



Development and evaluation of barley mini-core collection for salinity tolerance and identification of novel haplotypic variants for *HvRAF*

Sonia · Vikender Kaur ·
Shashank Kumar Yadav · Sunder Singh Arya ·
J. Aravind · Sherry R. Jacob · Raj Kumar Gautam

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Abstract

Background and aims India is one of the countries being projected as the salinization hotspots in the near future. To identify novel donors imparting salt tolerance, barley mini-core collection comprising 107 *Hordeum vulgare* germplasm and 3 wild accessions from the Indian National Genebank were evaluated under salt stress.

Methods Barley accessions were screened under salt stress (200 mM NaCl) at early growth and the adult plant stage based on morpho-physiological traits including salt uptake parameters. Further, the possible role of candidate gene *Hordeum vulgare* *root*

abundant factor (*HvRAF*) was studied by deciphering allelic variation and expression analysis in selected salt tolerant and susceptible accessions.

Results Analysis of variance revealed a significant effect of salinity on all traits and elucidated profound genotypic variation. Salinity caused a drastic reduction in growth and severely affected ion homeostasis resulting in decline in grain yield by 65.35% compared to control. Accessions EC0578359, EC0578251, IC0547723, EC0123148, EC0299361, EC0177250 and IC0247671 were identified as the most promising salt-tolerant genotypes. Further investigation of allelic variation in *HvRAF* revealed a total of 26 SNPs, of which 10 were non-synonymous, 8 were synonymous and 5 were conserved. Haplotype variant analysis indicated two major haplotypic groups (Hap 1 and Hap 2) for *HvRAF*, of which the Hap 2 was found to be more prevalent than Hap 1.

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Sonia · S. S. Arya
Department of Botany, Maharishi Dayanand University,
Rohtak, Haryana, India
e-mail: soniaharshit89@gmail.com

S. S. Arya
e-mail: aryasunder.hau@gmail.com

V. Kaur (✉) · S. K. Yadav · R. K. Gautam
Division of Germplasm Evaluation, Indian Council
of Agricultural Research- National Bureau of Plant
Genetic Resources (ICAR-NBPGR), New Delhi 110012,
India
e-mail: Vikender.kaur@icar.gov.in; kaurvikender@gmail.com

S. K. Yadav
e-mail: shashank86livinz@gmail.com

R. K. Gautam
e-mail: Raj.Gautam@icar.gov.in

J. Aravind · S. R. Jacob
Division of Germplasm Conservation, Indian Council
of Agricultural Research- National Bureau of Plant
Genetic Resources (ICAR-NBPGR), New Delhi 110012,
India
e-mail: J.Aravind@icar.gov.in

S. R. Jacob
e-mail: Sherry.Jacob@icar.gov.in

Conclusions Salt-tolerant phenotype exhibited the physiological basis of tolerance and upregulated expression of *HvRAF*, although none of the identified Hap groups could be associated with salt tolerance suggesting the governance of trait by multiple loci.

Keywords Barley germplasm · Gene expression · Genetic variability · Root system architecture · Salt tolerance

Introduction

Salinity is one of the most challenging and rapidly growing abiotic stresses that hampers crop production worldwide. The world map of salt-affected soils (with the current information from 118 countries covering 85% of global land area) indicates that more than 424 million hectares of top soil (0.30 cm) and 833 million hectares of subsoil (30–100 cm) are salt-affected (FAO 2023). Hossain (2019) reported that one-fifth of the irrigated lands are salt-affected with an annual addition of 1.5 million hectares becoming affected by high salinity. With the latest projected global population of 9.7 billion in 2050 and 10.4 billion in 2100 (United Nations Department of Economic and Social Affairs, Population Division 2022), ensuring sustainable food production as included in the Sustainable Development Goals (UN 2023) is challenging and the most pressing issue. The situation is expected to be exacerbated further as the arable land is decreasing at the rate of 1–2% annually, while to maintain the current level of food supply, agriculture production has to be escalated by around 57% (Hossain 2019; Wild 2003). Under these circumstances, an increase in crop tolerance to salt stress is perceived to be the best option to meet exacerbated food demands in the future (Amna et al. 2021; Ma et al. 2022).

Barley (*Hordeum vulgare* L.), is a resilient crop for future nutritional security owing to its natural tolerance to various abiotic stresses, particularly salt and drought stress (Gürel et al. 2016). Besides, it is one of the best-suited model experimental plants to predict crop response to changing climatic conditions (Dawson et al. 2015; Kishore et al. 2016). Although considered a hardy crop owing to grain filling capacity, early anthesis nature and more extensive root system, its growth and productivity are adversely affected under salinity (Jamshidi and Javanmard

2018; Pour-Aboughadareh et al. 2021). Salinity leads to enforced Na^+ uptake and causes Na^+ toxicity in plants, limiting the uptake of other nutrients (K^+ , Ca^{2+} , P, N) required for plant growth (Arif et al. 2020). It leads to physiological drought in plants by accumulating excessive salt in the root zone and thus reducing the osmotic potential of the soil solutes affecting the acquisition of water and nutrients. The study of root traits and optimization of root system architecture (RSA) is, therefore, the much-focused research attention for improved stress tolerance in field crops (Tuberosa et al. 2002; Price et al. 2006; De Dorlodot et al. 2007; Lynch 2007; Serraj et al. 2004; Kaur et al. 2020; Manju et al. 2023). Stress response in plants involves a complex network of various receptors, kinases, phosphatases, and transcription factors (Gutterson and Reuber 2004; Chinnusamy et al. 2005; Mwando et al. 2020). Several stress-responsive genes impart cellular tolerance either through modulation of downstream genes or morphophysiological plant architecture. *HvRAF* (*Hordeum vulgare* root abundant factor), is an ethylene response factor (ERF)-type transcription factor (TF) (Sakuma et al. 2002; Gutterson and Reuber 2004), which has been found to regulate transcriptional induction of various biotic and abiotic stress-responsive genes in plants (Yi et al. 2004; Lee et al. 2004). It is known to play an important role in conferring salt tolerance by promoting seed germination and root growth under salinity (Jung et al. 2007; Gürel et al. 2016). Jung et al. (2007) reported more transcripts abundance of *HvRAF* in the roots than in leaves of barley seedlings indicating that it is a root-abundant TF having a role in multiple signaling pathways including high salinity stress. Therefore, to understand molecular dynamics underlying changes in root architecture in response to salinity stress, the Root abundant factor (*HvRAF*) gene was selected to unravel allelic diversity.

The development of salt-tolerant varieties is one of the most reliable approaches of crop improvement programs, but a prerequisite for plant breeding has been the search for donor parents. Barley genetic resources from the natural diversity-rich areas maintained in *ex-situ* germplasm repositories are expected to harbour genetic variants and thus form crucial resources for breeding. The Indian National Genebank (INGB) located in the premises of Indian Council of Agricultural Research–National Bureau of Plant Genetic Resources (ICAR-NBPGR), New Delhi,

maintains around 7,500 genetically and geographically diverse accessions of barley as base collection. A wide range of genetic variability has been reflected and trait-specific promising germplasm has been identified through the large-scale phenotypic characterisation studies performed involving INGB barley collection (Kaur et al. 2018, 2022; Manju et al. 2019a, b, 2023). The global predictions of primary soil salinization in twenty-first century has projected India as one of the salinization hotspots (Hassani et al. 2021), which necessitates the screening of genebank germplasm to identify novel donors for future breeding and climate resilience. In this context, a diverse set of 110 accessions representing barley mini-core collection was employed for the evaluation of salinity tolerance for key root-shoot traits including RSA. A subset of salinity tolerant and susceptible accessions selected based on seedling stage traits evaluated in hydroponics was further tested for adult plant stage tolerance in field conditions using agro-morphological, physiological and salt uptake parameters. Further, the possible role of *HvRAF* in salt tolerance was also studied by deciphering allelic variation in selected salt tolerant and susceptible accessions.

Materials and methods

Plant materials

Plant materials for this study included the INGB barley mini-core collection comprising 107 accessions and 3 wild barley accessions namely *H. murinum* ssp. *leporinum*, *H. spontaneum* (K. Koch.) and *H. maritimum* (Huds) (*ssp gussoneanum*) (Parl.). The details of accessions examined in the present study along with their passport data and biological status are provided in Table S1.

Development of barley mini-core collection

The mini-core collection was extracted from a multipurpose core collection of barley (678 accessions) representing maximum diversity and representativeness of the whole collection conserved as base collection at INGB (Kaur et al. 2022). The mini-core collection comprised 107 accessions, of which approximately 10% (67 accessions) were extracted from the core set germplasm considering

the neutral allele theory (Brown 1989) and 40 trait-specific accessions were additionally included for superior expression in the field and or limited representation from the INGB. The assembly was done using R front-end for Core Hunter 3 software (De Beukelaer and Davenport 2018; De Beukelaer et al. 2018) by maximizing both average genetic distances between each accession and nearest entry in the core (A-NE) and the average distance between each entry and nearest neighbouring entry (E-NE) with equal weightage of 1:1 as described earlier (Odong et al. 2013; Kaur et al. 2022). The quality of the mini-core was assessed using various quality evaluation indices based on differences in the mean, range and variance in comparison to the core collection using the R package EvaluateCore (Aravind et al. 2020).

Experimental conditions for phenotyping of root and shoot traits

To evaluate the salt tolerance potential of barley germplasm at the seedling stage, a laboratory experiment was carried out in the year 2019–20 at ICAR-NBPGR, New Delhi, India. The seeds of all 110 accessions and two released cultivars- NDB1173 and RD2794 (zonal checks for saline/alkaline soils) were surface sterilized with 2% Bavistin (w/v for 10 min) followed by Sodium Hypochlorite 2% (v/v for 10 min) using a horizontal shaker and then rinsed twice in double distilled water before being kept in aseptic Petri dishes (diameter 15 cm) paved with water-soaked damp germination paper. Post-germination, all accessions were tested in a hydroponic system using the germination paper roll method as explained by Manju et al. (2023). One set of seedlings was maintained as control/non-stressed (0 mM NaCl) and received only half-strength Hoagland solution (Hoagland and Arnon 1950) as a nutrient medium, while the other set received salinity stress treatment by adding 200 mM NaCl supplemented with Hoagland solution. To prevent stress shock, the stress treatment was imposed gradually in a phased manner by exposing the seedlings to 100 mM NaCl initially (post-germination) and then the concentration of the salt solution was increased to 150 mM on 3rd day before arriving at the chosen concentration of 200 mM NaCl on 6th day. Each set was kept at optimal growing photoperiod (16 h light/ 8 h dark) and day/night temperature (25/20 ± 2 °C) and 65–75%

relative humidity (RH) conditions for 21 days. The experiment was performed in a randomized complete block design (RCBD) with three replications in each treatment. Each paper roll containing ten seedlings constituted an experimental unit and five healthy and homogenous seedlings per accession made one replicate. Proper aeration was maintained and changing of solution was done at weekly intervals during the entire period of seedling growth and development. After 21 days of growth, seedlings were dissected at root-shoot junction and phenotypic data on various root and shoot traits and relative change in them under saline treatment were recorded. Root samples were then scanned in greyscale at 300 dpi using a flatbed scanner (EPSON 11000XL) equipped with Win-RHIZO™ Pro software (LA-2400 series, Regent Instruments, Montreal, QC, Canada) to measure total root length (TRL, cm), total root surface area (TRSA, cm²), total root volume (TRV, cm³), total root diameter (TRD, mm), fine roots of ≤ 0.5 mm diameter (percent contribution of total root length (FR0.5)), fine roots of ≤ 1.0 mm diameter (percent contribution of total root length (FR1)), no. of root tips and forks. In addition, other root-shoot traits such as seminal root number (SRN), coleoptile length (CL, cm), total plant biomass (TPB, mg), root shoot ratio (RSR), shoot dry weight (SDW, mg), and root dry weight (RDW, mg) were also recorded. SDW and RDW were recorded using a digital balance (Sartorius; BSA224S-CW) with an accuracy of ± 0.001 g after drying the samples in a hot air oven at 70 °C for 72 h.

Statistical data analysis for identification of a subset of salinity tolerant and susceptible accessions for outdoor evaluation experiment

Analysis of Variance (ANOVA) was performed on all root-shoot traits according to RCBD design using 'R' software 4.3.0 to test the effects of treatment (control and salinity) and their interaction. The correlation coefficients between all traits were computed using PAST software (v4.04) (Hammer et al. 2001). The box plots for all root-shoot traits were created and visualised using the 'ggplot2' package of 'R' software (R Core Team 2020). The relative change (RC) in all the observed traits were calculated as the average of the stress treatment divided by that of the control. Principal component analysis (PCA) was performed on the RC value to detect the inter-relationships

among measured traits using 'factoextra' package of 'R'. To group the investigated barley accessions into different categories based on salinity tolerance, hierarchical clustering was done on criteria of Euclidean distance using 'ggdendro' package of 'R'. A subset of 27 accessions comprising of salt-tolerant and susceptible genotypes including two check varieties (NDB1173 and RD2794) was selected on the basis of PCA and cluster analysis for field evaluation employing agro-morphological and physiological traits.

Outdoor evaluation experiment

The outdoor experiments were performed in pots at ICAR-NBPGR farm, IARI, New Delhi during the cropping seasons of two consecutive years (2020–21 and 2021–22). The experimental site is located at latitude 28°38'23''N, longitude of 77°09'27''E and altitude 228.61 m above the mean sea level in the sub-tropical climatic zone of New Delhi, India. All the selected 27 accessions were sown in pots containing normal homogenized field soil. Each accession had three replication pots for control as well as for salinity treatment. Thus, a total of 162 pots (27 accessions \times 2 treatments \times 3 replicates) arranged in a completely randomized design constituted the experimental setup. In each pot, a measured amount of water was given to bring the moisture level to field capacity before sowing. The electrical conductivity of soil and irrigation water was measured before sowing. Initially, 8 seeds were sown in each pot and later 4 uniformly grown seedlings were further retained for recording observations. First irrigation was applied at the three-leaf stage using normal irrigation water (2 dS m⁻¹) in control pots and saline water (200 mM NaCl) irrigation in treatment pots. Thereafter, the salinity levels were maintained at 20 dS m⁻¹ in treatment pots with the help of alternate cycles of irrigation using saline and normal water. To prevent the accumulation of salts at the bottom of the soil in the treatment pots, a specialized apparatus consisting of a plastic tube (2.5 cm diameter and 45 cm length) with perforation at both ends was inserted in the middle of each pot under salinity treatment and used for supplying normal irrigation water. Observations were recorded on 6 phenological and agronomic traits viz. days to spike emergence (DSE), plant height (PH; cm), spike length (SL; cm), number of spike triplet groups per spike (STG), hundred-grain weight

(HGW, g), and grain yield per plant (GY, g) under both control (non-stress) and salinity stress conditions as per Mahajan et al. (2000). In addition, physiological traits such as relative water content (RWC, %), membrane stability index (MSI, %), chlorophyll concentration index (CCI), and chlorophyll fluorescence (Fv/Fm) were recorded in fully expanded flag leaves at post-anthesis stage at around 70–75 days after sowing under both control and saline conditions. RWC was measured from fully expanded flag leaves of each genotype as described by Barrs and Weatherley (1962). MSI was measured as described by Ibrahim and Quick (2001) with the conductivity meter (Cyber Scan CON11 Eutech Instruments, Thermo Fisher). CCI was measured by using a portable hand-held chlorophyll concentration meter (CCM-200, Opti-Sciences, Inc., USA). Chlorophyll fluorescence (Fv/Fm) was measured by using a portable hand-held Chlorophyll Fluorometer (OS30P + Opti-Sciences, Inc.). Shoot Na^+ and K^+ content was measured at maturity by using a flame photometer (PFP7, Bibby Scientific, UK) and expressed on mg/g dry weight basis. The data on all the recorded traits were analysed as per RCBD design using 'R'. The correlation plots between all traits under control and salinity treatment were prepared using Past3 software. The relative stress tolerance of the accessions under field evaluation was characterized using Stress Susceptibility Index (SSI) as per Fischer and Maurer (1978) and they were categorised as highly salt tolerant ($\text{SSI} \leq 0.50$), tolerant ($0.50 < \text{SSI} \leq 1.00$) and susceptible ($\text{SSI} > 1.00$). Based on SSI, 13 germplasm comprising 7 salt tolerant and 6 salt sensitive accessions were chosen for sequence variation analysis of the candidate gene, *HvRAF*. The relative performance of tolerant and susceptible germplasm was depicted using violin cum box plots visualised through the 'ggplot2' package of 'R'.

Study of allelic variation in candidate gene

DNA was isolated from 14-day-old seedlings following the CTAB method (Doyle and Doyle 1987) with minor modifications. The total genomic DNA was quantified using NanoDrop™ One/One^C (Thermo Scientific). Gene-specific primers were designed using the Primer 3 tool (Untergasser et al. 2012) with barley *HvRAF* gene (NCBI Genbank: accession number DQ102384) as reference sequence (Table S2). PCR amplification

was done following conventional cycling conditions with a reaction volume of 40 μl having a final concentration of DNA adjusted to 50 ng/ μl using Hi-Proof™ DNA Polymerase (HiMedia) as per the manufacturer's protocol in a thermocycler (BLUE-RAY BIOTECH). The amplified PCR product was run on 1.5% TAE Agarose gel electrophoresis and visualized on a Gel-Documentation system (Alpha Biotech). The desired amplicon was cut and gel purified using QIAquick® gel elution kit (Qiagen) to be used further for Sanger sequencing using gene-specific primers. Percentage sequence similarity was confirmed using the BLASTn program (NCBI; <http://www.ncbi.nlm.nih.gov/gorf/gorf.html>) and multiple sequence alignment was executed following the Clustal Omega program (Sievers et al. 2011).

Phenotyping of selected accessions contrasting in stress response

To evaluate salt responsiveness of *HvRAF* gene expression in a spatiotemporal manner, four selected genotypes (one tolerant and one susceptible accession from each of the two haplotype groups) were grown hydroponically as described previously in the Section 'Experimental conditions for phenotyping of root and shoot traits' and phenotyping for the root-shoot traits (total root length, total root surface area, total root volume, root diameter, primary root length, coleoptile length, root and shoot biomass) was conducted in six biological individual replicates. These accessions were further assayed for physiological-biochemical parameters (RWC, MSI, chlorophyll content, proline, Malondialdehyde (MDA) and H_2O_2 content). RWC and MSI were measured from the leaves of the control and stressed plants as mentioned previously in the Section 'Outdoor evaluation experiment'. The chlorophyll content was quantified spectrophotometrically (Hiscox and Israelstam 1979). Proline estimation was done as per Bates et al. (1973). MDA content was estimated following the method by Heath and Packer (1968) and H_2O_2 content was determined as per Alexieva et al. (2001).

Total RNA extraction and quantitative gene expression analysis

Total RNA was isolated from the shoot tissue of selected four barley accessions post 12 h of salt

treatment (200 mM NaCl) using RNeasy® Plant Mini Kit (Qiagen). DNA contamination was removed by using DNase I treatment (Qiagen). About 1 µg of total RNA was used to synthesize first-strand cDNA using PrimeScript™ cDNA synthesis kit (TaKaRa). Details of primers used for quantitative expression profiling of *HvRAF* are provided in Table S2. qRT-PCR was performed using the KAPA superfast SYBR mix (KAPA Biosystems) in a total volume of 10 µL on the StepOne Real-Time PCR system (Applied Biosystems). All the PCR reactions were performed on three independent biological samples with three technical replicates. Data were normalized to the barley *Actin* gene, and the relative expression levels of the target genes were calculated using the formula $2^{-\Delta\Delta Ct}$ (Livak and Schmittgen 2001). Relative fold change was calculated for the NaCl-treated plants compared to the control (non-stress conditions). The expression data were analysed using Student's t-test.

Results

In the present study, the salt tolerance potential of barley collection conserved at INGB was investigated in a diverse and representative set of accessions in the form of a mini-core collection. The evaluation was done at the seedling and adult plant stage which helped identify novel potential donors for imparting salt tolerance. Allelic variation was unraveled in the candidate gene (*HvRAF*) in a sub-set of selected salt-tolerant and susceptible genotypes. The salt-tolerant phenotype variation was further evaluated for morpho-physiological adaptation under stress and gene expression analysis to understand stress response. The findings are presented under respective sub-heads in the following section:

Root and shoot growth under salinity stress

The two-way analysis of variance (ANOVA) for various root and shoot traits at the seedling stage showed that the effect of salinity was highly significant ($P \leq 0.05$) for all the examined traits except for number of root tips (Table 1). Significant differences were observed for all traits among the investigated accessions. Similarly, the interaction effect between the genotypes and salinity stress was significant for all root and shoot features. The statistical analysis

showing mean, range and coefficient of variation (CV%) for all traits under both control and salt treatments and % reduction due to stress conditions is shown in Table 1. The phenotypic means were higher for all traits under control conditions except for RSR, TRD, Tips, Forks and FR0.5. The range was wider for all traits under control and stress conditions except TRD and forks under salt stress. The coefficient of variation ranged from 10.65% to 63.87% under control and 15.75% to 74.21% under stress conditions. Salinity caused the reduction (7.39–62.02%) in all parameters except RSR, TRD, Tips, Forks and FR0.5, where stress-induced increase was noticed. CL reduced drastically (62.02%) in all barley accessions when exposed to 200 mM NaCl compared to control conditions. The minimum reduction was recorded in SRN (7.39%). Differential stress response and profound genotypic variation among the investigated barley mini-core accessions were observed for all the root and shoot traits under control and saline stress conditions as depicted in box plots (Fig. 1). Salinity caused a decline in all parameters as could be visualised through box plots, however, RSR, TRD, tips, forks and fine roots (FR0.5) showed stress-induced increase in comparison to control conditions.

Correlation matrix for root and shoot traits at the seedling stage

Correlation analysis revealed highly significant and positive correlations among different shoot and root traits under control and saline conditions (Fig. 2 and Table S3). Major root traits such as TRL, TRSA and TRV were significantly and positively correlated with CL, TPB and RDW under control as well as saline stress conditions. Fine roots possessing diameters ≥ 0.5 mm and ≤ 1.0 mm were also positively and significantly correlated with all major root and shoot traits such as RDW, TPB, CL, SRN, TRSA, TRD and TRV under both environments. RSR was positively correlated ($r=0.64$, $p \leq 0.05$) with RDW under control conditions and saline conditions although the magnitude was comparatively low ($r=0.32$, $p \leq 0.05$) under salinity. A negative and highly significant correlation was observed among RSR and CL ($r=-0.52$), SDW ($r=-0.41$) and FR0.5 ($r=-0.39$) under saline conditions, however no relationship was observed among these traits under non-stress environment. TRD was found to be negatively correlated with the number

Table 1 Analysis of variance, descriptive statistics, coefficient of variation (CV%) and percent reduction for various root-shoot traits at seedling stage in 110 barley accessions under control and saline conditions

Traits	Mean sum of squares				Control				Salinity stress				% reduction
	Genotype (G)	Treatment (T)	G X T	Residual	Range	Mean ± S.E	SD	CV (%)	Range	Mean ± S.E	SD	CV (%)	
CL	90.68**	41,664.36**	59.10**	3.70	12.87–41.73	27.01 ± 0.58	6.17	22.84	2.38–21.12	10.26 ± 0.37	3.88	37.81	62.02
SDW	1320.95**	38,215.24**	369.14**	56.77	5.33–122.67	60.05 ± 1.70	18.02	30.01	5.33–103.67	44.09 ± 1.57	16.63	37.71	26.58
RDW	291.22**	4037.92**	139.14**	67.75	2.33–83.67	22.69 ± 1.01	10.68	47.09	1.33–43.00	17.48 ± 0.57	6.04	34.57	22.97
TPB	2514.43**	67,097.48**	679.52**	127.99	7.67–165.33	82.74 ± 2.48	26.2	31.67	6.67–146.67	61.57 ± 2.01	21.25	34.51	25.59
RSR	0.08**	0.23**	0.06**	0.02	0.18–1.55	0.38 ± 0.02	0.16	42.45	0.18–0.90	0.42 ± 0.01	0.13	31.55	-10.52
SRN	3.99**	21.54**	0.71**	0.15	1.17–7.08	5.28 ± 0.09	0.91	17.33	1.00–6.53	4.89 ± 0.09	0.92	18.74	7.39
TRL	923.22**	26,174.52**	291.94**	29.89	6.77–89.31	51.56 ± 1.50	15.91	30.85	6.22–74.04	38.34 ± 1.27	13.43	35.02	25.64
TRSA	20.86**	496.86**	5.27**	0.59	0.66–13.88	7.72 ± 0.23	2.49	32.21	0.60–10.58	5.90 ± 0.17	1.82	30.86	23.58
TRD	0.02**	0.15**	0.01**	0.00	0.31–0.60	0.48 ± 0.00	0.05	10.65	0.31–0.74	0.51 ± 0.01	0.08	16.42	-6.25
TRV	0.00**	0.05**	0.00**	0.00	0.01–0.19	0.09 ± 0.00	0.03	35.82	0.01–0.15	0.07 ± 0.00	0.02	32.53	22.23
Tips	4359.83**	568.26	1177.77**	158.75	8.58–252.83	50.07 ± 3.02	31.98	63.87	10.17–160.67	52.04 ± 2.82	29.88	57.42	-3.93
Forks	6617.82**	28,039.16**	1972.37**	217.95	3.17–132.58	49.31 ± 2.56	27.04	54.85	4.33–262.08	63.18 ± 4.43	46.88	74.21	-28.12
FR0.5	601.94**	597.59**	2155.81**	16.31	37.12–95.50	69.59 ± 1.10	11.60	16.67	45.24–94.77	71.77 ± 1.07	11.31	15.75	-3.13
FR1	504.31**	141.42**	111.90**	14.51	3.06–58.65	27.42 ± 1.07	11.28	41.19	4.31–47.15	23.55 ± 0.90	9.57	40.65	14.11

*, **Significance at the P ≤ 0.05 level and P ≤ 0.01 level, respectively; CL, Coleoptile Length (cm); SDW, Shoot Dry Weight (mg); RDW, Root Dry Weight (mg); TPB, Total Plant Biomass (mg); RSR, Root Shoot Ratio; SRN, Seminal Root Number; TRL, Total Root Length (cm); TRSA, Total Root Surface Area (cm²); TRD, Total Root Diameter (mm); TRV, Total Root Volume (cm³); Tips, No. of Root Tips; Forks, No. of Root Forks; FR0.5, Fine Roots of Diameter ≤ 0.5 mm (percent contribution of total root length); FR1, Fine Roots of Diameter ≤ 1.0 mm (percent contribution of total root length)

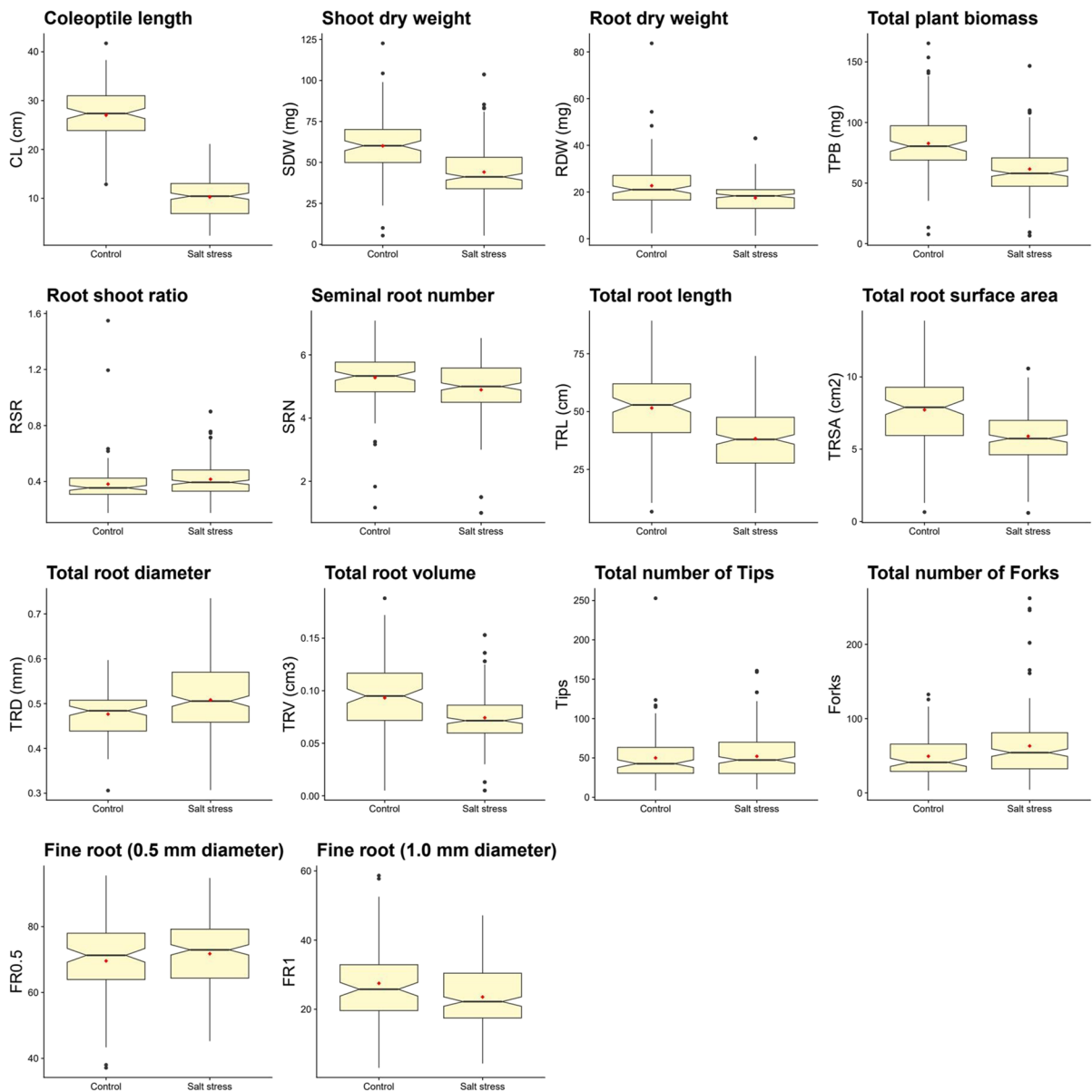


Fig. 1 Box plots showing the genotypic variation among barley mini-core accessions and stress response for different root and shoot traits under control and saline stress conditions. Each

box represents interquartile range. Whiskers show the range and horizontal bar in each box shows the median. Red dot represents the mean

of root tips, forks and fine roots (≤ 0.5 mm diameter) under both non-stress and stress environments.

Principal component analysis and hierarchical clustering

The principal component analysis (PCA) was computed on the dataset of all 14 root-shoot traits

recorded in 112 barley accessions including two check varieties to group the measured traits based on the relationship among germplasm under salinity stress. The results showed that the first four principal components (PCs) with Eigen value > 1 accounted for 76.66% of total variations (Table S4). The first PC (PC1) accounted for 40.91% of the total variations in the data and was significantly

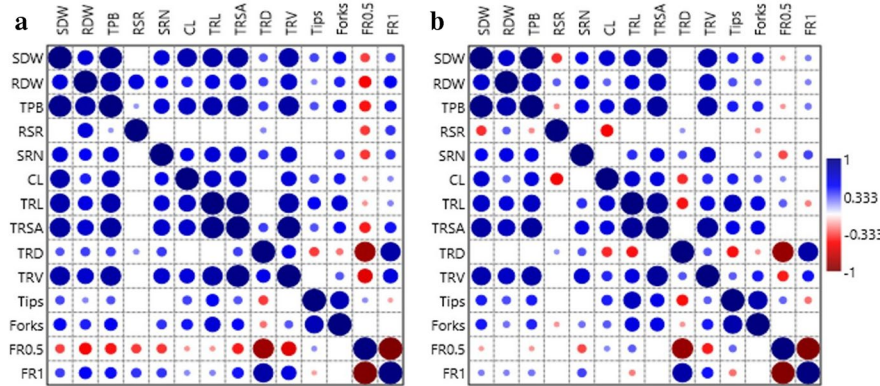


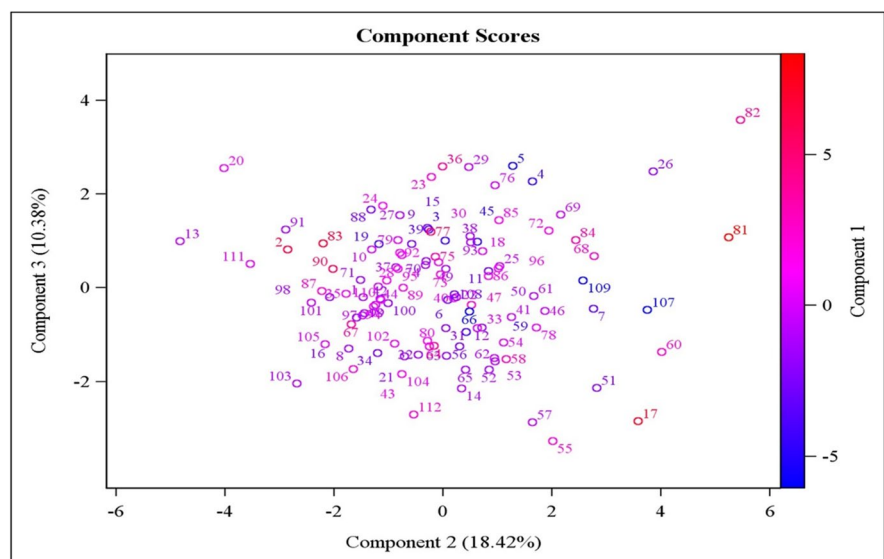
Fig. 2 Correlation matrix between the root-shoot traits showing significant interrelationship under control (a) and salinity stress (b) conditions among 110 barley germplasm. Colour gradient of the circle from blue to red denotes positive to negative

values of correlation, respectively. Size of the circle represents the strength of p value. Correlation between the traits was considered significant at p value ≤ 0.05 . The upper and lower diagonal from left to right are mirror images of each other

correlated with CL, SDW, RDW, TPB, SRN, TRL, TRSA, TRV, Tips and Forks. PC2 explained 18.42% of total variations and was mainly inferred by TRD, FR0.5 and FR1. PC3 accounted for 10.38% of total variation and was influenced by RSR, while PC4 accounted for 6.95% of total variation. To visualise the accessions based on their relative salt tolerance, PCA plot was drawn on the relative change in different seedling traits under salinity stress compared to the control (Fig. 3). Further, the dendrogram obtained from cluster analysis grouped all the investigated barley accessions into 4 major sub-clusters

(Table S5, Fig. S1). Group I being highly salinity tolerant had 13 accessions, group II being salinity tolerant had 45 accessions including all 3 wild barley germplasm included in the study, while group III and group IV accommodated 37 and 17 accessions which were susceptible and highly susceptible to salinity, respectively. Based on PC1 scores and clustering pattern, a sub-set of 27 accessions comprising salinity tolerant and susceptible germplasm was selected for field evaluation at the adult plant stage using physiological and agronomic traits (Table S5).

Fig. 3 Principal component analysis (PCA) plot of various root and shoot growth parameters in barley germplasm accessions investigated under salinity stress conditions. The relative values of all traits were used for PCA. Component 1, Component 2 and Component 3 represent PC1, PC2 and PC3 scores, respectively. Red colour indicates high PC1 scores (highly salt tolerant accessions) and blue colour indicates low PC1 score (highly salt sensitive accessions)



Outdoor evaluation at the adult plant stage

Amongst the 27 accessions of barley germplasm selected for an outdoor evaluation, salinity tolerance at the seedling stage was validated in adult plants in pot evaluation. Table 2 presents the summary statistics of physiological and morpho-agronomic traits under control and salinity stress imposed in pots. Under salinity stress conditions the mean value of all traits across the 27 investigated germplasm was reduced in the range of 5.94% to 80.04% compared with their respective values in control conditions. All tested germplasm showed a high rate of reduction for physiological parameters ranging from 5.94% for RWC to 13.78% for MSI. The relative chlorophyll content (CCI) and chlorophyll fluorescence (Fv/Fm) also showed salinity-induced decline by 10.73% and 7.10%, respectively. About ionic concentrations, the shoot Na⁺ content was significantly increased (170.11%) owing to NaCl treatment. The pattern of K⁺ concentration in shoot tissue was different from the pattern in Na⁺ content. Salinity treatment severely decreased SK (45.73%) across all the 27 tested barely accessions. The ratio of SK to SN in barley shoots decreased by 80.04% under salinity stress compared

to control conditions. Spike emergence was significantly delayed owing to stress. The reduction was more severe for yield and its attributes under saline conditions. Salinity reduced PH (23.39%), SL (20.08%), STG (21.46%) and NGS (28.12%), while mean HGW and GY were reduced drastically by 48.62% and 65.35%, respectively. The accessions susceptible during initial growth were found to be susceptible during field trial, however, two accessions namely, EC0578761 and EC0578517 showed tolerance to salinity stress at the seedling stage but turned out to be susceptible during reproductive phase and maturity during field evaluation (Table 3 and S5). The comparative differences in the mean and range of physiological and morpho-agronomic traits recorded under control and salinity stress conditions were visualized through violin cum box plots (Fig. 4), depicting the differential stress response *vis-a-vis* genotypic variability in studied germplasm.

Inter-relationships between physiological and agronomic traits at the adult-plant stage

Pairwise correlation coefficients were computed for all the traits recorded in the selected panel of

Table 2 Range, mean and percent reduction in different traits assessed in a sub-set of selected 27 accessions grown under control and saline conditions in an outdoor cultivation experiment

Trait	Control		Salinity stress		% reduction
	Range	Mean + SE	Range	Mean + SE	
RWC	73.29–88.00	80.92 + 2.42	69.35–83.00	76.11 + 2.38	5.94
MSI	68.32–92.23	77.85 + 4.97	59.00–84.12	67.12 + 5.18	13.78
CCI	20.32–39.67	29.77 + 3.68	17.25–39.43	26.57 + 4.27	10.73
CF	0.69–0.79	0.75 + 0.02	0.61–0.75	0.69 + 0.03	7.10
SN	5.00–17.60	9.56 + 2.07	15.00–37.50	25.87 + 4.34	-170.11
SK	14.00–31.00	23.18 + 3.02	7.95–17.85	12.58 + 1.64	45.73
SKN	0.91–5.44	2.63 + 0.63	0.23–1.19	0.53 + 0.15	80.04
DSE	63.00–98.50	89.46 + 5.19	63.00–109.50	91.24 + 6.16	-1.99
PH	45.25–88.12	65.33 + 7.69	30.75–70.00	50.05 + 6.42	23.39
SL	1.73–8.67	6.47 + 1.13	1.87–7.03	5.17 + 0.84	20.08
STG	8.33–22.67	15.65 + 2.65	6.50–18.67	12.29 + 2.11	21.46
NGS	10.50–48.60	24.73 + 7.89	1.67–43.00	17.78 + 6.97	28.12
HGW	0.55–4.81	2.80 + 0.69	0.41–3.28	1.44 + 0.47	48.62
GY	1.86–8.29	6.04 + 1.21	0.22–4.82	2.09 + 0.77	65.35

RWC, Relative Water Content (%); MSI, Membrane Stability Index (%); CCI, Chlorophyll Concentration Index; CF, Chlorophyll Fluorescence (Fv/Fm); SN, Shoot Na⁺ content (mg/g DW); SK, Shoot K⁺ content (mg/g DW); SKN, Shoot K⁺/Na⁺ ratio; DSE, Day to Spike Emergence; PH, Plant Height (cm); SL, Spike Length (cm); STG, No. of Spike Triplet Groups per Spike; NGS, Number of Grains per Spike; HGW, Hundred Grain Weight (g); GY, Grain Yield per Plant (g)

Table 3 Subset of salt tolerant and sensitive barley germplasm accessions for identification of allelic variants of *HvRAF*

S. no	Stress response group	National id	Spike row type	Grain type	SSI
1	Salt Tolerant	EC0578359	Six-row	Hulless	0.37
2	Salt Tolerant	EC0578251*	Two-row	Hulled	0.42
3	Salt Tolerant	IC0547723	Six-row	Hulless	0.39
4	Salt Tolerant	EC0123148 [#]	Two-row	Hulled	0.35
5	Salt Tolerant	EC0299361 [§]	Two-row	Hulled	0.74
6	Salt Tolerant	EC0177250	Two-row	Hulled	0.55
7	Salt Tolerant	IC0247671	Six-row	Hulled	0.61
8	Salt Sensitive	EC0578761	Six-row	Hulless	1.41
9	Salt Sensitive	EC0177257	Two-row	Hulled	1.22
10	Salt Sensitive	RD2794	Six-row	Hulled	1.07
11	Salt Sensitive	IC0533161	Six-row	Hulless	1.35
12	Salt Sensitive	EC0578517	Six-row	Hulled	1.21
13	Salt Sensitive	IC0355879	Two-row	Hulled	1.43

**H. murinum* ssp. *leporinum*; [#]*H. marinum* (Huds) (ssp. *gussoneanum*) (Parl.); [§]*H. spontaneum* (K. Koch.)

27 barley accessions evaluated in field conditions for salinity tolerance. Only the traits showing significant correlation ($p \leq 0.05$) are shown in the correlogram (Fig. 5). In general, the grain yield and its attributes SL, HGW, STG and PH were found to be positively and significantly correlated with each other under both non-stress and stress environments. NGS responded differently under control and saline conditions. It had a highly positive significant correlation with SN and PH and a negative correlation with SKN under control conditions. However, it was negatively associated with SK under saline conditions. Shoot Na^+ content was found to be negatively correlated with RWC ($r = -0.40$, $p \leq 0.05$), MSI ($r = -0.52$, $p \leq 0.01$) and GY ($r = -0.39$, $p \leq 0.05$). SKN was negatively and significantly correlated with PH ($r = -0.39$, $p \leq 0.05$) and SL ($r = -0.56$, $p \leq 0.01$) under salinity stress (Table S6).

Haplotypic variant analysis of *HvRAF* gene in salinity tolerant and susceptible barley genotypes

A subset of 7 salt tolerant and 6 salt-sensitive genotypes was constituted based on SSI for studying the allelic variation in candidate gene, *HvRAF* for salt tolerance (Table 3). SSI was computed on grain yield to assess the relative stress tolerance potential of studied germplasm and it was low ranging from 0.35 to 0.74 in salt tolerant accessions, while high SSI values (1.07 to 1.43) were recorded in susceptible germplasm. Salt tolerant accession- EC0123148 had the lowest SSI=0.35, while the most susceptible

accession- IC0355879 had the highest SSI=1.43. Diverse germplasm with varying different genetic background in spike morphology (two-rowed/six-rowed) and grain type (hulled or naked/hulless) were accommodated in this panel to identify allelic variants. This subset had 7 accessions of six-rowed barley (*H. vulgare* ssp. *hexastichon*) and 6 accessions of two-rowed barley (*H. vulgare* ssp. *distichon*), while grain type in 4 accessions was hulless or naked and remaining 9 accessions had hulled grains. In the selected salinity tolerant and susceptible accessions, 26 SNP loci were identified across the two exonic regions leading to 10 non-synonymous and 8 synonymous SNPs, while 5 SNPs were conserved (Fig. 6). Further, the non-synonymous and synonymous SNPs led to formation of two major haplotypic groups- a smaller haplotype (Hap 1), comprising four genotypes namely IC0547723, EC0299361 and IC0355879 including the reference sequence- *Hordeum vulgare* ssp. *vulgare* cv. Jinkwang (GenBank accession DQ102384.2) and the larger haplotype (Hap 2) constituted by 9 genotypes namely, EC0578359, EC0578761, EC0578251, EC0177250, IC0247671, EC0177257, RD2794, IC0533161 and EC0578517.

Salt tolerant phenotype variation and morpho-physiological changes during salt stress adaptation

Salinity stress was found to notably reduce the overall physiological growth in four selected accessions from two haplotype groups. The tolerant genotypes namely

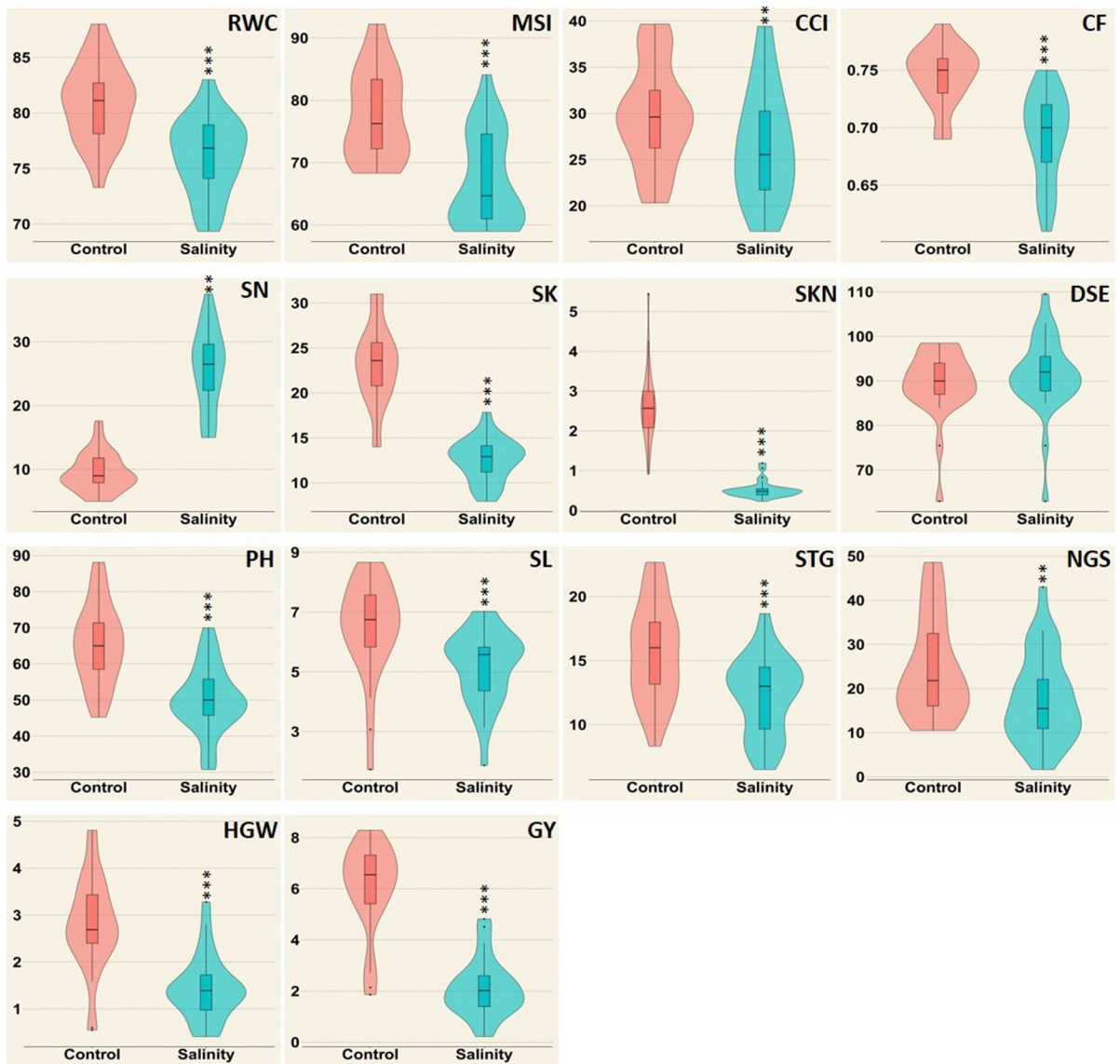


Fig. 4 Violin cum box plots depicting the comparative difference in physiological traits, ionic concentrations and morpho-agronomic parameters of selected 27 barley accessions evaluated under control and salinity stress. RWC, Relative Water Content (%); MSI, Membrane Stability Index (%); CCI, Chlorophyll Concentration Index; CF, Chlorophyll Fluorescence (Fv/Fm); SN, Shoot Na⁺ content (mg/g DW); SK, Shoot K⁺

content (mg/g DW); SKN, Shoot K⁺/Na⁺ ratio; DSE, Day to Spike Emergence; PH, Plant Height (cm); SL, Spike Length (cm); STG, No. of Spike Triplet Groups per Spike; NGS, Number of Grains per Spike; HGW, Hundred Grain Weight (g); GY, Grain Yield per Plant (g). *, **, *** indicate significant differences between control and salinity stress at $P \leq 0.05$, $P \leq 0.01$ and $P \leq 0.001$, respectively

EC0578359 and EC0299361 maintained significantly higher RWC (74 and 79%, respectively) compared to susceptible accessions IC0355879 and EC0578517 wherein it was around 68% (Fig. 7A). Similarly, the chlorophyll content was significantly higher compared to the susceptible accessions (Fig. 7B). The

MSI decreased to about 50% in susceptible accessions, while tolerant lines maintained significantly higher membrane stability (Fig. 7C). Upon quantification, the content of osmolyte proline was found to be higher in EC0578359 (11.97 $\mu\text{g/g}$ FW) and EC0299361 (12.6 $\mu\text{g/g}$ FW) compared to susceptible

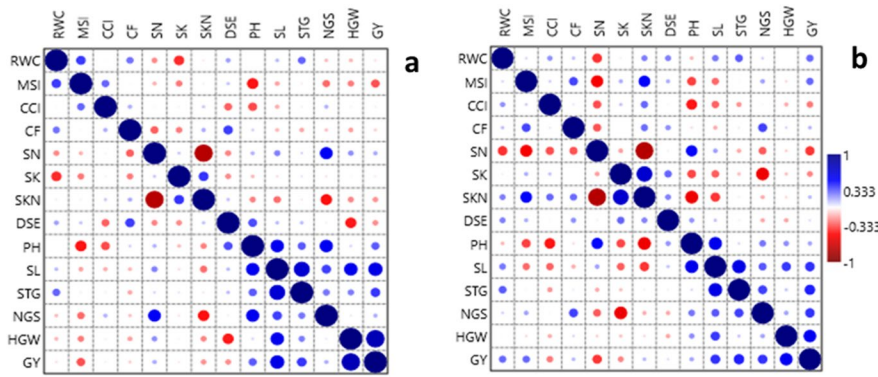


Fig. 5 Pairwise correlation between the morpho-physiological traits showing significant interrelationship under control (a) and salinity stress (b) conditions among 27 barley germplasm. Colour gradient of the circle from blue to red denotes positive to negative values of correlation, respectively. Size of the cir-

cle represents the strength of p value. Correlation between the traits was considered significant at p value ≤ 0.05 . The upper and lower diagonal from left to right are mirror images of each other

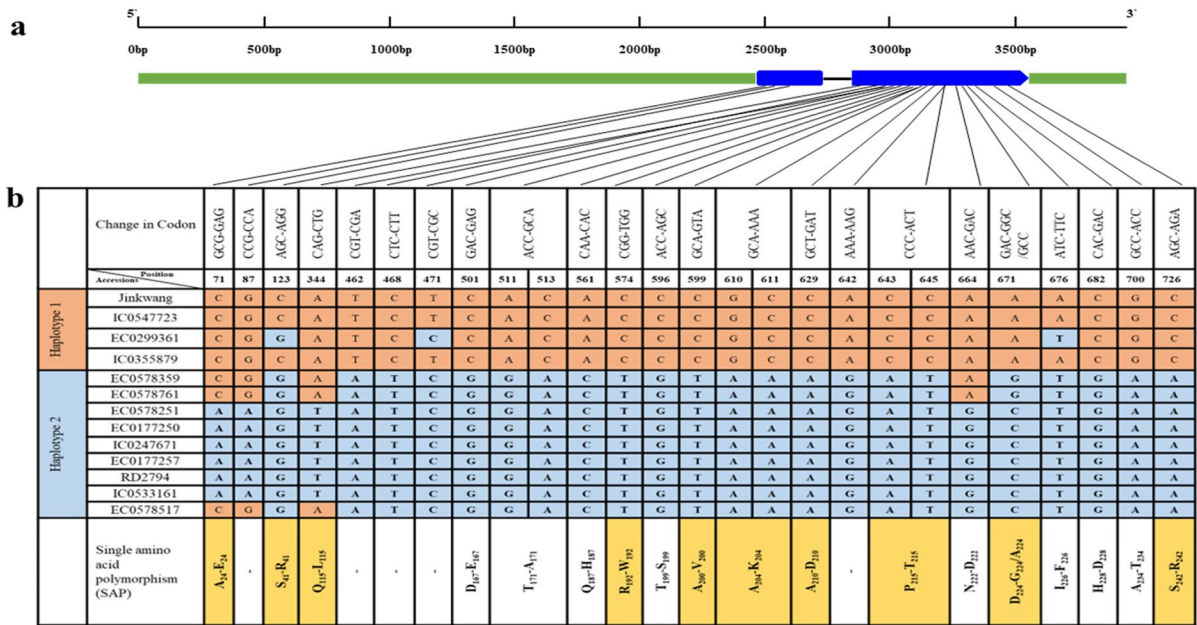
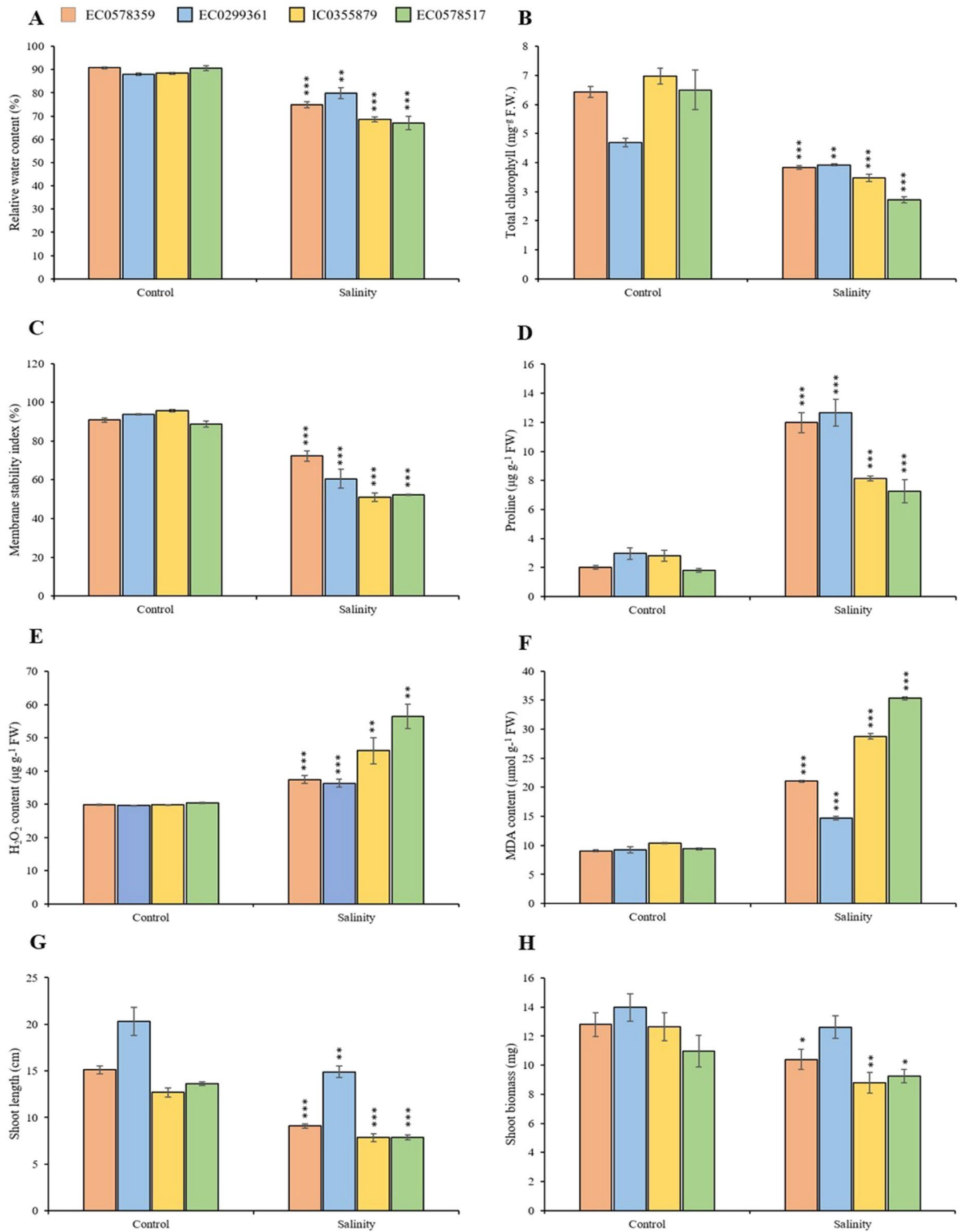


Fig. 6 Schematic representation of identification of two major haplotypes reconstructed for *HvRAF* gene across the selected panel of salinity tolerant and susceptible Barley accessions. (a) Gene structure of *HvRAF* representing the positional localization of SNPs in the exonic region; (b) The header of each col-

umn refers to base pair position of the variant relative to the start codon of *HvRAF* while the lowermost row represents the synonymous and non-synonymous single amino acid polymorphism (SAPs) highlighted in yellow coloured

accessions IC0355879 and EC0578517 with 8.14 and 7.2 $\mu\text{g/g}$ FW, respectively (Fig. 7D). The tolerant genotypes had lower MDA and H_2O_2 content compared with susceptible accessions under salinity stress (Fig. 7E and F). Contrasting phenotypic variation

was also evident in root morphological traits amongst salinity tolerant and susceptible accessions under stress conditions (Fig. S2). Salt tolerance phenotype exhibited an overall reduction of 26–39% in shoot length under salt stress compared to a 38.32–42%



◀**Fig. 7** Morpho-physiological and biochemical analysis in selected accessions under control (non-stress) and salinity stress (200 mM NaCl). **A** Relative water content estimation; **B** Quantification of total chlorophyll; **C** Assessment of membrane stability (MSI); **D** Quantification of Proline content; **E** Quantification of hydrogen peroxide; **F** Quantification of MDA (malondialdehyde) levels; **G** Estimation of Shoot length and **H** Shoot biomass. Bars indicate mean \pm SE. *, **, *** indicate significant differences between control and salinity stress at $P \leq 0.05$, $P \leq 0.01$ and $P \leq 0.001$, respectively

decline in susceptible germplasm (Fig. 7G). Consequently, the overall biomass was reduced proportionally (Fig. 7H). The tolerant genotypes exhibited an overall reduction of 26 and 32% in biomass EC0578359 and EC0299361 compared to 30 and 34.8% in IC0355879 and EC0578517 respectively (Fig. 8A). Our results significantly illustrated a stress-induced decline in total root biomass, length, surface area and root volume while the root diameter showed marginal increase under salt stress (Fig. 8B, C, D, E and F). Thus, the tolerant accessions were able to maintain a better root architecture compared to the susceptible accessions under saline growth environment indicating better adaptation.

Salinity stress-responsive expression profiling of *HvRAF*

The transcript abundance of *HvRAF* was assessed in selected four barley accessions post 12 h of salt treatment. Salt stress upregulated *HvRAF* expression by 15- and 1.35-folds in the identified tolerant genotypes namely EC0578359 and EC0299361, respectively. Contrarily, the expression was downregulated to 0.01- and 0.87 folds in the susceptible accessions IC0355879 and EC0578517, respectively (Fig. 9).

Discussion

Variation of root-shoot traits at early growth under salinity stress

Seedling stage evaluation indicated that all the genotypes were severely affected by salinity stress when compared to control conditions (Table 1). A significant effect of NaCl treatment was evident on various root-shoot parameters (Table 1, Fig. 1). Earlier, Rasel et al. (2020) reported that shoots were more

sensitive to salinity stress in comparison to roots. Present research findings indicated that shoot growth was more severely affected (reduction by 62.02%) compared to root growth (25.64% reduction in TRL) under salinity stress (Table 1). Similarly, a reduction of 25.59% was recorded in root and shoot biomass, while RSR, forks and fine roots of diameter ≤ 0.5 mm were increased under salinity stress, which is likely to be a result of plant adopting to physiological and architectural changes to cope up with stress. Similar reports for salinity-induced reduction in root/shoot biomass have also been reported in wheat (Ahmadi et al. 2020), barley (Pour-Aboughadareh et al. 2021; Ali and Abbas 2003) and rice (Rasel et al. 2020).

Plants respond differently under salt stress conditions depending on species, genotype, and adaptability. Also, the stress response differs with regard to the growth stage of the plant. Multivariate approaches such as PCA, cluster analysis and selection indices (SSI, Stress Susceptibility Index) are widely used to group the measured parameters and to select the best-suited genotypes. The present study also employed PCA and clustering pattern to select a subset of 27 salinity tolerant and susceptible accessions for validation at the adult plant stage in outdoor trials. SSI developed by Fischer and Maurer (1978) was used to characterize the relative stress tolerance potential of selected barley accessions under control and saline conditions. SSI is an inclusive and precise measure and has been well advocated as a selection index for choosing better-performing barley lines under salinity (Jamshidi and Javanmard 2018).

Morpho-physiological basis of tolerance – impact and potential of genotypes

Salt stress led to a severe reduction ranging from 5.94% to 80.04% in all physiological and agronomic traits in comparison to control in the examined set of 27 accessions (Table 2, Fig. 4). The reduction in physiological parameters ranged from 5.94% for RWC to 13.78% for MSI. RWC has been valued as an important physiological indicator of abiotic stress particularly drought and salinity tolerance in plants (Kaur et al. 2020; Manju et al. 2023). A reduction of 5.94% in RWC under salinity conditions in comparison to control conditions was observed (Table 2), which is in congruence with the study of Pour-Aboughadareh et al. (2021) although they reported

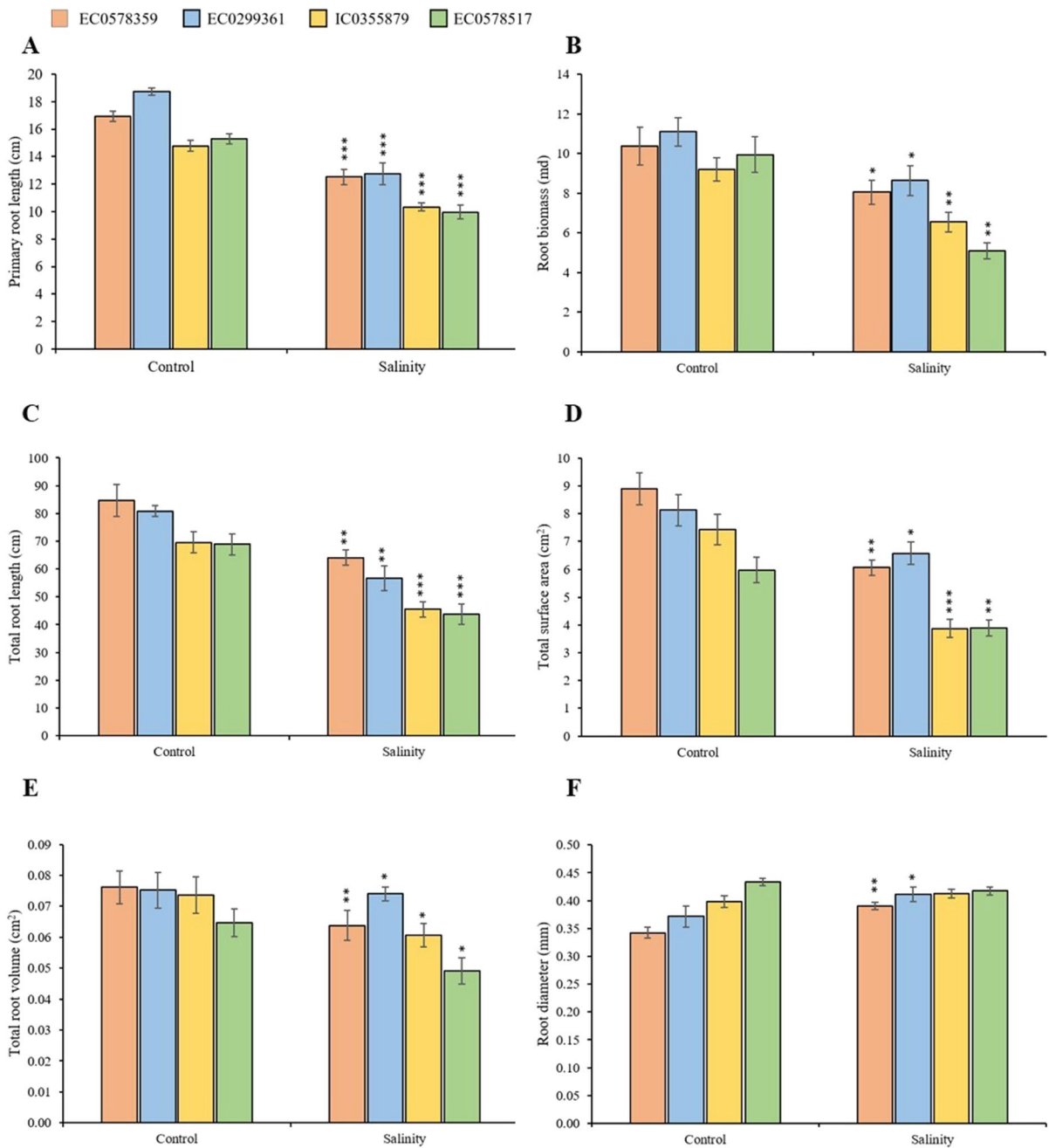


Fig. 8 Comparative analysis of root architecture under control (non-stress) and salinity stress (200 mM NaCl). **A** Estimation of primary root length curated manually; **B** Root biomass; **C** Assessment of total root length; **D** Total surface area; **E** Total

root volume and **F** Bars indicate mean ± SE. *, **, *** indicate significant differences between control and salinity stress at $P \leq 0.05$, $P \leq 0.01$ and $P \leq 0.001$, respectively

reduction of higher magnitude (up to 15.53%) in barley genotypes. Membrane stability is another physiological parameter that has been employed to estimate

salinity tolerance in different plants including barley (ElBasyoni et al. 2017; Ebrahim et al. 2019; Abrar et al. 2020). Furthermore, MSI is a moderately

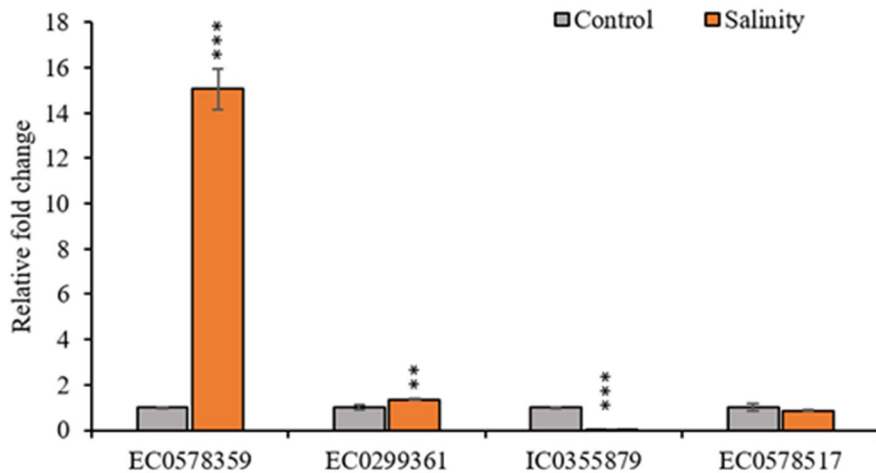


Fig. 9 The *HvRAF* expression level as measured by qRT-PCR across the four accessions at seedling stage under control (non-stress) and salinity stress (200 mM NaCl). Expression levels were normalised against that in control seedlings, which was set to 1. The experiments were repeated three times with simi-

lar results. Error bars indicate SE of three technical replicates. Bars indicate standard error and *, **, *** indicate significant differences between control and salinity stress at $P \leq 0.05$, $P \leq 0.01$ and $P \leq 0.001$, respectively

heritable trait and has been found to correlate with grain yield (Hemantaranjan et al. 2014). However, our findings suggest that MSI was not correlated with GY but with salt uptake. MSI had a positive correlation with SKN and a negative correlation with SN under salinity. Overall, MSI changed significantly when plants were exposed to salinity. On a similar note, the relative chlorophyll content declined (10.73%) under salt treatment, which may be ascribed to the disturbance in structural stability at various points of PSII and chloroplast membrane injury/destabilization. This result was in congruence with earlier reports in extreme halophyte *Salvadora persica* (Rangani et al. 2016) and barley (Pour-Aboughadareh et al. 2021; Akhter et al. 2021).

Salt-tolerant plants have evolved a variety of adaptive strategies to resist this water deficit condition (physiological drought caused by salinity) and to maintain ion homeostasis in the cell (Hasegawa 2013; Zhu 2003; Flowers and Colmer 2008). Selective uptake of essential ions like K^+ and Ca^{2+} and keeping a tolerable range of Na^+ concentration in tissue and thus maintaining ion homeostasis is the most important mechanism for plants to sustain growth under salt stress (Kumar et al. 2018). Numerous studies have reported that plants tend to maintain high K^+ concentration and low Na^+ content in shoots and roots and thus decrease Na^+ toxicity (Tao et al. 2021;

Pour-Aboughadareh et al. 2021; Akhter et al. 2021; Chaurasia et al. 2022; Javed et al. 2022). Under saline conditions, Na^+ ions can easily enter through the cell membrane via K^+ channels (owing to similar hydration energy and ionic structure with Na^+). This leads to higher cytoplasmic concentration of Na^+ and K^+/Na^+ ratio which negatively affects the growth and development of plants. In this study, the shoot Na^+ content was significantly increased (170.11%). In comparison, SK content decreased (45.73%) owing to NaCl treatment (Table 2, Fig. 4). Furthermore, the ratio of shoot K^+ to Na^+ content decreased drastically by 80.04% under salinity stress. The SN content was negatively correlated with physiological parameters RWC, MSI and GY under saline conditions. Among the selected panel of 7 salt-tolerant and 6 salt-sensitive accessions (Table 3), the mean SN content of tolerant accessions was 17.61 mg/g DW, which is much lower than the Na^+ content of salt-susceptible accessions (29.12 mg/g DW). In addition, the tolerant accessions were able to maintain a higher SKN ratio of 0.78 compared to susceptible germplasm (0.46) under saline conditions. Therefore, these accessions can serve as superior salinity-tolerant lines due to the better maintenance capability of K^+ ions in the tissue. Following earlier studies, it may be ascribed to the plant's defense strategy to minimize the negative effects of excessive Na^+ and to maintain a high $K^+/$

Na⁺ ratio (Adolf et al. 2013). Regarding, yield and its attributes under stress conditions, salinity reduced PH (23.39%), SL (20.08%), STG (21.46%) and NGS (28.12%), HGW (48.62%) and GY (65.35%). Several studies have earlier reported similar results in barley for ion uptake and growth restriction under salinity (Tavakkoli et al. 2011; Rajeswari et al. 2019; Pour-Aboughadareh et al. 2021; Akhter et al. 2021). Overall, based on cumulative stress response in terms of better root-shoot traits, physiological parameters including ionic concentrations and grain yield, accessions EC0578359, EC0578251, IC0547723, EC0123148, EC0299361, EC0177250 and IC0247671 were identified as the most promising salt-tolerant germplasm.

Unraveling the salt-tolerant phenotype variation and morpho-physiological adaptation in the haplotypic variants of the *HvRAF* gene

Scarce information is available regarding the involvement of *Root abundant factor (RAF)* in regulating stress-responsive genes in plants (Yi et al. 2004; Lee et al. 2004; Jung et al. 2007). In the present study, allele mining for the *HvRAF* gene in the selected salt-susceptible and tolerant accessions led to the identification of a total of 26 SNPs across the two exonic regions of the gene. The analysis of SNP polymorphism revealed the 10 non-synonymous and 8 synonymous SNPs thus constituting two major haplotypic groups- a smaller haplotype (Hap 1) comprising four genotypes and the larger haplotype (Hap 2) constituted by 9 genotypes, although haplotypic variants could not be associated either with salinity tolerance or susceptibility. These findings suggest trait governance by more genes/multiple loci. However, the physiological and biochemical assays in representative accessions from each Hap group indicated that the salt-tolerant genotypes (EC0578359 and EC0299361) were able to maintain significantly higher RWC, MSI, chlorophyll content and proline compared to susceptible accessions (IC0355879 and EC0578517) (Fig. 7). The tolerant genotypes had lower MDA and H₂O₂ content compared with susceptible accessions under salinity stress (Fig. 7E and F). Also, the tolerant accessions were able to maintain a better root architecture compared to the susceptible accessions under saline growth environment indicating better

adaptation (Fig. 8, Fig. S2). Similar findings were also reported in the evaluation of wheat germplasm (Chaurasia et al. 2022) and in barley under salinity stress (Javed et al. 2022). Furthermore, salt stress upregulated *HvRAF* expression in tolerant genotypes (Fig. 9). Therefore, it will be interesting to identify more haplotypic variants in a bigger panel of diverse germplasm to establish and validate trait association. Data from the coding regions are regularly used for the identification of stress-responsive genes. The identification of allelic variations in salt-responsive candidate gene from diverse phenotypes can provide genomic resources given the scarce information available for *HvRAF*. Further exploration of novel haplotypes and germplasm resources will be instrumental in the adoption of haplotype-led breeding programs for promoting trait-tailored parental selection to maximize genetic gains.

Conclusion

Soil salinization is a major global constraint for agricultural contemporary productivity as it is threatening sustainable food production from limited land and water resources amid climate change concerns. The identification of novel allelic variants from diverse germplasm can assist in obtaining desirable genotypes through breeding. This study evaluated a diverse set of germplasm in the form of barley mini-core collection for root system architecture, physiological efficiency and agronomic performance under salinity stress and identified salt-tolerant germplasm which may be useful to develop salt-tolerant varieties and also aid in developing mapping populations for the detection of underlying QTLs for salinity tolerance. The haplotype variant analysis unraveled allelic diversity associated with two haplotype groups, of which one was more prevalent (Hap 2). None of the identified SNPs and haplotypic variants could be linked with salinity tolerance/susceptibility, however, salt-tolerant phenotype exhibited the physiological and biochemical basis of tolerance. Also, the expression of *HvRAF* was upregulated in tolerant genotypes. The present findings provide a comparative account of the candidate gene variation and morpho-physiological traits for salt tolerance in barley, which can be used in improving stress tolerance.

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Authors' contributions S: Investigation, data curation, writing original draft; VK: Conceptualization, resources, supervision, writing, review and editing; SKY: Investigation, formal analysis and writing original draft; SSA: Conceptualization, Supervision, JA: Resources, formal analysis; SRJ: Resources; RKG: review and resources.

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Data availability The original contributions presented in the study are included in the article/supplementary information. The full-length *HvRAF* gene sequences from barley germplasm accessions were submitted to NCBI (<https://www.ncbi.nlm.nih.gov/nucleotide/>) under GenBank ids: OR077393, OR077394, OR077395, OR077396, OR077397, OR077398, OR077399, OR0773400, OR0773401, OR0773402, OR0773403, and OR0773404 and are also included in the manuscript under supplementary information (Table S7). Further enquiries may be directed to the corresponding author.

Declarations

Conflict of interest Authors declare that they have no conflict of interest.

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