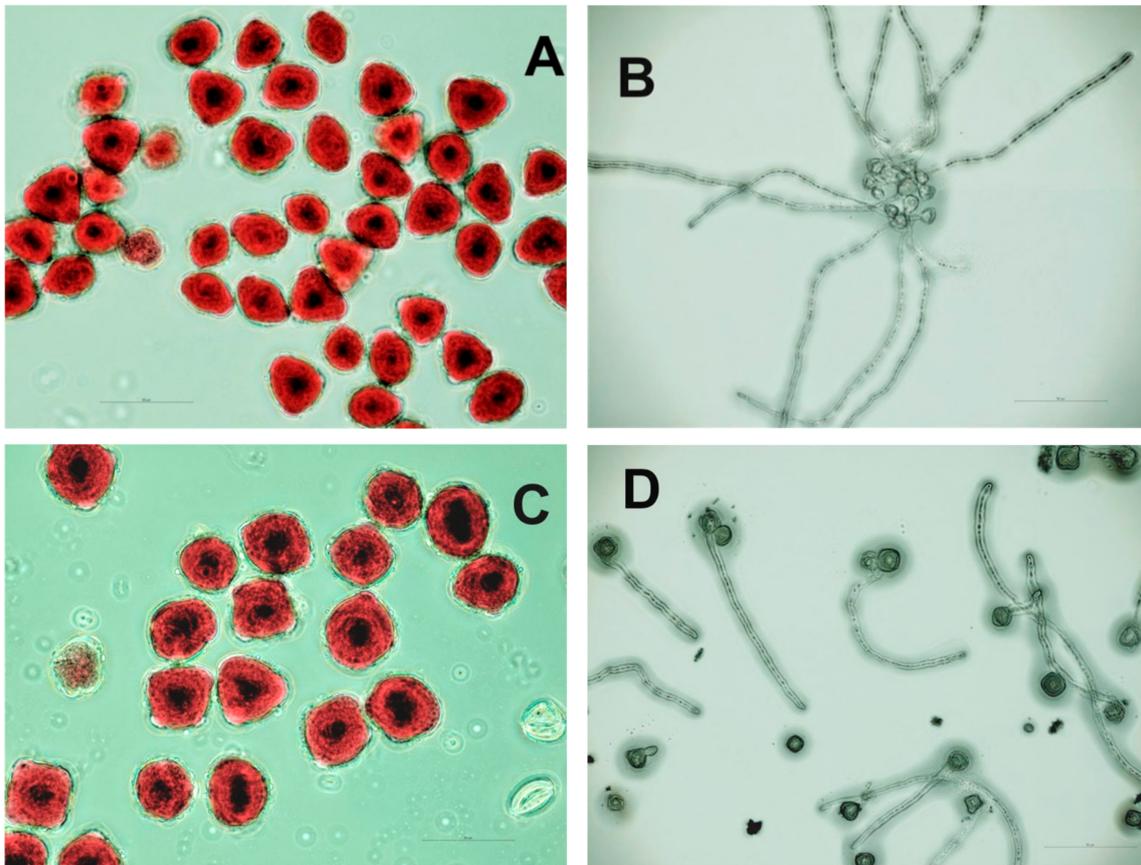


Figure 5. Differences between size and germination of pollen grains of diploid (A,B) and tetraploid (C,D) apple cv. ‘Redchief’; scale bars = 50 μ m.

3.2. Testing for Drought Tolerance

3.2.1. Physiological Parameters under Drought

Based on the measurements of the water potential in the leaves, it was found that the plants growing with limited water supply were under drought stress. Fifteen days after stress induction, the mean values of the water potential in control and stressed plants were higher in tetraploids than in diploids; for diploids, they were -0.71 MPa (control) and -2.19 MPa (drought), and for tetraploids, -0.43 MPa (control) and -1.12 MPa (drought). At the next time point, on the 29th day of the experiment, these values for diploids were -1.22 and -1.91 MPa (control vs. drought) and for tetraploids were -1.02 and -2.93 MPa (control vs. drought).

Physiological parameters (photosynthesis rate and transpiration rate) were higher in tetraploids growing under water stress (Figure 6). Under drought, a significant decrease in the values of both parameters was observed compared with the control, but for tetraploids, the differences were smaller (Figure 6). For example, on the 15th day of the experiment, the photosynthesis rate in diploids growing under drought was 3.5 times lower compared with the control plants; for tetraploids, the decrease was 2.5-fold. In the case of transpiration rate, the differences were even greater; on the 15th day, the decrease was 5.5-fold and 2.9-fold for diploids and tetraploids, respectively.

3.2.2. Growth Parameters under Drought

In diploids, drought caused a significant reduction in the growth of the main shoot. The increase in the height of the main shoot was approx. 60% lower in the stressed plants compared to the optimally irrigated plants (Figure 7A), while the increase of the main shoot diameter was reduced by 36% in stressed plants compared with control (Figure 7B). In tetraploids, the increase in the main shoot height was reduced by almost 20% in stressed

plants compared with control but the difference was not statistically significant (Figure 7A). No differences in the increase in main shoot diameter between the stressed and the control tetraploids were observed (Figure 7B). A significant reduction in the leaf area was observed in the diploids subjected to drought stress, while the leaf area of the tetraploids growing under drought was almost unchanged compared with the control plants (Figure 7C).

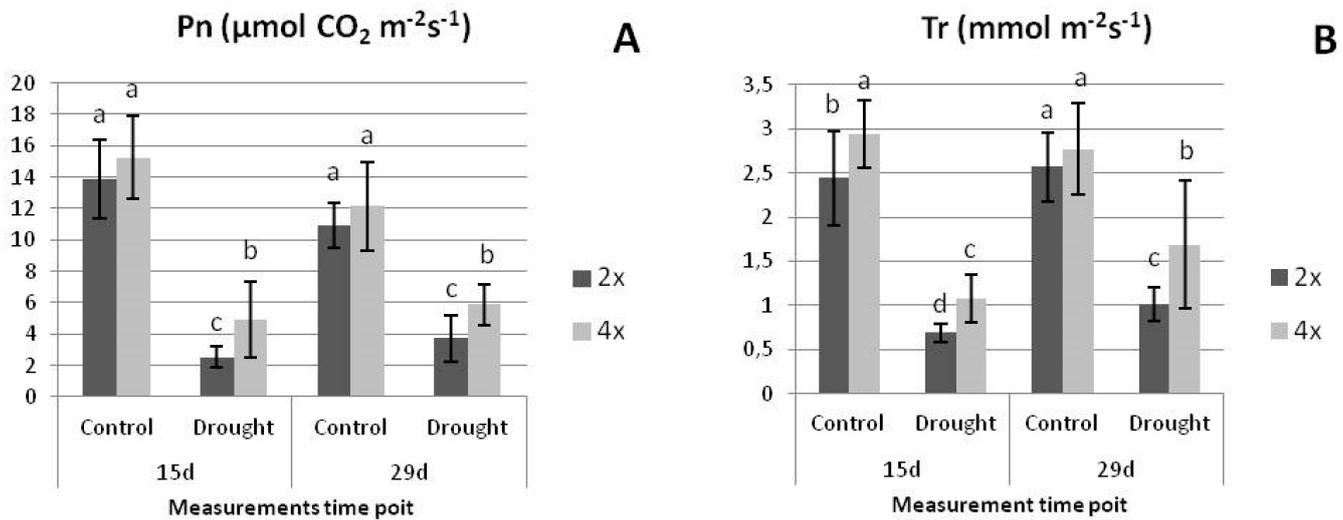


Figure 6. Physiological parameters of diploid (2x) and tetraploid clone 4x-25 of the apple cv. ‘Redchief’, optimally irrigated and subjected to drought stress, 15 and 29 days after drought induction: (A) net photosynthesis rate (Pn) and (B) transpiration rate (Tr). Bars marked with the same letter do not differ significantly at $p = 0.05$; Duncan’s test.

3.2.3. Gene Expression Analysis

Under limited water supply, the expression level of all studied genes increased, it reached the highest level on the 15th day of the experiment (Figure 8). However, only small differences in gene expression levels were observed between diploids and tetraploids. Seven days after drought was imposed, expression of *SOD* was significantly higher in tetraploids than in diploids, but at subsequent time points, there were no differences in the expression of this gene between diploids and tetraploids. Fifteen days after stress induction, it was observed that the expression of *APX* was over 2 times higher in tetraploids than in diploids (Figure 8C). In the case of three aquaporin genes—*PIP2;1*, *PIP2;3*, and *TIP1;1*—the expression level analysed 15 days after drought stress induction was lower in tetraploids than in diploids (Figure 8E–G).

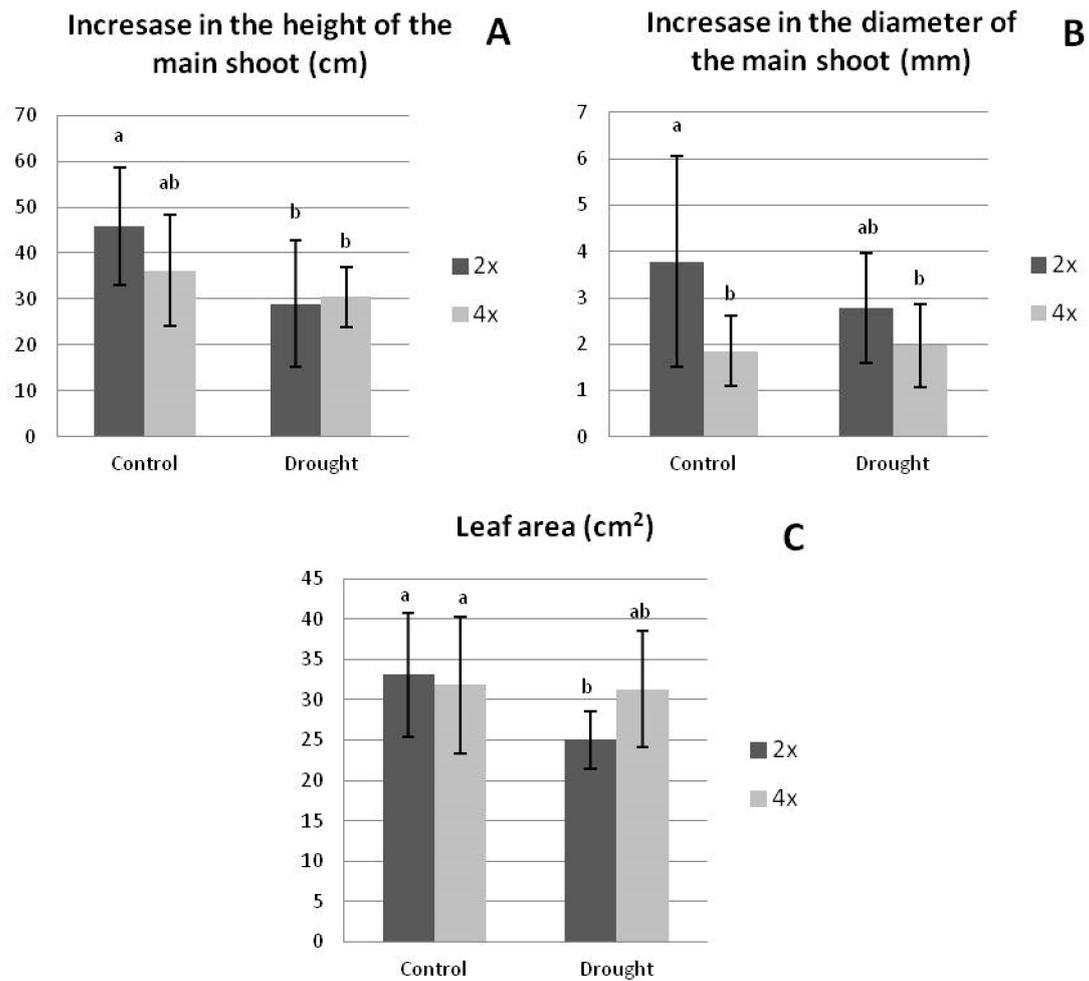


Figure 7. Growth parameters of diploid (2x) and tetraploid clone 4x-25 of the apple cv. ‘Redchief’, optimally irrigated and subjected to drought stress: (A) increase in the height of the main shoot, (B) increase in the diameter of the main shoot, (C) leaf area. Bars marked with the same letter do not differ significantly at $p = 0.05$; Duncan’s test.

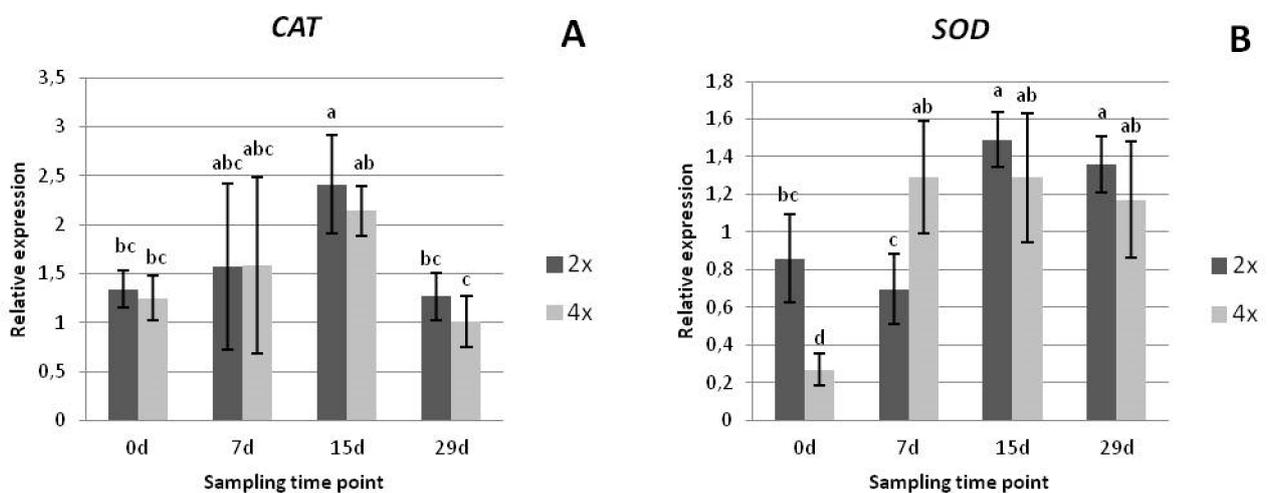


Figure 8. Cont.

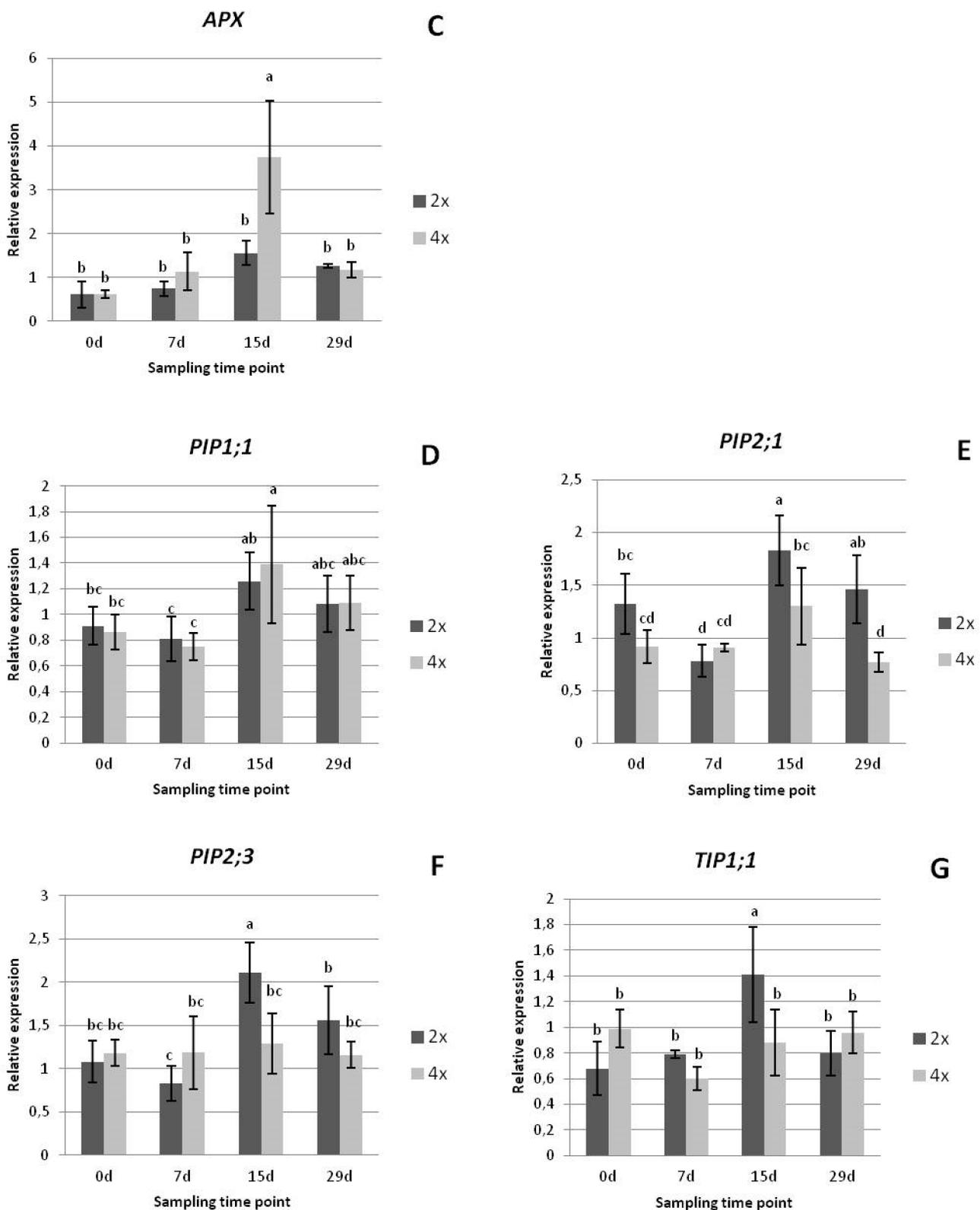


Figure 8. Relative expression analysis using qRT-PCR of the genes (A) *CAT*, (B) *SOD*, (C) *APX*, (D) *PIP1;1*, (E) *PIP2;1*, (F) *PIP2;3*, (G) *TIP1;1* in the apple cv. ‘Redchief’, diploid (2x) and tetraploid clone 4x-25 (4x), before (0 d) and 7, 15, and 29 days after inducing drought. Bars marked with the same letter do not differ significantly at $p = 0.05$; Duncan’s test.

4. Discussion

4.1. Phenotypic Characteristics of ‘Redchief’ Tetraploids

Newly generated tetraploids of apple exhibit a dwarf phenotype and weak growth [34,40,46,58]. Phenotypic observations of 6–8-month-old own-rooted tetraploids of apple cv. ‘Free Redstar’ showed that, compared with their diploid counterparts, they have shorter shoots, fewer branches, and smaller stem diameter [46]. In our study, the observations were carried out on five-year-old own-rooted and M9-grafted trees growing in the experimental orchard. As in the studies cited above, tetraploid own-rooted ‘Redchief’ trees showed much weaker growth than diploids; all growth parameters for tetraploids were significantly lower. According to the findings of Ma [58], dwarfism in polyploids may be caused by decreased levels of endogenous plant growth regulators. In these studies, using digital gene expression (DGE) and qRT-PCR methods, the authors showed that in the autotetraploid apple, the expression of a number of genes involved in the biosynthesis of plant hormones was changed compared with diploids. Moreover, the levels of indoleacetic acid (IAA) and brassinosteroids (BRs) were reduced in three- and five-year-old tetraploid apple plants [58]. Podwyszyńska [46] postulated that the weak growth of young, own-rooted apple tetraploids may have a transient character and may be connected with a high degree of DNA methylation. Xue et al. [40] observed the growth of tetraploid clones of apple cv. ‘Hanfu’ for five years; they reported that tetraploids showed increased vigour, larger organs, and enhanced physiological parameters after reaching maturity (reproductive phase) while growth of diploid plants decreased once they started flowering and fruiting. Our observations show that own-rooted five-year-old tetraploid trees still show significantly reduced growth; however, they have not yet reached the reproductive phase, unlike the own-rooted diploids, so it is necessary to monitor their further growth to confirm the results obtained by Xue [40].

In M9-grafted trees, the differences between ‘Redchief’ diploids and tetraploids were small, although for the total length of current season shoots and the number of current-season shoots per tree, the means were still significantly lower in the tetraploids. These observations are, in part, consistent with the results obtained by Podwyszyńska [46] in the study on ‘Free Redstar’ tetraploids. When tetraploid ‘Free Redstar’ clones were grafted on M9 rootstock, no significant differences in morphological and physiological parameters were observed between the three-year-old diploid and tetraploid trees growing in the orchard [46]. ‘Malling 9’ (‘M9’) is the best characterised and one of the most widely used dwarfing apple rootstocks [59]. The complex interaction between the scion and the rootstock, which includes the nutrient uptake, hormonal communication, and carbohydrate distribution, regulates the growth and development of the scion [60]. It is postulated that the main causes of rootstock-induced dwarfism are changes in the level and transport of hormones between the scion and rootstock, and anatomical factors such as proportion of cortex and the number and diameter of xylem cells [59]. The dwarfing effect of M9-grafting was clearly visible for diploid ‘Redchief’ trees; all growth parameters were reduced in ‘Redchief’ diploids grafted on M9 rootstock compared with own-rooted trees. Unlike in diploids, grafting on M9 rootstock improved the growth of ‘Redchief’ tetraploid plants. It can be assumed that hormone influx from the rootstock or other rootstock-dependent factors improved the growth of the tetraploid scions. This issue requires further research.

Phenotypic changes in tetraploid ‘Redchief’ clones also concerned other traits—leaf shape and anatomy, stomata size and frequency, and pollen characteristics—and were analogous to those reported by Sedysheva and Gorbacheva [36], Podwyszyńska [46], and Xue [40] for other apple tetraploids. Similar to ‘Free Redstar’ and ‘Hanfu’ tetraploids, the leaves of ‘Redchief’ tetraploids were reshaped, ticker, and contained more chlorophyll; the stomata were larger but less frequent compared with diploids. Increase in stomata size and chlorophyll content is commonly observed in the polyploids of many plant species, e.g., daylily [61], rhododendron [62], *Lycium ruthenicum* [63], and mango [64]. Analogous to the ‘Redchief’ tetraploids, the stomatal density was significantly reduced in polyploids of *Rhododendron fortunei* [62] and *Gladiolus grandiflorus* [65], and tetraploids of the apple ‘Hanfu’ [40]. Our research showed that leaf anatomical structure was different in ‘Redchief’

diploids and tetraploids, and the abaxial and adaxial epidermal layers and both types of mesophyll were significantly thicker in tetraploids. The enlargement of plant organs is considered characteristic of polyploids. The leaves, flowers, and fruits of ‘Hanfu’ tetraploids were larger than those of diploids [40]. Leaves of tetraploid clones of ‘Redchief’ and ‘Free Redstar’ [46] were not larger than those of their diploid counterparts, while in tetraploid and octoploid *Rhododendron fortunei* the leaves were even smaller than in diploids [62]. On the other hand, in tetraploid ‘Redchief’, the cells of leaf tissues were enlarged, which contributed to thicker individual layers of mesophyll and epidermis, and the entire thickness of the leaves. Thicker leaves were also observed in the polyploids of other apple cultivars [40,46] as well as in *Rhododendron fortunei* [62] and *Lycium ruthenicum* [63]; moreover, tetraploids of *R. fortunei* had more abundant and longer epidermal hairs than those of diploids. Pollen grains were significantly larger in the ‘Redchief’ tetraploids, but pollen viability and germination were reduced, which was consistent with what was observed in *Gladiolus grandiflorus* polyploids [65].

4.2. Drought Tolerance Assessment

The evaluation of the susceptibility of apple tetraploids to stress factors showed that polyploids are characterised by increased resistance to both biotic and abiotic stresses [37–39,41,46,47]. Similar results were obtained in our study on ‘Redchief’ tetraploids. Our previous findings revealed that the tetraploid clones of ‘Redchief’ are characterised by increased resistance to *E. amylovora* infection [47]. Recent results may indicate that ‘Redchief’ tetraploids were more tolerant to drought than their diploid counterparts. Under limited water supply, the growth of the main shoots of tetraploids was less inhibited and no reduction in leaf area was observed, in contrast to diploids. Moreover, the ratios of net photosynthesis and transpiration were less affected than in diploids. Under drought, tetraploids had over a two-fold higher level of *APX* expression, but no significant differences were observed between diploids and tetraploids in the expression of the other two genes encoding the major antioxidant enzymes (CAT and SOD) and aquaporin genes. This may be due to the moderate level of drought that was applied; the irrigation was limited but not withheld, and no severe symptoms such as leaf wilting or dropping were observed during the course of the experiment. Superior drought tolerance was also observed in autotetraploids of apple ‘Hanfu’ and ‘Gala’ [38]. Symptoms of drought occurred earlier in ‘Hanfu’ and ‘Gala’ diploids than in tetraploids; under drought, tetraploids had higher relative water content and chlorophyll content but less proline and MDA than diploids. Expression of key aquaporin genes, *PIP1;1* and *TIP1;1*, under drought was much induced, but the level was lower in tetraploids than in diploids. A similar result was observed in ‘Redchief’ tetraploids, three out of four tested aquaporin genes showed lower expression in tetraploids than in diploids under drought stress.

Bai [11] postulated that the superior drought tolerance observed in the apple cv. ‘Honeycrisp’ can be related to anatomical and morphological features of their leaves. In ‘Honeycrisp’, the leaves and the cuticle were thicker and palisade mesophyll cells were longer than in the less-drought-tolerant cultivar ‘Yanfu 3’. The increased thickness of leaves and their epidermis and mesophyll layers may play a role in the elevated drought tolerance of ‘Redchief’ tetraploids. Features such as stomatal size and density also affect the level of drought tolerance. In the case of ‘Redchief’ tetraploids, the stomata were larger than in diploids, but their density decreased, so that the total pore space on a leaf could be lower [66].

Our research showed that the autotetraploids of ‘Redchief’ are characterised by an altered phenotype compared with their diploid counterparts. Data from the experiment in which plants grew with limited water supply suggest that ‘Redchief’ tetraploids may have increased tolerance to drought. Taking into account previous findings indicating an elevated level of resistance to fire blight, it is postulated that ‘Redchief’ tetraploid clones could provide valuable genetic resources for breeding of new apple cultivars. Further research is needed, especially to assess their ability to cross with diploid apple cultivars.

Author Contributions: Conceptualisation, D.W., M.P. and K.K.; funding acquisition, D.W.; investigation, D.W., M.M., A.M.-C., K.K., Z.B., P.P.T., K.W. and J.F.; methodology, D.W., M.M., A.M.-C., K.K., Z.B., K.W. and W.T.; data curation, visualisation, D.W., M.M., A.M.-C., K.K., Z.B., P.P.T. and K.W.; statistical analysis, D.W., M.M., K.K. and Z.B.; project administration, D.W.; writing—original draft, D.W., A.M.-C. and K.K.; writing—review and editing, D.W. All authors have read and agreed to the published version of the manuscript.

Funding: This research was supported by the Polish Ministry of Agriculture and Rural Development as a grant Biological Progress in Crop Production, Task No. 49.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: The data presented in the study are available on request from the corresponding author.

Conflicts of Interest: The authors declare no conflict of interest.

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