

Article

Drought and Saline Stress Tolerance Induced in Somatic Hybrids of *Solanum chacoense* and Potato Cultivars by Using Mismatch Repair Deficiency

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Abstract: Global climate change, especially when involving drought and salinity, poses a major challenge to sustainable crop production, causing severe yield losses. The environmental conditions are expected to further aggravate crop production in the future as a result of continuous greenhouse gas emissions, causing further temperature rise and leading to increased evapotranspiration, severe drought, soil salinity, as well as insect and disease threats. These suboptimal growth conditions have negative impact on plant growth, survival, and crop yield. Potato is well known as a crop extremely susceptible to drought, which is primarily attributed to its shallow root system. With potato being the fourth major food crop, increasing potato productivity is thus important for food security and for feeding global population. To maintain a sustainable potato production, it is necessary to develop stress tolerant potato cultivars that cope with the already ongoing climate change. The aim of our study is to analyze the response of potato somatic hybrids to drought and salt stress under in vitro conditions; the somatic hybrids studied are the wild relative *Solanum chacoense* (+) *Solanum tuberosum*, with or without mismatch repair deficiency (MMR). Upon this selection of drought and salt tolerant genotypes, somatic hybrids and their parents were phenotyped on a semi-automated platform, and lines tolerant to medium water scarcity (20% compared to 60% soil water capacity) were identified. Although none of the parental species were tolerant to drought, some of the MMR-deficient somatic hybrids showed tolerance to drought and salt as a new trait.

Keywords: drought stress; salt stress; potato somatic hybrid; proline; photosynthesis



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1. Introduction

Freshwater shortage has become an increasing problem worldwide, which is the result of climate change, increased pollution, and overconsumption of water. At present, more than 40% of humanity suffers from water scarcity. The lack of fresh water does not only affect the accessibility to drinking water but also leads to food shortage [1]. Around 85% of freshwater is utilized in agriculture. Out of the total land used for agricultural production, 40% constitutes irrigated areas [2]. However, Meier et al. [3] estimated 18% more irrigated area than the reported data. In the beginning, irrigation increases two to four times the crop yield production of an area compared with rain fed farming, but during long term usage it has a substantial drawback, i.e., it induces salt accumulation in soil, which adversely affects the production of various crops [4]. Approximately 6% of the total land area and 50% of irrigated lands are under the threat of salinity worldwide [5].

Since both drought and salinity induce water stress in the cells of the plants, both induce similar effects in the early stages of plant development and growth, causing osmotic imbalance, cell dehydration, and ROS production [6,7]. Plants suffer from water deficit when the rate of transpiration is higher than the water uptake [8], which influences the physiology of plants. Related stress-responses of drought-tolerant plants induced by water scarcity are summarized in Table 1. When the roots of plants sense soil dryness, the level of abscisic acid (ABA) in plant increases, which leads to stomatal closure. Therefore, the first physiological response to drought stress is the limitation of gas exchange, which leads to reduction in transpiration. Stomatal closure entails a reduction in water absorption through the root system, which affects the plant mineral-nutrient supply [9]. As a result of declined stomatal aperture, the CO₂ assimilation of plants is also reduced.

Table 1. Synthetic presentation of physiological and molecular changes of drought-tolerant plants induced during water shortage.

Physiological Responses	Biochemical Responses	Molecular Responses
Loss of turgor and osmotic regulation	Decrease in photosynthetic efficiency	Stress response gene expression (ABA)
Reduced leaf water potential	Accumulation of stress solutes in extracellular matrix and cytosol	Synthesis of specific protective proteins
Reduced CO ₂ concentration due to stomatal closure	Increase the antioxidative enzyme production	
Reduced growth		

When water deficit persists for a long time, cell division, enlargement, and differentiation is limited, which reduces the growth of the plant, but the root-to-shoot ratio increases in order to facilitate water absorption [10]. As a negative effect of drought stress, the total leaf area decreases, which reduces crop yield through a reduction in CO₂ assimilation [11]. During long exposure to salinity stress, plants experience ionic stress, accumulating high amounts of Na⁺ and K⁺, which affects photosynthetic components (enzymes and pigments) and increases oxidative stress [12]. Furthermore, a decrease of photosynthetic activity disrupts the balance between generation and utilization of electrons, which leads to reactive oxygen species production (ROS: O₂[−], ¹O₂, H₂O₂). During long-lasting drought conditions, the accumulated ROS have irreversible deleterious effects such as amino acid oxidation, DNA nicking and lipid peroxidation [13]. However, before inhibition of photosystem II (PSII) of the photosynthetic apparatus occurs, which triggers the cell-damaging cascade, several neutralizing mechanisms try to protect the reaction center of photosynthesis by eliminating the excess energy through cyclic electron transport [6]. These mechanisms detoxify harmful ROS, which can be accomplished through enzymatic (superoxide dismutase, catalase, ascorbate peroxidase or peroxidase) or non-enzymatic processes (flavones, anthocyanins, and ascorbic acid) [14]. During water stress, the accumulation of solutes in extracellular matrix and cytosol increases, which contain ions such as K⁺, Na⁺ and Cl[−], or organic compounds such as different amino acids (e.g., proline), polyamines, and glycine betaine to prevent water loss of cells and to maintain the turgor of the leaves [15].

Cultivated potato uses water in a relative efficient way, but it is considered to be sensitive to moderate levels of water deficit, which causes significant yield losses. Its sensitivity is attributed to superficial location of roots [16]. During drought stress, the length of potato leaves have a positive correlation with the water potential of the leaf [17]. The leaves, which expand in water-deficit periods, grow smaller and have lower specific leaf area than the control ones. Moreover, linear correlations between CO₂ assimilation, potato plant height, and tubers yield were observed [18,19]. Water limitation prevents the development of apical branches and reduces the number of stolons [20]. A water shortage after stolon and tuber initiation greatly reduces tuber size and yield [21].

The increase in drought periods affecting the most agriculturally important areas motivate the breeders to select drought-tolerant cultivars to avoid yield losses. Generally, the

stress selection of plants begins with a laborious in vitro prescreening of drought-tolerant plants, followed by an ex vitro selection imitating the naturally occurring conditions. The selection agent during in vitro prescreening process is frequently polyethylene glycol (PEG), a hydrophilic molecule with a high affinity to bind water molecules in culture media and therefore induces water stress [22,23]. PEG proved to be effective in selecting drought-tolerant potato cultivars (e.g., Agria, Kennebec, Sante) [23] and resistant transgenic potato varieties [24].

In the case of transgenic potato varieties, the high proline content in stressed tissues has beneficial effects on water deficit induced stress tolerance [24]. In drought conditions, high amounts of proline accumulation are caused by the activation of proline biosynthesis and also by the inactivation of proline degradation mechanisms [25,26]. Proline has osmoprotective properties when the water content of cells decreases. This amino acid protects the subcellular structures, macromolecules, and membranes; it also reduces the photodamage in thylakoid membranes by inhibiting the production of $^1\text{O}_2$ in drought conditions [6,27].

A DNA mismatched repair (MMR) system reduces recombination between homologous sequences (similar but not identical DNA sequences). A function known as anti-recombination prevents the transfer of resistance genes from wild related species into economically important crops. To induce MMR deficiency, *S. chacoense* was transformed using the *AtMSH2* gene in antisense orientation (S) or with a dominant negative mutant sequence (DN) [28].

Phenotypic characterization of water-stressed plants helps to determine the morphological and physiological effects of the induced stress. Phenotyping platforms made it possible to monitor the development of plants during water deficit by determining biomass accumulation without physiological damage [29,30]. Green biomass of plants provides information about stress-induced response such as plant height, leaf number, and size, and it also makes possible to determine the effectiveness of photosynthesis [31]. The biomass accumulation is monitored with image analysis, which ensures an easier and non-laborious evaluation of stressed plants.

The goal of our research is to determine the drought and salt tolerance of somatic hybrids and backcross progenies between potato and wild type or MMR-deficient *S. chacoense*, respectively, using in vitro stress selection with PEG and salt. Plant response to drought stress was also evaluated using phenotypic characterization on a semi-automated phenotyping platform and by the photosynthesis status of drought-stressed plants.

2. Materials and Methods

2.1. Plant Material

For this study, we used two types of somatic hybrids (SHs) regenerated after mesophyll protoplast electrofusion and hybrid plant regeneration and selection [28]. The first type was obtained by protoplast electrofusion between *S. tuberosum* cv. Delikat (Dk) or Desiree (De) and the wild *S. chacoense* accession GLKS 30138 (Gross Lüsewitz Potato Collections, Genbank, Germany) (*S.chc* 1G) or a high leptine producer *S. chacoense* accession PI 458310 (*S.chc* HL). The second type was obtained by protoplast electrofusion between *S. tuberosum* cv. Delikat (Dk) or Desiree (De) and the high leptine producer (HL), transgenic mismatch repair (MMR) deficient *S. chacoense* (Table 2). The hybrid status of SHs was confirmed by phenotypic characterization, simple sequence repeat (SSR) marker analysis [28], and by determination of genomic composition using the genomic in situ hybridization (GISH) method (Molnar et al., article in preparation).

Table 2. Plant material used in the experiments: parents, somatic hybrids with or without MMR deficiency and derived backcrosses (BC₁, BC₂).

Parent Lines	Combination	SHs and BCs without MMR Deficiency	Combination	SHs with MMR Deficiency
<i>S. tuberosum</i> cv. Delikat (4×)	<i>S. tub</i> cv. Desiree + <i>S. chc</i> HL	De. C4 (6×)	<i>S. tub</i> cv. Desiree + <i>S. chc</i> HL DN gene (AtMSH2)	DeDN5.5 (4×)
<i>S. chacoense</i> HL (2×)		De. C7 (4–5×)		DeDN11.5 (4×)
<i>S. chacoense</i> 1G (2×)	<i>S. tub</i> cv. Delikat + <i>S. chc</i> 1G	1913/6 (5×)	<i>S. tub</i> cv. Delikat + <i>S. chc</i> HL DN gene	DeDN11.29 (4×)
		1913/10 (4–5×)		DkDN5.3 (6×)
		1552/1 * (5×)		DkDN5.6 (4×)
		1552/1/1 (4–5×)		DkDN5.11 * (6×)
	BC ₁ : <i>S. tub</i> cv. Delikat + <i>S. chc</i> 1G × <i>S. tub</i> cv. Romanze	1552/1/2 * (4–5×)		DkDN5.17 (6×)
		1552/1/3 (4–5×)		DkDN5.25 (4–6×)
		1552/1/4 (5×)		DkDN11.24 (6×)
		1552/1/7 (4×)		DkDN11.26 (5–6×)
		1552/1/18 (4×)		DkDN11.34 (4–5×)
		1553/1/7 (3–4×)		DkS10.5 (4×)
	BC ₂ : BC ₁ × <i>S. tub</i> cv. Sonate	1552/1/7/1 (4×)		DkS10.11 (4×)
		1552/1/7/2 (4–5×)		DkS10.13 * (4×)
				DkS10.20 (4–6×)
				DkS10.35 (4–5×)
			<i>S. tub</i> cv. Delikat + <i>S. chc</i> HL AS gene	DkS10.40 * (4×)
				DkS10.43 (4×)
				DkS10.47 (4×)
				DkS10.51 (4×)
				DkS10.61 (4×)

All of the listed genotypes were tested for in vitro stress selection, genotypes marked with * were included in ex vitro water stress experiment on a phenotyping platform; De = cv. Desiree; Dk = cv. Delikat; number coding such as 1552/1 refers to somatic hybrids (SHs) with *S. chc* 1G, while back-crosses (BCs) are indicated by 1552/1/1 as BC₁, 1552/1/7/2 as BC₂; MMR-deficient SHs are noted with Dk or De for potato cultivar; S means *AtMSH2* gene in antisense orientation and DN is the dominant negative *AtMSH2* gene in the transgenic *S. chacoense* used in somatic hybridization, while the number stands for each transgenic clone (e.g., 5 or 11), followed by the number of SH clone; in bold = genotypes used in ex vitro experiment; * = multiple resistance to the biotic (CPB) and abiotic traits (drought, salt). More details on the SHs characterization can be also found in previous publications [28,32].

2.2. In Vitro Drought and Saline Stress

In vitro drought-stress of somatic hybrids with or without MMR deficiency and back-cross progenies was performed using RMB5 media (Murashige and Skoog (MS) salts and Gamborg B5 vitamin medium—Duchefa Biochemie, Harleem, Netherlands), supplemented with polyethylene glycol (PEG 6000, AppliChem GmbH, Darmstadt, Germany). Two types of drought conditions were established: a moderate stress, simulated with 5% PEG, and a severe drought condition, obtained by adding 15% PEG to culture media.

In the case of salinity stress, plants were exposed to four different concentrations of NaCl (Sigma-Aldrich, St. Louis, MO, USA): 40 mM, 80 mM, 120 mM and 160 mM, which simulated moderate and severe salt stress.

The genotypes presented in Table 2 (5 individuals for each) with 5 replicates were maintained for three weeks in a growth chamber at 21 °C with a photoperiod of 16 h and a light intensity of 90 µmol·m²·s^{−1}. After three weeks under stress, the viability, regeneration ability, shoot, and root growth rate of the tested plants were evaluated.

The proline content of plants was determined according to Bates et al. [33].

2.3. Plant Cultivation and Ex Vitro Drought Stress Treatment

Drought-tolerant plant selection in ex vitro conditions was performed using the phenotyping platform HAS-RSDS-SSDS at the Biological Research Centre Szeged, Hungary. Two wild type and five MMR deficient SHs, as well as the parents were selected from previously analyzed genotypes (Table 2, genotypes in bolt). The one-week-old in vitro grown plants were planted in 50% sandy Maros soil and 50% Tera peat soil, using 3 replicates for each tested genotype (3 plants/genotype). The dimensions of the pots were 30 cm in height and 15 cm in diameter, and the pots contained unique radio-frequency identifier chips (RFID). In the first two weeks, plants were acclimatized to greenhouse conditions using plastic beaker covers and were watered equally. In drought conditions plants were watered only up to 20%, while control plants were watered up to 60% compared to 100% soil water capacity. The adjusted water limitation corresponded to moderate drought conditions. Every second day the growth of plants was photographed from eleven sideway positions using Olympus C-7070WZ (Olympus Ltd., Southend-on-Sea, UK) digital cameras. Differences in biomass accumulation of stressed and control plants were used to select drought-tolerant genotypes. Plant biomass was calculated using green pixel numbers of photos with MATLAB software, Image Processing Toolbox (The MathWorks Inc., Natick, MA, USA). The plants were monitored for six weeks and then harvested; tuber number and yield were recorded.

2.4. Determination of Photosynthesis Efficiency

Chlorophyll fluorescence emission was measured with the Pocket PEA (plant efficiency analyzer) chlorophyll fluorimeter (Hansatech Instruments, Pentney, UK) and pulse amplitude modulation fluorometer (PAM-2000 Heinz Walz GmbH, Pfullingen, Germany). The upper surface of the third completely developed leaf was used in both measurements. In the case of the Pocket PEA, leaves were dark-adapted for 15 min in order to determine the minimal and maximal fluorescence levels (F_0 , F_0' and F_m , F_m'). Other parameters such as potential photochemical efficiency, effective quantum yield, water splitting complex activity, membrane integrity, and non-photochemical quenching were also measured.

The pulse–amplitude–modulation (PAM) measurements were carried out with a Mini-PAM instrument. Pulses were maintained for 3 μ s and repeated at a frequency of 600–2000 Hz. Mini-PAM offers the possibility to measure maximal fluorescence yield, photosynthetically active radiation (PAR), the apparent rate of electron transport (ETR), and effective quantum yield.

2.5. Statistical Analysis

Statistical analysis was performed using Microsoft Excel 2013 (Microsoft Corporation) and R statistical software. All data were expressed as mean \pm standard error (SE). Comparison of the phenotypic variations, proline content differences, and photosynthesis changes of stressed and control plants was performed using unpaired Student's *t*-tests. Statistical analyses of the biomass accumulation were performed using one-way ANOVA; the data were normally distributed and variance homogeneous. In our analysis, a *p* value below 0.05 was interpreted as indicating a significant difference.

3. Results and Discussion

The frequency of drought periods increases yearly, which is one of the undisputable results of climate change. Unfortunately, in the near future this situation is unlikely to improve; on the contrary, drought periods are likely to increase and larger areas likely affected by water shortage. As an effect of drought stress, the sustainability of crop production has been compromised. Lobell et al. [34] predicted that until 2030 in Southern Africa, maize production is expected to decrease by up to 30%, and South Asia could lose more than 10% yield of economically important crops (rice, millet, and maize). Potato is considered as a drought-sensitive crop, with a high quantity of yield reduction during water shortage [35]. Therefore, the development of drought-tolerant cultivated plants with

more efficient water-usage is indispensable in order to reduce the devastating agronomical and social effects of drought [30].

Stress selection of drought-tolerant SHs with or without MMR deficiency and BC progenies was performed with both in vitro and ex vitro conditions (Table 2). In vitro stress selection was induced with different concentrations of PEG (5% and 15%) and also different concentrations of NaCl (40 mM, 80 mM, 120 mM, and 160 mM), which simulated mild and severe water and salt stress conditions.

3.1. In Vitro Drought-Stress Selection

During moderate drought stress (5% PEG), the majority of the genotypes developed more weakly compared to control group plants: the shoot length of stressed plants was significantly smaller (t -test, $p < 0.05$) (Figures 1 and 2) and also significantly lower number leaves developed (t -test, $p < 0.05$). Shao et al. [36] revealed similar results: they associated the height of the plant and the leaf area reduction during drought stress with a decrease of cell enlargement and with increased leaf senescence. Furthermore, reduction of leaf area resulted in decreased crop production due to the reduced photosynthesis.

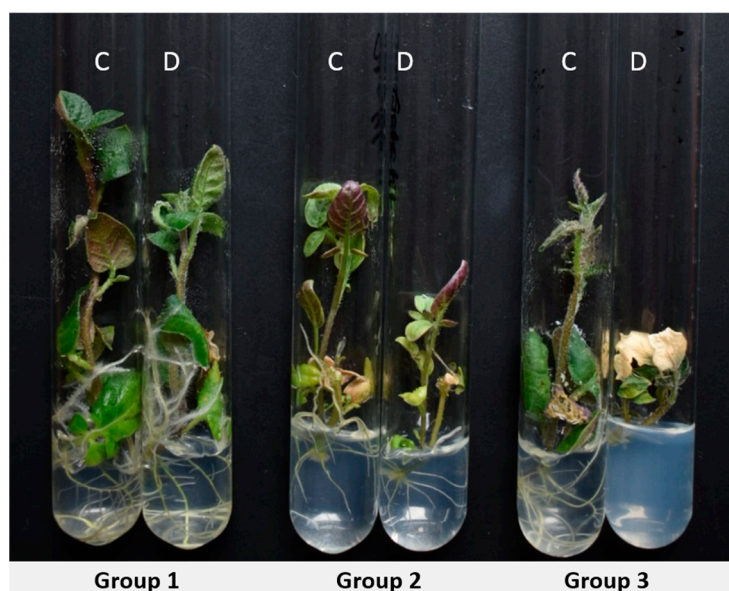


Figure 1. Illustration of morphological differences between members of the three groups after three weeks on 5% PEG media. C—control; D—drought-stressed plants. The representative genotype of Group 1 is DkS10.13, of Group 2 is DeDN11.29, and of Group 3 is DkDN11.26.

The analyzed genotypes responded differently to the induced drought stress; therefore, they could be classified into three distinct groups (Figures 1 and 2 and Supplementary Table S1). Group 1 contains plants whose leaf development was similar to the control plants; no significant differences were observed between root (t -test, $p = 0.683$) and shoot (t -test, $p = 0.491$) length in the treated and control group. Group 2 includes genotypes with a well-developed root system (t -test, $p = 0.129$); no significant differences between stressed and control plants root length were observed, but the shoot length of plants was significantly shorter (t -test, $p < 0.05$) compared to the shoot length of control plants (the average length of control plants was 8.52 cm, while the stressed plants length was approximately 6.15 times shorter, they had an average length of 1.38 cm). Group 3 contains plants that developed very weakly. Both the shoot and root lengths were significantly smaller than in the case of control plants.

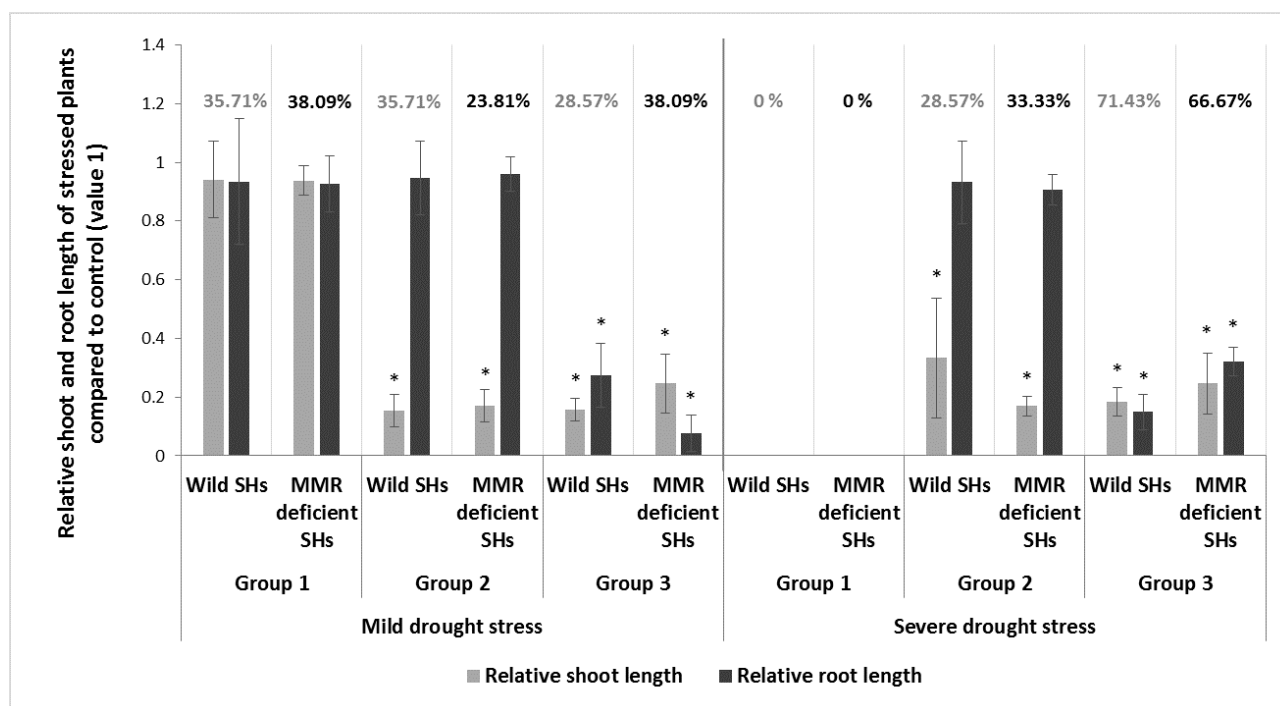


Figure 2. Classification of wild type somatic hybrids and their progenies (indicated as wild SHs), and MMR-deficient SHs (indicated as MMR-deficient SHs) by morphological adaptation to moderate and severe drought stress. Root and shoot lengths of stressed plants were compared to the same properties of control plants (value 1). * indicates significant differences ($p < 0.005$) compared to control. The percentage values show the percentage of hybrids of different origins present in a given category (total number of wild SHs was 14, while MMR-deficient SHs was 21).

Genotypes from Group 1 managed the water deficit efficiently during moderate drought stress. In their case, only the number of leaves was significantly lower than that of the control group (the stressed group had, on average, 1.86 times fewer leaves than the control group). Members of the second group show a weaker tolerance of water shortage. They invest all of their reserves in the development of their root systems to increase the possibility of reaching the water stores located in deeper layers of the soil before the adverse effects of drought stress damage the plant. Although the genotypes in the third group survived the drought stress, they were not capable of overcoming the negative effects of water deficit. These plants were sensitive to moderate drought conditions. In this case, if the water deficit had persisted for a longer time, the plants would have most probably died. The shoot and root systems of the plants did not develop; the development of these systems is essential for surviving, and in many instances, the bottom leaves and the edges of the upper leaves were withered, while some of the stressed plants died (Figures 1 and 2).

In the second experiment, the plants were exposed to severe drought conditions induced by 15% PEG supplementation in culture media. All of the genotypes developed more weakly; their shoot lengths were significantly shorter compared to the ones in the control group (average shoot lengths of stressed plants was 4.28 times shorter than those of the control plants, which had an average of 8.52 cm). The root system of stressed plants was poorer in ramification, while a large part of the analyzed genotypes did not develop more than 0.5 cm long roots (Figures 2 and 3).

During severe drought stress, the analyzed genotypes can be classified only in two groups (Group 2 and 3), because none of the plants developed similarly as the control plants did. The majority of the stressed plants belonged to the third group, and only one-third of the analyzed SHs and BC₁ progenies were included in Group 2 (Figure 2, Supplementary Table S2).

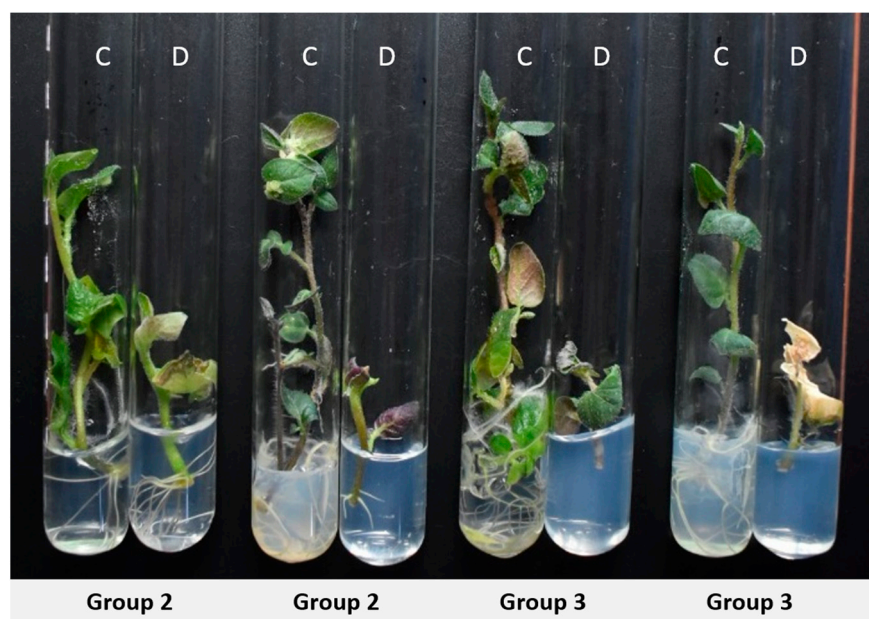


Figure 3. Morphological differences of severe drought-stressed (D) and control (C) plants after three weeks on 15% PEG media. Representative genotypes of Group 2 shown from left to right: DkS10.13, DkDN5.11; of Group 3: DkDN5.6 and DkDN11.26.

In this experiment, more plants withered and died compared to those in moderate drought condition. Parental lines were sensitive to severe water shortage as well. Genotypes of the second group (i.e., developed root system, reduced shoot length) after moderate drought stress have a weaker drought tolerance strategy and were classified as a Group 3 member (reduced root and shoot length) after severe drought stress; most of the plants from Group 1 preserved their resistance during severe water shortage, as they were only mildly affected and were therefore considered in Group 2 during severe water shortage (Figure 2, Supplementary Table S2).

Based on the different morphological responses of stressed parental lines and resistant SHs and BC clones, we concluded that during somatic hybridization, drastic genetic modifications may take place within the fused cells, which may result in the appearance of new, useful properties of the regenerated plant. The parental lines were susceptible to drought stress, but quite a large number of their hybrids and backcrosses were highly resistant to moderate and severe drought conditions. The effectiveness of drought-tolerance properties is higher in the case of MMR-deficient SHs than in the case of genotypes without MMR deficiency. MMR-deficient SHs developed significantly more richly (t -test, $p < 0.05$) and on average 2.14 times longer (t -test, $p < 0.05$) root systems during severe drought stress than MMR-proficient SHs and BC clones. There was no correlation between drought tolerance and ploidy level, as established in previous analyses [28] and presented for each genotype used in the present experiments (see Figure 2 and Supplementary Tables S1 and S2). The ploidy level determined by flow cytometry and chromosome counting varied in the somatic hybrids with the wild type SHs being predominantly $4\times$ to $5\times$ or $5\times$, while the MMR-deficient SHs were mostly $4\times$ [28]. The SHs and BCs selected for the present study are also with variable ploidy and represent the abovementioned tendency. However, the development of the plants presents no correlation to ploidy levels, especially in MMR-deficient SHs, which are tetraploids, hexaploids, or $4\times$ to $5\times$. In many cases, their growth was not following a linear correlation to ploidy [28]. This analysis helped us to identify the SHs having a “mutator” phenotype caused by MMR deficiency from the effects of somatic hybridization.

After evaluating the phenotypic responses of stressed plants, the accumulated proline concentration in the control group and both types of drought-stressed groups was

determined. The degree of proline accumulation during drought stress influences the ability of the plant to tolerate the effects of stress on a higher level. Proline protects the integrity of essential proteins and enzymes and has an important role in harmful ROS detoxification [6,37,38]. Choudhary et al. [39] and Szabados and Savoure [40] demonstrated, respectively, that drought-tolerant plants accumulated high amount of proline during drought stress, while sensitive plants accumulated this amino acid in a lower amount. The survival rate of tested genotypes with a high concentration of proline was significantly higher than in the case of drought-susceptible plants [24].

The proline content of the control group plants was genotype dependent (the proline concentration of control plants varied between 0.41 and 4.67 $\mu\text{mol/g}$ plant tissue). Therefore, to compare without distortion the drought responses of different genotypes, the proline concentration levels of stressed and control plants were used, and the proline concentration changes were followed accordingly.

All genotypes, except DeDN5.5, accumulated significantly more (t -test, $p < 0.05$) proline as a response to moderate water stress, but the proline level changes were considerably different between the analyzed genotypes (Figure 4). Some hybrids accumulated 7 to 8-fold more proline during water deficit (1552/1, DkDN5.11), while others accumulated only 2 to 3-fold more proline than control plants (DkDN5.17, DeDN11.29).

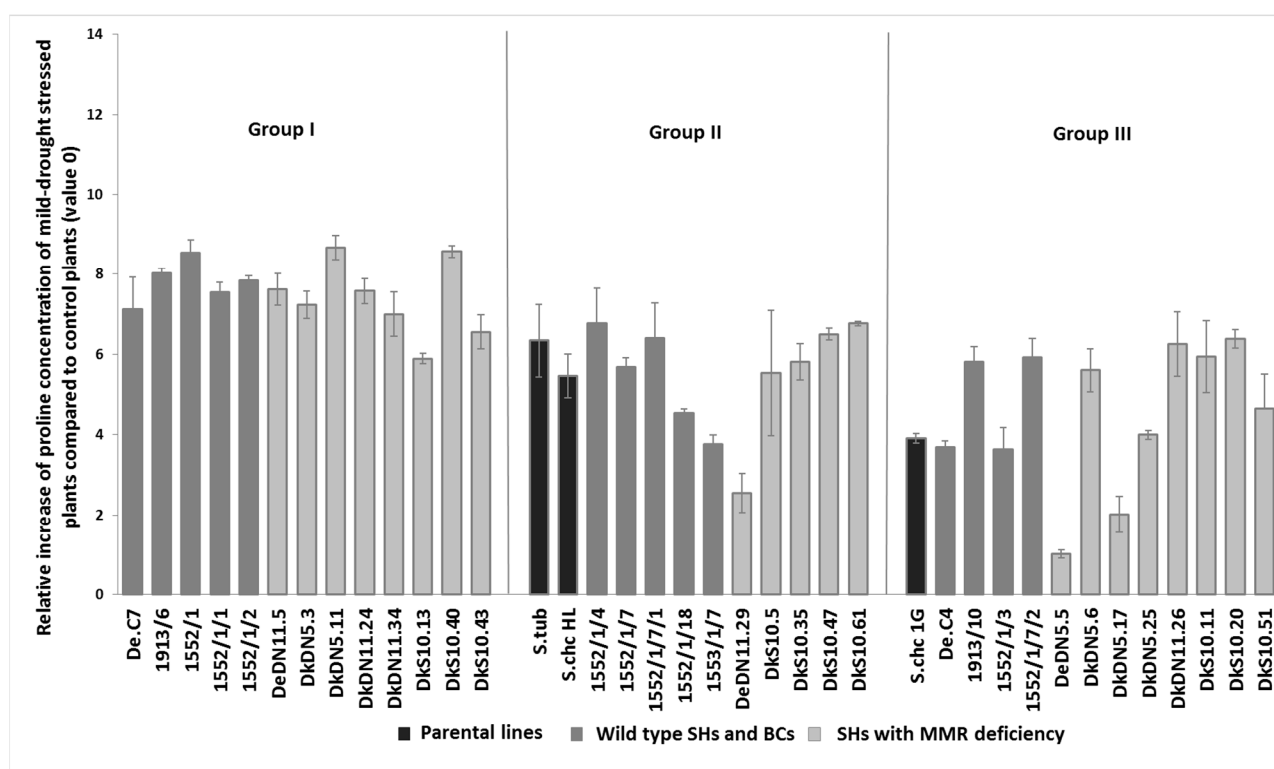


Figure 4. Relative proline concentration changes (mean \pm SE, $n = 5$) during moderate water stress of different SHs with or without MMR deficiency, BC progenies, and parental lines (*S. tuberosum* cv. Delikat and *S. chacoense*—*S. chc* HL or 1G).

To establish the role of proline in the drought tolerance of *Solanum* SHs, the relationship between morphological responses and proline concentration was determined.

During moderate drought stress, the analyzed genotypes were classified in three groups based on morphological responses to the stress. Among the created three groups a significant difference in proline accumulation can be observed: the members of the first group accumulated the most proline, i.e., an average of 7.55-fold more than control ones, and the second group 5.5-fold more than control plants, while the third group accumulated the lowest concentration of proline, i.e., only 4.52-fold more compared to control plants.

Genotypes from Group 1 accumulated significantly more proline during moderate water stress than susceptible plants from Group 3 (t -test, $p < 0.05$) (Figure 4).

During severe water deficit, a large part (two-thirds) of the stressed plants were not able to efficiently manage their resources; an efficient management would have helped in overcoming the negative effects of drought. Therefore, these genotypes became susceptible to severe water stress. The drought-tolerant group (Group 2, based on morphological traits) accumulated significantly more proline than the susceptible genotypes during severe drought stress (t -test, $p < 0.05$) (Group 3) (Figure 5). During severe drought condition, the tolerant genotypes accumulated 8.94 times more proline in their tissue, while the susceptible plants accumulated only 3.21 times more proline than the control plants.

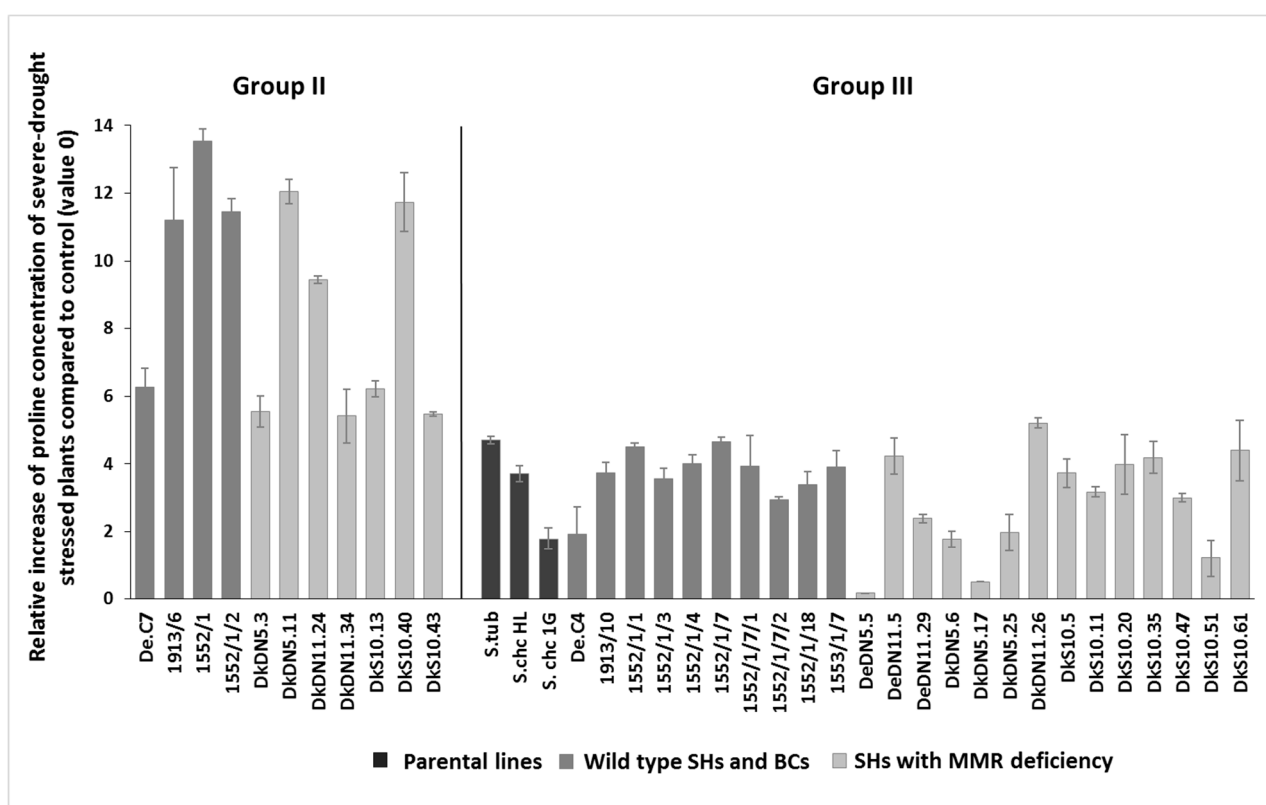


Figure 5. Relative proline concentration changes (mean \pm SE, $n = 5$) during severe water stress of different SHs with or without MMR deficiency, BC progenies, and parental lines (*S. tuberosum* and *S. chacoense*—*S.chc* HL or 1G).

Among the resistant plants, the SHs of 1913/6, 1552/1, DkDN5.11, DkDN11.24 and DkS10.40 and BC₁ 1552/1/2 accumulated the most proline during severe water stress, approximately twice as much as the other genotypes. Some SHs from the susceptible group (DeDN5.5 and DkDN5.17) synthesized less proline than control ones; these genotypes apparently suffered (lacking root system, yellowed leaves) from the severe water stress, and therefore these plants were unable to carry out the vital metabolic processes, and several of them withered.

Based on the obtained results, we can conclude that proline accumulation during water stress positively correlates with the tolerance ability of the plant. Increased proline biosynthesis in chloroplasts maintains the photosynthetic electron transport by holding the NADPH: NADP⁺ ratio at a low level, which allows for the carbon assimilation in the plants during water shortage [41]. The obtained results support this phenomenon because during moderate drought stress, the resistant plants, which accumulated high amount of proline, developed normally, while in severe drought condition the tolerant plants were able to grow roots, which is essential for withstanding water shortages in natural conditions.

3.2. In Vitro Salinity-Stress Selection

During salinity stress, plant biomass generally decreased with the increase of salt levels, and the majority of the analyzed lines developed more weakly than the control ones (Figure 6).



Figure 6. Phenotypic changes of DkDN11.34 during increasing salt concentration of the medium with the control plant (C) and plants in 40, 80, 120, 160 mM concentration of NaCl in culture medium. This genotype tolerated the medium salt-stress (40 mM, 80 mM) but was sensitive to severe salt concentrations of medium (120 mM, 160 mM).

During salinity stress, resistant plants accumulated higher amount of proline, while sensitive plants synthesized this amino acid in a lower concentration. Genotypes SH 1552/1 and BC₁ 1552/1/2 and the somatic hybrids with MMR deficiency (DkDN5.11, DkDN11.24, DkS10.13, and DkS10.40) highly tolerated salinity stress, and with the exception of DkDN5.11, DkS10.40 accumulated significantly more proline during moderate salinity stress than the control plants. This allowed them to survive and develop relatively well during the stress period (Figure 7). In the case of DkDN5.11 and DkS10.40, instead of proline accumulation, another mechanism provided resistance to salt stress. To determine the properties of this mechanism, further investigations are required.

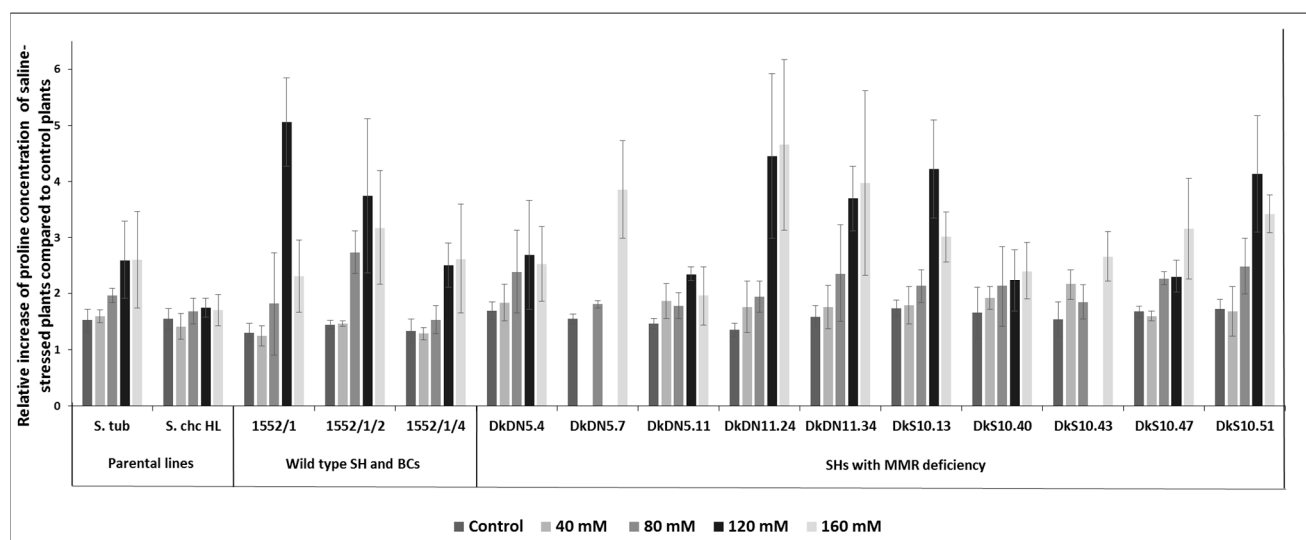


Figure 7. Relative proline concentration changes (mean \pm SE, $n = 5$) during severe salt stress of different SHs with or without MMR deficiency, BC progenies, and parental lines (*S. tuberosum* and *S. chacoense*—*S. chc* HL).

All salinity-tolerant genotypes have a deficiency in their DNA repair system, and therefore MMR deficiency increases the transfer of salinity-tolerance traits. Besides proline, plants also synthesize other compounds with a protective role: soluble sugars, sugar alcohols, glycine betaine, calcium, and potassium ions. As a result of accumulation of these osmotically active compounds, the osmotic potential of cells decreases, which allows for easier water intake of the cells. This helps to maintain the turgor of the cells [42]. In the case of some resistant somatic hybrids, the proline proved to be the main contributor in maintaining the macromolecule integrity and osmotic pressure, while in the case of DkDN5.11, DkS10.40, and 1552/1/4, other osmoprotectants similar to proline may contribute to salinity-tolerance ability (Figure 7). These results open future perspectives in the analysis of other osmoprotectants with a potential role in water-stress tolerance in potato SHs.

3.3. Plants Biomass Accumulation under Drought Condition

Based on in vitro stress selection results from the drought-tolerant group (i.e., 6 SH and 1 BC₁) and from the sensitive group (parental lines *S. tuberosum*, *S. chacoense* HL, 1G), 2 SH and 1 BC₁ were selected. The drought resistance of the selected plants was analyzed further in ex vitro conditions (genotypes in bold in Table 2). Among the analyzed SHs, both types (i.e., MMR-deficient and MMR proficient) were selected. Several studies proved the effectiveness of phenotyping platforms in monitoring morphological and physiological changes of plants during water stress [43], but to the best of our knowledge, this is the first-time that somatic hybrids of potato are analyzed on a phenotyping platform.

In our experiment, the impact of drought on morphological traits of stressed plants was determined using biomass accumulation differences between the control and the drought-stressed plants. Biomass accumulation of plants was calculated from green pixel quantities of RGB images, which correspond to the surface area of the shoots and leaves of plants. Therefore, plants with higher amounts of green pixels developed an extended surface area of their shoot and leaves (Figures 8 and 9), which is directly proportional with the fresh biomass. The extent of biomass decreases under drought, therefore, the amount of green pixels can be considered as an indicator of drought tolerance.

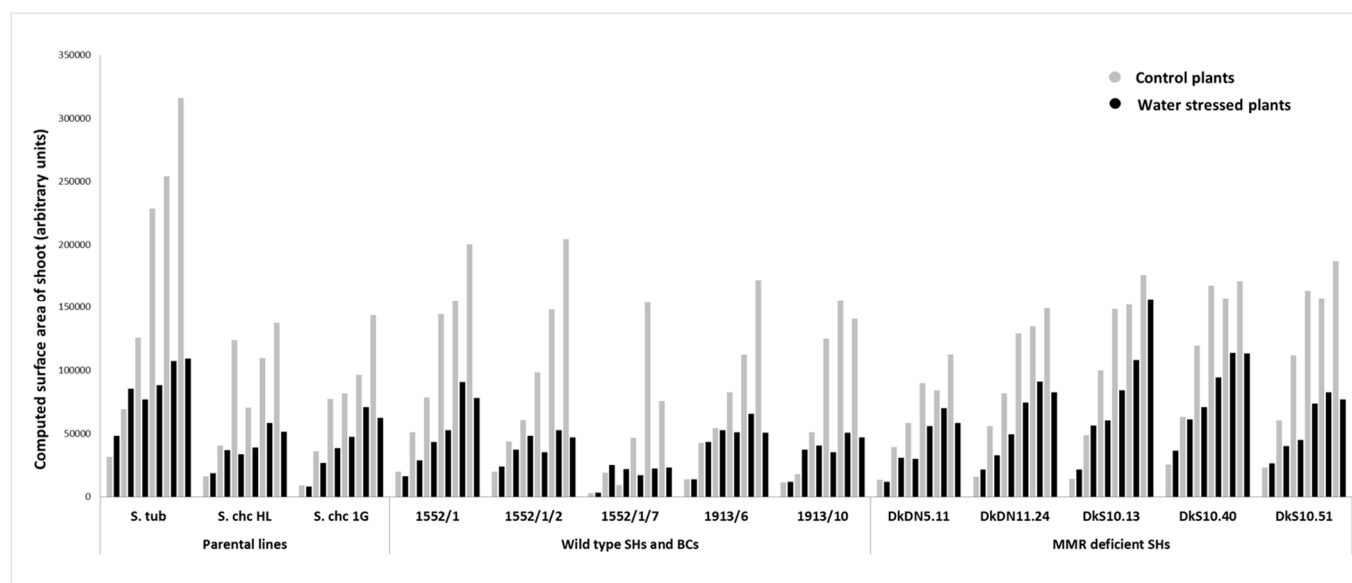


Figure 8. Biomass accumulation changes of the analyzed *Solanum* genotypes during the six-week drought stress, as tracked on the phenotyping platform. The biomass accumulation of plants was recorded once per week as detailed in Section 2.

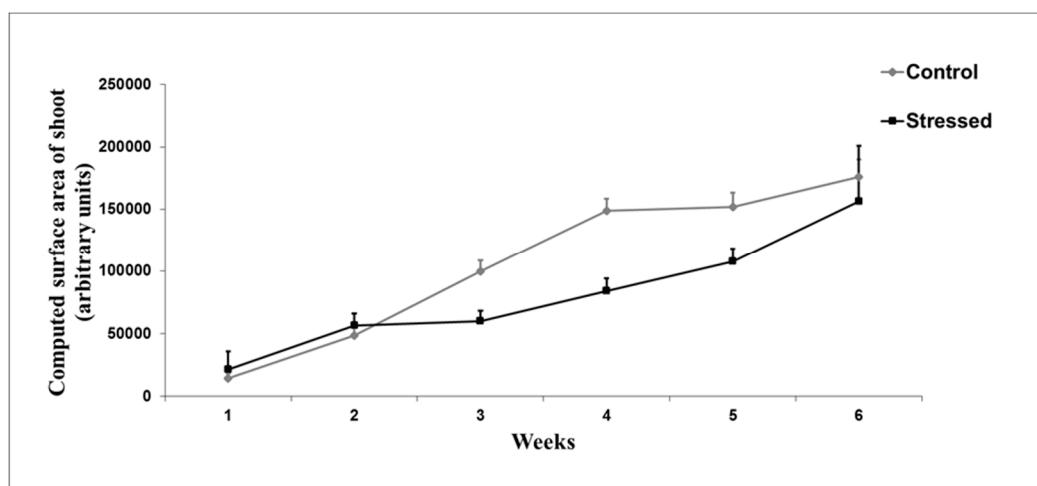


Figure 9. Biomass accumulation (mean \pm SE, $n = 3$) in MMR-deficient DKS10.13 during moderate drought conditions in the greenhouse, as tracked on the phenotyping platform.

Based on the biomass of the control plants, the cultivated potato had the most extended surface area of green shoot, while for SHs and BC₁ progenies the biomass accumulation was between the biomass levels of the parental lines. This behavior proves their hybridity, namely, that these genotypes were regenerated from a fused (combined) cell of *S. chacoense* and cultivated potato and that they had an intermediate biomass accumulation. The results support the previous reports of intermediate morphology of somatic hybrids between wild relatives and potato crop [44–46].

As an effect of drought stress, all of the analyzed plants, excluding DKS10.13, accumulated an average of 2.68 times less biomass (ANOVA, $p < 0.05$) than the control ones. Obidiegwu et al. [47] obtained similar results: drought negatively affected plant development, which resulted in reduced foliage extension and decreased tubers yield and quality. Water deficit had negative effects on DKS10.13 as well, but this genotype was able to catch up to the biomass level of the control plants until the sixth week of the investigation (ANOVA, $p = 0.122$). Therefore, this genotype proved to be the most resistant among the analyzed plants (Figure 9).

The smallest difference between the biomasses of the stressed and the control plants was observed in the case of DkDN5.11, DkDN11.24, DkS10.13, and DkS10.40 (Figure 8). These genotypes responded better to drought stress than their parental lines did. Their shoot surface biomass greatly approached the size measured in the case of control plants, which proves their drought-tolerance ability. All of the drought-tolerant plants have a deficiency in their DNA repair system: none of them were from MMR-proficient plant group, which suggests that MMR deficiency increases the mutation rate, contributing to a new drought-tolerance trait.

3.4. Drought Stress Effects on Tuber Production

Drought stress negatively affects the foliar extension of plants, which leads to tuber yield reduction. Not only can the number of tubers be affected, but also other properties, such as their weight, which can be reduced as a consequence of drought condition during their formation [48,49]. Therefore, tuber development and yield can be considered the most economically important drought-tolerance indicator of water stressed plants.

After 6 weeks of water shortage, the tubers of control and stressed plants were harvested and their mean weight determined (Figure 10).

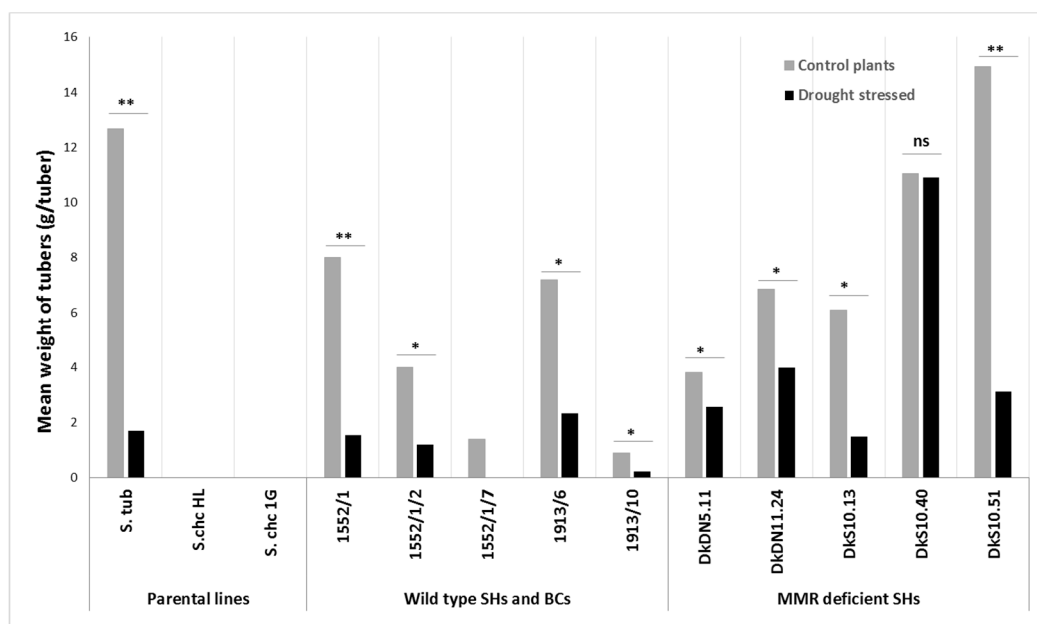


Figure 10. Mean weight of tubers of different genotypes (3 plants/genotype) after six weeks of water shortage in the greenhouse. Symbol * and ** denotes significant differences (* indicates $p < 0.005$, while ** indicates $p < 0.001$) in tuber weight accumulation between stressed and control plants; ns indicates a $p > 0.005$.

S. chacoense HL and 1G did not develop tubers even under control conditions because this *Solanum* species is weaker in tuber formation, and they are more sensitive to artificial conditions (e.g., being grown in pots) compared to the cultivated potato. *S. tuberosum*.DkS10.40 and DkS10.51 developed the largest tubers in control conditions. However, under drought stress, only DkS10.40 was able to develop large tubers (mean weight of 10.906 g per tuber). Only in the case of this hybrid did the mean weights of the tubers not show significant differences compared to the measurements in the case of control plants (t -test, $p = 0.348$) (Figure 10).

Furthermore, in the case of 1913/10, DkDN5.11, and DkDN11.24, a slight decline in tuber weight was recorded during water stress. Unfortunately, even in control conditions, 1913/10 developed only small sized tubers, and therefore this genotype was not considered to be suitable for further breeding programs.

Comparing tuber yield and biomass accumulation changes during water stress, those plants that suffered less from drought and accumulated approximately as much green biomass than control plants were able to develop good quality tubers, while plants with reduced foliar extension developed small-sized tubers. The only exception was DkS10.13, which accumulated the same level of green biomass as the control plants, but the tuber yield was much less. During water shortage, the stressed plants have to invest their energy into shoot development, and therefore other parts of plants grow more weakly. In our case, it resulted in a decline in the quality of tubers. Similar results were obtained by Basu et al. [49]. They showed that potato plants with excised tubers exhibit a higher accumulation of leaf carbohydrates compared to the ones with tubers under drought stress conditions.

After ex vitro stress selection, we can conclude that all of the analyzed genotypes tolerated the induced moderate drought stress. None of them withered during the experiment, but significant differences in biomass accumulation, tuber yield, and quality were observed between drought-sensitive (*S. tuberosum*, *S. chacoense*, 1552/1, 1552/1/2, 1552/1/7, 1913/6, 1913/10, DkS10.51) and drought-tolerant or -resistant (DkDN5.11, DkDN11.24, DkS10.13, DkS10.40) genotypes (ANOVA, $p < 0.05$). The results of ex vitro stress selection correspond to the results of the in vitro experiments. Plants sensitive to water stress in the in vitro conditions were also sensitive in ex vitro stress selection, but among the drought-resistant genotypes from the in vitro experiment, only MMR-deficient genotypes were tolerant to water shortage in the ex vitro experiment. Moreover, biomass accumulation is in accordance with that of the previous morphological variation of MMR-deficient somatic hybrids [28].

3.5. Drought Stress Effects on Photosynthesis

Photosynthesis is the most important biological process of plants, and it is essential in biomass accumulation [49,50]. The first response of plants after drought perception is stomatal closure, which has the role of reducing the transpiration and increasing the water use efficiency [51]. As a result of this defensive mechanism, the intercellular CO₂ concentration is reduced, leading to declined photosynthetic carbon assimilation.

Chlorophyll fluorescence measurements are rapid, non-destructive methods that provide information about the integrity and functionality of the electron transport chain [52]. In our study, plants were exposed ex vitro to long-term moderate drought stress, and its impact on chlorophyll fluorescence was investigated at the beginning of the stress exposure period (2nd week), as well as at the end of the experiment (6th week). After both measurements, the maximal quantum efficiency of stressed plants was not decreased under drought conditions. In all analyzed genotypes, the calculated ratio fell within the optimal range (0.75–0.84), similar to the case of the control (unstressed) plants. Therefore, the induced moderate water stress had no negative effect on the PSII photochemistry on dark-adapted leaves. Usually, the quantum efficiency of PSII is reduced only in the case of drastic water shortage [53]. The experiments conducted by Lu and Zhang [54] also support our findings. They observed that drought does not destabilize the PSII functionality in drought-stressed wheat plants. Jefferies et al. [55] obtained similar results in the case of potato; the F0 and Fv parameters were reduced in drought conditions, but the intensity of fluorescence was not affected.

Photosynthesis occurs in thylakoid membranes, and therefore the integrity of these membranes is essential for the normal, healthy functioning of electron transport. Thylakoid membranes of plants grown in drought condition were unaffected; the Fm/F0 ratio did not decrease to the value of 1, but rather it remained between 4 and 5, which is characteristically the expected value of a healthy plant.

Moreover, the effectiveness of the photosynthesis was evaluated by calculating the performance index, which proved to react more sensitively to drought stress than the maximal quantum efficiency [56]. In the first investigation, the performance index of drought-stressed plants—in most cases—was similar to the values obtained for the control group (except for *S.chc* HL, 1G, 1552/1/2 and DKS10.40). While this index was significantly

higher (t -test, $p < 0.05$) in the second measurements, the only exception was DkD11.24, but in this case an increase was similarly observed. However, this was not significantly higher than the control (Figure 11).

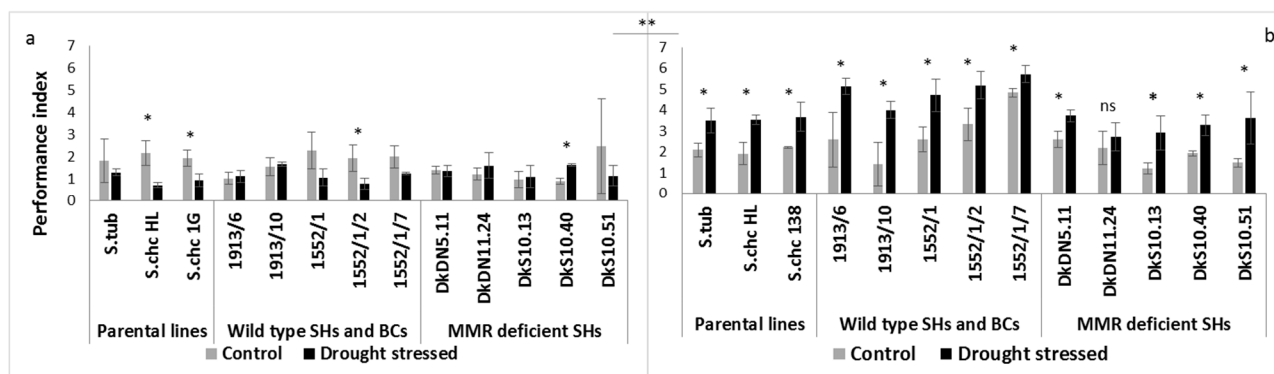


Figure 11. Photosynthesis performance index (PI) (mean \pm SE, $n = 3$) of SHs, BC1 clones, and parental lines (*S. tuberosum* and two accessions of *S. chacoense*—*S. chc*) in control and drought conditions: (a) first fluorescence measurements at two weeks, (b) second measurements on six week old plants. ** indicates significant differences ($p < 0.001$) in the total PI indexes of the first measurements compared to second measurements; * indicates significant differences ($p < 0.05$) between control and drought stressed plants; ns means there was no significant differences ($p > 0.05$) between control and stressed plants.

This index provides a complex image about the entire process of photosynthesis [57] by quantifying the most important steps in the PSII reaction center complex, i.e., light energy absorption, the trapping of the excitation energy, and the conversion and transmission of this energy to the electron transport chain [52]. The increase of the PI index in the second measurements shows that the analyzed plants were able to adapt, with time, to the applied moderate drought stress (Figure 11b).

In the next step, the light-adapted values of initial and maximal fluorescence were used to determine the effective quantum yield (F_v'/F_m'), providing information about the effectiveness of light energy transformation into chemical energy [58] (Figure 12).

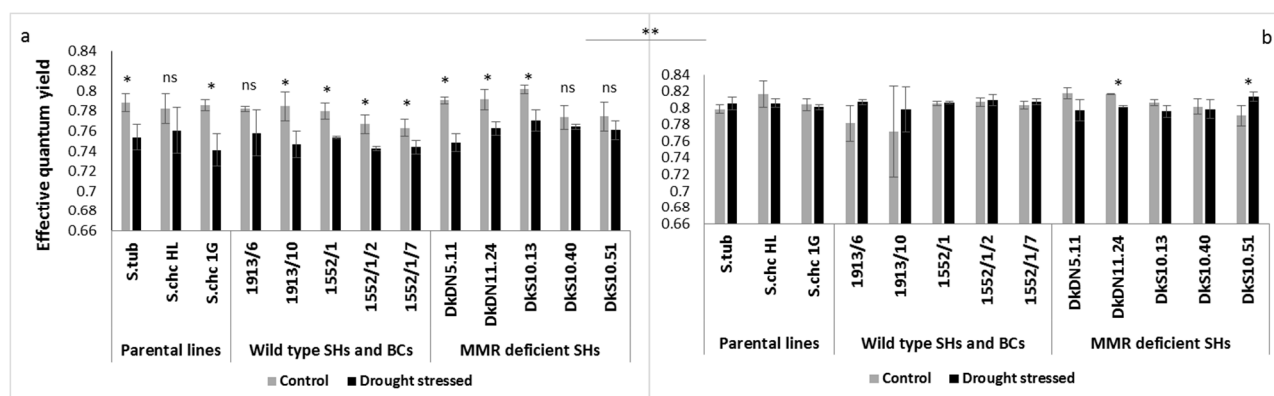


Figure 12. Effective quantum yield (mean \pm SE, $n = 3$) of SHs, BC1 clones, and parental lines in control and drought conditions: (a) first fluorescence measurements, (b) second measurements. ** indicates significant differences ($p < 0.001$) in the total PI indexes of the first measurements compared to second measurements; * denotes significant differences ($p < 0.05$) between control and drought stressed plants; ns means no significant differences ($p > 0.05$) between control and stressed plants.

After the first measurements, a significant decrease of F_v'/F_m' rates in drought stressed plants was observed (Figure 12a), which can be explained by increased light-induced, non-photochemical quenching or by photochemical quenching such as photorespiration [59]. Both types of mechanisms have the role of dissipating the excess of

non-radiative energy in the PSII system, thereby providing protection against photodamage of the electron transfer chain [60]. In the second measurements, the effective quantum yield of stressed plants in almost all cases (except for DkDN11.24 and DkS10.51) approached the F_v'/F_m' rates measured in the case of the control plants; no significant differences (t -test, $p > 0.05$) between the two experimental groups were observed (Figure 11b). The obtained results can be explained by the fact that the plants successfully adapted to the induced drought condition and were able to utilize more effectively the energy of light during the CO_2 assimilation process, compared to the earlier stage.

Overall, the induced moderate drought conditions did not affect the maximum quantum yield of photosynthesis, which proves that the PSII was not damaged. The increased performance index (PI) values during the second measurements suggests that the stressed plants were able to adapt with time to the moderate drought condition.

4. Conclusions

The application of the in vitro and ex vitro stress selection proved to be an effective method for selecting moderate and/or severe drought- and salt-tolerant *Solanum* somatic hybrid genotypes. This stress selection was based on the evaluation of morphological traits and proline content of stressed plants. Parental lines were sensitive to water shortage; despite this, drought-tolerant SHs with or without MMR deficiency and BC clones were observed. A higher proportion of MMR-deficient SHs tolerated the induced severe drought stress more effectively than *Solanum* genotypes without MMR deficiency.

Based on the obtained results, we concluded that somatic hybrids between *S. tuberosum* and *S. chacoense* with or without MMR deficiency and their BC progenies represent a valuable plant material for potato breeders and also for experimental biologists. The unique character of these genotypes lies in the fact that these plants were obtained by the fusion of two genetically different plant cells, which causes mixing of the genetic material of the parent lines in the SHs. In this work, we used an approach of combining different biotechnological tools (also termed as combinatorial biotechnology [61,62]); specifically, we combined protoplast electrofusion and genetic transformation for MMR deficiency to increase the possibility of integrating stress-resistance genes into the potato gene pool, the consequently producing these SHs and BCs [28,63,64]. Overall, the results suggest that the somatic hybridization process and MMR deficiency influenced the development of new traits in the somatic hybrids.

Amongst drought-tolerant genotypes, several proved to be resistant also to the Colorado potato beetle attack (CPB) [32]. This result reveals a new role of the concept of combinatorial biotechnology because this strategy may be important in the formation of new resistance properties during somatic hybridization processes. A total of 6 SHs with and 1 without MMR deficiency, and 1 BC₁ line could be selected with a combined multiple resistance to the biotic (CPB) and abiotic traits, i.e., drought and salt (these are marked with * in Table 2). These genotypes might also be resistant to other stress factors because plants use the same signaling pathways for activating defense responses. For example, exposure of the plants to a mild stress triggers immune responses and enhances their resistance against a range of stress factors [65]. These results open new possibilities for the integration in breeding programs of resistant or tolerant somatic hybrid clones.

Supplementary Materials: The following are available online at <https://www.mdpi.com/article/10.3390/agriculture11080696/s1>, Table S1: Classification of parental lines, SHs and BC clones by morphological appearance after moderate drought stress. *S. tub* = *S. tuberosum* cv. Delikat, *S. chc* = *S. chacoense*. Ploidy level is indicated in parenthesis, Table 2: Classification of parental lines, SHs and BC clones by morphological appearance after severe drought stress. *S. tub* = *S. tuberosum* cv. Delikat, *S. chc* = *S. chacoense*. Ploidy level is indicated in parenthesis.

Author Contributions: E.R.-T. was involved in planning and supervised the project. I.M. and L.C. carried out the in vitro experiments. I.M. performed the ex vitro experiments and I.V. supervised the work. I.M., T.-É.D. and I.-Z.V. performed the photosynthesis measurements. I.M. performed the

calculations and wrote the manuscript. All authors discussed the results and contributed to the final format of manuscript. All authors have read and agreed to the published version of the manuscript.

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