Proline accumulation has been reported in a diversity of plant species and organs (Abbas et al. 2014, Vendruscolo et al. 2007, Verbruggen and Hermans 2008, Yamada et al. 2005). Proline concentration can increase several folds in response to different abiotic constraints (Hare and Cress 1997, Siripornadulsil et al. 2002) and is considered as a good marker of osmotic stress associated to drought and salinity (Boscaiu et al. 2013, Sayed et al. 2012). A high genetic variability exists among and within crop species for their ability to accumulate proline (Sharma and Verslues 2010). Genotypes with high proline concentration under stress environment are generally considered to be tolerant to a number of abiotic constraints (Abbas et al. 2014, Vendruscolo et al. 2007, Yamada et al. 2005), suggesting the use of this trait as an indirect selection criterion. Proline concentration has however been poorly used in plant breeding programs (Ashraf and Foolad 2013, Sayed et al. 2012, van Rensburg et al. 1993). Successful exploitation of a trait in breeding highly depends on the information available concerning its genetics (Kalyar et al. 2014, Rauf et al. 2009). An indirect selection criterion needs to be positively associated with yield under drought, genetically variable and highly heritable (Edmeades et al. 1997). High narrow sense heritability results in higher genetic gains in early segregating populations and could speed up the development of breeding material (Kalyar et al. 2014). Additive effects of minor alleles generally facilitate the selection due to their contribution to heritability (Kalyar et al. 2014). In barley for instance, epistatic and dominance effects have been reported for proline concentration that limited the potential exploitation of this trait in breeding (Sayed et al. 2012).

Handling large scale screening of germplasm in field experiments is laborious and difficult due to soil heterogeneity and additional field induced injuries such as nutrient deficiencies, high temperature and pest infestation (Rauf et al. 2015). On the other hand, in vitro culture was found useful for screening any number of genotypes in the presence of...
osmotic stress (Rai et al. 2011). Polyethylene glycol (PEG) induced osmotic stress is constant, can be precisely controlled and results are repeatable (Rai et al. 2011, Rauf et al. 2015). Therefore, in vitro techniques are very useful for discriminating large number of genotypes for their capacity of osmotic adjustment mediated by various compatible solutes such as proline (Rai et al. 2011).

The genetics of proline accumulation have been analyzed in this study in seedlings of 60 sunflower hybrids and 17 their parental lines, submitted to osmotic stress under in vitro conditions. The association of this trait with osmotic potential, osmotic adjustment, growth parameters, the accumulation of compatibles solutes like potassium, calcium and total soluble sugars and the presence of stress induced symptoms has also been examined.

**Materials and Methods**

**Plant material**

A total of 19 sunflower lines, extracted from a segregating drought tolerant population developed by the Department of Plant Breeding and Genetics of the University College of Agriculture of Sargodha, Pakistan, were used in this study. These 19 breeding lines included twelve cytoplasmic male sterile lines (CMS) and seven fertility restorer (FR) lines. Each of the CMS lines was crossed (as female parent) to each of the FR lines (as male parent) to potentially develop 84 full and half sib hybrids. Due to inability of reproductive synchronization between female and male lines in some cross combinations, only 75 hybrids were obtained and used for the analysis of association between proline concentration and the other measured traits. As a complete set of some crosses were missing, only 60 crosses, between twelve CMS lines and five FR lines, were available for genetic analysis. Heads of breeding lines were covered with a bag to avoid pollen contamination. Pollen from FR lines was collected early in the morning. CMS lines were pollinated daily until stigma withers away. Sunflower heads were harvested, dried and seeds were threshed. All seeds were kept in kraft paper bags, stored in cool condition and protected from sunlight.

**Evaluation under osmotic stress conditions**

Healthy seeds of the 19 parental lines and full or half sib 75 hybrids were disinfected with a 15% sodium hypochlorite solution for 8–10 minutes followed by one rinse with 70% ethanol and three rinses with doubled distilled water in a laminar airflow cabinet. Seeds of parental lines and crosses were germinated after removing seed coats in Petri dishes at 25°C/15°C day and night, under photoperiod of 16 hours and 60% relative humidity.

The experiment was carried out in a completely randomized design with two factors (genotypes and treatments) and three replications. Germinated seedlings were shifted to culture boxes (8 × 8 × 10 cm) containing 100 ml of 50% diluted MS media (Murashige and Skoog 1962). Two osmotic conditions were created by adding polyethylene glycol (PEG) molecular weight of 6000 at the concentration of 0 and 50 g L\(^{-1}\) before solidification of the medium using 8% agar. Addition of 50 g L\(^{-1}\) (PEG) created a difference of osmotic stress of –2.42 ± 0.06 MPa.

**Traits measurement**

After three weeks, the seedlings were harvested and carefully dried to remove any moisture on the surface of seedlings. Seedlings root and shoot length were measured. Root and shoot weights were determined on digital analytical balance (GC 2502, Sartorius, Göttingen, Germany).

Proline concentration (Pro) was assessed according to Bates et al. (1973). Acid ninhydrin solution was prepared by heating 1.25 g of ninhydrin in 20 ml of 6 M phosphoric acid and 30 ml of glacial acetic acid with continuous stirring until mixture was completely homogenized. 0.5 g of fresh leaf sample taken from each genotype was homogenized in 10 ml of 3% aqueous sulfosalicylic acid. Homogenized samples were centrifuged (Universal 320 R, Hettich Zentrifugen, Tuttlingen, Germany) at a temperature of 25°C and 4000 RPM for 10 minutes. 2 ml of extract from each centrifuged sample was carefully taken and put in separate test tubes. 2 ml glacial acetic acid and 2 ml acid ninhydrin were also added in these test tubes. Prepared samples were kept at 100°C for 1 hour and reaction was terminated in ice bath. 4 ml of toluene was added in the reaction mixture and stirred for 15–20 seconds with test tube stirrer. Chromophore developed was carefully aspirated, warmed to 25°C and absorbance was read at wavelength of λ = 520 nm with a UV-2600 spectrophotometer (Shimadzu, Kyoto, Japan) by using sulfosalicylic acid as blank and standards of known concentration of proline. Capacity to accumulate proline (CAP) was calculated as 

\[
\frac{\text{[proline concentration under osmotic stress]}}{\text{[proline concentration under non-stress]}}
\]

Potassium concentration was determined by collecting fresh leaf samples in Eppendorf tubes. Samples were freeze-dried and thawed after 2 days for extraction of cell sap. Thawed samples were pressed and centrifuged (Universal 320 R, Hettich Zentrifugen, Buckinghamshire, England) at a temperature of 25°C and 9000 RPM for 10 minutes. Supernatant was carefully extracted and diluted 30 times by adding deionized water. Potassium concentration of the solution was analyzed using a flame photometer (PFP 7, Jenway, Staffordshire, UK). The diluted sap solution used for potassium content determination was also utilized for the analysis of calcium concentration on atomic absorption spectrophotometer (Spectra AA 220, Varian, Santa Clara, USA). Total soluble sugars (TSS) were determined by filling the detector lens of refractometer (OPTI 38-02, Bellingham and Stanley, Kent).

Osmotic potential was determined on cell sap, extracted from the frozen samples. Samples were freeze-dried and thawed after 2 days for extraction of cell sap. Thawed samples were pressed and centrifuged (Universal 320 R, Hettich
Zentriugen, Buckinghamshire, England) at a temperature of 25°C and 9000 RPM for 5 minutes to remove debris. Supernatant was carefully extracted through micropipette and osmotic potential of cell sap from every sample was estimated using an osmometer (Vapro 5520, Viescor, Utah, USA). Osmotic potential of all plant samples (under stress and non-stress) were determined after humidifying them at 25°C over night to regain full turgor (Blum and Sullivan 1986). Cell sap was extracted according to the method described above and osmotic potential was determined on osmometer (Vapro 5520, Viescor, Utah, USA). Osmotic adjustment was measured according to the rehydration method, i.e., by subtracting the osmotic potential under non-stress regime to the osmotic potential under osmotic stress after recuperation of full turgor (Babu et al. 1999) through humidifying the sample overnight.

Injuries due to osmotic stress were visually evaluated on basis of extent of calli and other deformation on the seedlings. Plant were visually rated from 0–4, where 0 was the absence of any injury, 1 = minute lower injury, 2 = partial callus (whitish green) formation with significant reduction in growth when compared with non-stress, 3 = full (whitish/black) callus formation with no growth under osmotic stress, 4 = complete death of plant.

Data analysis

Genetic analysis and combining ability effects were estimated according to Kempthorne (1957). Heterosis shown by hybrids was calculated as [(F1–MP)/MP] × 100, where F1 is the mean performance of each of the crosses and MP is mid-parent value, (P1 + P2)/2. The degree of dominance was estimated as (F1−MP)/(BP−MP) where BP is the best parent estimated as 

\[
\frac{(1 + F)}{4} \times \sigma^2 \]

by hybrids was calculated as 

\[
\frac{(F1−MP)}{MP} \times 100
\]

where F1 is the sum of i-th CMS lines values over all FR lines, Xj is the sum of j-th FR lines values over all CMS lines, Xij is the grand total of all i and j cross combinations, and where and l = no. of CMS lines, t = number of fertility restorer lines, r = number of replications.

Correlations among traits were calculated within the set of 75 hybrids from values obtained under osmotic stress. The significance (two-tailed p-values) of Pearson correlation coefficients between two traits was calculated using the correlation value r and the sample size.

The osmotic treatment highly significantly (P ≤ 0.01) affected proline concentration (Table 1). A highly significant (P ≤ 0.01) genotype effect was also noted for this trait. This effect remained significant within the set of lines and the set of hybrids. Significant interactions between genotypes and treatments suggested a different ranking of genotypes for proline concentration in the two contrasting osmotic conditions (Table 1). The difference in proline concentration between lines and hybrids was highly significant (P ≤ 0.01). A significant variation (P ≤ 0.01) was noted for this trait within the set of CMS lines but not within the set of FR lines. CMS lines differed from FR lines for this trait.

Genotypic effect was significant for proline concentration in each treatment and for capacity to accumulate proline, within 60 hybrids used for genetic analyses. Genotypic and phenotypic variances of these traits sharply increased under osmotic stress. In absence of osmotic stress, additive variance was nil for proline concentration (Table 2). Additive and dominance effects for this trait increased and became almost similar under osmotic stress (Table 2). Genetic variance and broad sense heritability estimates were higher under osmotic stress compared to non-stress conditions.

| Source of variation | Degrees of freedom | Mean sum of square |
|---------------------|--------------------|--------------------|
| Genotype (G)        | 93                 | 0.67**             |
| Line (L)            | 18                 | 0.29**             |
| Hybrid (H)          | 74                 | 0.74**             |
| L vs. H             | 1                  | 2.86**             |
| Treatment (T)       | 1                  | 30.19**            |
| G × T               | 93                 | 0.32**             |
| L × T               | 18                 | 0.07**             |
| H × T               | 74                 | 0.35**             |
| FR line             | 4                  | 0.71NS             |
| CMS line            | 11                 | 1.14**             |
| CMS line × FR line  | 44                 | 0.41**             |
| Error               | 376                | 0.02               |

**P ≤ 0.01, NS not significant.
Dominance effects were higher than additive effects for the capacity to accumulate proline. Therefore, low narrow sense heritability was estimated for the capacity to accumulate proline (Table 2).

Proline concentration was higher in hybrids, compared to inbred lines (Table 3) and osmotic stress increased the differences in proline concentration between these two sets of genotypes. Proline concentration was similar in CMS lines (0.36 μg g⁻¹) and hybrids (0.35 μg g⁻¹) under non-stress conditions (Table 3). However, hybrids showed a wide range of mean values than their parental lines. Hybrids had the highest ability to accumulate proline under osmotic stress followed by FR parental lines. Heterosis and degree of dominance were low under non-stress conditions but considerably increased with osmotic stress for both proline concentration and capacity to accumulate proline.

Proline concentration of inbred lines under osmotic stress was not associated to their capacity to accumulate proline in response to osmotic stress (Fig. 1). For example, quadrate 1 included three CMS lines (M-2, M-3, M-5, M-20) with high proline concentration and low capacity to accumulate proline while quadrate IV included lines with low proline contents but high capacity to accumulate proline under osmotic stress. The relationship between dominance and the capacity to accumulate proline was positive (Fig. 2). Over dominance type of gene action (reflected by potence ratio > 1) was significantly correlated to heterosis (Fig. 3). No relationship was noted between proline concentration and the general combining ability (GCA) of parental lines (Fig. 4). For example, quadrate I included breeding lines with high capacity to produce proline under osmotic stress and low general combining ability while quadrate IV included lines with low proline concentration and high GCA. Quadrate III grouped lines (R-8 and M-9) with high GCA and proline concentration.

Within hybrids proline concentration was positively associated to osmotic adjustment but negatively to potassium concentration and growth traits such as root length, shoot length, root weight and shoot weight (Table 4). Capacity to

### Table 2. Genetic analysis of proline concentration and capacity to accumulate proline within the two contrasting osmotic treatments

| Source of variation | Mean sum of square | Proline concentration | Capacity to accumulate proline |
|---------------------|--------------------|-----------------------|--------------------------------|
|                     | Control            | Osmotic stress        |                                |
| \(\sigma^2_g\)      | 0.04               | 0.28                  | 3.10                           |
| \(\sigma^2_p\)      | 0.06               | 0.34                  | 3.94                           |
| \(\sigma^2_{additive}\) | 0.00               | 0.12                  | 0.80                           |
| \(\sigma^2_{dominance}\) | 0.03               | 0.15                  | 2.26                           |
| \(h^2_{bs}\)        | 0.72               | 0.82                  | 0.79                           |
| \(h^2_{ns}\)        | 0.02               | 0.35                  | 0.20                           |

\(\sigma^2_g\) = genotypic variance, \(\sigma^2_p\) = phenotypic variance, \(h^2_{bs}\) = broad sense heritability, \(h^2_{ns}\) = narrow sense heritability.

### Table 3. Mean and ranges of proline concentration in lines and hybrids, heterosis and degree of dominance

| Genotypes | Proline \(\mu g g^{-1}\) | Capacity to accumulate proline |
|-----------|---------------------------|-------------------------------|
|           | Control Range             | Osmotic stress Range          |                                |
| Lines     | 0.31±0.03 0.12–1.02       | 0.55±0.07 0.22–1.14           | 1.07±0.21 –0.14–3.19           |
| CMS lines | 0.36±0.03 0.14–1.02       | 0.61±0.06 0.27–1.14           | 0.99±0.26 –0.14–2.67           |
| FR lines  | 0.22±0.03 0.12–0.32       | 0.45±0.08 0.22–0.74           | 1.20±0.31 0.11–3.19            |
| Hybrids   | 0.35±0.08 0.10–1.19       | 0.87±0.21 0.14–2.15           | 1.84±0.21 –0.59–8.37           |
| Heterosis | 0.11 0.59                | 0.72                            |
| Degree of dominance (potence ratio) | 0.67 5.55 | 10.18 |

Values sharing similar letter are statistically similar at 0.05%. AVG is average of all lines, Range indicate the minimum and maximal values over replications CMS, FR or hybrids and STD is standard deviation estimated over replications.
accumulate proline was negatively correlated with osmotic potential, shoot weight and proline contents and significantly positively associated to osmotic adjustment, potassium concentration and total soluble sugars concentration. Stress induced injury, estimated from the presence of calli on the seedlings, was significantly (P ≤ 0.05) negatively associated to the capacity to accumulate proline. Moreover, stress induced injury was significantly (P ≤ 0.05) negatively correlated with osmotic adjustment, proline contents, root length, shoot length and shoot weight. These associations showed that hybrids with higher values of osmotic adjustment, capacity to accumulate proline and proline contents per se had lower stress injury. Hybrids with lower stress injury also had higher root length, shoot length and shoot weight.

**Discussion**

**Effects of osmotic stress on proline concentration**

A significant concentration of proline was noted in the control treatment indicating that the accumulation of this amino acid may also occur in non-stressed conditions, as previously reported by Mattioli et al. (2009). The concentration of proline however significantly increased with osmotic stress. The effects of water stress on proline concentration were reported in many crops including sunflower under random field condition where irrigation was skipped throughout the reproductive stage (Pourmohammad et al. 2014). The accumulation of proline in response to osmotic stress was however relatively modest in the present study. Concentration was doubled under osmotic stress, however a maximum of 8 times increased of proline was noted in some genotypes compared to unstressed situation, while some authors reported levels of accumulation 100 times greater than in control situation (Verbruggen and Hermans 2008).

**Genetic variation in proline concentration and capacity to accumulate proline**

Significant genotypic differences in proline concentration have been previously reported in sunflower (Canavar et al. 2014, Cechin et al. 2006, Oraki et al. 2012, Pourmohammad et al. 2014). In the present study, proline concentration was higher in hybrids than in parental lines, as previously noted by Petrović et al. (1992) and Pourmohammad et al. (2014) and a significant genotype effect was noted for this trait within both lines and hybrids. The capacity to accumulate proline was negatively correlated with osmotic potential, shoot weight and proline contents and significantly positively associated to osmotic adjustment, potassium concentration and total soluble sugars concentration. Stress induced injury, estimated from the presence of calli on the seedlings, was significantly (P ≤ 0.05) negatively associated to the capacity to accumulate proline. Moreover, stress induced injury was significantly (P ≤ 0.05) negatively correlated with osmotic adjustment, proline contents, root length, shoot length and shoot weight. These associations showed that hybrids with higher values of osmotic adjustment, capacity to accumulate proline and proline contents per se had lower stress injury. Hybrids with lower stress injury also had higher root length, shoot length and shoot weight.

### Table 4. Pearson correlation between osmotic potential (OP), osmotic adjustment (OA), potassium concentration (K), calcium concentration (Ca), root length (RL), shoot length (SL), root weight (RW), shoot weight (SW), total soluble sugars (TSS), capacity to accumulate proline (CAP) and callus intensity (callus) under osmotic stress in hybrids. The significance (two-tailed p-values) of Pearson correlation coefficients were calculated using the correlation value r, and the sample size.

| Trait | OP | OA | K | Ca | Pro | RL | SL | RW | SW | TSS | CAP |
|-------|----|----|---|----|-----|----|----|----|----|-----|-----|
| OA    | -0.67** |    |   |    |     |    |    |    |    |     |     |
| K     | 0.30**  | -0.22** |    |    |     |    |    |    |    |     |     |
| Ca    | 0.43**  | -0.02NS | 0.47** |    |     |    |    |    |    |     |     |
| Pro   | -0.06NS | 0.26** | -0.19* | 0.06NS |    |    |    |    |    |     |     |
| RL    | 0.19*   | -0.31** | 0.43** | 0.30** | -0.27** |    |    |    |    |     |     |
| SL    | 0.26**  | -0.41** | 0.44** | 0.05NS | -0.27** | 0.73** |    |    |    |     |     |
| RW    | 0.21*   | -0.45** | 0.05NS | -0.11NS | -0.29** | 0.63** | 0.37** |    |    |     |     |
| SW    | 0.31**  | -0.30** | 0.71** | 0.45** | -0.27** | 0.69** | 0.67** | 0.39** |    |     |     |
| TSS   | 0.19*   | -0.39** | -0.17NS | 0.23** | -0.04NS | -0.03NS | -0.04NS | 0.12NS | 0.24** |    |     |
| CAP   | -0.29** | 0.21* | 0.19* | 0.06NS | -0.19* | -0.08NS | -0.11NS | -0.16NS | -0.36** | 0.33** |     |
| Callus| -0.26** | -0.28** | 0.14NS | 0.11NS | -0.37** | -0.39** | -0.23* | 0.22* | -0.24* | 0.13NS | -0.44* |

* P ≤ 0.05, ** P ≤ 0.01, NS not significant.
proline in response to osmotic stress was also much higher in hybrids than in inbred lines. Significant interactions between genotypes and treatments suggested a different ranking for proline concentration in the two contrasting osmotic conditions as reported by Pourmohammad et al. (2014). Genotype, treatment and interaction effects were also noted for the capacity to accumulate proline which was also higher in hybrids, compared to their parental lines (Table 2).

**Association with osmotic adjustment**

The highly significant positive association between proline concentration and osmotic adjustment suggests that the accumulation of this free amino-acid is playing an important role in this osmotic stress avoidance mechanism (Table 4). Proline has been repeatedly reported as an important component of osmotic adjustment in various crop species including sunflower (Boscaiu et al. 2013, Sharmaand Verslues 2010, Vendruscolo et al. 2007). The capacity to accumulate proline was also positively associated with osmotic adjustment suggesting that osmotic adjustment is related to the capacity of the plant to specifically accumulate the amino-acid under osmotic stress.

**Association with growth traits**

Whereas many authors have reported a positive association between proline accumulation and growth under drought or salinity stress, this is not corroborated by other studies (Deau-Jeney and Verma 1993). The sign and magnitude of the correlation are likely to depend on the stage of assessment of proline concentration as proline accumulation does not start quickly after stress imposition (Ünyayar et al. 2004) but rather when cell injury is evident (Moftah and Michel 1987). The significant negative correlation between proline concentration and morphological traits such as root length, shoot length, root weight and shoot weight indicate that proline accumulation does not directly translate in increased growth (Table 4). Osmotic adjustment and proline accumulation could be related with improved survivability of seedling under osmotic stress. Proline has been reported to minimize the effects of cell damage (Caplan et al. 1990) by protecting enzymes (Thomas 1990), scavenging ROS (Smirnoff and Cumbes 1989), stabilizing the structure of proteins, buffering cytosolic pH and balancing cell redox status. It could also constitute a carbon and nitrogen source for stress recovery (Hare and Cress 1997, Trotel et al. 1996). The hybrids with high capacity to accumulate proline also showed highly significantly lower frequency of calli, reflecting a lower level of osmotic stress induced injury.

**Heritability and gene action**

In both treatments, genotypic variance of proline concentration was high, leading to high broad sense heritability, an important condition for the trait to be used in breeding (Tables 1, 2). Moreover, broad sense heritability was higher under osmotic stress than under non-stress conditions suggesting to select for this trait under stressed conditions. In both treatments narrow sense heritability values were however much lower than those reported by Pourmohammad et al. (2014) suggesting that selection for proline concentration may not be effective under the present experimental conditions in early segregating populations. This could be related in part to the lower stress induced accumulation of proline and genetic variation noted in our conditions, compared to this reported by this author.

In sunflower, several studies regarding gene action of agronomic traits has been carried out, mainly under optimal conditions (Bajaj et al. 1997, Ghaffari et al. 2011, Hussain et al. 1998, Pourmohammad et al. 2014, Radhika et al. 1999). In absence of osmotic stress, dominance effects explained here most of the genetic variation for proline concentration. This result disagrees with Pourmohammad et al. (2014) who reported in field conditions a predominance of additive effects. Under osmotic stress both dominance and additive variance were high (Table 2). The increase of additive and dominance effects with osmotic stress indicated that osmotic stress treatment enhanced the additivity of alleles related to proline concentration and also favored intral allelic interactions. Stress regime was found to represent a better environment for selection since it promoted additive effects of alleles associated with proline concentration and capacity to accumulate proline under osmotic stress. The importance of dominance effects noted along with additive effects suggested that several genomic regions are controlling these traits, as previously reported in barley through a QTL analysis (Sayyed et al. 2012). The relationship between dominance and the capacity to accumulate proline was positive. Capacity to accumulate proline showed dominance effect along with small additive effects. Moderate narrow sense heritability was noted for capacity to accumulate proline contents indicating the possibility to select for this trait in early segregating generations.

**General combining ability and heterosis**

General combining ability helps to identify the parent with the highest proportion of positive additive alleles associated with trait of interest (Townsend et al. 2013). As such it is not necessarily associated with mean values since the effects of negative alleles may be masked by positive alleles in breeding lines and uncovered in the hybrid if the combined line also carries negative alleles for the same trait (Dabholkar 2006). Parents with high proportion of positive additive alleles may be recombined through various types of breeding procedures to develop transgressive segregants carrying most of the positive alleles in single genotypes (Dabholkar 2006). Unsurprisingly, no relationship was noted between proline concentration and the general combining ability (GCA) of parental lines (Fig. 4). General combining ability, the average performance of a line in a series of crosses, and a good predictor of heterotic performance (Kadkol et al. 1984) and information about this genetic characteristic has proven to be useful in the production of
high yielding sunflower hybrids (Škorić 1992). The breeding lines M-7 is a good example of general combiners, carrying probably a high proportion of positive additive alleles affecting proline concentration. Heterosis was significantly correlated to dominance (reflected by potence ratio > 1). The higher capacity of hybrids to accumulate proline may be due to heterozygous genetic background which induces dominance type of gene action. Crosses with high degree of dominance could be exploited commercially. Heterosis for proline concentration was much higher under osmotic stress, reflecting that the advantage of hybrids over their parental lines for this trait increased with stress. 

**Correlation between traits**

Capacity of hybrids to accumulate proline showed a negative and significant relationship with proline concentration and a significant positive relationship with osmotic adjustment, potassium and total soluble sugars concentration, confirming the role of proline accumulation as a component of osmotic adjustment. Other osmolytes such as potassium, calcium and total soluble sugars could contribute to osmotic adjustment passively as their increased concentration may be due to the decreased moisture contents in the plants subjected to the osmotic stress. Despite lack of potassium and calcium contribution as active osmolytes in osmotic adjustment, their concentration was positively related to seedling biomass suggesting their involvement in some other mechanism of drought adaptation. For instance calcium may be involved in an alternative pathway that switches on stress-adaptable genes (Chinnusamy et al. 2004, Reddy et al. 2011). Different stresses are known to cause signal-specific changes in cellular calcium concentration which in turn serves as a messenger for modulating various physiological adaptive processes under abiotic stresses. Several Ca++ or Ca++/calmodulin-binding factors which are paying an important role in stress signaling pathways have been identified in plants (Reddy et al. 2011).

**Conclusion**

Overall negative relationship with morphological traits indicated that proline concentration may not be related with enhancing growth in sunflower but could increase survivability under stress by acting as active osmotic contributing to the osmotic adjustment, and could participate in rapid recovery. Genotypic variance and broad sense heritability of proline concentration were high, particularly under osmotic stress, allowing utilization of this trait in breeding programs. The low narrow sense heritability values of proline concentration suggested that selection for proline concentration in seedlings and under the present experimental conditions may not be effective in early segregating populations over both regimes. Conversely, selection in early generations may be possible for capacity to accumulate proline or direct selection for proline contents under osmotic stress, traits which showed higher narrow sense heritability. The importance of additive or dominance effects reflected by high additivity of alleles and intra-allelic interactions suggested that several genomic regions are controlling proline concentration. Transgressive effects and heterosis could be enhanced using lines with high general combining ability. 

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**Literature Cited**

Abbas, S.R., S.D. Ahmad, S.M. Sabir and A.H. Shah (2014) Detection of drought tolerant sugarcane genotypes (*Saccharum officinarum*) using lipid peroxidation, antioxidant activity, glycine-betaine and proline contents. J. Soil Sci. Plant Nutr. 14: 233–243.

Allard, R.W. (1999) Principles of plant breeding, 2nd ed. John Wiley & Sons, New York.

Ashraf, M. and M.R. Foolad (2013) Crop breeding for salt tolerance in the era of molecular markers and marker-assisted selection. Plant Breed. 132: 10–20.

Babu, R.C., M.S. Pathan, A. Blum and H.T. Nguyen (1999) Comparison of measurement methods of osmotic adjustment in rice cultivars. Crop Sci. 39: 150–158.

Bajaj, R.K., K.K. Aujla and G.S. Chalal (1997) Combining ability studies in sunflower (*Helianthus annuus* L.). J. Crop Improv. 24: 50–54.

Bates, L.S., R.P. Waldren and I.D. Teare (1973) Rapid determination of free proline for water-stress studies. Plant Soil 39: 205–207.

Blum, A. and C.Y. Sullivan (1986) The comparative drought resistance of landraces of sorghum and millet from dry and humid regions. Ann. Bot. 57: 835–846.

Boscaiu, M., C. Lull, J. Llinares, O. Vicente and H. Boira (2013) Proline as a biochemical marker in relation to the ecology of two halophytic *Juncus* species. J. Plant Ecol. 6: 177–186.

Canavar, Ö., K.P. Götz, F. Tellimer, F.M. Chmielewski and M.A. Kaynak (2014) Determination of the relationship between water use efficiency, carbon isotope discrimination and proline in sunflower genotypes under drought stress. Aust. J. Crop Sci. 8: 232–242.

Caplan, A., B. Claes, R. Dekeyser and M. Van Montagu (1990) Salinity and drought stress in rice. In: Sangwan, R.S. and B.S. Sangwan-Norreel (eds.) The impact of biotechnology in agriculture, Kluwer Academic, Dordrecht, the Netherlands, pp. 391–402.

Cechin, I., S.C. Rossi, V.C. Oliveira and T.F. Fumis (2006) Photosynthetic responses and proline content of mature and young leaves of sunflower plants under water deficit. Photosynthetica 44: 143–146.

Chinnusamy, V., K. Schumaker and J.K. Zhu (2004) Molecular genetic perspectives on cross-talk and specificity in abiotic stress signaling in plants. J. Exp. Bot. 55: 225–236.

Comstock, R.E., A. Robinson and J.W. Gowen (1952) Estimation of average dominance of genes. In: Gowen, J.W. (ed.) Heterosis, Ames, la.: Iowa State College Press, pp. 494–516.

Dahbolkar, A.R. (2006) General plant breeding, Concept Publishing Company, New Dehli, India.

Delauney, A.J. and D.P.S. Verma (1993) Proline biosynthesis and osmoregulation in plants. Plant J. 4: 215–223.

Edmeades, G.O., J. Bolaños and S.C. Chapman (1997) Value of selection for proline concentration in *Helianthus annuus* and gene action for agronomic traits and oil content in sunflower (*Helianthus annuus* L.) using F1 hybrids. Crop Breed. J. 1: 75–87.
Hare, P.D. and W.A. Cress (1997) Metabolic implications of stress-induced proline accumulation in plants. Plant Growth Reg. 21: 79–102.

Hayman, B.I. (1957) Interaction, heterosis and diallel crosses. Genetics 42: 336–355.

Hussain, M.K., S.N. Muhammad and O.U. Rehman (1998) Combining ability estimates in some salt tolerant inbreds of sunflower (Helianthus annuus L.). Helia 21: 35–40.

Kadkol, G.P., I.J. Anand and R.P. Sharma (1984) Combining ability and heterosis in sunflower. Ind. J. Genet. Plant Breed. 44: 447–451.

Kalyar, T., S. Rauf, J.A. Teixeira da Silva and M. Shahzad (2014) Handling sunflower (Helianthus annuus L.) populations under heat stress. Arch. Agron. Soil Sci. 60: 655–672.

Kemphorne, O. (1957) An introduction of genetic statistics, John Willey & Sons Inc. New York, USA, pp. 468–473.

Mattioli, R., P. Costantino and M. Trovato (2009) Proline accumulation in plants. Not only stress. Plant Signal Behav. 4: 1016–1018.

Mofarah, R. and B.E. Michel (1987) The effect of sodium chloride on soluble potential and proline accumulation in soybean leaves. Plant Physiol. 83: 238–240.

Murashige, T. and F. Skoog (1962) A revised medium for rapid growth of tobacco tissue cultures. Physiol. Plant. 15: 473–497.

Oraki, H., F. Parhizkarkhajani and M. Aghaalkhah (2012) Effect of water deficit stress on proline contents, soluble sugars, chlorophyll and grain yield of sunflower (Helianthus annuus L.) hybrids. Afr. J. Biotechnol. 11: 164–168.

Petrović, M., R. Kastori, D. Škorić and N. Petrović (1992) Effects of lead on water relations in sunflower and sugar beet plants. Helia 15: 57–64.

Pourmohammad, A., M. Toorchi, S.S. Alavikia and M.R. Shakiba (2014) Genetic analysis of yield and physiological traits in sunflower (Helianthus annuus L.) under irrigation and drought stress. Not. Sci. Biol. 6: 207–213.

Radhika, P., K. Jagadeshwar and P.S. Sharma (1999) Genetic analysis of seed yield and certain physiological parameters in sunflower. J. Res. Angrau 27: 5–17.

Rai, M.K., R.K. Kalia, R. Singh, M.P. Gangola and A.K. Dhawan (2011) Developing stress tolerant plants through in vitro selection—an overview of the recent progress. Environ. Exp. Bot. 71: 89–98.

Rauf, S., H.A. Sadaqat, I.A. Khan and R. Ahmed (2009) Genetic analysis of leaf hydraulics in sunflower (Helianthus annuus L.) under drought stress. Plant Soil Environ. 55: 62–69.

Rauf, S., J.M. Al-Khayri, M. Zaharieva, P. Monneveux and F. Khalil (2015) Breeding strategies to enhance drought tolerance in crops. In: Al-Khayri, J.M., S.M. Jain and D.V. Johnson (eds.) Advances in plant breeding strategies; Agronomic, abiotic and biotic stress traits, Springer, Heidelberg, pp. 1–54.

Reddy, A.S., G.S. Ali, H. Celesnik and I.S. Day (2011) Coping with stresses: roles of calcium-and calcium/calmodulin-regulated gene expression. Plant Cell 23: 2010–2032.

Sayed, M.A., H. Schumann, K. Pillen, A.A. Naz and J. Léon (2012) AB-QTL analysis reveals new alleles associated to proline accumulation and leaf wilting under drought stress conditions in barley (Hordeum vulgare L.). BMC Genet. 13: 61.

Sharma, S. and P.E. Verslues (2010) Mechanisms independent of abscisic acid (ABA) or proline feedback have a predominant role in transcriptional regulation of proline metabolism during low water potential and stress recovery. Plant Cell Environ. 33: 1838–1851.

Škorić, D. (1992) Achievements and future directions of sunflower breeding. Field Crops Res. 30: 231–270.

Smirnoff, N. and Q.J. Cumbes (1989) Hydroxyl radical scavenging activity of compatible solutes. Phytochemistry 28: 1057–1060.

Thomas, H. (1990) Osmotic adjustment in Lolium perenne, its heritability and the nature of solute accumulation. Ann. Bot. 66: 521–530.

Townsend, T., V. Segura, G. Chigeza, T. Penfield, A. Rae, D. Harvey, D. Bowles and I.A. Graham (2013) The use of combining ability analysis to identify elite parents for Artemisia annua F1 hybrid production. PLoS ONE 8: e61989.

Trotel, P., A. Bouchereau, M.F. Niogret and F. Larher (1996) The fate of osmo-accumulated proline in leaf discs of rape (Brassica napus L.) incubated in a medium of low osmolarity. Plant Sci. 118: 31–45.

Ünyayar, S., Y. Keleş and E. Ünal (2004) Proline and ABA levels in Artemisia annua under water deficit stress. J. Plant Physiol. 161: 1367–1376.

Van Rensburg, L., G.H.J. Krüger and H. Krüger (1993) Proline accumulation as drought-tolerance selection criterion: its relationship to membrane integrity and chloroplast ultrastructure in Nicotiana tabacum L. J. Plant Physiol. 141: 188–194.

Vendruscolo, E.C.G., I. Schuster, M. Pileggi, C.A. Scapim, H.B.C. Molinari, C. Marur and L.G.E. Vieira (2007) Stress-induced synthesis of proline confers tolerance to water deficit in transgenic wheat. J. Plant Physiol. 164: 1367–1376.

Verbruggen, N. and C. Hermans (2008) Proline accumulation in plants: a review. Amino Acids 35: 753–759.

Yamada, M., H. Morishita, K. Urano, N. Shiozaki, K. Yamaguchi-Shinozaki, K. Shinozaki and Y. Yoshida (2005) Effects of free proline accumulation in petunias under drought stress. J. Exp. Bot. 56: 1975–1981.