LOCALNUTLEG project is part of the PRIMA programme supported by the European Union's Horizon 2020 research and innovation programme

One publication in a peer-reviewed open access journal regarding the response of pumpkin genotypes under abiotic stress conditions

DELIVERABLE D1.7

PulpIng

Developing of **Pu**mpkin Pu**lp** Formulation using a Sustainable **In**te**g**rated Strategy

Index

Document Information

Deliverable Number	1.7
Deliverable name	One publication in a peer-reviewed open access journal regarding the response of pumpkin genotypes under abiotic stress conditions
Contributing WP	WP1: Defining agronomic conditions for pumpkin production
Contractual delivery date	M36, August 2023
Actual delivery date	M36, August 2023
Requested delivery date	August 2023
Dissemination level	Public
Responsible partner	UTH
Reviewers	All partners
Version	1

1. Summary

PulpIng project aims at the development of a high-quality pumpkin pulp product enriched and preserved by added-value compounds obtained from pumpkin by-products, fostering an integrative and sustainable strategy. Defining the best agronomic conditions for sustainable pumpkin production, is the main goal of the WP1 – "Defining agronomic conditions for pumpkin production". This report refers to deliverable D1.7 – "One publication in a peer-reviewed open access journal regarding the response of pumpkin genotypes under abiotic stress conditions" of the WP1. One publication is presented regarding the assessment of salt tolerance among 15 local squash (*Cucurbita maxima* Duchesne) landraces from Tunisia (ANNEX D1.7, published in Plants journal (MDPI). We also published one more paper regarding the effects of drought stress on on the germination and early seedling growth traits, physiological parameters and phytochemicals content of Tunisian squash (Cucurbita maximaDuch.) landraces in Frontiers in Plant Science (Frontiers Media S.A.) (ANNEX D1.7).

ANNEX D1.7

plants

MDPI

Article

The Effects of Salt Stress on Germination, Seedling Growth and Biochemical Responses of Tunisian Squash (*Cucurbita maxima* Duchesne) Germplasm

Neji Tarchoun [1,*], Wassim Saadaoui [1], Najla Mezghani [1,2], Ourania I. Pavli [3], Hanen Falleh [4] and Spyridon A. Petropoulos [3,*]

[1] High Agronomic Institute of Chott Mariem, University of Sousse, Sousse 4042, Tunisia; wessaadaoui@gmail.com (W.S.); najla_mezghani@yahoo.fr (N.M.)
[2] National Gene Bank, Boulevard du Leader Yasser Arafat, ZI Charguia 1, Tunis 1080, Tunisia
[3] Department of Agriculture, Crop Production and Rural Environment, University of Thessaly, Fytokou Street, 38446 Volos, Greece; ouraniapavli@uth.gr
[4] Centre of Biotechnology of Borj Cedria, BP 901, Hammam-lif 2050, Tunisia; hanenfalleh@gmail.com
* Correspondence: nejitarchoun@yahoo.fr (N.T.); spetropoulos@uth.gr (S.A.P.)

Abstract: Salt stress is considered as one of the most common abiotic stresses reducing the productivity and fruit quality of crop plants. The present study was carried out to assess the salt tolerance among 15 local squash (*Cucurbita maxima* Duchesne) landraces. Different salt (NaCl) concentrations of 0, 100, 200 and 300 mM were selected in order to evaluate the response of the study germplasm to salt stress based on 12 agronomic parameters and 3 biochemical traits, proline, malondialdehyde (MDA) and chlorophylls. A varied effect of the salt stress level was observed among the studied landraces based on germination potential, as well as on growth and biochemical parameters at seedling stage. Results showed that all landraces were drastically affected at high stress level with a significant variation in their stress response, indicating the existence of considerable genetic variability. Landraces "746" and "747" were the best performing cultivars across stress levels, whereas "1007", "1008" and "1009" were the most negatively affected. Based on the tested landrace performance, four landraceswere selected and further evaluated at biochemical level, focusing on the determination of compounds that play a key role in the ability to withstand salt stress. The mean MDA content across landraces was generally increased in stressed plants, as compared to the control treatment; the increase was attributed to a peak in MDA content at specific stress levels. In particular, "746" and "1007" showed the maximum content at 100 mM NaCl, while in landrace "751", MDA content reached its peak at 300 mM NaCl. In addition, the response of most landraces to salt stress involved an increase in free proline content, with the exception of "746", with the maximum content being observed either at 200 mM ("748" and "751" landraces) or at 300 mM NaCl, where only "747" expressed the highest content. These findings can be extrapolated into efforts to develop more salt-tolerant squash landraces and exhaust the possibilities of using saline water or soils under changing climate conditions.

Keywords: salinity stress; proline; seed germination; MDA; *Cucurbita* sp.; landrace

Citation: Tarchoun, N.; Saadaoui, W.; Mezghani, N.; Pavli, O.I.; Falleh, H.; Petropoulos, S.A. The Effects of Salt Stress on Germination, Seedling Growth and Biochemical Responses of Tunisian Squash (*Cucurbita maxima* Duchesne) Germplasm. *Plants* **2022**, *11*, 800. https://doi.org/10.3390/plants11060800

Academic Editors: Pirjo Mäkelä, Mercè Llugany, Peter A. Roussos and Mumtaz Cheema

Received: 18 February 2022
Accepted: 16 March 2022
Published: 17 March 2022

1. Introduction

The genus *Cucurbita*, belonging to the family Cucurbitaceae, includes several economically and nutritionally important vegetable crops cultivated worldwide [1]. It contains five domesticated species, namely *C. argyrosperma* Huber, *C. ficifolia* Bouché, *C. maxima* Duch., *C. moschata* Duch. and *C. pepo* L. [2]. *Cucurbita maxima* Duch. is an allogamous and extremely diverse species that originated in South America from the wild progenitor *C. maxima* ssp. *andreana* (Naud.). The species contains a series of squash ecotypes, whose

dispersal is attributed to Spain, which acted as a bridge between South America and Europe, and from there dispersed into other continents [3,4], while its domestication has been regulated by hybridization, introgression and gene flow processes [4].

In Tunisia, squash (*Cucurbita maxima* Duch.) is usually cultivated in small farms. At a local level, the germplasm employed for cultivation refers to landraces produced by open pollination or farmer mass selection and maintained by local farmers. Tunisia is considered to be one of the most important diversity centers of cultivated cucurbits [5], with squash landraces being produced in various cultivation zones (North East, Central West, Cap-Bon, Sahel, Siliana, Monastir, Tunisia). Given the importance of local squash production, in recent years, several efforts have addressed the issues of collection, ex-situ characterization and maintenance of the local germplasm accessions [6]. In this concept, the studies of ISA CM (High Agronomic Institute of Chott Mariem) revealed important parameters related to the agronomic performance and valorization of local landraces [7], while the National Gene Bank of Tunisia is currently conducting a breeding program focusing on collecting squash landraces and wild relatives from selected areas that represent the cultivating zones of Tunisia. Recent studies have revealed that the local germplasm of *C. maxima* is highly adaptive to diverse agro-climatic conditions and possesses considerable genetic variability for important agronomic traits as well as traits related to fruit size and fruit quality [6,7].

Many authors reported that salinity is considered as one of the most common abiotic stresses reducing the productivity and fruit quality of crop plants [8–10]. The major contributing factors to soil salinization include climate change, leading to land degradation and desertification [11], as well as the poor quality of irrigation water and irrational fertilization management, which results in reduced productivity in either irrigated or rainfed farming systems [12]. The compromised crop performance is the result of a combined osmotic and ionic stress that induces complex interactions at morphological, physiological, biochemical and molecular level [13,14], thus leading to altered photosynthetic activity, detoxification capacity, energy state and plant cellular homeostasis [15–17]. In particular, salt stress is interlinked with lipid peroxidation in cellular membranes, DNA damage, protein denaturation, carbohydrate oxidation, pigment breakdown and impairment of enzymatic activity, as well as metabolic adaptations, mainly involving the accumulation of osmolytes [16]. Osmolyte accumulation acts in favor of cell water uptake and cell turgor maintenance, stabilization of membranes, enzymes and proteins and the reduction of oxidative damage due to decreased Reactive Oxygen Species (ROS) levels, thereby contributing to redox balance [18]. Well known examples of metabolites with an osmoprotective function under salt stress conditions are certain amino acids, mainly referring to proline, and glycine betaine, belonging to the group of quaternary amines [19].

In salt-sensitive species, the stress effects vary considerably depending on the extent and duration of the stress but also on factors relating to plant characteristics [20–24]. Although most plant species are salt-sensitive at all stages of their lifecycle, their sensitivity differs among growth stages [21], with seed germination being viewed as the most critical stage when salt stress impairs water absorption during seed imbibition and turgescence [25]. At this stage, salt stress is expressed through the reduction in germination percentage, delayed germination rate and inhibited tissue elongation [23,26]. The increased Na+ and Cl- ion concentration induces ionic toxicity, oxidative stress and nutritional imbalance as well as water stress by lowering the osmotic potential of soil solution, ultimately leading to the inhibition of germination in many species [8,27,28]. Salt stress further affects the ultrastructure of root cells and severely inhibits root growth during early seedling growth [29], as compared to shoot growth, in various plant species, including tomato [30], chickpea [31], lentil [32] and lettuce [33]. However, such salt stress responses are highly subjected to both species and cultivar dependency, with great variability being reported [34–36].

In Tunisia, soil salinization is gradually increasing due to the scarcity of rains and the increase in evapotranspiration, adversely affecting plant germination, growth, development and fruit setting in salt-affected soils, as is the case of semiarid regions [37]. In squash, salinity is a factor that severely limits crop growth and productivity while at the same time

deteriorating fruit quality. Despite the acquired knowledge in relation to the salt stress response in a plethora of plant species [33,38,39], there is a gap in relevant research fields for squash. Considering that the development of salt-tolerant germplasm is one of the most effective means for enhancing squash production in saline soils, this study aimed at investigating the response of squash germplasm to salt stress at the germination stage and examining the potential of selecting salt-tolerant genotypes at the laboratory level early. As such, 15 Tunisian squash landraces were subjected to NaCl-induced salinity stress at varying stress levels (0, 100, 200 and 300 mM of NaCl), and their response was assessed on the basis of traits related to seed germination and seedling growth potential. Moreover, based on the results related to salt stress response, four landraces were selected and further assessed in terms of biochemical parameters that are routinely employed as indicators of salt tolerance, namely malondialdehyde (MDA), free proline and chlorophyll a and chlorophyll b content.

2. Results

2.1. Effect of Salt Stress Level on Traits Related to Seed Germination and Seedling Growth Potential

In order to evaluate the response of 15 Tunisian squash germplasm to salt stress (see the corresponding Table in Section 4), seeds were germinated on solutions differing in NaCl concentration (0, 100, 200 and 300 mM), and seed and seedling parameters were evaluated based on 12 criteria (see the corresponding Table in Section 4). The obtained data revealed a significant effect of the salt stress level ($p < 0.001$) on the germination and seedling growth potential of the tested squash landraces, with the stress effects being in most cases analogous to the stress level applied, therefore leading to most drastic effects at high stress levels for all the traits under study (Tables 1 and 2).

Table 1. Analysis of variance (mean of squares) for traits related to seed germination and seeding growth in squash germplasm (accession) under different salt stress conditions (Salinity).

S.O.V.	DF	GP	SL	RL	SFW	RFW	SRR	GR	SLR	RLR	GSTI	SLSTI	RLSTI
Accession	14	4066.8	341.0	152.9	2.6	0.05	54.8	3801.8	487.9	115.3	0.39	26,109.5	18,310.1
Salinity	3	17,405.8	1103.6	697.3	8.4	0.25	139.3	7553.0	22.9	227.9	0.98	1484.6	44,510.7
Accession x Salinity	42	620.4	46.7	14.1	0.2	0.004	18.9	455.4	9.1	6.6	0.05	669.1	1321.4
CV (%)		13.3	9.0	11.3	17.0	24.9	20.6	10.2	20.7	21.7	29.69	15.4	16.6

S.O.V.: source of variance; DF: degree of freedom. CV: coefficient of variance; GP: germination percentage; SL: shoot length: RL: root length; SFW: shoot fresh weight; RFW: root fresh weight; SRR: shoot length/root length ratio; GR: germination reduction; SLR: shoot length reduction; RLR: root length reduction; GSTI: germination stress tolerance index; SLSTI: shoot length stress tolerance index; RLSTI: root length stress tolerance index.

Table 2. Mean effect of the salt stress level (0, 100, 200 and 300 mM NaCl) on germination potential and seedlings characteristics (mean ± SD), regardless of the squash accession.

NaCl Concentration (mM)	GP (%)	SL (mm)	RL (mm)	SFW (g)	RFW (g)	SRR	GR (%)	SLR (%)	RRL (%)	GSTI (%)	SLSTI (%)	RLSTI (%)
Control	86.3 ± 1.08 a	12.3 ± 0.24 a	7.4 ± 0.18 a	0.9 ± 0.02 a	0.14 a ± 0.004 a	1.3 ± 0.02 c	-	-	-	-	-	-
100	23.3 ± 1.50 b	7.1 ± 0.33 b	4.7 ± 0.24 b	0.6 ± 0.03 b	0.08 ± 0.004 b	1.7 ± 0.06 b	63.0 ± 1.49 c	5.2 ± 0.37 b	2.7 ± 0.22 c	0.27 ± 0.016 a	60.1 ± 2.83 a	63.8 ± 2.94 a
200	15.0 ± 1.08 c	6.4 ± 0.31 c	3.3 ± 0.20 c	0.5 ± 0.05 c	0.06 ± 0.003 c	1.8 ± 0.10 b	71.3 ± 1.18 b	5.9 ± 0.35 a	4.1 ± 0.19 b	0.17 ± 0.011 b	54.2 ± 2.58 b	44.0 ± 2.34 b
300	8.4 ± 0.67 d	6.3 ± 0.30 c	2.0 ± 0.14 d	0.3 ± 0.01 d	0.04 ± 0.003 d	3.6 ± 0.27 a	77.9 a ± 1.02 a	5.9 ± 0.39 a	5.3 ± 0.16 a	0.09 ± 0.006 v	54.6 ± 2.84 b	27.5 ± 1.77 c
F Value	8816 **	2141.20 **	2879.11 **	815.17 **	649.38 **	745.38 **	362.56 **	143.95 **	791.03 **	296.89 **	19.75 **	16.58 **

** Means in the same column followed by the same letter are not significantly different at $p < 0.05$, according to Duncan's Multiple Range test; Parameters from GR to RLSTI were evaluated for all landraces compared to the control (see material and methods); GP: germination percentage; SL: shoot length: RL: root length; SFW: shoot fresh weight; RFW: root fresh weight; SRR: shoot length/root length ratio; GR: germination reduction; SLR: shoot length reduction; RLR: root length reduction; GSTI: germination stress tolerance index; SLSTI: shoot length stress tolerance index; RLSTI: root length stress tolerance index.

The final germination percentage, evaluated on the seventh day after salt stress initiation, was strongly affected by the stress level, thus showing decreasing trends as NaCl increased (GP: 86.35%, 23.33%, 15.00% and 8.40% at 0 mM, 100 mM, 200 mM and 300 mM NaCl, respectively) (Table 2). The analysis of data further revealed significant differences in shoot (SL) and root length (RL) among the stress levels applied, with most profound effects being noted at the high stress level (Table 2). Accordingly, the shoot/root ratio (SRR) was considerably affected by the stress level, presenting a gradual increase as NaCl concentration increased (ranging from 1.28 to 3.58), which reflects the fact that root length was more profoundly affected than shoot length under stress conditions (Table 2). Such drastic effects were also noted in shoot (SFW) and root fresh weight (RFW), which were significantly affected by the stress level. In particular, both SFW and RFW showed decreasing trends as NaCl concentration increased, with the respective values ranging between 0.90–0.31 g and 0.14–0.04 g for shoots and roots (Table 2).

In addition, a significant effect of the salt stress level on the germination stress tolerance index (GSTI) was also recorded, with the effects being proportional to the stress level applied. In particular, GSTI decreased as NaCl concentration increased (ranging from 0.29% to 0.09%), thus reflecting the differential effects of the various stress levels on seed germination potential (Table 2). Accordingly, the shoot length tolerance index and root length stress tolerance index (SLSTI and RLSTI) declined with increasing stress level. The effects of stress on root length were profoundly evidenced at higher stress levels where significant differences between the levels of 200 mM and 300 mM of NaCl were recorded, whereas in the case of shoot length, no significant differences in SLSTI values were recorded between the indicated stress levels (e.g., 200 mM and 300 mM of NaCl). In agreement with the abovementioned data for shoot and root fresh weight, the most drastic effects were noted at RLSTI compared to SLSTI, as a result of the most severe effects of salt stress on seedling root length, which are expressed with lower values of the RLSTI index (Table 2).

2.2. Effect of Landrace on Traits Related to Seed Germination and Seedling Growth Potential

The data underline the significant effect of landrace ($p < 0.001$) on the germination and seedling growth potential of squash under salinity stress conditions (Tables 1 and 3). The differential response of the landraces to salt stress was evidenced for all the traits under study, while the analysis showed RFW, SRR, SLR, RRL and GSTI as the most variable traits (CV > 20%) (Table 1).

Germination was considerably affected by the landrace, as evidenced by the mean GP of landraces under study (Table 3), which ranged from 49.33% to 16.75% in landraces "747" and "1008", respectively. Although such values suggest a high salt tolerance ability and sensitivity for "747" and "1008" landraces, respectively, they are also attributed to their innate germination potential, as evidenced by the differences in GP in the respective control treatments (97.33% and 67.00% for "747" and "1008" respectively) (Table 4). Moreover, the landraces "746", "752", "745", "748", "749" and "750" were characterized by a mean GP of 39.50%, while the other landraces presented intermediate GP values ranging from 31.25% to 21.50% (Table 3). In relation to seedling growth potential, the effects of stress were consistently more severe on roots than on shoots (Table 3). Although the stress effects were obvious in all landraces, their response to salinity differed substantially. As such, "1006" presented the highest values for both SL and RL, while the lowest respective values were noted in "1007" and "1008". Regarding the SRR, "753" and "1007" presented the highest and lowest values, respectively. Accordingly, significant differences were also observed for SFW and RFW, with "752" showing the highest value for both traits, while the lowest values were recorded in "1008" and "746" landrace, respectively (Table 3).

Table 3. The mean effect of the squash germplasm accessions on traits related to seed germination and seedling growth (mean ± SD), regardless of the salinity level (NaCl concentration).

Accession	GP (%)	SL (mm)	RL (mm)	SFW (g)	RFW (g)	SRR	GR (%)	SLR (%)	RRL (%)	GSTI (%)	SLSTI (%)	RLSTI (%)
"745"	42.0 ± 4.72 bc	8.0 ± 0.18 gh	4.7 ± 0.29 de	0.6 ± 0.02 de	0.07 ± 0.008 e	1.8 ± 0.58 h	74.6 ± 3.55 c	6.0 ± 1.11 d	2.0 ± 0.56 h	0.24 ± 0.04 e	56.67 ± 7.45 e	68.9 ± 7.47 b
"746"	48.4 ± 5.36 a	8.3 ± 0.32 g	4.5 ± 0.24 e	0.6 ± 0.007 de	0.02 ± 0.005 h	2.4 ± 0.26 e	67.0 ± 3.15 de	2.1 ± 0.69 h	3.6 ± 0.74 e	0.32 ± 0.03 ab	80.85 ± 5.66 a	49.8 ± 7.55 e
"747"	49.3 ± 4.81 a	7.8 ± 0.16 h	3.0 ± 0.26 g	0.6 ± 0.03 de	0.03 ± 0.005 fg	3.6 ± 0.36 b	64.0 ± 2.64 ef	1.6 ± 0.25 h	2.5 ± 0.61 fh	0.34 ± 0.07 a	82.25 ± 7.23 a	44.3 ± 8.50 ef
"748"	41.5 ± 7.02 cd	9.3 ± 1.11 d	4.7 ± 0.66 de	0.8 ± 0.05 b	0.10 ± 0.008 cd	2.1 ± 0.72 f	64.6 ± 2.49 ef	4.8 ± 0.89 e	2.0 ± 0.55 gh	0.29 ± 0.02 cd	64.20 ± 5.15 d	67.6 ± 8.47 b
"749"	40.0 ± 4.93 cd	9.3 ± 0.29 de	4.0 ± 0.41 f	0.6 ef ± 0.01 ef	0.10 ± 0.006 cd	2.9 ± 0.32 d	60.0 ± 3.56 g	4.1 ± 0.81 f	4.1 ± 0.64 d	0.30 ± 0.02 bc	72.54 ± 6.80 b	42.9 ± 7.89 gh
"750"	39.5 ± 5.05 d	5.3 ± 0.81 i	3.0 ± 0.51 g	0.8 ± 0.05 c	0.10 ± 0.006 cd	3.3 ± 0.24 c	71.3 ± 4.40 c	2.0 ± 0.66 h	2.8 ± 0.52 f	0.23 ± 0.03 e	73.00 ± 6.77 b	44.7 ± 8.12 fg
"751"	31.2 ± 4.83 e	9.8 ± 0.35 c	8.0 ± 0.36 b	0.9 ± 0.02 b	0.09 ± 0.004 d	1.2 ± 0.23 i	65.0 ± 5.06 ef	10.6 ± 1.35 c	5.3 ± 0.79 c	0.19 ± 0.05 f	40.54 ± 5.44 f	57.4 ± 7.36 c
"752"	42.7 ± 4.10 b	8.8 ± 0.37 f	5.8 ± 0.36 c	1.1 ± 0.03 a	0.15 ± 0.006 a	1.5 ± 0.34 h	71.0 ± 2.64 cd	3.8 ± 0.85 f	2.3 ± 0.50 gh	0.26 ± 0.02 de	67.43 ± 6.70 cd	70.5 ± 5.47 b
"753"	28.7 ± 5.47 f	9.0 ± 0.24 ef	3.0 ± 0.31 g	0.6 ± 0.05 d	0.11 ± 0.005 b	4.5 ± 0.52 a	61.7 ± 4.04 fg	3.0 ± 0.80 h	3.4 ± 0.71 e	0.18 ± 0.04 f	81.11 ± 8.30 a	32.6 ± 4.55 h
"1004"	23.2 ± 5.09 gh	10.9 ± 0.24 b	4.7 ± 0.57 d	0.5 ± 0.02 f	0.10 ± 0.006 cd	3.1 ± 0.21 c	66.3 ± 3.20 e	3.6 ± 0.84 f	4.1 ± 0.82 d	0.09 ± 0.03 h	73.98 ± 6.21 b	50.8 ± 8.24 de
"1005"	22.0 ± 3.23 gh	10.7 ± 0.22 b	5.9 ± 0.62 c	0.6 ± 0.04 de	0.10 ± 0.006 cd	1.9 ± 0.18 fg	54.7 ± 6.80 h	4.2 ± 0.91 f	4.2 ± 0.80 d	0.13 ± 0.20 g	69.99 ± 5.41 bc	54.6 ± 4.91 cd
"1006"	28.1 ± 4.95 f	13.4 ± 0.85 a	8.3 ± 0.38 a	0.4 ± 0.03 g	0.10 ± 0.006 cd	1.7 ± 0.16 gh	92.3 ± 2.77 a	2.8 ± 0.60 g	2.1 ± 0.58 gh	0.15 ± 0.01 i	81.98 ± 7.16 a	81.6 ± 7.93 a
"1007"	21.5 ± 6.76 h	2.7 ± 0.34 k	1.8 ± 0.30 i	0.2 ± 0.03 h	0.04 ± 0.007 f	0.4 ± 0.10 j	86.0 ± 2.58 b	10.7 ± 1.42 c	7.4 ± 0.88 b	0.03 ± 0.01 i	0.00 ± 0.00 g	0.0 ± 0.00 i
"1008"	16.7 ± 6.30 i	2.9 ± 0.78 k	1.3 ± 0.54 j	0.1 ± 0.06 i	0.03 ± 0.001 h	0.5 ± 0.13 j	67.0 ± 4.99 de	11.6 ± 1.54 b	5.3 ± 0.84 c	0.04 ± 0.01 i	0.00 ± 0.00 g	0.0 ± 0.00 i
"1009"	24.0 ± 4.94 g	3.8 ± 0.38 j	2.3 ± 0.21 h	0.2 ± 0.04 h	0.03 ± 0.006 h	0.4 ± 0.10 j	96.0 ± 4.40 a	15.1 ± 1.66 a	9.1 ± 0.96 a	0.03 ± 0.01 i	0.00 ± 0.00 g	0.0 ± 0.00 i
CV %	13.54	8.96	11.32	16.98	23.92	20.63	10.23	20.69	21.74	26.69	15.39	16.62

Means in the same column followed by the same letter are not significantly different at $p < 0.05$, according to Duncan's multiple range test; GP: germination percentage; SL: shoot length: RL: root length; SFW: shoot fresh weight; RFW: root fresh weight; SRR: shoot length/root length ratio; GR: germination reduction; SLR: shoot length reduction; RLR: root length reduction; GSTI: germination stress tolerance index; SLSTI: shoot length stress tolerance index; RLSTI: root length stress tolerance index.

Table 4. Response of squash germplasm accessions to varying salt stress levels (0, 100, 200 and 300 mM NaCl) in relation to traits (means ± SD) related to seed germination and seedling growth.

NaCl Concentration (mM)	Accession	GP (%)	SL (mm)	RL (mm)	SFW (g)	RFW (g)	SRR
Control	"745"	97.9 ± 1.5	12.5 ± 1.6	6.2 ± 0.8	0.9 ± 0.2	0.15 ± 0.02	2.0 ± 0.4
	"746"	98.7 ± 1.5	9.9 ± 1.8	7.2 ± 0.7	0.8 ± 0.1	0.04 ± 0.01	1.4 ± 0.2
	"747"	97.3 ± 2.3	9.1 ± 0.7	4.9 ± 0.5	0.7 ± 0.4	0.07 ± 0.03	1.9 ± 0.2
	"748"	90.0 ± 8.7	12.9 ± 1.8	6.2 ± 0.6	1.1 ± 0.3	0.15 ± 0.02	2.1 ± 0.4
	"749"	85.0 ± 4.3	12.4 ± 1.6	7.0 ± 1.2	0.76 ± 0.06	0.15 ± 0.01	1.8 ± 0.4
	"750"	93.0 ± 2.6	6.8 ± 0.7	5.1 ± 0.3	1.1 ± 0.1	0.16 ± 0.02	1.3 ± 0.2
	"751"	80.0 ± 4.3	17.8 ± 1.8	12.0 ± 2.0	1.3 ± 0.2	0.14 ± 0.02	1.5 ± 0.1
	"752"	96.0 ± 2.6	11.7 ± 1.1	7.5 ± 0.6	1.7 ± 0.2	0.19 ± 0.05	1.4 ± 0.2
	"753"	75.0 ± 4.3	10.4 ± 0.4	5.6 ± 0.3	0.83 ± 0.03	0.18 ± 0.02	1.9 ± 0.1
	"1004"	73.0 ± 5.7	13.5 ± 1.1	7.7 ± 1.4	0.63 ± 0.05	0.15 ± 0.01	1.8 ± 0.3
	"1005"	63.0 ± 6.7	13.9 ± 1.0	9.1 ± 1.1	0.8 ± 0.1	0.15 ± 0.03	1.5 ± 0.2
	"1006"	97.3 ± 1.9	15.5 ± 0.5	9.9 ± 0.4	0.69 ± 0.07	0.16 ± 0.02	1.6 ± 0.1
	"1007"	86.0 ± 5.2	10.7 ± 0.4	7.4 ± 0.4	0.86 ± 0.03	0.17 ± 0.02	1.4 ± 0.1
	"1008"	67.0 ± 5.7	11.6 ± 0.8	5.3 ± 0.3	0.52 ± 0.03	0.16 ± 0.02	2.2 ± 0.2
	"1009"	96.0 ± 2.6	15.1 ± 0.8	9.1 ± 0.3	0.73 ± 0.09	0.10 ± 0.01	1.7 ± 0.1
100	"745"	50.0 ± 5.7	7.7 ± 0.6	5.0 ± 0.5	0.7 ± 0.04	0.08 ± 0.01	1.5 ± 0.2
	"746"	40.0 ± 4.3	9.0 ± 0.6	5.7 ± 0.5	0.6 ± 0.2	0.03 ± 0.05	1.6 ± 0.2
	"747"	45.0 ± 4.3	8.0 ± 0.4	3.8 ± 0.4	0.68 ± 0.04	0.04 ± 0.01	2.1 ± 0.2
	"748"	35.0 ± 3.3	8.6 ± 0.5	5.3 ± 0.2	0.9 ± 0.2	0.10 ± 0.03	1.6 ± 0.1
	"749"	40.0 ± 4.8	9.0 ± 0.9	4.4 ± 0.3	0.64 ± 0.03	0.10 ± 0.01	2.0 ± 0.3
	"750"	30.0 ± 5.3	4.8 ± 0.9	4.5 ± 0.3	0.92 ± 0.06	0.11 ± 0.01	1.1 ± 0.2
	"751"	30.0 ± 5.3	8.5 ± 0.5	9.3 ± 0.3	1.00 ± 0.04	0.11 ± 0.01	0.9 ± 0.1
	"752"	35.0 ± 4.3	8.9 ± 0.5	6.3 ± 0.3	1.29 ± 0.08	0.17 ± 0.01	1.4 ± 0.1
	"753"	25.0 ± 3.3	7.6 ± 0.4	3.5 ± 0.3	0.71 ± 0.04	0.12 ± 0.01	2.2 ± 0.2
	"1004"	25.0 ± 3.3	10.4 ± 0.7	6.2 ± 0.3	0.58 ± 0.02	0.11 ± 0.01	1.7 ± 0.1
	"1005"	15.0 ± 2.6	11.1 ± 0.8	6.5 ± 0.2	0.69 ± 0.02	0.10 ± 0.01	1.7 ± 0.2
	"1006"	10.0 ± 4.4	12.2 ± 0.5	9.4 ± 0.3	0.44 ± 0.03	0.10 ± 0.01	1.3 ± 0.1
	"1007"	0	0	0	0	0	0
	"1008"	0	0	0	0	0	0
	"1009"	0	0	0	0	0	0
200	"745"	15.0 ± 4.3	6.6 ± 0.4	4.1 ± 0.2	0.53 ± 0.03	0.04 ± 0.00	1.6 ± 0.1
	"746"	35.0 ± 4.3	7.7 ± 0.6	3.4 ± 0.3	0.50 ± 0.04	0.01 ± 0.00	2.3 ± 0.3
	"747"	35.0 ± 4.3	7.5 ± 0.4	2.2 ± 0.3	0.55 ± 0.03	0.01 ± 0.00	3.5 ± 0.5
	"748"	25.0 ± 3.2	8.2 ± 0.3	4.1 ± 0.2	0.74 ± 0.03	0.08 ± 0.03	2.0 ± 0.1
	"749"	25.0 ± 3.2	8.2 ± 0.7	3.0 ± 0.3	0.49 ± 0.14	0.08 ± 0.01	2.8 ± 0.3
	"750"	25.0 ± 3.2	3.7 ± 0.5	1.6 ± 0.2	0.64 ± 0.02	0.08 ± 0.01	2.3 ± 0.3
	"751"	10.0 ± 4.2	7.2 ± 0.4	6.4 ± 0.3	0.85 ± 0.04	0.08 ± 0.01	1.1 ± 0.1
	"752"	25.0 ± 3.3	7.6 ± 0.4	5.9 ± 0.3	0.88 ± 0.03	0.13 ± 0.01	1.3 ± 0.1
	"753"	10.0 ± 3.3	8.3 ± 0.3	2.2 ± 0.2	0.57 ± 0.03	0.09 ± 0.03	3.9 ± 0.3
	"1004"	5.0 ± 1.7	9.8 ± 0.7	3.4 ± 0.3	0.53 ± 0.02	0.08 ± 0.01	2.9 ± 0.4
	"1005"	10.0 ± 4.2	9.8 ± 0.3	4.7 ± 0.1	0.51 ± 0.03	0.08 ± 0.01	2.1 ± 0.1
	"1006"	5.0 ± 1.7	10.8 ± 0.5	8.5 ± 0.4	0.33 ± 0.02	0.08 ± 0.01	1.3 ± 0.1
	"1007"	0	0	0	0	0	0
	"1008"	0	0	0	0	0	0
	"1009"	0	0	0	0	0	0

Table 4. *Cont.*

NaCl Concentration (mM)	Accession	GP (%)	SL (mm)	RL (mm)	SFW (g)	RFW (g)	SRR
300	"745"	5.0 ± 1.7	5.3 ± 0.6	3.5 ± 0.3	0.33 ± 0.03	0.02 ± 0.00	1.5 ± 0.2
	"746"	20.0 ± 3.3	6.7 ± 0.7	1.6 ± 0.3	0.50 ± 0.01	0.01 ± 0.00	4.0 ± 0.9
	"747"	25.0 ± 3.3	6.7 ± 0.4	1.0 ± 0.2	0.47 ± 0.03	0.01 ± 0.00	7.0 ± 1.4
	"748"	17.0 ± 4.1	7.7 ± 0.3	3.0 ± 0.3	0.5 ± 0.1	0.07 ± 0.01	2.6 ± 0.2
	"749"	10.0 ± 3.2	7.5 ± 0.5	1.6 ± 0.2	0.35 ± 0.03	0.05 ± 0.01	5.0 ± 1.0
	"750"	10.0 ± 3.2	6.0 ± 1.0	0.7 ± 0.1	0.32 ± 0.04	0.06 ± 0.02	8.5 ± 1.3
	"751"	5.0 ± 1.7	5.6 ± 0.6	4.4 ± 0.3	0.35 ± 0.03	0.04 ± 0.01	1.3 ± 0.1
	"752"	15.0 ± 4.3	6.9 ± 0.4	3.6 ± 0.2	0.64 ± 0.03	0.12 ± 0.01	1.9 ± 0.1
	"753"	5.0 ± 1.7	9.4 ± 0.3	0.9 ± 0.1	0.38 ± 0.02	0.05 ± 0.02	10.3 ± 1.5
	"1004"	5.0 ± 1.7	9.8 ± 0.7	1.6 ± 0.2	0.35 ± 0.02	0.05 ± 0.01	6.2 ± 0.8
	"1005"	5.0 ± 1.7	8.1 ± 0.5	3.5 ± 0.2	0.3 ± 0.1	0.05 ± 0.03	2.3 ± 0.1
	"1006"	5.0 ± 1.7	15.2 ± 0.5	5.4 ± 0.3	0.21 ± 0.02	0.06 ± 0.02	2.8 ± 0.1
	"1007"	0	0	0	0	0	0
	"1008"	0	0	0	0	0	0
	"1009"	0	0	0	0	0	0

GP: germination percentage; SL: shoot length: RL: root length; SFW: shoot fresh weight; RFW: root fresh weight; SRR: shoot length/root length ratio.

In agreement with the abovementioned findings, a considerable variation for the GSTI, SLSTI and RLSTI was also recorded (Table 3). Based on GSTI, "747" followed by "746" were proved to be the best performing landraces. In relation to SLSTI, "747" followed by "1006", "753" and "746" showed the highest tolerance to salt stress, whereas "1006" ranked as the best performing landrace in terms of RLSTI. In contrast, "1007", "758" and "759" were proved to be the most salt-sensitive based on GSTI, SLSTI and RLSTI values (Table 3).

2.3. Effect of the Landrace and the Salt Stress Level on Traits Related to Seed Germination and Seedling Growth Potential

Based on the analysis of variance applied on individual data, all traits related to germination and seedling growth potential under salt stress conditions were differentially affected by the landrace and the salt stress level applied (Table 4). In the absence of stress, germination was considerably affected by the landrace, thus substantiating a variable germination potential per se, which could be mainly attributed to the median longevity of seeds whose fruits were harvested at different periods (2018 to 2020). Among landraces, "746" and "1005" presented the highest and lowest GP under normal conditions (98.66 % and 63.00 %, respectively) which also justifies that seed longevity is a genotype-dependent variable (Table 4). Upon stress, the germination of all landraces was severely affected, with the effects of stress being in general analogous to its level. At 100 mM NaCl, "745" proved to be the most capable landrace of retaining a high germination rate (50.00%), while "746", "747" and "749" also showed relatively high GP values ($\geq$40%). In contrast, "1007", "1008" and "1009" landraces, which were of medium-high innate germinability (67.00–96.00%), were incapable of germination at all stress levels (Table 4). Moreover, at 200 mM NaCl, "746" and "747" presented the highest GP (35.00 %), whereas "754" and "1006" ranked as the landraces with the lowest GP values. Finally, at 300 mM NaCl, "747" followed by "746" proved to be the best performing landraces (25% and 20%, respectively). Interestingly, "745", although showing superior performance at 100 mM NaCl, suffered great losses at both 200 mM and 300 mM NaCl (15.00% and 5.00%, respectively). Furthermore, the response of most landraces to salt stress involved a drastic reduction in both SL and RL, with the latter parameter being more pronouncedly affected in the majority of the tested landraces (Table 4). At all stress levels, "1006" presented the highest values for SL and RL, thus proving its ability to withstand even high levels of salt stress. In contrast, "750" consistently showed low values for SL and RL—a finding that could be partly attributed to its low growth potential per se, as evidenced by the respective values in the control

treatments (6.83 and 5.10 mm, respectively) (Table 4). In agreement with these findings, the SFW and RFW followed a decreasing trend as NaCl concentration increased. The highest values for these traits were recorded in "752" both in control and stress conditions, whereas the lowest values were noted in "1006" and "746" for SFW and in "747" for RFW. Although affected at all stress levels, the SRR was more profoundly affected at 300 mM NaCl, with the landraces showing varying values ranging from 10.19 to 1.27. The highest SRR was noted in "753", thus reflecting the more drastic effect of salt stress to the roots than in shoots (Table 4).

The seeds of the selected landraces representing the four types of cultivated squash are illustrated in Figure 1. Regarding the different salt concentrations, landrace "746" (Batati orange) showed the highest germination percentage at all salt levels and was distinguished for its good tolerance to salt stress. Moreover, landrace "751" (Bejaoui green) expressed the lowest values especially at 200 and 300 mM, while landrace "748" (Kerkoubi orange) and "747" (Galaoui) could be also considered as similarly tolerant landraces and would be recommended for cultivation in saline soils or with irrigation water with a high salt level.

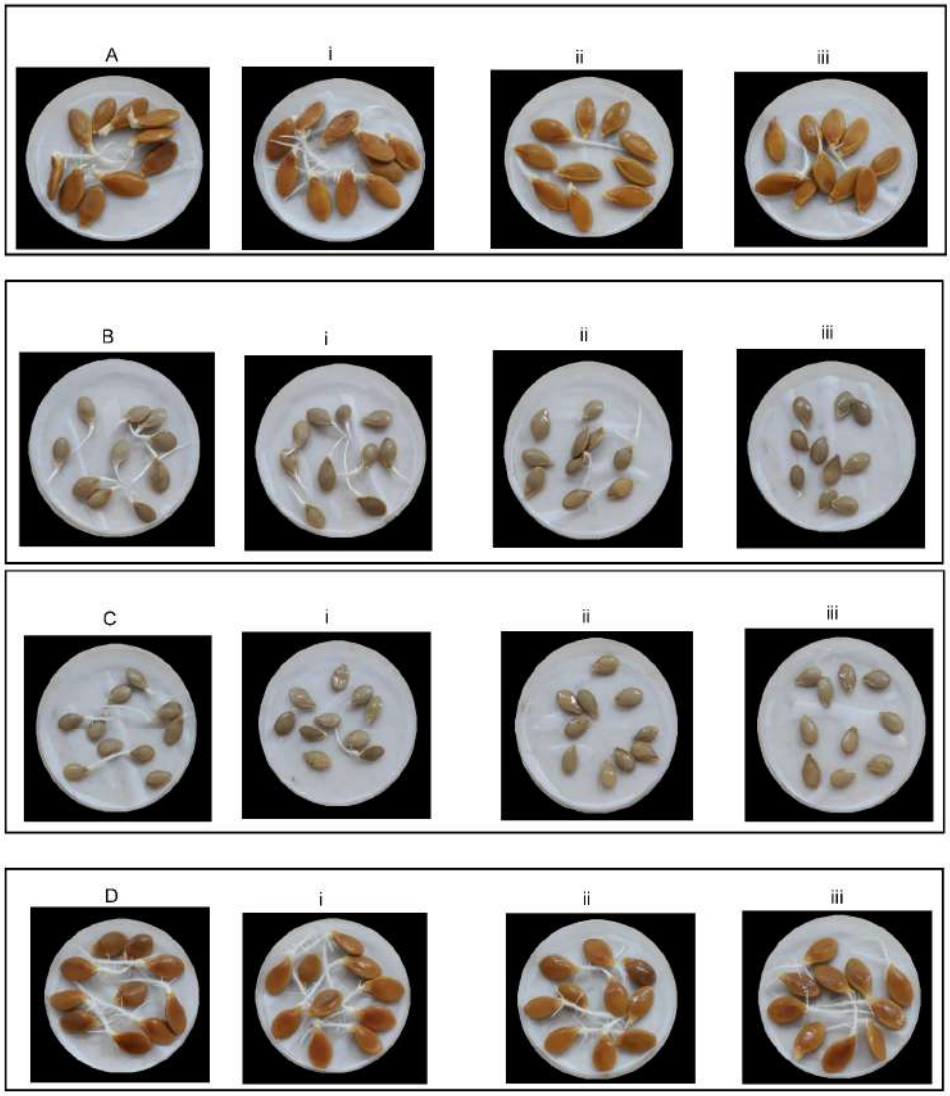

Figure 1. Effect of salt stress treatment on four selected landraces representing the main types of cultivated squash: (**A**): "748" control 0 mM NaCl, (**i**) 100 mM NaCl, (**ii**) 200 mM NaCl, (**iii**) 300 mM NaCl; (**B**): "747" control 0 mM NaCl, (**i**) 100 mM NaCl, (**ii**) 200 mM NaCl, (**iii**) 300 mM NaCl; (**C**): "751" control 0 mM NaCl, (**i**) 100 mM NaCl, (**ii**) 200 mM NaCl), (**iii**) 300 mM NaCl; (**D**): "746" control 0 mM NaCl, (**i**) 100 mM NaCl, (**ii**) 200 mM NaCl, (**iii**) 300 mM NaCl).

2.4. Effect of the Salt Stress Level on the Content of Malondialdehyde (MDA), Free Proline and Chlorophyll a and b

The results indicate that the different stress levels significantly affected the content of MDA, free proline and chlorophylls of squash seedlings (Table 5). Regarding MDA content, a significantly increased content was noted in stressed seedlings as compared to the control treatment (1.16 μmol mg^{-1} FW). The highest MDA content was recorded at 100 mM NaCl (1.75 μmol mg^{-1} FW), while the respective values for 200 and 300 mM NaCl did not differ significantly (1.43 and 1.34 μmol mg^{-1} FW). The content of free proline was the highest at the highest salinity level (0.92 μg mg^{-1} FW), whereas no significant differences were observed between the control and the 200 mM NaCl treatments (Table 5). An exception to this trend was noted at 100 mM NaCl, which showed the lowest values of free proline content (0.53 μg mg^{-1} FW). As expected, chlorophyll a (chla) and b (chlb) were reduced in stressed plants, as compared to the control treatment (Table 5), although the salt level of 100 mM NaCl showed a differential trend of increased chla and chlb (35.47 and 69.88 mg g^{-1} FW, respectively). The chlorophyll a values ranged from 25.70 to 35.47 mg g^{-1} FW, while chlorophyll b ranged from 52.44 to 69.88 mg g^{-1} FW.

Table 5. Mean effect of the salt stress level (0, 100, 200 and 300 mM NaCl) on the content of malondialdehyde (MDA), free proline and chlorophyll a and b (mean ± SD), regardless of the pumpkin accession.

NaCl concentration (mM)	MDA (μmol g^{-1})	Proline (μg mg^{-1})	Chl a (mg mg^{-1})	Chl b (mg mg^{-1})
Control	1.17 ± 0.53 b	0.82 ± 0.06 a	30.09 ± 2.87 b	60.64 ± 5.96 b
100	1.75 ± 0.30 a	0.51 ± 0.02 b	35.47 ± 3.90 a	69.88 ± 7.68 a
200	1.43 ± 0.21 b	0.82 ± 0.04 a	25.70 ± 2.43 d	52.44 ± 5.50 d
300	1.34 ± 0.18 b	0.92 ± 0.05 a	28.91 ± 2.46 c	56.34 ± 5.98 c
F Value	33.6 **	471.2 **	552.3 **	737.5 **
SD				

** Means in the same column followed by the same letter are not significantly different at $p < 0.05$, according to Duncan's multiple range test.

2.5. Effect of Landrace on the Content of Malondialdehyde, Free Proline and Chlorophyll a and b

The obtained results point to a significant effect of the landrace on the content of *MDA*, free proline and chlorophyll in squash seedlings (Table 6). In relation to MDA, the highest content was recorded in landrace "746" (1.60 μmol g^{-1} FW), while the values of all the other landraces did not differ significantly. The landrace "746" was further distinguished by the highest content of free proline (1.00 μg mg^{-1} FW). On the other hand, landrace "748" showed the lowest content of free proline (Table 6). With respect to chla and chlb, the lowest values were obtained in the landrace "746" (12.49 and 25.69 mg g^{-1} FW of chla and chlb, respectively), followed by "747" (14.81 and 27.89 mg g^{-1} FW of chla and chlb, respectively), whereas the highest content was noted in "751" (51.07 and 108.74 mg g^{-1} FW of chla and chlb, respectively) (Table 6).

Table 6. Mean effect of the squash germplasm accession on the content of malondialdehyde (MDA), free proline and chlorophyll a and b (mean ± SD), regardless of the salinity level (NaCl concentration).

Accession	MDA (μmol g^{-1})	Proline (μg mg^{-1})	Chl a (mg mg^{-1})	Chl b (mg mg^{-1})
"746"	1.6 ± 0.12 a	1.0 ± 0.06 a	12.5 ± 1.50 d	25.7 ± 2.08 d
"747"	0.8 ± 0.19 b	0.8 ± 0.02 b	14.8 ± 1.16 c	27.9 ± 2.15 c
"748"	0.8 ± 0.12 b	0.5 ± 0.04 d	41.8 ± 1.50 b	77.0 ± 2.08 b
"751"	0.9 ± 0.26 b	0.6 ± 0.05 c	51.1 ± 1.29 a	108.7 ± 3.82 a
F Value	15.5 **	1466.3 **	12473.9 **	21337.9 **
SD				

** Means in the same column followed by the same letter are not significantly different at $p < 0.05$, according to Duncan's multiple range test.

2.6. Effect of Landrace and Salt Stress Level on the Content of Malondialdehyde, Free Proline and Chlorophyll a and b

Our findings indicate that the contents of MDA, proline and chlorophyll were differentially affected by the landrace and the salt stress level applied (Table 7). In relation to MDA, "748" landrace showed a decreasing trend as NaCl increased. Furthermore, all the other landraces exhibited a decreased content upon stress, yet they showed a notable increase either at 100 mM NaCl ("747" and "746") or 300 mM NaCl ("751"). Accordingly, the response of these four landraces ("747", "746", "748" and "751") to the different stress levels varied considerably in terms of the free proline content (Table 7). The stress response of most landraces involved an increased content of free proline, with the exception of landrace "746", whose content was maximized in control plants. In relation to the stress level effects, the application of 200 mM NaCl induced proline accumulation in "748" and "751", whereas "747" presented a peak at 300 mM NaCl (Table 7). Similarly, the content of chla and chlb was differentially affected both by the landrace and the stress level. Chlorophyll a and b were generally reduced in stressed plants, as compared to the control treatment, yet deviations from such a decreasing trend were observed depending on the landrace and stress intensity (Table 7). Specifically, "748" and "751" landraces showed the highest content of chla and chlb at 100 mM NaCl, while "747" presented the highest values at 300 mM NaCl. For the "746" landrace, the highest and lowest values for chla and chlb were noted at 0 mM and 200 mM NaCl, respectively.

Table 7. Response of squash germplasm accession to varying salt stress levels (0, 100, 200 and 300 mM NaCl) in relation to the content of malondialdehyde (MDA), free proline and chlorophyll a and b (mean ± SD).

Accession	NaCl Concentration (mM)	MDA (μmol g^{-1})	Proline (μg mg^{-1})	Chl a (mg mg^{-1})	Chl b (mg mg^{-1})
"746"	Control	0.88 ± 0.03	1.42 ± 0.03	14.7 ± 0.2	31.1 ± 0.7
	100	4.24 ± 0.03	0.74 ± 0.01	11.6 ± 0.2	24.4 ± 0.5
	200	0.58 ± 0.08	1.13 ± 0.05	9.9 ± 0.2	20.7 ± 0.3
	300	0.68 ± 0.09	0.71 ± 0.08	13.7 ± 0.1	26.6 ± 0.8
"747"	Control	0.9 ± 0.1	0.59 ± 0.02	13.7 ± 0.1	26.0 ± 0.3
	100	1.9 ± 0.6	0.44 ± 0.01	13.2 ± 0.2	25.8 ± 0.2
	200	0.36 ± 0.06	0.56 ± 0.02	15.6 ± 0.4	29.5 ± 0.5
	300	0.5 ± 0.2	0.8 ± 0.009	16.8 ± 0.4	30.2 ± 1.1
"748"	Control	1.8 ± 0.4	0.47 ± 0.05	37.5 ± 0.6	72.2 ± 0.9
	100	0.65 ± 0.03	0.48 ± 0.04	57.0 ± 0.8	103.6 ± 1.5
	200	0.60 ± 0.04	0.55 ± 0.05	30.2 ± 0.6	55.2 ± 0.6
	300	0.20 ± 0.07	0.40 ± 0.01	42.5 ± 0.8	76.9 ± 0.9
"751"	Control	1.1 ± 0.2	0.63 ± 0.09	54.5 ± 0.5	113.3 ± 1.7
	100	0.2 ± 0.1	0.46 ± 0.01	60.0 ± 3.8	125.7 ± 3.9
	200	0.20 ± 0.03	1.06 ± 0.05	47.1 ± 0.4	104.3 ± 3.5
	300	1.56 ± 0.20	0.98 ± 0.03	42.7 ± 0.3	91.7 ± 2.4

3. Discussion

The efficient selection of a salt-tolerant germplasm is considered of utmost importance in all breeding programs aimed at the development of salt-tolerant varieties. Given that salinity poses severe constraints to crop growth and productivity as well as to fruit quality in squash, this study aimed at determining the response of Tunisian squash germplasm to salinity stress at the stage of germination and early seedling growth. Plant response was assessed on the basis of selected traits related to seed germination and seedling growth potential. Based on their performance, four landraces were selected and further evaluated at the biochemical level, focusing on the determination of compounds that play a key role in the ability to withstand salt stress (e.g., MDA, free proline and chlorophylls).

Our findings revealed that salinity stress substantially affects all traits associated with germination and early seedling growth, with the effects of stress being in most cases analogous to the stress level applied. Differences in germination potential were reflected at the final germination percentage, on the seventh day after salt stress initiation, as well as at the decrease in germination over the control treatment. These findings are consistent with previous reports regarding the effects of salt stress on the germination of various plant species, including lettuce [33,40], rice [41], chickpea [31] and wheat [42]. Indeed, plants' sensitivity to salinity varies among growth stages [21], while during germination, the stress effects are aggravated due to the reduced seed water uptake that limits imbibition and seed turgescence [25]. Our data point to a gradual increasing severity of effects depending on stress intensity, thus supporting previous conclusions that the varying stress levels differently affect germination and seedling growth in various species, such as sugar beet and cabbage [43], soybean [44] and lentil [32]. Although all landraces were drastically affected, especially at high stress levels, their stress response varied significantly, thus indicating the existence of considerable genetic variation related to salt tolerance in the germplasm under study. As such, "746" and "747" were the best performing cultivars across stress levels, whereas "1007", "1008" and "1009" landraces, despite their relatively high innate germinability, were incapable of germination at all stress levels.

In addition to seed germination, the increasing level of salinity led to a gradually decreasing tissue elongation, expressed as reduced root and shoot lengths, probably as a result of toxicity as well as limited nutrient and water uptake due to osmotic stress [45,46]. Such data further support previous evidence that root and shoot lengths are the most suitable traits in terms of evaluating salt tolerance [31,47], since roots are responsible for water absorption and shoots for supplying aboveground tissues with water. In our study, seedling growth was inhibited at all salt stress levels, while root length was more severely affected than shoot length, most probably due to the fact that roots are directly and for a longer period exposed to salinity [46]. Indeed, such restricting effects have been attributed to the reduced water and nutrient absorption capacity from soil solution, which limits cell elongation and plant tissue development [48–51]. These findings are in agreement with the observed severe effects of salinity in roots of sugar beet, cabbage and amaranth [43] as well as soybean [44] and lentil [32]. Despite the fact that salinity led to a drastic reduction in seedling growth in all the tested landraces, a large variation between landraces was observed. At all stress levels studied, "1006" showed the highest SL and RL, therefore providing evidence for its salt tolerance ability, whereas the lowest values were noted in landraces "1007" and "1008" (in the case of SL) or "1008" (in the case of RL), indicating high susceptibility to salt stress. Accordingly, our data support a trend of gradually decreasing seedling fresh weight with increasing stress intensity, which could be mainly attributed to ionic effects occurring as a result of a proportional increase in Na+ concentration. Among landraces, "752" showed the highest growth potential as expressed by SFW and RFW parameters, both under normal and salt stress conditions, whereas "1008" and "747", "1008" and "1009" were the most drastically affected in relation to SFW and RFW, respectively. Concomitant with the abovementioned findings, all the recorded physiological indices were severely affected by salinity stress, especially at high stress levels. The adverse effect of salt stress on GSTI, SLSTI and RLSTI has been previously evidenced in maize germplasm subjected to drought stress [52], while previous reports further underline the significant effect of the genotype on the response to salt stress for several crop species [43,51,53]. Our findings support the notion that stress effects are differentially expressed in the germplasm under study. As such, "747" proved to be the most superior landrace in terms of GSTI and SLSTI, while "1006" was characterized as the most tolerant landrace based on RLSTI. In contrast, the combined data of GSTI, SLSTI and RLSTI classified "1007", "1008" and "1009" as the most salt-sensitive landraces.

In addition to germination and seedling growth potential under stress conditions, an indicator routinely employed to assess the degree of damage to plant cells caused by abiotic stress is MDA accumulation—a natural product of lipid peroxidation due to oxidative

stress [54–56]. Our data revealed a significant variation both in the landraces tested and the stress level applied. Although the mean MDA content across landraces was generally increased in stressed plants, as compared to the control treatment, such an increase was attributed to a peak in MDA content at specific stress levels. Interestingly, "746" and "747" showed the maximum content at 100 mM NaCl, while in landrace "751", MDA content reached its peak at 300 mM NaCl. Such findings are in total agreement with the previously reported inconclusive differences in MDA level in purslane plants subjected to varying salt stress levels, where the MDA content showed a peak after exposure at 300 mM NaCl for 6 or 9 days [57]. In general, the increased MDA and O2- synthesis under salt stress has been associated with damage to cell membrane integrity and protein activity, although the damage recorded depends on the stress period [58]. It has been suggested that long-term exposure to high salinity may lead to the destruction of cell membranes, adversely affecting SOD, POD and CAT activities, whereas oxidative stress-mediated lipid peroxidation does not occur within a short period; e.g., up to 5 week exposure to salt stress [59]. Our data further point to inconclusive differences in free proline content at various stress levels. Although the response of most landraces to salt stress involved an increase in free proline, with the exception of "746", the maximum content was observed either at 200 mM ("748" and "751") or at 300 mM NaCl ("747"). Such observations further support the previously reported negative correlation between MDA and free proline content [54], yet they partly deviate from the suggested positive association between proline content and stress intensity [57]. In our study, this association was only evidenced in landrace "747", whose overall performance classified it as salt-tolerant, thus supporting previous conclusions related to the increased proline content in salt-tolerant genotypes of various plant species, including potato [60], melon [61] and tomato [62]. It is well evidenced that plants' response to high salinity adversely affects photosynthesis activity, thus involving a reduction in chlorophyll content. Our data showed a general decreasing trend of chla and chlb in stressed plants, as compared to the control treatment, which indicates that the response to high salinity may involve a decrease in chlorophyll content in various plant species, including pepper [63] and winter squash [64]. However, deviations from this trend were noted among the tested landraces and the stress levels applied, indicating a genotype-depended response. Interestingly, the content of both chla and chlb was the maximum at 100 mM NaCl across landraces, with this peak being mostly evidenced in landraces "748" and "751", thus probably reflecting an adaptive stress response governing tolerance to low-medium salinity.

Overall findings provide conclusive evidence that the evaluation of squash genotypes at germination phase shows a great potential for revealing genetic variability related to salt tolerance. Addressing the classification of genotypes in terms of salt tolerance, our data related to germination and seedling growth potential under salt stress point to the superiority of landraces "746" and "747", followed by "1006", at all stress levels applied. In contrast, "1007", "1008" and "1009" landraces were incapable of germination at all stress levels, thus proving their sensitivity to salinity even at relatively low stress levels. Our findings further support previous reports highlighting that the innate genotypic high growth potential is associated with salt sensitivity and vice versa [65]. Mostly relevant to this argument is the performance of landrace "751": although it exhibited an enhanced seedling growth under normal conditions, it suffered a significant decrease after exposure to salt stress. Addressing the evaluation of salt tolerance based on biochemical parameters, the observed cumulative patterns for MDA, free proline and chla and chlb deviate considerably from the general stress response profiles that usually involve an increased MDA and proline content as well as a decreasing chlorophyll content with increasing salt concentration. Despite such deviations, which further strengthen previous reports on inconclusive differences in MDA and chlorophyll content in various plant species subjected to salt stress [57,65,66], the overall data support the feasibility of conducting the early selection of salt-tolerant squash germplasm on the basis of germination and seedling growth potential under salt stress.

4. Materials and Methods

4.1. Plant Material and Growth Conditions

The studied genetic material consisted of 15 Tunisian squash landraces, collected from different geographic regions of Tunisia during the period extending from 2018 to 2020 (Table 8). Each landrace was assigned passport data and an inventory number, according to the National Gene Bank of Tunisia, while full details are available at the Germplasm Resources Information Network—GRIN (http://www.tn-grin.nat.tn/gringlobal/search.aspx, accessed on 15 February 2022). The description of fruit morphology was performed based on the European Cooperative Program for Plant Genetic Resources (ECPGR) list of descriptors for *Cucurbita* spp. [67].

Table 8. Description of the Tunisian squash landraces employed in this study.

Landrace Inventory Number	Local Name	Origin	Latitude	Longitude	Short Description
NGBTUN745 (“745”)	Batati Green	Ariana (Kalaat Andalous)	37°033″ N	10°11′7″ e	Globular fruit, light green skin, green flesh
NGBTUN746 (“746”)	Batati orange	Siliana (SidiHamada)	35°57′28″ N	9°32′57″ e	Globular fruit, orange skin, light orange flesh
NGBTUN747 (“747”)	Galaoui	Ariana (Kalaa Andalous)	37°033″ N	10°11′7″ e	Raised fruit with basal tip, green skin, green flesh
NGBTUN748 (“748”)	Karkoubi orange	Sousse (SidiBouali)	35°54′22.21″ N	10°32′47.81″ e	Flattened fruit, dark yellow skin, yellow flesh
NGBTUN749 (“749”)	Batati yellow spotted with white	Siliana (SidiHamada)	35°57′28″ N	9°32′57″ e	Globular fruit, orange skin spotted with white, orange flesh
NGBTUN750 (“750”)	Batati white	Monastir (Sahline)	35°45′05″ N	10°42′39″ e	Globular fruit, white skin, white flesh
NGBTUN751 (“751”)	Bejaoui Green	Siliana (SidiHamada)	35°57′28″ N	9°32′57″ e	Flattened fruit, dark green skin, light green flesh
NGBTUN752 (“752”)	Batati yellow	Siliana (North)	35°57′28″ N	9°32′57″ e	Globular fruit, yellow skin, light orange flesh
NGBTUN753 (“753”)	Béjaoui Green	Siliana (South)	35°57′28″ N	9°32′57″ e	Flattened fruit, dark green skin, light green flesh
NGBTUN1004 (“1004”)	Galaoui large seeds	Ariana (Kalaat Andalous)	37°033″ N	10°11′7″ e	Turbinate interior fruit with basal tip, green skin, white green flesh
NGBTUN1005 (“1005”)	Galaoui smoll seeds	Ariana (Kalaat Andalous)	37°033″ N	10°11′7″ e	Turbinate interior fruit with a big basal tip, green skin, white green flesh
NGBTUN1006 (“1006”)	Karkoubi orange	Monastir (Sahline)	35°45′05″ N	10°42′39″ e	Flattened fruit, dark yellow skin, yellow flesh
NGBTUN1007 (“1007”)	Batati Green	Siliana	35°57′28″ N	9°32′57″ e	Rounded fruit, green skin, green flesh
NGBTUN1008 (“1008”)	Batati Green	Monastir (Teboulba)	35°45′05″ N	10°42′39″ e	Globular fruit, flat stem end, green skin, light green flesh
NGBTUN1009 (“1009”)	Bejaoui spotted with yellow	Siliana (SidiHamada)	35°57′28″ N	9°32′57″ e	Globular fruit with flat stem end, spotted with yellow light green skin, light green flesh

4.2. Salinity Stress Treatments

The experiment was carried out at the Department of Horticulture, High Agronomic Institute of Chott Mariem-Sousse-Tunisia. Following the selection of seeds for size homogeneity, 50 seeds per landrace (five petri dishes with 10 seeds each, $n = 5$) were surface-sterilized for 5 min in 10% H_2O_2 (v/v) and rinsed twice in sterile dH_2O. Sterilized seeds

were primed via exposure to an eliciting solution of 1.5 mM gibberellic acid (GA_3) for 24 h to stimulate germination and subsequently rinsed in sterile dH_2O. Five to ten seeds, according to size, were placed on sterile petri dishes containing two layers of filter paper moistened daily with 5 mL of appropriate solutions: dH_2O (control), 100, 200 and 300 mM NaCl. Seedlings were grown under controlled conditions for 7 days (25 ± 2 °C, 50 ± 5% relative humidity, 18 h light/6 h dark photoperiod under white fluorescent light (40 µmol m^{-2} s^{-1}).

4.3. Determination of Germination and Seedling Growth Potential under Salt Stress

Salt tolerance was evaluated on the basis of various parameters related to seed germination and seedling growth potential under salt stress conditions, measured daily until no more germinated seeds were recorded (first–seventh day) (Table 9). Seeds were considered germinated when the protruding radicle was at least 2 mm long. The parameters germination reduction (GR), root length reduction (RLR) and shoot length stress tolerance index (SLSTI) express the decreased values of salt-stressed plants over the control treatment.

Table 9. Description of the evaluation criteria for salinity tolerance employed in this study.

Trait	Unit	Description/Formula	Reference
Germination percentage (GP)	%	$GP = \frac{\text{number germinated seeds}}{\text{number of total seeds}} \times 100$	Scott et al. (1984)
Shoot length (SL)	mm	At the day of germination	Sivakumar et al. (2020)
Root length (RL)	mm	At the day of germination	Sivakumar et al. (2020)
Shoot fresh weight (SFW)	g	Recorded by using a sensitive balance (Sartorius AC 1215, Germany)	Jamil et al. (2006)
Root fresh weight (RFW)	g	Recorded by using a sensitive balance (Sartorius AC 1215, Germany)	Jamil et al. (2006)
Shoot length/Root length Ratio (SRR)	-	Ratio of SL to RL	Thabet et al. (2018)
Germination reduction (GR)	%	GR = GP of controls − GP of stress plants	Thabet et al. (2018)
Shoot length reduction (SLR)	mm	SLR = SL of controls − SL of stress plants	Partheeban et al. (2017)
Root length reduction (RLR)	mm	RLR = RL of controls − RL of stress plants	Thabet et al. (2018)
Germination stress tolerance index (GSTI)	%	$GSTI = \frac{\text{GP under salt stress conditions}}{\text{GP under normal conditions}} \times 100$	Partheeban et al. (2017)
Shoot length stress tolerance index (SLSTI)	%	$SLSTI = \frac{\text{SL under salt stress condtions}}{\text{SL under normal conditions}} \times 100$	Partheeban et al. (2017)
Root length stress tolerance index (RLSTI)	%	$RLSTI = \frac{\text{RL under salt stress condtions}}{\text{RL under normal conditions}} \times 100$	Partheeban et al. (2017)

4.4. Evaluation of Salinity Tolerance Based on Biochemical Parameters

Based on the obtained data related to the response of the 15 landraces to salinity stress, 4 landraces representing the main types of cultivated squash were selected for further evaluation. As such, the landraces "748" (Karkoubi), "751" (Bejaoui), "747" (Galaoui) and "746" (Batati) were assessed, employing the content of seedling tissues in MDA (MDA), free proline and chlorophyll a and b as evaluation criteria.

The content of MDA (MDA) was determined using the method applied by Hnilickova et al. [57] with minor modifications. Briefly, 200 mg of leaf samples were homogenized with liquid nitrogen and, following the addition of 80% ethanol, samples were centrifuged for 5 min at 6000 rpm. Aliquots of 0.7 mL of the supernatant solution were mixed with 0.7 mL of 0.65% thiobarbituric acid (TBA) in 20% TCA (trichloroacetic acid) and 0.01% BHT (butylated hydroxytoluene). A second set of 0.7 mL samples was mixed with 0.7 mL of 20 % TCA and 0.01 % BHT. Following incubation at 95 °C for 25 min and subsequent cooling, samples were centrifuged for 5 min at 6000 rpm. The content of MDA was determined at 532 nm using a UV–Vis spectrophotometer (Evolution 210, Thermo Scientific, Abingdon, UK) and expressed in µmol g^{-1} of fresh weight (FW).

The content of free proline was measured according to the method described by Monneveux and Nemmar [68]. Leaf samples (100 mg) were homogenized in 10 mL of 3% sulfosalicylic acid and, following filtration, the homogenate was heated to 85 °C in a water bath for 60 min. After cooling, 1 mL of ninhydrin reagent was added (ninhydrin reagent consisted of 120 mL distilled water, 300 mL of acetic acid, 80 mL acetic orthophosphoric acid at a density of 1.7, and 25 mg of ninhydrin). The samples were boiled for 30 min and, after cooling, 5 mL of toluene was added and samples were vortexed. The upper phase was recovered and was measured using a UV-Vis spectrophotometer (Evolution 210, Thermo Scientific, Abingdon, UK) at 528 nm. A proline standard curve ranging from 0 to 2.5 mg mL^{-1} of L-proline was used to determine the proline content, expressed in μg mg^{-1} of FW.

For chlorophyll content determination, the extraction of samples was performed as described by Curtis and Shetty [69]. Briefly, 50 mg of leaf tissue (in triplicate) was extracted into 3 mL of methanol and stored at 23 °C in darkness for 2 h. Absorption of extracts (1.5 mL) was measured at 650 and 665 nm using a spectrophotometer (Evolution 210, Thermo Scientific, Abingdon, UK). Chlorophyll a and chlorophyll b were expressed in mg g^{-1} FW.

4.5. Statistical Analysis

The experimental layout was completely randomized with three replications. Data were analyzed using ANOVA tests ($p \leq 0.05$), according to the experimental design, combining salt concentrations and genotypes. Differences between means were compared using the Duncan Multiple Range test (DMRT). Statistical analyses were performed using SAS software V9 (SAS Institute, Cary, CA, USA).

5. Conclusions

Soil salinization is gradually increasing due to the scarcity of rains and the increase in evapotranspiration, adversely affecting plant germination, growth, development and fruit setting in salt-affected soils, as is the case of semiarid regions. Most vegetable species are salt-sensitive at all stages of their lifecycle, while germination and seedling growth have been viewed as the critical stage under salt conditions. In particular, salinity is a factor that severely limits squash growth and productivity while, at the same time, deteriorating fruit quality. Despite the scientific evidence in relation to the salt stress response in a plethora of plant species, there is a lack of information in relevant research fields for squash. The development of a salt-tolerant germplasm is one of the most effective means for enhancing squash production in saline soils. The results of our study suggest the feasibility of conducting an early selection of salt-tolerant squash germplasm on the basis of germination and seedling growth potential under salt stress. Such an approach may considerably upgrade all procedures aimed at selecting salt-tolerant germplasm to be exploited for cultivation in saline soils. In this context, landraces "746" and "747" were the best performing cultivars for the tested salinity levels in terms of germination percentage, germination percentage stress index and shoot length stress index, which indicates that they could be integrated as valuable germplasm material in breeding programs targeted at improving salt tolerance in squash through the selection of elite genotypes.

Author Contributions: Conceptualization, N.T. and S.A.P.; methodology, W.S., N.M. and H.F.; formal analysis, W.S., N.M. and H.F.; investigation, W.S., N.M. and H.F.; data curation, W.S., N.M. and H.F.; writing—original draft preparation, N.T. and O.I.P.; writing—review and editing, N.T., N.M., O.I.P. and S.A.P.; visualization, N.T. and S.A.P.; supervision, N.T. and S.A.P.; project administration, N.T. and S.A.P.; funding acquisition, S.A.P. All authors have read and agreed to the published version of the manuscript.

Funding: The project is funded by the General Secretariat for Research and Technology of the Ministry of Development and Investments under the PRIMA Program. PRIMA is an Art.185 initiative supported and co-funded under Horizon 2020, the European Union's Program for Research and

Innovation (Prima2019-08). This research was also jointly founded by Prima project and National research laboratory LR21AGR05.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: Not applicable.

Conflicts of Interest: The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

References

1. Abdein, M.A.E.-H. Genetic Diversity between Pumpkin Accessions Growing in the Northern Border Region in Saudi Arabia Based on Biochemical and Molecular Parameters. *Egypt. J. Bot.* **2018**, *58*, 463–476. [CrossRef]
2. Brown, C.H.; Luedeling, E.; Wichmann, S.; Epps, P. The paleobiolinguistics of domesticated squash (Cucurbita spp.). In *Explorations in Ethnobiology: The Legacy of Amadeo Rea*; Quinlan, M., Lepofsky, D., Eds.; Society of Ethnobiology, Department of Geography, University of North Texas: Denton, TX, USA, 2013; pp. 132–161.
3. Ferriol, M.; Picó, B.; Nuez, F. Morphological and Molecular Diversity of a Collection of *Cucurbita maxima* Landraces. *J. Am. Soc. Hortic. Sci.* **2004**, *129*, 60–69. [CrossRef]
4. Decker-Walters, D.S.; Walters, T.W. *The Cambridge World History of Food*; Kiple, K., Ornelas, K.C., Eds.; Cambridge University Press: Cambridge, UK, 2000.
5. Le Floch, E.; Boulos, L.; Véla, E. *Catalogue Synonymique Commenté de la Flore Tunisie*; Ministère de l'Environnement et du Développement durable: Tunis, Tunisie, 2010; p. 500.
6. Hamdi, K.; Palma, D.; Angelini, P.; Acciarri, N.; Tarchoun, N.; Sestili, S. *Cucurbita maxima* Duch. population analysis: Relationship between Tunisian and Italian germplasm. *J. Hortic. Sci. Biotechnol.* **2020**, *95*, 496–505. [CrossRef]
7. Hamdi, K.; Ben-Amor, J.; Mokrani, K.; Mezghanni, N.; Tarchoun, N. Assessment of the genetic diversity of some local squash (*Cucurbita maxima* Duchesne) populations revealed by agro-morphological and chemical traits. *J. New Sci.* **2017**, *42*, 2306–2317.
8. Shahid, M.A.; Sarkhosh, A.; Khan, N.; Balal, R.M.; Ali, S.; Rossi, L.; Gómez, C.; Mattson, N.; Nasim, W.; Garcia-Sanchez, F. Insights into the physiological and biochemical impacts of salt stress on plant growth and development. *Agronomy* **2020**, *10*, 938. [CrossRef]
9. Sium, A.; Shawon, A.; Swapan, K.R.; Sun, H.W.; Kailas, S.D.; Abdullah, S.M. Effect of salinity on the morphological, physiological and biochemical properties of lettuce (*Lactuca sativa* L.) in Bangladesh. *Open Agric.* **2019**, *4*, 361–373. [CrossRef]
10. Rouphael, Y.; Petropoulos, S.A.; Cardarelli, M.; Colla, G. Salinity as eustressor for enhancing quality of vegetables. *Sci. Hortic.* **2018**, *234*, 361–369. [CrossRef]
11. Rogel, J.A.; Ariza, F.A.; Silla, R.O. Soil salinity and moisture gradients and plant zonation in Mediterranean salt marshes of Southeast Spain. *Wetlands* **2000**, *20*, 357–372. [CrossRef]
12. Rubio, J.S.; García-Sánchez, F.; Rubio, F.; Martínez, V. Yield, blossom-end rot incidence, and fruit quality in pepper plants under moderate salinity are affected by K+ and Ca2+ fertilization. *Sci. Hortic.* **2009**, *119*, 79–87. [CrossRef]
13. Soltabayeva, A.; Ongaltay, A.; Omondi, J.O.; Srivastava, S. Morphological, physiological and molecular markers for salt-stressed plants. *Plants* **2021**, *10*, 243. [CrossRef] [PubMed]
14. Giordano, M.; Petropoulos, S.A. Response and Defence Mechanisms of Vegetable Crops against Drought, Heat and Salinity Stress. *Agriculture* **2021**, *11*, 463. [CrossRef]
15. Noctor, G.; Foyer, C.H. Ascorbate and glutathione: Keeping Active Oxygen Under Control. *Annu. Rev. Plant. Physiol. Plant. Mol. Biol.* **1998**, *49*, 249–279. [CrossRef] [PubMed]
16. Saibi, W.; Feki, K.; Ben Mahmoud, R.; Brini, F. Durum wheat dehydrin (DHN-5) confers salinity tolerance to transgenic *Arabidopsis* plants through the regulation of proline metabolism and ROS scavenging system. *Planta* **2015**, *242*, 1187–1194. [CrossRef] [PubMed]
17. Sazzad Hossain, M.; Persicke, M.; Elsayed, A.I.; Kalinowski, J.; Dietz, K.J. Metabolite profiling at the cellular and subcellular level reveals metabolites associated with salinity tolerance in sugar beet. *J. Exp. Bot.* **2017**, *68*, 5961–5976. [CrossRef]
18. Chaves, M.M.; Oliveira, M.M. Mechanisms underlying plant resilience to water deficits: Prospects for water-saving agriculture. *J. Exp. Bot.* **2004**, *55*, 2365–2384. [CrossRef]
19. Kaur, G.; Asthir, B. Proline: A key player in plant abiotic stress tolerance. *Biol. Plant.* **2015**, *59*, 609–619. [CrossRef]
20. Ashraf, M.; Harris, P.J.C. Photosynthesis under stressful environments: An overview. *Photosynthetica* **2013**, *51*, 163–190. [CrossRef]
21. Acosta-Motos, J.R.; Ortuño, M.F.; Bernal-Vicente, A.; Diaz-Vivancos, P.; Sanchez-Blanco, M.J.; Hernandez, J.A. Plant responses to salt stress: Adaptive mechanisms. *Agronomy* **2017**, *7*, 18. [CrossRef]
22. Mahlooji, M.; Seyed Sharifi, R.; Razmjoo, J.; Sabzalian, M.R.; Sedghi, M. Effect of salt stress on photosynthesis and physiological parameters of three contrasting barley genotypes. *Photosynthetica* **2018**, *56*, 549–556. [CrossRef]
23. Orlovsky, N.; Japakova, U.; Zhang, H.; Volis, S. Effect of salinity on seed germination, growth and ion content in dimorphic seeds of *Salicornia europaea* L. (Chenopodiaceae). *Plant. Divers.* **2016**, *38*, 183–189. [CrossRef]

24. Petropoulos, S.A.; Daferera, D.; Polissiou, M.G.; Passam, H.C. The effect of salinity on the growth, yield and essential oils of turnip-rooted and leaf parsley cultivated within the Mediterranean region. *J. Sci. Food Agric.* **2009**, *89*, 1534–1542. [CrossRef]
25. Golbashy, M.; Ebrahimi, M.; Khavari Khorasani, S.; Mostafavi, K. Effects of drought stress on germination indices of corn hybrids (*Zea mays* L.). *Electrnonic J. Plant. Breed.* **2012**, *3*, 664–670.
26. Almansouri, M.; Kinet, J.M.; Lutts, S. Effect of salt and osmotic stresses on germination in durum wheat (*Triticum durum* Desf.). *Plant. Soil* **2001**, *231*, 243–254. [CrossRef]
27. Flowers, T.J.; Munns, R.; Colmer, T.D. Sodium chloride toxicity and the cellular basis of salt tolerance in halophytes. *Ann. Bot.* **2015**, *115*, 419–431. [CrossRef] [PubMed]
28. Khan, N.; Bano, A.; Babar, M.A. The stimulatory effects of plant growth promoting rhizobacteria and plant growth regulators on wheat physiology grown in sandy soil. *Arch. Microbiol.* **2019**, *201*, 769–785. [CrossRef] [PubMed]
29. Savvas, D.; Lenz, F. Effects of NaCl or nutrient-induced salinity on growth, yield, and composition of eggplants grown in rockwool. *Sci. Hortic.* **2000**, *84*, 37–47. [CrossRef]
30. Parvin, K. Response of Tomato Plant Under Salt Stress: Role of Exogenous Calcium. *J. Plant. Sci.* **2015**, *10*, 222–233. [CrossRef]
31. Janghel, D.K.; Kumar, K.; Sunil, R.; Chhabra, A.K. Genetic Diversity Analysis, Characterization and Evaluation of Elite Chickpea (*Cicer arietinum* L.) Genotypes. *Int. J. Curr. Microbiol. Appl. Sci.* **2020**, *9*, 199–209. [CrossRef]
32. Foti, C.; Khah, E.M.; Pavli, O.I. Germination profiling of lentil genotypes subjected to salinity stress. *Plant. Biol.* **2019**, *21*, 480–486. [CrossRef]
33. Shin, Y.K.; Bhandari, S.R.; Jo, J.S.; Song, J.W.; Cho, M.C.; Yang, E.Y.; Lee, J.G. Response to salt stress in lettuce: Changes in chlorophyll fluorescence parameters, phytochemical contents, and antioxidant activities. *Agronomy* **2020**, *10*, 1627. [CrossRef]
34. Othman, Y.; Al-Karaki, G.; Al-Tawaha, A.R.; Al-Horani, A. Variation in Germination and Ion Uptake in Barley Genotypes under Salinity Conditions. *World J. Agric. Sci.* **2006**, *2*, 11–15.
35. Mehr, Z.S. Salt-induced changes in germination and vegetative stages of *Anethum graveolens* L. *J. Stress Physiol. Biochem.* **2013**, *9*, 189–198.
36. Kandil, A.; Shareif, A.; Gad, M. Effect of Salinity on Germination and Seeding Parameters of Forage Cowpea Seed. *Res. J. Seed Sci.* **2016**, *10*, 17–26. [CrossRef]
37. Viegas, D.X.; Piñol, J.; Viegas, M.T.; Ogaya, R. Estimating live fine fuels moisture content using meteorologically-based indices. *Int. J. Wildl. Fire* **2001**, *10*, 223–240. [CrossRef]
38. Yildirim, E.; Dursun, A.; Kumlay, M.A.; Güvenç, Í. The effects of different salt, biostimulant and temperature levels on seed germination of some vegetable species. *Acta Agrobot.* **2013**, *55*, 75–80. [CrossRef]
39. Azeem, A.; Javed, Q.; Sun, J.; Nawaz, M.I.; Ullah, I.; Kama, R.; Du, D. Functional traits of okra cultivars (Chinese green and Chinese red) under salt stress. *Folia Hortic.* **2020**, *32*, 159–170. [CrossRef]
40. Pavli, O.; Kempapidis, K.; Maggioros, L.; Foti, C.; Panagiotaki, P.; Khah, E. Response of Lettuce Germplasm to Salt Stress at Different Developmental Stages. *Ann. Agric. Crop Sci.* **2021**, *6*, 1088. [CrossRef]
41. Srivastava, S.; Sharma, P.K. Effect of NaCl on chlorophyll fluorescence and thylakoid membrane proteins in leaves of salt sensitive and tolerant rice (*Oryza sativa* L) varieties. *J. Stress Physiol. Biochem.* **2021**, *17*, 35–44.
42. Hasan, B.; Higano, Y.; Yabar, H.; Devkota, M.; Lamers, J.P.A. Conservation Agriculture Practices in Salt-Affected, Irrigated Areas of Central Asia: Crop Price and Input Cost Variability Effect on Revenue Risks. *Sustain. Agric. Res.* **2015**, *4*, 1. [CrossRef]
43. Jamil, M.; Rha, E.-S. The Effect of Salinity (NaCI) on the Germination and Seedling of Sugar Beet (*Beta vulgaris* L.) and Cabbage (*Brassica oleracea* L.). *Plant. Resour.* **2004**, *7*, 226–232.
44. Pavli, O.I. Effect of Salinity on Seed Germination and Seedling Development of Soybean Genotypes. *Int. J. Environ. Sci. Nat. Resour.* **2021**, *27*, 556210. [CrossRef]
45. Abdoli, M.; Saeidi, M.; Azhand, M.; Jalali-honarmand, S.; Esfandiari, E.; Shekari, F. The Effects of Different Levels of Salinity and Indole-3-Acetic Acid (IAA) on Early Growth and Germination of Wheat Seedling. *J. Stress Physiol. Biochem.* **2013**, *9*, 329–338.
46. Ouji, A.; El-Bok, S.; Mouelhi, M.; Younes, M.; Kharrat, M. Effect of salinity stress on germination of five Tunisian lentil *Lens culinaris* L. genotypes. *Eur. Sci. J.* **2015**, *11*, 63–75.
47. Shin, Y.K.; Bhandari, S.R.; Cho, M.C.; Lee, J.G. Evaluation of chlorophyll fluorescence parameters and proline content in tomato seedlings grown under different salt stress conditions. *Hortic. Environ. Biotechnol.* **2020**, *61*, 433–443. [CrossRef]
48. Abbas, W.; Ashraf, M.; Akram, N.A. Alleviation of salt-induced adverse effects in eggplant (*Solanum melongena* L.) by glycinebetaine and sugarbeet extracts. *Sci. Hortic.* **2010**, *125*, 188–195. [CrossRef]
49. Kaya, M.D.; Ipek, A.; Öztürk, A. Effects of different soil salinity levels on germination and seedling growth of safflower (*Carthamus tinctorius* L.). *Turk. J. Agric. For.* **2003**, *27*, 221–227. [CrossRef]
50. Benidire, L.; Daoui, K.; Fatemi, Z.A.; Achouak, W.; Bouarab, L.; Oufdou, K. Effect of salt stress on germination and seedling of *Vicia faba* L. *J. Mater. Environ. Sci.* **2015**, *6*, 840–851.
51. Alom, R.; Hasan, M.A.; Islam, M.R.; Wang, Q.F. Germination characters and early seedling growth of wheat (*Triticum aestivum* L.) genotypes under salt stress conditions. *J. Crop Sci. Biotechnol.* **2016**, *19*, 383–392. [CrossRef]
52. Partheeban, C.; Chandrasekhar, C.N.; Jeyakumar, P.; Ravikesavan, R.; Gnanam, R. Effect of PEG Induced Drought Stress on Seed Germination and Seedling Characters of Maize (*Zea mays* L.) Genotypes. *Int. J. Curr. Microbiol. Appl. Sci.* **2017**, *6*, 1095–1104. [CrossRef]

53. Hannachi, S.; Van Labeke, M.C. Salt stress affects germination, seedling growth and physiological responses differentially in eggplant cultivars (*Solanum melongena* L.). *Sci. Hortic.* **2018**, *228*, 56–65. [CrossRef]
54. Du, F.; Shi, H.; Zhang, X.; Xu, X. Responses of reactive oxygen scavenging enzymes, proline and malondialdehyde to water deficits among six secondary successional seral species in Loess Plateau. *PLoS ONE* **2014**, *9*, e98872. [CrossRef]
55. Ma, J.; Du, G.; Li, X.; Zhang, C.; Guo, J. A major locus controlling malondialdehyde content under water stress is associated with *Fusarium* crown rot resistance in wheat. *Mol. Genet. Genom.* **2015**, *290*, 1955–1962. [CrossRef] [PubMed]
56. Zhou, C.; Wang, Z.W.; Han, X.G.; Yang, Y.G.; Busso, C.A.; Zhang, Z.; Yang, Y.F. Effect of mixed salt stress on malondialdehyde, proteins and antioxidant enzymes of *Leymus chinensis* in three leaf colors. *Phyton* **2017**, *86*, 205–213.
57. Hnilickova, H.; Kraus, K.; Vachova, P.; Hnilicka, F. Salinity stress affects photosynthesis, malondialdehyde formation, and proline content in *Portulaca oleracea* L. *Plants* **2021**, *10*, 845. [CrossRef]
58. Xing, J.-C.; Dong, J.; Wang, M.-W.; Liu, C.; Zhao, B.-Q.; Wen, Z.-G.; Zhu, X.-M.; Ding, H.-R.; Zhao, X.-H.; Hong, L.-Z. Effects of NaCl stress on growth of *Portulaca oleracea* and underlying mechanisms. *Braz. J. Bot.* **2019**, *42*, 217–226. [CrossRef]
59. Borsai, O.; Al Hassan, M.; Negruşier, C.; Raigón, M.D.; Boscaiu, M.; Sestraş, R.E.; Vicente, O. Responses to salt stress in portulaca: Insight into its tolerance mechanisms. *Plants* **2020**, *9*, 1660. [CrossRef]
60. Jaarsma, R.; de Vries, R.S.M.; de Boer, A.H. Effect of Salt Stress on Growth, Na+ Accumulation and Proline Metabolism in Potato (*Solanum tuberosum*) Cultivars. *PLoS ONE* **2013**, *8*, e60183. [CrossRef] [PubMed]
61. Sarabi, B.; Bolandnazar, S.; ghaderi, N.; ghashghaie, J. Genotypic differences in physiological and biochemical responses to salinity stress in melon (*Cucumis melo* L.) plants: Prospects for selection of salt tolerant landraces. *Plant. Physiol. Biochem.* **2017**, *119*, 294–311. [CrossRef]
62. De la Torre-González, A.; Montesinos-pereira, D.; Blasco, B.; Ruiz, J.M. Influence of the proline metabolism and glycine betaine on tolerance to salt stress in tomato (*Solanum lycopersicum* L.) commercial genotypes. *J. Plant. Physiol.* **2018**, *231*, 329–336. [CrossRef]
63. Zhani, K.; Mariem, B.F.; Fardaous, M.; Cherif, H.; Zhani, K.; Mariem, B.F.; Fardaous, M.; Cherif, H. Impact of salt stress (NaCl) on growth, chlorophyll content and fluorescence of Tunisian cultivars of chili pepper (*Capsicum frutescens* L.). *J. Stress Physiol. Biochem.* **2012**, *8*, 236–252.
64. Seroczyńska, A.; Antczak, A.; Kamińska, K.; Korytowska, M.; Korzeniewska, A.; Niemirowicz-szczytt, K.; Radomski, A.; Zawadzki, J. Evaluation of the selected forms of winter squash (*Cucurbita maxima* Duch.) for the content of free sugars and polysaccharides. *Polish J. Agron.* **2014**, *16*, 69–73.
65. Xu, C.; Mou, B. Evaluation of lettuce genotypes for salinity tolerance. *HortScience* **2015**, *50*, 1441–1446. [CrossRef]
66. Ekinci, M.; Yildirim, E.; Dursun, A.; Turan, M. Mitigation of salt stress in lettuce (*Lactuca sativa* L. var. Crispa) by seed and foliar 24-epibrassinolide treatments. *HortScience* **2012**, *47*, 631–636. [CrossRef]
67. ECPGR. Minimum descriptors for *Cucurbita* spp., cucumber, melon and watermelon. *Cucurbits Work. Gr.* **2008**, *15*, 1–13.
68. Monneveaux, P.; Nemmar, M. Contribution à l'étude de la résistance à la sécheresse chez le blé tendre (*Triticum aestivum* L.) et chez le blé dur (*Triticum durum* Desf.): Étude de l'accumulation de la proline au cours du cycle de développement. *Agronomie* **1986**, *6*, 583–590. [CrossRef]
69. Curtis, O.F.; Shetty, K. Growth medium effects on vitrification, total phenolics, chlorophyll, and water content of in vitro propagated oregano clones. *Acta Hortic.* **1996**, *426*, 489–504. [CrossRef]

frontiers | Frontiers in Plant Science

TYPE Original Research
PUBLISHED 31 July 2023
DOI 10.3389/fpls.2023.1215394

OPEN ACCESS

EDITED BY
Marco Landi,
University of Pisa, Italy

REVIEWED BY
Angeles Calatayud,
Valencian Institute for Agricultural Research (IVIA), Spain
Cristina Patanè,
National Research Council (CNR), Italy

*CORRESPONDENCE
Neji Tarchoun
nejitarchoun@yahoo.fr
Spyridon A. Petropoulos
spetropoulos@uth.gr

RECEIVED 01 May 2023
ACCEPTED 07 July 2023
PUBLISHED 31 July 2023

CITATION
Saadaoui W, Tarchoun N, Msetra I, Pavli O, Falleh H, Ayed C, Amami R, Ksouri R and Petropoulos SA (2023) Effects of drought stress induced by D-Mannitol on the germination and early seedling growth traits, physiological parameters and phytochemicals content of Tunisian squash (*Cucurbita maxima*Duch.) landraces. *Front. Plant Sci.* 14:1215394. doi: 10.3389/fpls.2023.1215394

Effects of drought stress induced by D-Mannitol on the germination and early seedling growth traits, physiological parameters and phytochemicals content of Tunisian squash (*Cucurbita maxima*Duch.) landraces

Wassim Saadaoui[1], Neji Tarchoun[1]*, Insaf Msetra[1], Ourania Pavli[2], Hanen Falleh[3], Chadha Ayed[1], Roua Amami[1], Riadh Ksouri[3] and Spyridon A. Petropoulos[4]*

[1]Research Laboratory LR21AGR05, High Agronomic Institute of ChottMariem, University of Sousse, Sousse, Tunisia, [2]Laboratory of Genetics and Plant Breeding, Department of Agriculture, Crop Production and Rural Environment, University of Thessaly, Volos, Greece, [3]Laboratory of Aromatic and Medicinal Plant, Centre of Biotechnology of Borj Cedria, Tunis, Tunisia, [4]Laboratory of Vegetable Production, Department of Agriculture, Crop Production and Rural Environment, University of Thessaly, Volos, Greece

Introduction: Drought stress is one of the most devastating environmental stressors, especially in the arid and semi-arid regions of the world. Considering the major constraints that drought stress poses to crop production and the consequent yield losses in food crops, breeding for climate-resilient crops is an efficient means to mitigate stress conditions.

Materials and methods: This study aimed at evaluating the response of four squash (*Cucurbita maxima* Duchesne) landraces to drought stress at germination and at plant stage. Drought stress was induced by different concentrations of D-mannitol (-0.24, -0.47 and -0.73 MPa). The tested parameters at germination stage included germination percentage, seedling vigor index, seed water absorbance and seedling growth potential. At the plant stage, leaf chlorophyll and carotenoids content, chlorophyll fluorescence, evapotranspiration, photosynthesis activity and several biomarkers, namely malondialdehyde, proline, total phenols content, total flavonoids content and DPPH radical scavenging activity were evaluated in both roots and leaves.

Results and discussion: Our results indicate a magnitude of drought stress effects reflected via repression of germination and seedling growth as well as adjustments in physiological functions at later growth stages, in a genotype depended manner. Among landraces, "751" and "746" showed better

performance, as evidenced by higher seed germination and seedling growth potential even at high stress levels (-0.47 and - 0.73 MPa), whereas "747" was the most sensitive landrace to drought stress at both tested stages. In conclusion, our findings highlight the importance of squash landraces selection for the identification of elite genotypes with increased tolerance to drought stress.

KEYWORDS

water stress, *Cucurbita maxima* Duchesne, seed germination, seedling vigor, total phenols content, chlorophyll fluorescence, carotenoids

1 Introduction

Over the last decades, climate change substantially intensifies drought incidences as a result of warming and the increased evaporation. Drought is undoubtedly one of the most important environmental stressors that severely affects a wide range of major food crops, leading to considerable yield losses, especially in the arid and semi-arid regions. Considering these adverse effects of drought to both plant growth and productivity, breeding for drought tolerant varieties is generally considered as an effective and sustainable means of ensuring economic viability of crops under water deficit conditions. The mitigation of yield losses under drought conditions primarily relies on improving traits that enhance drought tolerance, such as water consumption and water use efficiency (WUE) (Hatfield and Dold, 2019). WUE refers to plant's ability to cope with moderate or severe soil water deficit and is considered as an important indicator of plant survival under limited water availability (Schulz et al., 2021). The response of plants to water deficit conditions includes a series of morphological, physiological, and biochemical modifications, including changes in photosynthesis, respiration, transpiration processes as well as the accumulation of reactive oxygen species (ROS) (Chevilly et al., 2021; Giordano et al., 2021; Nalina et al., 2021; Zhou et al., 2021). The molecular responses to drought include the activation of antioxidant enzymes and osmolyte accumulation which serve towards the stabilization of membranes, enzymes and proteins, as well as to the protection against oxidative damage through scavenging of ROS (Hasanuzzaman et al., 2020; Begum et al., 2022). Regarding the plant organs level, leaf morphology and functions may promote water use efficiency, thus contributing to mitigation of stress effects, while root characteristics such as length, weight, volume, and density, play a pivotal role in drought tolerance (Kapoor et al., 2020).

In the search to identify eligible strategies to improve drought tolerance of crops, several studies highlight the complexity of plant responses to drought, involving numerous factors such as the growth stage, the duration and severity of stress (Golldack et al., 2014), as well as the polygenic nature of quantitative tolerance traits (Mahmood et al., 2020). Considering that most plant species are drought-sensitive at all stages of their lifecycle, seed germination is considered as the most critical stage since water deficit impairs germination by limiting water imbibition and further reduces seedling vigor, via repressing root emergence and shoot elongation, thus preventing the establishment of a uniform stand (Queiroz et al., 2019; Rosero et al., 2020). As such, the most common plant response to reduced osmotic potential refers to delayed and reduced germination rates(Saberali and Shirmohamadi-Aliakbarkhani, 2020). Such adverse effects have been reported for a plethora of crop species, including maize (Queiroz et al., 2019), sorghum (Cokkizgin et al., 2019), faba bean (Li et al., 2018), cucumber(Mombeini et al., 2021), and melon (Saroj and Choudhary, 2020). Therefore, it is suggested that seed germination and seedling growth potential under stress conditions may enable the early selection of tolerant genotypes, thus enhancing the efficiency of selection procedures for suitable germplasm material to be cultivated or exploited in relative breeding programs (Tarchoun et al., 2022).

In the context of improving complex traits, such as drought tolerance, the research interest is progressively focused on the search of functional markers to be employed in breeding procedures aimed at early selecting for tolerant genotypes. Well known examples of stress indicators are certain amino acids of the group of quaternary amines with osmoprotective functions under water stress conditions, such as proline and glycine betaine(Bankaji et al., 2019). In the same line, plant protection against oxidative damage involves the induction of the activity of antioxidant enzymes(Gill and Tuteja, 2010). Carotenoids serve in multiple functions in plants, including photosynthesis and regulation of redox status(Gill and Tuteja, 2010), while phenolic compounds contribute to plant defense mechanisms against abiotic stress factors (Cheynier et al., 2013).

Given that photosynthesis is one of the most critical metabolic processes, its repression under drought stress adversely affects plant growth and development (Al Hassan et al., 2015). Upon severe drought stress, both in terms of duration and intensity, inhibition of photosynthesis is manifested by changes in chlorophyll content, mainly due to ROS-induced damage of chloroplasts, as well as damages in the whole photosynthetic apparatus (Porcar-Castell et al., 2014). Accordingly, ROS production is mainly driven by excess energy absorption in the photosynthetic apparatus, that might be reversed by degrading the absorbing pigments. Therefore, chlorophyll fluorescence is considered a fast, accurate and non-invasive tool to monitor photosystem II (PSII) (Baker, 2008), while photosynthetic activity can also be assessed since chlorophyll fluorescence can describe and investigate the photosynthetic light processes and quantum conversion by chlorophyll and accessory pigments of chlorophyll-protein complexes (Porcar-Castell et al., 2014).

Among the cultivated *Cucurbita* species, e.g. *C. argyrosperma,C. ficifolia, C. maxima*, *C. moschata* and *C. pepo*, the latter three are the most important in terms of worldwide production. In Tunisia, the squash (*C. maxima*Duch.) germplasm used for commercial cultivation is essentially derived from local landraces, produced by open pollination or farmer mass selection. Given the importance of local squash production, an increasing number of studies focuses on collection, *ex-situ* and *in-situ* conservation, characterization and maintenanceof local germplasm accessions (Hamdi et al., 2017; Hamdi et al., 2019; Tarchoun et al., 2022). Local landraces are often characterized by particularly broad leaves, thus leading to high evapotranspiration and high crop water requirements, since cultivation usually takes place during the warmer months of the year (Hamdi et al., 2017).

In this context, breeding efforts are focused on the selection of drought tolerant landraces so as to ensure economic viability of squash cropping in arid and semi-arid regions, such as Tunisia. Although several studies aimed at understanding the drought stress response in Cucurbita species, mainly *C. pepo* and *C. moschata*, so far (Biareh et al., 2022), there is a gap in relevant research for *C. maxima*. Therefore, this study aimed at (i) investigating the response of four squash local landraces of Tunisia to drought stress at germination stage, (ii) examining the possibilities of early selecting drought tolerant genotypes and (iii) determining the effects of drought stress at later plant growth stages. For this purpose, drought stress was imposed by different water potential (0, -0.24, -0.47 and -0.73 MPa). During germination, the evaluation of drought tolerance was based on traits related to germination and seedling growth (germination percentage, seedling vigor index, root and shoot length, root and shoot fresh weight, root volume and seed water absorbance). At later growth stages, the response of plants to stress was assessed using physiological (chlorophyll fluorescence, chlorophylls and carotenoid content, evapotranspiration, photosynthesis activity) and biochemical parameters (malondialdehyde (MDA), free proline, total phenolic compounds and total flavonoid content) and DPPH radical scavenging activity of roots and leaves.

2 Materials and methods

2.1 Plant material

Four landraces were selected for further evaluation of their response to D-mannitol induced drought stress, based on relevant assessment data related to the response of fifteen squash landraces to varying salt stress levels (Tarchoun et al., 2022). The genotypes under study consisted of four local landraces representing the main types of cultivated squash, namely Batati orange ("746"), Galaoui ("747"), Karkoubi Orange ("748") and Bejaoui Green ("751") (Table 1). Each landrace was assigned with passport data and an inventory number, according to the National Gene Bank of Tunisia, while full details are available at the Germplasm Resources Information Network - GRIN (http://www.tn-grin.nat.tn/gringlobal/search.%20aspx, accessed on 15 February 2022). The description of fruit morphology was performed based on the European Cooperative Program for Plant Genetic Resources (ECPGR) list of descriptors for *Cucurbita* spp. (ECPGR, 2008).

2.2 Drought stress treatments at germination stage

The present research was conducted at the Department of Horticulture, Vegetable Laboratory, High Agronomic Institute of Chott Mariam, Tunisia. Drought stress was induced by D-mannitol at different stress levels (0, -0.24, -0.47 and -0.73 MPa), while the response of the studied *C. maxima* genotypes was assessed based on traits related to germination and seedling growth (Tarchoun et al., 2022). The osmotic potential of the tested treatments was as follows: 0 MPa; - 0.24 MPa; - 0.47 MPa and - 0.73 MPa. One hundred and sixty (160) seeds per landrace were collected from mother plants protected from cross-pollination based on size homogeneity. Then, they were subsequently surface-sterilized, using 1% hypochlorite/H_2O solution under gentle agitation for 5 min, and washed 4 times with excess of sterile water. Sterilized seeds were initially primed in gibberellic acid solution (1.5 mM GA_3) for 24h, to stimulate germination, and then rinsed in sterile water. Ten seeds of each accession were placed in a Petri dish (90 mm) and lined with two layers of filter paper soaked with the appropriate D-mannitol concentration, while four Petri dishes per treatment were used. Seeds were allowed to germinate under controlled conditions (28 ± 2°C, 16 h light/8 h dark, 50 ± 5% relative humidity). Seeds were regularly monitored and 3 mL of the respective D-mannitol solution were added daily to ensure continuous germination.

2.3 Parameters for evaluation of drought tolerance at germination stage

Drought tolerance was evaluated on the basis of various parameters related to seed germination and seedling growth potential under the tested drought stress levels (Tarchoun et al.,

TABLE 1 Description of the Tunisian squash landraces employed in this study.

Landrace Inventory	Local Name	Origin	Short Description
NGBTUN746 ("746")	Batati Orange	Siliana (Sidi Hamada)	Globular fruit, orange skin, light orange flesh
NGBTUN747 ("747")	Galaoui	Ariana (KalaaAndalous)	Raised fruit with basal tip, green skin, green flesh
NGBTUN748 ("748")	Karkoubi Orange	Sousse (SidiBouali)	Flattened fruit, dark yellow skin, yellow flesh
NGBTUN751 ("751")	Bejaoui Green	Siliana (Sidi Hamada)	Flattened fruit, dark green skin, light green flesh

2022). Specifically, genotype evaluation for drought tolerance was performed on the basis of germination percentage (GP), seed water absorbance (WU), seedling vigor index (SVI), root and shoot length (RL, SL), root and shoot fresh weight (RFW, SFW) and ratio of shoot length to root length (SL/RL).

Germination percentage was scored daily for a period of 15 days, until no more germinated seeds were recorded. Seeds were considered as germinated when the radicle had emerged from the seed coat and had a length of at least 2 mm. Seed germination percentage was determined according to the formula:

$$Germination\quad percentage\quad (GP) = \frac{number\quad germinated\quad seeds}{number\quad of\quad total\quad seeds} \times 100$$

(Scott et al., 1984).

WU (%) and SVI (%) were estimated at the 15th day, according to the formula:

$$Water\quad seed\quad absorbance\quad (WU) = \frac{initial\quad seed\quad weight}{seed\quad weight\quad following\quad water\quad absorbance} \times 100$$

(Partheeban et al., 2017),

Seedling vigor index (SVI) = (Root length + Shoot length) × GP % (Ashraf et al., 2021).

SL (cm), RL (cm), SL/RL ratio, as well as RFW (g) and SFW (g) were estimated at the 15th day after treatment initiation in ten seedlings per treatment and accession (Tarchoun et al., 2022). The fresh weight (FW) of shoots and roots was recorded at harvest, and shoot and root dry weight (DW) was measured after oven drying (Memmert, Buchenbach, Germanny) at 70°C for 48 h. For the determination of the root volume (RV) of untreated and stressed plants, a beaker was filled with dH_2Oin a constant volume (V_1) and the final volume (V_2) was recorded after placing the root in the beaker. Root volume (RV) was determined using the formula:

$$RV\quad (cm^3) = V_2 - V_1$$

2.4 Drought stress treatments at the plant stage

Fifteen days after the start of the experiment, 15 selected seedlings for each landrace and stress level (60 seedlings per accession) were planted into 2 L pots containing peat and soil to ensure good drainage and prevent water logging. Planted seedlings were transferred to the greenhouse under controlled conditions and watered regularly at 2-day intervals for approximately 7 weeks, with solutions differing in D-mannitol concentration (0, -0.24, -0.47 and -0.73 MPa). Once a week, plants were also fertilized with nutrient solution (20-20-20; N-P-K; 2g/L/plant). After 45 days, the plant response to drought stress was assessed on the basis of physiological parameters, e.g. chlorophyll fluorescence, content of chlorophylls a, b and carotenoids, evapotranspiration (RET) and photosynthetic activity (PA), and biochemical parameters, e.g. malondialdhehyde (MDA), free proline, total phenols and total flavonoids content and DPPH radical scavenging activity, the latter determined in both roots and shoots.

2.4.1 Chlorophyll fluorescence, PhotosyntheticActivity and Real Evapotranspiration

Chlorophyll fluorescence was measured on fully expanded healthy leaves using a fluorometer (Plant Stress Kit, Opti-Sciences model, NH, USA). The dark adaptation period and the level of saturating light were determined before measurements. After dark adaptation of leaves for 15 min, the maximum fluorescence yield (F_m), evaluated at 6000 µmol saturation pulse, and minimum leaf fluorescence yield (F_0) of the leaves were first determined. The values of minimum (F_0) and maximum (F_m) fluorescence,as well as those for the F_v/F_m quantum ratio were also determined using Fluorometer (Plant kit stress, Opti-Sciences model, NH, USA).Photosynthetic activity (PA) (µmol photons m^{-2} s^{-1}) and real evapotranspiration (RET) (mm H2O/day)were measured on the fourth fully formed leaf from the apex in a clear day using Fluorometer (Plant kit stress, Opti-Sciences model, NH, USA).

2.4.2 Chlorophyll and carotenoids content

Chlorophyll content was determined according to the method of (Porcar-Castell et al., 2014). Briefly, 0.1 g of leaf tissue were ground with 10 mL of 80% acetone. Following filtration, the solutions were incubated in the dark to avoid photo-oxidation. The chlorophyll content (Chlorophyll a (Chla) and Chlorophyll b (Chlb)) of the filtered solution was measured using a UV-visible spectrophotometer (Evolution 210, Thermo Scientific, Abingdon, UK) at 645 and 663 nm, respectively, whereas carotenoids content was determined at 470 nm (Curtis and Shetty, 1996). Calibration of the apparatus was performed using 80% acetone. The relationship between concentration (mg/g fresh matter) and optical density was determined according to the following formulas (Arnon, 1949):

$$Chla\quad (mg/g) = 12.7 \times D_{663} - 2.59 \times D_{645}$$

$$Chlb\quad (mg/g) = 22.9 \times D_{645} - 4.68 \times D_{663}$$

$$Ccar\quad (mg/g) = [(5 \times D_{470}) \times (3.19 \times Chla)) + (130.3 \times Chlb)]/200,$$

where D refers to absorbance at the corresponding nanometers.

2.4.3 Free proline content

Free proline content was determined using the method of (Monneveaux and Nemmar, 1986). Briefly, 100 mg of fresh leaf tissue were placed in 5 mL of 40% methanol and the mixture was heated for 1 h in a water bath at 85°C. After cooling, 1 mL of the extraction solution was added to 1 mL of acetic acid, 25 mg of ninhydrin and 1 mL of the mixture [dH_2O+ acetic acid + orthophosphoric acid of density 1.7 (120:300:80, v/v/v)]. Following incubation for 30 min in a water bath (100°C) and subsequent cooling, 5 mL of toluene were added to the samples. The upper phase containing proline was recovered and its optical density was determined at 528 nm using a UV-Vis spectrophotometer (Evolution 210, Thermo Scientific, Abingdon,

UK). Proline concentration was calculated using a standard curve created using stock solutions ranging from 0 to 2.5 mg/mL of L-proline. Proline contents were expressed as µg/mg FW.

2.4.4 Malondialdehyde

Two hundred mg of fresh leaves were ground and homogenized in 1 mL of 0.1% trichloroacetic acid (TCA) (Hnilickova et al., 2021). Following centrifugation at 15000 rpm for 10 min at 4°C, 0.5 mL of the supernatant was mixed with 1 mL of 0.5% thiobarbituric acid (TBA). The solution was heated to 95°C for 30 min, cooled and centrifuged at 500 rpm for 30 min and the absorbance was measured at 532 nm using a UV-Vis spectrophotometer (Evolution 210, Thermo Scientific, Abingdon, UK). The calibration curve was prepared using TBA (0.5%) and TCA (20%). MDA content was expressed as µmol g^{-1} FW.

2.4.5 Total phenolic compounds content

Total phenolic content was assayed using Folin-Ciocalteu colorimretic method according to (Blainsk et al., 2013). Briefly, 0.125 ml of methanolic extract solution was mixed with 0.5 ml of dH_2O and 0.125 ml of the Folin-Ciocalteu reagent. Following incubation for 1 min, 1.25 mL of7% sodium carbonate (Na_2CO_3) solution were added. After incubation for 120 min in the dark, the absorbance was measured at 760 nm using a UV-Vis spectrophotometer (Evolution 210, Thermo Scientific, Abingdon, UK). The TPC was expressed as mg gallic acid equivalent (GA) per 100 mg dry weight (DW).

2.4.6 Total flavonoid content

Total flavonoids content of the methanolic extract solution was determined using the aluminum chloride assay (Zhishen et al., 1999). A total of 0.5 mL methanolic extract was mixed with 2.5 mL of dH_2O and 0.15 mL of 5% sodium nitrite ($NaNO_2$) solution. The mixture was incubated for 5 min before adding 0.3 ml of 10% aluminum chloride ($AlCl_3$) and 1 mL of 1 M sodium hydroxide (NaOH) solution. Following incubation for 15 min, the absorbance was measured at 510 nm using a UV-Vis spectrophotometer (Evolution 210, Thermo Scientific, Abingdon, UK). The TFC was expressed as mg quercetin equivalent (QE) per 100 mg dry weight (DW).

2.4.7 DPPH radical scavenging activity

Antioxidant activity was evaluated as the scavenging activity of 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical (Ferreira et al., 2007). A total of 0.5 mL of 0.2 mM DPPH methanolic solution was added to shoot and root extracts of squash landraces. Briefly 0.25 mM solution of DPPH radical (0.5 mL) was added to the sample solution in ethanol (1 mL) at a concentration (300 µg/ml). The reaction mixture was incubated in dark for 30 min and absorbance was measured at 517 nm using a UV-Vis spectrophotometer (Evolution 210, Thermo Scientific, Abingdon, UK). The antiradical activity was expressed as (%), which represent the concentration of the extract (mg/mL) required to inhibit 50% of the radicals.

2.5 Statistical analysis

Data were analyzed by a two-ANOVA according to the experimental design. The experimental design was *Completely Randomized Design* (CRD) with three replicates (Petri dishes). The effect of stress level was assessed across genotypes; the genotype performance was comparatively assessed across stress levels, while the landrace x stress level interaction effects were also determined. The significance of differences between pairs of means was assessed by the Duncan's Multiple Range test ($p< 0.05$). Statistical analyses were performed using the Statistical Analysis System Software (v.9.0) (SAS Institute S.A., Cary, NC, USA).

3 Results

3.1 Response of squash landraces to drought stress at germination stage

Overall findings indicate that drought stress affected all traits related to germination and seedling growth, as evidenced by the mean values of GP, SVI, RL, RFW, SL, SFW, SL/RL and WU in controls and stressed plants. Further, the analysis of variance pointed as the most variable traits those directly linked to germination (WU, GP, SVI) (Table 2).

3.1.1 Effect of the drought stress level across landraces

Seed germination varied significantly between the tested stress levels, while a decreasing trend was observed in GP. It is worth noting that at - 0.24 MPa and - 0.47 MPa squash landraces reached GP values >70%, indicating their high germination potential under drought stress conditions (Supplementary Table S1).

A similar trend was also recorded in seedling growth traits, with significantly decreased values at the highest level induced by D-mannitol (-0.73 MPa) (Supplementary Table S1). In particular, SL and RL were considerably affected by the stress level, showing values that ranged from 9.87 cm to 2.62 cm and from 6.82 cm to 3.04 cm in the control and at - 0.73 MPa, respectively. Accordingly, the shoot/root ratio (SL/RL) ranged from 1.53 to 0.91, indicating that shoot length was more profoundly affected than root length under drought stress conditions. Furthermore, at -0.47 MPa both SL and SFW were more pronouncedly affected than RL and RFW (decreased by 48.5% and 21.9%, compared to 37.9% and 16.2%, respectively), whereas at - 0.73 MPa only SL showed a higher decrease than RL (73.4% compared to 55.0%). Such values were also reflected in SVI, indicating that high drought stress significantly affects the overall growth of seedlings. Finally, RV was significantly decreased in stressed plants regardless of the stress level, as compared to the control treatment (11.40 cm^3), although this decrease was proportional to the stress level applied (5.47 cm^3 at - 0.73 MPa).

TABLE 2 Analysis of variance (mean squares) for traits related to seed germination and seedling growth of the studied squash landraces (accessions) under the different drought stress levels (0, -0.24, -.0.47 and - 0.73 MPa).

S.O.V.	DF	GP	SVI	RL	RFW	SL	SFW	SL/RL	WU
Accession	3	3269.87	59065.66	11.84	3.17	13.60	0.85	1.64	3919.46
Treatment	3	10103.12	10331613.85	89.52	0.39	363.27	1.24	3.18	5476.76
Accession x Treatment	9	561.09	211024.01	16.13	0.16	2.70	0.03	0.80	376.076
CV (%)	–	11.74	10.88	4.63	5.47	2.01	1.94	6.56	14.086

S.O.V., source of variance; CV, coefficient of variance; DF, degree of freedom; GP, germination percentage; SVI, seedling vigor index; RL, root length; RFW, root fresh weight; SL, shoot length; SFW, shoot fresh weight; SL/RL, ratio shoot length to root length; WU, seed water absorbance.
–, not applicable.

3.1.2 Performance of landraces across stress levels

In terms of GP, landraces showed values ranging from 63.75% to 84.97%, indicating a variable response to drought stress (Supplementary Table S2). Landrace "751" showed the highest GP (84.97), thus providing evidence for its better adaptation to drought stress, whereas landrace "747" showed the lowest GP values (63.75%). Landraces "746" and "748" showed a mean GP of 79.41% and 69.69% respectively, suggesting a moderate drought tolerance. The superiority of landrace "751" was further depicted in SVI values, reflecting the rapidity of germination compared to other landraces. In contrast, the same landrace ("751") presented the lowest values for SL (4.71 cm) and relatively low also for RL (4.61 cm), whereas the highest values for these parameters were recorded in landrace "747" (5.52 and 5.98 cm for RL and SL, respectively). In addition, drought stress had a more severe impact on root length than shoot length in the case of "746" and "747", whereas landraces "748" and "751" showed the opposite trend. Significant differences were also observed for RFW and SFW, with "746" showing the highest value for both traits (Supplementary Table S2). Furthermore, landraces differed significantly in relation to WU, with "751" and "748" showing the highest and lowest values, respectively. Finally, the highest value of RV was noted in "746" landrace (11.54 cm^3), whereas the lowest values were recorded in "748" (5.20 cm^3) landrace.

3.1.3 Landrace × drought stress levels interaction effects

The two-way ANOVA revealed that landraces differentially responded to varying stress levels, as evidenced by the respective interaction effects (landrace × drought stress levels) for all traits related to germination and seedling growth potential (Table 3). In untreated seeds, germination was considerably affected by the landrace, thus indicating their different innate germination potential, which is probably attributed to differences in the median longevity of seeds. Among landraces, "751" and "748" presented the highest and lowest GP under control conditions (97.77% and 85.77%, respectively), thus justifying that seed longevity is a genotype-dependent variable. Under stress conditions, the landraces differed significantly in relation to their germination potential at the different stress levels. As such, "751" ranked as the most tolerant at - 0.24 MPa and - 0.47 MPa (96.55% and 83.33% GP, respectively), whereas"747" was consistently the most sensitive genotype to all stress levels (68.33% and 33.33% GP). Interestingly, at - 0.73 MPa, "746" showed the highest GP (66.77%), followed by "751" (63.33%).

The drastic effect of drought stress was further evidenced in both SVI and WU parameters, with landraces presenting varying responses to the different stress levels applied (Table 3). At both osmotic potentials of 0 MPa and - 0.24 MPa, "748"presented the highest values for SVI (1776.88% and 856.27%, respectively), whereas "751" showed the lowest values (1287.58% and 755.28% for 0 MPa and - 0.24 MPa, respectively). However, at higher stress levels, "751" was characterized by the highest SVI, while "746" and "748" were the most sensitive at - 0.47 MPa and - 0.73 MPa respectively. Similarly, "751" showed the highest WU values at -0.24 and - 0.47 MPa (96.55% and 83.33%, respectively), while at - 0.73 MPa the highest value was recorded in "746" (66.77%).

As far as traits related to seedling growth are concerned, the analysis also revealed significant interactions ($p<0.001$) among landraces and stress levels applied (Table 3). As expected, the response of all landraces to D-mannitol levels led a substantial reduction in both SL an RL. Although at lower stress levels "747"proved as most capable of retaining its growth ability, at -0.73 MPa "746"presented the highest values for both RL and SL, thus indicating its ability to withstand severe drought stress. In agreement with these data, the RFW and SFW were also drastically affected as D-mannitol concentration increased. The highest values for RFW were recorded in "746" both in controls and stressed plants, while for SFW the highest values were recorded in "748" and "746" in controls and stressed plants, respectively. In contrast, the lowest values were noted in "747" and "751" for both RFW and SFW. The SL/RL ratio was also profoundly affected by drought stress, especially at - 0.73 MPa, where landraces showed varying values ranging from 0.71 to 1.40. At this stress level, the highest SL/RL ratio was noted in "747", reflecting the more drastic effect of water stress on roots than shoots (Supplementary Table S1). Evaluating the root volume (RV) at different D-mannitol levels, all landraces showed a decreasing trend, analogous to the stress level applied, thus proving the severe effect of drought stress on roots. In relation to RV, "746" and "748"presented the highest and lowest values at all stress levels applied, with the former being capable of retaining its root elongation ability even at - 0.73 MPa (9.66 cm^3).

3.2 Physiological and biochemical response of squash landraces under drought stress at the plant stage

Our findings indicate that the response of the studied squash landraces to drought stress involved substantial changes occurring both at the physiological and biochemical level. As such, all traits related to

10.3389/fpls.2023.1215394

TABLE 3 Response of the studied squash landraces to different drought stress levels (0, -0.24, -.0.47 and - 0.73 MPa) in relation to germination and seedling growth traits (means ± SD).

Water potential (MPa)	Landrace	GP (%)	SVI (%)	RL (cm)	RFW (g)	SL (cm)	SFW (g)	SL/RL	WU	RV (cm^3)
Control	"748"	85.77 ± 2.64^{d*}	1776.88 ± 38.53^{a}	8.55 ± 0.20^{a}	0.67 ± 0.01^{b}	9.77 ± 0.08^{b}	0.84 ± 0.006^{a}	1.14 ± 0.02^{b}	88.77 ± 2.64^{d}	9.35 ± 0.25^{c}
	"751"	97.77 ± 1.44^{a}	1287.58 ± 40.90^{c}	6.66 ± 0.18^{c}	0.03 ± 0.001^{c}	9.68 ± 0.12^{c}	0.63 ± 0.003^{c}	1.45 ± 0.04^{b}	96.66 ± 1.44^{b}	12.38 ± 0.24^{a}
	"747"	96.66 ± 2.22^{b}	1581.41 ± 28.90^{b}	7.64 ± 0.14^{b}	0.01 ± 0.0004^{d}	10.53 ± 0.06^{a}	0.63 ± 0.003^{c}	1.37 ± 0.02^{b}	97.77 ± 2.22^{a}	11.51 ± 0.34^{b}
	"746"	92.22 ± 2.64^{c}	1572.39 ± 50.82^{b}	4.43 ± 0.22^{d}	0.75 ± 0.02^{a}	9.51 ± 0.03^{d}	0.81 ± 0.005^{b}	2.15 ± 0.10^{a}	92.22 ± 2.64^{c}	12.37 ± 0.37^{a}
-0.24	"748"	74.11 ± 3.86^{c}	856.27 ± 49.23^{a}	5.35 ± 0.22^{b}	0.56 ± 0.03^{b}	5.29 ± 0.02^{b}	0.62 ± 0.005^{b}	0.98 ± 0.04^{b}	74.11 ± 3.86^{c}	7.44± 0.28^{d}
	"751"	96.55 ± 1.05^{a}	755.28 ± 20.76^{d}	4.47 ± 0.21^{c}	0.018 ± 0.0004^{c}	3.59 ± 0.27^{c}	0.38 ± 0.003^{d}	0.80 ± 0.06^{d}	96.55 ± 1.05^{a}	9.33± 0.13^{c}
	"747"	68.33 ± 3.90^{d}	778.76 ± 9.76^{c}	6.69 ± 0.17^{a}	0.01 ± 0.0005^{c}	5.83 ± 0.05^{a}	0.40 ± 0.004^{c}	0.87 ± 0.02^{c}	68.33 ± 3.90^{d}	9.56± 0.10^{b}
	"746"	82.88 ± 1.73^{b}	789.74 ± 43.48^{b}	3.50 ± 0.23^{d}	0.66 ± 0.01^{a}	5.60 ± 0.05^{a}	0.78 ± 0.01^{a}	1.60 ± 0.10^{a}	82.88 ± 1.73^{b}	12.17 ± 0.33^{a}
-0.47	"748"	68.88 ± 2.68^{c}	548.13 ± 13.52^{c}	3.30 ± 0.19^{c}	0.15 ± 0.02^{b}	2.90 ± 0.09^{d}	0.41 ± 0.008^{b}	0.88 ± 0.04^{b}	68.88 ± 2.68^{c}	2.53± 0.13^{d}
	"751"	83.33 ± 4.63^{a}	676.69 ± 13.85^{a}	4.27 ± 0.21^{b}	0.008 ± 0.0007^{c}	3.39 ± 0.24^{c}	0.22 ± 0.002^{d}	0.79 ± 0.05^{c}	83.33 ± 4.65^{a}	7.46± 0.33^{b}
	"747"	55.55 ± 1.30^{d}	636.05 ± 29.70^{b}	5.46 ± 0.25^{a}	0.01 ± 0.0005^{c}	4.40 ± 0.04^{b}	0.35 ± 0.03^{c}	0.80 ± 0.04^{c}	55.55 ± 1.30^{d}	6.43± 0.23^{c}
	"746"	75.77 ± 1.35^{b}	426.03 ± 12.77^{d}	4.27 ± 0.16^{b}	0.54 ± 0.006^{a}	4.65 ± 0.08^{a}	0.61 ± 0.003^{a}	1.08 ± 0.03^{a}	75.77 ± 1.35^{b}	11.60 ± 0.19^{a}
-0.73	"748"	50.00 ± 4.08^{c}	183.54 ± 29.90^{d}	2.43 ± 0.26^{c}	0.07 ± 0.006^{b}	1.87 ± 0.04^{c}	0.31 ± 0.004^{b}	0.77 ± 0.08^{b}	50.00 ± 4.08^{c}	1.46± 0.13^{d}
	"751"	63.33 ± 1.44^{b}	512.61 ± 13.53^{a}	3.03 ± 0.15^{b}	0.008 ± 0.0004^{c}	2.17 ± 0.06^{b}	0.18 ± 0.004^{c}	0.71 ± 0.03^{d}	63.33 ± 1.44^{b}	6.47± 0.34^{b}
	"747"	33.33 ± 5.27^{d}	330.08 ± 9.11^{b}	2.28 ± 0.27^{c}	0.005 ± 0.0004^{c}	3.18 ± 0.03^{a}	0.17 ± 0.003^{d}	1.40 ± 0.16^{a}	33.33 ± 5.27^{d}	4.28± 0.17^{c}
	"746"	66.77 ± 1.47^{a}	215.34 ± 18.59^{c}	4.40 ± 0.37^{a}	0.52 ± 0.006^{a}	3.27 ± 0.05^{a}	0.58 ± 0.006^{a}	0.74 ± 0.05^{c}	66.77 ± 1.47^{a}	9.66± 0.21^{a}

* Means in the same column and for the same D-mannitol concentration followed by the same letter are not significantly different at $p< 0.05$, according to Duncan's Multiple Range test; GP, germination potential; SVI, seedling vigor index; RL, root length; RFW, root fresh weight; SL, shoot length; SFW, shoot fresh weight; SL/RL, ratio shoot length to root length; WU, seed water absorbance; RV, root volume.

chlorophyll fluorescence and contents of chlorophylls a and b and carotenoids, as well as real evapotranspiration and photosynthetic activity were significantly affected. The analysis further revealed significant changes in the accumulation of osmoprotectant compounds, such as MDA, free proline, phenolic compounds and flavonoids content and antioxidant activity assayed with DPPH method. It is worth noting, that among traits employed for evaluation of drought tolerance, chlorophyll b, F_m, PA and the free proline content were the ones with the highest variability (Table 4).

3.2.1 Effect of drought stress on chlorophyll fluorescence parameters

Our findings indicate that the content of chlorophyll fluorescence parameters showed a decreasing trend at all stress levels, thus proving the adverse effects of drought stress (Table 3)

3.2.1.1 Effect of drought stress across genotypes

D-mannitol concentrations induced changes on chlorophyll fluorescence, with the respective values differing significantly among stress levels (Table 3). At - 0.73 MPa, the minimum fluorescence (F_0) and the variable fluorescence (F_v) parameters showed the highest values (549.63 and 4.89, respectively) as compared to the controls (373.09 and 3.97, respectively). In contrast, the values of maximum fluorescence (F_m) decreased as D-mannitol concentration increased (Table 3), leading to the highest decline at - 0.73 MPa (1722.97), whereas no significant differences were noted at -0.47 and - 0.24 MPa (1959.53 and

10.3389/fpls.2023.1215394

TABLE 4 Analysis of variance (mean squares) for traits related to the physiological and biochemical response of squash landraces (accessions) to different drought stress levels ((0, -0.24, -.0.47 and - 0.73 MPa).

S.O.V.	DF	Chla	Chlb	Car	F_0	F_m	F_v/F_m	F_v	PA	RET	MDA	Pro.	TPC.	TFC	DPPH
Accessions	5	115.09	34.75	0.16	44638.72	627992.87	0.03	16.26	4187363.34	43148.17	74.86	4.22	648.98	1017.37	281.53
Treatment	3	309.53	68.95	0.83	195511.73	2761160.96	0.008	5.54	1950444.81	27821.72	264.29	16.54	1315.92	1812.89	995.07
Accession × Treatment	9	5.26**	10.92	0.01**	23261.41**	466898.21**	0.01**	3.29**	1773157.56**	2870.03**	50.01**	2.41**	77.87**	127.025**	47.97**
CV (%)	–	12.84	19.33	16.54	17.28	19.74	12.29	15.89	19.62	18.13	10.77	21.53	15.92	13.70	13.81

S.O.V., source of variance; DF, degree of freedom; CV, coefficient of variance; Chl a, chlorophyll a; Chl b, chlorophyll b; Car, carotenoids; F_0, Minimal chlorophyll fluorescence intensity; F_m, Maximum chlorophyll fluorescence intensity; F_v/F_m, Maximum quantum efficiency of PSII photosystem; F_v, Variable chlorophyll fluorescence; PA, photosynthetic activity; RET, real evapotranspiration; MDA, Malondialdehyde; Pro, proline; TPC, Total phenolic compounds content; TFC, Total flavonoids content; DPPH, 1,1-diphenyl-2-picrylhydrazyl. –, Not applicable; **, significantly different at p<0.01.

1948.96, respectively). Interestingly, the maximum efficiency of PSII, expressed by the ratio of F_v/F_m, showed a significant reduction at - 0.47 MPa, as compared to the control (0.780 and 0.800, respectively), while the respective values significantly increased at - 0.73 MPa (0.830) (Table 3).

3.2.1.2 Performance of landraces across stress levels

Accordingly, the data underline the significance of landrace effect ($p< 0.001$) on the chlorophyll fluorescence parameters under drought stress conditions (Table 4). The variable response of the landraces to drought stress was evidenced for all fluorescence parameters, while the F_m was the most varied trait (CV > 19%) (Table 4).

Chlorophyll fluorescence was considerably affected by the landrace, as evidenced by the mean F_v/F_m values (Supplementary Table S4), ranging from 0.76 to 0.82, with landraces "748",and "747" showing the lowest and highest values, respectively, which are indicative of drought tolerance and drought susceptibility. Furthermore, all the landraces except for "748", they could be characterized as drought tolerant since they showed mean F_v/F_m values within the range of 0.79 - 0.82.

3.2.1.3 Landrace x drought stress levels interaction effects

As depicted in Figure 1, all the parameters related to chlorophyll fluorescence under drought stress conditions were variably affected by the landrace and the studied stress levels. In the absence of stress, F_0, F_m, F_v and F_v/F_m were considerably affected by the landrace. Among landraces, "748" and "746" showed the lowest and the highest F_0 values under control conditions (357.72 and 651.55, respectively; Figure 1A), while at the same time presenting the highest and the lowest F_m under the same conditions (2520.87 and 2089.11, respectively; Figure 1B). As expected, drought stress severely affected all chlorophyll fluorescence parameters for all landraces, with the severity of effects being increased with increasing stress intensity. At - 0.24 MPa, "746", "747"and "751", landraces were the most affected, representing a quantum ratio (F_v/F_m) of 0.85. Given that such values are outside the range of 0.79 - 0.82, their performance is classified as inferior in relation to stress tolerance. On the other hand, at -0.47 and - 0.73 MPa all the landraces presented a quantum ratio (F_v/F_m) outside of the mentioned range (Figure 1C) which indicates a significant effect of drought on the studied landraces. Furthermore, the response of most landraces to drought stress involved a drastic reduction in F_m. In agreement with these findings, the F_v followed an increasing trend as D-mannitol concentration increased in landrace "751", while the highest values for F_v were recorded in "746" both in control and - 0.24 MPa. As expected, the F_v was more profoundly affected at - 0.73 MPa with the landraces showing varying values ranging from 3.48 to 5.42 (Figure 1D).

3.2.2 Effect of drought stress on the content of chlorophyll a, chlorophyll b and carotenoids

Similar to the effects of drought stress on chlorophyll fluorescence parameters, the content of pigments such as chlorophyll a and b and carotenoids, was drastically affected (Supplementary Figure S1).

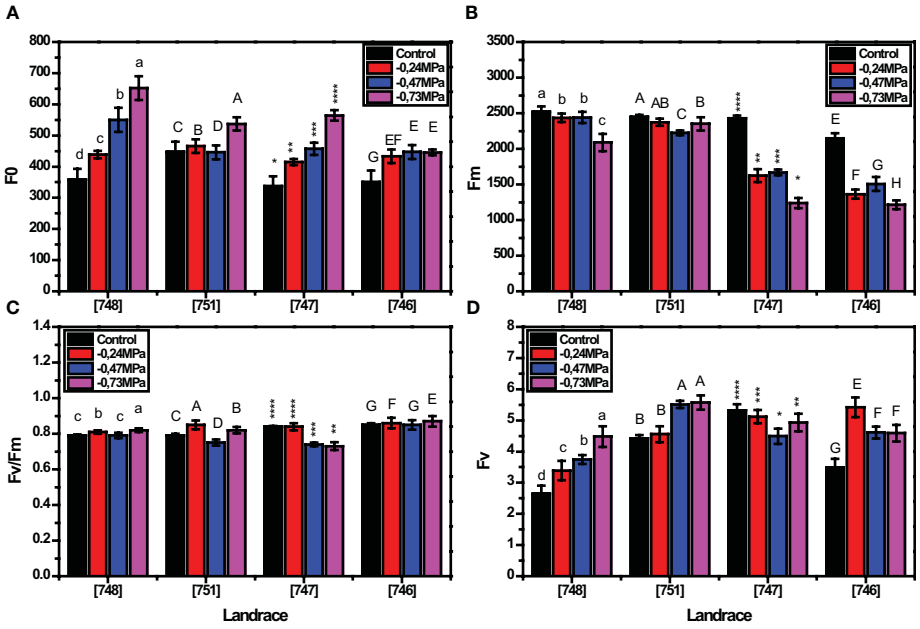

FIGURE 1
Response of squash landraces to different drought stress levels ((0, -0.24, -.0.47 and - 0.73 MPa) in relation to chlorophyll fluorescence parametersF_0 **(A)**, F_m **(B)**, F_v/F_m **(C)** and F_v **(D)**, 45 days after transplantation. Different letters above vertical bars of the same landrace indicate significant differences between the tested drought stress levels according to Duncan's Multiple Range test at $p< 0.05$. Significance was indicated by small letter (a, b, c, d) for [748], by big letter (A, B, C, D) for[748], by stars for [747], and by big letters (E, F, G, H) for [746] landrace. The different number of asterisks (e.g., *, **, ***, ****) above the bars of genotype 747 indicate significant difference between the means of this genotype for each of the tested parameters.

3.2.2.1 Effect of drought stress across genotypes

Chlorophyll a and carotenoids content decreased significantly as D-mannitol concentration increased. In particular, chlorophyll a values decreased from 13 mg g^{-1} FW in the control treatment to 6.41 mg g^{-1} FW in plants treated with - 0.73 MPa, while the respective decrease in carotenoids content was from 0.8 mg g^{-1} FWto 0.46 mg g^{-1} FW for the control treatment and the potential of - 0.73 MPa, respectively (Supplementary Figure S1). On the other hand, chlorophyll b presented a different trend where its content decreased only at high drought intensity (- 0.47 and 0.73 MPa) compared to the control treatment and the potential of - 0.24 MPa.

3.2.2.2 Performance of landraces across drought stress levels

Our results revealed a significant effect of the landrace on the content of chlorophyll a, chlorophyll b and carotenoids under drought stress conditions (Supplementary Table S5). In relation to chlorophyll a and clorophyll b, the highest overall content was recorded in landrace "746" (11.24 mg g^{-1} FW and 4.94 mg g^{-1} FW, respectively), whereas the lowest value was recorded in "748"(7.48 mg g^{-1} FW and 2.67 mg g^{-1} FW). Regarding carotenoids, the highest content was recorded in landrace "751" (0.69 mg g^{-1} FW), while the lowest value was observed in landrace "748" (0.53 mg g^{-1} FW).

3.2.2.3 Landrace × drought stress interaction effects

Our findings indicate that the contents of chlorophyll a, chlorophyll b and carotenoids were variably affected depending on the landrace and the stress level (Figure 2). In relation to chlorophyll a, all landraces showed a decreasing trend as D-mannitol increased (Figure 2A). A similar trend was recorded in the case of chlorophyll b, although "747" landrace seemed to be less affected with increasing severity of drought stress (Figure 2B). Furthermore, the content of carotenoids was differentially affected both by the landrace and the stress level, showing a general reduction in highly stressed plants (- 0.47 and 0.73 MPa) (Figure 2C).

3.2.3 Effect of drought stress on real evapotranspiration and photosynthetic activity

3.2.3.1 Effect of drought stress across genotypes

The real evapotranspiration (RET) and photosynthetic activity (PA) were evaluated on the 4th fully expanded leaf, 45 days after transplantation. The analysis revealed the significant effects ($p<$ 0.001, Table 4) of the studied drought stress levels on these variables (Supplementary Figure S2). Both RET and PAparameters recorded a significant reduction as D-mannitol concentrations increased. For RET, the stress effect was manifested through a decrease by 9.5%, 18% and 40% in plants subjected to -0.24, - 0.47 and - 0.73 MPa respectively, as compared to the control treatment (Supplementary Figure S2A). On the other hand, PA was less affected by drought stress, showing a 29% reduction at - 0.73 MPa, as compared to the controls, while no significant difference was noted between - 0.47 and - 0.73 MPa (Supplementary Figure S2B).

3.2.3.2 Performance of landraces across drought stress levels

A significant effect of the landrace on both RET and PA parameters was also recorded (Supplementary Table S6). For

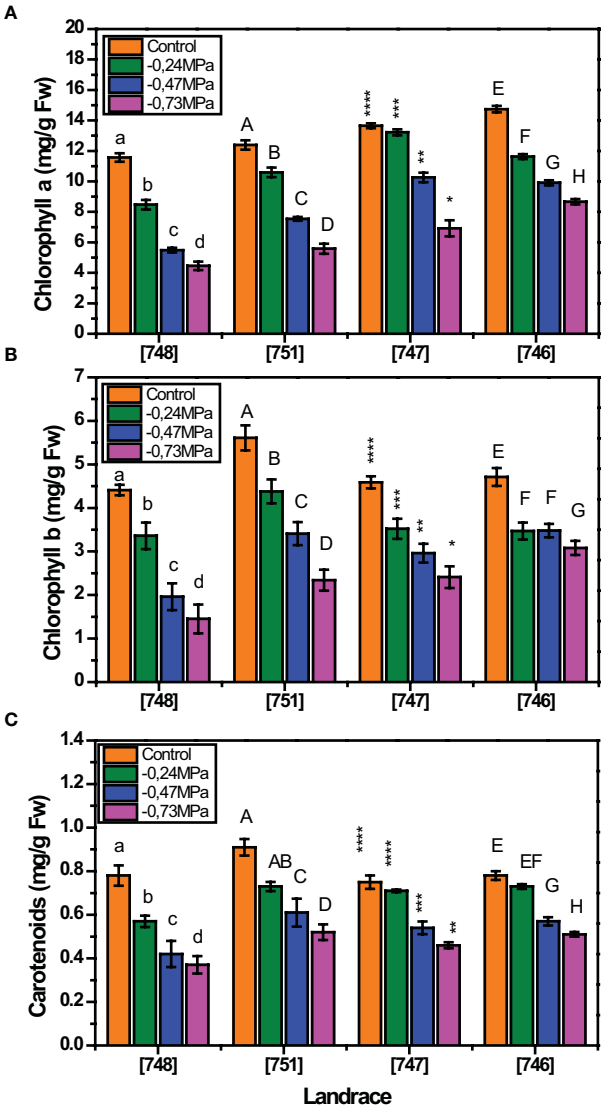

FIGURE 2
Response of squash landraces to different drought stress levels (0, -0.24, -0.47 and - 0.73 MPa) in relation to chlorophyll a **(A)**, chlorophyll b **(B)** and carotenoids **(C)**, measured at 45 days after transplantation. Different letters above vertical bars of the same landrace indicate significant differences between the tested drought stress levels according to Duncan's Multiple Range test at $p< 0.05$. For [747] landrace, significance is indicated by the asterisk (*) symbol.

RET, the highest value was noted in "746" landrace (179.44 mm/day), followed by "747" (137.99 mm/day), whereas "751" and"748"landraces showed lower values (104.08 and 108.94 mm/day, respectively) with no significant differences between them. Such findings are indicative of the differential response of landraces to drought stress, which is probably regulated through adjustments in stomatal closure. It is worth noting that the response of landraces to drought stress in relation to transpiration is similar to their photosynthetic response. Accordingly, the highest and lowest PA values were noted in landraces "746" and "751" respectively (1790.80 and 957.83 µmol photons m^{-2} s^{-1}).

3.2.3.3 Landrace × drought stress interaction effects

Our results further revealed significant interaction effects between landraces and drought stress levels, thus indicating the variability of the landraces under study to withstand varying drought stress levels (Table 5). All the tested landraces showed a general decreasing trend for both RET and PA parameters as D-mannitol concentration increased, indicating that stomatal closure which regulates photosynthesis by restring gas exchange between the atmosphere and the leaves is the first response when plants are subjected to drought stress. In relation to RET, all landraces were significantly affected by drought stress especially at high stress levels, yet their response varied considerably. As such, landraces "748" and "747"suffered the highest reduction, even at mild stress potential (e.g., - 0.24 MPa), whereas "751" and "746" were least affected since the reduction was more profound at potential higher than -0.47 MPa. For PA, a more pronounced decrease was noted at -0.47 and - 0.73 MPa for all the tested landraces. In particular, landraces "748" showed the better adaptability to high drought

TABLE 5 Response of squash landraces to different drought stress levels ((0, -0.24, -.0.47 and - 0.73 MPa) in relation to the real evapotranspiration and photosynthetic activity, measured at 45th day after plantation.

Landrace	Water potential (MPa)	RET (mm H_2O/day)	PA (µmol photons m^{-2} s^{-2})
"748"	Control	135.67 ± 3.00[a]*	1551.05 ± 22.86[a]
	-0.24	104.60 ± 2.37[b]	1328.61 ± 19.08[b]
	-0.47	101.55 ± 0.97[c]	1269.66 ± 26.67[c]
	-0.73	93.94 ± 2.85[d]	1160.16 ± 30.77[d]
"751"	Control	120.17 ± 40.16[b]	1471.88 ± 30.60[a]
	-0.24	125.00 ± 3.15[a]	1328.51 ± 19.08[b]
	-0.47	115.23 ± 2.98[c]	954.33 ± 27.01[c]
	-0.73	55.92 ± 2.95[d]	358.11 ± 25.80[d]
"747"	Control	189.99 ± 8.69[a]	1935.49 ± 34.63[a]
	-0.24	155.00 ± 3.09[b]	1838.33 ± 29.74[b]
	-0.47	131.42 ± 3.85[c]	1256.72 ± 25.27[c]
	-0.73	75.52 ± 2.82[d]	934.66 ± 25.20[d]
"746"	Control	194.52 ± 6.51[a]	1846.16 ± 30.49[a]
	-0.24	192.55 ± 3.05[b]	1659.49 ± 26.84[b]
	-0.47	176.61 ± 3.24[c]	1160.16 ± 30.77[c]
	-0.73	154.06 ± 2.39[d]	1080.17 ± 24.94[d]

* Means in the same column and for the same D-mannitol concentration followed by the same letter are not significantly different at $p< 0.05$, according to Duncan's Multiple Range test; RET, real evapotranspiration; PA, photosynthetic activity.

stress since RET values were reduced by 18.2% and 25.2% at - 0.47 and - 0.73 MPa, respectively. On the other hand, the rest of the landraces showed a similar response at - 0.47 MPa with reduction rates between 35.0% and 37.2%, while "751" landrace was the most severely affected at - 0.73 MPa showing a reduction of 75.7%.

3.2.4 Effect of drought stress on MDA, free proline, total phenols, total flavonoids and DDDH scavenging activity

The content of osmoprotective compounds, such as MDA, free proline, total phenols, total flavonoids and DPPH, was significantly affected by D-mannitol-induced drought stress (Supplementary Table S7).

3.2.4.1 Effect of drought stress across genotypes

At - 0.24 MPa, squash landraces presented a considerable increase in the content of MDA, free proline, total phenols, total flavonoids and DPPH, ranging to 23.27%, 63.33%, 16.13%, 16.24% and 19.69%, as compared to the control treatment, respectively (Supplementary Table S7). As expected, a further increase in concentration of D-mannitol at - 0.47 and - 0.73 MPa induced the accumulation of osmoprotectants, thus leading to significantly increased values for all the tested parameters, except for the case of MDA where no significant increase was observed at potential higher than -0.47 MPa. In particular, at - 0.47 MPa, the increase in the content of MDA, free proline, total phenols, total flavonoids and DPPH ranged to 39.77%, 86.66%, 25.60%, 26.02% and 28.54%, as compared to the control treatment, whereas at - 0.73 MPa the respective contents increased by 41.18%, 127.77%, 31.77%, 27.62% and 32.27%, over the control (Supplementary Table S7).

3.2.4.2 Performance of landraces across drought stress levels

The results presented in Supplementary Table S8 indicate a significant effect of the landrace on the content of MDA, free proline, total phenols, total flavonoids and DPPH. In relation to MDA, the highest content was recorded in landrace "747" (14.08 µmol g^{-1} FW) without being significantly different from landrace "746", whereas the lowest value was recorded in landrace "748". On the other hand, landrace "746" recorded the highest content of free proline (1.86 µg mg^{-1} FW), while the lowest respective values were noted in landraces "747" and "751" (1.32 and 1.38 µg mg^{-1} FW, respectively). Regarding TP and TF content and DPPH, the lowest values were detected in landrace "751" (33.28 mg GA/100 mg DW, 42.36 mg QE/100 mg DW and 26.64%, respectively), whereas "748" was the landrace with the highest overall content in phenolic compounds and the antioxidant activity (40.42 mg GA/100 mg DW, 51.78 mg QE/100 mg DW and 31.38%, respectively).

3.2.4.3 Landrace × drought stress interaction effects

From a breeding perspective, our findings indicate the existence of significant genotype × drought stress interaction effects in relation to the content of osmoprotectants (Table 6). In relation to MDA content, landraces "751", "747"and "746"showed an increasing trend

as D-mannitol concentration increased, whereas MDA content in "748" landrace showed fluctuating values with a notable increase at - 0.24 and - 0.47 MPa and a decrease at - 0.73 MPa. On the other hand, all the landraces showed an increased content over the control treatment when D-mannitol concentration increased, especially in landraces "746" and"748" where a gradual increase with increasing content of D-mannitol was recorded. Similar trends were detected in the case of TP and TF content which also showed a gradual increase with increasing D-mannitol content. Moreover, it has to be mentioned the pronounced increase in TP and TF over the control for "748", especially at - 0.73 MPa where TP and TF content increased by 207.7% and 41.2%, respectively. This particular trend was also confirmed in the case of DPPH antioxidant activity where the highest activity was recorded at the highest potential (-0.73 MPa) for all the tested landraces.

3.2.5 Variation in the contents of MDA, FP, TP, TF and DPPH activity in the shoots and the roots of squash landraces subjected to drought stress

Significant differences in the content of osmoprotectants were also recorded between the roots and shoots (Table 7). Specifically, roots showed an increased content of MDA, free proline, TF and DPPH as compared to the shoots (13.78 *vs* 12.71 µmol g^{-1} FW, 2.10 *vs* 0.95 µg mg^{-1} FW, 47.20 *vs* 46.56 mg QE/100 mg DW and 28.98% *vs* 28.38%, respectively), whereas only TP content was higher in the shoots than roots (37.57 *vs* 36.22 mg GA/100 mg DW, respectively).

The accumulation of MDA was also affected by the stress level applied, with its content being proportional to D-mannitol concentration in both roots and shoots (Figure 3A). In roots, the MDA content ranged from 10.47 to 16.10 µmol g^{-1} FW at 0 mM and - 0.73 MPa respectively, while in shoots the values ranged from

TABLE 6 Response of squash landraces to different drought stress levels ((0, -0.24, -.0.47 and - 0.73 MPa) in relation to the content of malondialdhehyde (MDA), free proline (FP), total phenolic compounds (TP), total flavonoids (TF) and DPPH activity (means ± SD).

Landrace	Water potential (MPa)	MDA (µmol g^{-1} FW)	Free proline (µg mg^{-1} FW)	TP (mg GA/100 mg DW)	TF (mg QE/100 mg DW)	DPPH (%)
"748"	Control	11.00 ± 0.48^{c*}	0.64 ± 0.14^{d}	30.97 ± 0.17^{d}	40.15 ± 0.43^{d}	22.90 ± 1.04^{d}
	-0.24	12.23 ± 0.34^{b}	1.45 ± 0.29^{c}	41.59 ± 1.22^{c}	54.29 ± 0.94^{c}	32.48 ± 1.73^{c}
	-0.47	13.04 ± 1.49^{a}	1.79 ± 0.19^{b}	43.18 ± 0.53^{b}	55.96 ± 0.57^{b}	34.62 ± 1.30^{b}
	-0.73	10.17 ± 1.00^{d}	2.29 ± 0.31^{a}	95.31 ± 2.13^{a}	56.71 ± 0.56^{a}	35.52 ± 1.31^{a}
"751"	Control	9.83 ± 0.71^{d}	0.66 ± 0.07^{d}	28.09 ± 1.88^{d}	39.22 ± 0.82^{c}	21.17 ± 1.94^{d}
	-0.24	12.54 ± 0.93^{c}	1.77 ± 0.16^{b}	33.03 ± 2.59^{c}	42.15 ± 0.89^{b}	26.89 ± 1.63^{c}
	-0.47	13.96 ± 1.45^{b}	1.82 ± 0.14^{a}	34.90 ± 3.53^{b}	44.26 ± 0.99^{a}	28.85 ± 1.51^{b}
	-0.73	17.11 ± 1.05^{a}	1.46 ± 0.17^{c}	37.10 ± 3.25^{a}	44.83 ± 0.93^{a}	29.64 ± 1.54^{a}
"747"	Control	10.78 ± 0.14^{d}	1.15 ± 0.42^{d}	32.33 ± 1.03^{d}	39.19 ± 1.45^{d}	24.21 ± 0.98^{c}
	-0.24	14.42 ± 1.37^{c}	1.67 ± 0.25^{a}	35.24 ± 1.15^{c}	45.89 ± 1.69^{c}	27.30 ± 1.04^{b}
	-0.47	15.27 ± 1.16^{b}	1.48 ± 0.10^{c}	41.48 ± 1.07^{b}	50.84 ± 1.51^{b}	30.35 ± 0.85^{a}
	-0.73	15.86 ± 1.84^{a}	1.57 ± 0.13^{b}	42.03 ± 1.01^{a}	51.41 ± 1.45^{a}	30.84 ± 0.60^{a}
"746"	Control	10.83 ± 0.09^{d}	1.15 ± 0.12^{d}	33.29 ± 1.26^{d}	41.38 ± 1.26^{c}	25.55 ± 1.41^{d}
	-0.24	12.41 ± 1.12^{c}	1.59 ± 0.27^{c}	34.91 ± 1.73^{c}	43.21 ± 1.41^{b}	28.03 ± 1.68^{c}
	-0.47	15.65 ± 1.42^{b}	1.81 ± 0.11^{b}	36.43 ± 1.74^{b}	50.02 ± 1.62^{a}	29.36 ± 1.16^{b}
	-0.73	16.16 ± 1.88^{a}	2.87 ± 0.28^{a}	39.39 ± 1.05^{a}	50.62 ± 1.33^{a}	31.18 ± 1.36^{a}

* Means in the same column and for the same D-mannitol concentration followed by the same letter are not significantly different at $p< 0.05$, according to Duncan's Multiple Range test.

10.75 to 13.55 µmol g^{-1} FW. It is worth noting that in all cases stressed plants showed a higher MDA content in roots than in shoots. A similar trend was also noted for free proline content which was maximum at - 0.73 MPa in both roots and shoots, corresponding to an increase of 125% and 132.81% compared to the control treatment, respectively (Figure 3B).

Moreover, drought stress caused an increase in the contents of total phenolic compounds (TP), total flavonoids (TF) and DPPH in both shoots and roots of squash landraces (Figures 4A–C). The content of phenolics was proportional to the stress level applied, thus showing a gradual increase in both shoots and roots (- 0.24 MPa: 12.79% and 19.73%, - 0.47 MPa: 22% and 29.53%, - 0.73 MPa: 27.83% and 35.25% increase over the control treatment in shoots and roots, respectively), as D-mannitol concertation increased (Figure 4A). A similar trend was also observed for total flavonoids (TF), whose content presented a significant increase in both shoots and roots (- 0.47 MPa: 18.06% and 33.94%, -0.73 MPa: 19.75% and 35.42% increase over the control treatment in shoots and roots, respectively) (Figure 4B). Finally, drought stress induced a considerable increase in DPPH contents, compared with the respective control, which ranged between 24.68% and 31.08% and from 22.24% to 32.52% in shoots and roots, respectively (Figure 4C).

4 Discussion

The results of the present study underline the drastic effects of drought stress depending on the landrace, the stress level as well as their interaction. During germination, all traits related to both germination ability and rate (GP, WU, SVI), as well as seedling growth (RL, SL, RFW, SFW, RV) were negatively affected in all the studied landraces, with the effects being proportional to the stress level applied, thus providing evidence for their relative sensitivity or tolerance to drought stress. Such findings are further supportive of previous reports related to the severe effects of drought stress during germination in a plethora of crop species, including maize (Queiroz

TABLE 7 Mean effect of shoot and root tissue on the content of MDA, FP, TP, TF and DPPH activity.

Organ	MDA (µmol g^{-1} FW)	Free proline (µg mg^{-1} FW)	TP (mg GA/100 mg DW)	TF (mg QE/100 mg DW)	DPPH (%)
Shoots	12.71_b*	0.95^b	37.57^a	46.56^b	28.38^b
Roots	13.78^a	2.10^a	36.22^b	47.20^a	28.98^a

* Means in the same column followed by the same letter are not significantly different at p< 0.05, according to Duncan's Multiple Range test; MDA: Malondialdhehyde; TP: Total phenols; TF: Total flavonoids; DPPH: 2,2-diphényl 1-picrylhydrazyle.

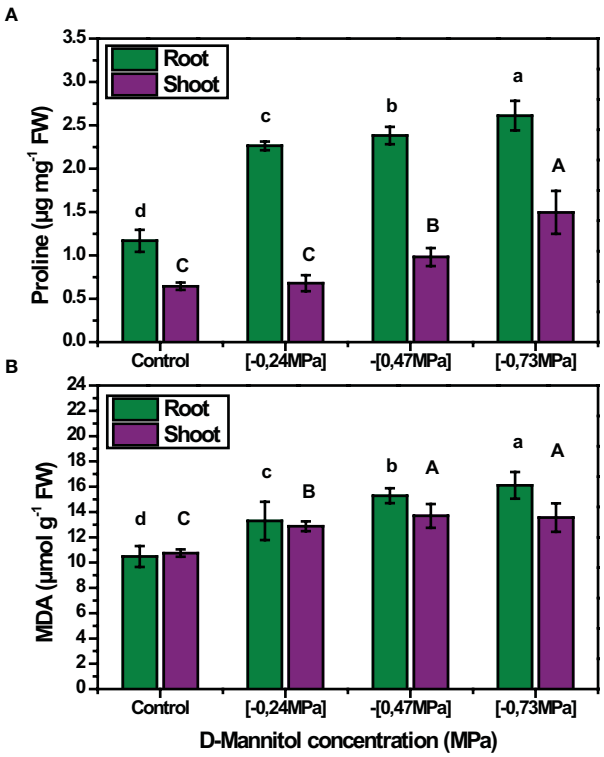

FIGURE 3
Mean effect of drought stress level (0, -0.24, -0.47 and - 0.73 MPa) on proline content **(A)** and on MDA **(B)** of squash landraces. Different letters above the bars (shoots: A, B, C, D and roots: a, b, c, d) indicate significant differences according to Duncan Multiple Range test at $p< 0.05$.

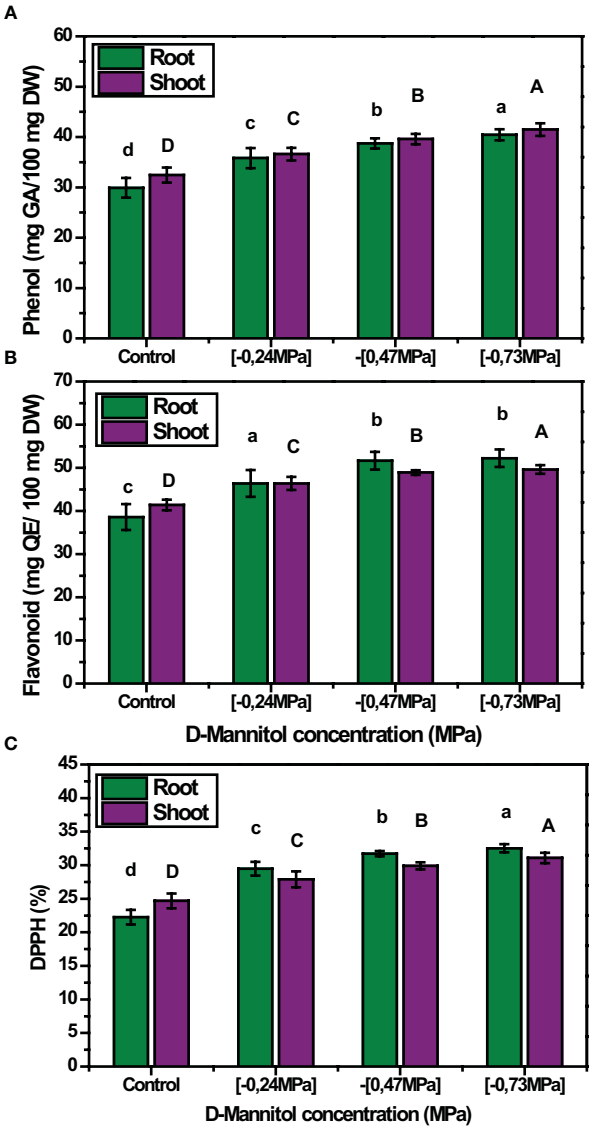

FIGURE 4
Mean effect of drought stress level (0, -0.24, -0.47 and - 0.73 MPa) **(A)** on total phenolic compounds content (mg GA 100 g^{-1} FW); **(B)** total flavonoids content (mg QE 100 g^{-1} FW); and **(C)** DPPH activity (%) of the studied squash landraces. Different letters above the bars (shoots: A, B, C, D; and roots: a, b, c, d) indicate significant differences according to Duncan Multiple Range test at $p< 0.05$.

et al., 2019), sorghum (Cokkizgin et al., 2019), faba bean (Mombeini et al., 2021), cucumber (Rahmani et al., 2019), as well as pumpkin (*Cucurbita pepo* L.; Biareh et al., 2022). The drought stress effects during germination are widely attributed to the fact that this stage is considered as the most sensitive throughout the plant life cycle since water uptake, referred to as imbibition, is a prerequisite to initiate germination. Accordingly, the amount of the absorbed water depends both on the level of the initial seed moisture content, as well as on its chemical composition (McDonald et al., 1988). In fact, protein and pectin are hydrophilic colloids, thus requiring more water than starch to promote imbibition and germination processes (Queiroz et al., 2019). In addition to germination potential, our findings further pointed to the drastic effects of drought stress on seedling growth, with the increasing concentration of D-mannitol leading to gradually decreased elongation in both roots and shoots. Such seedling growth inhibitory effects have been also reported in a wide range of plant species and are mainly attributed to osmotic stress effects (Queiroz et al., 2019). In our study, the findings which suggest that roots were more drastically affected than shoots are in agreement with previous reports which also suggest that the aboveground parts are more affected than the aerial parts in several species when the plant is subjected to drought stress, broad been (Cokkizgin et al., 2019) and tomato plants (Al Hassan et al., 2015), but also in squash germplasm under salt stress conditions (Tarchoun et al., 2022). Despite the fact that drought stress posed a limiting factor in germination and growth potential of squash landraces, it is worth noting that the tested landraces showed a differential response to varying stress levels. Among landraces, "751" and"746"exhibited a higher germination potential at all stress levels, thus indicating their superiority in terms of drought

tolerance. Given that these particular landraces were capable of germination and seedling growth even under severe stress conditions (-0.47 and - 0.73 MPa), it could be assumed that they consist a promising genetic material to be further exploited for breeding purposes.

Furthermore, our data underlined the significant effect of drought stress in all the studied physiological and biochemical traits, since chlorophyll b, F_m, PA and free proline content were the most variable traits, thus providing evidence for their suitability as screening criteria for drought tolerance among the tested landraces. Chlorophyll fluorescence analysis, which is routinely employed to assess the status of plant photosynthesis, revealed significant variations not only for the landrace and the drought stress level but also for their interactions. Changes in the minimum fluorescence yield (F_O) values suggest differential response to the transfer of excitation energy between pigments molecules (Liang et al., 2020). In our study, maximum fluorescence (F_m), whose values are maximum in healthy non-stressed leaves that have been dark-adapted (Huang et al., 2019), showed a decreasing trend as D-mannitol concentration increased. Furthermore, the F_v/F_m ratio, which refers to the maximum quantum yield of PSII photosynthetic apparatus and is commonly used as indicator of stress sensitivity (Shin et al., 2021), presented the lowest value at - 0.47 MPa (0.78), thus reflecting the existence of stress which lead to inactivation damage of PSII, referred to as photoinhibition. However, the F/Fm ratio showed higher values at - 0.73 MPa (0.83) which are indicative of unstressed leaves. Similar drought stress effects in photosynthesis activity were also reported in other plant species, including lettuce (Inoue et al., 2021), tomato and pepper (Parkash and Singh, 2020). According to previous studies, such disturbances due to abiotic stress, including water deficit, are attributed to changes in the activity of pigments in the course of photochemical and enzymatic reactions in thylakoids and the chloroplast, resulting in low amount of energy absorbed by PSII, as well as in changes in the membrane permeability of chloroplasts (Wang et al., 2018). Apart from the obvious stress level effects, our data pointed to differences in chlorophyll fluorescence attributed to the effect of landrace. Interestingly, landraces "747" and "746" showed a notable decrease at - 0.47 MPa, which is indicative of a stress state as a result of the initial plant damage closely related to PSII, whereas "751" and "748"exhibited an increased F_v/F_m ratio at both - 0.47 and - 0.73 MPa.

Moreover, our results suggested the drastic effect of drought stress on the content of chlorophylls a, b and carotenoids as well as on real evapotranspiration (RET) and photosynthetic activity (PA), while it was evidenced that the stress effects were analogous to its intensity. Our findings also confirm that the decreased content of chlorophylls and carotenoids, coupled with the decreased values for RET and PA, reflect the first defense reaction of leaves, involving stomatal closure as a means to reduce transpiration and photosynthesis as well as to limit CO_2 exchange, as previously reported in other vegetable crops (Parkash and Singh, 2020). Although the observed reduction in RET and PA values under D-mannitol stress conditions is in accordance with previous reports in other plant species, such as s, cucumber (Li et al., 2008) and watermelon (Mo et al., 2016), it is worth noting that landrace "746", despite of its high RET values, also exhibited the highest PA values (1790.84 μmol photons m^{-2} s^{-1}) at - 0.73 MPa, thus reflecting an ability to maintain its photosynthetic activity even under severe drought stress. In contrast, landrace "751" was characterized by the lowest values for both RET and PA, probably as a result of adjustments in stomatal closure. Such regulation of stomatal control under drought stress conditions consists the main physiological factor in the context of optimizing water use and preventing excessive water loss, as it has already been proven in soybean (Liu et al., 2005). In the same line, it has been evidenced that wheat's response to drought stress involved a decreased rate of photosynthesis and transpiration as well as decreased stomatal conductance (decrease by 78.4%, 85.4% and 92%, respectively), the latter being more correlated with the transpiration rate than the photosynthetic rate (Farooq et al., 2014).

It is well evidenced that plants' response to abiotic stress involves the enhanced accumulation of osmolytes, such as free proline, MDA, total phenols and flavonoids, as well as increased radical-scavenging activity to mitigate oxidative stress (Kapoor et al., 2020; Parkash and Singh, 2020; Yadav et al., 2021). Among compounds with osmoprotective function, free proline is one of the most well studied indicators for drought tolerance as it plays a pivotal role in regulating osmotic pressure in stressed plants (Yadav et al., 2021). Similarly, MDA generated by the peroxidation of polyunsaturated fatty acids of the cell membranes as a response to ROS generation (Zhou et al., 2017), is routinely employed as an indicator to evaluate the degree of plasma membrane damage and the ability of plants to cope with drought stress. In relation to both free proline and MDA, our findings indicate a differential response of roots and shoots, with the former showing an increased content thus suggesting more intense stress conditions. Such observations are in agreement with the fact that under drought stress conditions roots play a fundamental role both in the perception of water deficit status and hormone signal transduction, mediated by abscisic acid (ABA), the latter triggering signals to aerial organs and tissues through vascular bundles, causing morphological/anatomical alterations, such as the root to shoot ratio (Takahashi et al., 2020). As the first organ to drought perception, roots respond with a series of morphological changes in the growth and architecture, including their length, weight and volume, while, at the same time, constituting the primary site of osmolyte accumulation (Fang and Xiong, 2015). Such drought adaption mechanisms in roots regulate water loss and promote water use efficiency, which are considered as key functional traits that contribute to drought tolerance (Takahashi et al., 2020). In accordance with the drought responses in typical roots, our findings suggest a more pronounced increase in total phenolic compounds and total flavonoids content as well as in DPPH activity in roots than in shoots, especially at high stress levels, which is indicative of the essential role of these parameters in mitigating the water deficit effects.

5 Conclusions

Conclusively, our findings contribute towards understanding the response of squash landraces to drought stress and further provide evidence regarding the tolerance ability of the landraces under study. Among landraces, "751" and"746"were classified as better performing in terms of drought tolerance both at germination and later plant growth stages. Considering the small number of landraces under study, it is concluded that squash germplasm exhibits considerable variation for drought tolerance traits, thus providing ground both for selecting appropriate landraces for cultivation under water deficit conditions and applying breeding approaches targeted at improving crop's drought tolerance. However, further studies are needed with a broader group of germplasm accessions of squash germplasm that will help to valorize the existing variability through the selection of elite genotypes in targeted breeding programs.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Author contributions

WA: methodology; formal analysis; investigation; data curation; NT: conceptualization; writing-original draft preparation; writing-review and editing; visualization; supervision; project administration; IM and RA: methodology; formal analysis; investigation; data curation; OP: writing-original draft preparation; HF: methodology; formal analysis; investigation; data curation; CA: methodology; formal analysis; investigation; data curation; RK: writing-review and editing; visualization; supervision; project administration; SAP: conceptualization; writing-review and editing; visualization; supervision; project administration; funding acquisition. All authors contributed to the article and approved the submitted version.

Funding

The project is funded by the General Secretariat for Research and Technology of the Ministry of Development and Investments under the PRIMA Programme. PRIMA is an Art.185 initiative supported and co-funded under Horizon 2020, the European Union's Programme for Research and Innovation (Prima2019-08). This research was also jointly founded by Prima project and National research laboratory LR21AGR05.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2023.1215394/full#supplementary-material

References

Al Hassan, M., Martínez Fuertes, M., Ramos Sánchez, F. J., Vicente, O., and Boscaiu, M. (2015). Effects of salt and water stress on plant growth and on accumulation of osmolytes and antioxidant compounds in cherry tomato. *Not. Bot. Horti Agrobot. Cluj-Napoca* 43, 1–11. doi: 10.15835/nbha4319793

Arnon, D. I. (1949). Copper enzymes in isolated chloroplasts. Polyphenoloxidase in beta vulgaris. *Plant Physiol.* 24, 1–15. doi: 10.1016/B978-0-12-409548-9.11130-3

Ashraf, F., Zargar, T. B., and Veres, S. (2021). Comparison between germinating parameters of basils (Ocimum basilicum L.) and pumpkin (*Cucurbita pepo* L.) under drought stress conditions. *Rev. Agric. Rural Dev.* 10, 100–106. doi: 10.14232/rard.2021.1-2.100-106

Baker, N. R. (2008). Chlorophyll fluorescence: A probe of photosynthesis *in vivo*. *Annu. Rev. Plant Biol.* 59, 89–113. doi: 10.1146/annurev.arplant.59.032607.092759

Bankaji, I., Sleimi, N., Vives-Peris, V., Gómez-Cadenas, A., and Pérez-Clemente, R. M. (2019). Identification and expression of the *Cucurbita* WRKY transcription factors in response to water deficit and salt stress. *Sci. Hortic.* 256, 108562. doi: 10.1016/j.scienta.2019.108562

Begum, N., Wang, L., Ahmad, H., Akhtar, K., Roy, R., Khan, M. I., et al. (2022). Co-inoculation of arbuscular mycorrhizal fungi and the plant growth-promoting rhizobacteria improve growth and photosynthesis in tobacco under drought stress by up-regulating antioxidant and mineral nutrition metabolism. *Microb. Ecol.* 83, 971–988. doi: 10.1007/s00248-021-01815-7

Biareh, V., Shekari, F., Sayfzadeh, S., Zakerin, H., Hadidi, E., Beltrão, J. G. T., et al. (2022). Physiological and qualitative response of *Cucurbita pepo* L. @ to salicylic acid under controlled water stress conditions. *Horticulturae* 8, 1–13. doi: 10.3390/horticulturae8010079

Blainsk, A., Lopes, G. C., and de Mello, J. C. P. (2013). Application and analysis of the folin ciocalteu method for the determination of the total phenolic content from l*imonium brasiliense* L. *Molecules* 18, 6852–6864. doi: 10.3390/molecules18066852

Chevilly, S., Dolz-Edo, L., Martínez-Sánchez, G., Morcillo, L., Vilagrosa, A., López-Nicolás, J. M., et al. (2021). Distinctive traits for drought and salt stress tolerance in melon (*Cucumis melo* L.). *Front. Plant Sci.* 12. doi: 10.3389/fpls.2021.777060

Cheynier, V., Comte, G., Davies, K. M., Lattanzio, V., and Martens, S. (2013). Plant phenolics: Recent advances on their biosynthesis, genetics, andecophysiology. *Plant Physiol. Biochem.* 72, 1–20. doi: 10.1016/j.plaphy.2013.05.009

Cokkizgin, A., Girgel, U., and Cokkizgin, H. (2019). Mannitol (C6H14O6) effects on germination of broad bean (*Vicia faba* L.) seeds. *For. Res. Eng.* 3, 20–22. doi: 10.15406/freij.2019.03.00073

Curtis, O. F., and Shetty, K. (1996). Growth medium effects on vitrification, total phenolics, chlorophyll, and water content of in *vitro* propagated oregano clones. *Acta Hortic.* 426, 489–504. doi: 10.17660/ActaHortic.1996.426.53

ECPGR (2008) *Minimum descriptors for Cucurbita spp., cucumber, melon and watermelon* Available at: http://www.ecpgr.cgiar.org/fileadmin/templates/ecpgr.org/upload/NW_and_WG_UPLOADS/Cucurbits_DescriptorLists.pdf

Fang, Y., and Xiong, L. (2015). General mechanisms of drought response and their application in drought resistance improvement in plants. *Cell. Mol. Life Sci.* 72, 673–689. doi: 10.1007/s00018-014-1767-0

Farooq, M., Hussain, M., and Siddique, K. H. M. (2014). Drought stress in wheat during flowering and grain-filling periods. *CRC. Crit. Rev. Plant Sci.* 33, 331–349. doi: 10.1080/07352689.2014.875291

Ferreira, I. C. F. R., Baptista, P., Vilas-Boas, M., and Barros, L. (2007). Free-radical scavenging capacity and reducing power of wild edible mushrooms from northeast Portugal: Individual cap and stipe activity. *Food Chem.* 100, 1511–1516. doi: 10.1016/j.foodchem.2005.11.043

Gill, S. S., and Tuteja, N. (2010). Reactive oxygen species and antioxidant machinery in abiotic stress tolerance in crop plants. *Plant Physiol. Biochem.* 48, 909–930. doi: 10.1016/j.plaphy.2010.08.016

Giordano, M., Petropoulos, S. A., and Rouphael, Y. (2021). Response and defence mechanisms of vegetable crops against drought, heat and salinity stress. *Agriculture* 11, 1–30. doi: 10.3390/agriculture11050463

Golldack, D., Li, C., Mohan, H., and Probst, N. (2014). Tolerance to drought and salt stress in plants: Unraveling the signaling networks. *Front. Plant Sci.* 5. doi: 10.3389/fpls.2014.00151

Hamdi, K., Ben-Amor, J., Mokrani, K., Mezghanni, N., and Tarchoun, N. (2017). Assessment of the genetic diversity of some local squash (*Cucurbita maxima* Duchesne) populations revealed by agro-morphological and chemical traits. *J. New Sci.* 42, 2306–2317.

Hamdi, K., Mokrani, K., and Tarchoun, N. (2019). Agro-morphological and biochemical characterization of squash (*Cucurbita maxima* Duchesne) accessions cultivated in Tunisia. *Int. J. Agric. Environ. Biores.* 04, 1–10. doi: 10.35410/ijaeb.2019.0110

Hasanuzzaman, M., Bhuyan, M. H. M. B., Zulfiqar, F., Raza, A., Mohsin, S. M., Al Mahmud, J., et al. (2020). Reactive oxygen species and antioxidant defense in plants under abiotic stress: Revisiting the crucial role of a universal defense regulator. *Antioxidants* 9, 1–52. doi: 10.3390/antiox9080681

Hatfield, J. L., and Dold, C. (2019). Water-use efficiency: Advances and challenges in a changing climate. *Front. Plant Sci.* 10. doi: 10.3389/fpls.2019.00103

Hnilickova, H., Kraus, K., Vachova, P., and Hnilicka, F. (2021). Salinity stress affects photosynthesis, malondialdehyde formation, and proline content in *Portulaca oleracea* L. *Plants* 10, 845. doi: 10.3390/plants10050845

Huang, H., Ullah, F., Zhou, D. X., Yi, M., and Zhao, Y. (2019). Mechanisms of ROS regulation of plant development and stress responses. *Front. Plant Sci.* 10. doi: 10.3389/fpls.2019.00800

Inoue, T., Sunaga, M., Ito, M., Yuchen, Q., Matsushima, Y., Sakoda, K., et al. (2021). Minimizing VPD fluctuations maintains higher stomatal conductance and photosynthesis, resulting in improvement of plant growth in lettuce. *Front. Plant Sci.* 12. doi: 10.3389/fpls.2021.646144

Kapoor, D., Bhardwaj, S., Landi, M., Sharma, A., Ramakrishnan, M., and Sharma, A. (2020). The impact of drought in plant metabolism: How to exploit tolerance mechanisms to increase crop production. *Appl. Sci.* 10 (16), 5692. doi: 10.3390/app10165692

Li, S., Gu, S., Liu, W., Han, H., and Zhang, Q. (2008). Water quality in relation to land use and land cover in the upper Han River Basin, China. *Catena* 75, 216–222. doi: 10.1016/j.catena.2008.06.005

Li, J., Zeng, L., Cheng, Y., Lu, G., Fu, G., Ma, H., et al. (2018). Exogenous melatonin alleviates damage from drought stress in *Brassica napus* L. (rapeseed) seedlings. *Acta Physiol. Plant* 40, 1–11. doi: 10.1007/s11738-017-2601-8

Liang, G., Liu, J., Zhang, J., and Guo, J. (2020). Effects of drought stress on photosynthetic and physiological parameters of tomato. *J. Am. Soc Hortic. Sci.* 145, 12–17. doi: 10.21273/JASHS04725-19

Liu, F., Andersen, M. N., Jacobsen, S. E., and Jensen, C. R. (2005). Stomatal control and water use efficiency of soybean (*Glycine max* L. Merr.) during progressive soil drying. *Environ. Exp. Bot.* 54, 33–40. doi: 10.1016/j.envexpbot.2004.05.002

Mahmood, T., Khalid, S., Abdullah, M., Ahmed, Z., Shah, M. K. N., Ghafoor, A., et al. (2020). Insights into drought stress signaling in plants and the molecular genetic basis of cotton drought tolerance. *Cells* 9, 1–30. doi: 10.3390/cells9010105

McDonald, M. B., Vertucci, C. W., and Roos, E. E. (1988). Soybean seed imbibition: water absorption by seed parts. *Crop Sci.* 28, 993–997. doi: 10.2135/cropsci1988.0011183x002800060026x

Mo, Y., Wang, Y., Yang, R., Zheng, J., Liu, C., Li, H., et al. (2016). Regulation of plant growth, photosynthesis, antioxidation and osmosis by an arbuscular mycorrhizal fungus in watermelon seedlings under well-watered and drought conditions. *Front. Plant Sci.* 7. doi: 10.3389/fpls.2016.00644

Mombeini, M., Alamzadeh Ansari, N., Abdossi, V., and Naseri, A. (2021). Reducing destructive effects of drought stress on cucumber through seed priming with silicic acid, pyridoxine, and ascorbic acid along with foliar spraying with silicic acid. *Agric. Conspec. Sci.* 86, 35–49.

Monneveaux, P., and Nemmar, M. (1986). Contribution à l'étude de la résistance à la sécheresse chez le blé tendre (Triticum aestivum L.) et chez le blé dur (*Triticum durum* Desf.): étude de l'accumulation de la proline au cours du cycle de développement. *Agronomie* 6, 583–590. doi: 10.1051/agro:19860611

Nalina, M., Saroja, S., Chakravarthi, M., Rajkumar, R., Radhakrishnan, B., and Chandrashekara, K. N. (2021). Water deficit-induced oxidative stress and differential response in antioxidant enzymes of tolerant and susceptible tea cultivars under field condition. *Acta Physiol. Plant* 43, 1–17. doi: 10.1007/s11738-020-03174-1

Parkash, V., and Singh, S. (2020). A review on potential plant-basedwater stress indicators for vegetable crops. *Sustain.* 12 (10), 3945. doi: 10.3390/SU12103945

Partheeban, C., Chandrasekhar, C. N., Jeyakumar, P., Ravikesavan, R., and Gnanam, R. (2017). Effect of PEG induced drought stress on seed germination and seedling characters of maize (*Zea mays* L.) genotypes. *Int. J. Curr. Microbiol. Appl. Sci.* 6, 1095–1104. doi: 10.20546/ijcmas.2017.605.119

Porcar-Castell, A., Tyystjärvi, E., Atherton, J., van der Tol, C., Flexas, J., Pfündel, E. E., et al. (2014). Linking chlorophyll a fluorescence to photosynthesis for remote sensing applications: Mechanisms and challenges. *J. Exp. Bot.* 65, 4065–4095. doi: 10.1093/jxb/eru191

Queiroz, M. S., Oliveira, C. E. S., Steiner, F., Zuffo, A. M., Zoz, T., Vendruscolo, E. P., et al. (2019). Drought stresses on seed germination and early growth of maize and sorghum. *J. Agric. Sci.* 11, 310–318. doi: 10.5539/jas.v11n2p310

Rahmani, F., Sayfzadeh, S., Jabbari, H., Valadabadi, S. A., and Hadidi Masouleh, E. (2019). Alleviation of drought stress effects on safflower yield by foliar application of zinc. *Int. J. Plant Prod.* 13, 297–308. doi: 10.1007/s42106-019-00055-7

Rosero, A., Granda, L., Berdugo-cely, J. A., Šamajov, O., Šamaj, J., and Cerkal, R. (2020). Crops into production systems to enhance their resilience to water deficiency. *Plants* 9, 1–21. doi: 10.3390/plants9101263

Saberali, S. F., and Shirmohamadi-Aliakbarkhani, Z. (2020). Quantifying seed germination response of melon (*Cucumis melo* L.) to temperature and water potential: Thermal time, hydrotime and hydrothermal time models. *South Afr. J. Bot.* 130, 240–249. doi: 10.1016/j.sajb.2019.12.024

Saroj, P., and Choudhary, B. (2020). Improvement in cucurbits for drought and heat stress tolerance- a review. *Curr. Hortic.* 8, 3–13. doi: 10.5958/2455-7560.2020.00013.8

Schulz, P., Piepenburg, K., Lintermann, R., Herde, M., Schöttler, M. A., Schmidt, L. K., et al. (2021). Improving plant drought tolerance and growth under water limitation through combinatorial engineering of signalling networks. *Plant Biotechnol. J.* 19, 74–86. doi: 10.1111/pbi.13441

Scott, S. J., Jones, R. A., and Williams, W. A. (1984). Review of data analysis methods for seed germination 1. *Crop Sci.* 24, 1192–1199. doi: 10.2135/cropsci1984.0011183x002400060043x

Shin, Y. K., Bhandari, S. R., Jo, J. S., Song, J. W., and Lee, J. G. (2021). Effect of drought stress on chlorophyll fluorescence parameters, phytochemical contents, and antioxidant activities in lettuce seedlings. *Horticulturae* 7, 1–16. doi: 10.3390/horticulturae7080238

Takahashi, F., Kuromori, T., Urano, K., Yamaguchi-Shinozaki, K., and Shinozaki, K. (2020). Drought stress responses and resistance in plants: from cellular responses to long-distance intercellular communication. *Front. Plant Sci.* 11. doi: 10.3389/fpls.2020.556972

Tarchoun, N., Saadaoui, W., Mezghani, N., Pavli, O. I., Falleh, H., and Petropoulos, S. A. (2022). The effects of salt stress on germination, seedling growth and biochemical responses of Tunisian squash (*Cucurbita maxima* Duchesne) germplasm. *Plants* 11, 1–18. doi: 10.3390/plants11060800

Wang, Y., Reiter, R. J., and Chan, Z. (2018). Phytomelatonin: A universal abiotic stress regulator. *J. Exp. Bot.* 69, 963–974. doi: 10.1093/jxb/erx473

Yadav, B., Jogawat, A., Rahman, M. S., and Narayan, O. P. (2021). Secondary metabolites in the drought stress tolerance of crop plants: A review. *Gene Rep.* 23, 101040. doi: 10.1016/j.genrep.2021.101040

Zhishen, J., Mengcheng, T., and Jianming, W. (1999). The determination of flavonoid contents in mulberry and their scavenging effects on superoxide radicals. *Food Chem.* 64, 555–559. doi: 10.1016/S0308-8146(98)00102-2

Zhou, Y., Wen, Z., Zhang, J., Chen, X., Cui, J., Xu, W., et al. (2017). Exogenous glutathione alleviates salt-induced oxidative stress in tomato seedlings by regulating glutathione metabolism, redox status, and the antioxidant system. *Sci. Hortic.* 220, 90–101. doi: 10.1016/j.scienta.2017.02.021

Zhou, J., Zhou, J., Ye, H., Ali, M. L., Chen, P., and Nguyen, H. T. (2021). Yield estimation of soybean breeding lines under drought stress using unmanned aerial vehicle-based imagery and convolutional neural network. *Biosyst. Eng.* 204, 90–103. doi: 10.1016/j.biosystemseng.2021.01.017