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Evaluation of Introgressed Lines of Sunflower (*Helianthus annuus* L.) under Contrasting Water Treatments

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Abstract: Drought stress is a major production constraint in crops globally. Crop wild relatives are important sources of resistance and tolerance for both biotic and abiotic stresses, respectively. A breeding program was initiated to introgress drought tolerance in sunflowers through hybridization between the wild species *Helianthus argophyllus* and the cultivated pool of *H. annuus*. Selection was carried out from the F_2 to F_5 segregating populations for the silver canopy, high cuticular wax, small leaf area, single heading and high oil content. Cuticular wax ranged between 8.72 µg g^{-1} and 17.19 µg g^{-1} in the F_5 offspring. The selected F_5 breeding lines were self-pollinated to obtain the F_6 generation. Thereafter, this F_6 was compared with the non-adapted elite sunflower germplasm in a factorial complete randomized design with different water treatments; i.e., comparing fully irrigated (100%, T0) versus 75% (T1), 50% (T2) and 25% (T3) of total irrigation. The comparison between the two types of the germplasm showed that drought-tolerant breeding lines had a comparatively lesser decrease in leaf area (0, 11, 22%) and shoot length (4, 21, 28%) than the elite germplasm, which experienced a decrease in leaf area (21%, 33% and 40%) and shoot length (17, 27 and 34%) under the various drought treatments. Moreover, drought-tolerant breeding lines had 100% more root shoot ratios than the elite germplasm (20%) in T3 when compared with control. Several drought-tolerant promising lines (D-2, D-5 and D-27) were selected due to their high leaf area, great root length and increased root to shoot ratio under T3. Some of the lines could be directly used for the development of drought-tolerant hybrids. Combining ability testing indicated that D-27 (F_7) was a good general combiner for seed yield plant^{-1} and oil content after mating with male-line RSIN.82. Resulting hybrids could help to minimize seed yield loss due to water stress and to achieve profitable cultivation of sunflowers in arid regions of Pakistan.

Keywords: drought stress; introgression; leaf area; root to shoot ratio; wild species; yield loss

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1. Introduction

Drought is one of the major production constraints worldwide and a key yield limiting factor accounting for at least a 70% yield loss worldwide [1]. Hence, improvement of crop responses to this particular abiotic stress continues to be a major objective. Development of drought-tolerant cultivars is a cost-effective option for field crops [2].

The sunflower (*Helianthus annuus* L.) is an important oilseed crop that ranks sixth among them [3]. Yields have been threatened by various biotic and abiotic stresses. Among the stress factors, water scarcity has a significant impact on sunflowers [4]. For example, sunflower seed yield and oil content were about 16 and 22% lower in field trials under water stress over 3 years and four irrigational treatments, respectively [5,6]. However, sunflowers had a higher yield loss than other oilseed crops such as the safflower and sesame due to a decrease in seed mass and capitula number [5]. Drought-tolerant sunflower hybrids reduced a yield loss due to overaccumulation of osmotica such as proline, which occurred at the cost of plant energy [6]. Development of drought-tolerant inbred lines is therefore one of the priority breeding objectives for sunflowers. Genetic variability amongst the sunflower and experimental hybrids has shown that the improved germplasm for drought tolerance may be developed through selection [7–9]. Introgression of traits such as high cuticular wax, leaf hairiness and maintenance of the root shoot ratio tends to decrease the water loss through leaf transpiration, thus enabling plants to remain hydrated under stress [8,10].

Introgression of drought-tolerant genes in crop species is one of the key germplasm enhancement methods used to improve the elite germplasm [7,9]. Wild species are important sources of stress-tolerant genes [6]. *Helianthus argophyllus* is known to bear drought-tolerant as well as powdery mildew and charcoal rot-resistant genes [9–13]. This wild relative shows drought adaptability traits such as a small leaf area, high cuticular wax and intense hairiness, which are known to improve tolerance against drought stress [10,12]. These traits help to reduce the transpiration loss, thus inducing a water saving mechanism in sunflowers to maintain plant turgor under water stress [10,12].

Wild species are sources of resistant novel genes in the sunflower accessions and several molecular tools are utilized to identify genetic variation in drought-related traits. Moreover, quantitative trait loci (QTL) related to complex traits were found using simple sequence repeats (SSR), inter-simple sequence repeats (ISSR) or the inter-retrotransposon amplified polymorphism (IRAP) under water stress in sunflowers [14]. Furthermore, transcriptomics and metabolomics may assist to understand the basis of drought tolerance in the wild and cultivated germplasm [14,15]. Wild species may not be directly taken into the cultivation, and introgression may be required to transfer useful genes into the cultigen pool. Sunflower breeding may use gene editing through CRISPR/Cas9 to remove several undesirable traits from the genome of wild species [16].

The aim of our research was to develop sunflower drought-tolerant breeding material through introgression from wild species through selection of traits such as a small leaf area, hairiness and cuticular wax. These traits may reduce transpiration losses in sunflowers and improve survival of cultivated lines under water stress. The drought-tolerant germplasm can be further used for the development of hybrids or to improve elite sunflower breeding populations.

2. Materials and Methods

2.1. Development of Drought-Tolerant Breeding Lines

The College of Agriculture of the University of Sargodha began sunflower breeding for drought-prone environments in 2014. Crossings were made between *H. argophyllus* accessions ARG-1802, ARG-1805 and ARG-1806 and *H. annuus* lines CMS-10 and CMS-24. Offspring from CMS-10 × ARG-1802, CMS-10 × ARG-1805, CMS-10 × ARG-1806, CMS-24 × ARG-1802, CMS-24 × ARG-1805 and CMS-10 × ARG-1806 became available. *H. argophyllus* accessions were initially planted in the pot and later transferred to the field at plant-to-plant distance of 30 cm. Afterwards, two elite lines (CMS-10 and CMS-24) belonging to *H. annuus* were planted at 20-day intervals to synchronize flowering with the species. F_1 seeds were harvested in 2015 and sown in the field in August 2015 to obtain F_2 seed (Figure 1). The F_2 populations of 412 plants were planted in February 2016. However, only 72 plants were selected and self-pollinated by bagging their capitula to grow and the resulting F_3 offspring was evaluated (Figure 1). The F_3 populations were re-sown in August 2016 and the resulting offspring was compared for silver canopy traits, seed setting, pollen fertility and oil content. Among the F_3 offspring, 52 of them were the best and selected. Within each progeny, single best plants were self-pollinated to obtain F_4 progenies (Figure 1). Offspring with good pollen fertility and single heading were self-pollinated. Plants with better oil content were selected thereafter, and the F_4 was evaluated during February 2017. These selections were evaluated in the field for single heading type, leaf cuticular wax, leaf hairiness, leaf area, oil content (%) and days to maturity. Thirty-one lines with small leaf area, high cuticular waxes, small seed size and high oil content were selected and their seed was harvested to evaluate the F_5. The breeding lines were self-pollinated to develop the F_6 offspring used in this study (Figure 2).

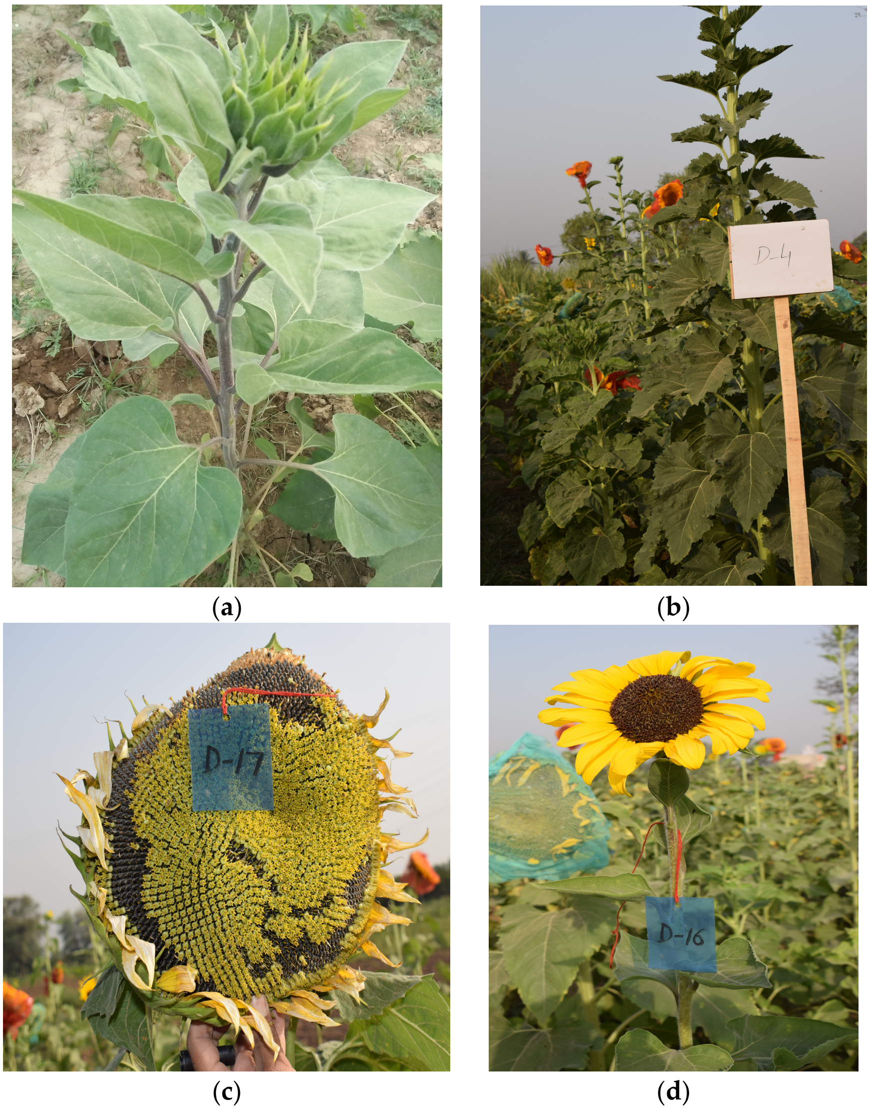


Figure 1. (**a**) Introgressed F_5 plant with silver canopy with intense hairiness and small leaf area; (**b**) D4 line with introgression traits but undesirable plant height; (**c**) head development in D-17 of F_7 population following combining ability test; and (**d**) peculiar drought-tolerant phenotype of D-16 breeding line with silver canopy.

2.2. Evaluation of Drought-Tolerant Plant Selections under Contrasting Water Treatments in Polythene Bags (Figure 3)

Thirteen F_6 drought-tolerant breeding lines were compared against randomly selected sunflower elite lines along with two hybrids (Hysun 33, FH 331) as susceptible checks (Table 1). Polythene bags (20 cm × 45 cm) were filled with equal amount of sand and loam soil. Farmyard manure (5%) was added to improve the texture and water holding capacity of the soil. Each bag was filled with 30 kg of soil and brought to a field capacity at 18%. At planting, 2 g of urea was applied to raise soil fertility. The soil moisture content in the control treatment was kept at field capacity by regularly irrigating each polythene bag. Four contrasting water regimes were obtained by differential irrigation of the polythene bags. The control treatment (T0) involved a total of 14.5 L of water in 8 split irrigations.

Irrigation treatments were 75% (T1), 50% (T2) and 25% (T3) of the control. Soil moisture content was measured through the gravimetric method [17]. All treatments were otherwise treated the same with a constant air temperature of 25 ± 2 °C, relative humidity set at 40%, day length at 14 h and photosynthetic active radiation of 850 µmol m^{-2} s^{-1}. There were three replications and 10 plants for each accession and water treatment. Two seeds were sown in each bag, which was thinned to single seedling. Temperature and soil moisture content was regularly monitored throughout the growing phase.

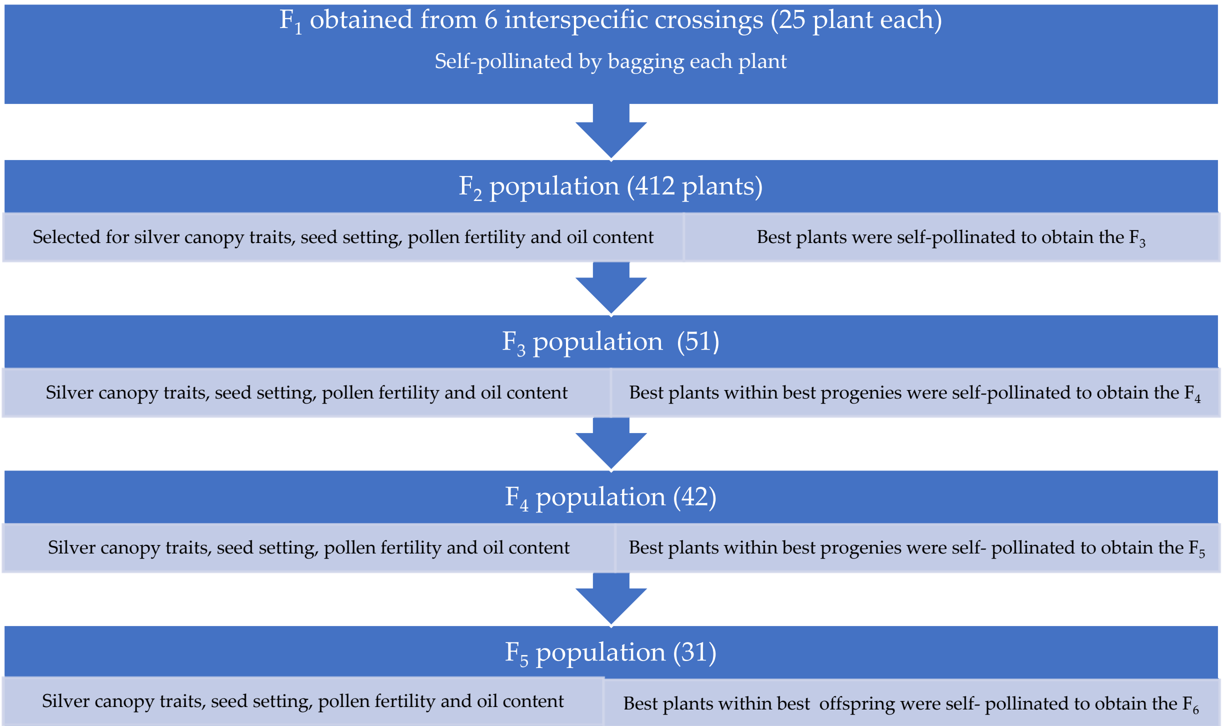


Figure 2. Steps taken for developing drought-tolerant sunflower breeding lines.

All plants were evaluated for the following traits:

1. Leaf area: It was determined using a CID Bio-Science CI-202 portable leaf area meter. Leaf area of three leaves from each plant was used to determine the average leaf area $plant^{-1}$. There were five plants within each replication.
2. Root length (cm): Polythene bags were gently dissected and roots were washed with constant gentle water pressure to avoid breakage of roots. The roots were dried to remove any surface moisture and the primary root length was measured.
3. Shoot length (cm): It was determined from the base to the tip of the meristematic tissues.
4. Fresh root and shoot biomass: The above ground shoot biomass and root biomass of five plants within each replication were recorded.
5. Root shoot ratio: The root biomass was divided by shoot biomass to estimate the root shoot ratio.
6. Drought resistance index (DRI): It was calculated using the following formula:

$$DRI = (LAS/LANS)/(LAGS/LAGNS)$$

where LAS is the leaf area under stress, LANS is the leaf area under non-stress, LAGS is the average leaf area of all genotypes under stress and LAGNS is the average leaf area of all genotypes under non-stress.

7. Cuticular wax: It was determined following work by Hussain et al. [10]. Leaf disc of 8 cm^2 was obtained from 15-day-old leaf from top of canopy. Adaxial leaf discs

were dipped in chloroform for 15 s at 25 °C. Extract was filtered and chloroform was evaporated. About 5 mL of reagent was prepared using 20 g of potassium dichromate dissolved in 40 mL of distilled water. The reagent solution was then mixed in concentrated H_2SO_4 for 1 h. Thereafter, 1 mL of reagent was added to develop chrome. Readings were obtained at optical density of 590 nm and noted. A standard solution was prepared by mixing known concentration of cuticular wax in the solution ranging from 0 to 100 µg L^{-1}.

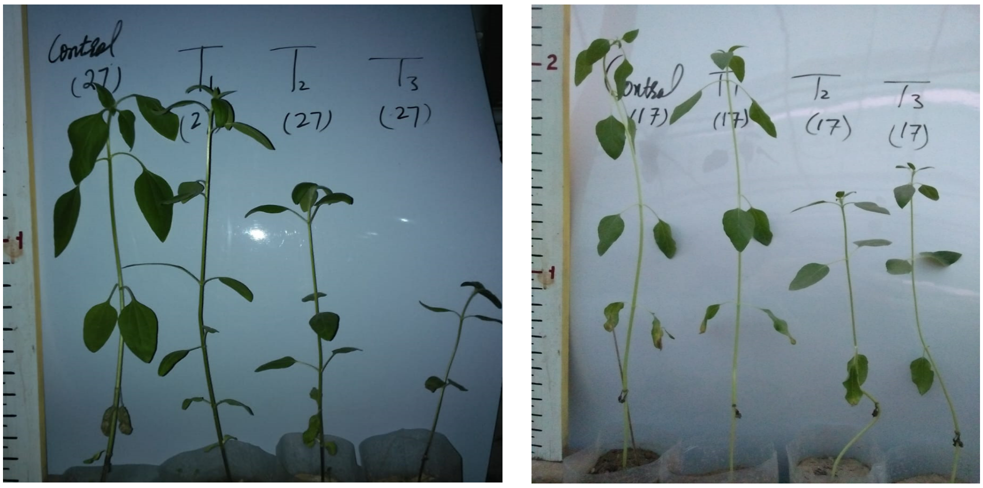
Table 1. List of the breeding material and their main features along with randomly selected elite sunflower populations and advanced lines.

Breeding Material	Status	Oil Content (%)	Days to Flowering	Canopy Color	Cuticular Wax (µg g^{-1})
578874	OPV *	34.12	52.46	Green	3.19
B.224	B-Line **	33.38	50.58	Green	3.3
B.64	B-Line	34.12	50.13	Green	2.8
Pervenent	OPV	35.24	53.39	Green	2.14
B.385	B-Line	40.15	56.34		3.65
B.12	OPV	38.63	52.37	Green	2.13
UCA-1-DR	F_5 Progeny ***	37.83	68.36	Silver	12.13
UCA-2-DR	F_5 Progeny	31.29	64.37	Silver	9.89
UCA-3-DR	F_5 Progeny	35.13	70.35	Silver	13.14
UCA-4-DR	F_5 Progeny	38.17	72.34	Silver	12.16
UCA-5-DR	F_5 Progeny	38.24	70.31	Silver	10.24
UCA-6-DR	F_5 Progeny	36.58	62.36	Silver	17.19
UCA-10-DR	F_5 Progeny	36.13	69.34	Silver	12.34
UCA-11-DR	F_5 Progeny	35.19	70.32	Silver	10.09
UCA-14-DR	F_5 Progeny	28.35	62.32	Silver	11.12
UCA-15-DR	F_5 Progeny	38.91	71.38	Silver	10.16
UCA-16-DR	F_5 Progeny	36.19	73.27	Silver	13.12
UCA-20-DR	F_5 Progeny	35.15	61.29	Silver	8.72
UCA-27-DR	F_5 Progeny	35.23	70.35	Silver	9.12
Hysun 33		34.56	74.35	Green	10.13
FH 331	Hybrid	35.38	70.18	Green	9.34

* OPV = open pollinated variety; ** B-Line = maintainer line in hybrid development; *** F_5 = 5th filial generation.

2.3. Combining Ability Test

Promising F_5 lines were self-pollinated to raise F_6 and F_7 progenies (Figure 2). Few plants within each F_7 progeny (D5, D22, D26, D27) and susceptible lines (B.224 and 577874) were manually emasculated and pollinated with male lines (R.365, R.SIN.82) to test their combining ability for seed yield plant^{-1}, oil content and head diameter under semi-arid conditions. All offspring was sown at two locations (Faisalabad, Sargodha) during spring 2020 with plant-to-plant distance of 22 cm, and row-to-row distance of 75 cm on ridges. Each progeny was sown in three rows, while length of each row was 3 m. The experimental design was a randomized complete block design with three replications. Each field was fertilized with 100 kg ha^{-1} of diammonium phosphate, while pre-emergence herbicide S-metolachlor (dual gold, Syngenta) was sprayed (1 L ha^{-1}) to eliminate weeds. Soils had field capacity of 18%, and single irrigation of canal water was performed to gain uniform seed germination, while both trials were raised on rainfall, which was 160 mm and 170 mm at Faisalabad and Sargodha, respectively. Most of the rainfall fell, however, during the vegetative phase.



(**a**) (**b**)

Figure 3. Response of promising breeding lines (**a**) D-27 and (**b**) D-17 to various water regimes in polythene bag trials.

Seed yield plant^{-1} (g) was determined by harvesting floral heads of 5 plants within each row after maturity. Floral heads were threshed and dried to constant moisture content and mass of each floral head was determined on digital balance. Head diameter was measured with a scale. Means of 5 plants were computed for oil content as determined through soxhlet apparatus. A 10 g seed sample was crushed gently and put in thimble. Oil content was determined using n-hexane as solvent.

2.4. Statistical Analysis

The additive linear model for the analysis of variances was that of a completely randomized block design with two factors (irrigation treatments and breeding lines). Heritability was estimated using the approach outlined by Acquaah [18] in which broad sense heritability was defined as $H = V_G/V_P$, where V_G and V_P are the genetic and phenotypic variances, respectively. Combining ability analysis was performed following what Kempthrone [19] described for assessing breeding values.

3. Results

There was significant ($p \leq 0.05$) variation due to breeding lines, water treatments and the interaction lines × water treatments (Table 2). The magnitude of the sum of squares for the interaction lines × treatments was, however, lower than that of breeding lines, thus indicating that breeding lines in all traits except for leaf area (LA) did not change significantly across water treatments (Table 2). LA showed very high lines × treatments, which suggests that breeding lines changed their relative ranking across contrasting water regimes (Table 2). Heritability estimates for the shoot length (SL) and root to shoot ratio (R/S) were moderately high, showing that breeding lines may be selected in a single treatment for these traits. The root length (RL), shoot weight (SW) and root weight (RW) had low heritability estimates, thereby indicating that selection should be in the target population of the environment for the improvement of these traits.

Mean values, ranges and the phenotypic coefficient of variation (PCV, %) of all traits are given in Tables 3–5. Drought-tolerant breeding lines (DRL) showed the lowest LA under control conditions. Such results may be due to selection for a lower leaf area in the segregating populations. However, the leaf area of DRL was maintained and showed a lower decrease than the non-selected sunflower elite germplasm (SEG) and hybrids in T2 and T3 treatments. DRL experienced a decrease of 0%, 11% and 22% in LA while SEG showed a decrease of 21%, 33% and 40%, under three water stress treatments. Hybrids showed a relative decrease of 18% and 47% for LA. Differences in PCV (%) were lower in DRL, which may be due to the sharing of common parentage and selection for similar traits among DRL. The differences within PCV (%) increased with stress treatments for both types of the germplasm (Table 3).

Table 2. Mean sum of squares and heritability estimates of leaf area (LA, cm^2), root length (RL, cm), shoot length (SL, cm), shoot weight (SW, g), root weight (RW, g) and root shoot ratio (R/S).

Source of Variation	Degrees of Freedom	Mean Sum of Squares					
		LA	RL	SL	SW	RW	R/S
Lines	19	14.81 **	10.54 **	608.08 **	18.67 **	0.07 **	0.03 **
Water regimes (DL)	3	489.57 **	10.14 **	2235.11 **	251.43 **	3.03 **	0.14 **
Lines × DL interaction	57	24.00 **	4.77 **	99.13 **	6.82 **	0.05 **	0.03 **
Residual	160	5.41	1.12	36.47	1.27	0.01	0.00
σ^2 Genotype		0.00	0.64	56.55	1.32	0.00	0.01
σ^2 Environment		5.41	1.12	36.47	1.27	0.01	0.01
σ^2 Phenotype		10.59	2.98	113.91	4.44	0.03	0.03
Broad-sense heritability		0.00	0.22	0.50	0.30	0.10	0.58

LA: leaf area, RL: root length, SL: shoot length, SW: shoot weight, RW: root weight, R/S: root shoot ratio. ** indicates that sources of variation was highly significant at $p < 0.01$.

Table 3. Mean values, ranges and variation for leaf area and root length within drought-tolerant inbred lines compared with elite sunflower germplasm and hybrids under control, low (T1), medium (T2) and high (T3) water stress.

Breeding Lines	Leaf Area (cm^2)				Root Length (cm)			
	Control	T_1	T2	T3	Control	T1	T2	T3
Tolerant inbred lines	22.63b	22.98a	20.60ab	17.72a	6.12b	6.53ab	6.61a	7.19b
Phenotypic coefficient of variation (%)	9.04	11.11	12.90	15.82	13.38	20.82	21.57	20.85
Range	19.13–26.21	19.97–28.57	17.16–25.33	13.50–23.18	5.00–7.37	3.87–8.70	3.60–8.67	4.50–9.89
Elite germplasm	28.71a	22.76a	19.10b	17.22a	6.10b	5.45a	6.63a	6.55b
Phenotypic coefficient of variation (%)	10.16	15.92	12.50	23.37	27.50	19.48	32.36	23.37
Range	23.12–29.56	17.67–27.80	15.04–21.29	15.31–19.36	4.30–8.10	2.83–7.73	4.67–7.90	3.67–9.40
Hybrids	29.12a	24.95a	23.85a	15.58a	7.83a	8.07a	6.93a	9.37a

Means followed by the same letter in the same column indicate that the average trait value for lines, elite germplasm and hybrids is non-significantly different ($p > 0.05$).

Hybrids had the highest LA under controlled treatments, while RL tended to increase with stress treatments. An increase in RL suggests it is a responsive trait. An increase in the root elongation has been noted as a mechanism for water exploration into lower soil profiles (Table 3). PCV (%) increased with drought treatments in DRL while it decreased for the SEG (Table 4).

SEG showed greater PCV than DRL in the control and T3 (Table 3). RW of SEG was the highest under the control, while hybrids showed the highest SW in all treatments. DRL was significantly higher than SEG for SW in all treatments. SEG showed higher PCV (%) under control treatment while DRL showed higher phenotypic variability under water stress treatments as indicated by the PCV (%). SL was the highest for hybrids under all treatments, including the control (Table 5).

Table 4. Mean values, ranges and variation for shoot weight and root weight within drought-tolerant inbred lines compared with elite sunflower germplasm and hybrids under control, low (T1), medium (T2) and high (T3) water stress.

Breeding Lines	Shoot Weight (g)				Root Weight (g)			
	Control	T1	T2	T3	Control	T1	T2	T3
Tolerant inbred lines	7.49a	5.66b	3.61a	2.09a	0.63b	0.29b	0.25a	0.24a
Phenotypic coefficient of variation (%)	33.34	40.41	26.93	30.88	18.91	44.74	40.19	34.69
Range	4.23–11.67	2.57–9.63	2.00–4.93	1.37–3.50	0.47–0.80	0.10–0.56	0.13–0.43	0.13–0.42
Elite germplasm	6.31a	5.26b	3.40a	1.67a	0.83a	0.26b	0.21a	0.23a
Phenotypic coefficient of variation (%)	39.44	38.61	25.12	48.64	34.88	30.94	18.99	29.74
Range	2.93–9.23	3.13–8.00	2.57–4.67	0.45–2.70	0.47–1.27	0.13–0.36	0.16–0.25	0.14–0.42
Hybrids	6.13a	8.00a	4.83a	2.33a	0.58b	0.48a	0.27a	0.29a

Means followed by the same letter in the same column indicate that the average trait value for lines, elite germplasm and hybrids is non-significantly different ($p > 0.05$).

Table 5. Mean values, ranges and variation for shoot length and root to shoot ratio within drought-tolerant inbred lines compared with elite sunflower germplasm and hybrids under control, low (T1), medium (T2) and high (T3) water stress.

Breeding Lines	Shoot Length (cm)				Root to Shoot Ratio			
	Control	T1	T2	T3	Control	T1	T2	T3
Tolerant inbred lines	50.35a	45.55b	39.97b	36.22ab	0.15a	0.05a	0.06a	0.30a
Phenotypic coefficient of variation (%)	17.67	21.25	9.83	10.29	23.21	60.48	69.98	78.56
Range	35.73–65.62	29.17–59.17	35–49.17	27.92–41.67	0.05–0.16	0.03–0.14	0.03–0.17	0.07–0.26
Elite germplasm	48.83a	40.29b	35.56b	32.13b	0.10a	0.06a	0.07a	0.12b
Phenotypic coefficient of variation (%)	25.27	22.16	30.47	35.30	35.45	22.08	30.63	89.71
Range	26.33–60.28	31.83–58.75	15.00–47.08	9.58–46.67	0.11–0.21	0.04–0.07	0.05–0.10	0.07–0.26
Hybrids	51.78a	56.25a	52.83a	45.21a	0.12a	0.07a	0.08a	0.10b

Means followed by the same letter in the same column indicate that the average trait value for lines, elite germplasm and hybrids is non-significantly different ($p > 0.05$).

DRL showed higher values than SEG under stress treatments. PCV (%) was high for SEG while it tended to decrease with stress treatments (Table 5). R/S of DRL was higher than SEG and commercial hybrids. DRL also showed higher PCV (%) than SEG (Table 5).

A biplot analysis of genotype characterization for drought resistance is shown in Figure 4. Breeding lines having a drought resistance index (DRI) equal to or above 1 were regarded as tolerant. The biplot includes the breeding lines in quadrants III and IV showing superior performance under T1, T2 and T3 treatments (Figure 4). A genotype such as D5 was tolerant under mild (T1) water stress treatment. D3, D6, D10, D11 and D14 were tolerant in all treatments, while genotypes such as D15 and D27 showed drought tolerance under high (T2) and medium (T3) stress treatments (Figures 4 and 5).

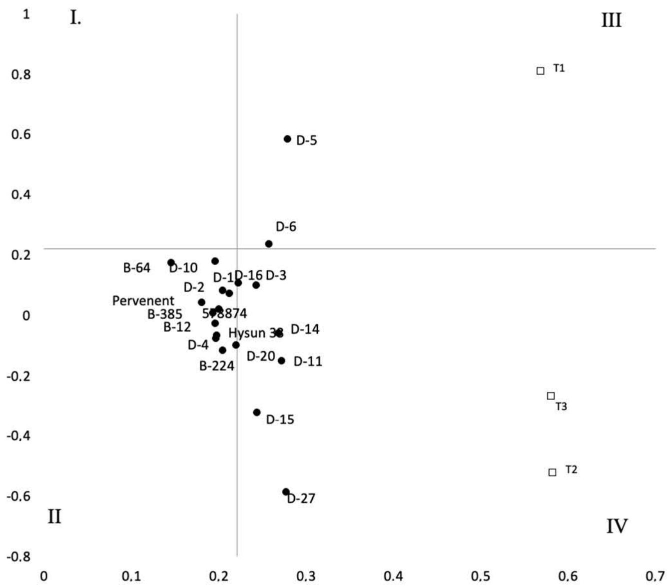


Figure 4. Biplot analysis of drought tolerance index of breeding lines and hybrids due to three levels of water stress (T1 = mild stress, T2 = medium stress, T3 = high stress). Breeding lines were grouped on the basis of four quadrants (I, II, III, IV) in scatter plot.

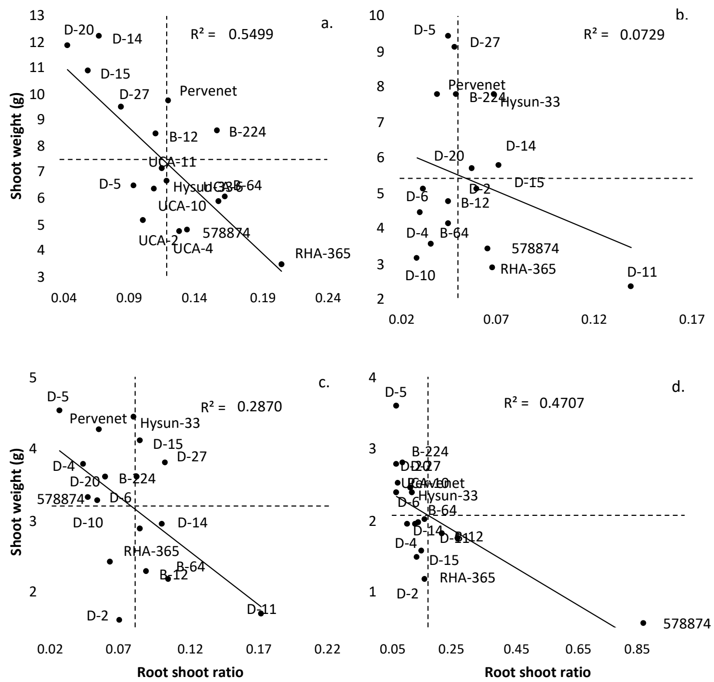


Figure 5. Relationship between the shoot weight and root shoot ratio traits under control (**a**), low water stress treatment (**b**), medium water stress treatment (**c**) and high-intensity water stress treatment (**d**).

There was a positive correlation between LA and RL. Breeding lines were grouped on the basis of four quadrants in scatter plots (Figure 4). Quadrant III was marked by the higher LA and RL of genotypes. Accession B12 and hybrid Hysun 33 had the highest LA and RL under control treatments (Figure 5). Moreover, B-224, D27 and Hysun 33 were highest under T1, D11 and D27 were highest under T2, and D2 and D5 were highest under T3 (Figure 5).

The relationship between SW and R/S (Figure 5) could be explained by the breeding lines under T0, T2 and T3. There was a negative relationship shown for both traits, indicating that R/S increased at the expense of SW and breeding lines partitioned a higher biomass to roots under stress treatment. B.224 showed the highest SW and R/S under T0 and T1. D44 and Hysun 33 also had the highest SW and R/S under T1. D15 and D27 had higher SW and R/S under T2. The breeding line D-5 shows promise but it was present in quadrant II with higher SW but lower RSR (Figure 4).

Field trials were conducted to determine the potential of promising drought-tolerant breeding lines for hybrid breeding (Table 6). Selected drought-tolerant lines (D2, D5, D20, D26, D27) and susceptible lines (B.224 and 577874) were mated with two males (R.365 and R.SIN.82) and analyzed for yield and components. The breeding line D26 had the highest head diameter (HD) when mated with R.SIN.82 at Sargodha (Table 6). The susceptible accession 578874 and B.224 showed higher HD at Faisalabad when mated with R.SIN.82. D20, D26 and D27 were positive general combiners for HD at Sargodha, and B.224 and 577874 were the best combiners for HD at Faisalabad (Table 6).

Table 6. Mean (±standard error) of half-sib offspring and general combining ability (GCA) of various traits at two locations.

Breeding Lines	Sargodha		GCA *	Faisalabad		GCA
	R.365	R.SIN.82		R.365	R.SIN.82	
			Head Diameter (cm)			
D2	19.33 ± 1.53	22.33 ± 0.58	−1.95	18.33 ± 2.08	21.0 ± 1.21	−1.21
D5	17.67 ± 2.08	22.00 ± 2.65	−2.95	14.00 ± 1.35	22.0 ± 2.65	−2.88
D20	23.00 ± 1.13	26.00 ± 1.39	1.71	19.67 ± 1.53	21.7 ± 1.53	−0.21
D26	23.33 ± 2.11	23.67 ± 0.58	0.71	18.33 ± 2.10	21.3 ± 1.61	−1.05
D27	23.00 ± 1.00	26.00 ± 1.32	1.71	19.00 ± 1.50	22.0 ± 1.52	−0.38
B.224	22.67 ± 1.53	22.67 ± 1.53	−0.12	22.00 ± 1.25	23.7 ± 0.58	1.95
577874	24.00 ± 1.39	23.33 ± 1.69	0.88	24.00 ± 1.35	25.3 ± 1.19	3.79
			Oil content (%)			
D2	33.00 ± 2.00	34.33 ± 2.08	−1.95	35.00 ± 1.39	35.7 ± 0.58	−0.36
D5	36.67 ± 1.53	37.33 ± 1.53	1.38	35.67 ± 1.53	36.3 ± 0.67	0.31
D20	36.00 ± 1.57	36.67 ± 1.69	0.71	36.67 ± 1.61	35.0 ± 1.35	0.14
D26	35.67 ± 2.08	37.67 ± 1.53	1.05	36.67 ± 2.15	35.7 ± 0.89	0.48
D27	35.67 ± 1.65	37.00 ± 1.21	0.71	36.00 ± 2.03	36.3 ± 0.63	0.48
B.224	34.33 ± 0.58	33.67 ± 1.53	−1.62	35.33 ± 0.58	35.0 ± 1.21	−0.52
577874	35.67 ± 1.08	35.00 ± 1.19	−0.29	35.00 ± 1.35	35.3 ± 0.61	−0.52
			Seed yield (g plant^{-1})			
D2	42.22 ± 3.61	54.21 ± 2.80	−8.71	46.97 ± 1.20	55.9 ± 1.38	−8.30
D5	47.40 ± 1.82	49.63 ± 2.93	−8.41	55.30 ± 1.94	60.3 ± 0.97	−1.93
D20	54.97 ± 1.56	56.67 ± 1.49	−1.11	60.00 ± 0.58	62.4 ± 1.02	1.45
D26	65.96 ± 3.46	63.95 ± 2.03	8.03	66.49 ± 4.05	68.7 ± 1.76	7.88
D27	62.67 ± 3.53	65.33 ± 3.64	7.07	62.89 ± 2.62	58.0 ± 1.57	0.71
B.224	51.92 ± 1.47	59.31 ± 0.94	−1.31	56.31 ± 2.00	58.6 ± 1.95	−2.29
577874	58.61 ± 3.69	64.10 ± 1.56	4.43	62.17 ± 2.70	62.3 ± 2.13	2.48

* GCA = general combining ability.

D5, D20, D26 and D27 were positive combiners for oil content at both locations. D.26 showed the highest mean value for oil content at Sargodha, whereas D5 and D27 had the highest values at Faisalabad when mated with R.SIN.82 (Table 6). D26 and D27 were the best general combiners for seed yield plant^{-1} (SYP) at Sargodha and Faisalabad (Table 6). D26 showed the highest SYP when mated with R.365 at Faisalabad and the same line showed the highest SYP at Sargodha when mated with R.SIN.82 (Table 6).

4. Discussion

Drought stress had a negative impact on morphological traits such as the shoot length, leaf area and shoot weight. Nine major traits (including the leaf area, leaf number, collar diameter, plant height, leaf water content, transpiration or osmotic potential) are known to be affected as a result of the drought response of sunflowers [20]. Plants exposed to water stress treatments may maternally transfer water stress signals to the seed such as accumulation of abscisic acid and polyphenols that may help to provide an adaptive mechanism against stress during its germination [21].

Exogenous application of compatible solutes such as trehalose and salicylic acid decrease the yield loss by maintaining relative water content, proteins, the organellar membrane structural integrity and the induced antioxidative enzyme activity that may help to scavenge harmful radicals [22,23]. Moreover, zinc foliar application (3 μ mol) under water stress also improved morphological and leaf gas exchange traits and reduced the yield loss [24]. However, agronomic recommendation of foliar sprays for inducing water stress tolerance is laborious, thus increasing production costs and it may not always translate into high yield potential [1].

Water stress tolerance is a complex trait and several loci are known to affect the plant performance under stress. Putative genes related to these traits were identified through microarrays with 32,423 probe sets [25]. Drought stress also up-regulated several regions within sunflower loci such as HaTIP proteins and real-time PCR showed several fold increases in the expressions of proteins [26].

The sensitivity of morphological traits may be used to distinguish between tolerant and susceptible genotypes under water stress. The leaf area could differentiate sunflower genotypes under water stress. The leaf area in control treatment may not be used as an index of its resistance to water stress [1]. A smaller leaf area may be an adaptive mechanism against water stress, reducing the evapotranspiration loss. The drought-tolerant germplasm may be selected for a lower leaf area, intense hairiness and cuticular waxes to protect against transpiration loss and exposure to high temperatures [12,25]. The leaf area of the drought-tolerant germplasm was stable under stress treatments, whereas the elite germplasm showed a significant decline in the leaf area, which was an indication of water stress sensitivity [8].

The contrasting impact of water stress treatments over the magnitude of genetic variation in both types of the germplasm was also noted. Drought-tolerant breeding lines showed a higher magnitude of PCV under stress than the control. Genetic variances due to drought-tolerant inbred lines increased under water stress. Functional diversity for drought tolerance was helpful to keep the variability among the germplasm [7]. Hence, crop diversity conservation has a significant role in enhancing environmental sustainability [26]. On the other hand, low functional diversity leads to the absence of phenotypic plasticity that results in the reduction in overall genetic variance in the elite germplasm [27].

A positive relationship between the root length and leaf area was indicative of leaf area expansion due to root elongation. Hence, it may be feasible to obtain water from the soil profile more efficiently in drought-tolerant lines [12]. Based upon our results, the drought-tolerant lines D27 and D11 were promising with the highest leaf areas and root lengths. Traits such as the shoot weight and root shoot ratio showed a negative correlation, thus indicating that higher root shoot ratios occurred at the expense of reduction in shoot weight [2]. Breeding lines such as D2, D5 and D27 showed a better performance under water stress treatments.

Combining ability testing was undertaken with two male lines to determine the suitability of developed hybrids based on their performance in semi-arid environments. Breeding lines with positive GCA had a high frequency of positive alleles for target traits and may show potential for their further use in a hybrid breeding program [7,28]. D26 and D27 were promising lines due to their positive GCA at both locations for oil content and SYP.

The transcriptomic analysis of the sunflower accessions and hybrids was compared to identify genes and transcripts involved in drought tolerance mechanisms [29,30] or to understand the molecular mechanism involved in physiological processes such as leaf senescence [31]. Hence, these developed tolerant lines may also be an interesting research germplasm for transcriptomic and metabolic analyses to understand the molecular mechanisms and genes involved in the expression of drought tolerance of the introgressed bred germplasm.

5. Conclusions

Drought is a major production constraint across the globe. This study used the wild relative of sunflower *H. argophyllus* to introgress its genes into the breeding pool and thereafter select for drought tolerance into the elite Pakistan sunflower germplasm. The developed material was evaluated under various water treatments and found to be superior to the available bred germplasm in terms of sustained morphological traits under water stress treatments. These new breeding lines have traits such as high cuticular wax and a smaller leaf area. Drought-tolerant breeding material had a lower comparative decrease in the leaf area and plant height and a higher capacity to elongate the root and root shoot ratio under water stress treatments. The developed breeding material may enhance drought tolerance in sunflower breeding lines and hybrids. These lines will be shared for further utilization with breeding programs worldwide. The general combining ability analysis showed the utility of the introgression lines D26 and D27 for hybrid breeding. All drought-tolerant lines in this study are maintained and available for future research on drought-tolerant gene expression.

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