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Variation in Morpho-Physiological Responses of Desi Chickpea (*Cicer arietinum* L.) Seedlings to Progressive Water Stress

Saeedreza Vessal[*1], Narjes Amirchakhmaghi[1], Mehdi Parsa[2]

[1]*Department of Biotechnology and Plant Breeding, Faculty of Agriculture, Ferdowsi University of Mashhad, Mashhad, Iran*
[2]*Department of Agronomy and Plant Breeding, Faculty of Agriculture, Ferdowsi University of Mashhad, Mashhad, Iran*

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ABSTRACT

Water deficit stress is one of the key determinants causing crop yield losses globally. The present study was conducted to effectively screen Desi chickpea genotypes based on early dehydration tolerance-related traits as a tool for further evaluation in field experiments. Sixty-four genotypes of Desi chickpea were assessed under progressive water deficit stress, resulting in high variability in early growth characteristics and traits. The clustering analysis with UPGMA, separated the genotypes into three major groups, in accordance with biplot analysis grouping. The highest root length density was observed in the MCC438 genotype with an 18.6-fold increase compared to MCC884 which showed the lowest root length density among all analyzed chickpea genotypes. The genotypes MCC32 and MCC539 produced the higher shoot and root dry weight, while MCC884 showed the lowest value (with 12- and 32.5-fold differences, respectively). Ten genotypes showed differences in terms of their overall response to the water deficiency stress, including eight tolerant genotypes (MCC320, MCC418, MCC425, MCC438, MCC539, MCC540, MCC560, MCC576) and two susceptible ones (MCC433 and MCC897), were selected for further investigation of various growth and physio-biochemical traits based on drought response indices. A clear distinction was observed among ten analyzed genotypes for some physio-biochemical traits, indicating their tolerant responses to drought stress. Drought-tolerant candidate genotypes showed higher indices of seedling growth parameters, proline content, RWC, membrane stability, and root-to-shoot ratio in comparison to drought-susceptible candidate genotypes. The genotypes MCC425, MCC438, MCC418, and MCC539 were found more drought tolerant in the seedling stages, whereas genotype MCC433 was more sensitive. These results were consistent with what was obtained in our preliminary study. However, these results should be addressed further in the field conditions.

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1. Introduction

Chickpea (*Cicer arietinum* L.) is one of the annual Cicer species within the Fabaceae family (Maqbool *et al.*, 2017; Pundir *et al.*, 1985), growing in the subtropical to Mediterranean regions of Western Asia, Northern Africa, and Southern and Southwestern Europe (Canci and Toker, 2009a, b; Ganjeali *et al.*, 2011; Hosseinzadeh *et al.*, 2018). As suggested by Vavilov and recent genome sequencing-based studies, primary centers of chickpea origin are placed in the Fertile Crescent (Southeastern Turkey and Syria) and the Mediterranean areas, while South Asia and Ethiopia are the secondary centers of origin (Arriagada *et al.*, 2022).

Chickpea, the world's second most important grained legume, is mostly grown as a dry weather crop in semi-arid zones or a rainfed crop in cool climates (Arif *et al.*, 2021; Kakaei *et al.*, 2024), dividing into two main types of "Kabuli" and "Desi" (Maqbool *et al.*, 2017). It is a valuable crop for soil fertility improvement and increasing the yield of arable land, especially in rainfed or dry regions according to features such as nitrogen-fixing, effective use of rainwater, and root length elongation (Ganjeali *et al.*, 2011; Hosseinzadeh *et al.*, 2016; Hosseinzadeh *et al.*,

* Corresponding author.
E-mail address: vessal@um.ac.ir

2018; Katerji *et al.*, 2001). This crop is cultivated in over 54 countries of the world on an area of 13.20 million hectares (Mha) with an annual production of 12 million tons (FAO, 2018).

About 90% of the total annual global production of chickpea is grown in arid and semi-arid zones under rainfed conditions in which the main source of available water is stored soil moisture (Kashiwagi *et al.*, 2006; Kumar and Abbo, 2001; Lokhande *et al.*, 2019). India, Turkey, Pakistan, and Iran are four leading countries classified as arid or semi-arid regions and this crop is generally cultivated under rainfed conditions in these regions (Sani *et al.*, 2018; Talebi *et al.*, 2013).

In the last decades, climate change and its consequences such as irregularity of rainfall, increase in heat wave frequency, drought and flood have had a devastating impact on agriculture and crop yield (Varshney *et al.*, 2014). The most substantial constraints to chickpea productivity are drought and high temperature stresses which can lead to annual yield losses of up to 50% (Devasirvatham and Tan, 2018; Gaur *et al.*, 2013; Ramamoorthy *et al.*, 2016).

All growth stages of chickpea crops can be adversely affected by drought as a complex abiotic stress. The adverse effects can be determined based on features such as seed emergence, seedling establishment, physiological and biochemical parameters (relative water content, leaf temperature, stomatal conductance, proline, malondialdehyde, etc.), osmotic regulation, molecular regulation, nutrient uptake, level of antioxidants and yield and its components (Daws *et al.*, 2008; Joseph and Jini, 2011; Mafakheri *et al.*, 2010; Maqbool *et al.*, 2017). Under drought stress, several morphological, physiological, and biochemical changes occur in various plant species including limited growth, decreased chlorophyll contents, ascorbic acid and relative water content (RWC), increase in proline accumulation, excised leaf water loss (ELWL) and hydrogen peroxide (Araghi and Assad, 1998; Denčić *et al.*, 2000; Dhanda and Sethi, 1998; Maqbool *et al.*, 2017; Mukherjee and Choudhuri, 1983; Shah *et al.*, 2020).

The most promising characteristics in chickpea breeding for terminal drought tolerance are root traits including root depth, root biomass, and total root length density which can improve the extraction of soil moisture and increase drought tolerance (Kashiwagi *et al.*, 2006; Varshney *et al.*, 2011; Varshney *et al.*, 2014).

Depending on the genotypes and the growth stages, mechanisms of responses to progressive water deficit stress are varied in individual plants (Ashraf and Harris, 2004). Knowledge of the physiological and biochemical responses of individual species can offer insight into developing useful ways for screening large numbers of genotypes, in a short period to select drought-tolerant individuals in crop species (Talebi *et al.*, 2013).

The present study, aimed (1) at the application of a simple method to analyze "Desi" chickpea genotypes' response to a progressive decrease of water levels; (2) to determine the extent of variability of the shoot and root traits under progressively drought stress in chickpea genotypes and (3) to investigate changes in some critical physiological and biochemical parameters as a potential screening tool for drought tolerance.

2. Materials and methods

2.1. Plant materials

Sixty-four 'Desi' chickpea (*Cicer arietinum* L.) genotypes, harvested in the past two years, were collected for this study from Pluses Seed Bank of Research Center for Plant Sciences at Ferdowsi University of Mashhad, Iran (Table 1). Chickpea seeds were germinated in the petri dishes containing filter papers and distilled water was added. These germinated seeds were sown in one-liter pots, containing a mixture of field soil and fine cocopeat (2:1, v/v) and a final bulk density of 0.9 $g.cm^{-3}$.

2.2. Root and shoot sampling

2.2.1. Experiment 1

The experiment was conducted in a completely randomized design (CRB) with four replications for one level of water deficit treatment under controlled conditions. After growing in the greenhouse with controlled temperature of 23/20°C day/night, relative moisture of 45%, and 15/9 h photoperiod (day/night) under natural light with regular irrigation to keep moisture level around 70% water holding capacity (WHO) for 3 weeks, pots were exposed to water withdrawal to create progressive water deficit condition for seedlings until 5 weeks after emergence. Then, seedlings were harvested and plant growth traits

such as shoot and root fresh and dry weights, shoot and root length and the number of leaves were recorded. The shoot fresh weight was measured immediately after harvesting, while root dry weight was determined by drying in the oven at 60°C for 48 h after image acquisition.

For the image acquisition, plant roots were extracted from the soil by washing carefully with running water to separate soil particles from the root tissues, and an image was acquired by scanner (DELTA-T Scan AT) to assess the root morphological parameters. Finally, the total root length, mean root diameter and total area of the root were measured using built-in image analyzer software (DF2). After recording root fresh weight and image acquisition, the samples were placed in a paper bag and oven-dried at 60°C for 48 h.

2.2.2. Experiment 2

Ten out of 64 primary chickpea genotypes, eight drought-tolerant and two drought-susceptible genotypes determined in experiment 1, were selected for further analysis in the second experiment through some additional physiological and biochemical parameters.

Eight uniform germinated seeds of each genotype were sown in eight separated pots as replicates with the same mixture and density as experiment 1. The Plants were grown for 5 weeks in the greenhouse condition with temperature regulated at 25/20°C day/night, photoperiod at 17/7 h (day/night) natural light, and relative moisture of nearly 45%. The experiment was conducted in a completely randomized block design with four replications for one-level progressive water deficit treatment. Pots were regularly irrigated to keep moisture level around 70% water holding capacity (WHO) for up to 3 weeks. Then half of the plants received no water after 3 weeks of sowing and were exposed to progressive drought until 5 weeks after emergence while the other four pots were used as a control group. The shoot and root traits were evaluated using the same methodology as described in the previous experiment. Additional biochemical and physiological changes were also measured in the following sections.

2.3. Proline content

Proline content was calculated by using fresh chickpea leaves according to (Bates *et al.*, 1973). Briefly, 100-mg fresh leaf was homogenized in 10 mL of 3% aqueous solution of sulphosalicylic acid. The solution was centrifuged at 12,000 rpm for 10 min and the supernatant was added to a mixture of reactive ninhydrin (1.25 g ninhydrine in 30 ml glacial acetic acid and 20 ml 6 M orthophosphoric acid) in glacial acetic acid. After incubation at 100°C water for 1 h, 4 ml toluene was added to each sample and shaken vigorously. The colored phase was used to read absorbance at 520 nm by spectrophotometer (SP-3000 Plus) using toluene as a blank. Finally, the proline content was calculated based on the standard curve (obtained from different concentrations of proline) using the following formula (Equation 1):

(1) Proline (µmole g^{-1} fresh weight) = [(proline (µg mL^{-1}) × toluene (mL)) / 115.5 (µg $µmol^{-1}$)] / [(sample (g) / 10)]

2.4. Cell membrane stability

Cell membrane stability was evaluated according to the method developed by (Sairam, 1994) based on electrical conductivities. Leaf samples (100 mg) from each plant were washed and placed in the test tubes containing 15 mL of distilled water. After 24 h, the initial electrical conductivity of liquid was measured (EC1). Then, solutions were autoclaved for 20 min and the other conductivity was recorded at 25°C (EC2). Cell membrane stability was calculated as follows (Equation 2):

(2) Cell membrane stability (%) = [1 – (EC1 / EC2)] × 100

2.5. Relative water content

The relative water content (RWC) of the leaf was measured according to the method developed by (Barrs and Weatherley, 1962). 100 mg leaf tissue of each plant was excised and kept in a Petri dish containing double distilled water for 4 h, then the turgid weight of the leaf tissue was measured. Eventually, samples were oven-dried at 65°C for 24 h, and the dry weight of the leaf materials was recorded. RWC was calculated using the following formula (Equation 3):

(3) RWC (%) = [(Fresh weight of sample - Dry weight of sample) / (Turgid weight of sample - Dry weight of sample)] ×100

Various data sets obtained from this experiment were used to calculate stress tolerance indices including proline content stress tolerance index (PCSI),

relative water content stress tolerance index (RWCSI), membrane stability stress tolerance index (MSSI), shoot length and root length density stress tolerance index (SLSI and RLDSI, respectively), shoot and root and total plant dry weight stress tolerance index (SDWSI, RDWSI and TDWSI, respectively) and root to shoot ratio (dry weight basis) stress tolerance index (RTSSI) to test genotypes as follows (Equations 4-12):

$$(4)\quad \text{SLSI} = \frac{\text{Shoot length of stressed plants}}{\text{Shoot length of control plants}} \times 100$$

$$(5)\quad \text{RLDSI} = \frac{\text{Root length density of stressed plants}}{\text{Root length density of control plants}} \times 100$$

$$(6)\quad \text{SDWSI} = \frac{\text{Shoot dry weight of stressed plants}}{\text{Shoot dry weight of control plants}} \times 100$$

$$(7)\quad \text{RDWSI} = \frac{\text{Root dry weight of stressed plants}}{\text{Root dry weight of control plants}} \times 100$$

$$(8)\quad \text{TDWSI} = \frac{\text{Total dry weight of stressed plants}}{\text{Total dry weight of control plants}} \times 100$$

$$(9)\quad \text{PCSI} = \frac{\text{Proline content of stressed plants}}{\text{Proline content of control plants}} \times 100$$

$$(10)\quad \text{RWCSI} = \frac{\text{Relative water content of stressed plants}}{\text{Relative water content of control plants}} \times 100$$

$$(11)\quad \text{MSSI} = \frac{\text{Membrane stability of stressed plants}}{\text{Membrane stablity of control plants}} \times 100$$

$$(12)\quad \text{RTSSI} = \frac{\text{Root to shoot ratio of stressed plants}}{\text{Root to shoot ratio of control plants}} \times 100$$

2.6. Statistical data analysis

All data acquired from different experiments were statistically analyzed using IBM SPSS software (version 20). One-way analysis of variance (One-Way ANOVA) at 5% and 1% probability level in Duncan's multiple range test were employed for comparison of means with 4 replicates. The relative data and indices were normalized by converting to arc sin and square root transformation. Principal component analysis (PCA) on different seedling growth parameters data obtained in experiment 1, were performed using Statgraphics version 18. Dendrograms were generated using the similarity matrix, and unweighted pair-group average (UPGMA) method and it was calculated by NTSYSpc version 2.1(Rohlf, 2000). A goodness-of-fit of the clusters on data was evaluated by comparing the original similarity matrix with the cophenetic value which was derived from the similarity matrix using COPH program, using Mantel matrix correspondence test (Mantel, 1967).

3. Results and discussion

3.1. Experiment 1: Prompt comparing of genotypes

Basic summary statistics and genotypic means were estimated for all studied seedling traits to assess the variability in all data obtained from four replications. Genotypes differed significantly ($p \leq 0.01$) in terms of shoot length and shoot dry weight under drought stress conditions. Means of shoot length and shoot dry weight were 7.66cm and 0.051 gr, and ranged from 0.7 to 13.6 cm and 0.004 to 0.129 gr, respectively. Limited water availability adversely affected germination and seedling establishment of stressed seeds and all later morphological traits, such as plant height, shoot and root dry weight, root length, root density, and leaf number in which the variation rate depended also on the genotype (Hussain *et al.*, 2015; Randhawa *et al.*, 2014; Soltani *et al.*, 2006; Weber *et al.*, 2005).

Also, root length density and the average root diameter values for the studied genotypes ranged from 0.019 to 0.353 $cm.cm^{-3}$ and 0.184 to 0.774 mm with the mean values of 0.148 $cm.cm^{-3}$ and 0.555 mm, respectively. Root dry weight had a mean of 0.0127g and a range of 0.0015 to 0.024 g.

Analysis of variance (ANOVA) revealed a significant genotypic variability ($p \leq 0.01$) among sample sets in terms of shoot and root length, shoot and root dry weight, the average diameter of the root and the number of leaves among the chickpea genotypes under progressive dehydration. The highest number of leaves was observed in MCC540 followed by the MCC425 genotype, being about 9-fold more than MCC884 with the least number of leaves. The genotype MCC320 and MCC198 had the maximum shoot length and root length density whereas these traits were at minimum in MCC775 and MCC884. The highest root length density occurred in MCC438 and MCC198 with an18.6-fold increase than MCC884 with the lowest value. Regarding the shoot length variability, the mean value was about 19.4-fold more in MCC320 compared to MCC775. The genotypes MCC32 and MCC539 produced the maximum shoot and root dry weight, while MCC884 had the lowest value of both traits under dehydration stress conditions (Table 1). Drought treatment resulted in a 12-fold

higher root dry weight in MCC539 compared to MCC884. Whereas the highest shoot dry weight belonged to the MCC32 with a 32.5-fold increase compared to MCC884. The comparisons related to various traits showed a large variation in terms of early seedling growth and development among the genotypes in response to drought stress. This genotypic variability can be a key breeding tool for improvement of chickpea genotypes against drought.

Table 1. Effect of progressive dehydration on various seedling traits among 64 chickpea genotypes. Data are means ± SE (n=4).

No.	Genotype MCC*	Shoot length (cm)		Leaves (no.)		Shoot dry weight (g)		Root length density ($cm.cm^{-3}$)		Average root diameter (mm)		Root dry weight (g)	
		mean	SE (±)	mean	SE (±)	mean	SE (±)	mean	SE (±)	mean	SE (±)	mean	SE (±)
1	10	6.28	±1.08	3.75	±0.85	0.046	±0.019	0.126	±0.023	0.46	±0.18	0.011	±0.003
2	20	6.73	±0.70	4	±0.91	0.078	±0.039	0.127	±0.024	0.63	±0.04	0.013	±0.003
3	29	7.83	±1.16	3.5	±0.65	0.074	±0.029	0.175	±0.029	0.71	±0.09	0.018	±0.006
4	32	10.38	±1.55	5	±0.91	0.130	±0.050	0.152	±0.017	0.51	±0.06	0.015	±0.003
5	34	8.00	±1.02	4	±0.91	0.055	±0.012	0.130	±0.017	0.67	±0.04	0.012	±0.004
6	49	8.00	±1.08	3.5	±0.65	0.050	±0.018	0.174	±0.025	0.57	±0.10	0.015	±0.002
7	59	8.05	±0.70	3.5	±0.65	0.046	±0.017	0.102	±0.009	0.67	±0.03	0.012	±0.003
8	88	7.33	±1.12	4.75	±0.75	0.068	±0.028	0.245	±0.024	0.56	±0.12	0.018	±0.004
9	96	5.88	±0.32	4.5	±0.87	0.043	±0.013	0.147	±0.022	0.61	±0.09	0.013	±0.003
10	100	6.43	±0.89	4.75	±0.48	0.049	±0.020	0.104	±0.010	0.62	±0.11	0.011	±0.003
11	104	4.88	±0.72	3.75	±0.48	0.028	±0.014	0.281	±0.018	0.46	±0.09	0.011	±0.003
12	122	8.50	±1.24	4.75	±0.85	0.061	±0.022	0.202	±0.032	0.58	±0.09	0.013	±0.005
13	125	5.55	±0.76	2.75	±0.48	0.031	±0.016	0.084	±0.014	0.73	±0.08	0.010	±0.002
14	148	8.00	±0.65	5.5	±0.50	0.047	±0.002	0.224	±0.026	0.43	±0.04	0.015	±0.002
15	155	8.63	±0.90	5.25	±0.85	0.058	±0.019	0.163	±0.025	0.71	±0.13	0.016	±0.003
16	187	5.75	±0.60	3.25	±0.85	0.047	±0.025	0.180	±0.040	0.37	±0.24	0.012	±0.006
17	195	5.75	±0.72	3.5	±0.65	0.027	±0.012	0.090	±0.013	0.59	±0.10	0.006	±0.003
18	198	8.05	±1.14	4.25	±0.75	0.054	±0.020	0.353	±0.049	0.44	±0.07	0.023	±0.007
19	205	8.38	±0.88	4	±0.91	0.058	±0.017	0.100	±0.020	0.72	±0.05	0.012	±0.003
20	207	5.00	±0.54	3.75	±0.48	0.027	±0.006	0.077	±0.014	0.58	±0.05	0.008	±0.000
21	212	3.63	±0.24	3.5	±0.29	0.026	±0.010	0.083	±0.018	0.54	±0.18	0.010	±0.005
22	291	5.48	±0.74	3.5	±0.96	0.049	±0.024	0.154	±0.025	0.37	±0.13	0.013	±0.006
23	320	13.60	±1.31	5.5	±0.65	0.072	±0.011	0.252	±0.039	0.47	±0.08	0.016	±0.002
24	335	6.70	±0.72	3.5	±0.65	0.034	±0.015	0.079	±0.010	0.56	±0.12	0.007	±0.002
25	364	8.45	±0.75	5.5	±0.65	0.063	±0.020	0.109	±0.016	0.68	±0.06	0.016	±0.002
26	368	9.38	±1.09	4.25	±0.63	0.044	±0.008	0.074	±0.013	0.64	±0.08	0.008	±0.002
27	376	7.63	±0.66	4.75	±0.48	0.041	±0.016	0.098	±0.012	0.62	±0.05	0.009	±0.004
28	382	7.00	±0.56	4.5	±0.65	0.048	±0.012	0.099	±0.012	0.61	±0.11	0.011	±0.002
29	385	10.78	±0.48	5.25	±0.48	0.062	±0.012	0.151	±0.020	0.53	±0.05	0.016	±0.003
30	394	8.18	±0.74	4.25	±0.48	0.052	±0.016	0.107	±0.016	0.56	±0.05	0.013	±0.002
31	408	8.00	±0.74	5	±0.58	0.050	±0.011	0.142	±0.025	0.56	±0.05	0.013	±0.003
32	411	9.63	±0.94	5.25	±0.63	0.048	±0.004	0.146	±0.017	0.48	±0.11	0.014	±0.001
33	416	6.58	±0.67	4.5	±0.65	0.049	±0.011	0.131	±0.019	0.57	±0.08	0.016	±0.004
34	418	12.88	±1.40	6.5	±0.65	0.092	±0.009	0.258	±0.031	0.48	±0.05	0.024	±0.003
35	425	13.50	±1.22	6.5	±0.65	0.084	±0.011	0.231	±0.038	0.53	±0.07	0.018	±0.004
36	430	7.60	±1.05	5.25	±0.63	0.045	±0.010	0.132	±0.015	0.34	±0.07	0.006	±0.001
37	433	5.50	±0.74	3.5	±0.65	0.033	±0.015	0.077	±0.015	0.66	±0.03	0.009	±0.002
38	435	11.13	±1.31	4.5	±0.65	0.060	±0.009	0.130	±0.027	0.57	±0.01	0.014	±0.002
39	436	2.25	±0.25	1.5	±0.29	0.014	±0.008	0.065	±0.012	0.56	±0.20	0.006	±0.003
40	437	10.15	±0.46	5.25	±0.63	0.059	±0.013	0.213	±0.043	0.58	±0.11	0.014	±0.005
41	438	12.35	±0.56	6.25	±0.48	0.083	±0.012	0.352	±0.041	0.45	±0.07	0.019	±0.001
42	439	10.18	±0.46	5.75	±0.48	0.058	±0.007	0.178	±0.035	0.57	±0.10	0.014	±0.004
43	440	12.13	±0.52	5.75	±0.25	0.061	±0.004	0.177	±0.033	0.44	±0.08	0.012	±0.002
44	539	11.50	±0.65	5.5	±0.65	0.078	±0.029	0.190	±0.024	0.65	±0.05	0.024	±0.006
45	540	11.43	±0.69	6.75	±0.48	0.076	±0.008	0.198	±0.035	0.51	±0.03	0.018	±0.002
46	555	7.33	±1.04	3.5	±0.65	0.052	±0.028	0.125	±0.021	0.74	±0.08	0.012	±0.004
47	560	9.63	±0.97	5.75	±0.75	0.064	±0.012	0.201	±0.025	0.53	±0.04	0.017	±0.004
48	567	9.13	±1.21	4	±0.41	0.057	±0.027	0.150	±0.037	0.61	±0.08	0.015	±0.005
49	576	11.50	±0.94	5.5	±0.65	0.075	±0.014	0.171	±0.029	0.65	±0.06	0.017	±0.003
50	584	3.38	±0.55	2.5	±0.50	0.030	±0.023	0.104	±0.015	0.31	±0.19	0.009	±0.006
51	634	7.80	±0.73	3.75	±0.48	0.044	0.015	0.146	±0.023	0.51	±0.09	0.010	±0.003
52	639	8.08	±0.67	4.25	±0.48	0.048	±0.012	0.255	±0.037	0.41	±0.12	0.012	±0.002
53	643	5.13	±0.31	4.25	±0.63	0.047	±0.020	0.168	±0.033	0.63	±0.07	0.019	±0.003
54	649	3.25	±0.32	2.25	±0.48	0.026	±0.009	0.056	±0.002	0.74	±0.04	0.012	±0.000

Continuation of Table 1. Effect of progressive dehydration on various seedling traits among 64 chickpea genotypes. Data are means ± SE (n=4).

No.	Genotype MCC*	Shoot length (cm)		Leaves (no.)		Shoot dry weight (g)		Root length density (cm.cm^{-3})		Average root diameter (mm)		Root dry weight (g)	
		mean	SE (±)	mean	SE (±)	mean	SE (±)	mean	SE (±)	mean	SE (±)	mean	SE (±)
55	680	8.50	±1.10	4.75	±0.85	0.059	±0.017	0.173	±0.027	0.55	±0.05	0.017	±0.006
56	755	0.70	±0.44	1	±0.58	0.005	±0.005	0.086	±0.014	0.77	±0.15	0.008	±0.002
57	829	9.50	±0.96	4.5	±0.29	0.056	±0.021	0.142	±0.029	0.59	±0.05	0.013	±0.004
58	864	4.83	±0.46	3.75	±0.48	0.032	±0.006	0.174	±0.029	0.59	±0.13	0.011	±0.002
59	872	3.58	±0.33	2.75	±0.48	0.030	±0.012	0.070	±0.009	0.59	±0.08	0.010	±0.001
60	884	1.83	±0.64	0.75	±0.25	0.004	±0.004	0.019	±0.002	0.18	±0.18	0.002	±0.002
61	897	5.50	±0.65	2.5	±0.29	0.033	±0.019	0.088	±0.010	0.23	±0.14	0.008	±0.004
62	914	4.20	±0.62	2.25	±0.25	0.022	±0.010	0.072	±0.012	0.46	±0.19	0.006	±0.003
63	917	11.25	±0.66	5.25	±0.48	0.065	±0.009	0.152	±0.015	0.52	±0.04	0.012	±0.002
64	931	6.38	±0.83	4.5	±0.65	0.034	±0.012	0.072	±.0010	0.74	±0.03	0.010	±0.001

* MCC: Mashhad Chickpea Collection

Correlation analyses indicated that among different chickpea genotypes, shoot length had significantly the highest correlation with number of leaves (0.87**), followed by shoot dry weight (0.82**). However, root length density was negatively correlated with average root diameter (Table 2); Shoot dry weight was positively correlated with root length density, root dry weight and number of leaves as well ($p\leq0.01$). Interestingly, leaf number was also correlated significantly with two important root parameters i.e. root dry weight (0.70) and root length density (0.60) which may imply the importance of leaf surface in terms of photosynthesis for plant root growth and development.

Table 2. Correlation values between each pair of seedling growth parameters among different genotypes of chickpea at 28 DAS seedlings under water deficit stress condition.

	Shoot length (cm)	Shoot dry weight (g)	Root length density (cm.cm^{-3})	Average root diameter (mm)	Root dry weight (g)	Leaves (no.)
Shoot length (cm)	1					
Shoot dry weight (g)	0.822**	1				
Root length density (cm.cm^{-3})	0.576**	0.561**	1			
Average root diameter (mm)	0.008	0.056	-0.227	1		
Root dry weight (g)	0.646**	0.738**	0.729**	0.150	1	
Leaves (no.)	0.870**	0.759**	0.603**	0.034	0.669**	1

**. Correlation is significant at the 0.01 level

The relationships among various seedling traits of 64 chickpea genotypes in response to progressive water deficit are graphically displayed in the biplot of two main principal components in Fig. 1. Overall, 81.8% of the total variations between traits were justified using the first and second components, with first principal component (PC1) accounting for 63.31% and PC2 for 18.49% of the total variation. Interrelationships among the different traits can be inferred from the cosine of the angle between the vectors in the biplot diagram. Two axes separated average root diameter in a single group and other factors (leaves number, shoot length, root length density, root dry weight and shoot dry weight) in another group. The biplot vectors for the shoot growth traits, shoot length and dry weight remained between the root dry weight and root density vectors, indicating that these indices are very similar for drought selection (Fig. 1).

Hierarchical cluster analysis of genotypes using an unweighted pair group method with arithmetic mean (UPGMA) procedure based on Euclidean distance, separated 64 studied genotypes into three major groups (Fig. 2). The Mantel test indicated that the cophenetic matrix of UPGMA cluster dendrogram was moderately high (r=0.73) and provides a good representation of the similarity matrix.

The results of the dendrogram from UPGMA cluster analysis (Fig. 2) were consistent with those of biplot analysis (Fig. 1). The genotypes with the highest shoot and root length and dry weight were grouped in one cluster (Fig. 2- cluster B) and therefore these genotypes can be considered to be the most desirable in response to drought conditions. While the susceptible genotypes with low seedling growth values grouped in the one cluster (Fig. 2- cluster C).

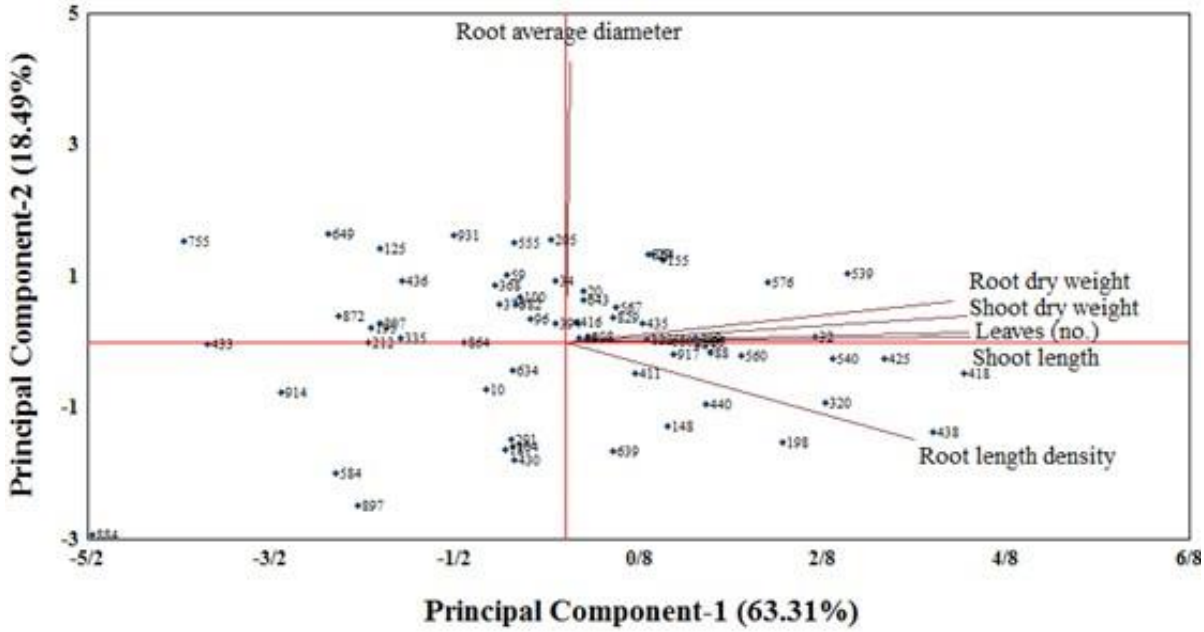

Figure 1. Biplot diagram of two main principal components for different seedling traits among 64 chickpea genotypes in response to water deficit stress. Numbers in the plot area correspond to the genotypes serial numbers in the Mashhad Chickpea Collection (MCC).

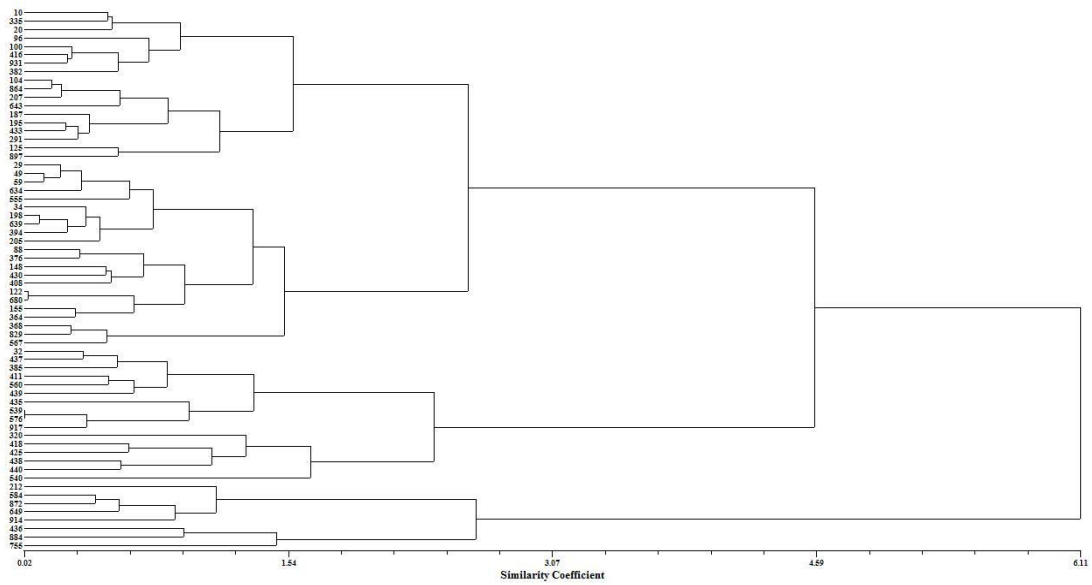

Figure 2. Dendrogram from UPGMA cluster analysis of genotypes based on the different seedling traits in response to water deficit stress. Numbers in Y-axis correspond to the genotypes serial numbers in the Mashhad Chickpea Collection (MCC).

3.2. Experiment 2: Contrastive genotypes re-evaluation

Based on the overall and initial results of genotype screening for dehydration stress from different shoot and root traits in the experiment 1, 10 chickpea genotypes differing in dehydration tolerance including eight more tolerant genotypes (MCC320, MCC418, MCC425, MCC438, MCC539, MCC540, MCC560, MCC576) and two of dehydration-susceptible ones (MCC433 and MCC897), were selected as representatives of each class for more precise investigation in the experiment 2 (Table 1 and Fig. 3).

Highly significant genotypic differences were observed for all indices in this experiment ($p \leq 0.05$ and $p \leq 0.01$). Biochemical and physiological traits indices were subjected to summary statistical analysis. Data analysis from progressive dehydration treatment resulted in increased proline content of leaves in all genotypes with mean value 14.37 $\mu molg^{-1}$ fresh weight within the range of 4.7 to 33.8 $\mu molg^{-1}$ fresh weight (Fig. 3a). The highest value for PCSI (proline content stressed to tolerance index) was 443.5 % and the lowest was 204.5 % in MCC418 and MCC433 genotypes, respectively (Fig. 3b).

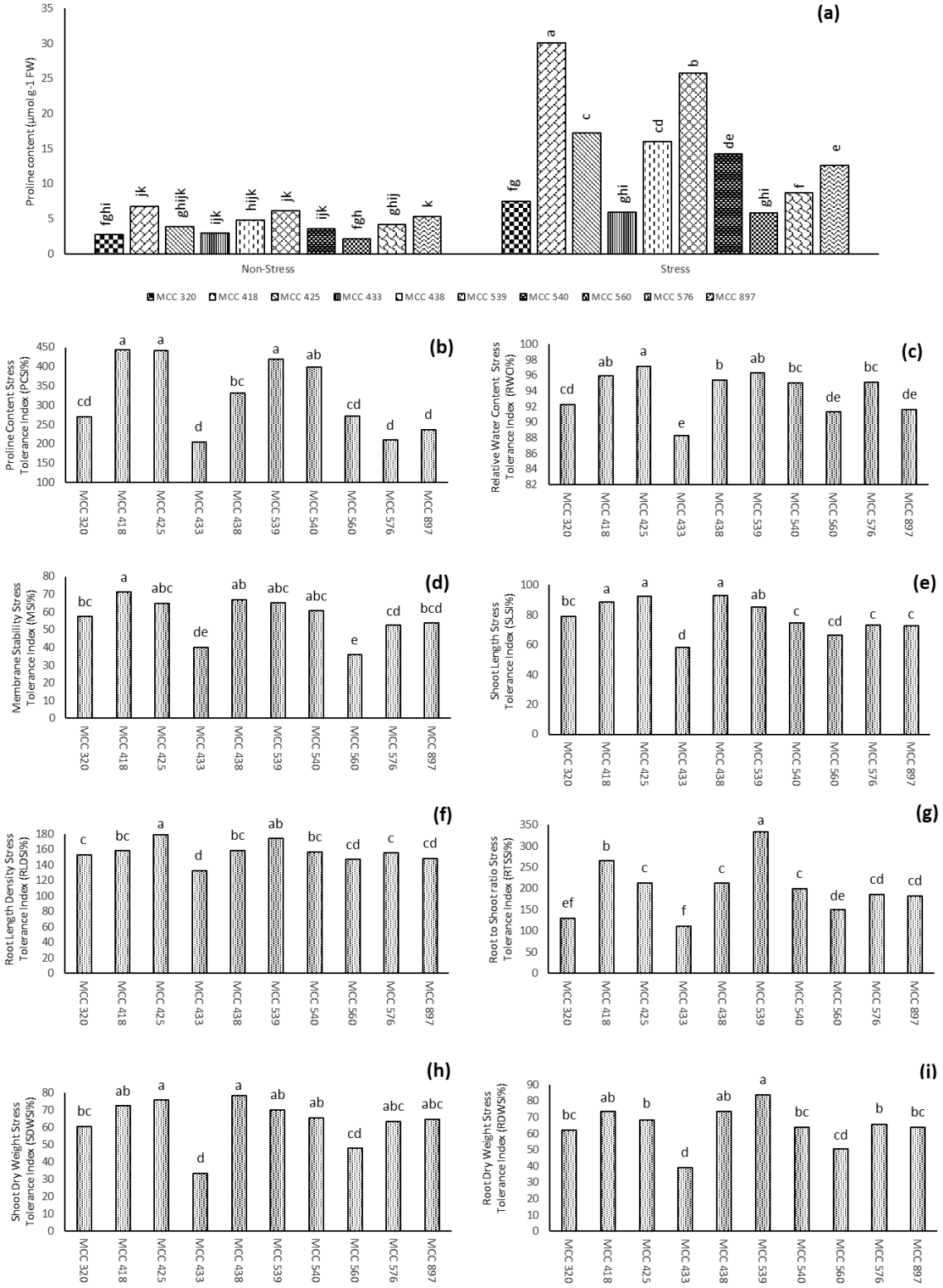

Figure 3. The physiological and biochemical response of selected chickpea genotypes under drought stress. a) Proline content changes among genotypes under stress. b) Proline content stress tolerance index (PCSI), c) Relative water content stress tolerance index (RWCSI), d) Membrane stability stress tolerance index (MSSI), e) Shoot length stress tolerance index (SLSI), f) Root length density stress tolerance index (RLDSI), g) Root to shoot ratio (dry weight basis) stress tolerance index (RTSSI), h) Shoot dry weight stress tolerance index (SDWSI) and i) Root dry weight stress tolerance index (RDWSI). Means that columns with at least one letter in common in the range are not significantly different based on Duncan's multiple range test (p≤0.05).

The relative water content mean across all studied chickpea genotypes was 67.57%. MCC 425 had higher mean value 71.66% with an RWCSI value of 97.18% followed by MCC418 with RWC and RWCSI values of 69/83 and 95.92, respectively. But, the minimum observed RWC value was 62.21% in MCC433 with

RWCSI equivalent to 88.33% (Fig. 3c). Among all genotypes, MCC418 showed relatively less decrease in membrane stability percentage compared to control under dehydration stress, ranging from 18.75 % to 41.92 % among genotypes. Maximum and minimum MSSI values were 71.39% in MCC418 and 35.93% in MCC560, respectively (Fig. 3d).

Significant ($p \leq 0.05$) difference was observed in shoot length of all genotypes under dehydration stress conditions. Genotype MCC425 had the highest (17.12cm, 92.57%) whereas MCC433 had the minimum shoot length and SLSI (7cm, 58.33%) (Fig. 3e and 4).

The mean root length density reached a higher value of 1.27 (cm/cm3) in MCC539 under dehydration stress conditions, but the highest value of the root length density stress tolerance index (RLDSI) was achieved in MCC425 with 178%. In contrast, the lowest value (0.238 cm/cm3 equivalent to 132.8% RLDSI) was observed in MCC433 genotype (Fig. 3f). Root/shoot ratio (based on dry weight) also revealed great range of variation. MCC539 exhibited significantly ($p \leq 0.05$) higher value of RTSSI (333.69%) among all genotypes and MCC433 had lower value (110.49) in response to stress (Fig. 3g).

Genotype MCC539 also hit the highest shoot dry weight (0.28 g) and root dry weight (0.083 g), and the MCC433 was the lowest (0.07g and 0.02g, respectively) under drought conditions. MCC438 and MCC539 genotypes had also the largest value of 78.37 and 83.59 percent in SDWSI and RDWSI, respectively. The lowest value in both parameters (33.26 and 39.26 percent, respectively) was observed in MCC433 genotype (Fig. 3h and 3i).

Figure 4. Representative growth view of chickpea seedlings (45 days old) in the extreme genotypes after two weeks from imposing progressive drought stress.

Roots are primarily exposed to drought stress and in response reduce drought effects by extracting higher soil moisture (Cutforth *et al.*, 2013; Varshney *et al.*, 2014). Various studies demonstrated that root traits, such as root biomass and root length density play an important role in chickpea drought tolerance (Kashiwagi *et al.*, 2006; Serraj *et al.*, 2004; Varshney *et al.*, 2011; Varshney *et al.*, 2014). Additional statistical parameters like genotypic means and standard error of mean for various seedling traits were also given in Table 1.

Understanding the wide range of plant responses (such as physiological, biochemical, and ecological) to drought stress is absolutely necessary for better stress management (Fahad *et al.*, 2017; Lesk *et al.*, 2016). To avoid adverse effects of drought stresses on plants, development of drought-tolerant cultivars is the most effective way (Shah *et al.*, 2020). Drought tolerance studies in chickpea showed that germplasm screening based on the physiological and biochemical parameters is required for effective crop growth and productivity improvements (Hosseinzadeh *et al.*, 2018; Kashiwagi

et al., 2006; Ramamoorthy *et al.*, 2016; Shah *et al.*, 2020; Talebi *et al.*, 2013). In the current study, the biplot diagram based on various traits could differentiate genotypes MCC320, MCC418, MCC425, MCC438, MCC539, MCC540, MCC560 and MCC576 as tolerant, and also genotypes MCC433 and MCC897 as sensitive to water deficit stress at seedling stage under controlled condition (Fig. 1).

The amplitude of proline as an osmolyte, a free radical scavenger, nitrogen-storage compound and a hydrophilic protectant for enzymes and cellular structures in plants, is a major metabolic acclimation mechanism against abiotic stress condition (Filippou *et al.*, 2013; Kaur *et al.*, 2017; Khan *et al.*, 2015). Proline plays a substantial function in water deficit stress tolerance, and increases in leaves at both vegetative and reproductive stages (Mafakheri *et al.*, 2010). Better accumulation of the proline facilitates maintaining the tissue water content and membrane stability, resulting in improved drought resistance (Farooq *et al.*, 2009; Nezhadahmadi *et al.*, 2013). In the present study, water deficit caused proline content to be more than 4-fold higher relative to the control (based on the PCSI index) in MCC418, MCC425 and MCC539, as well as about 2-fold increase compared with the genotypes containing lowest amount of proline. Stronger defense response of these genotypes to the dehydration stress than other genotypes, through higher level of proline accumulation, might be an indication so that they can be considered tolerant candidate genotypes against the stress. Furthermore, the lowest proline accumulation was significantly ($p \leq 0.05$) observed in MCC433 which was also indicated as susceptible candidate genotype according to other indices as well.

Based on the studies on chickpea response to water deficit stress it has been approved that electrolyte leakage augmented under drought stress conditions as result of damage to the cell walls caused by the plant temperature increase (Keerthi Sree *et al.*, 2023; Pouresmael *et al.*, 2013). Also, during seedling and early flowering stages, membrane stability and relative water content of tolerant genotypes remain higher than susceptible ones in response to decreasing water availability conditions, being consistent with our observation (Rahbarian *et al.*, 2011; Talebi *et al.*, 2013). Relative decrease in shoot elongation compared with root growth leads to enhancement in the root-to-shoot ratio in chickpeas under drought conditions (Kumar *et al.*, 2012; Ramamoorthy *et al.*, 2017).

Similar studies (about shoot dry weight and root dry weight) confirmed that genotypes having values for growth indices equal to 100, were showing less sensitivity to stress conditions, whereas values for growth indices near zero reflected the highest sensitivity in response to stress (Ramamoorthy *et al.*, 2016; Shah *et al.*, 2020). In the present study, evaluation of different morphological and physiological traits in the early growth stages of chickpea genotypes revealed the significant variations in these traits and proved that these may be an effective tool for assessing drought resistance. These results are consistent with the findings presented in the previous investigation (Bibi *et al.*, 2009; Maqbool *et al.*, 2017).

4. Conclusion

The present investigation revealed a high variation in growth characteristics and traits among Desi-type chickpea genotypes under progressive water deficit stress conditions. Also, there was a clear distinction in some physiobiochemical traits among these genotypes, explaining their tolerance responses to drought stress. Tolerant candidate genotypes selected from large numbers in the initial experiment also showed higher values of seedling growth characters, proline content, RWC, membrane stability and root-to-shoot ratio in the second experiment in comparison to susceptible candidates. Based on biplot diagram and clustering analysis in the first experiment (Fig. 1 and 2), and also various tolerance indexes in the second experiment (Fig. 3) collectively, genotypes MCC425, MCC438, MCC418, MCC539 were found to be high drought tolerant in the seedling stages, and MCC433 was sensitive one. Using these succeeding experiments, we could delegate the preliminary reaction of the genotypes to drought stress, and also narrow down the number of contrastive genotypes under controlled conditions, but these findings should be authenticated under real drought conditions at mature plants in the field. For this purpose, the selected genotypes will be further evaluated for their response to real drought conditions in the field experiments.

Conflict of interests

All authors declare no conflict of interest.

Ethics approval and consent to participate

No human or animals were used in the present research.

Consent for publications

All authors read and approved the final manuscript for publication.

Availability of data and material

All the data are embedded in the manuscript.

Authors' contributions

All authors had an equal role in study design, work, statistical analysis and manuscript writing.

Informed consent

The authors declare not to use any patients in this research.

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