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Communication

In Vitro Assessment of Salt Stress Tolerance in Wild Potato Species

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Abstract: Proof of concept salt tolerance and plasticity. Wild germplasm may represent a precious source of genetic variability for salt tolerance. This study evaluated the morphological changes occurring under controlled and saline conditions in tuber-bearing *S. bulbocastanum*, *S. commersonii*, *S. chomatophyllum*, *S. multidissectum*, *S. pinnactisectum*, *S. phureja*, and cultivated *S. tuberosum*. An in vitro screening method was employed. Significant phenotypic variations were observed for all phenotypic traits analyzed at all NaCl levels (0, 40, 60, and 120 mM). In addition, a significant correlation between root plasticity and salt tolerance was found. Further, changes in proline and total phenolic content were assessed to envisage the metabolic adjustments of tolerant clones towards salinity. The most promising sources of tolerance were identified in *S. commersonii* and *S. multidissectum* and information obtained is discussed from a breeding perspective.

Keywords: salinity stress; root plasticity; salt tolerance index

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1. Introduction

Abiotic stresses, such as salinity, drought, high temperature, and flooding, severely threaten agriculture. They account for significant crop yield losses worldwide that can reach very high thresholds [1]. Soil salinization in particular is reducing total arable land worldwide, with severe consequences for food security. Given the seriousness of such a problem, research is increasingly pushing toward developing salt-tolerant plant varieties through breeding [2]. A critical step to accomplish such a goal is understanding the mechanisms of plant adaptation to this stress. Recent studies proved that developmental plasticity is a critically important plant ability to adapt to and cope with environmental changes [3]. When it occurs in crops in response to environmental signals, breeders need to target such a trait to increase agricultural productivity [4].

Under salt-affected soils, root systems can exhibit enormous plasticity and promote biomass, morphology, and/or physiology modifications to mitigate the impact of stress and maintain greater plant productivity [5]. Due to the limited genetic diversity within cultivated germplasm, wild relatives have been identified as potential sources of salt tolerance in several crops, such as rice, barley, wheat, tomato, and potato [6–10]. Despite current understanding, the research is not substantial. Furthermore, given the demonstrated involvement of radical plasticity in the salinity response, it is crucial to investigate this response mechanism in wild potato species. Identification of a source of tolerance would open new possibilities for both conventional and molecular breeding strategies.

Among major crops, the potato (*Solanum tuberosum* L.), ranks third after rice and wheat in terms of human consumption. Over a billion people eat potatoes, and the global total crop

production exceeds 300 million tonnes [11]. The potato is intensively irrigated in many semi-arid and arid regions, which may cause major salinity problems. Although *S. tubersoum* is classified as moderately sensitive to salinity, its growth and yield are markedly influenced by salinity stress [12]. Salinity drastically affects tuber quality parameters making them unsuitable for processing and consumption [13].

Leveraging the untapped diversity of wild potato relatives offers excellent potential to develop tolerant varieties. The section Petota of the genus Solanum includes approximately 200 tuber-bearing species [14–16]. Most of them are diploid (2n = 2x = 24), and others have a somatic chromosome complement ranging from 2n = 3x = 36 to 2n = 6x = 72. The cultivated potato *S. tuberosum* is a tetraploid (2n = 4x = 48). Due to their extensive geographical and ecological adaptation range, wild potato species have often developed strong tolerance to the major threats affecting potato cultivation, such as late blight (sources of tolerance have been found in *S. bulbocastanum*, *S. demissum*, *S. chacoense*, and *S. phureja*), PVX, PVY, PLRV (in *S. commersonii*, *S. chacoense*, *S. demissum*, and *S. acaule*), bacterial disease (in *S. commersonii*, *S. chacoense,* and *S. yungasense*), and cold (*S. commersonii* and *S. acaule*) [17–21]. However, despite their potential significance in breeding efforts, only a few reports have been published on exploring salinity tolerance among wild potatoes.

In this research, we screened wild potato germplasm to identify promising salt-tolerant clones to be introduced in pre-breeding programs and study the involvement of root plasticity in their response to increased levels of salt stress. Currently, several in vivo and in vitro methods are available to assess salt stress tolerance in plants. Among the former methods, field trials and controlled environment experiments (e.g., greenhouse and growth chambers involving pots or hydroponic) are often used [22,23]. Regarding the in vitro techniques, several effective methods for assessing stress tolerance have already been reported, such as cell and apical cultures through micropropagation. In this work, we employed an in vitro screening approach that owns several advantages over in vivo methods, including fast evaluation of a large number of clones in a reduced space and controlled environment free from confounding variables inherent in field conditions [10,13,24,25]. Notably, the high association between in vitro and field trials in stress response assessments has been reported [26,27]. Data obtained allowed us to identify sources of tolerance and make hypotheses on their tolerance mechanisms, finding a high correlation between salt resistance and the plasticity of the root system.

2. Materials and Methods

2.1. Plant Material

The plant material was selected based on previous knowledge, mainly related to their stress tolerance [14,18]. Plant materials included two *S. tuberosum* cultivated varieties (Désirée and Tasso, with Désirée tolerant control as reported by several authors [23,28], and two clones of *S. phureja* Juz. et Buk. (S. Ph. DM1-3-516-R44 and 85/16), and six clones from five wild potato species, namely, *S. bulbocastanum* Dunal (BLB1C), *S. commersonii* Dunal (CMM1T, CMM6-6), *S. chomatophyllum* Bitter (CHM1B), *S. multidissectum* Hawkes (MLT1A), and *S. pinnactisectum* Dunal (PNT04). The Inter-regional Potato Introduction Project (IR-1) provided wild species as true-seed, (Sturgeon Bay, WI, USA). DM1-3-516-R44 (hereafter S. Ph. DM) was provided by the James Hutton Institute (Dundee, Scotland). S. Ph 85/16 (GRIN No. 07S0300152), held in work collection in vitro of Dept. of Genetics and Breeding of FAFNR CZU Prague, was obtained from Gene Bank in vitro of Potato Research Institute Ltd. In Havlíčkův Brod (Czechia). The potato varieties were available at the Department of Agricultural Sciences of the University of Naples Federico II (Portici, Italy). Additional details of plant materials used are reported in Table S1. Plants of each clone were maintained in vitro on Murashige and Skoog (MS) medium (Sigma-Aldrich, St. Louis, Missouri, United States. http://www.sigmaaldrich.com, accessed on 1 January 2023) with 3% (*w*/*v*) sucrose and 0.8% (*w*/*v*) agar at 24 °C with an Irradiance of 200 µmol m^2 s^{-1}, under a 16/8 h (light/dark) photoperiod.

2.2. Salinity Tolerance Evaluation

Plant materials were screened for salt tolerance, growing them in vitro on an MS medium supplemented with various concentrations of NaCl, according to [28], with some modifications. Briefly, plantlets were maintained in vitro for up to three weeks before being used in the experiment. At that time, for each clone, five 1.0–1.5 cm long stem cuttings—with one auxiliary bud—were laid out in plastic squared Petri dishes 12 × 12 cm with 50 mL of MS medium containing the following four NaCl levels: 0, 40, 60, and 120 mM (Figure S2). Petri dishes were placed at a 45 degrees inclination to facilitate the positive geotropism of the roots. Cultures were incubated at 25 ± 2 °C for four weeks with an irradiance of 200 µmol m^2 s^{-1} under a 16/8 h (light/dark) photoperiod. In total, 40 explants (10 replicates of each treatment) in 8 Petri dishes were used for each clone. The experiment was designed as a completely randomized design [29] and replicated three times.

2.3. Phenotyping

To observe the stress-induced effect, shoot and root lengths were measured every five days. After four weeks, all in vitro plantlets were washed in distilled water to remove media, dried on filter paper, and separated into shoots and roots. Based on Gelmesa et al. (2017) and Zaman et al. (2015) [28,30], the evaluated traits were:

- Shoot height, SH (mm): the length of the main stem from the base to the tip of the plantlet;
- Root length, RL (mm): the maximum length of the root produced per plantlet;
- Leaf number, LN: the total number of new leaves produced by each plantlet;
- Root number, RN: the total number of roots (all types of roots) produced at approximately one cm from the basal tip of the plantlet;
- Total fresh weight, TFW (mg): plantlet weight;
- Root fresh weight, RFW (mg): root weight;
- Shoot fresh weight, SFW (mg): shoot weight;
- Root dry weight, RDW (mg): root weight after freeze-drying;
- Shoot dry weight, SDW (mg): shoot weight after freeze-drying;
- Total dry weight, TDW (mg): plantlet weight after freeze-drying;
- Days of roots (DR) and shoots (DS) emission: number of roots and shoots differentiated from the beginning of the salt stress.

For a better understanding of the collected data, we have calculated a salinity tolerance index (STI), which is a tolerance score for each of the traits mentioned above as the ratio between the trait performance at 40, 60, and 120 mM NaCl over the trait performance at 0 mM NaCl for each parameter [24,31]. Successively, to evaluate the clone performance for all tolerance traits analyzed, an evaluation index (EI) was calculated as the sum of all STI values according to the following formula: $EI = \Sigma\ STI_x$, where x is the trait described above. To observe the stress adaptation of the root system, the root mass ratio (RMR) was measured as described by [32]: RMR = root weight/(shoot weight + root weight). Then, the root plasticity was calculated using the following equation: root plasticity = $RMR_{stress}/RMR_{control}$.

2.4. Determination of Proline and Total Phenol Content

Lyophilized powdered samples (20 mg) of expanded leaves from five in vitro plantlets per each clone were mixed with 1 mL of ethanol:water in the ratio 40:60 (*v*/*v*), incubated overnight at 4 °C and centrifuged at 14,000× *g* for 10 min at 4 °C. The supernatants were pooled and used for the analyses. Proline was determined according to a procedure previously described by [33]. Total phenols content was determined using the Folin–Ciocalteu (FC) colorimetric method, according to [34]. TPC content was estimated from the calibration curve of Gallic acid (GA) (range = 0.02–2 mg/mL^{-1}, seven levels; Abs 0.0006 × µg·GA/mL + 0.0392; R^2 = 0.9999) used as the reference standard. The results were expressed as GA equivalents (mg GAE) per gram in dry weight.

2.5. Data Analysis

Pearson correlation, Student's *t*-test, analysis of variance (ANOVA), and Duncan multiple range test were conducted using the SPSS computer package [10].

3. Results

3.1. Evaluation Index (EI)

Individual STI values derived from the 12 parameters measured (SH, RL, LN, RN, FW, SDW, SFW, RDW, TDW, DW, DR, and DS) were calculated for each clone (Table S2), and the EI computed (Figure 1A–C) to classify the clones. For each saline concentration, clones with above-average EI were classified as tolerant. At 40 mM, the average EI (EIavg) was 9.08, ranging from 5.83 (S. Ph. DM) to 12.09 (CMM1T) (Figure 1A). At this concentration, the most tolerant clones (EI > 9.08) were CMM1T (12.09), MLT1A (11.04), CMM6-6 (9.60), and Désirée (9.50). These clones were classified as tolerant at 40 mM NaCl concentration. At 60 mM, the EIavg (Figure 1B) was 9.46, ranging from 5.75 (S. Ph. 85/16) to 13.00 (MLT1A). At this concentration, clones displaying the highest tolerance (EI > 9.46) were MLT1A (13.00), CMM1T (11.52), Désirée (11.36), CMM6-6 (10.92), and BLB1C (9.98). These clones were classified as tolerant at 60 mM NaCl concentration. At 120 mM, all STIs dropped dramatically down (Table S2). As a consequence, also the EIs fell significantly, with an average value of 2.94 (Figure 1C). Despite the strong impact of the high salt concentration, CMM6-6 (5.60) and MLT1A (5.49) performed better than all other clones. Therefore, these clones were classified as tolerant at 120 mM NaCl concentration.

3.2. Root Plasticity and Correlation Analyses

By applying the formula by Reddy et al. (1982) [31], we calculated the root plasticity and found a general decrease in response to salt treatment (Figure S1). There was no significant difference between clones analyzed between the 40 and 60 mM levels. Only clones S. Ph 85/16 and S. Ph DM showed an increase in this trait, although not significant, from 40 to 60 mM NaCl concentration. Moreover, at 60 mM, 50% of them did not root at all (root plasticity = 0), while others developed short roots and no shoots (high root plasticity). This behavior was not observed in tolerant clones, which developed both shoots and roots, and their root plasticity values were high with low data spread around the mean (low SE). From our point of view, the high variability observed in S. Ph DM, S. Ph 85/16, and BLB1C further outlines a different behavioral pattern between tolerant and susceptible clones. At a higher salinity level (120 mM), all clones showed a significant decrease in root plasticity. For each salt concentration, the differences among the tolerant clones identified here (CMM1T, CMM6-6, and MLT1A) and the tolerant control Désirée were evaluated to characterize better their diversified response (Figure 2). At the salt concentration of 40 mM, the differences in root plasticity level were significant ($p < 0.01$) among the clones CMM1T and CMM6-6. At 60 mM, the differences were significant ($p < 0.01$) between CMM1T and CMM6-6, CMM1T, and MLT1A. Finally, at 120 mM, the differences were significant ($p < 0.05$) between CMM6-6 and CMM1T, CMM6-6, and Désirée, CMM1T and MLT1A. At this concentration, the differences in root plasticity were significant ($p < 0.01$) between MLT1A and Désirée. A scatterplot was built to understand the relationship between EI and root plasticity (Figure 3). The most tolerant clones with a high EI (*y*-axis) were located at the top right of the plot. In contrast, the most susceptible ones were at the bottom right. EI was positively correlated with root plasticity ($R^2 = 0.750$), and the linear regression model was significant for $p < 0.01$ (Table S3).

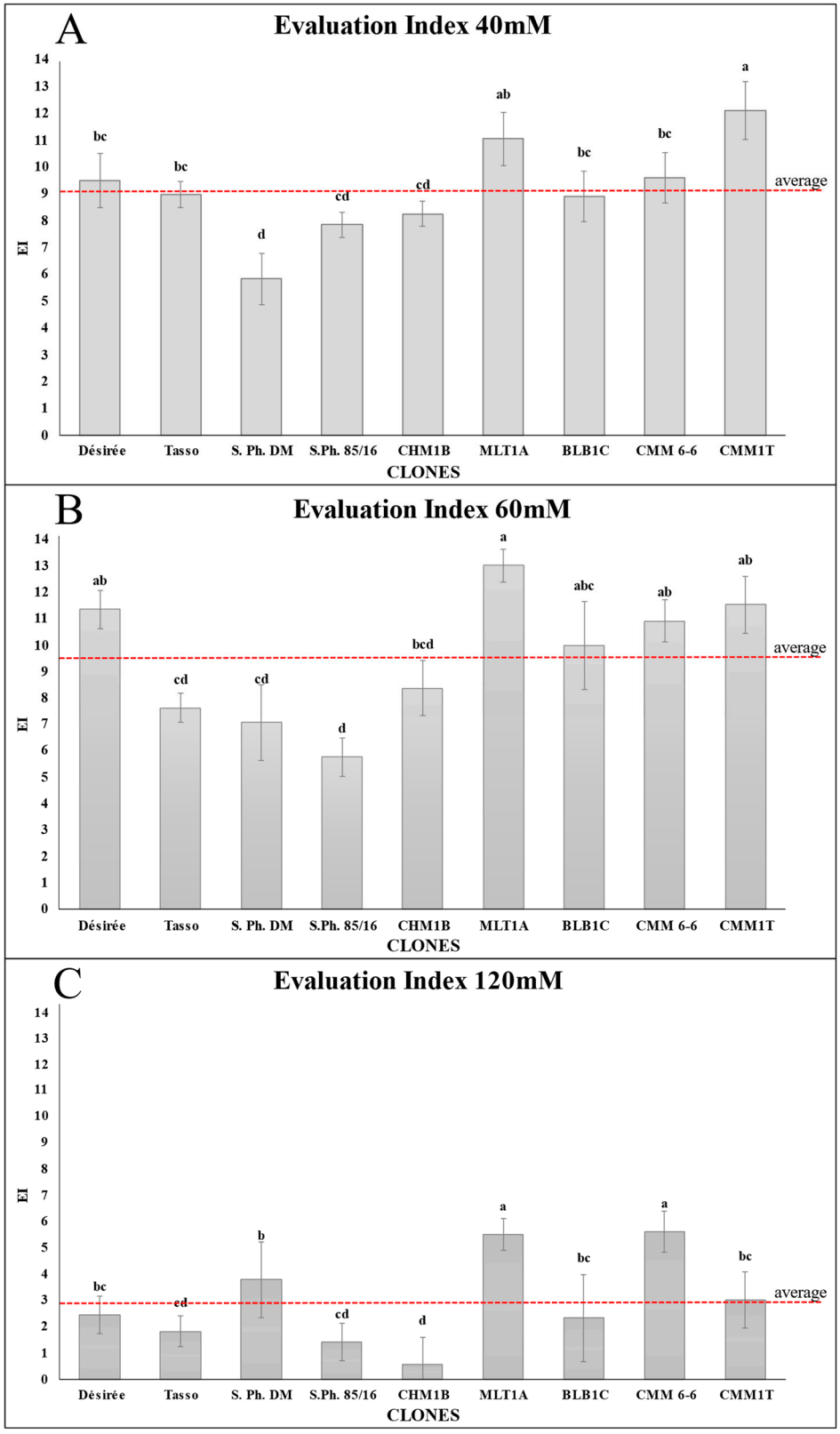

Figure 1. Evaluation index (EI) of nine potato clones at each salt concentration. EI was calculated for each salt concentration according to the formula: EI = Σ STI_x, where x is one of the 12 phenotypic parameters evaluated (see Section 2). The red line indicates the average value of all clones. Data were analyzed by two-way analysis of variance following Duncan's test. Error bars with different letters represent a statistical difference ($p < 0.05$, Duncan's test).

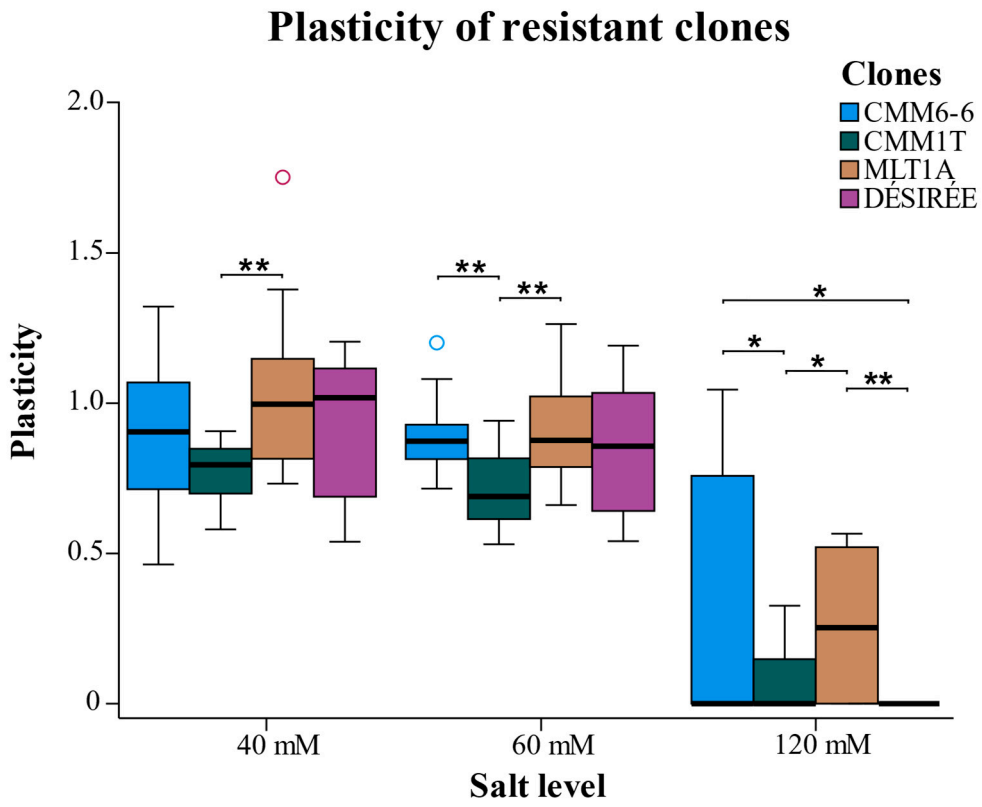

Figure 2. BoxPlots of salinity-resistant clones. BoxPlots shows the levels of plasticity of three selected tolerant clones and the control Désirée at each salt concentration. Bold asterisks indicate the significant differences between clones determined by the Student's *t*-test (* $p < 0.05$, ** $p < 0.01$). Purple and blue circles represent outliers.

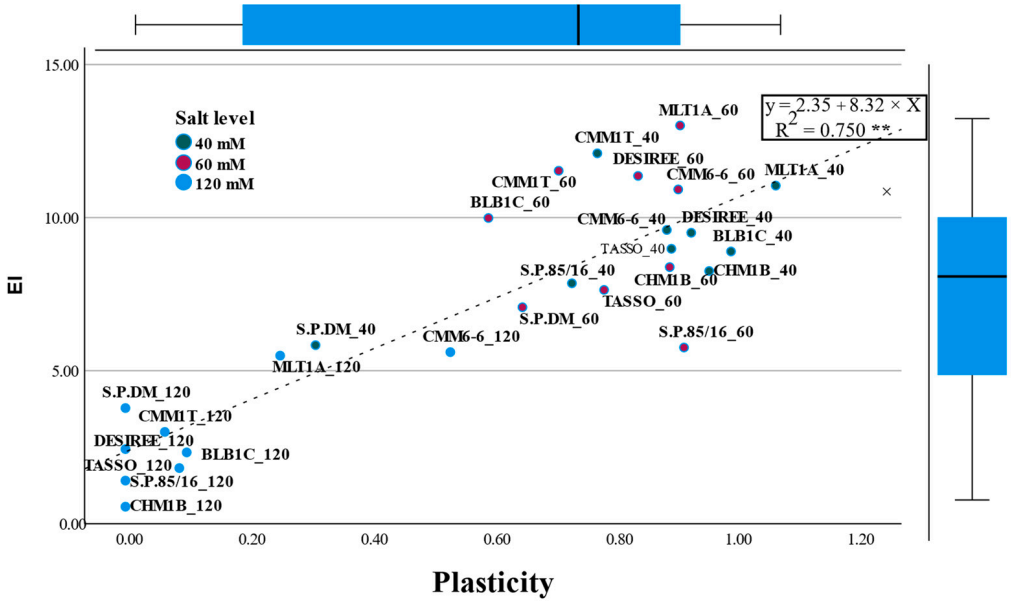

Figure 3. Scatter plot showing the correlation between evaluation index (EI) and plasticity. The different salt concentrations are identified by different colors. Data were analyzed by linear regression; the coefficient of determination R^2 shows the correlation between EI and plasticity. Bold asterisks indicate significant differences (** $p < 0.01$). Blue bars provide a summary of the variability of dataset values. They show the median, the upper and lower quartiles, the minimum, and the maximum values.

3.3. Proline and Total Phenol Content

The analysis of the proline and total phenol content was carried out on three tolerant clones (CMM1T; CMM6-6; MLT1A) and the control Désirée (Figures 4 and 5). It was observed that the increase in salt concentration caused a significant increase in proline content in CMM1T, MLT1A, and Désirée; in CMM6-6 this increase was not significant. The analysis of the total phenol content showed that the increase in salt concentration caused a significant increase in total phenol content in MLT1A and Désirée.

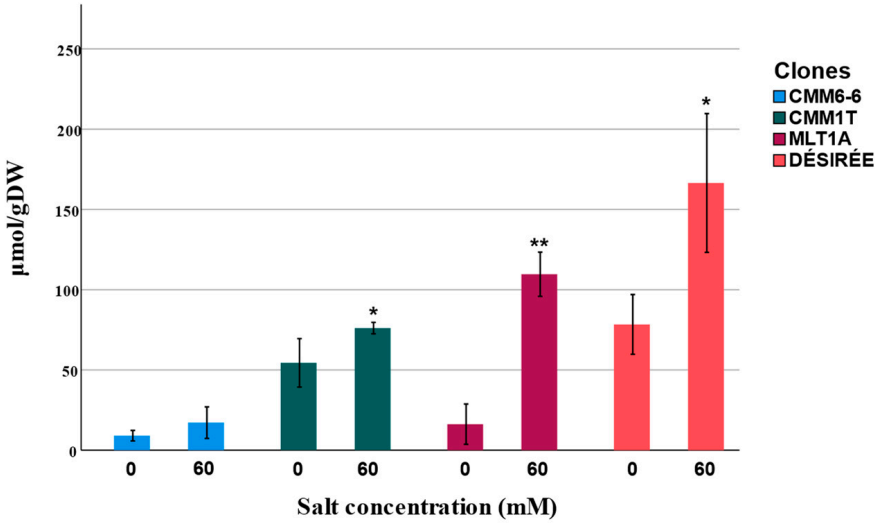

Figure 4. Proline content of three selected tolerant clones and the control Désirée at 0 mM NaCl and 60 mM NaCl. Bars represent the standard error, while bold asterisks indicate the significant differences between clones determined by the Student's *t*-test (* $p < 0.05$, ** $p < 0.01$).

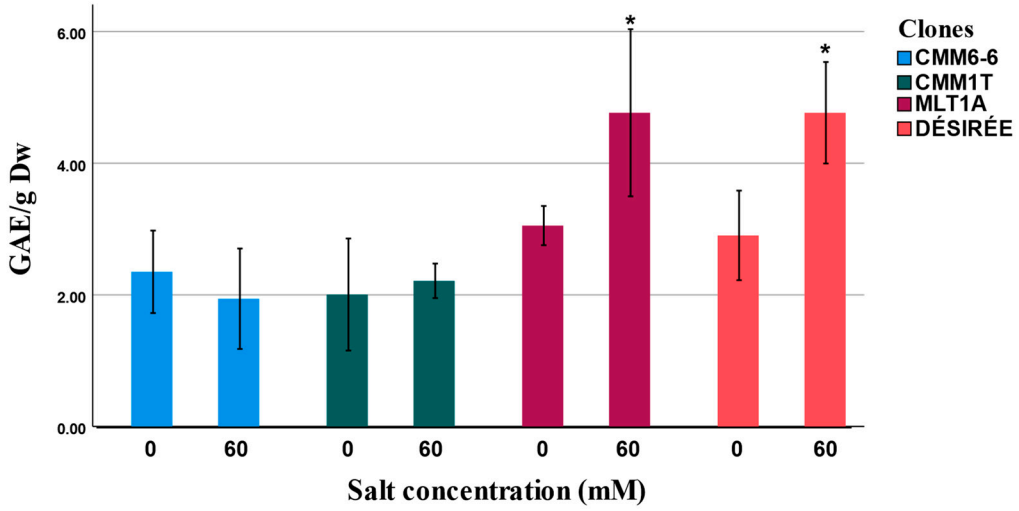

Figure 5. TPC (total phenol content) of three selected tolerant clones and the control Désirée at 0 and 60 mM NaCl. Bars represent the standard error, while bold asterisks indicate the significant differences between clones determined by the Student's *t*-test (* $p < 0.05$). GAE (Gallic acid equivalents).

4. Discussion

We studied a set of wild potato species to ascertain their phenotypic variability concerning salt tolerance, one of the most important potato production. Toward this goal, we used an in vitro screening method previously employed for potato germplasm evaluation and proved effective and reproducible in detecting subtle differences among clones [25,28,30]. A decrease in the number of roots and leaves and the diameter and length of stems and roots have been observed in our salt-stressed plants. The same response was also reported in

other species [29], where symptoms of salt toxicity have been reported to affect both plant roots and apices. To identify the most promising clones, we calculated a tolerance index (EI) that allowed us to identify three tolerant clones, namely, the MLT1A clone of *S. multidissectum* and clones CMM1T and CMM 6-6 of *S. commersonii*. They showed above-average EI values at all saline concentrations and significantly higher STI than susceptible clones. The lack of reduction of STI may be linked to the well-known ability of wild germplasm to adapt better to environmental constraints. Within the potato tuber-bearing relatives, sources of salt-stress tolerance have been identified by [10] in *S. acaule* Bitt., a sexually-isolated potato relative. Among the three tolerant clones, proline increased significantly in CMM1T and MLT1A, but to a lower extent as compared to Désirée. Total phenols content raised only in MLT1A in a similar trend as for Désirée. Based on the metabolic investments upon stress, our results suggest that MLT1A might share a metabolic behavior to salinity similar to Désirée. By contrast, CMM clones showing a more moderate response to salinity can either have a wider constitutive reservoir of molecular and metabolic resources to face stress or could perceive salinity stress more moderately.

In tuber crops, such as the potato, it has been reported that salt tolerance is linked to a variation in the relative growth rate of roots compared to shoots [35]. Therefore, we tested whether the salinity stress affected the root plasticity of our samples. Overall, at all salt levels tested, we observed a significant diversified response in root plasticity among species and between clones of the same species (i.e., *S. commersonii*). The three most susceptible clones, S. Ph. DM; S. Ph. 85/16, and BLB1C, demonstrated significant variability at the individual level (Figure S1). At 60 mM, 50% of these clones did not root at all (root plasticity = 0), while the surviving plants exhibited a high root-to-shoot ratio in response to salinity. Interestingly, this behavior was not observed in tolerant clones. Other authors also observed this diversified response to salt stress adaptation. In *S. tuberosum*, [29] reported an increase in root length at moderate saline stress (25 and 50 mM). Recently, a maximum root dry weight was recorded in the tolerant potato genotypes CIP112 and CIP117 under different concentrations of NaCl [36]. The importance of root:shoot ratio modulation in salt stress response has also been reported in other species, such as tomatoes [24]. The authors found that root fresh weight increased with salt concentrations in *S. peruvianum* and *S. pimpinellifolium.* Root plasticity plays a significant role in the salt resistance of adult plants. The ability of roots to adapt to environmental conditions is crucial for water and nutrient absorption, regulation of salt accumulation, reduction of oxidative stress, and adaptation to environmental variability. Plants with more remarkable root plasticity have an advantage in survival and growth in saline environments. We found a highly significant correlation between root plasticity and tolerance to salinity, suggesting that in the wild potato species we tested, salinity tolerance may be related to the ability of the root system to adapt to environmental constraints. Further research is needed to determine the mechanisms underlying root:shoot ratio regulation under salinity stress.

Overall, we found new sources of tolerance using an arbitrary evaluation index of resistance (EI). We think that it may be employed in pre-breeding programs to evaluate and select noteworthy germplasm for additional tolerance traits in potatoes and other crops. Our study also allowed us to hypothesize different strategies of response to salt stress between species and clones of the same species (i.e., *S. commersonii*). This latter finding is most likely related to the high heterozygosity of outcrossing *Solanum* species and the allelic and genetic diversity of loci [37]. It is worth highlighting that most of the tolerant wild potato species we found have additional noteworthy traits. For example, CMM1T combines tolerance to multiple stresses, such as cold, tuber soft rot, bacteria wilt, and PVY [18,38]. Such multiple stress tolerance is a major challenge for most crops [39]. Introgression of stress-tolerance traits from our clones into elite cultivars via genetic crosses can represent a suitable strategy to exploit this germplasm. For example, MLT1A is cross-compatible with *S. tuberosum* haploids and can be used in what is known as analytic breeding [40]. CMM1T and CMM6-6 are not directly crossable with *S. tuberosum* haploids. They should be used in breeding strategies based on bridge crosses [41]. In addition, the

genome sequences of tuber-bearing potato species are becoming available, opening an exciting new era for mapping and identifying genes, dissection of stress resilience and recovery mechanisms, and downstream varietal development. In this regard, the published genomes of *S. tuberosum* [42–46] and those of some wild potato relatives [47], including *S. commersonii* [48], offer an outstanding resource to access tolerance genetic determinants and help scientists to convert successful research into practical applications through the exploitation of the most recent genome editing technologies.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/agronomy13071784/s1, Table S1: Plant materials assessed for salinity tolerance. Table S2: Salinity tolerance index (STI) derived from 12 parameters measured on nine clones grown at three NaCl concentrations (40, 60, 120 mM). Leaf number (LN), Root number (RN), Shoots fresh weight (SFW), Shoots dry weight (SDW), Roots fresh weight (RFW), Roots dry weight (RDW), day of shoot (DS), Day of root (DR), Total fresh weight (TFW), Total dry weight (TDW), Shoot height (SH), Root length (RL). Figure S1: Root Plasticity of nine clones for each salt concentration, calculated as the ratio between the trait performance at 40, 60, 120 mM over the trait performance at 0 mM NaCl. Outliers are identified by different shapes and colors (blue dot = 40 mM; green dot = 60 mM; purple asterisk = 120 mM). Means denoted by the same letter did not significantly differ at $p < 0.05$ according to the Duncan's multiple range test. Table S3: The linear regression model was constructed between Root Plasticity data and the EI values of all nine clones at each salinity level tested. Figure S2: Plants after 30 days of growth laid out in plastic squared Petri dishes 12 × 12 cm with 50 mL of MS medium containing 0, 40, 60, and 120 mM of NaCl.

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Data Availability Statement: Data is contained within the article and Supplementary Material.

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