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ORIGINAL ARTICLE

Identifying salt-tolerant traits in *Solanum pimpinellifolium* through in vitro screening to enhance crop resilience

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Abstract

Abiotic stresses are considered the primary cause of crop loss worldwide, with salinity being particularly significant. Excessive salt accumulation can impair photosynthetic efficiency, disrupt nutrient assimilation, reduce growth, and lower product quality. In response to these issues, genetic improvement has focused on a limited number of tomato cultivars, significantly reducing their biodiversity. Tomato is a moderately salinity-sensitive crop widely cultivated even with poor-quality irrigation water, making it an ideal candidate for this research.An innovative in vitro screening method was developed using wild tomato seedling accessions grown on plates with sucrose-free agarized medium containing increasing concentrations of sea salt, using marine salinity as a reference. This autotrophic approach allowed us to (i) evaluate biometric and biochemical parameters while closely resembling in vivo plant behaviour, (ii) expand knowledge on biometric traits relevant to adaptation to adverse environmental conditions (salinity), (iii) define innovative phenotyping methodologies to select wild genotypes, and (iv) simultaneously analyse numerous accessions in the confined space of a growth chamber. The results from the in vitro culture screening effectively discriminated the biometric and biochemical parameters involved in salt stress resistance. Notably, the *S. pimpinellifolium* L. accessions investigated thus far have proven to be more resistant to salinity than traditional cultivars when grown in media enriched with 60% seawater. This study aimed to explore the existing genetic variability in wild tomato species (*Solanum pimpinellifolium* L.) and highlights their potential as genetic resources for developing more resilient tomato cultivars to meet future agricultural challenges posed by climate change, by enhancing the genetic diversity of contemporary cultivar.

Key message

A rapid and scalable in vitro screening protocol identifies salt-tolerant *Solanum pimpinellifolium* accessions, supporting early selection of resilient germplasm for tomato breeding under salinity stress.

Keywords *S. lycopersicum* · *S. pimpinellifolium* · Sea salt · Abiotic · Screening · In vitro

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Introduction

Sustainable agriculture has emerged as a global imperative to address the growing demand for food and the various challenges affecting agricultural production, including both biotic and abiotic stresses (Cruz-López et al. 2024; Dai 2011; Janni et al. 2024). Modern agriculture faces two critical issues: firstly is the loss of arable land, driven by water scarcity for irrigation and the progressive salinization of soils (Lakhiar et al. 2024; Liang et al. 2024; Zhou et al. 2024); then the decline in agrobiodiversity, caused by the widespread use of genetically uniform varieties to boost productivity and the replacement of local species with high-yield crops (Jago and Borrell 2024; Marcelino et al. 2024). Soil salinization is a significant global challenge (ITPS FA 2015), particularly in arid and semi-arid regions where water scarcity limits agricultural productivity (Munns 2002; Yermiyahu et al. 2007). The depletion of freshwater resources and the impacts of climate change have intensified the search for alternative irrigation sources. Seawater, due to its abundance, presents a promising option; however, its exclusive use remains impractical due to the risk of salinization. Blending seawater with freshwater offers a sustainable approach to mitigate salinity-related issues while supporting agricultural productivity (Javeed et al. 2021). Given these issues, it may be valuable to explore the application and use of brackish water as a solution to address soil salinization. Tomato (*Solanum lycopersicum*) cultivation is affected by both of the aforementioned problems. *S. lycopersicum* stands out as one of the most important vegetable fruits globally, with an annual production reaching 100 million tons. Market demands have shaped genetic improvements, prioritizing traits like uniformity, shelf life, and appearance. However, this focus has raised concerns about a progressive reduction in genetic diversity (Molitor et al. 2024) —a critical factor for ensuring crop resilience and safeguarding nutritional value. Although domestication has boosted agricultural yields, it has also led to losses in genetic diversity, along with declines in nutritional content and flavour quality (Zsögön et al. 2018; Schouten et al. 2019). Tomato is moderately tolerant to salinity, and interestingly, mild salt stress can enhance its quality (Van de Vooren et al. 1986; Agius et al. 2022; Cela et al. 2024). In coastal cultivation areas, such as in the italian island, Sicily, or other Mediterranean regions, the slight salinity from seawater or saline irrigation has been shown to improve the flavour, sweetness, and nutritional content of the tomatoes (Meza et al. 2020). The moderate salt exposure can increase levels of sugars, antioxidants, and soluble solids, resulting in tomatoes with superior taste and higher quality (Saito and Matsukura 2015; El-Mogy et al. 2018). This tolerance to salinity, combined with the potential benefits for fruit characteristics, makes tomato a unique crop that thrives in mildly saline environments or under conditions of light salt (eu-)stress. *Solanum lycopersicum* belongs to the *Solanaceae* family and shares close genetic ties with its wild relatives, like *Solanum pimpinellifolium* (Gramazio et al. 2020). Wild tomato species originate from arid regions between the Andes and the Pacific coast, spanning Ecuador to Chile (Peralta and Spooner 2005), and are naturally adapted to survive and thrive in extreme climatic conditions, particularly those exposed to brackish environments. Their ability to withstand harsh environmental stressors makes them valuable for improving the resilience of cultivated crops and offer valuable genetic diversity, including traits for disease resistance and abiotic stress tolerance, such as salinity (Bohra et al. 2022). This study investigated different accessions of wild *S. pimpinellifolium* ecotypes to assess their potential for enhancing tomato genetic traits. A comprehensive evaluation identified accessions exhibiting the highest resistance to salt stress, as determined by morphological and physiological parameters that best discriminated superior performance under high salt conditions. Additionally, the research validated an in vitro screening protocol developed in previous work (Cialli et al. 2024) demonstrating that an autotrophic in vitro condition—achieved using a sugar-free culture medium (Lucchesini et al. 2001), combined with compound sea salt—accurately simulates in vivo growth conditions. This study not only provides tools to enhance tomato resilience to salinity but also contributes to sustainable agricultural strategies, leveraging natural genetic resources to address global challenges related to food security and climate change. The developed in vitro approach could serve as a model for future research, facilitating the identification of more salt-tolerant tomato varieties and promoting more sustainable agricultural practices.

Materials and methods

Plant material

In this experiment, seeds from two species of *Solanum* were used: *Solanum lycopersicum* L.and *Solanum pimpinellifolium* L. *Solanum lycopersicum* cultivar "Principe Borghese", provided by Gargini Sementi (Lucca, Italy), was selected as commercial cultivar characterised by salt sensitivity (Cialli et al. 2024). *Solanum pimpinellifolium* seeds were obtained from the Genebank Information System of the IPK (GBIS/I, Gatersleben, Germany, https://gbis.ipk-gatersleben.de/gbis2i/faces/index.jsf). Specifically, the IPK provided 21 acc essions of *S. pimpinellifolium* accessions originating from geographic regions considered to be within the centre of domestication of cultivated tomato, as detailed in Table 1.

Table 1 *S. pimpinellifolium* accession and their country of origin

S. *pimpinellifolium* accession number	Country of Origin	Assigned ID
LYC28365	Perú	WR1
LYC2824	Perú	WR2
LYC2722	Argentina	WR3
LYC2724	Argentina	WR4
LYC3517	Perú	WR5
LYC2803	Argentina	WR6
LYC2801	Argentina	WR7
LYC2739	Argentina	WR8
LYC2794	Argentina	WR9
LYC2750	Argentina	WR10
LYC2665	Argentina	WR11
LYC2797	Argentina	WR12
LYC2913	Guatemala	WR13
LYC2914	Guatemala	WR14
LYC2800	Argentina	WR15
LYC2667	Guatemala	WR16
LYC3348	Cuba	WR17
LYC3342	Cuba	WR18
LYC1825	México	WR19
LYC1835	Ecuador	WR20
LYC3516	Perú	WR21

In vitro screening protocol

The screening procedure was performed according to the protocol of Cialli et al. (2024) and is summarized in Fig 1. Seeds were rinsed under running tap water for 30 min, then surface-sterilized by immersion in 70% ethanol for 30 s. Subsequently, they were shaken in a 15% (v/v) sodium hypochlorite solution supplemented with two drops of TWEEN 20 for 15 min. Finally, seeds were rinsed three times with sterile distilled water under laminar flow hood conditions. Seeds were sown in Petri dishes containing half-strength Murashige and Skoog (MS) medium, supplemented with 15 g L^{-1} sucrose and solidified with 3 g L^{-1} Gelrite™. The pH of the medium was adjusted to 5.8 prior to autoclaving. Following a pre-germination cold priming at 4 °C for 24–48 h in the dark, the plates were transferred to a growth chamber maintained at 22 °C under an 8:16 h dark–light cycle (100 µmol m^{-2} s^{-1}) until seedling development. Subsequently, 7-day-old seedlings were transplanted into square plastic plates containing sucrose-free, agar-solidified MS (Murashige and Skoog 1962) medium supplemented with increasing concentrations of sea salt: 0 g L^{-1} (Control) with an EC of 5.35 mS cm^{-1}, 10.5 g L^{-1} (T30) with an EC of 19.2 mS cm^{-1}, and 21 g L^{-1} (T60) with an EC of 31.1 mS cm^{-1}, corresponding to 0%, 30%, and 60% of seawater in the medium, respectively. The seawater content in the growth medium was simulated by supplementing it with Instant Ocean® compound sea salt. Three dish replicates were studied for each accession/cultivar, for a total of 180 square dishes.

Data collections

Twelve days after transplanting (DAT) root elongation, root necrosis, number of true leaves, seedling fresh and dry weight, mineral and pigment content, were monitored. Root elongation, root necrosis, number of secondary roots and number of true leaves were analysed visually or using a stereomicroscope (Zeiss Stemi 2000-C stereo microscope). Figure 2 shows details of root tips with root necrosis (Fig. 2a) or without (Fig. 2b).

The Salt Injury Level (S. I. L.) was calculated by slightly modifying what was described by Wu et al. (2019) and Zaki, and Yokoi, 2016), employing the following formula (1):

$$S.I.L. = \frac{\sum (S.I.L. \ * \ plant\ number)}{higher\ S.I.L \ * \ total\ plant\ number} * 100 \qquad (1)$$

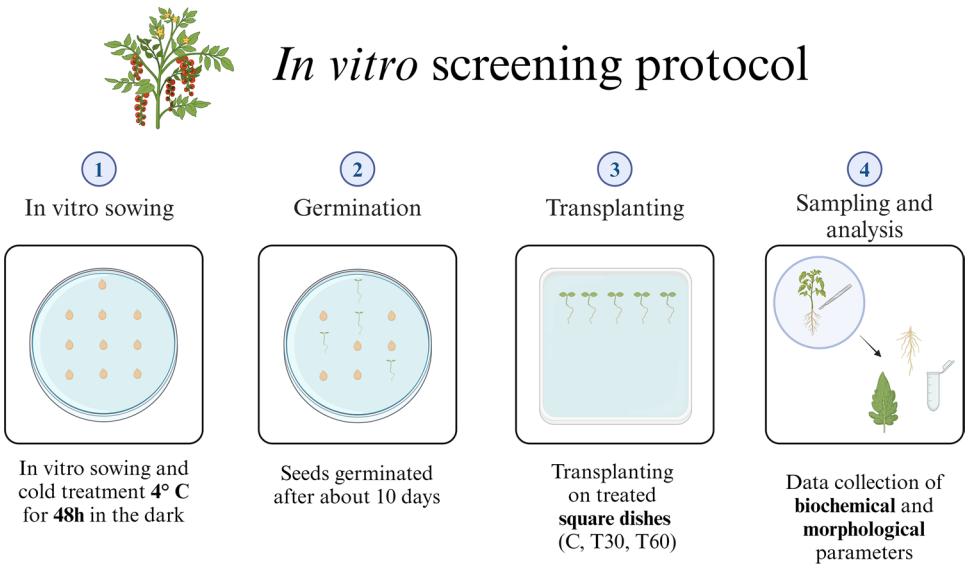


Fig. 1 In vitro screening protocol for *Solanum* spp.: (1) in vitro sowing, (2) seeds germinated after about 10 days, (3) transplanting on square plates with different salt content, (4) data collection of biochemical and morphological parameters. This illustration was created on BioRender.com

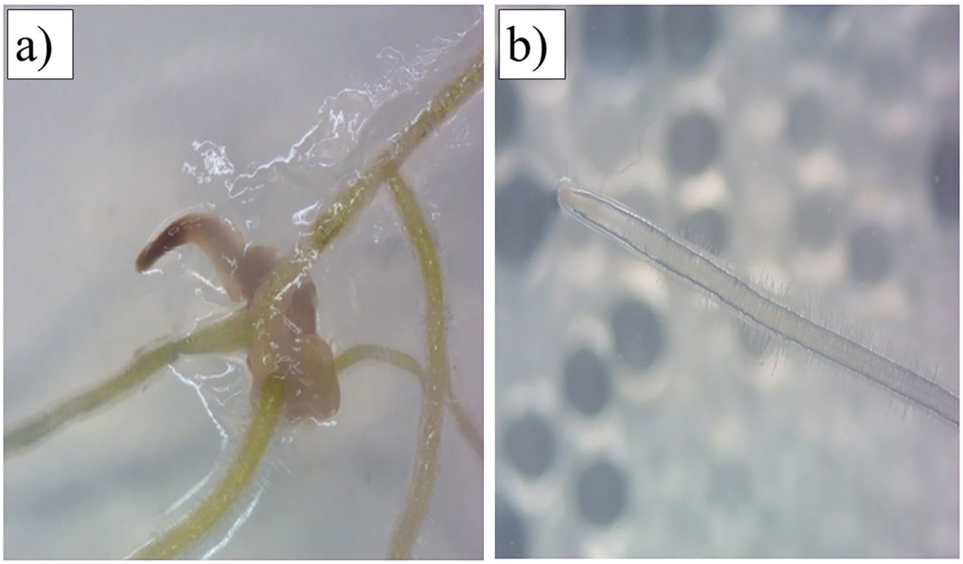


Fig. 2 Stereo microscope detail of apical necrosis (**a**) and apex without necrosis (**b**)

The observed damage levels were classified as follows:

- Level 0: no salt effect.
- Level 1: yellow leaves/no leaves.
- Level 2: root necrosis.
- Level 3: root necrosis + yellow leaves.
- Level 4: root necrosis + yellow cotyledons/no leaves.

At 12 DAT, the fresh weights of both shoots and roots were measured. Subsequently, the samples were dried in an oven at 70 °C for 7 days to determine their dry weights. Based on these measurements, the following indices were calculated: DWroots/DWshoots (DWR), Shoot Dry Residue (SDR), and Roots Dry Residue (RDR), using the formulas (2), (3), and (4).

$$DWR = \frac{roots\ dry\ weight\ (g)}{shoots\ dry\ weight\ (g)} \quad (2)$$

$$SDR = \frac{shoot\ dry\ weight\ (g)}{shoot\ fresh\ weight\ (g)} * 100 \quad (3)$$

$$RDR = \frac{roots\ dry\ weight\ (g)}{roots\ fresh\ weight\ (g)} * 100 \quad (4)$$

Macro and microelements determination

For each treatment, three dried biological replicates, with dry material ranging from 20 to 200 mg, were obtained from the aerial portion or the root portion of the seedlings. The material was mineralized (90 min at 220 °C) with nitric acid and 30% hydrogen peroxide. An atomic absorption spectrometer (Varian Model Spectra AA240 FS, Agilent Technologies Australia [M] Pty Ltd. Mulgrave, Australia) was employed to quantify sodium (Na), potassium (K), calcium (Ca), magnesium (Mg), copper (Cu), manganese (Mn), iron (Fe), and zinc (Zn). The accuracy of the measurements was tested using a tomato leaf, Certified Reference Material® 1573a (CRM 1573a), from the National Institute of Standards and Technology (Gaithersburg, MD, USA).

Chlorophyll and carotenoid analysis

A pure methanol solution was employed as the extraction solvent. The procedure, adapted from Maggini et al. (Maggini et al. 2013) with slight modifications, involved 0,0120 g of leaves tissue in 1 mL of solvent within 2-mL eppendorf tubes. The samples underwent two successive 30-minute sonication cycles in an ice bath, followed by overnight storage at −20 °C. After separating the supernatant, the extraction was repeated on the pellet using an additional 1.5 mL of fresh solvent. The two supernatant fractions were then combined and analysed within a few days, with all parameters expressed on a fresh weight (FW) basis. Absorbance readings were recorded using a spectrophotometer (Ultrospec 2100 Pro - GE Healthcare Ltd., Little Chalfont, UK) at 665.2 and 652.4 nm for chlorophylls and at 470 nm for total carotenoids. Pigment levels were calculated by (Lichtenthaler, and Buschmann, 2001) and expressed on a fresh weight basis of plant tissue. All measurements were performed using four independent biological replicates.

Statistical analysis

Data were subjected to statistical analysis using both GraphPad Prism (version 10.4.1 for Windows, GraphPad Software, La Jolla, San Diego, CA, USA) and R (version 4.3.1

for Windows, RStudio, PBC, Boston, MA, USA). To stabilize variance and normalize percentage data, the arcsine transformation was used. One-way ANOVA were carried out to analyse the effects of salt concentrations among the *S. lycopersicum* cultivar and *S. pimpinellifolium* accessions, and comparison among means values were separated using the Bonferroni multiple comparison test ($p < 0.05$). Values are means of 3 independent biological samples, considering each dish_replicate as a sample. Principal component analysis (PCA) was performed to reduce the dimensionality of the dataset and identify patterns between variables. All parameters used to assess salt stress resistance of all nineteen accessions of *S. pimpinellifolium* at T60 were analysed using PCA. Before PCA, the data were centered by subtracting the mean of each variable and the analysis was based on the covariance matrix. The number of principal components (PC) was determined by selecting those that explained at least 90% of the total variance.

Results

Data were collected from 19 accessions of wild relatives of *S. lycopersicum*, among which two *S. pimpinellifolium* accessions (LYC 1835 and LYC 3516) failed to germinate following in vitro sowing. A multivariate approach was employed to elucidate the salt treatment conditions that best characterized the responses of the *S. pimpinellifolium* accessions and to identify the parameters most indicative of salt stress resistance. This analysis was conducted exclusively on *S. pimpinellifolium*, based on preliminary evidence (Cialli et al. 2024) demonstrating its superior tolerance to salinity compared to *S. lycopersicum*. Principal component analysis (PCA) was performed to reduce data complexity and to elucidate the factors driving variance among the 19 *S. pimpinellifolium* accessions subjected to different salt stress conditions (Fig. 3). The first two principal components accounted for a cumulative 49% of the total variance, with PC1 and PC2 contributing 32.81% and 16.91%, respectively, as determined by the eigenvalue scree plot. The PCA score plot (Fig. 3a) revealed a clear separation of the T60 treatment from both the control (C) and T30 conditions, indicating that the T60 treatment induced the most pronounced variation among the accessions. In contrast, the substantial overlap between the C and T30 treatments suggested that T30 did not elicit significant changes relative to optimal growth conditions. The PCA analysis not only highlighted the distinct responses of the accessions to the various salt treatments but also underscored the relevance of diverse morphological, physiological, and biochemical parameters in describing the salt stress response. In the PCA loadings plot (Fig. 3b), the vectors illustrated the relative importance of these parameters with respect to the principal components. Dark purple markers indicated a strong negative contribution to PC1—specifically, Salt Injury Level and root necrosis—while yellow vectors denoted a robust positive correlation with PC1, as evidenced by the contributions of root elongation and the number of true leaves. The identical coloration for S.I.L. and necrosis underscored their strong positive interrelation, as did the similar representation of root elongation and true leaf number. Additionally, the number of secondary roots contributed positively to both PC1 and PC2. Among the biochemical parameters, chlorophyll content correlated positively with PC1, whereas the K^+/Na^+ ratio in roots and shoots loaded positively on PC2 and was positioned closer to the centre along PC1. Collectively, these findings underscored the utility of these parameters in characterizing the salt stress response of the accessions.

Figure 4 illustrates the Pearson correlation matrix for the parameters measured across different salt stress conditions. In this matrix, positive correlations are indicated by yellowish hues, while negative correlations are denoted by violet tones. Consistent with the PCA results (Fig. 3), Salt Injury Level and root necrosis exhibit a strong positive correlation. In contrast, these two parameters are negatively correlated with root elongation and the number of true leaves, which are positively interrelated. Additionally, chlorophyll content and secondary roots numbers show a positive correlation with both root elongation and the number of true leaves, but a negative correlation with root necrosis and S.I.L.

The cluster observed under the T60 treatment (Fig. 3a) reflects a more homogeneous response to salt stress; accordingly, only the variables that best characterize this response under T60 conditions are reported below.

The values of the parameters identified in the previous statistical analysis, which characterize the behavior of the 19 *S. pimpinellifolium* accessions and the *S. lycopersicum* cultivar, are presented in Figs. 5 and 6. Specifically, Fig. 5 shows the effect of salt stress on roots, while Fig. 6 focuses on salt stress symptoms in shoots or in the whole plant. Notably, with respect to root damage, the only *S. pimpinellifolium* accession that exhibiting significant differences compared to *S. lycopersicum* is WR2, as evidenced by both the percentage of root elongation (Fig. 5a) and root necrosis (Fig. 5b). Under salt stress, WR2 exhibited a significantly greater percentage of root elongation than both the cultivar and most other accessions, while also displaying reduced apical necrosis. Representative images of root development and necrosis in WR2 and the cultivated tomato are shown in Fig. 6, illustrating the contrasting phenotypic response under salt stress. In addition, the majority of the *S. pimpinellifolium* accessions produced a significantly higher number of secondary roots relative to the cultivar, with accessions

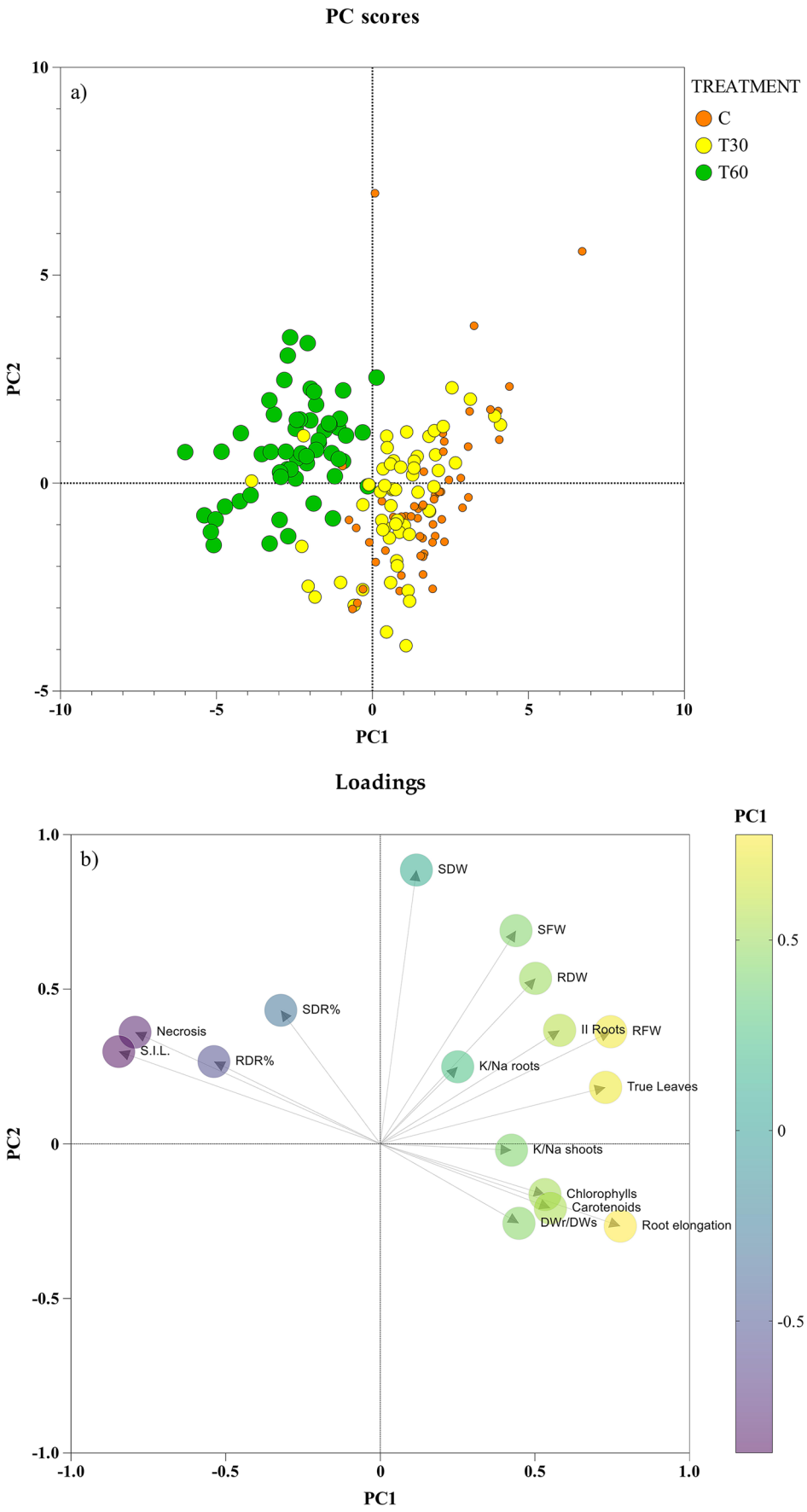
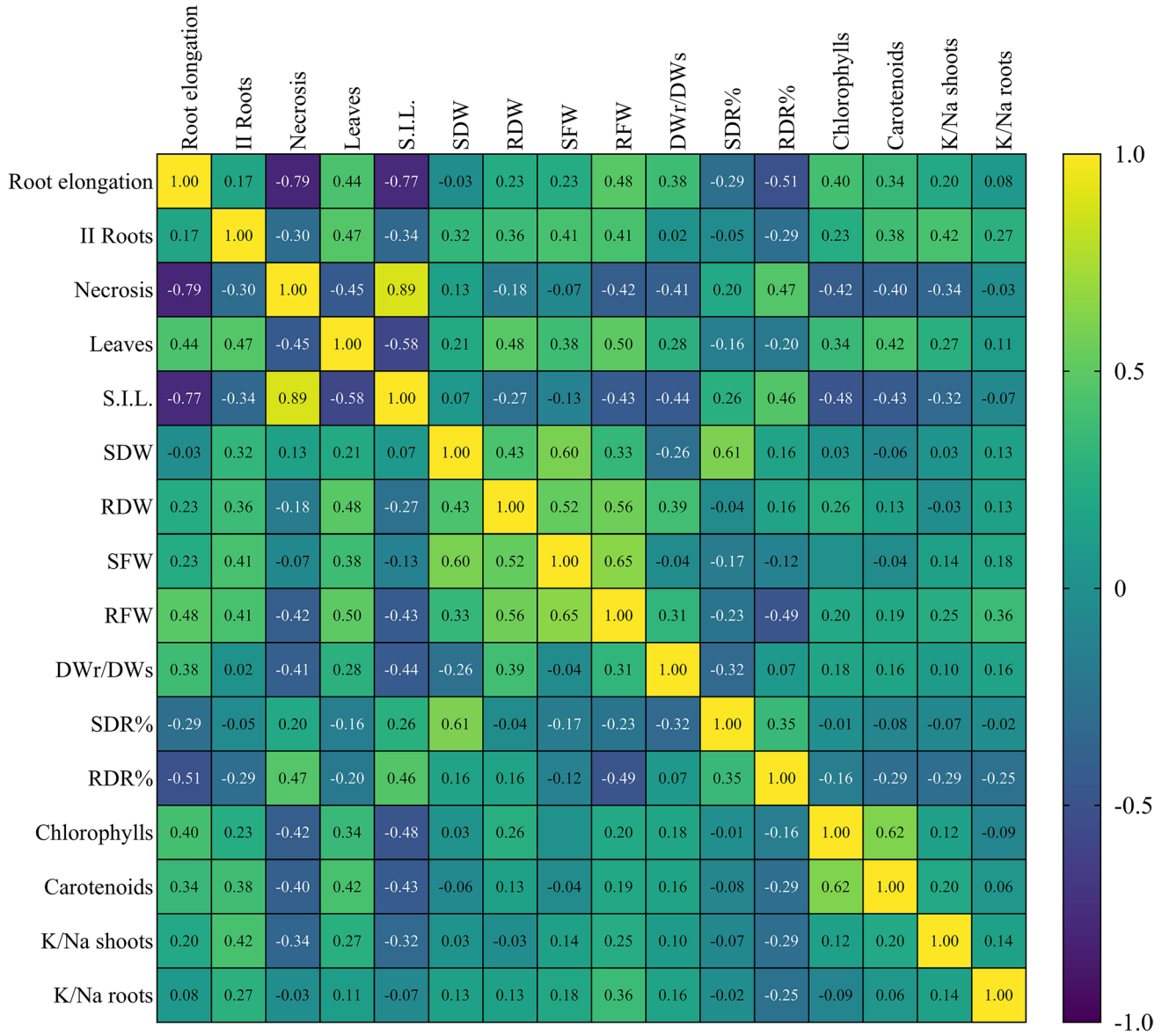


Fig. 3 Principal component analysis (PCA) (**a**) PC score and (**b**) loadings plot displaying variance between morphological and biochemical parameters based on all the sea salt treatment for all the *S. pimpinellifolium* accessions investigated. Data are plotted as a function of PC1 and PC2, which together accounted for 49% of cumulative variance 2. PC1 accounted for 32,81% of individual variance and PC2 accounts for 16,91% of individual variance, with a cumulative proportion of variance of 49%



	Root elongation	II Roots	Necrosis	Leaves	S.I.L.	SDW	RDW	SFW	RFW	DWr/DWs	SDR%	RDR%	Chlorophylls	Carotenoids	K/Na shoots	K/Na roots
Root elongation	1.00	0.17	-0.79	0.44	-0.77	-0.03	0.23	0.23	0.48	0.38	-0.29	-0.51	0.40	0.34	0.20	0.08
II Roots	0.17	1.00	-0.30	0.47	-0.34	0.32	0.36	0.41	0.41	0.02	-0.05	-0.29	0.23	0.38	0.42	0.27
Necrosis	-0.79	-0.30	1.00	-0.45	0.89	0.13	-0.18	-0.07	-0.42	-0.41	0.20	0.47	-0.42	-0.40	-0.34	-0.03
Leaves	0.44	0.47	-0.45	1.00	-0.58	0.21	0.48	0.38	0.50	0.28	-0.16	-0.20	0.34	0.42	0.27	0.11
S.I.L.	-0.77	-0.34	0.89	-0.58	1.00	0.07	-0.27	-0.13	-0.43	-0.44	0.26	0.46	-0.48	-0.43	-0.32	-0.07
SDW	-0.03	0.32	0.13	0.21	0.07	1.00	0.43	0.60	0.33	-0.26	0.61	0.16	0.03	-0.06	0.03	0.13
RDW	0.23	0.36	-0.18	0.48	-0.27	0.43	1.00	0.52	0.56	0.39	-0.04	0.16	0.26	0.13	-0.03	0.13
SFW	0.23	0.41	-0.07	0.38	-0.13	0.60	0.52	1.00	0.65	-0.04	-0.17	-0.12		-0.04	0.14	0.18
RFW	0.48	0.41	-0.42	0.50	-0.43	0.33	0.56	0.65	1.00	0.31	-0.23	-0.49	0.20	0.19	0.25	0.36
DWr/DWs	0.38	0.02	-0.41	0.28	-0.44	-0.26	0.39	-0.04	0.31	1.00	-0.32	0.07	0.18	0.16	0.10	0.16
SDR%	-0.29	-0.05	0.20	-0.16	0.26	0.61	-0.04	-0.17	-0.23	-0.32	1.00	0.35	-0.01	-0.08	-0.07	-0.02
RDR%	-0.51	-0.29	0.47	-0.20	0.46	0.16	0.16	-0.12	-0.49	0.07	0.35	1.00	-0.16	-0.29	-0.29	-0.25
Chlorophylls	0.40	0.23	-0.42	0.34	-0.48	0.03	0.26		0.20	0.18	-0.01	-0.16	1.00	0.62	0.12	-0.09
Carotenoids	0.34	0.38	-0.40	0.42	-0.43	-0.06	0.13	-0.04	0.19	0.16	-0.08	-0.29	0.62	1.00	0.20	0.06
K/Na shoots	0.20	0.42	-0.34	0.27	-0.32	0.03	-0.03	0.14	0.25	0.10	-0.07	-0.29	0.12	0.20	1.00	0.14
K/Na roots	0.08	0.27	-0.03	0.11	-0.07	0.13	0.13	0.18	0.36	0.16	-0.02	-0.25	-0.09	0.06	0.14	1.00

Fig. 4 Pearson correlation matrix for the analysed morphological and biochemical variables under the three treatment for all the *S. pimpinellifolium* accessions investigated. The correlation values (*r*) range from −1 to 1, where 1 indicates a perfect positive correlation, −1 a perfect negative correlation and 0 no correlation. The color scale reflects the intensity of these correlations, with positive values displayed in yellow and negative values in dark blue

WR1, WR2, WR9, WR11, WR14, WR16, WR17, and WR19 showing pronounced differences compared to the cultivar (Fig. 5c). Evaluating the whole-plant response through the Salt Injury Level (S.I.L.)—which integrates both aerial and root damage—revealed that accessions WR2, WR8, WR9, WR10, WR11, WR14, WR17, WR18, and WR19 registered significantly lower values than the cultivar (Fig. 7a). Assessment of the aerial components further indicated that only accession WR11 produced a significantly higher number of true leaves compared to the cultivar (Fig. 7b), while accessions WR2, WR5, WR8, WR9, WR11, WR14, and WR15 demonstrated significantly enhanced chlorophyll content (Fig. 7c).

Discussion

The primary objective of this study was to investigate the genetic variability among wild tomato (*S. pimpinellifolium*) ecotypes and to identify genetic resources for mitigating salinity stress in the context of Mediterranean climate change, based on biometric and biochemical parameters. The results revealed that *S. pimpinellifolium* accessions

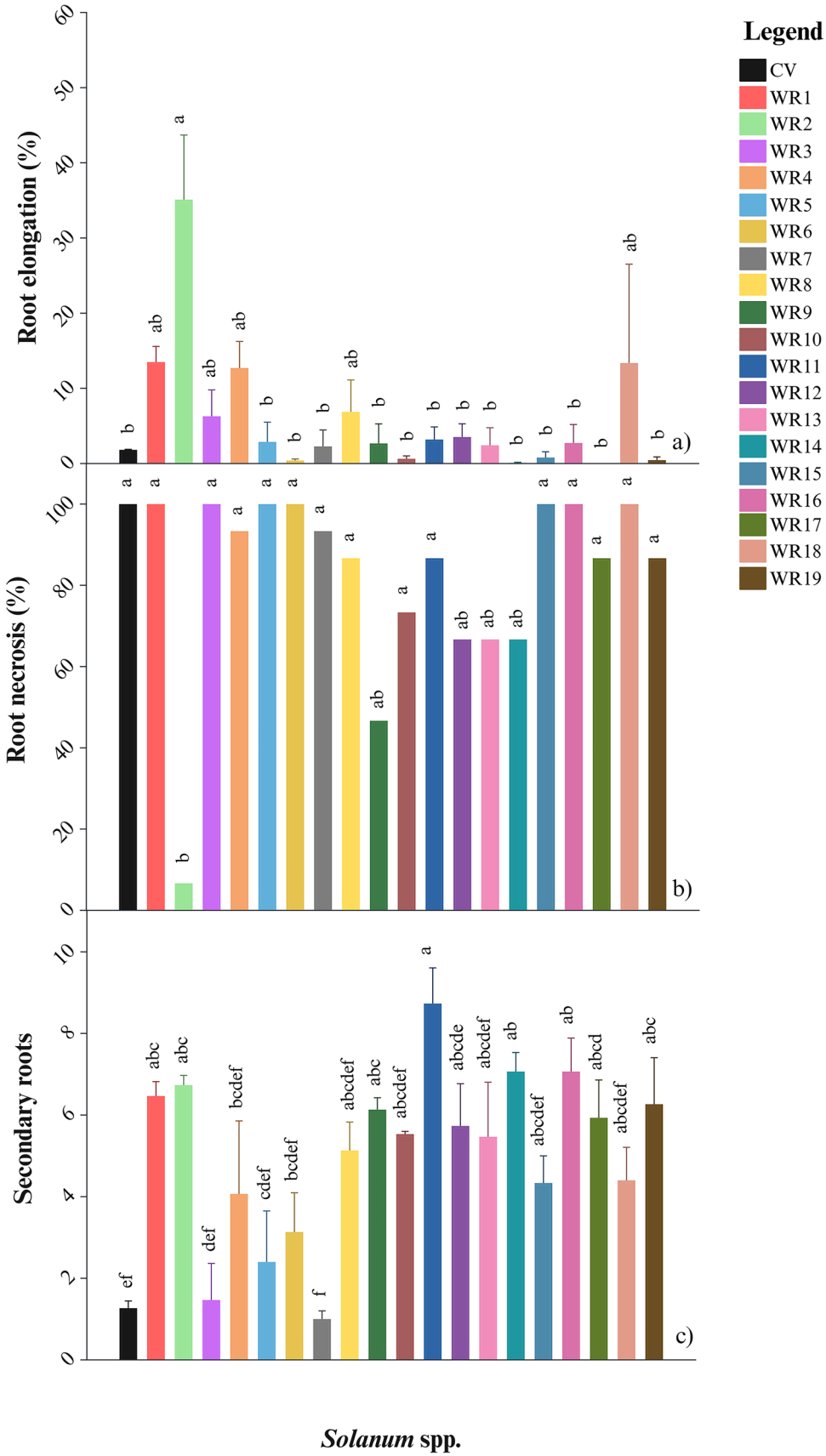


Fig. 5 **(a)** Root elongation (%), **(b)** Root necrosis (%) and **c** secondary roots for a modern cultivar (CV) and nineteen accessions of *S. pimpinellifolium* (WRs). All data were analysed using ANOVA and differences between the treatments for each *Solanum* spp. were analysed with Tukey's post hoc test. The results shown are the means ± SEs of three replicates. Different letters denote significant differences at $p \leq 0.05$



Fig. 6 Representative photos of *S. lycopersicum* and *S. pimpinellifolium* (accession WR2) grown in square plastic plates under Control and T60 salt stress conditions. **(a)** *S. lycopersicum* under Control condition, **(b)** S. *lycopersicum* under T60 treatment, **(c)** *S. pimpinellifolium* under Control condition and **(d)** *S. pimpinellifolium* under T60 treatment. Scale bar = 1 cm

were significantly more resistant to salinity stress than conventional *Solanum lycopersicum* cultivars, particularly when exposed to 60% seawater conditions. These findings, consistent with our previous research (Cialli et al. 2024), supported the refinement on the in vitro screening protocol, which enabled the evaluation of 21 *S. pimpinellifolium* accessions relative to *S. lycopersicum* based on additional morphological and biochemical parameters. Moreover, a substantial body of research has employed in vitro screening to assess resistance to various abiotic stresses and to evaluate the performance of different cultivated species (Aggarwal et al. 2024; Vijayan et al. 2003). Nonetheless, the majority of established protocols favour the use of callus or shoot explants as the main experimental materials (Maleki et al. 2019). Furthermore, the in vitro screening protocols proposed in the literature often involve long and laborious procedures (Zaman et al. 2015; Jurado-Mañogil et al. 2024), with numerous steps and the need for considerable space within growth chambers (Kripkyy et al. 2001; Wani 2019). Additionally, by relying solely on cell cultures or callus explants (Mishra et al. 2021; Massa et al. 2024), these protocols do not offer a complete and integrated view of the plant's overall response and performance, focusing instead on just a part of the plant and neglecting the functioning of the entire organism. The innovative approach of this study is to carry out the experiment in a growth chamber with a duration of only 12 days; although the growth conditions were reduced in terms of time and space, the seedlings developed sufficiently to be able to assess the response to salt stress in both shoots and the roots. The absence of sucrose in the growth medium did not impede seedling growth; rather, it promoted autotrophic conditions and approximates natural growth environments more closely, a phenomenon previously observed in the literature as a means to better simulate in vivo conditions (Mosadegh et al. 2018). Additionally, a novel experimental approach involves the use of sea salt to simulate the effect of brackish water, a method that has already been successfully applied to other crops (Asmaa et al. 2017; Tootoonchi and Gettys 2019). Sea salt is a compound salt, not just NaCl, and therefore provides a variety of elements to the nutrient solution (Atzori et al. 2019), which may contribute to a more complex and realistic simulation of environmental conditions. This provides a more sustainable perspective on the utilisation of this precious water resource, which could lead to savings in terms of economic and environmental costs (Awaad et al. 2020),

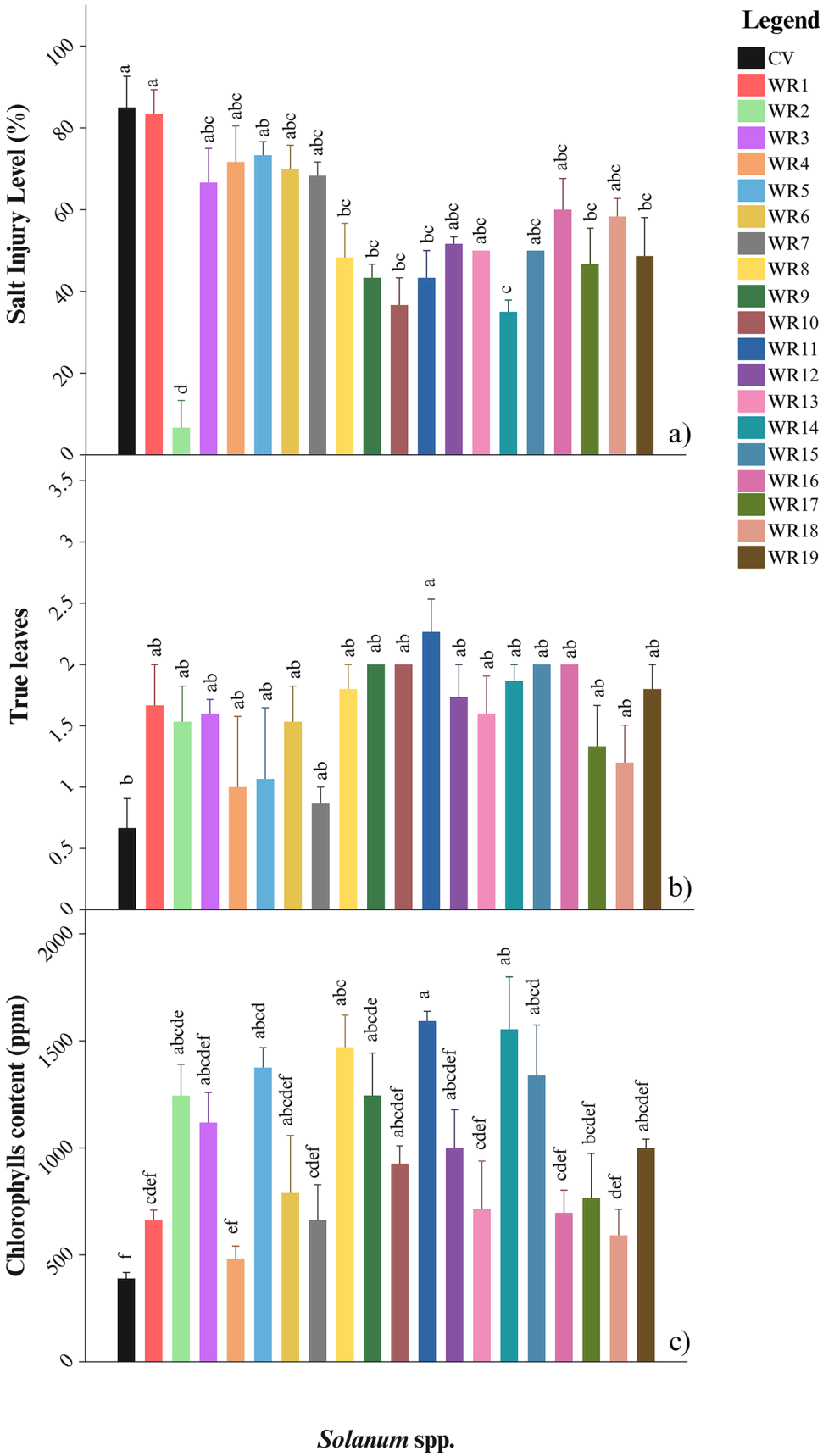


Fig. 7 **(a)** Salt Injury Level (%), **(b)** True leaves number and Chlorophylls content (ppm) for a modern cultivar (CV) and nineteen accessions of *S. pimpinellifolium* (WRs). All data were analysed using ANOVA and differences between the treatments for each *Solanum* spp. were analysed with Tukey's post hoc test. The results shown are the means ± SEs of three replicates. Different letters denote significant differences at $p \leq 0.05$

without excessively altering the yield of moderately salt-tolerant species (Puccinelli et al. 2022).

Principal Component Analysis (PCA) was employed as an initial exploratory method to identify patterns and correlations among variables related to salinity tolerance. This analysis helped to pinpoint the treatments that most effectively reflect plant's response to salt stress, as well as the parameters most pertinent for distinguishing *S. pimpinellifolium* accessions with enhanced tolerance to salinity. The clustering observed in the PCA score plot (Fig. 3a) reveals that, under high saline stress conditions, particularly with 60% seawater in the culture medium, *S. pimpinellifolium* accessions display distinct behaviour when compared to other experimental conditions and T60 was found to be the treatment that best screens *S. pimpinellifolium* behaviour under salt stress conditions. While cultivated tomato tolerates moderate salt stress, *S. pimpinellifolium*, naturally adapted to harsh (Zuriaga et al. 2009; Razali et al. 2018), may utilise specific mechanisms to counteract the water and osmotic imbalances caused by salinity (Mohamad Razali, et al., 2017; Aydin 2024). Biometric and biochemical analyses, evaluated through Principal Component Analysis (PCA), revealed significant correlations between various parameters and salinity tolerance. This analysis highlighted the importance of factors such as the Salt Injury Level (S.I.L.), root elongation, number of secondary roots and true leaves in assessing the plant's response to salt stress. The use of damage indices has proven to be highly effective, providing a comprehensive overview of the plant's condition at both root and foliar levels, as described in literature for some other species (Albiski et al. 2012; Aggarwal et al. 2024). Roots are the first organ to encounter saline stress, where the initial signaling pathways are activated to enable the export or compartmentalization of salt (Shabala et al. 2015). In contrast, leaves, as the primary photosynthetic organ (Zahra et al. 2022; Sudhir and Murthy 2004), represent a critical focus for investigation under salt stress conditions (Acosta-Motos et al. 2017). PCA results showed that root necrosis and S.I.L. were closely associated, reflecting similar patterns of salinity-induced damage. Similarly, root elongation and true leaves number were equally affected by salinity stress. The two response patterns under salt stress indicated that high root necrosis and S.I.L. reflected severe damage, while fewer true leaves and secondary roots suggested reduced growth and lower resilience. (Altaf et al. 2021; Roșca et al. 2023). These findings indicate that S.I.L., root elongation, secondary roots, and true leaves consistently reflect plant responses to salinity and represent reliable markers for screening salt tolerance and assessing adaptive traits. Using the Pearson correlation matrix, it was determined that chlorophyll content and the number of secondary roots were useful indicators for investigating the response to saline stress (Guo et al. 2022; Campobenedetto et al. 2021; Farooq et al. 2024). In fact, chlorophyll content was recognized as a critical marker of photosynthetic activity (Soltabayeva et al. 2021) and could be readily measured in miniaturized in vitro conditions, as several methods have been documented in the literature (Dobránszki and Mendler-Drienyovszki 2014; Limantara et al. 2015). Chlorophyll content positively correlated with root elongation and true leaf number, indicating healthy adaptation to salt stress, and negatively with damage indicators like root necrosis and S.I.L., confirming its reliability as a marker even in miniaturized systems. The number of secondary roots was significant, as it revealed the plant's capacity to explore the growth substrate (Julkowska et al. 2017). According to the literature, it has been reported that tomato plants under abiotic stress developed numerous lateral feeder roots, a phenomenon absent in plants grown under control conditions (Cuartero and Fernández-Muñoz 1998). Moreover, a robust number of secondary roots has been interpreted as indicative of the plant's ability to sustain active root biomass production (Hu et al. 2022). The positive correlations among secondary roots, root elongation, and true leaf number further support the idea that *S. pimpinellifolium* may adopt specific salt tolerance strategies, possibly involving distinct ion uptake or exclusion mechanisms linked to root architecture. (Morton et al. 2024; Martínez-Cuenca et al. 2020). Figure 2a and b illustrate the morphological damage induced by salinity, comparing a necrotic root apex under salt stress with a healthy one from control conditions. These visual cues, along with the negative correlations between S.I.L. and root necrosis with root elongation and true leaf number, provide an integrated view of *S. pimpinellifolium*' s response to salt stress. Under these conditions, the accessions showed reduced root necrosis and sustained shoot and root development, indicating higher tolerance—clearly exemplified by the WR2 phenotype shown in Fig. 6. *S. pimpinellifolium* originates from habitats characterized by proximity to marine and saline environments (Razali et al. 2018) and it may have developed specific physiological, morphological, and genetic adaptations, demonstrating a notable robustness and adaptability typical of wild species (Aydin 2024; Rao et al. 2013). These resistance traits could serve as a highly valuable resource for enhancing tolerance in modern tomato cultivars and, equally importantly, for increasing the biodiversity of this essential species. To further investigate the overall trends, a detailed examination of the histograms provided insight into the accessions' responses to salt stress. These graphical representations revealed distinct patterns and variations, offering valuable perspectives on their relative performance. Analysis of the data distribution across all treatments enabled the identification of accessions exhibiting the most promising traits for salt resistance. Specifically,

accession WR2 emerged as the most responsive among the *S. pimpinellifolium* samples, displaying adaptive responses to salinity across the studied parameters—with the exception of true leaf number. WR2 exhibits a significantly greater root percentage elongation and lower root necrosis compared to the cultivar, confirming that it does not suffer structural damage at the root level. Furthermore, secondary roots are more developed in this *S. pimpinellifolium* accession. While salinity typically impairs root growth, as consistently shown in previous studies (Bogoutdinova et al. 2023), our results indicate that WR2 maintains a strong level of tolerance under salt stress, as reflected by the three parameters evaluated. Salt stress resistance is also evident in the shoots of the seedlings, as WR2 demonstrates significantly higher chlorophyll production compared to the cultivar, which could be linked to the proper functioning of cellular structures involved in photosynthesis, even under severe salt stress (Wang et al. 2022). Additionally, the Salt Injury Level (S.I.L.), examined in this study as an integrative parameter combining both root and shoot responses, is significantly lower in WR2 than in *S. lycopersicum*, further confirming its greater tolerance to salinity. This approach has been validated in the literature as an effective method for evaluating plant stress tolerance (Wu at al., 2019), supporting its application in our research. Accessions WR9, WR11, and WR14 demonstrated enhanced resistance when evaluated based on secondary root number, Salt Injury Level (S.I.L.), and chlorophyll content. Although no in vivo data are presented in this study, the autotrophic in vitro screening system proved effective in identifying the most promising accessions for salt tolerance, based on a combination of morphological and biochemical traits. This approach allowed for the preliminary selection of genotypes for subsequent evaluation under greenhouse conditions. Ongoing validation of the autotrophic in vitro screening system in the greenhouse has yielded initial results that confirm its effectiveness as a tool for early genotype selection. In this experiment as well, the accessions selected through the in vitro screening proved to be more tolerant to salt stress than the cultivated variety. Follow-up experiments are currently underway and will include transcriptomic, metabolomic, volatilomic, physiological, morphological and biochemical analyses of the most tolerant accessions. These combined approaches aim to provide a comprehensive understanding of the mechanisms underlying the higher salt tolerance observed in wild species compared to modern cultivars.

Conclusions

The findings of this study highlight the significant potential of *Solanum pimpinellifolium*, particularly the WR2 accession, in adapting to salt stress. These results underscore the importance of both morphological and biochemical parameters in assessing salinity tolerance. Stress resistance was evaluated in both the aerial and root systems, providing a comprehensive understanding of the species' behaviour under severe salt stress. The identified accessions exhibiting enhanced resilience represent valuable candidates for further research and breeding programmes aimed at improving salt tolerance in crops. These findings confirm previous research on the moderate sensitivity of tomato to salinity and suggest that wild species can serve as a rich reservoir of valuable genes for enhancing salt tolerance in cultivated varieties. Moreover, the protocol developed in earlier studies has proven to be robust and reliable, delivering insightful results under miniaturised conditions, both in terms of space and time. This approach enabled the evaluation of numerous accessions within the limited space of a growth chamber, significantly reducing time requirements. The protocol also holds potential for studying other species and types of stress, making it a versatile tool for exploring plant responses to various environmental challenges. The accessions with the highest salt resistance will be further investigated in a greenhouse experiment to fully assess their behaviour in vivo, providing deeper insights into the mechanisms underlying salt resistance through morphological, physiological, phenological, metabolomic, and transcriptomic analyses. This research offers valuable insights into enhancing tomato resilience to salinity and introduces new tools for developing sustainable agricultural strategies. By tapping into natural genetic resources, this study addresses critical global challenges related to food security and climate change.

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Data availability The raw data supporting the conclusions of this article will be made available by the authors on request.

Declarations

Competing interests The authors have no relevant financial or non-financial interests to disclose.

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