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Selection for Some Functional Markers for Adaptability of *Helianthus argophyllus* × *Helianthus annuus* Derived Population under Abiotic Stress Conditions

https://doi.org/10.1515/helia-2017-0018
Received September 16, 2017; accepted December 15, 2017;
previously published online January 5, 2018

Abstract: Abiotic stresses including drought are major crop production constraints. However, specific functional phenotypic markers induce resistance against these stresses. Therefore, a study was initiated to study the variability, inheritance and selection of epicuticular waxes (EW) and leaf hairiness (LH) along with low cell membrane injuries (CMI) within F_2 populations derived by crossing *H. annuus* × *H. argophyllus* lines. These traits have been shown to be associated with drought tolerance of *Helianthus argophyllus* and thus study aims to introgress these traits in *Helinathus annuus*. The studied parent populations showed contrasting values of the traits. The drought susceptible line CMS-14 and CMS-20 showed lower epicuticular waxes (0.79, 0.69 mg g^{-1}), leaf hairiness (0.75, 1.53) and higher cell membrane injury (40.90, 55.76 %) respectively while drought resistant line Argo 1802 and 1806 showed higher epicuticular waxes (2.28, 3.18), leaf hairiness (3.71, 3.80) and lower cell membrane injury (14.22, 21.54 %) respectively. The F_1 hybrids had mean values

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of the three studied parameters i. e. epicuticular waxes (1.50 mg g^{-1}), cell membrance injury (32.54 %) and leaf hairiness (2.74) in the range of parent lines, but some of F_2 individuals extend beyond this range (Parents and F_1s). The two-step selections maintained high variability especially of LH for set of F_2 individuals (*H. annuus* CMS-20 × *H. argophyllus* 1806). Simultaneous selection of F_2 individuals with high values of LH or EW with low CMI was possible. The selected plants were further studied for narrow leaf, high fertility and silver canopy color. Selected material was promoted as the candidate of inbred line. Plant (F_4) having introgressed traits (silver canopy) showed lower yield (19 %) than green leafed plants (53 %) and commercial hybrids under drought stress (63 % and 53 %). The study could help to increase the abiotic stress tolerance, minimize the yield losses under drought stress and increase functional diversity within sunflower.

Keywords: inbred lines, leaf hairiness, epicuticular waxes, cell membrane injury, canopy color

Introduction

Drought is major production constraint in field. The vast global area was dry where agriculture was constrained due to un-availability of water (FAO, 2015). Drought was permnanent feature of the dry lands and caused significant reduction of yield potential of dry land crops (Rauf *et al.*, 2016). According to an estimate only 10 % of the global area was stress free (F.A.O., 2015). Plant breeder tends to introgress specific traits which help the crop species to cope with stress condition. Among these adaptability traits, leaf hariness, epicuticular waxes and smaller leaf area support the plant for their survival under insect infestation, drought and heat stress (Roy *et al.*, 1999; Holmes and Keiller, 2002; Zhang and Qi, 2012; Kalyar *et al.*, 2013; Niazi *et al.*, 2014). Leaf hariness reduced the transpiration and refelected radiation to avoid the exposure of the plant to the heat stress (Roy *et al.*, 1999; Holmes and Keiller, 2002). Moreover, epicuticular wax load also protected the plant from water losses (increase water use effeciency), reduces heat injuries and insect infestation (Eigenbrode, 2004; Zhang and Qi, 2012). These traits have been exploited for the development of inbred lines in sunflower (Rauf *et al.*, 2016).

Presence of adequate variation within primary gene pool has been reduced due to breeder selection for specific plant types. The problem of narrow genetic base of cultivated sunflower could be overcome by the introgression of genes for various morphological traits from wild sunflower species (Seiler *et al.*, 2006, Jan *et al.*, 2008). The crop wild relatives have the adaptability to specific condition and can supply resistant/tolerant genes for particular stresses (Seiler *et al.*, 2006).

Sunflower wild species such as *Helianthus argophyllus* was known to be heat and drought resistant (Škorić, 2009; Milton, 2013). The species contained heavy load of epicuticular waxes, leaf hariness and smaller leaf area when compared with cultivated germplasm which helped them to cope with abiotic stresss (Hussain *et al*., 2016). Therefore, a study was initaited to introgress these traits in cultivated germplasm. The study helped to eastablish segregating population, estimate variability within segregating population and estimate the inheritance of the functional markers.

Materials and methods

Experiment was conducted to evaluate *Helianthus annuus, Helianthus argophyllus* and their derived populations under drought stress conditions during the year 2014–16 at the research area of University College of Agriculture, University of Sargodha, Sargodha.

Two *H. argophyllus* accessions (ARG-1802 and ARG-1806), introduced from the USDA germplasm collection, and two lines of cultivated sunflower (*H. annuus*) were used in this study. The lines (CMS-14 and CMS-20) were crossed with *H. argophyllus* accessions to develop two F_1 hybrids. These hybrids were planted in the field and selfed pollinated by bagging their reproductive head to develop F_2 seed.

Development of F_1 and F_2 poulations

Seeds of *H. argophyllus* were germinated in large polythene bags containing equal volume of sand, silt and field soil. After thirty days *H. argophyllus* plants, sensible to the photoperiod, were transferred to the field for induction of flowering. At 120 days after emergence, plants were covered from 15:00 to 7:00 during 45 days with black cloth to provide 16 hour dark period. On the other hand, *H. annuus* lines were sown after the start of black cloth treatment to the *H. argophyllus* populations to synchronize flowering. Pollen of the *H. argophyllus* plants was collected at 7:00 in the morning and deposited with the help of a camel brush over the stigma of the lines. Meanwhile, heads of lines were covered with white cloth bags to avoid foreign pollen contamination. Several rounds of pollination were carried out until stigmas completely wither. Interspecific hybrid seeds, as well as seeds of *H. argophyllus* and lines were collected. CMS lines were maintained by tying the floral buds and pollinating the same plants with maintainer lines.

The obtained F_1 and their parents (*Helianthus annuus* L.) were resown during the same year in the field. Twenty plants of each cross/parent were sown in field to the maturity. The CMS lines were pollinated by the maintainers and *Helianthus argophyllus* pollen from plants already present in the field to develop fresh seed of parents and F_1. The F_1 plants were selfed pollinated to obtain F_2 seed.

Growth conditions

Seeds of *H. argophyllus*, Parental lines; CMS-14 and CMS-20, interspecific F_1 crosses and F_2 population were grown in large polythene bags containing 20 kg of a soil mixture containing an equal volume of sand and silt. Soil structure was improved by adding 5% farmyard manure. The fertility of soil was raised by adding 2 g of diammonium phosphate to the each polythene bag. Plants were given 2 g of Urea at regular interval of one month. Plant material were sown on 15th October 2014 under controlled conditions (27 °C; 16-h photoperiod; PPFD = 600 µmol m^{-1} s^{-1}). Two seeds of each cross/parents were sown within each polythene bag and thinned to a single plant after germination. Each of the parental line and interspecific hybrid was represented by 15 plants each while F_2 population was represented by 170 plants each. The experiment was laid out in completely randomized design where number of plants in parents and F_1 populations were considered as replications and variation among the plants was source of error terms. 15 days time interval for the reading was considred as factors and index of relablity for measurements of the traits. All the plants were subjected to the analysis of three plant traits relevant to abiotic stresses after every 15 days interval.

Establishment and selection among and within F_3 plant progenies

The selected F_2 single plant were selfed pollinated to obtain the F_3 seed. Each selfed pollinated F_2 plant was used to establish single F_3 plant progeny. There were 119 single plant progenies including silver canopy plant with small leaf area and green with broad leafed canopy. Each progeny was sown as a single row of 6 m having 20 plants within each row. The progenies were evaluated at the time of anthesis for traits such as silve canopy (hairiness, cuticular waxes),

leaf area (small or large), pollen fertility (presence of high pollen load), days to anthesis and head types. Multi-heading and late maturing (days to anthesis) plants were rejected from the progenies. Fertile plants were covered with netting bagg to avoid pollen contamination. The plants were evaluated for the achene formation and oil contents. Finally 36 single headed plant progenies with good fertility and differential canopy colour i. e. 18 progenies (silver + small leaf) and 12 progenies (green + large leaf) were selected.

Evalaution of F_4 plant progenies

Seeds of 36 F_4 progenies along with commercial hybrids (Hysun-33 and S-278) were sown in large polythene bags containing 25 kg of a soil mixture (equal volume of sand and silt). Soil structure was improved by adding 5 % farmyard manure. The fertility of soil was raised by adding 2 g of diammonium phosphate to the each polythene bag. Plants were given 2 g of Urea at regular interval of one month. Plant progenies were raised under controlled conditions (27 °C; 16-h photoperiod; PPFD = 600 µmol m^{-1} s^{-1}). Two seeds were sown within each polythene bag and thinned to a single plant after germination. The experiment was laid out in completely randomized design. Each of the plant progeny was represented by 5 plants within each replication. There were three replications and two regimes. Contrasting regimes was created by irrigating the control plants to field capacity (18 % by weight of soil) and maintaining 65 % of the field capacity in the stress regime. The moisture contents within each polythene bag were determined through the moisture meter at regular interval to maintain moisture contents close to field capacity and low moisture in stress regime. Leaf cuticular waxes were determined at the time of anthesis while seed yield per plant (g) and oil contents were determined at maturity.

Cell membrane injury (CMI)

Stress causes cell membrane dis-functioning and was estimated by cellular electrolyte leakage from effected tissues. CMI was measured according to Blum and Ebercon (1981). Leaf nodes at the top of canopy were tagged and two leaves from the same levels of node were detached from the plant after 15 days when leaves were fully expanded. Leaves were thoroughly washed with deionized water. Leaf discs (15 mm) were obtained by pressing the sharpen edges of disc maker over leaf. Three leaf disc from each plant were dipped in single glass vial

containing 25 ml of polyethelene glycol molecular weight, 6000 (30 %) solution as treatment and 25 ml deionized distilled water as control. Samples were incubated at 10 °C for 24 hours. After 24 hours samples were washed with deionized distilled water, and shifted to new glass vial and 25 ml deionized distilled water was again added to each sample. Samples were incubated at 10 °C for 18 hours. After the incubation period the samples were again kept for 1 h at 25 °C and electric conductivity (EC) was measured at 25 °C. After measuring EC, samples were autoclaved at 121 °C for 15 minutes to damage all tissues. The samples were cooled and incubating at 25 °C for 3 hours. The final electric conductivity was measured at 25 °C. Each plant was obsevred for electric conductivity three times with an interval of 15 days.

Cell membrane injury was calculated as;

$$CMS = [\{1 - (T_1/T_2)\}/\{1 - (C_1/C_2)\}]*100$$
$$\%CMI = 100 - CMS$$

Epicuticular leaf waxes

Epicuticular leaf waxes prevent evaporation from epidermal tissues and considered as an important marker for drought tolerance. Epicuticular leaf waxes were determined according to Ebercon *et al.* (1977). Leaves were tagged on the top of canopy and leaves were detached after 15 day when leaves were fully expanded. Leaf discs of 15 mm size from fully expanded leaves at top of the canopy were washed and dipped in glass vial containing 15 ml chloroform for 15 sec on rotary shaker. Chloroform was evaporated in water bath and 5 ml of reagent was added to each sample. Reagent was prepared by mixing 20 g of Potassium dichromate with 40ml distilled water and then heating with 1 L sulfuric acid. After adding reagent, solution was heated for 30 minutes in water bath and 12 ml of deionized distilled water wias added. Optical density (590 nm) of the sample was measured using spectrophotometer after standardizing it with de-ionized water and reagent. Standards were prepared by extracting 0, 5 mg, 7.5 mg, 10 mg and 15 mg waxes to 5 ml of reagent. Epiculticular waxes were determined three times with 15 days interval.

Hairiness

Hairiness helps in reflecting the light and thus help in the reduction of leaf temperature and transpiration. Leaves of similar age (15 days) was detecahed

from the canopy.Leaves were categorized for leaf pubescence under stereomicroscope. Leaf hairiness were chracterized as follows 0 = no hairs, 1 = less (30–50 hairs per oberved gallace), 2 = moderate (51–100 hairs per oberved gallace), 3 = dense (101–200 dense), 4 = highly dense (200 to above). Charcterization was carried out for three time per plants after 15 days interval.

Morphological traits

The seed yield per plant (g) was determined by drying the seed to uniform moisture contents (12%) and measuring the masses on weighing balance. The seed yield of each F_4 progeny was averaged over five plants. Oil% contents were determined over soxhlet appratus. All plant material was transfered to the field by digging pits of 60 × 40 cm. The plastic bags were gently removed and plants were shifted along with soil masses. The empty pit was filled with field soil and later on plants were irrigated to field capacity. The plants were grown to maturity in field. The selected individuals were further chracterized on the basis of canopy types such as branched or non branched, single or multiple headed, leaf shape or types, fertility and canopy color. The plants were evaluated at the time of anthesis 80 days after emergence (DAE). Branch type character was chracterized as non or multiple branching. Moreover, plants were evalauted as single or multiple headed. Leaf types were characterized on the basis of broad, medium or narrow. Plant hairiness was considered as high, medium, low or nil. Fertility was grouped on the basis of daily pollen production in floral head. The pollen intensity was chracterized as high, medium or low by touching a clean piece of cotton to the floral head and latter on obsereved the pollen under stereoscope. Canopy color was distinguished as silver, light green or green color. Some plants experienced damage to the canopy due to strong winds therefore they were not rated, while some plant did not initiate floral bud. Thus values were considered as missing.

Statistical methods

Statistical analysis was subjected to three traits, epicuticular waxes (EW), leaf hairiness (LH), and Cell Membrane Injury (CMI). The means and standard deviations were calculated for each plant population. F_2 variance was considered equal to the phenotypic variance (σ^2p). Variability within parental generation and F_1 was the source of environmental variance ($\sigma^2e = \sigma^2p1 + \sigma^2p2 + \sigma^2F_1/4$).

Genotypic variance (σ^2g) was estimated as $\sigma^2g = \sigma^2p - \sigma^2e$. Heritability was estimated as σ^2g/σ^2p. The linear mixed model was used to test the influence of R1, R2 and R3 (three time intervals) for different breeding lines or F1 hybrids. Only the lines and F_1 hybrids were considered during this step of analysis as the parental lines and F_1 generations were repeated while the F_2 generations consist of the single individuals with different genotype. Likelihood ratio test (LRT) was used to determine the significance of the fixed factors. The model contained fixed effects of genotype and three time intervals (R1, R2 and R3), the interaction between these two factors (also as fixed factor) and random factor connected with individual plants (nested factor in genotype). They were considered as fixed factors: i) the differences between genotypes, ii) the differences between trait values measured in different time iii) the differences between the response of genotype for the range of time. The calculations were made with the use of R software (R Core Team, 2015), the 'lmer' function from 'lme4′ package (Douglas *et al*., 2012).

Principal Component Analysis (PCA) was carried out for each trait. The PCA analysis and symmetrically scaled biplot were made with the objective to determine the time interval influence on studied trait. The PCA analysis of standardized variables (known also as Genotype and Genotype by Environment interaction analysis, GGE, Yan and Kang, 2003, Hussain *et al*., 2015) representing averaged traits values across time of observations was done. The PCA calculations were done (also with the use of R software) by 'svd' and 'scale' function (Sienkiewicz-Paderewska and Paderewski, 2015).

During the genotype selections of F_2 generations to the future breeding program the LH and EW were standardized. The cluster analysis was based on the Euclidean distance for both traits according to complete linkage method. If one F_2 individual had lack of the LH and other F_2 had lack of the EW, the distance can't be calculated. In such cases as the distance between those individuals the mean distance for all set of F_2 individuals was used. The cluster analysis was carried out with the use of R software by 'dist' 'hclust' and 'cutree' functions. It was done to determine relationship between the branching, head number, leaf shape, fertility, hairiness and canopy color for the selected F_2 individuals. The traits were transformed to the numeric values. The branched or non branched were chracterized as 1 or 0 respectively. The single headed plants were characterized as equal 1 and multi headed plant – 0. For the leaf type: narrow, medium narrow, broad or round the numeric equivalents were 1, 0.67, 0.33 and 0. Fertility was high, medium or low and the values were 1, 0.5 and 0. Plant hairiness described by cases: high, medium, low or nil had values equal 1, 0.67, 0.33 and 0 respectively. Canopy color such as silver, light green and green had values 1, 0.5 and 0. The dendogram was prepared through wards method.

Results

The main effects of genotypes were significant ($P \leq 0.001$) for EW whereas variability enclosed by CMI and LH was complex due to significance of interactions (Table 1). Therefore, results were interpreted according to the interactions in these traits. The values for the traits epi-cuticular waxes (EW), leaf hairiness (LH), cell membrane injury, CMI) averaged by the R1, R2 and R3 time intervals were calculated to obtain means and the standard deviations (Table 2). The mixed model was used to determine the significance of time interval influence (R1, R2 and R3) over various traits (EW, LH, and CMI) for different breeding lines.

Table 1: Analyses of variance for epicuticular waxes, cell membrane injury and leaf hairiness according mixed model.

Source of Variation	Df	Mean Sum of Squares		
		Epicuticular waxes	Cell membrane Injury	Leaf Hairiness
Plant	5	8.363**	1828.3**	44.52**
Time	2	0.007^{NS}	480.0**	0.10*
Plant × Time	10	0.024^{NS}	263.7**	0.47**

*Significant at level 0.05. **Significant at level 0.01. ^{NS} not significant.

Table 2: Mean values of the parental generations and their derived populations along with estimates of variability.

Variation	Epi-cuticular waxes (mg g^{-1})	Cell membrane injury %	Leaf hairiness
CMS-14	0.79 ± 0.11	40.90 ± 7.91	0.75 ± 0.29
Argo-1802	2.28 ± 0.25	14.22 ± 10.14	3.71 ± 0.25
CMS-14 × Argo-1802 (F_1)	1.05 ± 0.12	49.82 ± 15.92	3.64 ± 0.47
CMS-14 × Argo-1802 (F_2)	1.50 ± 0.79	32.54 ± 22.68	2.74 ± 0.86
σ^2 Phenotypic	0.62	514.17	0.73
σ^2 Environmental	0.08	167.97	0.14
σ^2 Genotypic	0.55	346.21	0.59
Heritability	0.87	0.67	0.80
CMS-20	0.62 ± 0.12	55.76 ± 8.77	1.53 ± 0.39
Argo-1806	3.18 ± 0.53	21.54 ± 7.55	3.80 ± 0.17
CMS-20 × Argo-1802 (F_1)	1.55 ± 0.15	46.50 ± 8.24	2.89 ± 0.41
CMS-20 × Argo-1802 (F_2)	1.89 ± 0.74	44.70 ± 16.47	2.74 ± 0.94
σ^2 Phenotypic	0.55	271.13	0.89
σ^2 Environmental	0.09	67.41	0.13
σ^2 Genotypic	0.46	203.72	0.76
Heritability	0.84	0.75	0.85

Mean values across time of parental and derived populations

The PCA based on standardized traits values, showed 56 % of variability for PC1 which dominated the pattern and the importance of PC2 and PC3 were similar (24 % and 20 %) for three traits. The PC1 identified F_2 individuals with low CMI and high LW or high EW and separated them from the F_2 individuals with high CMI and low LW and EW parameters (Figure 1).

There were significant differences among the mean values of the parental lines for all traits. The *Helianthus argophyllus* lines (line 1802 and line 1806) had higher values (Figure 1) for the LH and EW (Table 2) but significantly lower CMI than *Helianthus annuus* lines (CMS-14 and CMS-20) at the t-test $P < 0.001$ for each of the comparisons). On the basis of mean values of CMI, the parental lines of *Helianthus annuus* was considered susceptible than *Helianthus argophyllus* lines (Table 1).

F_1 generations means were according to additive hypothesis model for epicuticular waxes (Fritzz *et al.* 1994). However, differences between the F_1 generations and parent lines were significant at $P < 0.001$. The values of F_1 were closer to *H. annuus* parent lines. The F_1 generation of interspecific cross *H. annuus* CMS-14 × *H. argophyllus* 1802 had similar LH to its parent line *H. argophyllus* 1802 (according t-test at $P = 0.5097$), therefore LH seemed to be inherited from *H. argophyllus* parent line (the difference between the *H. annuus* CMS-14 was significant at $P \leq 0.001$). Moreover F_1 generations had high CMI values among which *H. annuus* CMS-14 × *H. argophyllus* 1802 had a slightly higher values than *H. annuus* CMS-14 parent line (Figure 1) but the difference was not significant ($P = 0.082$). The mean value of F_1 H. *annuus* CMS-20 × *H. argophyllus* 1806 was significantly lower at $P < 0.001$ to *Helianthus annuus* L. for CMI. It showed greater similarity to parental line CMS-20 than to *H. argophyllus*. The negative alleles of the parental lines had dominant effect masking effects over the positive alleles contributing to the drought tolerance. This pattern was according to the hybrid susceptibility hypothesis according to which interspecific hybrids tend to be show susceptibility to the stress due to negative interaction between the alleles at various loci. There were differences between the parental lines used in both crosses. The parental lines (*H. annuus* CMS-14, *H. argophyllus* 1802) had lower CMI values than parental lines (*H. annuus* CMS-20, *H. argophyllus* 1806) but F_1 generations were similar and did not separate on the biplot (Figure 1).

Both F_2 generations demonstrated a high variability due to genetic differentiation (Figure 1). The *H. annuus* CMS-14 × *H. argophyllus* 1802 had the

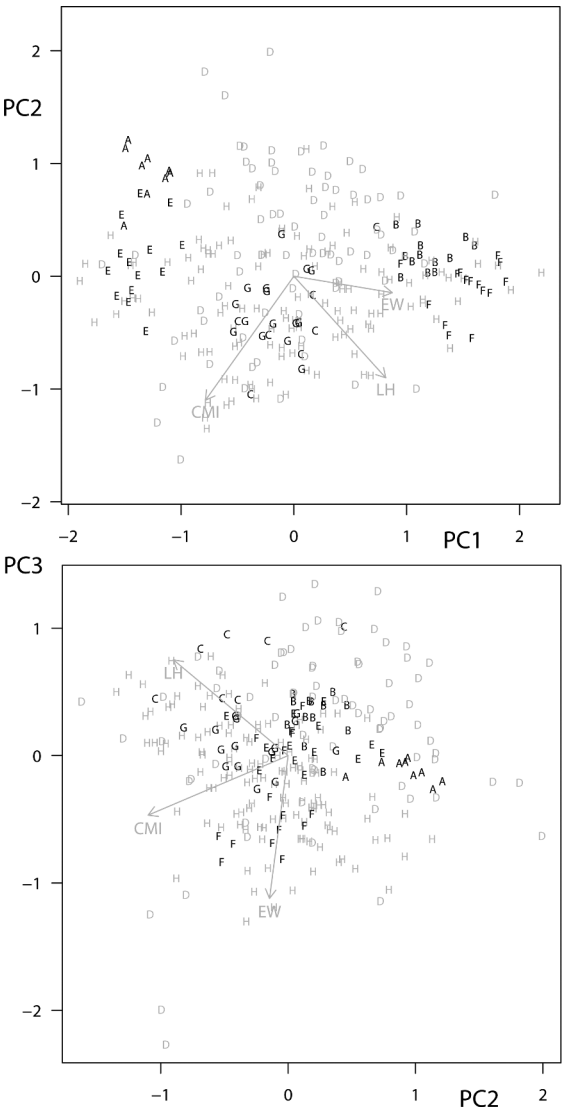

Figure 1: The biplots of a Principal Component analysis based on quantitative morphological data, Epicuticular Waxes (EW) Cell Membrane Injury (CMI) and Leaf Hariness (LH), averaged across three terms of observations of *Helianthus* parent lines, F_1 and F_2 generations. Components, PC1, PC2 and PC3, explained 56 %, 24 % and 20 % of the morphological variation respectively. The codes of parent lines and hybrids was written according the Table 3.

Table 3: Mean and standard deviation (in parenthesis) for PC scores of cell membrane injury of lines and hybrids.

Plant type (the code and the generation)	PC1	PC2	PC3
H. annuus CMS-14 (A)	−0.5	0.4	0.3
	(1.68)	(1.33)	(1.37)
H. argophyllus 1802 (B)	5.1	0.7	−0.3
	(0.95)	(0.89)	(1.13)
H. annuus CMS-14 *H.* × *argophyllus* 1802 (C, F_1)	−2.3	−0.3	1.7
	(3.35)	(2.64)	(0.86)
H. annuus CMS-14 × *H. argophyllus* 1802 (D, F_2)	1.3	0.4	0.1
	(4.75)	(1.22)	(1.41)
H. annuus CMS-20 (E)	−3.6	0.9	−2.4
	(1.84)	(2.12)	(1.81)
H. argophyllus 1806 (F)	3.6 (1.60)	1.0 (2.06)	0.1 (2.52)
H. annuus CMS-20 × *H. argophyllus* 1806 (G, F_1)	−1.7	2.6	−1.1
	(1.74)	(1.51)	(2.47)
H. annuus CMS-20 × *H. argophyllus* 1806 (H, F_2)	−1.3 (3.45)	0.3 (1.89)	0.1 (1.60)

highest range of CMI values. The individuals belonging to this F_2 generation had the highest extreme values for CMI. The F_2 *H. annuus* CMS-20 × *H. argophyllus* 1806 enclosed variability similar to both parents for CMI (as low as *H. argophyllus* 1806 or even higher than *H. annuus* CMS-20). Similar types of trends were also observed for the leaf hairiness and epicuticular waxes (Figure 1). The F_2 derived from *H. annuus* CMS-20 × *H. argophyllus* 1806 had higher ranges for leaf hairiness and epi-cuticular waxes. However, individuals having lower values of CMI were less frequent. The F_2 generation *H. annuus* CMS-14 × *H. argophyllus* 1802 had slightly lower averaged CMI and LH values than its F_1 parent hybrid (at P 0.001 and P ≤ 0.001 respectively). The *H. annuus* CMS-20 × *H. argophyllus* 1806 also showed wide range for the traits but the mean CMI and LH was similar to F_1 (at P 0.49 and P 0.28 respectively). F_2 populations from both crosses had higher averaged EW values (at P ≤ 0.001) than their F_1 crosses. Therefore, positive alleles from the *Helianthnus argorphyllus* were introgressed in many of the F_2 individuals which increased the overall mean performance of F_2 individuals.

Plant types × time interval for cell membrane injury

The PCA analysis for CMI was based on parent lines, F_1 and F_2 generations (Table 3). The singular values are as follows: 614.1, 115.5 and 104.4. Analysis of

variance for CMI showed that interaction plant –by– time was significant (Table 1) but the influence of the PC1 was dominant (it described about 94% of variability). The PC1 described main effects of plants (in descending order, Figure 2). Thus, showing that main effects dominated the variability among individuals and the genotype ranking was almost not affected due to interaction effects with time intervals (and, in consequence, the CMI values were rather repeatable in time). The influence of PC2 and PC3 were about 3.3% and 2.7% respectively showing minor impact of these components over the total variability (high PC2 scores means relatively higher CMI values at R_1 and R_2 whereas negative PC2 scores means relatively higher CMI values at R_3; positive PC3 scores means relatively higher CMI values at R_2 whereas negative PC3 scores means relatively higher CMI values at R1; Figure 2). This is in accordance with variability estimates (Table 2) that showed the high influence of genotypic and phenotypic variability, which was the main source for plant ranking.

Plant types × Time interval for leaf hairiness

The PCA analysis for leaf hairiness was calculated based on parent lines, F_1 and F_2 generations (Table 4). The singular values were as follows: 30.6, 10.8, 9.9. Although the variance analysis showed that the interaction plant –by– time is significant but the influence of the PC1 was dominant (it described about 81% of variability) (Figure 3). Thus, the main effects dominated but the influence of PC2 and PC3 were slightly higher than for CMI. The PC2 and PC3 described about 10.2% and 8.5% of leaf hairiness variability so those two principal components have also similar size of the impact. The PC1 scores of individuals could be associated with the average LH across time intervals (with inverse order) whereas the high PC2 score denotes the relatively higher (than would result from the PC1 scores) LH at R1 and R3 time intervals (and negative PC2 denotes relative higher LH at R2). The PC3 distinguishes between relatively higher values during the first two time intervals of the high LH of the third (R3) time interval. The F_1 *H. annuus* CMS-14 *H. argophyllus* 1802 was very similar to *H. argophyllus* 1802 parent line according all three PCs. The F_1 and F_2 *H. annuus* CMS-14 × *H. argophyllus* 1802 generations had positive PC3 so the LH values decreased in time (inherited behavior from the *H. argophyllus 1802* breeding line). The same pattern was observed for F_2 *H. annuus* CMS-20 × *H. argophyllus* 1806 but F_1 *H. annuus* CMS-20 × *H. argophyllus* 1806 behaved in the opposite.

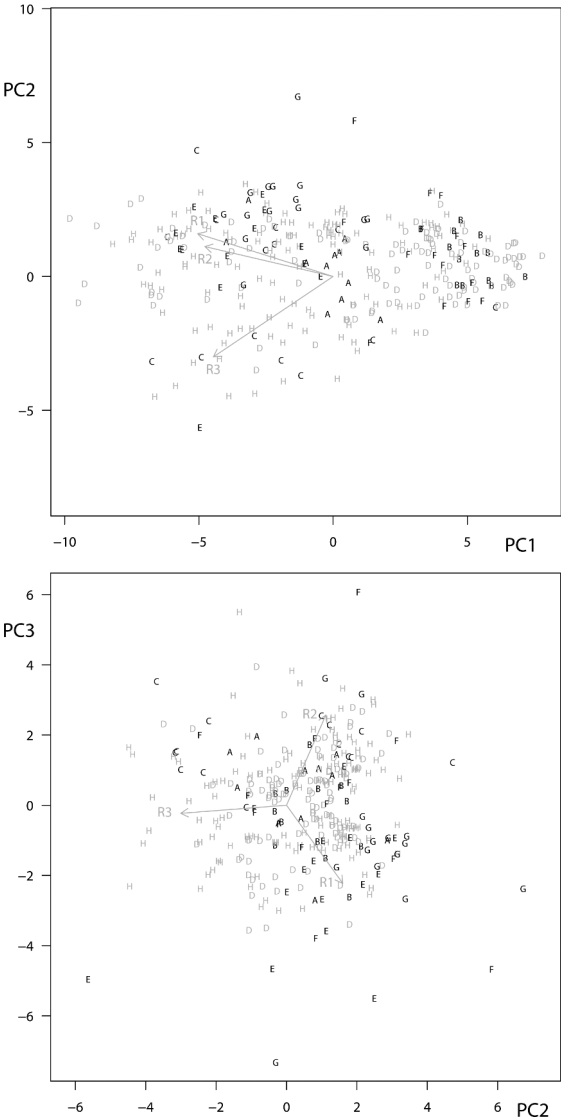

Figure 2: The proportional scaled biplots of a Principal Component analysis based on Cell Membrane Injury in R1, R2 and R3 terms of observations.
Components PC1, PC2 and PC3 explained 94.0 %, 3.3 % and 2.7 % of the CMI variation respectively. The codes of parent lines and hybrids according the Table 3.

Table 4: Mean and standard deviation (in parenthesis) for PC scores of leaf hairiness of lines and hybrids.

Plant type (the code and the generation)	PC1	PC2	PC3
H. annuus CMS-14 (A)	1.87	−0.11	−0.3
	(0.27)	(0.41)	(0.38)
H. argophyllus 1802 (B)	−0.89	0.13	0.47
	(0.24)	(0.36)	(0.37)
F1 *H. annuus* CMS-14 × *H. argophyllus* 1802 (C, F_1)	−0.83	0.09	0.22
	(0.29)	(0.44)	(0.46)
F2 *H. annuus* CMS-14 × *H. argophyllus* 1802 (D, F_2)	0.02	0	0.29
	(0.8)	(0.61)	(0.48)
H. annuus CMS-20 (E)	1,14	0.04	0.01
	(0.37)	(0.39)	(0.48)
H. argophyllus 1806 (F)	−0.99	0.06	0.08
	(0.16)	(0.46)	(0.39)
F1 *H. annuus* CMS-20 × *H. argophyllus* 1806 (G, F_1)	−0.15	−0.04	−0.29
	(0.39)	(0.28)	(0.53)
F2 *H. annuus* CMS-20 × *H. argophyllus* 1806 (H, F_2)	0.01	0.08	0.15
	(0.89)	(0.58)	(0.49)

Direction of selection

PCA analysis showed differential dominance of PC1 for each trait (in both cases the PC1 could be interpreted as the average value of each traits), therefore selection should be done according to the mean values of each traits (across time intervals). The base parameter that describes the stress tolerance was the CMI. The high LH and EW should imply higher drought tolerance but the relationship was weak (Figure 1). It was concluded that there were also some other factors that contribute toward low or high drought susceptibility of plants. These undefined parameters may be determined in next generations.

The sample with high variability

The observations were obtained on 268 individuals of F_2 generations. The 143 plants were belonging to *H. annuus* CMS-14 × *H. argophyllus* 1802 and 125 to *H. annuus* CMS-20 × *H. argophyllus* 1806. The 31 individuals had not defined CMI while 4 individuals having CMI was not measured for both EW and LH. These individuals were not taken into consideration as they were only 13 % of

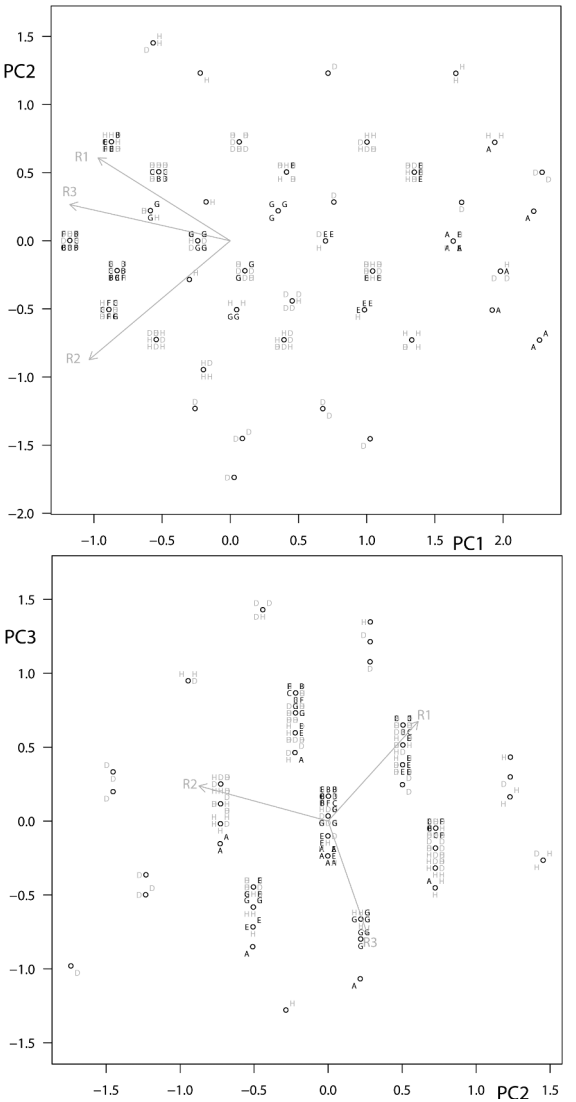

Figure 3: The proportional scaled biplots of a Principal Component analysis based on Leaf Hairiness in R1, R2 and R3 terms of observations.
Components PC1, PC2 and PC3 explained 81.3, 10.2 and 8.5 % of the LH variation respectively. The codes of parent lines and hybrids according Table 4.

the total observation during first step of genotype selection (that should provide a large variety of individuals). Thus 233 individuals were analyzed in the first step.

The individuals with higher CMI than average were discarded (rejected). In this way, 123 individuals were selected with the lowest CMI from both F_2 generations (it consist the set of the better individuals). Those individuals were grouped according to cluster analysis based on LH and EW parameters. The 40 homogenous groups were created. Each group comprised of one individual from each cross (*H. annuus* CMS-14 × *H. agrophyllus* 1802 and *H. annuus* CMS-20 × *H. agrophyllus* 1806). The 51 individuals had the lowest CMI values (Figure 4 and Table 5).

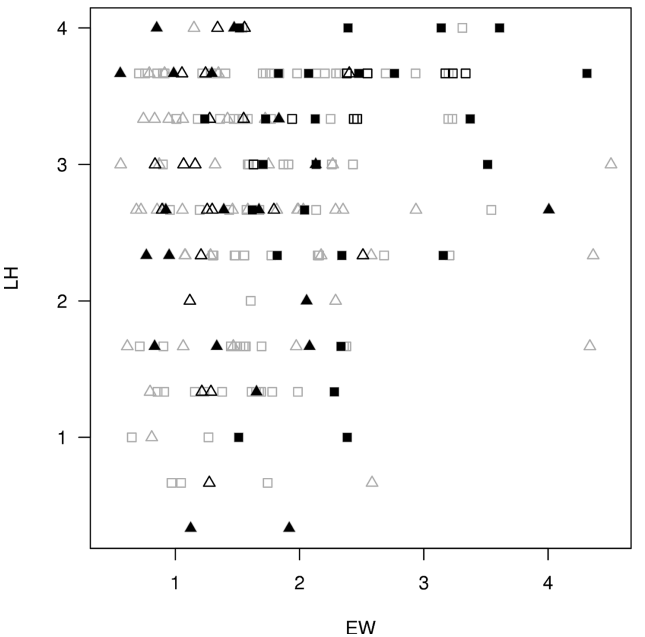

Figure 4: The diversity of selected F_2 individuals according EW and LH values.
Fulfill triangles: *H. Annuus* CMS-14 × *H. Argophyllus* 1802 with low CMI and high variability of LH and EW; Fulfill squares: *H. Annuus* CMS-20 × *H. Argophyllus* 1806 with low CMI and high variability of LH and EW; empty black triangles: *H. Annuus* CMS-14 × *H. Argophyllus* 1802 with low CMI that was not selected according to high variability of LH and EW; empty black squares: *H. Annuus* CMS-20 × *H. Argophyllus* 1806 with low CMI that was not selected according to high variability of LH and EW; empty grey triangles: *H. Annuus* CMS-14 × *H. Argophyllus* 1802 not selected to the future breeding program; empty grey squares: *H. Annuus* CMS-20 × *H. Argophyllus* 1806 not selected to the future breeding program.

Table 5: Means and standard deviations (in parenthesis) of the F_2 individuals selected to the future breeding program.

Parameter and the step of selections	*H. annuus* CMS-14 × *H. argophyllus* 1802	*H. annuus* CMS-20 × *H. argophyllus* 1806	Both F_2 generations
Number of individuals			
first step	26	25	51
second step	30	9	39
both steps	56	34	90
CMI			
first step	12.3 (7.6)	25.4 (8.7)	18.7 (10.4)
second step	18.7 (5.9)	23.1 (4)	19.7 (5.8)
both steps	15.7 (7.4)	24.8 (7.8)	19.1 (8.7)
EW			
first step	1.52 (0.73)	2.38 (0.77)	1.96 (0.86)
second step	1.34 (0.41)	2.57 (0.59)	1.68 (0.72)
both steps	1.43 (0.6)	2.43 (0.72)	1.85 (0.82)
LH			
first step	2.58 (1.08)	2.96 (0.93)	2.78 (1.01)
second step	2.83 (0.87)	3.48 (0.24)	3 (0.81)
both steps	2.71 (0.97)	3.1 (0.83)	2.87 (0.93)

During the second step, 4 individuals having only the CMI parameter were also considered. Diverse set of 51 F_2 individuals (coming from first step) was supplemented by the 39 individuals (that were not yet selected) with the best (lowest) values of CMI. In this way we selected a high diversity set of individuals (Figure 4) but with desirable values of CMI. The means and standard deviations for those individuals were calculated (Table 5).

F_2 individuals characterized by the low EW and high LH values were more frequent (Figure 4). There were less frequent individuals with high EW and low LH. These individuals independent of CMI were selected during the first step (which may give high diversity). The most F_2 individuals of *H. annuus* CMS-14 × *H. argophyllus* 1802 supplemented during the second step (that based on low CMI) had low EW. *H. annuus* CMS-20 × *H. argophyllus* 1806 had F_2 individuals with high EW and acceptable CMI.

The morphological traits of the selected individuals of both F2 generations were varied (Table 6). The cluster analysis was done to determine the relationship between the branching, head number, leaf shape, fertility, hairiness and canoy color. It was identified whether they were linked or those traits were inherited independently (and in consequence it was free to choose the type of appearance of new breeding lines on the basis of single or multiple morphological traits).

Table 6: Number of individuals that were characterized through morphological traits.

Traits	H. *annuus* CMS-14 × H. *argophyllus* 1802	H. *annuus* CMS-20 × H. *argophyllus* 1806
Head (Multi/Single/not defined)	(33/13/10)	(15/11/8)
Branching (Single/Multi/not defined)	(15/31/10)	(11/15/8)
Leaf type (Narrow/Medium/Broad/Round/ not def.)	(15/7/23/1/10)	(8/1/17/0/8)
Fertility (High/Medium/Low/not def.)	(15/8/9/24)	(8/2/2/22)
Hairiness (High/Medium/Low/NIL/not def.)	(24/9/5/2/16)	(18/3/3/0/10)
Canopy color (Silver/Light green/ Green/not def.)	(18/1/22/15)	(16/2/6/10)

If the cluster analysis describes the relationships between inheritance of studied traits based on all selected individuals then the relationships could be noised – this could be an effect of the selections of varied (according to EW and LH) individuals. If the selection wasn't be focused on the varied sample then the relationships will be slightly stronger. Individuals selected during the the second step had CMI eaqual to 28. The individuals with the CMI not greater than 28 were the sample that wasn't be focused on the variability of EW and LH. The head number (single or multi) was determned by the branching as shown by the denodogram (Figure 5). Moreover the hairiness showed relatonship with canopy color. However, the cluster analysis suggested that the traits were inherited independently. Thus the crosses had varied individuals (Table 6) that could be considered for further selection and new progenies may be selected solely on the bassis of individual plant traits for establishment of new inbreed lines.

Mean performance of F4 progenies under differential water regimes

The performance of F_4 progenies under stress and non-stress has been give in Table 7. The results showed that silver canopied progenies had higher cuticular waxes and leaf hairiness than green canopied progenies and commercial

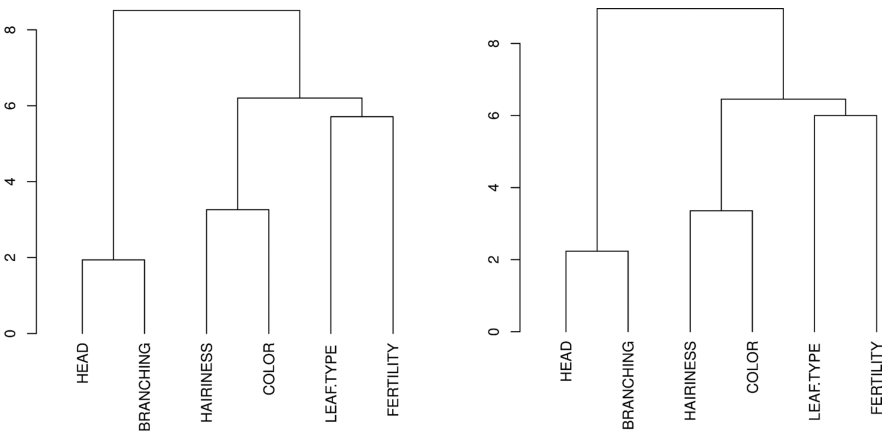

Figure 5: The dendrogram of cluster analysis for subset of 79 individuals (left one) and all 90 selected individuals (right one).

hybrids. Silver canopied progenies showed the lowest yield losses (19 %) when compared with green canopied and commercial hybrids. The green copied progenies had 53 % yield losses while commercial hybrids had yield losses of 65 % (Hysun-33) and 53 % (S-278) (Table 7). The commercial hybrid Hysun-33 had the highest yield under non-stress condition and silved canopied progenies had the highest yield under stress condition. Oil contents % of silver leafed canopy decreased by 12 % followed by 17 % decreased in green canopied progenies. The oil contents% decreased by 19 % and 26 % in Hysun-33 and S-278 respectively (Table 7). The Silver leafed progenies and S-278 had the oil% in non-stress conditions and silver leafed progenies had the highest oil content% under non-stress condition (Table 7).

Discussions

Drought has been major production constained in field crops (Rauf *et al*., 2016), which is atrributed due to the selection under optimum growth condition and absence of functional diversity among the traits associated with drought stress. It has been noted that genetic diversity within cultivated species has been decreased due to the selecton for similar plant types (Rauf *et al*., 2010). The cultivated sunflower germplasm has retained only 50 to 60 % of the genetic diversity (Liu and Burke, 2006; Mandel *et al*., 2011). Reduction in the genetic diversity reduces the adaptablity under diverse condition

Table 7: Average mean performance of F_4 plant progenies in control and stress condition.

Plant Progenies	Cuticular Waxes (mg g^{-1})		Hairiness		Seed Yield (g)		Oil Contents%	
	Control	Stress	Control	Stress	Control	Stress	Control	Stress
Silver leafed	$3.71^{a} \pm 0.64$	$4.52^{a} \pm 0.91$	$3.67^{a} \pm 0.64$	$3.83^{a} \pm 0.27$	$37.28^{d} \pm 4.16$	$30.27^{a} \pm 2.16$	$42.12^{a} \pm 1.11$	$37.19a \pm 1.42$
Green leafed	$0.69^{b} \pm 0.12$	$0.71^{c} \pm 0.17$	$0.54^{b} \pm 0.21$	$0.72^{b} \pm 0.18$	$41.34^{c} \pm 5.19$	$19.34^{b} \pm 1.48$	$36.34^{b} \pm 1.68$	$30.17^{b} \pm 1.24$
Hysun-33	$0.58^{c} \pm 0.14$	$0.51^{d} \pm 0.11$	$0.76^{b} \pm 0.17$	$0.82^{b} \pm 0.15$	$63.29^{a} \pm 3.64$	$22.15^{b} \pm 1.92$	$37.19^{b} \pm 1.04$	$30.24^{b} \pm 1.13$
S-278	$0.7^{5}b \pm 0.1$	$0.86^{b} \pm 0.22$	$0.94^{b} \pm 0.26$	$0.81^{b} \pm 0.24$	$49.39^{b} \pm 4.13$	$23.27^{b} \pm 2.23$	$43.24^{a} \pm 1.23$	$32.17^{b} \pm 1.34$

Values sharing similar letter as superscript are statistically similar $p \leq 0.05$ due to LSD test; mean values of 18 Silver leafed progenies and 12 green leafed progenies in the text.

environmental making susceptible to the biotic and abioti stresses (Rauf *et al*., 2010). Interspecifc hybridization has been used as a tool to introduce new genetic diversity within cultivated germplasm (Rauf *et al*., 2012). Therefore, two species of the sunflower were mated to introduce valuable genetic diversity within germplasm. The study have shown that parental species have contrasting forms of adapatability traits under study (Hussain *et al*., 2015). The F_1 showed significant heterosis with good fertility. However, F_1 was drought susceptible due to dominant masking effect of negative alleles over the positive alleles contributing to the drought resistance. This could be overcome by the selection of appropriate plant types in F_2 populattion having drought related trait introgression (Hussain *et al*., 2015).

The F_2 populations showed high variability for all the three traits i. e. CMI, LH and EW. Success of selection and breeding for improved traits of interest depended upon the magnitude and range of genetic variability (Basha and Sujatha, 2007). Variability of populations in breeding program facilitated selection by favoring expressions of traits (Hayward *et al*., 2012). Without high range of variability in crop plants success in breeding was not possible (Gepts, 2002). The F_2 populations had several plants with good fertility, phenology (single headed) and introgression of several *argophyllus* related traits such as smaller leaf size, silver canopy color, high leaf hariness and epicuticular waxes. These plants may be possible candidate for the development of progenies or inbred lines for the development of hybrids which may increase the genetic diversiy within the sunflower elite germplasm. These inbred line will be mated with appropriate male lines which may yield superior hybrids due to high heterosis as already observed in F_1 and may also have buffering capacity for the environmental vagaries (Rauf *et al*., 2010).

The traits under study showed high magnitude of heritability estimates. The magnistude of heritability also affects the selcetion gains (Kalyar *et al*., 2013). Therefore traits with high heritability were favoured for selection. Heritability measures the extent of genetic variation in total phenotypic variation. It was a useful measure to predict to consequences of artificial selection (Piepho and Möhring, 2007; Visscher *et al*., 2008; Silvertown and Charlesworth, 2009). Traits that show higher heritability were easier to modify by selection in successive generations (Holland *et al*., 2003; Russell and Sandall, 2006).

Cell membrane injury was used to differentiate drought tolerant or susceptible plant types (Blum and Ebercon, 1981). The drought stress induced significant membrane damage (Blum and Ebercon, 1981; ; Tripathy *et al*., 2000; Bajji *et al*., 2002; Farooq *et al*., 2009; Gomes *et al*., 2010.). Therefore, genotypes having stable cell membrane when subjected to the drought stress were regarded as drought resistant (Blum and Ebercon, 1981). However, cell

membrane stability was not strongly related with adaptability traits (leaf hairiness & epicuticular waxes). Thus showing that cell membrane stability was not a function of onlystudied adaptability traits. Cell membrane injury or adaptability traits depicted differential tolerance mechanism in plants. Leaf hairiness played an important role in plant protection from various stresses; i. e. high temperature, drought stress, herbivore's damage etc (Roy *et al*., 1999; Holmes and Keiller, 2002; Zhang and Qi, 2012; Kalyar *et al*., 2013). It increased the resistance of plant to drought stress by reducing transpiration. (Roy *et al*., 1999; Hameed *et al*., 2002, Bacelar *et al*., 2004; Liu *et al*., 2005). Epicuticular waxes also played an important role in controlling water loss from leaf surface and maintain plant integrity during stress condition (Jenks and Ashworth, 1999; Hameed *et al*., 2002; Liu *et al*., 2005; Kerstiens, 2006; Burghardt and Riederer, 2008). The plants with high leaf hairiness, high epicuticular waxes and low cell membrane injury were especially valuable for selection in further generations. These traits could help to act as buffer against yield reduction in sunflower which was later observed in F4 generation.

The study could help to develop a comprehensive breeding program for the development drought tolerant sunflower breeding material, reduction in the yield losses due to drought stress and expansion of functional diversity within sunflower germplasm.

Conclusions

Helianthus argophyllus is known for drought tolerance traits such as epicuticular waxes and leaf hairiness. The project aims to study the variability in F_2 generation and subsequent selection of drought tolerant transgressive segregant in F_2 population on the basis of the traits associated with drought tolerance. The *H. annuus* CMS-14 × *H. argophyllus* 1802 seems to be better than *H. annuus* CMS-20 × *H. argophyllus* 1806 based on CMI of both F_2 generations. The two-step selections maintained high variability especially of leaf hairiness for set of F_2 individuals *H. annuus* CMS-20 × *H. argophyllus* 1806. The F_1 hybrids had mean values of the three studied parameters in the range of parent lines and F_1s, but some of F_2 individuals extend beyond this range. The leaf hairiness depends on the time only with a small degree and cell membrane injury depends on the time a little more. The most of selected F_2 individuals were characterized by the low epicuticular waxes and high Leaf hairiness. The selection of F_2 individuals with high values of leaf hairiness or epicuticular waxes with low cell membrane injury simultaneously was possible. The high values of leaf hairiness and epicuticular waxes were not index of the low CMI value.

Plant having introgressed traits (silver canopy) showed better performance than green leafed plants and commercial hybrids under drought stress.

Conflict of interest Statement

All authors mentioned above certify that they have participated sufficiently in the work to take public responsibility for the content, including participation in the concept, design, analysis, writing, or revision of the manuscript. Furthermore, author (s) certifies that this material or similar material has not been and will not be submitted to or published in any other publication. we have not included an Acknowledgments in our manuscript, then that indicates that we have not received substantial contributions from non-authors.

The authors mentioned above certify that they have NO affiliations with or involvement in any organization or entity with any financial interest or non-financial interest in the subject matter or materials discussed in this manuscript.

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