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Physiological, Biochemical and Molecular Characterization of Potato (*Solanum tuberosum* L.*)* Genotypes under Salt Stress

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Abstract

Soil salinity is an alarming and an escalating threat to potato cultivation along Pakistan's Indus basin. It has burdened by millions of salt-affected hectares, making it most severely affected region globally. To address this challenge, five potato genotypes including 3 cultivars (Favorita, PRI-Red and Hermes) and 2 advance breeding lines (LS5 and GN3) were screened in *in-vitro* conditions. Seven NaCl concentration (0-140Millimolar) were assessed to record and evaluate the morpho-physiological parameters (plant height, root length, number of nodes, internode distance, fresh weight, dry weight), biochemical assays (chlorophyll content, proline content, Na^+ content, K^+ content, flavonoid and phenolics content) and nine SSR markers. At 140mM threshold, PRI-Red, GN3 and LS5 experienced severe degradation, lost 70-92% of their growth traits relative to control. However, Favorita and Hermes proved highly tolerant by maintaining superior growth and successfully regulated ion homeostasis, keeping their Na^+/K^+ ratio at or below 1.4 compared to the 9.5-26.5 ratio observed in the sensitive lines. Multivariate evaluations, including Principal Component Analysis (PCA analysis), correlation analysis and hierarchical clustering consistently divide these genotypes tolerant to sensitive. At molecular level 4 out of 9 markers proved polymorphic SSR markers, two of which (STM1053, STM2020) amplified in tolerant Favorita and Hermes perfectly following the phenotypical pattern. Favorite and Hermes emerge as a promising salt tolerant genotypes phenotypically and genotypically for future salt tolerance breeding programs. Furthermore, the strong alignment between diverse parameters shows that multi trait composite index provides a significantly more robust ranking for tolerance level than any single trait. Favorita and Hermes highlight as the most promising parental candidates for breeding programs to develop salt tolerant potato germplasm.

Keyword: Potato ·Salinity stress ·*in-vitro* screening ·Proline ·Stress tolerance index ·SSR markers

1 Introduction

Potato (*Solanum tuberosum* L.*)* ranked fourth staple crop after rice, wheat and maize globally, and it give more amount of edible energy and protein per unit of land and water than any of three crops (1, 2). Over the past three decades, its cultivation has expanded well above its traditional temperate range and much of that increase in production happen in South and Southeast Asia, where potato increasingly competes for the same irrigated land as rice and wheat (3, 4). In Pakistan, the potato crop has moved from a minor vegetable crop to a major cash crop over the last three decades, grown mostly under tube-well and canal irrigation system in Punjab, already carry heavy and significant salt loads across large areas of the Indus Basin.

Salinity stands one of the major global threats to irrigated soil which is impacting crop production. FAO stats indicated that several million hectares of global soil are currently affected by salinity, and this area continuously growing. This expansion is driven by a combination of a poor drainage system, sea water invading low-lying coastal deltas, excessive irrigational use of groundwater and fertilizers (5, 6). This condition impacts the several million hectors of Pakistan's irrigated agricultural land. Furthermore, potato crop is more sensitive to salinity than the other cereal crops (7). A similar trend occurs in the salinity impacted deltas of Bangladesh, where reduced potato yields due to soil salinity now pose a major threat to smallholder food security (8).

Salinity adversely impacts plants in two overlapping stages: an immediate osmotic phase and a slower ionic phase. During osmatic phase, high salt level makes it harder for roots to absorb water which instantly slower down cell growth and expansion. During ionic phase, toxic sodium Na^+ accumulate in the plant's leaves and shoots which kick out the essential potassium K^+ from its vital role in cell transport and enzyme function. This leads toward the destroying membrane structure and chloroplasts (9, 10). To survive this two-stage attack, salt tolerant genotypes mostly rely on combinations of defense mechanisms by limiting net Na^+ absorption or store it in the vacuole and maintain the K^+ levels at the plasma membrane. These tolerant genotypes regulate osmatic pressure using compatible solutes like proline and deploying an antioxidant system to control reactive oxygen species triggered by ionic imbalance (11, 12, 13, 14).

In potato crop, these mechanisms are typically documented individually. Yield and growth losses in NaCl stress are well described (15, 16), antioxidant and proline shifts have been measured in several cultivar panels (15, 17) and recent studies reported that the expression of ion-transport genes such as SOS1 and NHX1 is linked to tolerance ranking (18, 19). Two studies (8, 20) reported for comprehensive screening methods that simultaneously can evaluate plant growth, physiology, and biochemistry within a single

experiment. They emphasized that ranking derived on the basis of a single trait category often conflict with those from another, yet the majority of published studies only reported one or two of these trait classes for a given set of genotypes. A multi-level screening study of five Bangladeshi cultivars reported recently 2026, the only study to combine morphological, biochemical and gene-expression data for potato salinity tolerance in a single in vitro experiment (19). This study did not include a maker-based comparison or a composite index built from several independent tolerance formulas, both of which we have used in our study.

Potato germplasm is routinely evaluated and fingerprinted to check the genetic diversity by using simple sequence repeats (SSR) markers (21), with a limited number of studies specifically focusing on identifying SSR loci linked to salt tolerance in potato (22). Studies rarely test whether a marker panel designed for general diversity can also separate genotypes on the basis of their physiological tolerance. Doing this requires evaluating the same genotype for both traits, rather than comparing different germplasm across separate studies.

To address this gap, we have evaluated five potato genotypes including PRI-Red, LS5, GN3, Hermes, Favorita from the National Institute for Genomics and Advanced Biotechnology (NIGAB), Pakistan. These genotypes were chosen to represent a diverse working germplasm collection rather than being pre-classified by their environmental tolerance or sensitivity. To assess salt tolerance, plantlets were grown *in-vitro* across seven NaCl concentrations (0-140Mm) and evaluated using six growth traits, biochemical contents and physiological markers. The plantlets were then ranked using a composite score that integrated correlation, principal component analysis and cluster analysis with four independent salt tolerance indices. To evaluate whether genetic similarity correlates with phenotypic tolerance, we profiled same five genotypes using nine SSR markers. Therefor this study aimed to: (i) quantify the divergence in growth, physiology, and biochemistry among these genotypes under salinity stress. (ii) assess the consistency between the independent tolerance-ranking method and (iii) determine whether this consistency align with a SSR markers result.

2 Martials and Methods

2.1 Plant material and *In vitro* culture establishment

Five potato (*Solanum tuberosum* L) genotypes were obtained from the National Institute for Genomics and Advanced Biotechnology (NIGAB), National Agriculture Research Centre (NARC), Islamabad, Pakistan. These genotypes were included: Favorita, Hermes, LS-5, GN-3, and PRI-Red. Apical meristem were used as an explant, excised from the disease-free potato plants.

The explants were washed under tap water to remove debris and surface-sterilized in a laminar air flow cabinet. Sterilization process involved treatment with 20% (v/v) sodium hypochlorite solution containing 2-3 drops of tween-20 for 15 minutes followed by three to five rinses with autoclaved distilled water. The meristem then transferred to the Murashige and Skoog (MS) basal medium (44) containing vitamins, 30gL^{-1} sucrose, 1mg L^{-1} Gibberellic acid (GA3) and 2.2g L^{-1} gellan gum. The pH was adjusted at 5.8 prior to autoclaving. The cultures were kept in growth room at 23 ± 2 ℃ under a 16 h light/8 h dark photoperiod. The plantlets were subclustered after every six weeks before applying the salinity treatments 4 to 5 subculturing were performed.

2.2 Salinity treatments and experimental design

There were 35 combinations (5 genotypes × 7 NaCl levels) of single node cuttings were transferred to MS medium with different NaCl concentrations (0-control, 40, 60, 80, 100, 120 and 140mM). Three biological replicates were used in each combination (105 experimental units) in a completely randomized design (CRD). These cultures were maintained in growth room under conditions mentioned above.

2.3 Growth traits

After six weeks of stress exposure, different parameters i.e. plant height (cm), root length (cm), number of nodes per plantlet, internodal distance (cm), fresh weight (g) and dry weight (g) were recorded. Fresh weight of each plantlet was recorded immediately after taking out from glass tube. Drying weight was recorded by drying the plant tissue at 70 ℃ until it reached the constant weight.

2.4 Physiological and Biochemical Analysis

Proline content

The proline content was determined according to the method described by (23). Fresh leaf sample of 0.5g was homogenized in 10ml of 3% sulfosalicylic acid and filtered. After that 2ml of filtrate was then added to 2ml ninhydrin and 2ml glacial acetic acid and was heated at 100℃ for 1 hour and then the reaction was stopped by placing it on ice. The chromophore was extracted using 4ml of toluene and its absorbance was measured at 520nm by using spectrophotometer. The unit of proline content was recorded as [µg/ml].

Chlorophyll content

At the end of the 6 weeks of treatment the fully expanded leaves were measured from three different leaves per plant by a SPAD-502 chlorophyll meter (Konica Minolta, Japan) for relative chlorophyll content. The values were given in SPAD units [mg/cm^2].

Na^+/K^+ content

Flame photometry was used to determine the *Na^+/K^+ content.* The dried plant material of 0.5g was digested with nitric perchloric acid. The digests samples were filtered, diluted as necessary and compared with *Na^+ and K^+* standard solutions. The concentrations were recorded in units [mg/kg] and *Na^+/K^+* ratios were determined for each replicate.

Flavonoids and Phenolics content

The total flavonoids contents were measured; Plant extracts reacted with sodium nitrate, aluminum chloride and sodium hydroxide and its absorbance were recorded at 510nm. The units were presented as rutin equivalents [µg/ml]. The total phenolic content was determined by using Folin-Ciocalteu method. Sample absorbance was then recorded at 760nm after mixing with Folin-Ciocalteu and sodium carbonate. The unit was recorded in term of gallic acid equivalents [mg GAE/ml].

2.5 DNA extraction and Molecular (SSR based) analysis

The newly grown leaves were used for the extraction of genomic DNA using Cetyltrimethylammonium bromide (CTAB) method. The quality and integrity of DNA was checked by agarose gel electrophoresis and spectrophotometer. A set of nine SSR markers were used to report salt tolerance including: STM1106, STM0030, STM1031, STM0003, STM1053, STM2020, STM3010, STM 3016 and STM1104.

PCR reactions were performed using 12.5 µl of Green Taq Master Mix, forward and reverse primers, genomic DNA and nuclease free water. PCR product was separated in 3% agarose gel and photographed in gel documentation system. The step of PCR was used as denaturation at 94℃ for 3 minutes followed by 35 cycles of 94℃ for 1 minute, 56℃ for 45 seconds and 72℃ for 45 seconds and 10 minutes final extension at 72℃. The presence of bands was scored as 1 and their absence as 0.

2.6 Statistical analysis

These analyses were performed in R version 4.5.1 (24). Two-way analysis of variance (ANOVA) with genotype, NaCl concentration and their interaction as fixed effects was used to analyse the each of the 13 traits using agricolae package. Normality of the residual was verified in the 35 combinations by Shapiro-Wilk test and homogeneity of variance across the 35 combinations by Levene's test (median centered). Tukey's honestly significant difference (HSD) was used to separate the means at the 0.05 level using agricolae package in R (25). The 35 G×NS means were compared in all groups for each trait. Effect sizes are given as

$$\eta^2 = \frac{SS_{\text{effect}}}{SS_{\text{total}}} \times 100$$

All 105 individual observations were used for calculating Pearson correlation coefficients between pairs of the 13 traits using “corrplot’ package in R. Principal component analysis (PCA) was performed on the correlation matrix of the 35 genotypes × NaCl means (13 standarised traits) using packages “FactoMineR, and factoextra”. Standarised means of the traits were used to define genotypes by Hierarchical Agglomerative Clustering (HAC) which was based on the Euclidian distance and the Ward linkage (26). Each trait was standarised within the genotype, and the heatmap shows these z-scores using “pheatmap” package in R.

The highest salinity level was determined to be the tolerance level by calculating the means of each genotype grown in control (Y_p) and 140mM NaCl (Y_s) conditions. The tolerance ($TOL = Y_p - Y_s$) and mean productivity ($MP = (Y_p + Y_s)/2$) (27) the geometric mean productivity (GMP = $\sqrt{(Y_p \times Y_s)}$), the stress tolerance index ($STI = Y_p \times Y_s/Mp^2$) (28), the stress susceptibility index ($SSI = (1 - Y_s/Y_p)/SI$, with $SI = 1 - M_s/M_p$ (29), the yield index ($YI = Y_s/M_s$) and the yield stability index $(YSI = Y_s/Y_p)$ (30) were calculated using “emmeans” package. Here M_p and M_s are the average of the five genotypes under control and stress conditions respectively. The composite score was calculated using dendextend package in R as the scores of the first principal component and the genotypes were ranked by these scores. Genotypes were then categorized as tolerant, moderately tolerant or sensitive based on their composite scores.

3 Results

3.1 Assumption check

Normality of the residuals against 10 out of the 13 traits were rejected by the Shapiro-Wilk test ($P<0.05$). Leven’s test reveled that variances were homogeneous for all trait except Na^+/K^+ ratio ($P = 0.004$) and phenolic content ($P = 0.038$). The design was balanced, the main effects were very large relative to the residual error, and therefore we reported ANOVA for all traits. The results based on probabilities on the border were interpreted carefully.

3.2 Salinity and Growth responses

Genotype and Salinity level had a highly significant effect ($P < 0.001$) on all six growth traits (Table 1). The highest percentage level of variance for salinity level was observed as; for plant height (55.4%), root length (58.4%), number of nodes (63.8%) and fresh weight (71.8%). Variance in dry weight was best explained by genotype (58.2%) and the two factors accounted for similar proportions of variance in internode distance (genotype 44.2%, salinity 46.5%).

Table 1 Two-way analysis of variance of growth, physiological and biochemical traits of five potato genotypes grown at seven NaCl concentrations *in-vitro*

Trait	Genotype (df = 4)		NaCl level (df = 6)		Genotype × NaCl (df = 24)	
	F	**η² (%)**	***F***	**η² (%)**	***F***	**η² (%)**
Plant height (cm)	1352.0***	40.2	1241.5***	55.4	21.8***	3.9
Root length (cm)	2958.0***	39.1	2948.9***	58.4	28.5***	2.3
Number of nodes	137.5***	29.6	197.4***	63.8	1.5 ns	2.0
Internode distance (cm)	279.6***	44.2	196.1***	46.5	5.0***	4.8
Fresh weight (g)	906.9***	26.8	1616.4***	71.8	2.9***	0.5
Dry weight (g)	418.1***	58.2	169.7***	35.4	2.2**	1.9
Chlorophyll (SPAD)	353.4***	39.6	284.5***	47.8	15.7***	10.6
Proline content	1337.3***	76.9	222.3***	19.2	8.2***	2.8
Na^+ content	51.5***	0.4	8051.8***	99.2	3.4***	0.2
K^+ content	948.9***	50.9	506.4***	40.7	23.1***	7.4
Na^+/K^+ ratio	46.9***	16.5	77.7***	41.0	17.1***	36.2
Flavonoid content	3879.9***	69.6	811.6***	21.8	77.1***	8.3
Phenolic content	447.3***	21.6	680.5***	49.4	96.8***	28.1

Residual df = 68. *** $P < 0.001$; ** $P < 0.01$; * $P < 0.05$; ns, not significant. η^2 is the percentage of the total sum of squares attributable to each source.

As the concentration of NaCl increased, growth decreased gradually as indicted in (Fig. 1). The three sensitive genotypes including PRI-Red, LS-5, GN-3 responded immediately and lost 68-77% in plant height, 74-77% in root length. The response of Favorita and Hermes was more stable at 40mM and a difference in plant height observed for Favorita was -17% and for Hermes -28% as compare to their control plants which is significantly less as compare to sensitive genotypes (PRI-Red, LS-5, GN-3).

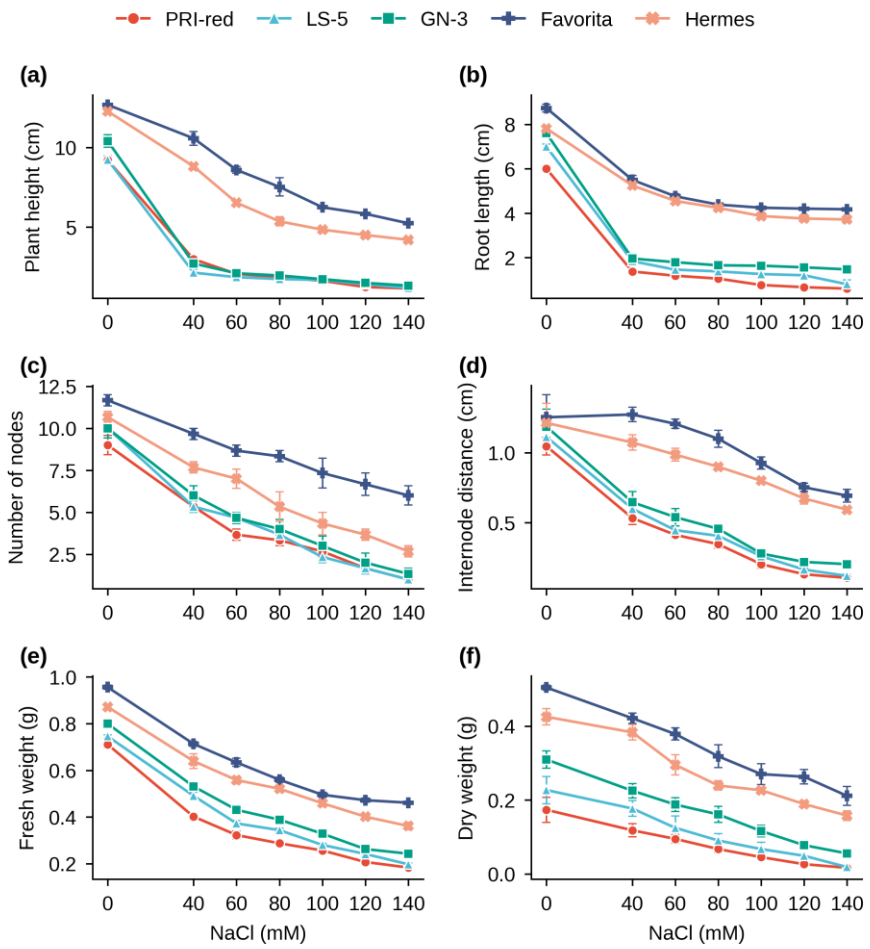


Fig. 1 Growth of five potato genotypes across NaCl concentrations in vitro after six weeks: (a) plant height, (b) root length, (c) number of nodes, (d) internode distance, (e) fresh weight and (f) dry weight. Symbols are means ± SE (n = 3); 0 mM is the control. Genotypes are identified in the legend

At 140mM the difference was greatest as indicated in (Table 2). In the sensitive genotypes, the plant height was reduced by 87-88% compared with control while it was reduced by 59% At in Favorita and 66% in Hermes. At 140mM Favorita had 6.0 noes/plantlet while PRI-Red, LS-5, GN-3 had 1.0-1.3 nodes/plantlet. The contrast was observed similar with respect to root length: 4.18 cm in Favorita and 3.72 cm in Hermes, while in sensitive group it was observed 0.60-1.47 cm. The same pattern was found for fresh and dry weight which clearly separated sensitive group from the tolerant group as eminent from (Table 2). Tukey HSD test differentiated all the six growth traits significantly among tolerant and sensitive genotypes.

Table 2. Genotypic variation in growth and biomass traits of five potato genotypes under control and salt stress (140 mM NaCl) conditions.

Trait	NaCl	PRI-Red	LS-5	GN-3	Hermes	Favorita
Plant height (cm)	Control	9.21 ± 0.22^{c}	9.23 ± 0.25^{c}	10.41 ± 0.39^{b}	12.28 ± 0.19^{a}	12.69 ± 0.04^{a}
	140 mM	1.13 ± 0.03^{l}	1.15 ± 0.04^{l}	1.31 ± 0.02^{l}	4.19 ± 0.01^{i}	5.23 ± 0.03^{fgh}
	Change (%)	−87.8	−87.5	−87.4	−65.9	−58.8
Root length (cm)	Control	6.00 ± 0.12^{d}	6.99 ± 0.12^{c}	7.60 ± 0.12^{b}	7.81 ± 0.14^{b}	8.73 ± 0.18^{a}
	140 mM	0.60 ± 0.02^{t}	0.80 ± 0.19^{rst}	1.47 ± 0.02^{lq}	3.72 ± 0.02^{j}	4.18 ± 0.01^{ghi}
	Change (%)	−89.9	−88.6	−80.7	−52.4	−52.2
Number of nodes	Control	9.0 ± 0.6^{be}	10.0 ± 0.6^{abc}	10.0 ± 0.6^{abc}	10.7 ± 0.3^{ab}	11.7 ± 0.3^{a}
	140 mM	1.0 ± 0.0^{p}	1.0 ± 0.0^{p}	1.3 ± 0.3^{op}	2.7 ± 0.3^{lp}	6.0 ± 0.6^{fj}
	Change (%)	−88.9	−90.0	−86.7	−75.0	−48.6
Internode distance (cm)	Control	1.05 ± 0.06^{ae}	1.11 ± 0.09^{ad}	1.19 ± 0.12^{abc}	1.21 ± 0.14^{abc}	1.25 ± 0.16^{ab}
	140 mM	0.11 ± 0.01^{q}	0.12 ± 0.01^{pq}	0.20 ± 0.02^{opq}	0.59 ± 0.02^{hm}	0.69 ± 0.05^{fk}
	Change (%)	−89.9	−89.2	−82.8	−51.1	−44.7
Fresh weight (g)	Control	0.711 ± 0.016^{de}	0.746 ± 0.007^{cd}	0.800 ± 0.012^{bc}	0.872 ± 0.007^{b}	0.956 ± 0.009^{a}
	140 mM	0.183 ± 0.010^{t}	0.197 ± 0.005st	0.243 ± 0.009^{qt}	0.361 ± 0.016^{lmn}	0.461 ± 0.005^{hk}
	Change (%)	−74.2	−73.5	−69.6	−58.5	−51.7
Dry weight (g)	Control	0.174 ± 0.034^{gm}	0.227 ± 0.037^{ei}	0.310 ± 0.024^{cde}	0.425 ± 0.022^{ab}	0.505 ± 0.007^{a}
	140 mM	0.017 ± 0.006^{p}	0.019 ± 0.005^{p}	0.056 ± 0.005^{nop}	0.158 ± 0.013^{ho}	0.211 ± 0.026^{ej}
	Change (%)	−90.4	−91.8	−82.1	−62.9	−58.1

Effect of salt stress (140 mM NaCl) on growth and biomass traits of five potato genotypes (PRI-Red, LS-5, GN-3, Hermes and Favorita). Values are means ± SE. Different letters within a trait indicate significant differences among genotype × treatment combinations ($P < 0.05$). “Change (%)” indicates the reduction under 140 mM NaCl relative to the respective control.

3.3 Physiological and Biochemical Responses

The 7 physiological and biochemical traits were studied (Table 1), and the interaction between genotypes and salinity level were significant ($P < 0.001$). Between salinity level and Na^+ content, 99.2% of the variance was explained by salinity. Genotype accounted for most of the variance for proline content (76.9%) and Flavonoid content (69.6%). The interaction between genotype and Na^+/K^+ ratio accounted for 36.2% of the variation, which is nearly as much as accounted by salinity (41.0%).

Chlorophyll and Proline Content

Chlorophyll content was recorded similar in all genotypes under control conditions (42.2-44.1) but showed variation as salinity level increased (Fig. 2a). At 140 mM, it had dropped by 68% in PRI-Red, 67% in LS-5, 44% IN gn-3, 28% in Hermes and just 17% in Favorita. GN-3 maintained significantly higher Chlorophyll content than PRI-Red, LS-5, but less than Hermes and Favorita. Favorita almost maintained its Chlorophyll content at 40 mM, just 2% decreased were recorded as compare to control.

Proline content increased with the salinity across all genotypes (Fig. 2b). Favorita showed the highest proline content at every NaCl concentration level it showed 1.41 in the control and 2.69 at 140mM, against 0.49 and 1.13 in PRI-Red. Hermes came second which showed 0.91 in control and 1.52 at 140mM. However, the larger relative gain was

recorded in the sensitive genotypes (PRI-Red, LS-5 and GN-3) as compare to tolerant genotypes (Hermes and Favorita) as evident from Table 3.

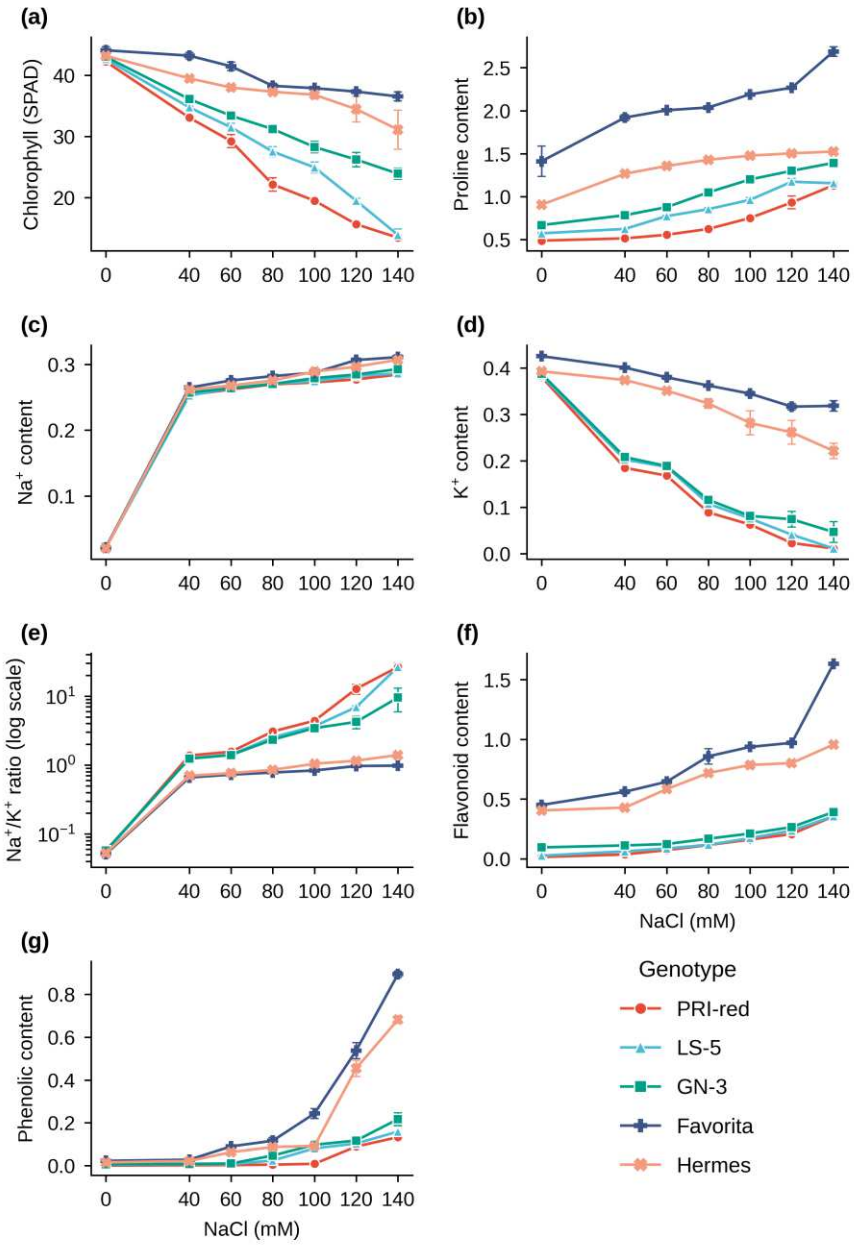


Fig. 2 Physiological and biochemical traits of five potato genotypes across NaCl concentrations *In vitro*: (a) chlorophyll content in SPAD units, (b) proline, (c) Na^+, (d) K^+, (e) Na^+/K^+ ratio (logarithmic axis), (f) flavonoid and (g) phenolic content. Symbols are means ± SE (n = 3); 0 mM is the control

Na^+, K^+ Content and Na^+/ K^+ Ratio

Na^+ content elevated roughly 12-13 folds from 0 to 40mM across all genotypes (from 0.021-0.022 to 0.25-0.26). It then increased 0.284-0.311 at 140Mm as evident from (Fig.

2c). Favorita (0.311) and Hermes (0.307) accumulated more Na^+ than the PRI-Red (0.284), LS-5 (0.286) and GN-3 (0.293). Favorita differ significantly from all the other three sensitive genotypes.

Potassium behaved differently from Na^+ as evident from (Fig. 2c & 2d) across the genotypes. The sensitive genotypes (PRI-Red, LS-5, GN-3) lost about half (46-51%) of their K^+ content at 40mM, while Favorita and Hermes just lost 5-6% of it. At 140mM, PRI-Red and LS-5 dropped its K^+ content by 97%, GN-3 by 88%, Hermes by 44% and Favorita by just 25%. In other words, Favorita and Hermes retained higher K^+ content against PRI-Red, LS-5 and GN-3 which separate them into tolerant and sensitive genotypes.

The Na^+ / K^+ ratio followed the both tends as shown in (Fig. 2e). At 140mM, PRI-Red and LS-5 reached at 26.5 and in GN-3 it was recorded 9.5 but stayed at 1.40 in Hermes and 0.98 in Favorita. In Favorita the ratio did not exceed 1.0 at any salinity level.

Flavonoids and Phenolics

The trend for both antioxidant classes showed increasing with increase in salinity in all genotypes (Fig. 2f, g). Favorita and Hermes showed higher flavonoids content as compare to the sensitive genotypes. At 140mM, Favorita showed 1.632 and Hermes 0.957, while PRI-Red, LS-5 and GN-3 stayed between 0.356 and 0.392.

Phenolics content remained low in all genotypes up to 80mM (about 0.12) and then diverged. At 140mM it was recorded 0.894 in Favorita and 0.682 in Hermes as compared to PRI-Red, LS-5 and GN-3 with 0.133-0.217.

Table 3 Physiological and biochemical traits of five potato genotypes in the control and at 140 mM NaCl

Trait	NaCl	PRI-Red	LS-5	GN-3	Hermes	Favorita
Chlorophyll (SPAD)	Control	42.2 ± 0.6^{ae}	42.6 ± 0.3^{ad}	43.0 ± 0.4^{abc}	43.2 ± 0.5^{ab}	44.1 ± 0.6^{a}
	140 mM	13.4 ± 0.6^{u}	13.9 ± 1.0^{u}	23.9 ± 0.9^{qrs}	31.1 ± 3.2^{ko}	36.5 ± 0.7^{fj}
	Change (%)	−68.1	−67.4	−44.4	−27.9	−17.1
Proline content	Control	0.49 ± 0.01^{r}	0.57 ± 0.01^{qr}	0.67 ± 0.01^{pqr}	0.91 ± 0.01^{mno}	1.41 ± 0.18^{efg}
	140 mM	1.13 ± 0.01^{im}	1.16 ± 0.02^{il}	1.39 ± 0.04^{eh}	1.52 ± 0.01^{e}	2.69 ± 0.06^{a}
	Change (%)	132.2	101.5	108.2	68.3	90.5
Na^+ content	Control	0.021 ± 0.001^{o}	0.021 ± 0.002^{o}	0.022 ± 0.002^{o}	0.021 ± 0.001^{o}	0.022 ± 0.002^{o}
	140 mM	0.284 ± 0.002^{cg}	0.286 ± 0.004^{cf}	0.293 ± 0.002^{bcd}	0.307 ± 0.004^{ab}	0.311 ± 0.003^{a}
	Change (×)	13.5	13.4	13.1	14.8	14.3
K^+ content	Control	0.381 ± 0.001^{abc}	0.384 ± 0.002^{abc}	0.387 ± 0.002^{abc}	0.393 ± 0.001^{abc}	0.425 ± 0.003^{a}
	140 mM	0.011 ± 0.002^{p}	0.011 ± 0.002^{p}	0.047 ± 0.022^{nop}	0.221 ± 0.016^{hi}	0.318 ± 0.011^{ef}
	Change (%)	−97.0	−97.1	−88.0	−43.7	−25.2
Na^+/K^+ ratio	Control	0.06 ± <0.01	0.06 ± 0.01	0.06 ± <0.01	0.05 ± <0.01	0.05 ± 0.01
	140 mM	26.48 ± 3.74	26.47 ± 3.74	9.51 ± 3.60	1.40 ± 0.08	0.98 ± 0.04
	Change (×)	480	477	165	26.6	19.2
Flavonoid content	Control	0.015 ± 0.003^{q}	0.026 ± 0.002^{q}	0.096 ± 0.003^{mq}	0.405 ± 0.011^{ij}	0.451 ± 0.015^{i}
	140 mM	0.356 ± 0.002^{j}	0.357 ± 0.002^{j}	0.392 ± 0.003^{ij}	0.957 ± 0.013^{b}	1.632 ± 0.007^{a}
	Change (×)	23.6	13.5	4.1	2.4	3.6

Trait	NaCl	PRI-Red	LS-5	GN-3	Hermes	Favorita
Phenolic content	Control	0.0002 ± <0.0001^{o}	0.0042 ± 0.0010^{o}	0.0060 ± 0.0003^{no}	0.0154 ± 0.0014^{mno}	0.0223 ± 0.0023^{ko}
	140 mM	0.1328 ± 0.0183^{gh}	0.1587 ± 0.0263^{fg}	0.2167 ± 0.0300^{ef}	0.6817 ± 0.0179^{b}	0.8939 ± 0.0146^{a}
	Change (×)	609	38.1	35.8	44.4	40.1

Physiological and biochemical responses of five potato genotypes (PRI-Red, LS-5, GN-3, Hermes and Favorita) to salt stress (140mM NaCl) relative to the control. Values are means ± SE. Different letters within a trait indicate significant differences among genotype × treatment combinations ($P < 0.05$). "Change" indicates the percentage change (%) or fold change (×) under 140mM NaCl relative to the respective control. Units of the biochemical traits are given in the Methods

3.4 Relationship Among Traits

Pearson correlation was calculated across all 105 0bervations (Fig. 3). Growth traits, chlorophyll content and K^+ content formed a tightly correlated block (r = 0.82-0.97), while the Na^+ was negatively correlated with every other trait of this block (r = -0.43 to -0.75). The Na^+/K^+ ratio was also observed negatively correlated with the block (r = -0.45 to -0.74).

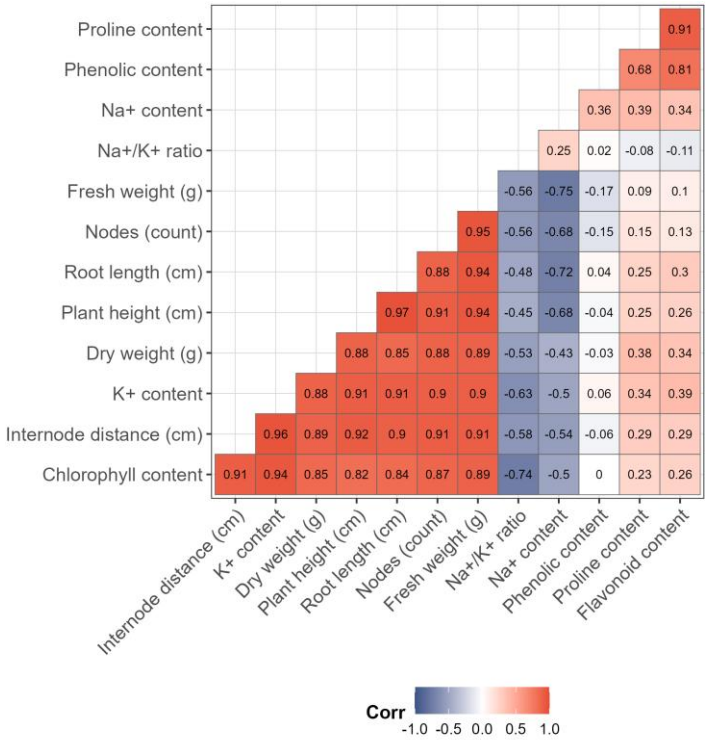


Fig. 3 Pearson correlation matrix for the 13 traits, calculated from all 105 observations. Numbers are correlation coefficients (*r*). Shading shows the sign and strength of *r*

3.5 Multivariate Analysis Among Genotypes

The first two principal components of PCA analysis explained 64.5% and 22.4% of the total variance (Fig. 4). PC1 was loaded strongly and positively with the growth-related traits, chlorophyll content and K^+ content (0.95- 1.00) and negatively by Na^+ content (-

0.64) and by the Na^+/K^+ ratio (- 0.66). PC2 was loaded by proline (0.92), phenolics (0.92) and a flavonoid (0.95), with a smaller Na^+ content (0.60) contribution.

Along PC1, the salinity score for the PRI-Red, LS-5, and GN-3 genotypes shifted from positive values in the control group to negative values under salt stress. In fact, all salt-treated samples for PRI-Red, LS-5, and GN-3 scored negatively along PC1. In contrast, Favorita maintained positive values across all NaCl concentrations. Hermes also stayed positive up to 120mM NaCl, only dipping slightly into the negative range (−0.53) at 140 mM. Furthermore, at the highest concentration of 140mM, Favorita and Hermes recorded the highest PC2 scores (5.56 and 2.91, respectively), indicating they accumulated the greatest amounts of proline, flavonoids, and phenolics.

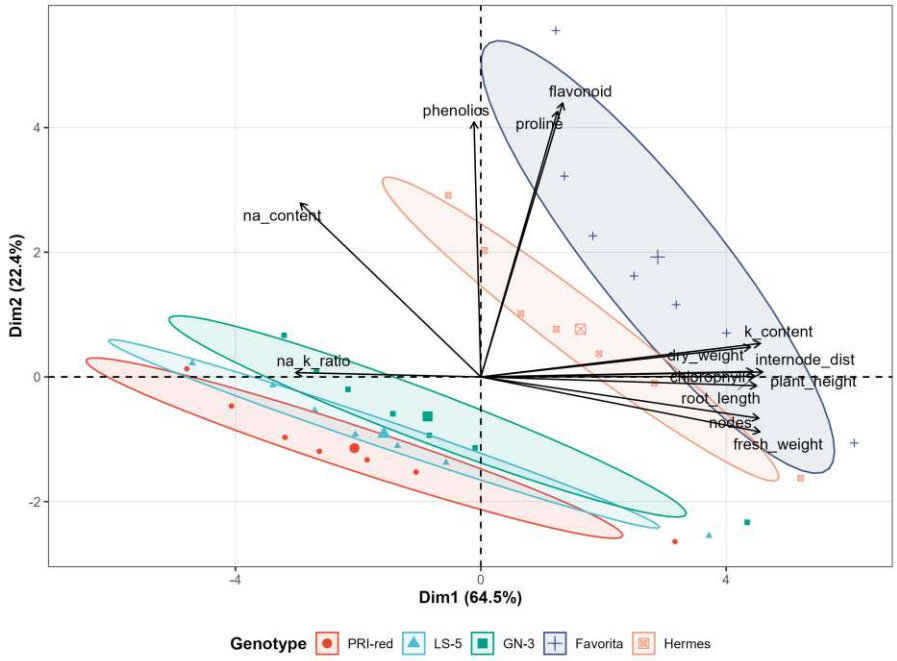


Fig. 4 Principal component analysis (PCA) biplot of genotype × NaCl means for 13 standarised traits. Each small symbol is one genotype × NaCl combination (seven per genotype), the large symbol marks the mean of each genotype, and ellipses group the points of each genotype. Arrows show trait loadings. Dim1 and Dim2 explain 64.5% and 22.4% of the total variance.

At 140mM NaCl treatment, two groups were formed by hierarchical clustering of the standarised trait profiles (Fig. 5). The one group was made up of Favorita and Hermes. The other group consisted of PRI-Red, LS-5 and GN-3, the two closest, LS-5 and GN-3, were at a moderately close distance, and GN-3 was further apart. High values of all traits were observed in the Favorita and Hermes, except for the Na+/K+ ratio that was lowest in these two genotypes. The PRI-Red, LS-5 and GN-3 group exhibited the opposite trends. The most moderate of the three was GN-3, the chlorophyll value was near the panel average.

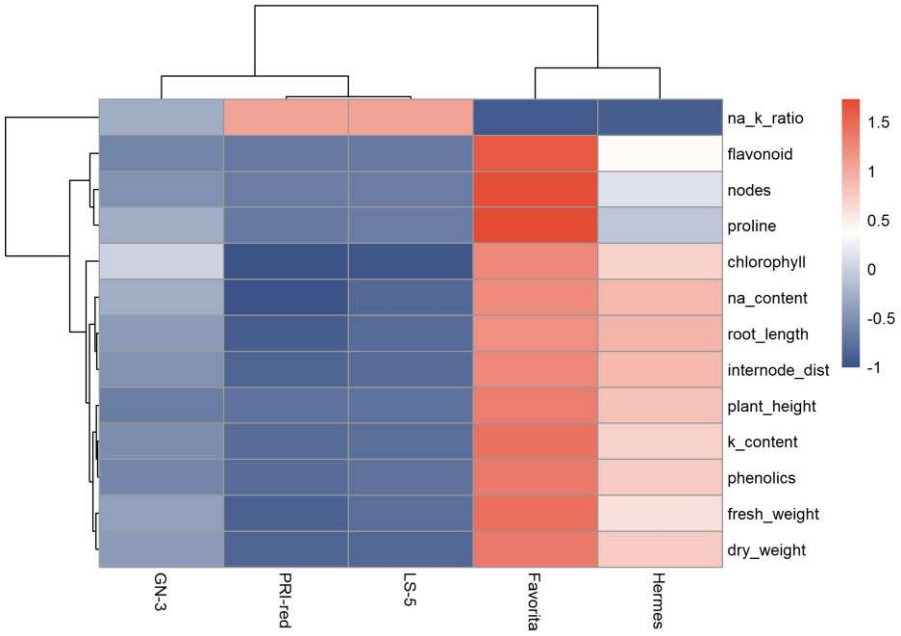


Fig. 5 Heatmap and hierarchical clustering (Euclidean distance, Ward's linkage) of standarised trait means at 140 mM NaCl. Values are z-scores calculated across the five genotypes for each trait

3.6 STI (Stress Tolerance Indices) score

Favorita had the highest and Hermes the second highest STI for all of the traits studied at 140mM (Fig. 6). Favorita's dry weight STI (0.99) was the largest single, while PRI-Red was the smallest (0.027). The only significant difference between GN-3 (STI 0.56) and the other two was chlorophyll (STI 0.31 and 0.32). Averaged over the six growth traits, the overall STI was 0.70 for Favorita, 0.48 for Hermes, 0.18 for GN-3, 0.11 for LS-5 and 0.09 for PRI-Red (Fig. 7).

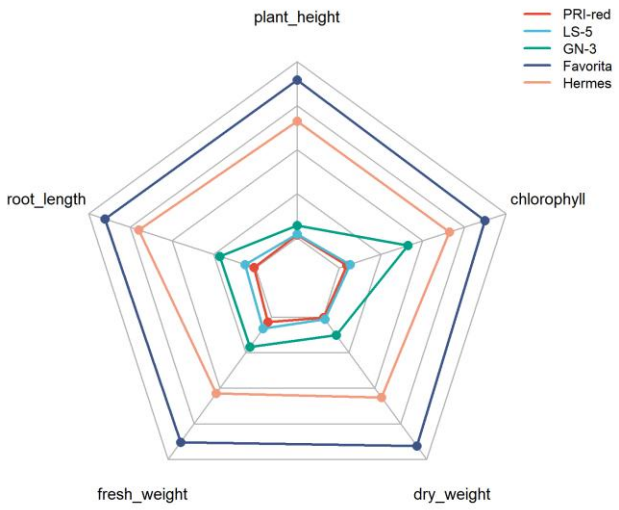


Fig. 6 Stress tolerance index (STI) at 140mM NaCl for five traits of five potato genotypes. Each axis is one trait, and distance from the center indicates STI

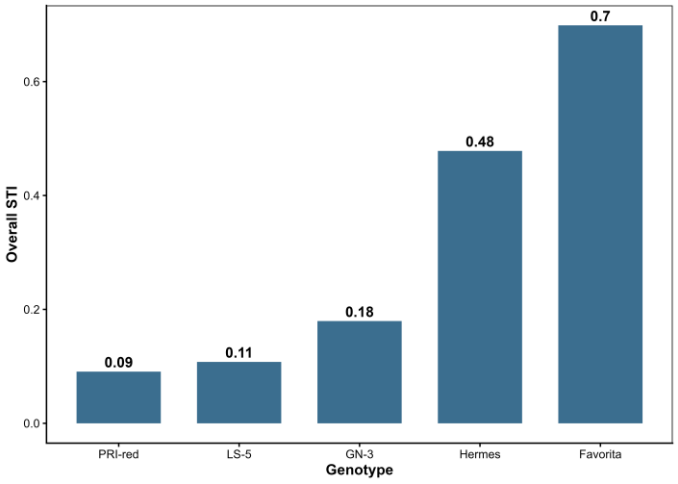


Fig. 7 Overall stress tolerance index (STI) of five potato genotypes at 140mM NaCl. Overall STI is the mean STI of six growth traits (plant height, root length, node number, internode distance, fresh weight and dry weight)

The first principal component of the 20 standarised index variables accounted for 97.7% of the variance, thus making it a composite score (Fig. 8; Table 4). Favorita's score was 6.54 and Hermes 3.83. GN-3, LS-5 and PRI-Red had negative scores (−1.75, −4.14 and −4.48). Favorita was accordingly classified as tolerant, Hermes as moderately tolerant and the other three genotypes as sensitive. The order was the same when the overall STI was used and when the composite score was recreated from all seven traits, or six growth traits. The score is relative and is used to rank these 5 genotypes against each other and is not an absolute measurement of tolerance.

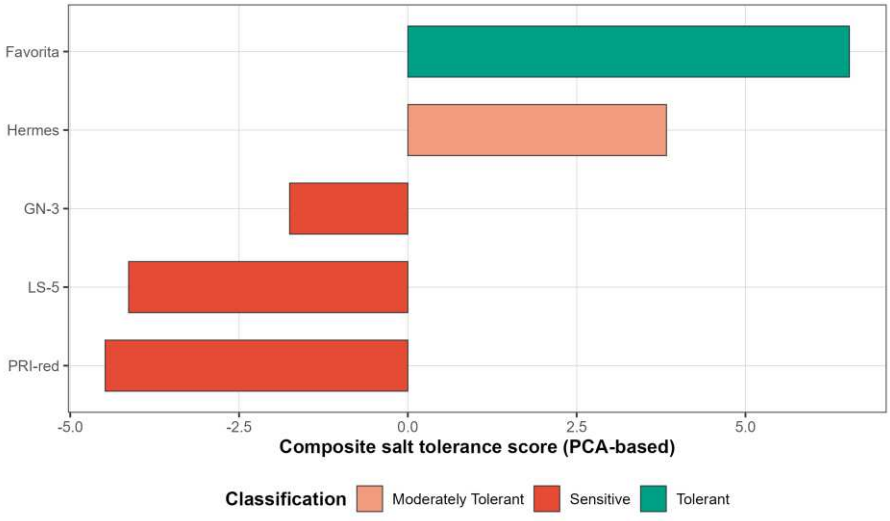


Fig. 8 Ranking of five potato genotypes by composite salt tolerance score. The score is the first principal component of the standarised GMP, STI, YI and YSI values of five traits (plant height, root length, fresh weight, dry weight and chlorophyll content). Bars are shaded by tolerance class.

Table 4 Overall stress tolerance index (STI), composite salt tolerance score, rank and classification of five potato genotypes at 140mM NaCl

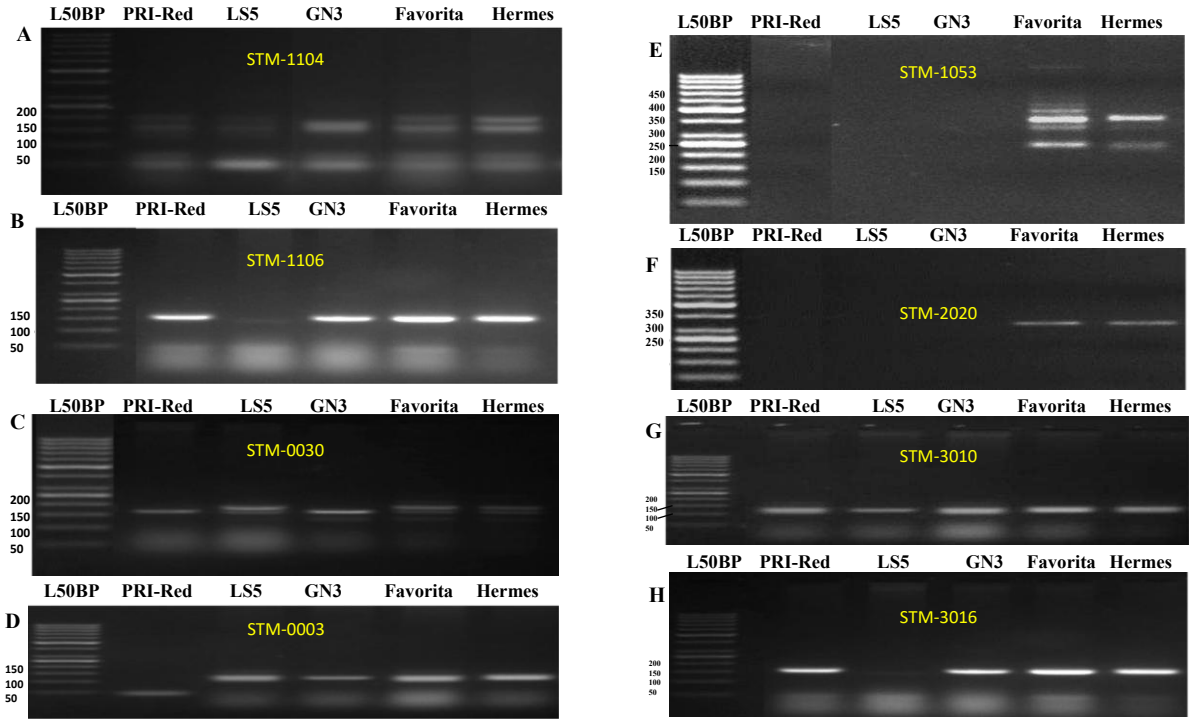
Genotype	Overall STI	Composite score	Rank	Classification
Favorita	0.70	6.54	1	Tolerant
Hermes	0.48	3.83	2	Moderately tolerant
GN-3	0.18	−1.75	3	Sensitive
LS-5	0.11	−4.14	4	Sensitive
PRI-Red	0.09	−4.48	5	Sensitive

Overall STI is the mean STI of six growth traits. The composite score is the first principal component of the standarised GMP, STI, YI and YSI values of five traits and is relative to this set of genotypes

3.7 SSR-based Molecular Analysis

The nine SSR markers produced bands on agarose gel that were reproducible and scoreable in all the genotypes (Table 5; Fig. 9). All five genotypes had five markers which were monomorphic (STM1104, STM1106, STM0030, STM1031 and STM3010). The four markers with the most genetic polymorphisms (44% of the panel) had a mean PIC of 0.40 (range 0.32–0.48) with a mean PIC of 0.18 for all nine markers.

Two markers (STM1053, ~350 bp and STM2020, ~250 bp) were only amplified in Favorita and Hermes and were not amplified in PRI-Red, LS-5 and GN-3, with the tolerant versus sensitive split matching that observed in the growth, physiological and tolerance-index data (Figs. 5, 8; Table 4). STM0003 was only missing in PRI-Red, while STM3016 was only missing in LS-5. The total number of bands scored for each genotype (Favorita and Hermes 9/9, GN-3 7/9, PRI-Red and LS-5 6/9) was in the same rank as the composite tolerance ranking (Table 4).



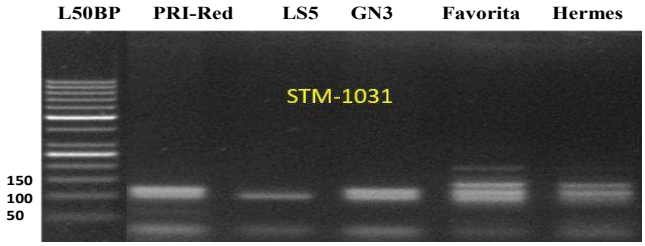


Fig 9. PCR amplification profiles of nine SSR/STM markers screened across five potato (*Solanum tuberosum*) genotypes (PRI-Red, LS-5, GN-3, Favorita, Hermes) to assess genetic diversity underlying differential salinity tolerance. **(A)** STM-1104, **(B)** STM-1106, **(C)** STM-0030, **(D)** STM-0003, **(E)** STM-1053, **(F)** STM-2020, **(G)** STM-3010, **(H)** STM-3016, **(I)** STM-1031. A 50 bp DNA ladder (L50BP) was used for fragment-size estimation. Four markers (STM-1104, STM-1106, STM-0030, STM-1031) and STM-3010 were monomorphic across all genotypes, whereas STM-0003, STM-1053, STM-2020, and STM-3016 revealed polymorphic banding patterns distinguishing tolerant from sensitive genotypes.

Table 5 SSR marker profile of five potato genotypes

Marker	PRI-Red	LS-5	GN-3	Favorita	Hermes	PIC	Information
STM1104	+	+	+	+	+	0.00	Monomorphic
STM1106	+	+	+	+	+	0.00	Monomorphic
STM0030	+	+	+	+	+	0.00	Monomorphic
STM1031	+	+	+	+	+	0.00	Monomorphic
STM0003	−	+	+	+	+	0.32	Polymorphic

Marker	PRI-Red	LS-5	GN-3	Favorita	Hermes	PIC	Information
STM1053	−	−	−	+	+	0.48	Polymorphic
STM2020	−	−	−	+	+	0.48	Polymorphic
STM3010	+	+	+	+	+	0.00	Monomorphic
STM3016	+	−	+	+	+	0.32	Polymorphic

+, band present; −, band absent. PIC, polymorphism information content, calculated as 2*pq*, where *p* and *q* are the frequencies of the presence and absence classes among the five genotypes

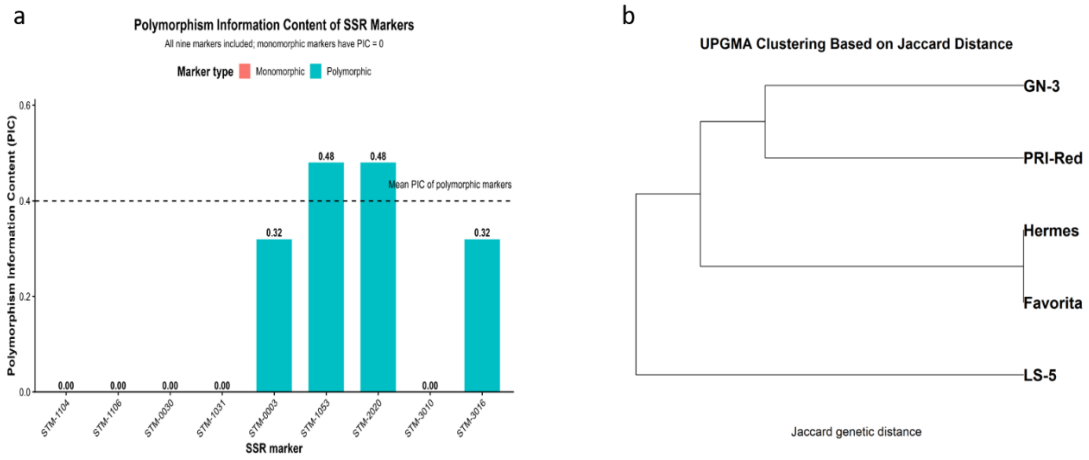


Fig. 9 Genetic diversity among the studied genotypes based on SSR markers. (a) Polymorphism information content (PIC) for each marker; the dashed line is the mean PIC of the four polymorphic markers, (b) UPGMA dendrogram of genetic similarity among genotypes, calculated with the Jaccard (43) coefficient from the four polymorphic markers, alongside the full nine-marker presence/absence matrix in the same genotype order.

4 Discussion

In this study we have used five genotypes including; PRI-Red, LS-5, GN-3, Favorita, Hermes which consistently separated out into two groups according to their responses to salinity. Favorita and Hermes maintained their growth and physiological parameters under salt stress and perform better than PRI-Red, LS-5 and GN-3. At 140mM NaCl, the sensitive genotypes showed a remarkable reduction of 70-92% across six growth traits, whereas Favorita and Hermes maintained their growth-related traits under same conditions. The experimentally proven results of growth traits, pigment retention, osmotic adjustment, ionic balance and multivariate analysis across five genotypes suggests that the difference in salt tolerance reflected coordinated physiological responses rather than variation in a single trait. Previous studies (17, 20) reported the differences in genotype rankings on the basis of traits used for evaluation. In contrast, the pattern observed in this study highlighted that several independent physiological indicators support the similar classification across five genotypes used in our study. These pronounced variations indicate that plant height could serve as an effective index for preliminary screening under

high salt stress. Nevertheless, confirming its validity as an independent selection criterion which requires further validation with larger germplasm panel.

Root length was mainly affected by increasing salinity level, with substantial reductions in the sensitive genotypes already showed at 40mM NaCl. At 140mM, root length was reduced to 0.60- 1.47 cm in PRI-Red, LS-5 and GN-3 as compare to Favorita with 4.18 cm and Hermes with 3.72 cm. Roots are directly exposed to saline conditions, making their growth highly susceptible to osmotic stress and cause ionic disturbances which limits the water and nutrients absorption, ultimately affects plant growth as reported by (31, 32). Similar results for roots growth under salinity stress were also reported by (16). The comparatively larger root length was maintained by Favorita and Hermes suggesting that these genotypes posses such traits which supported them for roots development under salt stress. However, further studies are required to investigate root architecture, water uptake and ion distribution to understand the mechanism responsible for this response.

Chlorophyll content further provided the evidence of variations in salinity tolerance across five genotypes. Although, they have a comparable SPAD readings under control conditions but their responses diverged on increasing NaCl concentrations. At 140mM NaCl, Favorita just dropped a 17% in chlorophyll content while sensitive genotypes (PRI-Red, LS-5 and GN-3) dropped 44-68%. Salinity induced chlorophyll loss is generally coupled with ionic toxicity, oxidate damage to chloroplast structures and restrictions on photosynthetic carbon assimilation (33, 34). The relatively stable SPAD values in Favorita suggestion its better preservation of leaf pigments under the salt stress. Gas exchange and chlorophyll-fluorescence measurements would help to determine either chlorophyll retention further translated into the improving photosynthetic performance.

Proline content accumulation revealed an important difference between constitutive levels and stress-induced responses. Favorita and Hemes maintained the higher proline content under salt stress as compare to sensitive genotypes (PRI-Red, LS-5 and GN-3). Proline contributes to the adjustment of osmotic pressure, redox regulation and cellular protection, but its accumulation may also reflect the stress level experienced by the plant (13). Through the proline content analysis, comparable differences between tolerant and sensitive cultivars were reported by Jaarsma et al. (15). It reported that, linked osmotic adjustment with improved cellular homeostasis in tolerant cultivar. Our findings indicated that the continuous accumulation of proline improve the tolerance level in Favorita and Hermes.

The Na^+/K^+ ratio provided the clearest distinction between the tolerant and sensitive genotypes. At 140mM NaCl, the sensitive genotypes (PRI-Red, LS-5 and GN-3) lost 88-97% of K^+, whereas Favorita and Hermes just lost 25-44%. Consequently, the Na^+/K^+ ratio for sensitive genotypes calculated as 26.48 in PRI-Red it remained at 0.98 in Favorita. Maintaining K^+ homeostasis under salt stress is integral for enzyme activity,

membrane stability and cellular process like opening and closing of stomata and is widely known as important player of salt tolerance (9, 35, 36). Another study by Mim et al. (19) reported similar associated salt response in potato genotype with the expression of *SOS1* and *NHX1*. Our findings support the importance of ionic balance but to find underlying the exact transport mechanisms further gene expression and transport activity analysis can be performed.

Salt stress induced an increase in phenolics and flavonoids concentrations across all genotypes; however, the highest absolute value was consistently observed in Favorita. At 140 mM NaCl, the reported values in Favorita and Hermes were 1.632 and 0.957 respectively, compared with 0.356- 0.392 in the sensitive genotypes. Phenolics and Flavonoids compounds serve as a vital component of antioxidant defense by scavenging the reactive oxygen species and maintaining the cellular redox balance (14, 37). A comparable difference between constitutive antioxidant level and activated responses has been observed in maize under salt stress (38). In our study, the elevated concentrations in Favorita and Hermes, along with their better growth and ionic balance, suggested that the constitutive antioxidant level plays a vital role in their overall performance.

The multivariate analysis further strengthens the patterns observed in individual traits. Principal component analysis (PC1) accounted for 64.5% of the total variance, revealing a distinct contrast between salt-tolerance associated traits (growth parameters, chlorophyll content and K^+ status) and salinity accumulation indicators (Na^+ accumulation and Na^+/ K^+ ratio). The negative ordination of salt-treated PRI-Red, LS-5 and GN-3 cohorts reflects their sensitivity and adverse physiological response to salt stress. Genotype rankings derived from the composite score of 4 tolerance indices including; GMP, STI, YI and YSI remains consistent regardless five, six or seven traits were tested. Consistent with aforementioned traits pattern, the overall STI score showed a clear gradient across all genotypes, ranking as: Favorita (0.70)> Hermes (0.48)> GN-3 (0.18)> LS-5 (0.11)> PRI-Red (0.09). These findings corroborate the assertions of Han et al. (39) and Dakal et al. (40), they reported that multi-trait integration provides a more reliable framework for assessing salinity tolerance than the single trait evaluation in potato.

The SSR results provided a clear picture of relationship between genetic variation and physiological performance across genotypes. Out of the nine SSR markers evaluated, only four were polymorphic. Notably, the exclusive presence of STM1053 and STM2020 in Favorita and Hermes implies that the allelic variation on these specific loci perfectly align with the observed physiological stratification. However, cluster analysis utilizing all polymorphic makers failed to recapitulate the two-group physiological pattern and LS-5 showed in separate clade from PRI-Red and GN-3. These findings in SSR resolution within localized cultivar panel aligns with previous studies that reported similar limitations in potato germplasm differentiation (21, 41). Therefore, a large panel of specific and

informative markers needed to be established more reliable genetic screening among the genotypes.

Overall, Favorita and Hermes emerges as a highly promising candidates for future potato salinity-tolerance breeding programs. Rather than relying on a single trait, multivariate analysis confirmed that their robust tolerance is governed by a coordinated response across multiple physiological traits, including better vegetative growth, maintain chlorophyll content retention, elevated proline accumulation and a well-regulated ionic balance. This highlights the importance of using of comprehensive physiological profile over individual traits to develop germplasm selection criteria under salt stress. While *in-vitro* screening offers a control environment for initial selection, it is limited to capture the multi factorial nature of open field condition which include fluctuating soil properties, ambient temperature, irrigation practices and nutrients dynamics. This limitation was also addressed by Rashid et al. (42), he evaluated *in-vitro* and field performance of Bangladeshi potato cultivars under salt stress and observed a strong correlation between them but not completely equivalent. This highlights the multi-year filed trial to accurately screen and select the salt tolerant cultivars. The future studies must utilize these promising genotypes under actual saline irrigation to check their effect on tuber yield and quality. To fully elucidate the physiological mechanisms responsible for salt tolerance in Favorita and Hemes, gene expression profiling targeted at ion-transport and antioxidant pathways must be investigated along with molecular profiling. Furthermore, the integration of filed based evaluation, high throughput phenotyping and targeted molecular analysis will be crucial to establish the breeding value of Favorita and Hemes. This will provide the framework to develop salt tolerant potato genotypes for Pakistan.

5 Conclusion

In conclusion, this study demonstrates that the genotypes Favorita and Hermes consistently exhibit superior salt tolerance compared to PRI-Red, LS-5 and GN-3. This ranking remained constant across evaluated parameters including physiological traits, chlorophyll retention, proline accumulation, ion balance and antioxidant content. These parameters were also confirmed by both multivariate ordination and a multi-formula composite tolerance index. Physiologically, the main mechanism driving this variation is differential potassium retention. Under 140 mM NaCl, the tolerant genotypes effectively maintained K^+ balance to keep Na^+/ K^+ ratio ≤ 1 whereas the sensitive lines exhibit severe K^+ decline with Na^+/ K^+ ratios exceeding 25. The SSR makers profiling successfully tracked the two loci which tracked the observed tolerance phenotypes. The nine-marker panel did not perfectly align with the phenotypic data of LS-5; therefore, a large panel of specific and informative markers was proposed to establish more reliable genetic screening among the genotypes. Ultimately, Favorita and Hermes highlight as the most promising parental candidates for breeding programs to develop salt tolerant potato germplasm. The future studies must utilize these promising genotypes under actual saline

irrigation to check their effect on tuber yield and quality. To fully elucidate the physiological mechanisms responsible for salt tolerance in Favorita and Hemes, gene expression profiling targeted at ion-transport and antioxidant pathways must be investigated along with molecular profiling.

Author contributions

Muhammad Rizwan Ali: performed data analysis and experiment, wrote the manuscript, Danish Abbasi: performed the experiments, Saima Noor: performed the experiments and reviewed manuscript, Muhammad Zeshan: designed the research activity, provided materials and proofreading of manuscript, Kazim Ali: designed the research activity, provided materials and proofreading of manuscript, Amber Imtiaz: performed the experiments, Raja Mohib Muazzam Naz: reviewed manuscript, Sarwat Batool: reviewed manuscript, Musab Imtiaz: reviewed manuscript, Muhammad Aqeel: reviewed manuscript, Shaukat Ali: reviewed manuscript. All authors have read and agreed to the published version of the manuscript.

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Not applicable

Data Availability:

The original contributions presented in the study are included in the article, further inquiries can be directed at the corresponding author.

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