Selection of Contrasting Parents for Drought Tolerance in Sunflower (Helianthus Annuus L.)

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Selection of contrasting parents for drought tolerance in sunflower (*Helianthus annuus* L.)

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**Abstract** The aim of this research was to select the best combination of contrasting parents to develop a mapping population for drought tolerance, based on phenotypic and genotypic data. Phenotyping was conducted in a greenhouse during 16 days at vegetative stage under well-watered (WW) and water-deficit (WD) conditions. Traits evaluated were: gain of leaf area (GLA), total water use (TWU), net assimilation rate (NAR), water use efficiency (WUE) and transpiration rate (TR) response to vapor pressure deficit (VPD) (slope and breakpoint). Genotyping was performed with 127 SSR markers and a cluster analysis was conducted. An important interaction was observed for NAR, WUE and breakpoint in the VPD response. Under WD conditions, all genotypes showed lower GLA and TWU, whereas NAR and WUE increased its values. All genotypes showed reduction of the slope and breakpoint in high VPD response on WD. PCA analysis explains the 80% of the total variability. PC1 discriminated HA89 and R419 due to a lower slope and higher breakpoint, while PC2 separated by water treatment based on the WUE and TWU values. Ninety nine SSR markers were amplified detecting 262 alleles. Cluster analyzes showed two main groups, one including HAR4 and B59 and the other one including five remaining genotypes. According to these results, only R419xHA64 and HA89xHAR4 had a greater genetic distance (1.08), besides a high polymorphism level between ILs (about 60%). Therefore, we conclude that these would be the best combination of contrasting parents to develop mapping populations for drought tolerance in sunflower.

**Keywords** Sunflower Inbred Lines, SSR markers, Drought Tolerance, Phenotyping, Water Use Efficiency, Vapor Pressure Deficit Response.
Introduction

Sunflower (*Helianthus annuus* var. *macrocarpus* (DC) Cockerell) is the fourth most important sources of vegetable oil in the world and the second most important in Argentina. In this sense, the country is the fourth producer with 6.2% of world oil production for the 2021/2022 season (USDA 2021). In this season, a harvested world area of 27 million hectares was recorded, with a seed production of 54.92 million tons; being Ukraine, Russia, European Union and Argentina the main producers for the 2021/2022 season (USDA 2021).

The cultivated sunflower is an annual plant and belongs to the family *Asteraceae*. It is a diploid species (2n=2x=34) with a haploid genome size of 3.6 Gb (Badouin et al. 2017). Is originally from the center-east region of the United States and from there it spread to the rest of the world. In Argentina, it was introduced in the 19th century by Jewish immigrants, which brought with them seeds of sunflower Russian varieties. Then, between 1930 and 1959 it expanded as an oilseed crop. From 1960, INTA through the genetic breeding, developed the first local varieties. This created a rich genetic variability that allowed the conformation of Argentinian germplasm from materials of Russian origin, local varieties and wild species (Vásquez 2002).

Actually, the sunflower breeding program of Instituto Nacional de Tecnología Agropecuaria (INTA) has released germplasm of phytotechnical value, not only in Argentina but also in the United States and Europe; thus contributing to increase the genetic gain of this species. Although the main objective is to increase the oil yield, they also conduct their efforts to obtain lines with a good behavior against *Verticillium*, Downy mildew and *Sclerotinia*, as well as industrial quality (high oleic acid) and drought tolerance (González et al. 2015; González 2016). In this context, the Active Germplasm Bank of the Manfredi Experimental Station (AGB-IM) of INTA hosts about 1200 accessions between cultivated and wild species; thus constituting an invaluable source of variability for the development of breeding program underway.

In the '90s, the sunflower crop was displaced towards areas with lower quality and agroecological aptitude. This caused that the yields have not experienced significant increases in the last five years, despite the technological changes incorporated (FAS USDA 2021). These marginal areas are characterized by a marked water deficit, due to the reduction in the frequency of annual rainfall. These changes in the seasonal distribution cause a discrepancy between crop cycles and water availability in the soil. Consequently, the water stress produced during this period causes significant yield losses, also affecting the content and chemical quality of the oil in the seed (D. Álvarez personal communication). Therefore, the increase in the drought tolerance in sunflower hybrids is our goal and for this, it is interesting to explore the genetic resources in order to identify genomic regions associated with this trait. In this way, since 2005 different studies were done with accessions from the AGB-IM, to obtain a better knowledge about drought tolerance in this crop (Andrade et al. 2014; Moreno et al. 2014; Escalante et al. 2014; Grandón 2018; Grandón et al. 2018a, b). In this sense, the aim of this study was to select the best combination of contrasting parents to develop mapping populations for drought tolerance, based on phenotyping in the greenhouse conditions at vegetative stage and genotyping with SSR markers.

Materials and methods

Plant materials, growth conditions and experimental design

Seven inbred lines (ILs) belong to an association-mapping panel (AMP) established by Fusari et al. (2012) (Table 1), were included in this study for drought tolerance phenotyping and molecular assay. Six of them were previously evaluated for drought tolerance in the field, under well-watered (WW) and water-deficit (WD) conditions during 2003-2004 and 2004-2005 seasons at INTA Manfredi, province of Córdoba, Argentina (31°49’12” S, 63°46’00” W) (Andrade et al. 2009). Inbred lines were classified as sensitive and drought-tolerant based on the seed yield ha$^{-1}$, oil yield ha$^{-1}$ and the relative germination percentage in manitol (200 and 400 mM) in a lab test. Pereyra-Irujo et al. (2007) classified HA64 as drought-tolerant in greenhouse experiment for evaluate the response of leaf growth to water deficit.

**Table 1** Description of sunflower inbred lines evaluated in this study

| Inbred lines | Pedigree          | Origen   | Mantainer/Restorer | Field assay (2003-2005) |
|--------------|-------------------|----------|--------------------|-------------------------|
| B59          | derived B85-9-7   | Argentina| Mantainer          | drought-sensitive       |
Three seeds of each ILs genotype were sown in PVC pipe pots filled with 4000 g of soil (typic Haplustoll, Serie Oncativo) and it were kept in the greenhouse conditions from September to October 2018 at INTA Manfredi. Those were arranged following a randomized complete block design with six replicas under two water treatments: well-watered (WW) and water-deficit (WD). Water deficit (70% of WW condition) was induced at eight-leaf stage for a period of 16 days. Gravimetric moisture contents at field capacity and permanent wilting point were measured initially. Based on these water constants the target weight was determined for each water condition, which were maintained with the irrigation applied daily. Seedlings were thinned to one plant per pot and were grown without water limitations until eight-leaf stage initiation in each IL. At this moment, soil water content was gradually decreased using the method described in Pereyra-Irujo et al. (2007). Then, the pots were covered with polyethylene to prevent evaporation from the ground. They were weighed and watered manually according to the water content in each treatment conditions. The growth conditions were 16 h photoperiod, 32/14 °C day/night, 39/79 % RH day/night and 3.24/1.43 kPa VPD day/night. Air temperature (T°C) and relative humidity (RH) were automatically recorded every hour with a data logger (Lascar, China). The value of vapor pressure deficits (VPD) was calculated daily as the difference between the saturated vapor pressure ($e_a$) and the actual vapor pressure ($e_d$), using daily maximum and minimum temperature ($T_{max}$ and $T_{min}$, respectively) and daily RHmax and RHmin, following the procedure described by Abbate et al. (2004):

\[
VPD = e_a - e_d
\]

\[
e_a = e_{a(T_{max})} (1 - \theta) + e_{a(T_{min})}(1 - \theta)
\]

\[
e_d = \frac{e_{d(T_{max})} + e_{d(T_{min})}}{2}
\]

\[
e_{d(T_{max})} = e_{a(T_{max})} \frac{RH_{min}}{100}
\]

\[
e_{d(T_{min})} = e_{a(T_{min})} \frac{RH_{max}}{100}
\]

\[
e_{a(T_i)} = 0.611 \exp\left(\frac{17.27 T_i}{T_i + 237.3}\right)
\]

where $\theta$ is a weighing parameter: 0.72 for $T_{max}$ and 0.28 for $T_{min}$; $T_i$ is $T_{max}$ or $T_{min}$; $T_{max}$: maximum temperature; $T_{min}$: minimum temperature; RHmax: maximum relative humidity; RHmin: minimum relative humidity.

Trait Measurements

Each seven days, the leaf area (LA), plant height and stem diameter were recorded as well as at the beginning and the end of the water stress period. The LA was recorded with scale as the width of each leaf and was estimated using the allometric relationships shown in Vega et al. (2001) and Druetta (2016). Gain of leaf area (GLA) was determined like differences between total leaf area per plant (TLAp) at the end and the beginning of the water stress period. Both dry weight (DW) and fresh weight (FW) were estimated at the beginning of the water stress period from the volume of the plants (plant height * stem diameter) and the TLAp, using the allometric relationships shown in Vega et al. (2001) and Druetta (2016). At the end of the experiment the shoots and roots were harvested and weighted and then were oven-dried at 105 °C for 24 h, to determine total DW. Water transpired (WT) per plant was estimated every day from the difference in the pot weight. Total water use (TWU) per plant was calculated at the end of the experiment by accumulating daily WT during the effective stress period. Net assimilation rate (NAR) was determined from the LA and total DW per plant (shoots + roots) with the following formula:
\[
NAR = \frac{(W_2 - W_1) \left( \log_e L_2 - \log_e L_1 \right)}{(L_2 - L_1)(t_2 - t_1)}
\]

where \(W_1\) and \(W_2\) are total dry weight and \(L_1\) and \(L_2\) total leaf area at times \(t_1\) and \(t_2\), respectively.

Water use efficiency (WUE) (on a whole plant basis) was determined at the end of the experiments as the ratio of dry weight gain (DWG = \(W_2 - W_1\)) to TWU during the effective water stress period. For daily transpiration rate (TR) response to vapor pressure deficit (VPD), slope and breakpoint were estimated from non-linear regression. The TR daily was calculated as the ratio of WT to LA per plant and VPD was estimated as detailed above.

Molecular assay

The genomic DNA from each IL was isolated from 10 mg of lyophilized material according to a modified CTAB method (Doyle and Doyle 1987), and quantified by spectrophotometry (NanoDrop 8000, Thermo Fisher Scientific, USA). A set of 127 SSR markers available on public basis (https://www.ncbi.nlm.nih.gov/nuccore) was selected and screened for polymorphisms among seven ILs. Genotyping was performed by multiplex PCR assays (two or three primers for reaction) designed with the Multiplex Manager software (Holleley and Geerts 2009); using a kit multiplex PCR (QIAGEN, Hilden, Germany). Amplifications were conducted in a GeneAmp PCR System 9700 thermocycler (Applied Biosystems, USA) following an initial denaturation step at 95 °C for 15 min, followed by 30 cycle of 94 °C for 30 s, 60 °C for 90 s and 72 °C for 60 s, with a final extension of 60 °C for 20 min. Separation of PCR products was performed in 6% denaturing polyacrylamide gel electrophoresis and visualized by silver nitrate stained according to Creste et al. (2001) and the fragment size was estimated using a DNA ladder 10 bp (Invitrogen, USA).

Data analysis

Principal components analysis (PCA), non-linear regression, LSD Fisher test and Pearson´s correlation coefficients were carried out using InfoStat (Di Rienzo et al. 2011). Mixed generalized linear models was performed using SAS University Edition 3.6 (SAS 2016). Genotypes, treatments and interactions were considered as a fixed factor and replicate nested in each table was considered as a random factor. The polymorphism was determined between the possible combinations of contrasting parental for drought tolerance. Cluster Analysis was performed from the Nei´s genetic distance (Nei 1972) and UPGMA algorithm. Molecular analysis was conducted with Infogen software (Balzarini and Di Rienzo 2011).

Results

Significant differences were observed between genotypes and water treatments for all traits. While the interaction was also significant for NAR (p=0.0022), WUE (p=0.0264) and the breakpoint to the VPD response (p=0.0021).

The genotype B59 presented higher average value for GLA (502.72 ± 109.16 cm²), while R419 showed lower average value (162.43 ± 100.39 cm²) for this trait. Significant differences between water treatments were also observed for plants under WD condition with an 18% average reduction (p=0.0004). However, HA64 showed a significant reduction (27%) between both conditions (p=0.0080) (Figure 1A). Regarding the total water use (TWU), significant differences was observed between genotypes (p<0.0001). For this trait, B59 presented highest average value (2524.00 ± 554.20 gH₂O.day⁻¹) whereas R419 showed the lowest one (1685.75 ± 399.72 gH₂O.day⁻¹) (Figure 1B). Significant differences were also observed between water treatments with a 23% average reduction for plants under WD. This situation was evident for HAR4 (reduction of 32%), B59 and R419 (both with a reduction of 29%) (p<0.05).
Fig. 1 a GLA and b TWU traits for seven inbred lines evaluated under well-watered (WW) (full spot) and water-deficit (WD) (empty spot) conditions. Data are means ± SE of six replicates. Values with the same letter are not significantly different (p ≥ 0.05)

NAR trait showed an increase in the WD condition (Figure 2A). Thus, both HA64 (p<0.0001) and HAR4 (p=0.0006) presented a significant difference between two water treatments, which increased under WD of 35% and 23%, respectively. Moreover, significant differences were observed between water treatments with an increase in efficiency under water-stress of 32% (p<0.0001) (Figure 2B). Besides, genotypic variability was observed for WUE trait under vegetative stage, being HA64 and HAR4 those that presented greater significant differences (p <0.05) between WW and WD conditions (55 and 39%, respectively). In addition, the other genotypes showed between 19 and 35% of increased (p<0.05) between both water conditions.

Significant differences were observed for genotypes (p<0.0001) and water treatments (p=0.0026) (Figure 3A) in the relation between daily transpiration rate (TR) and vapor pressure deficit (VPD). HA64 and R432 presented higher slope, whereas R419 and HA89 showed a lower slope in VPD response (Figure 3A and 4A). Although there was an average reduction of 6% between WW and WD conditions (Figure 4B), only R419 (p=0.0480) and R423 (p=0.0111) showed significant differences between both water conditions (Figure 3A). In addition, significant differences were observed for the breakpoint in VPD response both genotypes and water treatments (p=<0.0001), as well as significant
interaction (p=0.0021) (Figure 3B). Thus, HA64 and R432 showed lower breakpoint and reached a limited-transpiration rate (TRlim) at a lower VPD value, whereas R419 and HA89 showed, higher breakpoint both water contents (Figure 3B and 4A). Nevertheless, only HA64 (p=0.0493), R432 (p=0.0041) and HA89 (p<0.0001) showed significant differences between water treatments. Being the latter, which showed the biggest difference between both water conditions (an average reduction of 13%). Instead, HAR4 did not show significant differences between water treatments (p=0.9067), hence under WD maintained its TRlim (Figure 3B). In addition, an average reduction of 5% WD treatments with respect to WW condition was observed, thus decreasing the TRlim reached by each genotypes (Figure 4B).

The Principal Component Analysis (PCA) explains the 80% of the total variability (Figure 5). PC1 (43.8%) discriminated HA89 and R419 from the other genotypes due to a lower slope and higher breakpoint, both for WW and for WD treatments. Likewise, PC2 (35.9%) separated all genotypes by water treatment based on WUE and TWU values.
The correlation between traits was analyzed by Pearson correlation coefficients (Table 2). A higher significant positive correlation was found both GLA and TWU \( (r=0.60, p<0.0001) \) and NAR and WUE \( (r=0.77, p<0.0001) \). Whereas, a higher significant negative correlation was found between slope and breakpoint in the VPD response \( (r=-0.73, p<0.0001) \).

Fig. 5 PCA based on six traits evaluated in seven inbred lines under well-watered (WW) (full spot) and water-deficit (WD) (empty spot) conditions. GLA: Gain of Leaf Area, TWU: Total Water Use, NAR: Net Assimilation Rate, WUE: Water Use Efficiency.

Table 2 Pearson correlation coefficients \( (r) \) between six traits evaluated in seven inbred lines. Lower diagonal: \( r \)-values. Upper diagonal: \( p \)-value.

| Traits | WUE | Slope | Breakpoint | TWU | GLA | NAR |
|--------|-----|-------|------------|-----|-----|-----|
| WUE    | 1   | 0.957 | 0.003      | 0.004| 0.975| 0.000|
| Slope  | -0.01| 1     | 0.000      | 0.017| 0.172| 0.003|
| Breakpoint | -0.32| -0.73 | 1          | 0.029| 0.022| 0.001|
| TWU    | -0.32| 0.26  | -0.24      | 1   | 0.000| 0.292|
| GLA    | 0.00 | 0.15  | -0.25      | 0.60 | 1   | 0.786|
| NAR    | 0.77 | 0.32  | -0.36      | -0.12| 0.03 | 1   |

WUE: Water Use Efficiency; TWU: Total Water Use; GLA: Gain of Leaf Area; NAR: Net Assimilation Rate.

According to traits evaluated, ILs were classified as high (HA64 and HAR4), intermediate (B59, R423 and R432) and low transpiration efficiency (TE) (HA89 and R419). Additionally, HAR4 (high TE) and R419 (low TE) show the same behavior for drought-stress both in the greenhouse and in the field assay (Andrade et al. 2009); maintaining the same TE of each genotype both in the vegetative and the reproductive stages.

These seven ILs were genotyped with 127 SSR markers of which amplified 91 loci that allowed identifying 262 alleles, as well as a high level of polymorphism between the possible combinations of contrasting parents (Table 3). In addition, a neighbor-joining tree (Figure 6) was constructed based on the Nei Standard genetic distances (Nei 1972) calculated between pairs of ILs. At a distance of 1.05, two groups can be identified: one (group 1) composed of maintainer lines (HAR4 and B59) and the other (group 2) was dominated by the presence of restorer lines and separated into two subgroups. One of them contains R423, HA89 and R419 (subgroup 1) and the other subgroup consisting of R432 and HA64. However, based on the phenotyping, there were four possible combinations of contrasting parents, but only R419xHA64 and HA89xHAR4 had a greater genetic distance, besides a high polymorphism level between them (Table 3).
Table 3 Level of polymorphism between possible combinations of contrasting parents for 91 SSR markers analyzed

| Parental combination | Behavior against water-deficit in the greenhouse | Level of polymorphism | Nei Standard distance |
|----------------------|--------------------------------------------------|-----------------------|-----------------------|
| Female | Male | | |
| R419 | HA64 | low TE x high TE | 59% | 1.08 |
| R419 | HAR4 | low TE x high TE | 59% | 1.01 |
| HA89 | HA64 | low TE x high TE | 43% | 0.70 |
| HA89 | HAR4 | low TE x high TE | 60% | 1.08 |

TE: transpiration efficiency.

Fig. 6 Neighbor-Joining tree constructed based on the Nei Standard genetic distances and UPGMA algorithm for seven inbred lines. Cofenetic correlation coefficient: 0.71

Discussion

Based on these results, four combinations of contrasting parents were achieved, but only R419xHA64 and HA89xHAR4 showed the highest phenotypic contrast. Besides, a greater genetic distance (1.08) and a high polymorphism level between them (about 60%) (Table 3). That is why these are the most suitable combinations of contrasting parents to develop mapping populations for drought tolerance in sunflower.

Phenotyping assay allow finding a differential response to water stress, among those contrasting genotypes potential to be used as parents to build segregating mapping populations. Thus, the selection of highly contrasting genotypes increase the probability of finding genomic regions or allelic variants associated with the trait. Consequently, the phenotyping must be accurate and show an experimental design that allows finding significant differences between parental evaluated. In addition, a greater genetic distance and a high level of marker polymorphism between genotypes also increase the probability of finding QTLs associated with the trait. In this study, phenotyping for drought tolerance was made in a greenhouse during vegetative stage for 16 days. Based on these results, a wide genotypic variation of response was observed for traits and water stress level evaluated in seven inbred lines. Phenotyping in the greenhouse allows have greater control of the atmospheric demand generated and management issues such as water-stress level applied. This also allows determining the individual plant behavior at an early development stage and in short testing times (Casadebaig et al. 2008; Velázquez et al. 2017).

Although leaf expansion is the first morpho-physiological process affected by water-deficit due to the reduction in cell division and cell expansion (Pereyra-Irujo et al. 2008), the leaf conductance it would also be affected by stomatal closure. This mechanism is one of the main causes of reduction of transpiration rate under water-deficit; because of a reduction in leaf water potential causes a decrease in leaf conductance avoiding excessive water loss (Hsiao 1973). Likewise, Nardini and Salleo (2005) reported that stomatal opening was reduced between 28 and 50% when water-
stress increased from moderate to severe, respectively. Therefore, in our experiments all genotypes showed lower
GLA (average decreased of 18%) and TWU (average decreased of 23%) under WD conditions. For the GLA trait,
HA64 genotype was the unique IL, which showed significant differences between both water conditions (a decrease
of 27%) and consequently, a reduction of 18% of TWU. Moreover, HAR4, B59 and R419 also showed significant
differences (p<0.05) between WW and WD conditions for the last because of the lower GLA. In this sense, the
reduction on transpiration rate under water-stress is a consequence of the reduction of GLA and the decline of stomatal
opening. Similarly, Pereyra-Irujo et al. (2008) found variation for leaf expansion among genotypes analyzed,
suggesting that those with the greatest reduction in leaf expansion rate had a high osmotic adjustment (OA). In
agreement with this, Chimienti and Hall (1994) observed that sunflower genotypes with high OA showed less leaf
expansion. Thus, they conclude that there is a negative association between these traits, probably because of reduction
in leaf expansion is the most important mechanisms to avoid water loss.

The net assimilation rate (NAR) is an index that measures the photosynthetic efficiency and the net gain of
assimilated per unit leaf area and unit of time (Morales-Morales et al. 2015). This index reaches maximum values in
the vegetative stage and then decreases due to the increase of non-photosynthetic dry matter. In this study, NAR trait
showed an increase in the WD treatment with respect to control. Thus, HA64 and HAR4 were the unique genotypes
that showed a significant increase under WD (35% and 23%, respectively). This could be explained according to what
was reported by Velázquez et al. (2017), who observed an increase in the number of stomata per unit leaf area under
water-stress (75% more in HA64). Similarly, Carrera et al. (2021) found in soybean 28% more stomata under water-
stress compared to the non-stressed control. However, they did not observe that water-deficit significantly affected
the photosynthetic efficiency of photosystem II. Therefore, this would keep photosynthesis active without greatly
modifying the accumulation of biomass.

Water use efficiency (WUE) is defined as dry matter produced per unit of water transpired and expresses the
efficiency with which a crop fixes CO2 in relation to the water it loses (Dardanelli et al. 2003). Prieto et al. (2007,
2011) reported that WUE is a constitutive trait that shows intraspecific variation and increases in response to water-decit
during the vegetative stage in soybeans. For this reason, this trait has been considered as a selection criterion
due to its association with drought tolerance in other crops such as wheat, barley and soybean (Condon et al. 2004;
Prieto et al. 2007). In this study, the increase in WUE under water-deficit (32% with respect to control) would indicate
that stressed plants were more efficient and conservative with water in the tissues, producing more dry matter per
gram of water transpired. Similarly, Prieto et al. (2011) observed this behavior in soybean during the vegetative stage.
In addition, this difference between treatments was also observed by Velázquez et al. (2017) who detected between
15 and 30% increase in WUE under water-deficit in sunflower during the vegetative stage.

The transpiration rate (TR) is defined as the water transpired per unit leaf area per unit of time and depends on the
concentration gradient of water vapor (estimated from the Vapor Pressure Deficit or VPD) between the substomatic
cavity and the air surrounding the leaf. As the VPD increases, the TR also increases; however, this increase is not
unlimited since the TR reaches a limit (TRlim) above a threshold value of VPD or breakpoint (Turner et al. 1984). For
establish the relationship between daily TR and VPD response under two water conditions, the slope and the
breakpoint were determined. The evidence is that under WD there was a decrease in the slope and breakpoint (6% and
5%, respectively), decreasing the TRlim reached by each line. Thus, ILs with higher slope showed lower breakpoint
(HA64 and R432) and those with lower slope showed a higher breakpoint (R419 and HA89) (Figure 3). Therefore,
HA64 and R432 reached the TRlim at a lower VPD, reduced the stomatal conductance and consequently the water loss.
Instead, R419 and HA89 reached the TRlim at a higher VPD, probably due to a lower stomatal sensitivity to water
deficit (Turner et al. 1985). Although the photosynthetic rate is proportional to the TR (Tanner et al. 1983), it could be
higher at times of the day where the VPD is less than 2.46 kPa. The practical significance of the TR-VPD response
is that above the break point (specific for each genotype) there is a limitation in the TR due to an increase in stomatal
sensitivity, resulting in the conservation of water in the tissues. Thus, the results of this work contribute to demonstrate
the existence of significant differences between genotypes for this relationship under conditions of water limitation
during the vegetative stage in sunflower. According to Sinclair et al. (2008), the breakpoint as a response to a limited
hydraulic conductance (K) in the leaf, that constrains the flow of water from the xylem into the guard cells under high-evaporative conditions, i.e. high VPD. This would lead to a loss of turgor in the guard cells and a reduction of stomatal
conductance (Turner et al. 1985; Nardini and Salleo 2005). In this sense, Sadok and Sinclair (2010) and Nardini et al.
(2005), determined that the reduction in hydraulic conductance would be associated with the decrease in the expression
or activity of aquaporins sensitive to silver nitrate (AgNO3) in soybeans and to mercury chloride (HgCl2) in sunflower,
respectively. Therefore, these authors suggest that a reduction in the expression of these proteins would restrict the
flow of water causing the TR to reach a limit and remain stable at a high VPD. The changes in aquaporins transcripts
Adiredjo AL, Navaud O, Muños S, Langlade NB, Lamaze T, Grieu P (2014b) Genetic control of water use efficiency

Adiredjo AL, Navaud O, Lamaze T, Grieu P (2014a) Leaf carbon iso tope discrimination as an accurate indicator of

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In order to establish the genetic relationship between ILs evaluated, a cluster analysis was performed. Thus, a

neighbor-joining tree was constructed (Figure 6) and at a distance of 1.05, two well-defined groups can be identified.

One of them (group 1) was composed of maintainer lines of Argentinian origin (HAR4 and B59) and the other (group

2) was dominated by the presence of restorer lines and separated into two subgroups at a distance of 0.95. One of these

contains R423, HA89 and R419 (subgroup 1) which is expected since R419 derives from HA89. While grouping with

R423 was probably due to is a restorer line of Argentinian origin, just like R419. The second subgroup was conformed

by R432 and HA64 because of they share 45% of similarity among the SSR markers analyzed. Although in the records

HA64 is from USA and R432 is from Argentinian germplasm, these could have some common origin, which would

cause them to be located together in the neighbor-joining tree. These groups are coincident with that found by Filippi

et al. (2015) in their cluster analysis for 42 SSR in 170 sunflower ILs from the INTA – AMP.

Based on phenotyping results, four possible combinations between contrasting parents were achieved (Table 3).

However, only two (R419xHA64 and HA89xHAR4) showed a greater genetic distance (1.08) and a high level of

polymorphic markers between them (about 60%). In conclusion, these would be the best combinations to develop

mapping populations for drought tolerance in sunflower. Currently, the F1 populations were sowing in this 2021-2022

campaign. These will serve to identify new genomic regions or to validate SNPs variants associated with this trait,

previously identified in the association mapping population to which they belong. Our results suggest that it is relevant

a previously analysis with phenotypic and genotypic data to performs a correct selection of parental lines for the
develop of mapping population for quantitative traits like as drought tolerance and this combined evaluation might be

an important point that could be suggested to be applied in other breeding programs of the crop.

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Statements & Declarations

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Competing Interests

The authors have no relevant financial or non-financial interests to disclose.

Author Contributions

All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by Nancy Gabriela Grandón, Eugenia Alejandra Martin, Emanual Mauro Cicconi, Carolina del Pilar Díaz,
Eva María Celia Mamaní and María Valeria Moreno. The first draft of the manuscript was written by Nancy Gabriela Grandón and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

**Data Availability**

The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.